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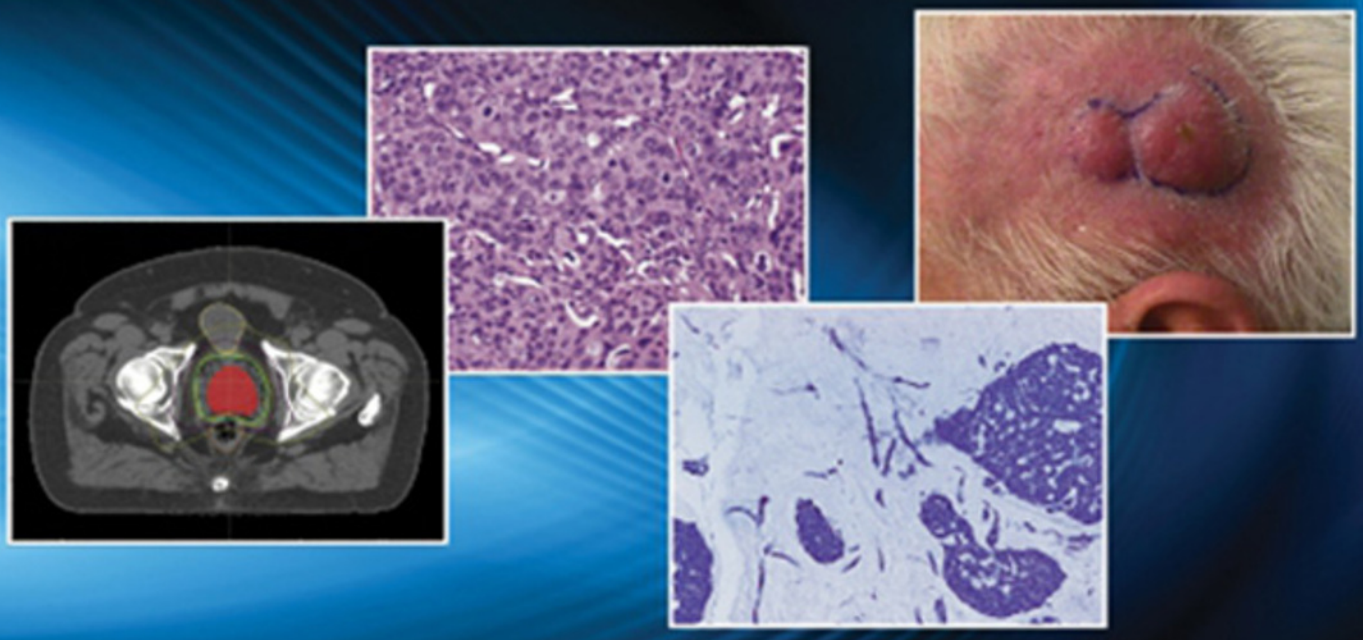

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To my son, Matthew, and my wife, Kathy, who have and continue to make sacrifices so that I might pursue my passions in medicine and research. To my colleagues at the National Cancer Institute, University of Virginia, Johns Hopkins, and across the country, whose selfless dedication to patient care and cancer research is truly an inspiration to all. To the many students who have trained with me over the years, to my patients, and to my colleagues at the Inova Translational Medicine Institute, who have given me the opportunity to have this tremendously rewarding career. Lastly, to Tracey, and to Marty, who, in memory, inspire all who knew them to work a little harder each day toward the elimination of the pain and suffering from this disease.
JOHN E. NIEDERHUBER, MD

To my wife, Nancy, for her love and support over 49 ½ years.
JAMES O. ARMITAGE, MD

To my wife, Robin Winkler Doroshow, MD, my classmate and greatest supporter, for her love, dedication, and commitment and for the remarkable joy and caring she brings to her patients and to all around her. To my remarkable daughter, Deborah Doroshow, MD, PhD, who is completing her training for a career in academic oncology; my fondest hope is that you will enjoy sharing with and learning from those you help as much as I have. To my patients and colleagues at the City of Hope and the National Cancer Institute who have all contributed so much of themselves to my continuing education as a physician and investigator, please accept my appreciation and utmost gratitude.
JAMES H. DOROSHOW, MD

To my wife, Kathy, and my sons, Benjamin, Nathaniel, and Jonathan. You are the lights of my life. I also acknowledge all of my mentors, colleagues, and patients, who have taught me so much. A special note of gratitude goes to Marty Abeloff, a mentor and an inspiring role model for career and for life.
MICHAEL B. KASTAN, MD, PhD

To my wife, Laurie, who has been my soul mate for many years and has constantly reminded me of life's priorities. To my family including my daughters, Miriam and Abigail, and my grandchildren, Zekariah, Zohar, Samuel, Marcelo, Jonah, and Aurelio. They have been an inspiration. To my many teachers through the years who have helped define and foster my professional career, but especially Herman Suit and Eli Glatstein.
JOEL E. TEPPER, MD

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Memoriam

Martin D. Abeloff, MD (1942-2007)

Martin D. Abeloff, a founding editor of *Clinical Oncology,* dedicated his life to caring for patients with cancer and to teaching his art to fellows, residents, and students. He was a brilliant and caring clinician, an extremely effective leader, and a beloved mentor to many trainees and young faculty.

Marty was born on April 4, 1942, in Shenandoah, Pennsylvania. He received his BA from The Johns Hopkins University in 1963 and his MD from The Johns Hopkins University School of Medicine in 1966. He spent the next year as an intern at the University of Chicago Hospitals and Clinics. His legacy in medicine was established on his return to Baltimore in 1971 as a fellow in clinical oncology. He would spend the rest of his career at The Johns Hopkins Hospital, achieving the rank of Professor of Medicine in 1990. At various times, he served as the fellowship training program director, chief of medical oncology, clinical director of the cancer center, oncologist in chief at The Johns Hopkins Hospital, and in 1992, was appointed the second director of The Johns Hopkins Oncology Center, later renamed, thanks to Marty's efforts, the Sidney Kimmel Comprehensive Cancer Center.

It was during his time as cancer center director that Marty brought to life the idea of a comprehensive, user-friendly textbook of oncology that would be as valuable to the practicing oncologist as to the primary care physician and physicians-in-training. The first edition of *Clinical Oncology* was published in 1995 to a gratifying response. It is now established as a cornerstone reference for those caring for patients with cancer.

In the sixth edition, we continue Marty's vision for an ever better, unique, and accessible text so that future generations of oncologists will remember his inspiration and leadership.

The editors again dedicate this text, which is already a recognized tangible aspect of his legacy in medicine, as a living memorial to him. *Abeloff's Clinical Oncology* will continue to serve as a reminder to all its users of this extraordinary person and exemplary physician who went before them.

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Preface

New insights into whole genome sequence variations and the genomic structural alterations associated with cancer, including their downstream effects on protein structure and function, are helping us to define specific communication pathway changes that drive cancer initiation, progression, metastasis, and resistance. We have learned that each individual and each tumor may be unique. Individual physiognomies in terms of path of progression and unique cellular communication pathway alterations are continuing to define the nature of specific cancers and offer greater opportunities for the development of highly prescriptive intervention(s).

In addition, we have a much greater understanding of the relationship of the host's tissues, the patient's immune system, and the broad tumor microenvironment, to the process of tumor development and progression and their impact on tumor control. This new body of knowledge on how the body's immune system and the tumor's microenvironment are altered to support disease growth, invasion, and distant spread is providing opportunities for the development of novel therapeutic interventions. There is exciting new evidence to support the presence of a special subclass of cells within the tumor that has properties of "stemness," which places them in the key role of maintaining tumor growth and tumor spread. The cumulative effect of these advances—where certain cancers can be prevented and where others will be detected earlier and controlled—promises to be transformative in our effort to conquer cancer.

The sixth edition of *Abeloff's Clinical Oncology* incorporates the exciting advances in basic, translational, clinical, and epidemiologic oncology. Each chapter begins with a summary highlighting the key points and comprises a critical analysis of the literature and updated clinical studies—authors present their own opinions in specially identified boxes and algorithms.

Despite significant progress, the diagnosis of cancer remains devastating to patients and their families. Our goal is to provide a reference textbook that is the most useful, understandable, attractive, and thorough in presenting the principles of clinical oncology. It is meant to be equally useful to students and trainees, experts in the various disciplines of oncology, and as a reference text for physicians from other medical disciplines and the various staff who regularly care for patients with cancer. It is our hope that readers will find this scholarly textbook properly balanced between the disciplines of science, clinical medicine, and humanism and that it will serve them well in their efforts to prevent, diagnose, and effectively treat their patients suffering from cancer.

The multidisciplinary nature of cancer care is, and will continue to be, reflected in our editors. Experts in cancer biology, surgical oncology, pediatric oncology, radiation oncology, medical oncology, and hematologic malignancies directed the development of the content. Reflecting the multispecialty approach necessary for optimal care of patients, the majority of chapters are the joint product of several of these disciplines. Engaging the very best subject matter authorities was a guiding principle for the editors and we are deeply indebted to our outstanding authors who, in a most diligent and thoughtful way, have brought their knowledge and skills to the sixth edition of *Abeloff's Clinical Oncology.*

ACKNOWLEDGMENTS

This sixth edition represents a highly collaborative and dynamic effort between the editors and Elsevier. We are greatly indebted to Laura Schmidt, Kathleen Schlom, and Kristi Batchelor for their creative input and guidance and for turning the principles behind this text into a reality. Finally, we want to express our gratitude to our many contributing authors for their dedication to this project, their generosity of time, and, of course, their very valuable friendship.

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PART I

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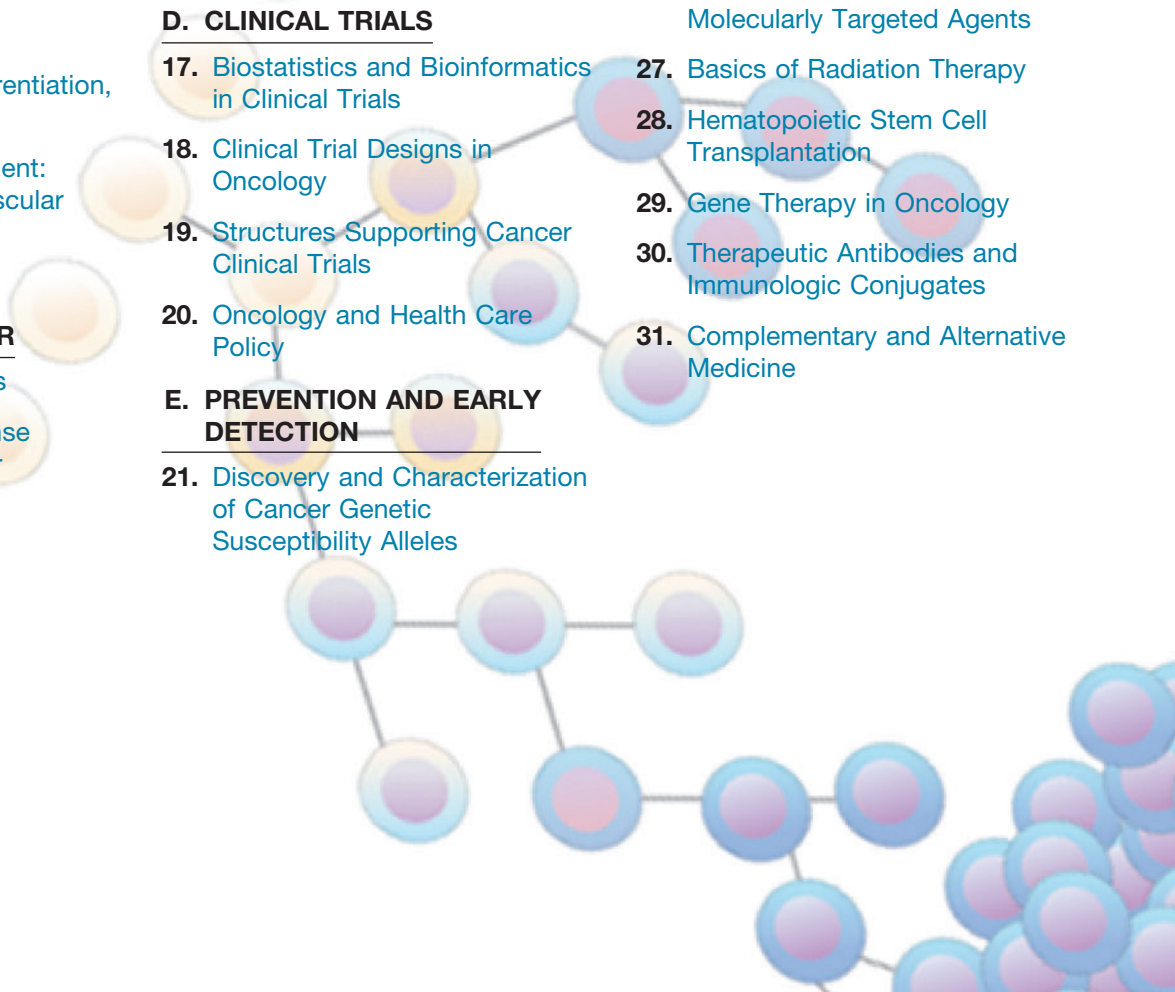

A. BIOLOGY AND CANCER

1 Molecular Tools in Cancer Research

Mauro W. Costa, Muneer G. Hasham, and Nadia Rosenthal

SUMMARY OF KEY POINTS

- Our understanding and treatment of cancer have always relied heavily on parallel developments in biologic research. Molecular biology provides the basic tools to study genes involved with cancer growth patterns and tumor suppression. An advanced understanding of the molecular processes governing cell growth and differentiation has revolutionized the diagnosis, prognosis, and treatment of malignant disorders.
- This introductory chapter relates basic principles of molecular biology to emerging perspectives on the origin and progression of cancer and explains newly developed laboratory techniques, including whole-genome analysis, expression profiling, and refined genetic manipulation in and use of genetically diverse animal models, providing the conceptual and technical background necessary to grasp the central principles and new methods of current cancer research.

Since the last edition of this book was published, advances in our understanding of the basic mechanisms of cancer have continued to inform and refine clinical approaches to prevention and therapy. New prognostic and predictive markers derived from molecular biology can now pinpoint specific genetic changes in particular tumors or detect occult malignant cells in normal tissues, leading to improved technologies for tumor screening and early detection. Diagnostic approaches have expanded from morphologic criteria and single-gene analysis to whole-genome technologies and single-cell genomics imported from other biologic disciplines. A new systemic vision of cancer is emerging, in which the importance of individual mutation has been superseded by an appreciation for higher-order organization and individual genetic background, disrupted by complex interactions of disease-associated factors and gene-environmental parameters that affect tumor cell behavior. Results from these cross-disciplinary investigations underscore the complexity of carcinogenesis and have profoundly influenced the design of strategies for both cancer prevention and advanced cancer therapy.

This overview will serve as a foundation of conceptual and technical information for understanding the exciting new advances in cancer research described in subsequent chapters. Since the discovery of oncogenes, which provided the first concrete evidence of cancer's genetic basis, applications of advanced molecular techniques and instrumentation have yielded new insights into normal cell biology. A basic fluency in molecular biology and genetics has become a necessary prerequisite for clinical oncologists because many of the new diagnostic and prognostic tools currently in use rely on these fundamental principles of gene, protein, and cell function.

OUR UNSTABLE HEREDITY

Cancer genetics has classically relied on the candidate-gene approach, detecting acquired or inherited changes in specific genetic loci accumulated in a single cell, which then proliferates to produce a tumor composed of its identical clonal progeny. During the early steps of tumor formation, mutations that lead to an intrinsic genetic instability allow additional deleterious genetic alterations to accumulate. These genetic changes confer selective advantages on tumor cell clones by disrupting control of cell proliferation. The identification of specific mutations that characterize a tumor cell has proved invaluable for analyzing the neoplastic progression and remission of the disease. The emergence of cancer cells is a byproduct of the necessity for continuous cell division and DNA replication to maintain organ functionality throughout the life cycle.

The highly heterogeneous nature of tumors, each composed of multiple cell types, led to the formulation of the "cancer stem cell" hypothesis, which posits that only a subpopulation of cancer cells is able to maintain self-renewal, unlimited growth, and capacity for differentiation into other, more specialized cancer cell types. Cancer stem cells display bona fide stem cell markers, in contrast to other cancer cells present in the tumor, which do not have tumorigenic potential. In fact, fewer than 1 in 10,000 cells present in human acute myeloid leukemia are capable of reinitiating a new tumor when transplanted into animals. Cancer stem cells have been identified in many solid tumors in the brain, colon, ovaries, prostate, and pancreas, suggesting that more effective cancer therapies would target these self-renewing cells, rather than the tumor as a whole. The cancer stem cell concept differs from the original clonal evolution hypothesis, which states that every cell in a tumor mass is capable of self-renewal and differentiation, and suggests that detecting and targeting subtle genetic and epigenetic differences that distinguish cancer stem cells may provide a more effective avenue to intervention in disease progression.

Heterogeneity can also arise as a result of stochastic mutational events that lead to cancer progression. Clastogenic insults to the genome, or genomic instability due to aberrant gene regulation, could lead to loss of heterozygosity (LOH) of tumor suppressor genes such as *TP53, RB1,* or *BRCA,* and can also lead to tumor heterogeneity and change in disease progression. Furthermore, activation of DNA or RNA editing enzymes in tumors could lead to *kataegis,* a DNA hypermutation process, and increase tumor heterogeneity. Although there are molecular biology tools currently available to detect aberrant but stable genomes, the later processes that lead to genomic instability make diagnosis and prognosis more challenging.

DETECTING CANCER MUTATIONS

Methods for mutation detection all rely on the manipulation of DNA, the basic building block of heredity in the cell. DNA consists of two long strands of polynucleotides that twist around each other clockwise in a double helix (Fig. 1.1). Nucleic acid bases attached to the sugar groups of each strand face each other within the helix, perpendicular to its axis. These comprise only four bases: the purines adenine and guanine (A and G) and the pyrimidines cytosine and thymine (C and T). During assembly of the double helix, stable pairings of nucleotides from either strand are made between A and T, or between G and C. Each base pair forms one of the billions of rungs in the long, unbroken ladder of DNA forming a chromosome.

The functional unit of inherited information in DNA, the gene, is most often represented by a discrete section of sequence necessary to encode a particular protein structure. Gene expression is initiated by forming a copy of the gene, messenger RNA (mRNA), constructed base by base from the DNA template by a polymerase enzyme. Once transcribed, an mRNA transcript is modified and the processed product is transported out of the nucleus. In the cytoplasm, proteins are then synthesized, or translated, in macromolecular complexes called ribosomes that read the mRNA sequence and convert the nucleic acid code, based on three-base segments or codons, into a 20–amino acid code to form the corresponding protein.

Although these canonic processes drive gene expression in all normal cells, cancer cells defy the rules. For instance, uracils, which are found

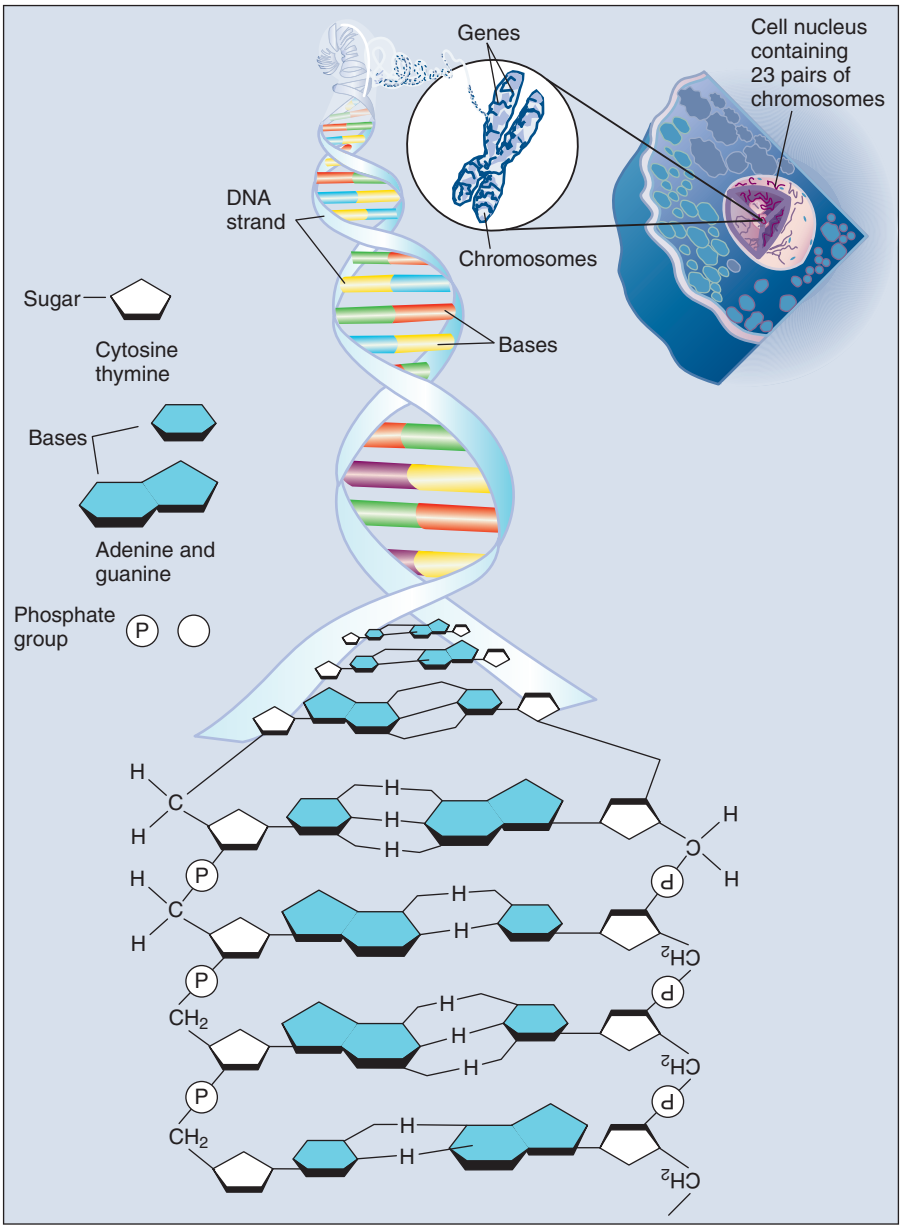

Figure 1.1 • DNA structure. Deoxyribonucleic acid *(DNA)* is the cell's genetic material, contained in single compacted strands comprising chromosomes within the cell nucleus. In the DNA double helix, the two intertwined components of its backbone, composed of sugar (deoxyribose) and phosphate molecules, are connected by pairs of molecules called *bases*. The sequence of four bases (guanine, adenine, thymine, and cytosine) in the DNA helix determines the specificity of genetic information. The bases face inward from the sugar-phosphate backbone and form pairs with complementary bases on the opposing strand for specific recognition. The arrangement of chemical groups is unique for each base pair, allowing base pairs to be specifically targeted by transcription factors, polymerases, restriction enzymes, and other DNA-binding proteins. (From http://www.terrapsych.com/dna.jpg.)

on RNA, can be detected in the DNA of cancer cells because of their high mutation rates. Paradoxically, these deviations from the norm allow the development of molecular biology tools to better diagnose and predict tumor progression.

GENERATING DIVERSITY WITH ALTERNATE SPLICING

In higher organisms, most protein coding gene sequences are interrupted by stretches of noncoding DNA sequences, called introns. In the nucleus, these introns are removed after mRNA transcription to produce a continuous chain of coding sequences, or exons, that subsequently undergo translation into protein. The splicing process requires absolute precision because the deletion or addition of a single nucleotide at the splice junction would throw the three-base coding sequence out of frame, or lead to exon skipping or addition, creating abnormal proteins.

The dramatic increase in genetic complexity conferred by alternate RNA splicing is underscored by the multiple splice patterns of many medically relevant genes, in which different combinations of exons are chosen for the final mRNA transcript, such that one gene can encode many different proteins (Fig. 1.2). The choice of protein isoform to be expressed from a gene with multiple splicing possibilities is a decision that can be perturbed in disease. Errors in splicing mechanisms have been associated with a large group of cancers. These include mutations in the oncogene *p53* in more then 12 different types of cancer, mutL homolog 1 protein (MLH1) mutation in hereditary nonpolyposis colorectal cancer, and several transcription factors and cell signaling and membrane proteins. When mutations in the splicing site lead to insertion of novel sequences in the mRNA, the encoded protein can be used as a potential clinical marker, as seen for the transcription factor NSFR in small cell lung cancer. Owing to their unique expression in cancer cells, these markers can be further explored as new cancer-specific therapeutic targets.

GENOMICS OF CANCER

The complete set of DNA sequences carried on all the chromosomes is known as the genome. Although the general map of the genome is shared by all members of a species, the recent sequencing of thousands of individual human genomes has given rise to the new field of genomics, providing us with new tools to reveal the more subtle variations that arise between individuals. These variations are critical, both as a natural engine driving heterogeneity within a species, and as a source of predisposition to cancer types. The most common forms of human genetic variations, or alleles, arise as single-nucleotide polymorphisms (SNPs). Because these allelic dissimilarities are abundant, inherited, and dispersed throughout the genome, SNPs can be used to track racial diversity, personal traits, and susceptibility to common forms of cancer (Fig. 1.3). Commercial entities have developed tools that can detect thousands of SNPs with relatively little sample material. Platforms such as MegaMUGA or GigaMUGA can allow mammalian genetic mapping that can aid in a number of diagnoses and can distinguish between predictive and prognostic markers.

How do SNPs arise between individuals? One source of variation in DNA sequence derives from deviations in the strict base-pairing rule underlying the structure, storage, retrieval, and transfer of genetic information. The duplicated genetic information in the two strands of DNA not only permits the repair of a damaged coding sequence but also forms the basis for the replication of DNA. During cell division, polymerase enzymes unwind the DNA strands and copy them, using the base sequences as a template for constructing a new helix so that the dividing cell passes its entire genetic content on to its progeny. Errors in this process are rare, and person-to-person differences comprise only about 0.1% of the human genome. SNPs are inherited if they occur in the germline. Many genetically inherited variations occur in regions that do not encode protein or alter the regulation of nearby genes. Given the disruptive effects even subtle genetic changes may have on cell function, it is important to distinguish SNPs that represent true mutations from benign polymorphisms.

Our ability to monitor hundreds of thousands of SNPs simultaneously is one of the most important advances in modern medical genetics. Relatively simple genotyping technologies for SNP detection rely largely on the polymerase chain reaction (PCR). In procedures that use this reaction, two chemically synthesized single-stranded DNA fragments, or primers, are designed to match chromosomal DNA sequences flanking the segment in which an SNP is positioned. With the addition of nucleotide building blocks and a heat-stable DNA polymerase, the

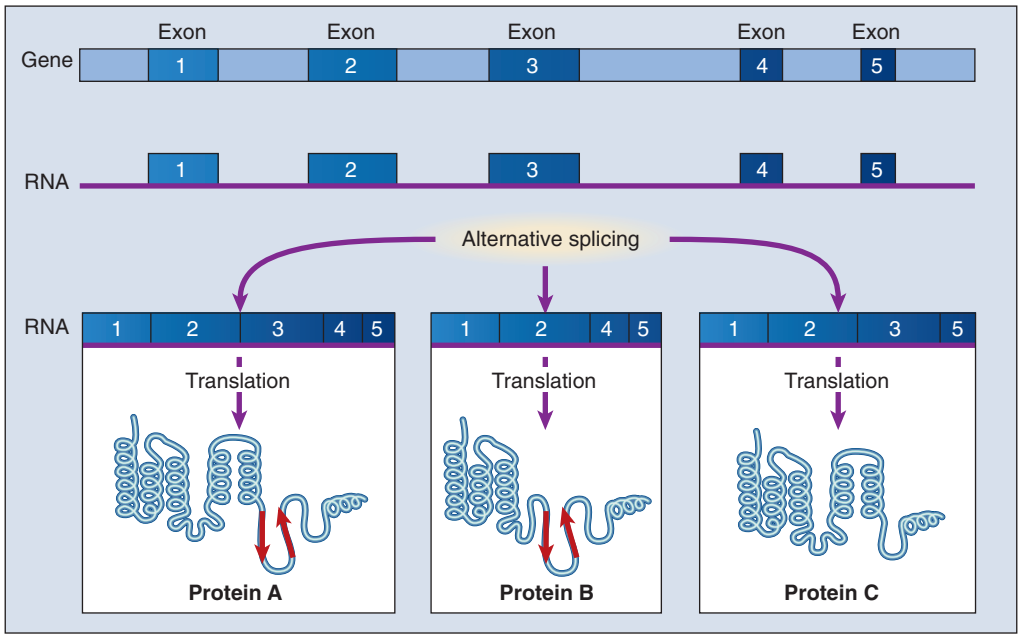

Figure 1.2 • RNA splicing. Alternate splicing produces multiple related proteins, or isoforms, from a single gene. (From Guttmacher AE, Collins F. Genomic medicine—a primer. *N Engl J Med.* 2002;347:1512–1520.)

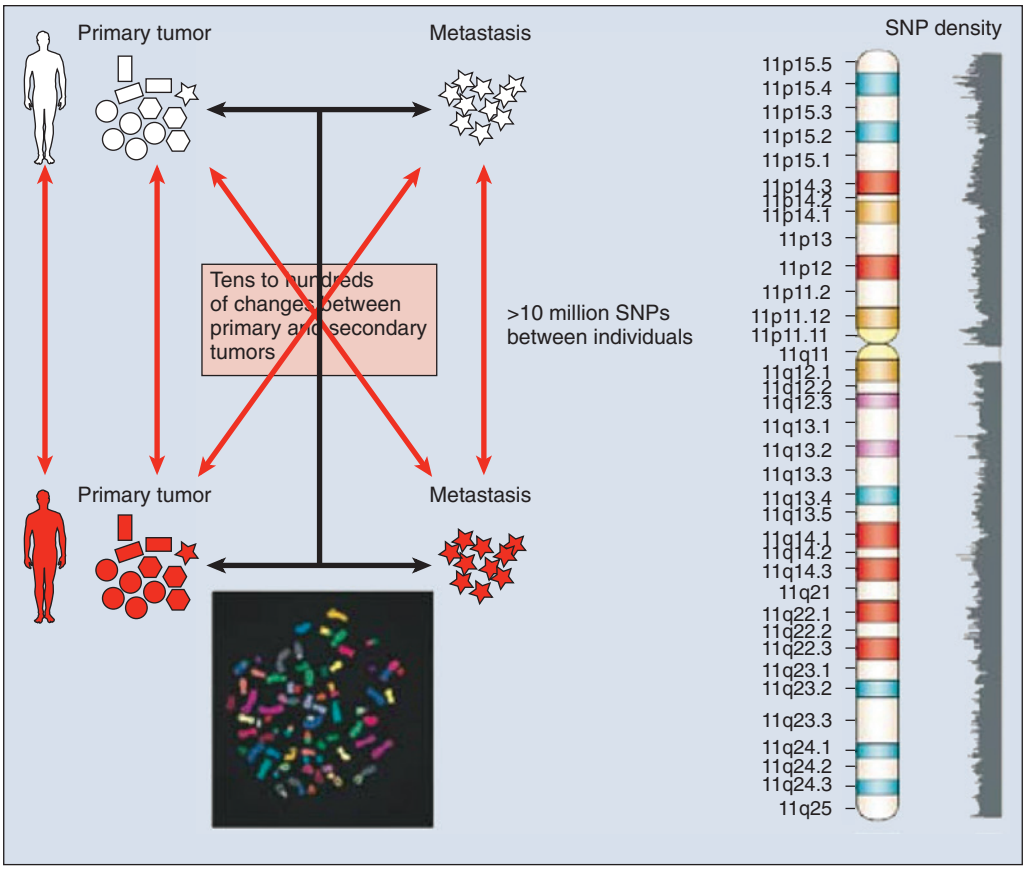

Figure 1.3 • Determining cancer susceptibility with single-nucleotide polymorphisms *(SNPs).* Millions of SNPs exist between individuals, as depicted by the red arrows and the SNP density map of human chromosome 11 *(right).* By contrast, point mutations, deletions, insertions, and rearrangements between normal tissues and tumors or between primary and secondary tumors probably number in the tens to hundreds (or potentially thousands), as depicted by the spectral karyotype image at the bottom of the figure. Because the constitutional genetic polymorphisms are present in all the tissues of the body, it might be possible to distinguish differences in metastatic versus nonmetastatic tumors and in nontumor tissues before they ever happen to develop a solid tumor. (From Hunter K. Host genetics influence tumour metastasis. *Nat Rev Cancer.* 2006;6:141–146.)

primer pairs, or amplicons, initiate synthesis of new DNA strands, using the chromosomal material as a template. Each successive copying cycle, initiated by "melting" the resulting double-stranded products with heat, doubles the number of DNA segments in the reaction (Fig. 1.4). The technique is exceptionally sensitive; millions of identical DNA copies can be generated in a matter of hours with PCR by using a single DNA molecule as the starting material.

Other novel methods for large-scale SNP detection include single-nucleotide primer extension, allele-specific hybridization, oligonucleotide ligation assay, and invasive signal amplification, which detect polymorphisms directly from genomic DNA without the requirement of PCR amplification. The International HapMap Project was established with the objective of identifying those variations (commonly thought to be on the order of 10 million in our genome) in the human population. This project is already in its third phase (HapMap3), now including both SNPs and copy number variations observed in 1184 samples from 11 different human populations. Regardless of the method used to characterize them, the collective SNPs in a selected genomic region characterize a haplotype, or specific combination of alleles at multiple linked genetic loci along a chromosome that are inherited together.

Even when the SNPs within a given haplotype are not directly involved in a disease, they provide markers for clonality and for the loss or rearrangement of specific chromosomal segments in growing tumors. In the human nucleus, each of the 23 tightly compacted chromosomes has a characteristic size and structure, and a distinctive base sequence that carries unique protein coding information. Other noncoding DNA sequences are used for directing the transcription of neighboring genes, through complex regulatory circuits involving protein binding and modification of the DNA itself, or shifting of its chromosomal packaging. Although genomic instability is generally considered a consequence of tumor formation rather than the initial trigger of cancer, the loss, gain, or rearrangement of chromosomal segments through deletion or translocation is a common form of neoplastic mutation, as protein coding segments from different genes are combined or regulatory sequences are brought into new proximity to genes they do not normally control, as seen in chronic myeloid leukemia (CML). In CML, recombination events lead to the fusion of *BCR* and *ABL* genes (Philadelphia chromosome). This results in constitutive activation of the fused gene, leading to loss of proliferative control in myeloid cells and consequently cancer. Gross changes in DNA arrangement can be detected by cytogenetic analysis of chromosomal features on metaphase spreads. Although fluorescence in situ hybridization (FISH) provides greater resolution by localizing specific chromosomal DNA sequences corresponding to fluorescently labeled probes (Fig. 1.5), and can be used to track specific alterations in chromosomal structure where known genes are involved, spectral karyotyping (SKY) is a powerful and more general tool that could aid diagnosis of cancer genomes. With each fluorescently labeled chromosome assigned a specific color, translocations and additions are revealed as multicolored chromosomes, or large deletions as pieces of missing chromosomes.

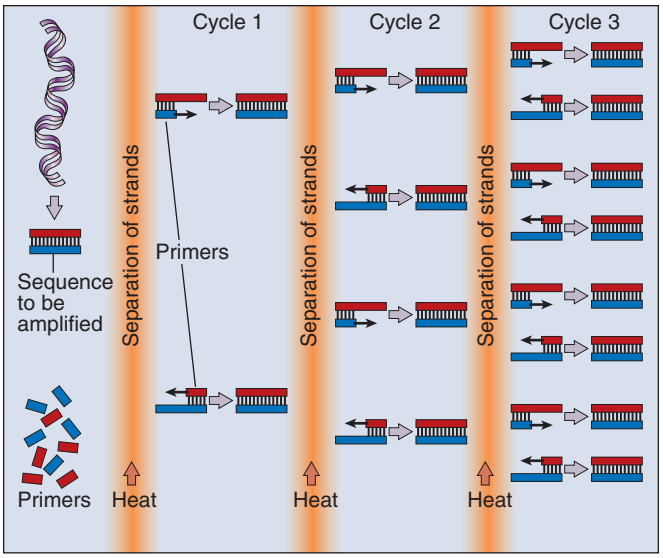

Figure 1.4 • Amplification of DNA by polymerase chain reaction (PCR). The DNA sequence to be amplified is selected by primers, which are short, synthetic oligonucleotides that correspond to sequences flanking the DNA to be amplified. After an excess of primers is added to the DNA, together with a heat-stable DNA polymerase, the strands of both the genomic DNA and the primers are separated by heating and allowed to cool. A heat-stable polymerase elongates the primers on either strand, thus generating two new, identical double-stranded DNA molecules and doubling the number of DNA fragments. Each cycle takes just a few minutes and doubles the number of copies of the original DNA fragment.

The plethora of data arising from genome-wide association studies using currently available techniques poses particular challenges to cancer researchers. Discerning the causal genetic variants among genotype-phenotype associations requires extensive replication, control for underlying genetic differences in population cohorts, and consistent classification of clinical outcomes. New technologies must be met with equivalently sophisticated and rigorous analytic methodologies for the true genetic cause of cancer to be teased out from our variable and often unstable heredity.

BUILDING GENE LIBRARIES

The engineering of genes by recombinant DNA technology evolved from methods initially devised to provide sequences in amounts sufficient for biochemical analysis. The original protocol involves clipping the desired segment from the surrounding DNA and inserting it into a bacterial or viral vector, which is then amplified millions of times in a host bacterium. Using recombinant DNA technology, genetic engineering can routinely produce industrial quantities of pure, clinically useful products in a cost-effective way. For diagnostic purposes, it is easier and faster to amplify a known genomic DNA sequence directly from a patient sample with PCR, but the classic approach is still applied to the construction of recombinant DNA libraries.

To be useful, a DNA library must be as complete as possible, with recombinant members, or clones, sufficiently numerous to include all the sequences in an individual genome. For certain kinds of gene-linkage analysis that require long, uninterrupted stretches of DNA, special vectors, such as bacterial or yeast artificial chromosomes, can carry foreign DNA fragments of enormous lengths. Chromosomal segments represented in genomic DNA libraries can contain the

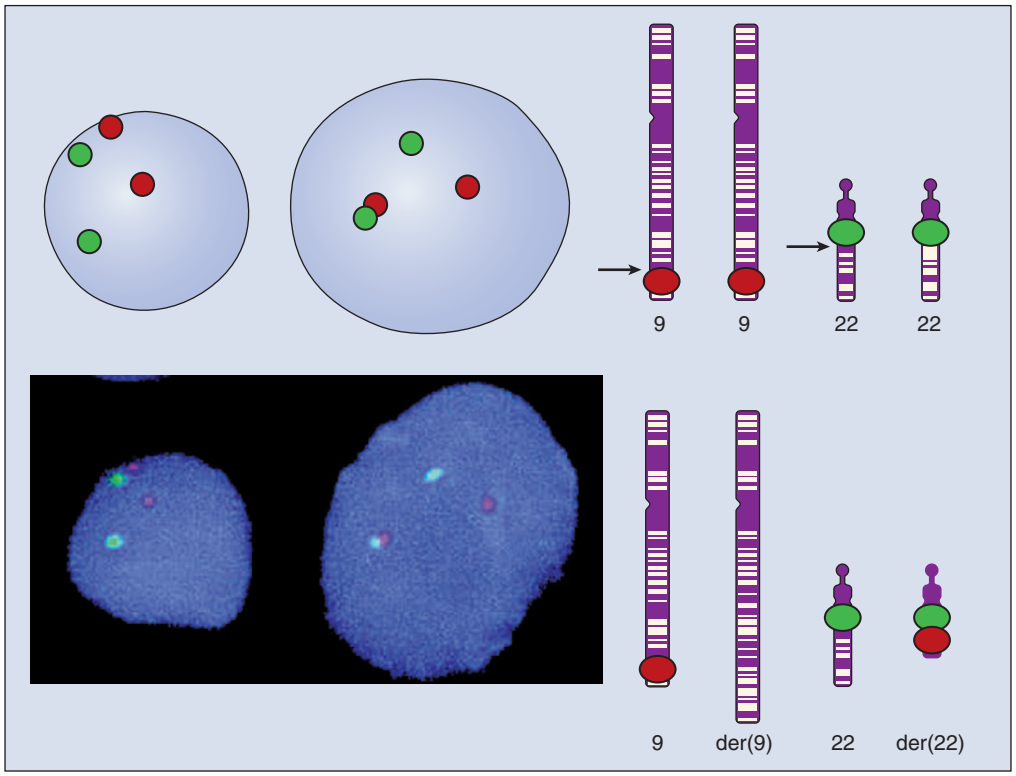

Figure 1.5 • Detection of chromosomal translocations. Fluorescence in situ hybridization (FISH) technology uses a labeled DNA segment as a probe to search homologous sequences in interphase chromosomes for the t(9;22)(q34;q11) translocation, associated with chronic myeloid leukemia. On the left, patient nuclei were hybridized with probes for chromosome 9 (labeled with SpectrumRed fluorophore) and chromosome 22 (labeled with SpectrumGreen). (Modified from Varella-Garcia M. Molecular cytogenetics in solid tumors: laboratorial tool for diagnosis, prognosis, and therapy. *Oncologist.* 2003;8:45–58.)

structure of an entire gene, including the information that regulates its expression, and formed the starting material for sequencing of the human genome.

Many cancer-associated genes were originally identified through use of partial DNA libraries, which contain only the DNA sequences transcribed by a particular tissue or type of cell. The starting material in this case is mRNA. For cloning purposes, the enzyme reverse transcriptase can convert mRNA into complementary DNA (cDNA). The number of clones in a cDNA library is much smaller than in a genomic library because a cDNA library represents only the genes expressed by the tissue of interest and contains exclusively the coding portion of genes. For this particular reason, this technique has now become obsolete for organisms whose genome has now been fully sequenced. New advances in PCR chemistry allowed for the direct cloning of increasingly larger cDNA fragments with high specificity and low error rates. Highly accurate PCR technology, coupled with the constantly evolving generation of genomic sequence maps in humans and model organisms, has exponentially expanded the availability of candidate genes to be tested in cancer biology.

LOSING CONTROL OF THE GENOME

Mutations that lead to oncogenic transformation of a cell invariably affect the expression of its genetic information that specifies functional products, either RNA molecules or proteins used for various cellular functions. The primary level of gene control is the transcription of DNA into RNA. Gene regulation, or the control of RNA synthesis, represents a complex process that is itself a frequent target of neoplastic mutation.

DNA regulatory sequences do not encode a product. However, without them a cell could not coordinate the expression of the hundreds of thousands of genes in its nucleus, select only certain genes for expression, and activate or repress them in response to precise internal or external signals. These control centers of the genome contain binding sites for multiple proteins, called transcription factors, which interact to form regulatory networks controlling gene transcription. Their function can be altered by signals that induce modifications such as phosphorylation, or by interactions with other regulators such as steroid hormones. Many of the cell's responses to a wide variety of external stimuli, such as neurotransmitters, antigens, cytokines, and growth factors, are mediated through transcription factors binding to DNA regulatory sequences.

Certain regulatory DNA sequences common to many genes are positioned upstream of the transcription start site (Fig. 1.6). Collectively called the "promoter" of a gene, these proximal sequences comprise binding sites for the RNA polymerase and its numerous cofactors. Whereas the position of the promoter with regard to the transcription start site is relatively inflexible, other DNA regulatory elements, known as enhancers, occur in unpredictable locations, often at a considerable distance from the genes they control. Some transcription factors bind to particular regions of enhancers and drive their associated genes in many types of cells, whereas others, active in only a limited variety of cells, maintain a tissue-specific pattern of gene expression. Enhancers are often responsible for the aberrant expression of genes induced by chromosomal translocation associated with specific forms of cancer: a normally quiescent gene promoting cell growth that is dislocated to a position near a strong enhancer may be activated inappropriately, resulting in loss of growth control.

Enhancers and promoters have been assigned specific roles by means of cell culture assays or in transgenic animals in which putative regulatory DNA sequences are linked to test or "reporter" genes, and are examined for their ability to activate expression of the reporter gene in response to the appropriate signals. Through assessment of the effects of deleting, adding, or changing DNA sequences within the regulatory element, the precise nucleotides that are critical for recognition by transcription factors can be determined.

The interaction between protein and DNA is increasingly used to identify transcription factor binding sites in a regulatory region. Whereas electrophoretic mobility shift assays (EMSAs), or DNA footprinting, were once standard techniques for determining protein-DNA interactions, emerging genome-wide technologies, such as chromatin immunoprecipitation on microarray chip (ChIP-chip) and chromatin immunoprecipitation on sequencing (ChIP-seq), are revolutionizing the way in which we see the interaction of a transcription factor complex with virtually all of its potential genomic targets in a particular cell state. These strategies involve the use of candidate protein–specific antibodies to pull down DNA targets regulated by them. These targets are further identified with the use of microarray ChIP-chip or next generation sequencing ChIP-seq technologies (see Fig. 1.14).

Our appreciation of oncogenic perturbations, by mutation of regulatory protein coding genes or by loss of controlled signaling by cell cycle switches or in the target sequences these proteins recognize, has recently extended to include posttranslational modifications that control protein activity, such as phosphorylation, ubiquitylation, and SUMOylation. Tumor-associated changes in these modifications underscore the multiple levels of control necessary to ensure correct gene expression that is so central to the normal function of the cell.

EPIGENETICS AND CANCER

Epigenetics refers to general control of gene expression that is inherited during cell division, although not part of the DNA sequence itself. Epigenetic regulation involves changes in chromatin, a higher-order building block of chromosomes that wraps DNA into coils with scaffolding proteins such as histones. Histones are a necessary component of chromosomal compaction, but also play a critical role in gene accessibility (Fig. 1.7). Active genetic loci are associated with loosely configured euchromatin, whereas silent loci are condensed in heterochromatin. The state of chromatin configuration (euchromatin or heterochromatin) both controls and is controlled by patterns of histone modifications such as methylation and acetylation on specific DNA sequences. This pattern relates the underlying genetic information to its higher-order structure that determines whether a particular gene regulatory element is available to transcription factors (on or off status). These epigenetic modifications of the nuclear environment that determine the accessibility of a gene can persist during cell division, because inherited epigenetic patterns provide permanent marks for altered chromatin configuration in daughter cells. The pattern of modifications generated by the epigenetic code rivals the complexity of the DNA code itself.

The accessibility of genomic regions can favor mutations. Enzymes such as the APOBEC family exploit this accessibility to induce C to U mutation, which is then converted to T or staggered single-strand breaks. If not rectified, these point mutations or breaks can lead to hypermutations. Kataegis is an example wherein such hypermutation occurs on the BRCA locus, generating neoplasia.

Research has linked rearrangement of chromatin and associated DNA methylation with the inactivation of tumor suppressor genes and neoplastic transformation. Defects that could lead to cancer involve perturbations in the "epigenotype" of a particular locus, through the silencing of normally active genes or activation of normally silent genes, associated with changes in DNA methylation, histone modification, and chromatin proteins (Fig. 1.8). Changes in the number or density of heterochromatin proteins associated with cancer-related genes such as *EZH2,* or of euchromatic proteins such as trithorax in leukemia, can also be associated with abnormal patterns of methylation in gene promoter regions and with higher-order chromosomal structures that are only beginning to be understood. Finally, it is increasingly evident that interactions among the "epigenome," the genome, and the environment are common targets for mutation and can have profound effects on the gene expression readout of a cancer cell.

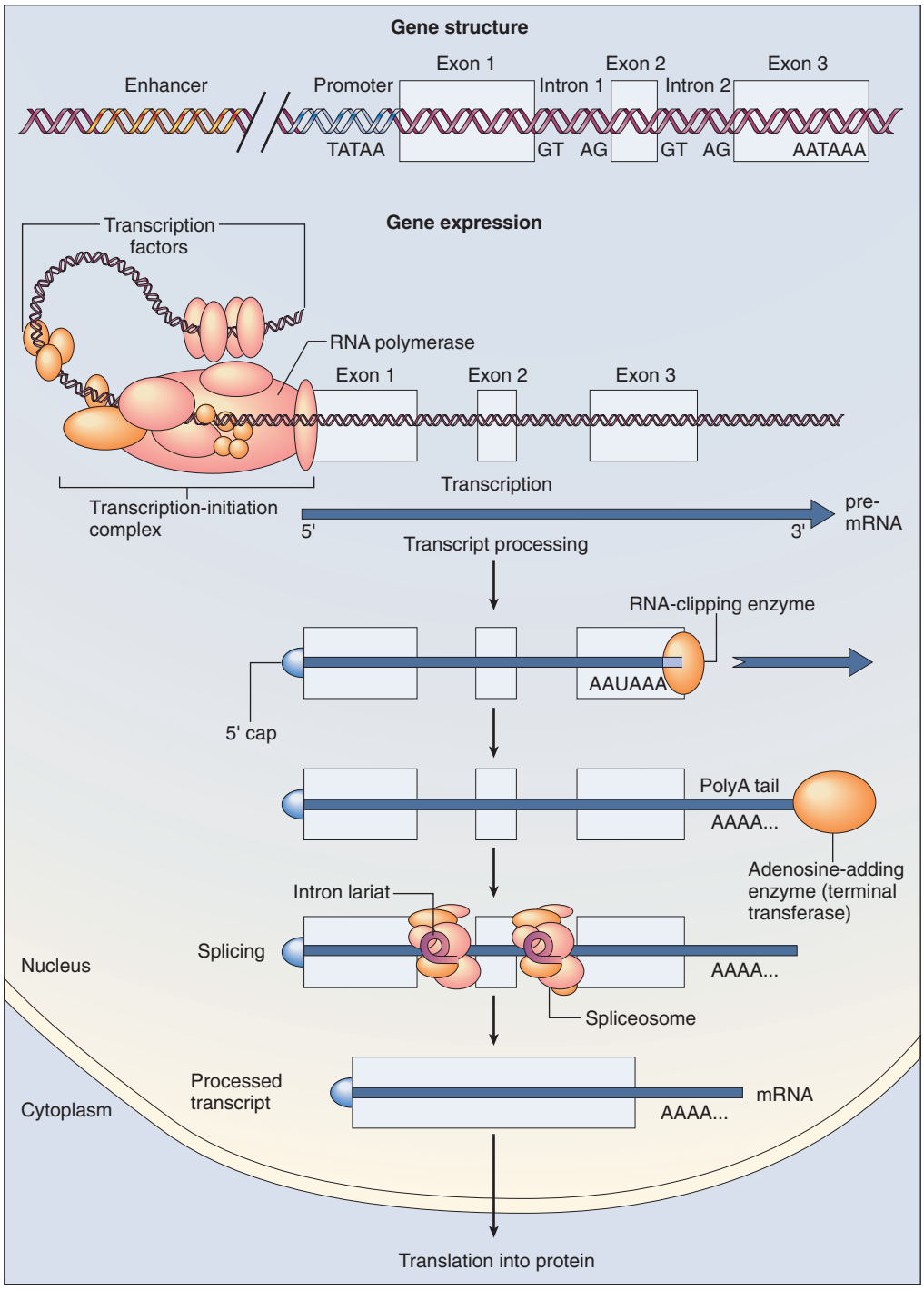

Figure 1.6 • Mammalian gene structure and expression. The DNA sequences that are transcribed as RNA are collectively called the *gene* and include exons (expressed sequences) and introns (intervening sequences). Introns invariably begin with the nucleotide sequence GT and end with AG. An AT-rich sequence in the last exon forms a signal for processing the end of the RNA transcript. Regulatory sequences that make up the promoter and include the TATA box occur close to the site where transcription starts. Enhancer sequences are located at variable distances from the gene. Gene expression begins with the binding of multiple protein factors to enhancer sequences and promoter sequences. These factors help form the transcription-initiation complex, which includes the enzyme RNA polymerase and multiple polymerase-associated proteins. The primary transcript (pre-mRNA) includes both exon and intron sequences. Post-transcriptional processing begins with changes at both ends of the RNA transcript. At the 5′ end, enzymes add a special nucleotide cap; at the 3′ end, an enzyme clips the pre-mRNA about 30 base pairs (bp) after the AAUAAA sequence in the last exon. Another enzyme adds a polyA tail, which consists of up to 200 adenine nucleotides. Next, spliceosomes remove the introns by cutting the RNA at the boundaries between exons and introns. The process of excision forms lariats of the intron sequences. The spliced mRNA is now mature and can leave the nucleus for protein translation in the cytoplasm. (From Rosenthal N. Regulation of gene expression. *N Engl J Med.* 1994;331:931–932.)

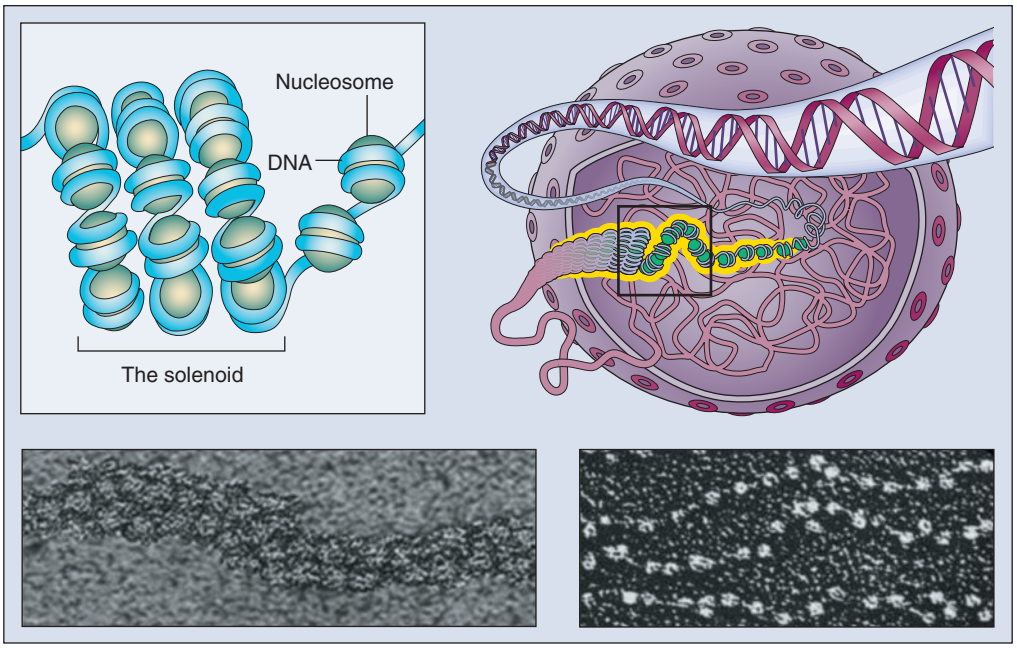

Figure 1.7 • Chromatin packaging of DNA. The 4 meters of DNA in every human cell must be compressed in the nucleus, reaching compaction ratios of 1 : 400,000. This is achieved by wrapping the DNA *(blue)* around histone protein complexes *(green),* forming nucleosomes connected by a thread of free linker DNA. Each nucleosome, together with its linker, packages about 200 bp (66 nm) of DNA. The nucleosomes are then coiled into chromatin, a rope of nucleoprotein about 30 nm thick *(bottom left electron micrograph).* To allow DNA to be accessed by transcription and replication apparatus, chromatin is relaxed *(bottom right electron micrograph).* (Courtesy Jakob Waterborg. www.umkc.edu/sbs/waterborg/chromat/chromatn.html. Copyright 1998 Jakob Waterborg.)

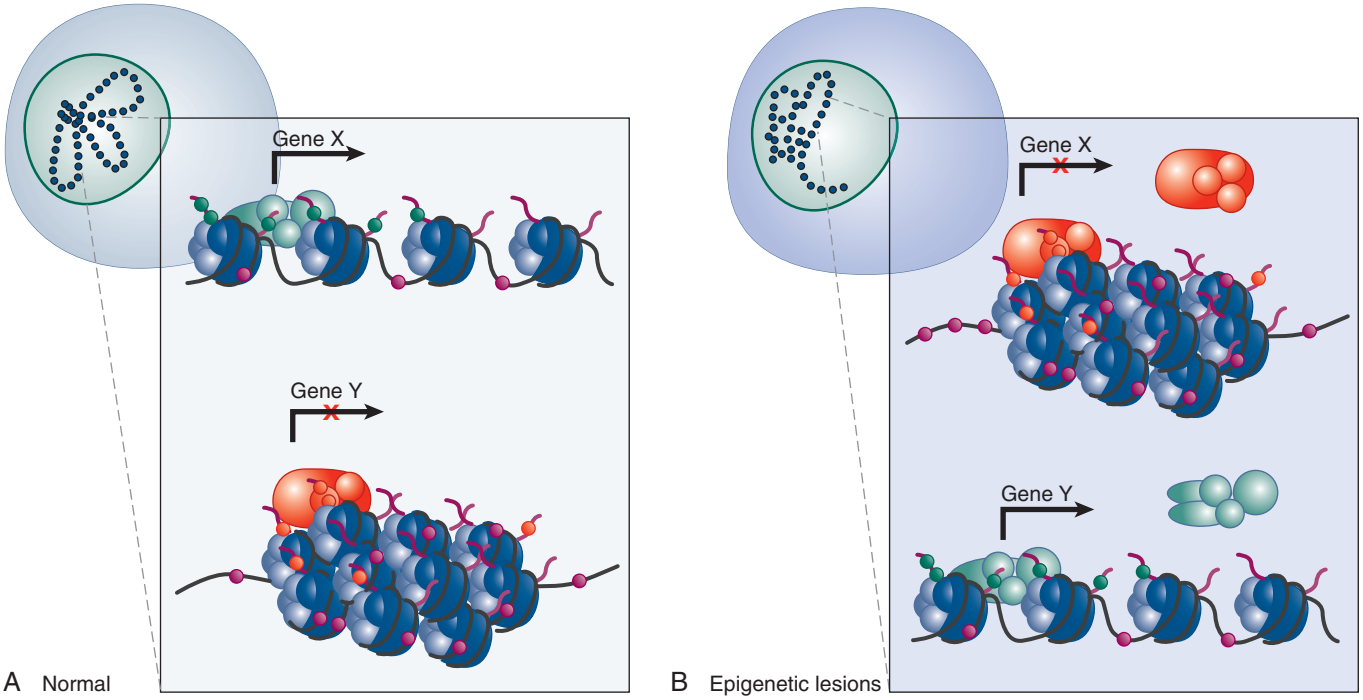

Figure 1.8 • Gene accessibility through epigenetics. Illustration depicts known and possible defects in the epigenome that could lead to disease. (A) Gene *X* is a transcriptionally active gene with sparse DNA methylation *(brown circles),* an open chromatin structure, interaction with euchromatin proteins *(green protein complex),* and histone modifications such as H3K9 acetylation and H3K4 methylation *(green circles).* Gene *Y* is a transcriptionally silent gene with dense DNA methylation, a closed chromatin structure, interaction with heterochromatin proteins *(red protein complex),* and histone modifications such as H3K27 methylation *(pink circles).* (B) The abnormal cell could switch its epigenotype through the silencing of normally active genes or activation of normally silent genes, with the attendant changes in DNA methylation, histone modification, and chromatin proteins. In addition, the epigenetic lesion could include a change in the number or density of heterochromatin proteins in gene *X* (such as EZH2 in cancer) or euchromatic proteins in gene *Y* (such as trithorax in leukemia). There may also be an abnormally dense pattern of methylation in gene promoters (shown in gene *X*), and an overall reduction in DNA methylation (shown in gene *Y*) in cancer. The insets show that the higher-order loop configuration may be altered, although such structures are currently only beginning to be understood.

PROFILING TUMORS

Monitoring global gene expression patterns of cells represents one of the latest breakthroughs in developing a molecular taxonomy of cancer. Although classic blotting and probe hybridization techniques (Northern blot) are still a reliable way to monitor expression of individual genes, these techniques have limitations, such as unequal hybridization efficiency of individual probes, sensitivity for low copy or small transcripts, and difficulty in detecting multiple RNAs simultaneously or in simultaneously analyzing a large number of targets. For cancer studies, it is important to be able to compare the expression pattern of all known RNAs, including noncoding RNAs, between cancer cells and normal cells. Thus new genome-wide analytic techniques are the state-of-the-art choice to detect mRNA expression profiles at a single point in time or cell state. Genome-wide profiling of gene expression in tumors delivers an unprecedented view into the biologic processes underlying tumor progression by following the changes in a tumor cell's transcriptional landscape.

With reliance on two-color fluorescence-based microarray technology (DNA microarray), simultaneous evaluation of thousands of gene transcripts and their relative expression can provide a snapshot of the "transcriptome," the full complement of RNA transcripts produced at a specific time during the progression of malignancy.

Transcriptional profiling with microarrays typically involves screens of mRNA expression from two sources (such as tumor and normal cells), using cDNA or oligonucleotide libraries that are arranged in extremely high density on microchips. These are probed with a mixture of fluorescently tagged cDNAs generated from the tumor and normal samples, which results in differential staining of each gene spot. The relative intensity of the two different colors reflects the RNA expression level of each gene; this is analyzed with a laser confocal scanner (Fig. 1.9). With microarrays, single genes that constitute diagnostic,

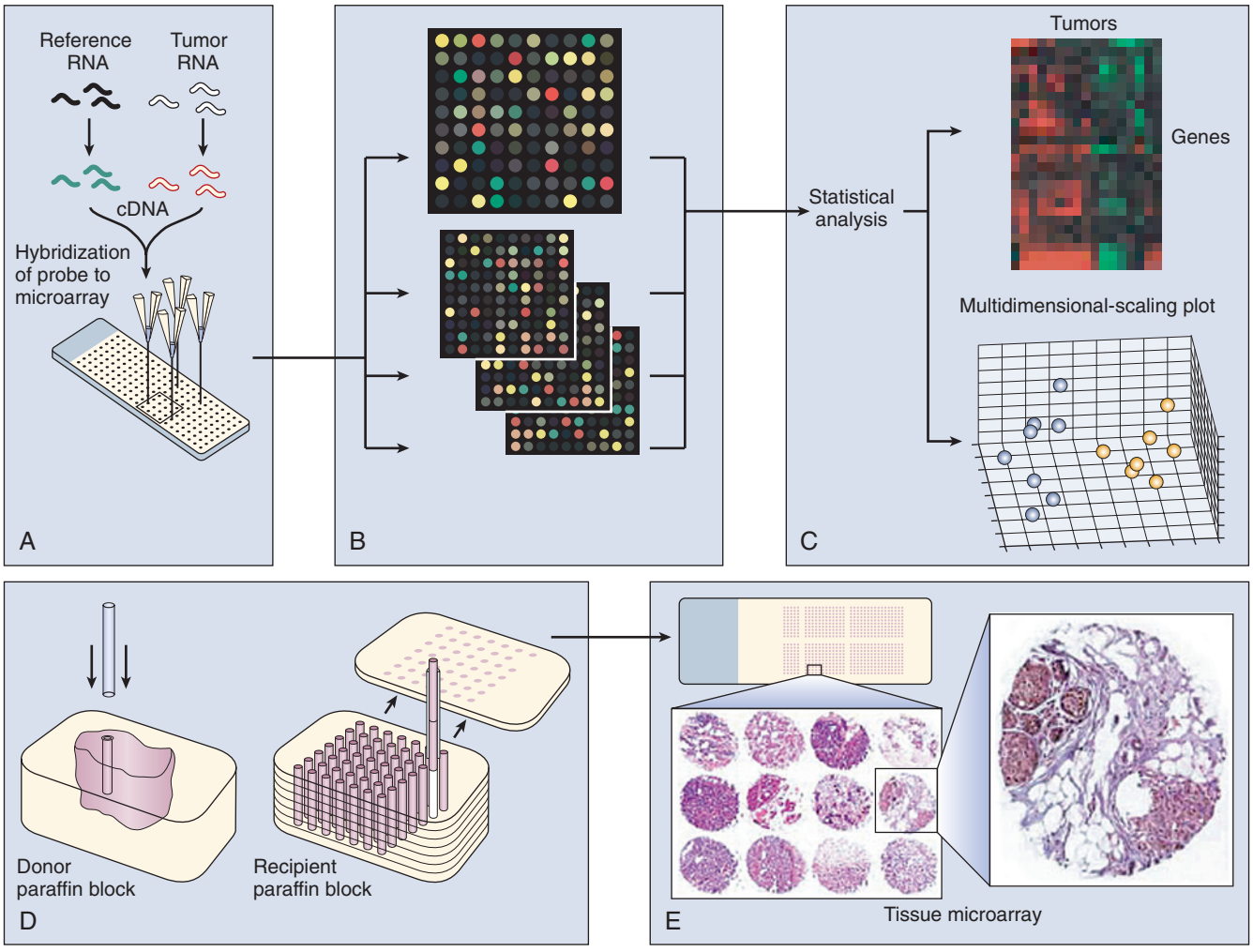

Figure 1.9 • Microarray-based expression profiling of tumor tissue. (A) Reference RNA and tumor RNA are labeled by reverse transcription with different fluorescent dyes (*green* for the reference cells and *red* for the tumor cells) and hybridized to a cDNA microarray containing robotically printed cDNA clones. (B) The slides are scanned with a confocal laser scanning microscope, and color images are generated with RNA from the tumor and reference cells for each hybridization. Genes upregulated in the tumors appear red, whereas those with decreased expression appear green. Genes with similar levels of expression in the two samples appear yellow. Genes of interest are selected on the basis of the differences in the level of expression by known tumor classes (e.g., BRCA1-mutation–positive and BRCA2-mutation–positive). Statistical analysis determines whether these differences in the gene expression profiles are greater than would be expected to occur by chance. (C) The differences in the patterns of gene expression between tumor classes can be portrayed in the form of a color-coded plot, and the relations between tumors can be portrayed in the form of a multidimensional-scaling plot. Tumors with similar gene-expression profiles cluster close to one another in the multidimensional-scaling plot. (D) Particular genes of interest can be further studied through the use of a large number of arrayed, paraffin-embedded tumor specimens, referred to as tissue microarrays. (E) Immunohistochemical analyses of hundreds or thousands of these arrayed biopsy specimens can be performed in order to extend the microarray findings. (From Hedenfalk I, Duggan D, Chen Y, et al. Gene expression profiles in hereditary breast cancer. *N Engl J Med.* 2001;344:539–548.)

prognostic, or therapeutically relevant markers can be systematically monitored. Alternatively, the entire set of expressed genes can be collectively analyzed through use of powerful statistical methods to classify tumors according to their transcriptional profile. Microarray analysis has already dramatically improved our ability to explore the genetic changes associated with cancer etiology and development and is providing new tools for disease diagnosis and prognostic assessment. For example, DNA microarray analysis of multiple primary breast tumor transcriptomes has revealed reproducible signature expression of 70 associated genes. These markers have been recently cleared by the US Food and Drug Administration (FDA) for PCR–based diagnostics showing that expression analysis of a relative small gene group can predict the prognosis of early stage breast cancers. When applied on a larger scale, these assays can predict response to chemotherapy, or optimize pharmaceutical intervention by targeting therapeutic approaches to specific patient populations and ultimately to individualized therapy.

A novel high-throughput approach for global transcriptome analysis has been made possible by advances in strategies that allow mass sequencing of DNA fragments. With this technique, called RNA-seq, it is now possible to obtain a comprehensive and unbiased analysis of all mRNA transcripts present in cells or tissues. (Fig. 1.10). The technique relies on the generation of small fragments of cDNA from any RNA sample, followed by sequencing of these expressed tags from one end (single-end sequencing) or both ends (pair-end sequencing), resulting in fragments of 30 to 400 base pairs (bp). The resulting sequences can be then mapped against the known reference genome or transcriptome of a certain species. Unlike microarray analysis of preselected gene sets, RNA-seq allows the unbiased identification of all genes, or even the presence of different isoforms, expressed in the sample, allowing a comprehensive comparison of transcript levels between normal and cancer cells.

The technologies just described can also be applied to the analysis of noncoding RNA species. In addition to the 20,000 protein coding transcripts used to classify a wide variety of human tumors, hundreds if not thousands of small, noncoding interference RNA species, with critical functions in multiple biologic processes, have been discovered; many of these RNA species are directly or indirectly involved in the control of cell proliferation. Known as microRNAs (miRNAs), these short transcripts arise from primary genome-encoded transcripts of variable sizes that are processed into 70- to 100-nucleotide hairpin-shaped precursors, which are processed into mature miRNAs of 21 to 23 bp RNA molecules (Fig. 1.11). miRNAs function by base-pairing

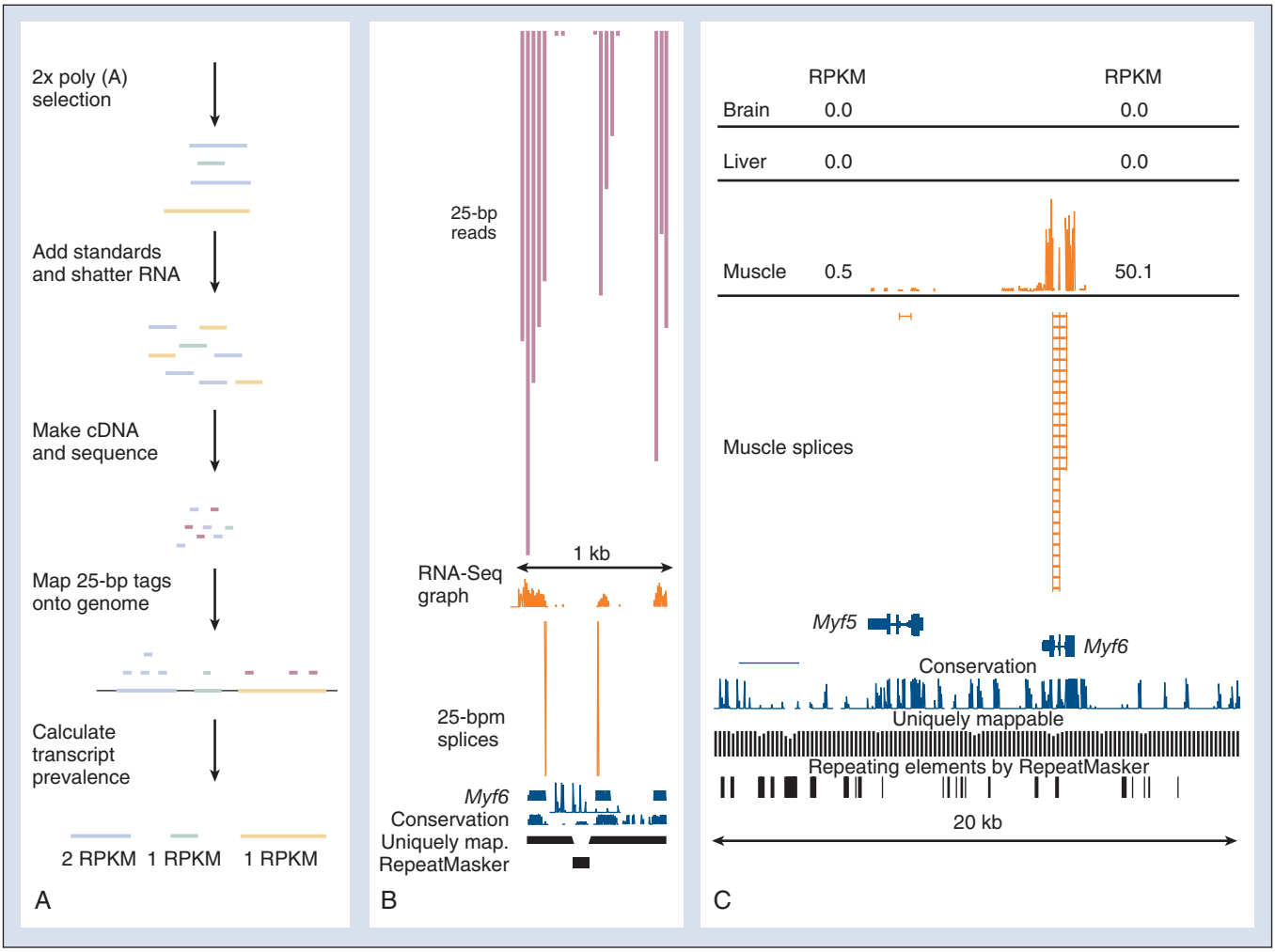

Figure 1.10 • Methods for high-throughput transcriptome analyses. (A) Schematics of regular protocol for RNA-seq sample preparation, showing poly-A tail specific mRNA isolation followed by fragmentation of RNA into smaller regions, further used for cDNA conversion. Polymerase chain reaction (PCR) fragments are then tethered by adaptors, sequenced by synthesis, and aligned to the reference genome or transcriptome to calculate relative prevalence of mRNAs (RPKM). (B) Target fragments can be used to map exon-intron boundaries and thus infer present and quantify different mRNA isoforms in the sample of interest, as shown for the muscle specific gene *Myf6* in this example. (C) Data generated with this method can also be compared with analysis of other tissues or samples, allowing assessment of relative quantification of targets, as exemplified here for a highly specific gene *(red peaks)* for muscle samples. (From Mortazavi A, Williams BA, et al. Mapping and quantifying mammalian transcriptomes by RNA-seq. *Nat Methods.* 2008;5:621–628.)

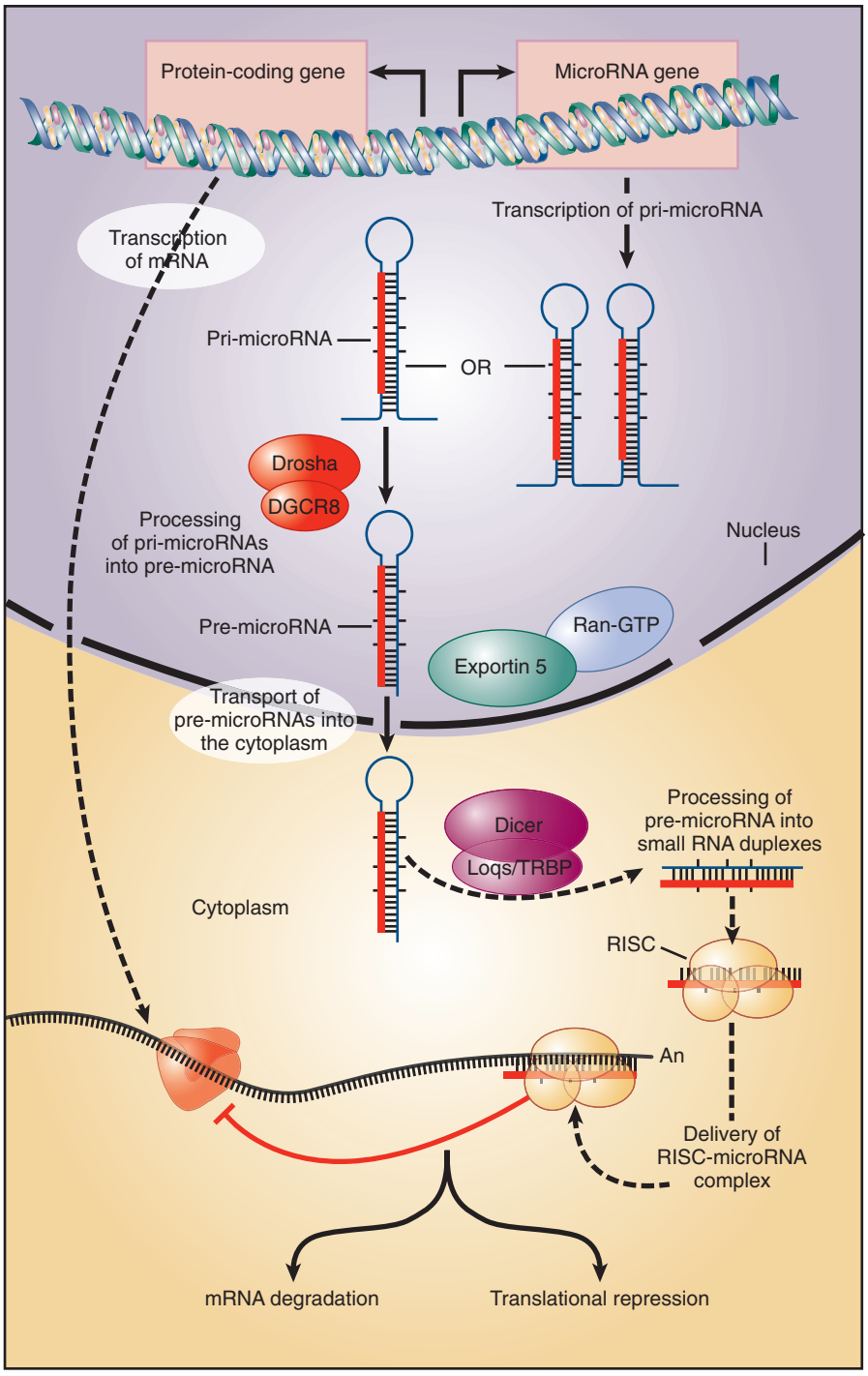

Figure 1.11 • MicroRNA production and gene regulation in animal cells. Mature functional microRNAs of approximately 22 nucleotides are generated from long primary microRNA *(pri-microRNA)* transcripts. First, the pri-microRNAs, which usually contain a few hundred to a few thousand base pairs, are processed in the nucleus into stem-loop precursors *(pre-microRNA)* of approximately 70 nucleotides by the RNase III endonuclease Drosha and DiGeorge syndrome critical region gene 8 *(DGCR8)*. The pre-microRNAs are then actively transported into the cytoplasm by exportin 5 and Ran-GTP and further processed into small RNA duplexes of approximately 22 nucleotides by the Dicer RNase III enzyme and its partner Loqacious *(Loqs)*, a homologue of the human immunodeficiency virus transactivating response RNA-binding protein. The functional strand of the microRNA duplex is then loaded into the RNA-induced silencing complex *(RISC)*. Finally, the microRNA guides the RISC to the target messenger RNA *(mRNA)* target for translational repression or degradation of mRNA. (Modified from Chen CZ. *New Eng J Med*. 2005;353:1768–71.)

with target mRNAs to inhibit translation and/or promote mRNA degradation. In the context of cancer, miRNAs may act in concert with other effectors such as p53 to inhibit inappropriate cell proliferation. A global decrease in miRNA levels is often observed in human cancers, indicating that small RNAs may have an intrinsic function in tumor suppression. The usefulness of monitoring the expression of miRNAs in human cancer is just now being explored, but preliminary findings reveal an extraordinary level of diversity in miRNA expression across cancers, and the large amount of diagnostic information encoded in a relatively small number of miRNAs. Significant technologic

advances facilitating the profiling of the miRNA expression patterns in normal and cancer tissues hint at the unexpected greater reliability of miRNA expression signatures than the respective signatures of protein coding genes in classifying cancer types. Along with their potential diagnostic value, miRNAs are also being tested for their prognostic use in predicting clinical behaviors of cancer patients.

Because probe specificity in miRNA microarray analysis is problematic owing to the small target size, hybridization can be performed first in solution, and then quantified with multicolor flow sorting. Real-time PCR can also be used to quantify specific miRNA sets, or to capture a more detailed picture of their changing expression profiles in tumor progression. Identification of the miRNAs involved in tumor pathogenesis and elucidation of their action in a specific cancer will be the next necessary steps for their manipulation in a therapeutic setting.

Advances in this field have revealed that miRNAs are also involved in cancer initiation and progression, and specific modulation of such RNAs may serve as a therapeutic strategy. Inhibition of key miRNAs using antagomirs (a class of chemically modified anti-miRNA oligonucleotides) has been effective in suppressing tumor growth in mouse models. It remains to be seen if these results can be extended to treatment of cancer in the clinic, but interference with miRNA function is an attractive new tool for the development of cancer therapies.

CANCER PROTEOME

The term *proteome* describes the entire complement of proteins expressed by the genome of a cell, tissue, or organism. More specifically, it is used to describe the set of all the expressed proteins at a given time point in a defined setting, such as a tumor. Like RNA transcription, the synthesis of proteins is a highly regulated process that contributes to the specific proteome of a particular cell and can be perturbed in diseases such as cancer.

Advances in protein analytic techniques over the last decades have progressed to the point that even small numbers of specific proteins expressed in tissues can be used to predict the prognosis of a cancer. The improvement of protein-based assays has made it possible to identify and examine the expression of most proteins, and to envision large-scale protein analysis on the level of gene-based screens. Various systematic methodologies have contributed to the current explosion of information on the proteome. These are now being compared for their suitability as platforms for the generation of databases on protein structural features, interaction maps, activity profiles, and regulatory modifications.

The yeast two-hybrid system has been a popular genetics-based approach for detecting protein-protein interactions inside a cell (Fig. 1.12). One protein fused to the DNA binding domain (bait) and a

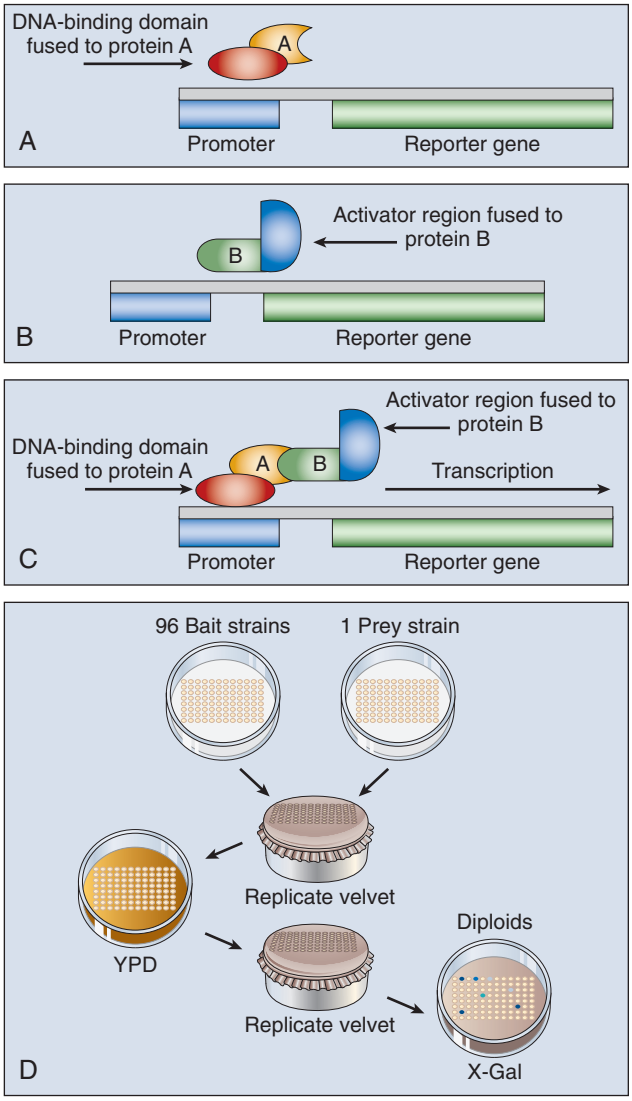

Figure 1.12 • Exploring protein-protein interactions with the yeast two-hybrid system. Two-hybrid technology exploits the fact that transcriptional activators are modular in nature. Two physically distinct functional domains are necessary to get transcription: a DNA-binding domain that binds to the DNA of the promoter and an activation domain that binds to the basal transcription apparatus and activates transcription. (A) The known gene encoding protein A is cloned into the "bait" vector, fused to the gene encoding a DNA-binding domain from some transcription factor. When placed into a yeast system with a reporter gene, this fusion protein can bind to the reporter gene promoter, but it cannot activate transcription. (B) Separately, a second gene (or a library of cDNA fragments encoding potential interactors), protein B, is cloned into the "prey" vector, fused to an activation domain of a different transcription factor. When placed into a yeast strain containing the reporter gene, it cannot activate transcription because it has no DNA-binding domain. (C) When the two vectors are placed into the same yeast, a transcription factor is formed that can activate the reporter gene if protein B, made by the second plasmid, binds to protein A. (D) Screening a yeast two-hybrid library. The plate on the left holds 96 different yeast strains in patches (or colonies), each of which expresses a different bait protein *(top)*. The plate on the right holds 96 patches, each of the same yeast strain (prey strain) that expresses a protein fused to an activation domain (prey). The plate of bait strains and the plate of prey strains are pressed to the same replica velvet, and the impression is lifted with a plate containing yeast extract peptone dextrose *(YPD)* medium. After 1 day of growth on the YPD plate, during which time the two strains mate to form diploids, the YPD plate is pressed to a new replica velvet, and the impression is lifted with a plate containing diploid selection medium and an indicator such as X-Gal. Blue patches *(dark spots)* on the X-Gal plate indicate that the lacZ reporter is transcribed, suggesting that the prey interacts with the bait at that location. (C from http://www.nature.com/…/journal/v403/n6770/full/403601a0_r.html. D from Bartel PL, Fields S, eds. *The Yeast Two-Hybrid System.* New York: Oxford University Press; 1997; Finley RL Jr, Brent R: Two-hybrid analysis of genetic regulatory networks. Retrieved from http://www.genetics.wayne.edu/finlab/YTHnetworks.html.)

different protein fused to the activation domain of a transcriptional activator (prey) are expressed together in yeast cells. If the bait and prey interact, transcription of a reported gene is induced and detected typically by a color reaction that reflects the transactivation of the reporter gene, and by proxy, the interaction of the two test proteins. The method can also be used for large-scale protein interactions, determination of RNA-protein interactions, and protein-ligand binding.

As a complementary proteomics tool, mass spectrometry (MS) is an accurate mass measurement of charged peptides isolated by two-dimensional gel electrophoresis, producing a mass-to-charge ratio of charged samples under vacuum that can be used to determine the sequence identity of peptides. Combined with a specific proteolytic cleavage step, mass spectroscopy can be used for peptide mass mapping. Automation of this process has made mass spectroscopy the analytic tool of choice for many proteomics projects. For diagnostic purposes, liquid chromatography and mass spectrometry (LC-MS/MS) have been combined to detect not only a single–amino acid change in the whole proteome, but also posttranslational protein modification such as phosphorylation, SUMOylation, or ubiquitination. These LC-MS/MS systems, such as the iTRAQ, allow for a more precise and individualized diagnosis of cancer.

Monoclonal antibodies (mAbs) have been a cornerstone of protein analysis in cancer research, and more recently have risen to prominence as cancer therapeutics based on their exquisite specificity for protein targets and their potent interference with protein function. Novel strategies have been developed that target not only antigens highly expressed in cancer cells but also to enhance the innate immune response against cancer cells. These antibodies can act via several mechanisms, including antibody-dependent cellular cytotoxicity (ADCC), complement-mediated cytotoxicity (CMC), and antibody-dependent cellular phagocytosis (ADCP) (Fig. 1.13). Laboratory mice have been the animal model of choice for generating a ready source of diverse, high-affinity and high-specificity mAbs; however, the use of rodent antibodies as therapeutic agents has been restricted by the inherent immunogenicity of mouse proteins in a human setting. The more recent application of transgenic mouse technology to introduce variable regions encoded by human sequences into the corresponding

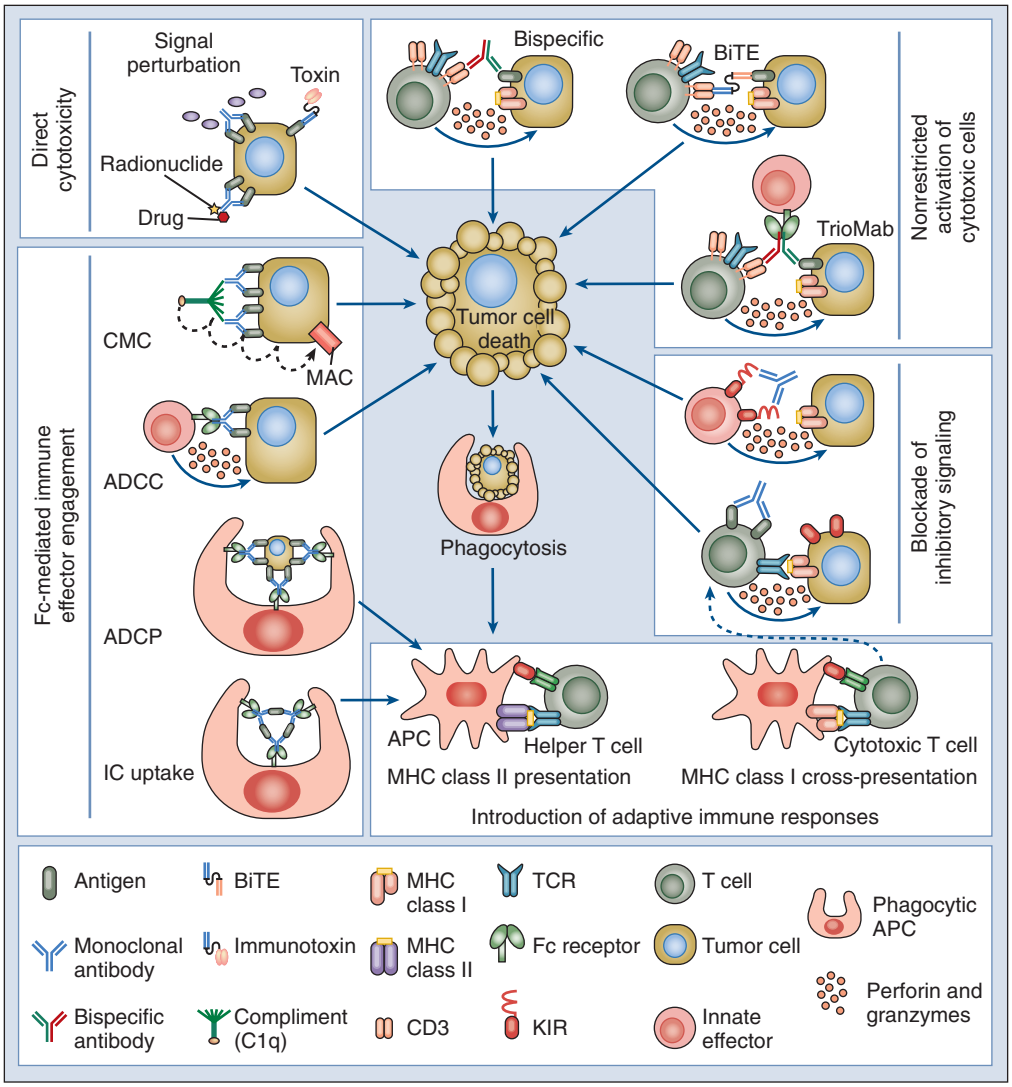

Figure 1.13 • Mechanisms for antibody-based therapies used against cancer cells. Multiple current approaches involve direct cytotoxicity, Fc-mediated immune effector engagement, nonrestricted activation of cytotoxic T cells, and blockade of inhibitory signaling. The diverse spectrum of action of these therapies will allow the inclusion of various anticancer targets in the near future (From Weiner LM, Murray JC, Shuptrine CW. Antibody-based immunotherapy of cancer. *Cell.* 2012;148:1081–1084.)

mouse immunoglobulin genes has enabled the generation of "humanized" therapeutic mAbs with reduced immunogenicity. In addition, bispecific antibodies (bsAbs) with dual affinity for tumor antigens, such as TriomAb, have been shown to effectively kill tumor cells by inducing memory T-cell protective immunity. In addition to the expected use of mAbs directed at extracellular epitopes (protein regions recognized by the antibody), evidence from mouse models has raised the possibility of using antibodies targeting intracellular epitopes for anticancer therapies. Targeting such antigens would enrich immunotherapy, allowing the use of tumor-specific intracellular mediators of cell survival and proliferation. Numerous mAb-based agents are currently in trial or in use as therapeutics for cancer, and the potential for further optimization of mAbs through genetic engineering promises to open new avenues for in vivo therapy.

A recent advancement in mAb-based cancer therapy is the generation of chimeric antigen receptor (CAR) T lymphocytes to target tumors in vivo. These are effector T-lymphocytes engineered to express a mAb that recognizes specific groups of cancer cells. The receptors are chimeric, composed of engineered molecules from diverse sources. The first generation of CAR-modified T cells (CAR T cells) showed success in preclinical trials and have entered phase I clinical trials in ovarian cancer, neuroblastoma, and various types of leukemia and lymphoma. Newer generations of these therapeutic lymphocytes are currently being developed that have increased specificity toward individualized cancers.

From an epigenetic perspective, new techniques are enabling the genome-wide characterization of protein-DNA interactions that can uncover novel transcription factor targets, histone modifications, and DNA methylation patterns within a cancer cell. Combining chromatin immunoprecipitation (ChIP) with microarray (ChIP-chip) allows genome-wide screening for the binding position of protein factors to their gene targets. In ChIP-chip assays or ChIP-seq, a cross-linking reagent is applied in vivo to proteins associated with DNA in the nucleus, which then can be coimmunoprecipitated with specific antibodies to the protein under analysis. The bound DNA and appropriate controls are then fluorescently labeled and applied to microscopic slides for microarray analysis, or directly sequenced, rendering a simultaneous profile of all the binding positions of specific proteins in the cancer cell's genome (Fig. 1.14). The global profiling of promoter occupancy of specific cancers, wherein protein-DNA interaction profiles discriminate patients with tumors from those presenting different clinical outcomes, is a promising predictive method.

After a decade of development, proteomics is still primarily a basic research activity, yet in the near future this technology is likely to have a profound impact on medicine. By defining the collective

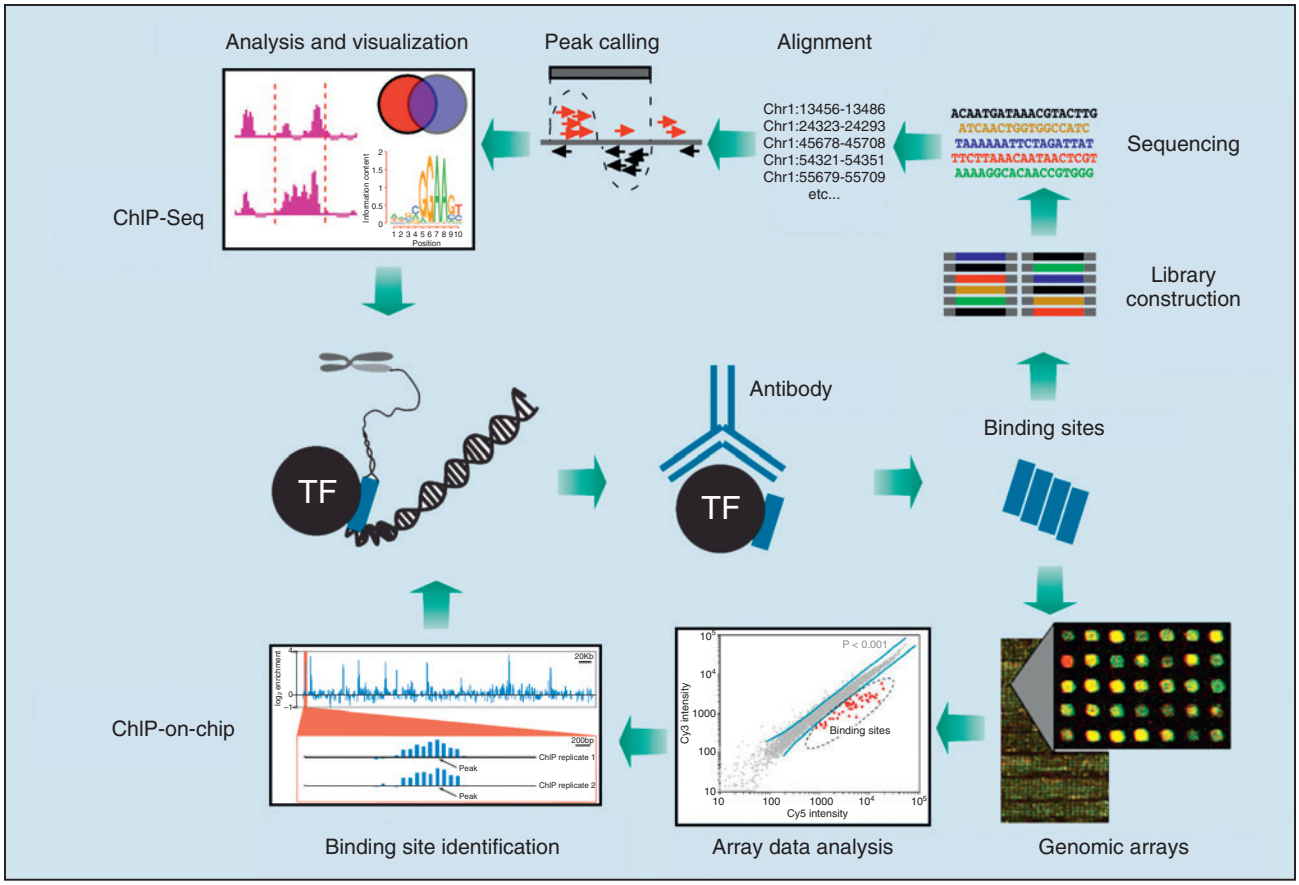

Figure 1.14 • Methods for unbiased identification of transcription factor binding sites. Chromatin immunoprecipitation on sequencing (ChIP-seq) and chromatin immunoprecipitation on microarray chip (ChIP-chip) can provide location, isolation, and identification of the DNA sequences occupied by specific DNA-binding proteins in cells. Proteins capable of DNA interactions are targeted with specific antibodies. DNA and the associated proteins are cross-linked; DNA is fragmented into 150 to 500 bp and immunoprecipitated. After reversion of the cross-link, DNA is isolated and either mass-sequenced (ChIP-seq) or used as probes in a genomic array (ChIP-chip), and binding sites occupied by the proteins can be identified in the genome. These binding sites may indicate functions of various transcriptional regulators and help identify their target genes during development and disease progression. The types of functional elements identified with these techniques include promoters, enhancers, repressor and silencing elements, insulators, boundary elements, and sequences that control DNA replication. (From Kim TH, Ren B. *Annu Rev Genomics Hum Genet*. 2006;7:81–102 and Liu et al. *BMC Biol.* 2010;8:56.)

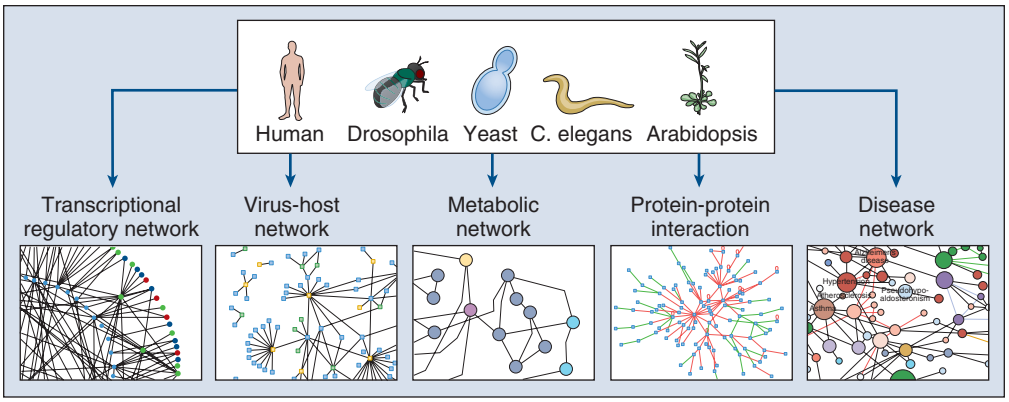

Figure 1.15 • Interactome networks and human disease. Networks are integrated sources of information obtained from biochemical, molecular, proteomic, and other high-throughput analyses. Different networks can be obtained for each organism, organ, or cell. In the first instance, central regulatory "nodes" identify important components in the network. These networks and their data can then be integrated and compared with healthy and disease models, allowing an integrative view of events that is much more powerful than isolated networks. (Modified from Vidal M, Cusick ME, Barabási A. Interactome networks and human disease. *Cell.* 2011;144:986–998.)

protein-protein interactions in a cancer cell (its "interactome"), functional relationships between disease-promoting genes may be revealed that provide novel candidates for intervention (Fig. 1.15). Networks of disorder-gene associations are already being built that offer a platform for describing all known phenotype and disease-gene associations, often indicating the common genetic origin of many diseases. A precise diagnosis of cancer through use of proteomics can be envisioned, based on highly discriminating patterns of proteins in easily accessible patient samples. Proteomics information also promises to provide sophisticated mathematical models of the molecular events underlying a process as complex as neoplastic transformation, which will capture the dynamics of the disease with unprecedented power.

MODELING CANCER IN VIVO

Once the mechanistic underpinnings of a particular cancer have been described, creating an animal model to test that mechanism becomes critical to understanding the pathophysiology and to design therapeutic strategies for treatment. Advances in manipulation of the mouse genome have resulted in more sophisticated models of human cancer. These methodologies can circumvent embryonic death by targeted alteration of gene expression only after a critical period in development, and reduce the complexity of gene functional analysis by restricting its pattern of activation. Inducible gene expression or silencing also allows acute, as opposed to chronic, effects to be assessed. Although species differences in tumor susceptibility and disease remission exist between mouse and man, the tools for genetic manipulation in mouse are superior to those in other mammals, and useful information about the function of oncogenes can be gained by targeted expression of mutant protein products in mouse tissues.

A major hurdle in generation of clinically relevant mouse models to develop cancer treatments stems from the lack of patient tailoring. Cancer cells present a highly heterogeneous population that varies with the genetic makeup of the individual patient. This shortcoming has been addressed with the advent of patient-specific avatars, also known as personalized mouse models or patient-derived xenograft (PDX) models (Fig. 1.16). Implanting patient biopsy specimens into immunodeficient mice allows growth of the tumor, generating in vivo precision models without further in vitro manipulation of the tumor tissue. These models show great promise for designing treatment and drug tests that should best target the patient-specific tumor. Most recently, PDX models have been further optimized with the use of humanized host mice that are modified to contain human immune systems.

TRANSGENIC MODELS OF CANCER

Integrating an oncogene that causes malignancy into the genome of a mouse without altering the mouse's own genes generates a transgenic, cancer-prone mouse that transmits this trait to its offspring with a dominant pattern of inheritance. The technology for producing transgenic mice joins recombinant DNA methodology with standard techniques that are used today by in vitro fertilization clinics, relying on the understanding of mammalian reproduction and the development of protocols to harvest, manipulate, and reimplant eggs and early embryos (Fig. 1.17). The transgene is constructed so that the gene product will be expressed under appropriate spatial and temporal control. In addition to all the standard signals necessary for efficient transcription and translation of the gene, transgenes contain a promoter, or regulatory region, that drives transcription in either a ubiquitous or a tissue-restricted pattern. This requires an extensive knowledge of genetic regulation in the target cells. A recent advance that circumvents this requirement involves embedding the transgene inside another gene locus that is expressed in the desired pattern. Held in a bacterial artificial chromosome (BAC) for easier manipulation, this long stretch of DNA surrounding the host gene is likely to carry all the necessary regulatory information to guarantee a predictable expression pattern of the introduced transgene.

The transgene DNA is then injected into the male pronucleus of a fertilized mouse egg, obtained from a female mouse in which hyperovulation has been hormonally induced. The injected eggs are cultured to the two-cell stage and then implanted in the oviduct of another recipient female mouse. Transgenic pups are identified by the presence of the transgene in their genomic DNA (obtained from the tip of the tail and analyzed with PCR assay). Typically, several copies of the transgene are incorporated in a head-to-tail orientation into a single random site in the mouse genome. About 30% percent of the resulting pups will have integrated the transgene into their germline DNA and constitute the founders of the transgenic lines. RNA analysis of their progeny determines the level of transgene expression, and whether the transgene is being expressed in the desired location or at the appropriate time. Given the variability in transgene number and chromosomal location, transgene expression patterns and levels can

Figure 1.16 • Mouse avatar (PDX) models. (A) Patient-derived xenograft (PDX) mice are generated by implanting patient tumors into immunodeficient/humanized mice. The tumors can then be propagated for several passages in fresh mice for a number of generations. (B) Usually, after the third generation the tumors can be isolated and characterized for further study. These mice can potentially be used for patient drug-specific testing and molecular characterization, therefore allowing for personalization of cancer treatment. (From http://www.the-scientist.com/?articles.view/articleNo/42470/title/My-Mighty-Mouse/.)

diverge considerably among different founder lines carrying the same transgene.

In general, transgenesis is optimal for modeling oncogenic mutations that cause a gain of function, producing disease even when they occur in only one of a gene's two alleles. For example, an activating mutation in a growth factor that causes abnormal cell proliferation can be mimicked by introducing a transgenic version of the mutated growth factor gene under the control of an appropriate regulatory sequence for expression in the tissue of interest. The relative susceptibility of such a transgenic mouse to tumorigenesis can help distinguish between a primary and secondary role of the mutant factor, and established lines of these animals can be used for testing new therapeutic protocols.

CONDITIONAL CONTROL OF ONCOGENE ACTIVATION

The genetic construction of cancer-prone transgenic mice with the capacity to induce oncogene expression in vivo provides a new avenue to modeling the role of oncogenes in tumor generation and maintenance. This technology relies on conditional mutagenesis. Producing conditional mutations in mice requires a DNA recombinase enzyme that does not recognize any mouse sequence, but rather targets short, foreign recognition sequences to catalyze recombination between them. By strategic placement of these recognition sequences in appropriate orientations either beside or within a mouse gene, the recombination results in deletion, insertion, inversion, or translocation of associated genomic DNA (Fig. 1.18). Two recombinase systems are currently in use: the Cre-loxP system from bacteriophage P1, and the Flp-FRT system from yeast. The 34 bp loxP or FRT recognition sequences do not occur in the mouse genome, and both Cre and Flp recombinases function autonomously, without the need for cofactors. Cre- or Flp-mediated recombination is not distance or cell-type dependent, and can occur in proliferating or differentiated tissues.

The general scheme involves two mouse lines, one carrying the recombinase either as a transgene driven by inducible regulatory elements or knocked into one allele of a gene expressed in the desired tissue. The other mouse line harbors a modified gene target including recognition sequences. Mating the two lines results in progeny carrying both the target gene and the recombinase, which interacts with the target gene only in the desired tissue.

A popular conditional methodology is based on the activation of nuclear hormone receptors to control gene expression. Two current systems involve activation of a mammalian estrogen receptor, estrogen analogue 4-hydroxy-tamoxifen, or an insect hormone receptor with the corresponding ligand ecdysone. Although several variations on these hormone-receptor systems are currently in use, the underlying principle is the same. The Cre recombinase gene, or another regulatory protein, such as a transcription factor, is fused with the ligand-binding domain (LBD) from a nuclear hormone receptor protein. The resulting chimeric transgene is placed under the control of a promoter that directs expression to the tissue of interest, and transgenic animals are generated. In the absence of the hormone or an analogue, the fusion protein accumulates in the desired tissue but is rendered inactive through its association with resident heat shock proteins. Hormone, administered either systemically or topically, binds to the LBD moiety of the fusion protein, dissociates it from the heat shock protein, and allows the transcriptional regulatory component to find its natural DNA targets and promote lox-P mediated recombination, or in the case of an inducible transcription factor, activate expression of the corresponding genes. If the LBD is fused to a recombinase, administration of hormone leads to the rearrangement of target sequences. This reaction is not reversible, but lends additional temporal control over recombinase-based mutation. If the LBD is fused to a transcription factor, removal of hormone leads to inactivation of the fusion protein and gene downregulation.

Another inducible method in use is the tetracycline (tet) regulatory system. In the classic design (tTA or tet-off), a fusion protein

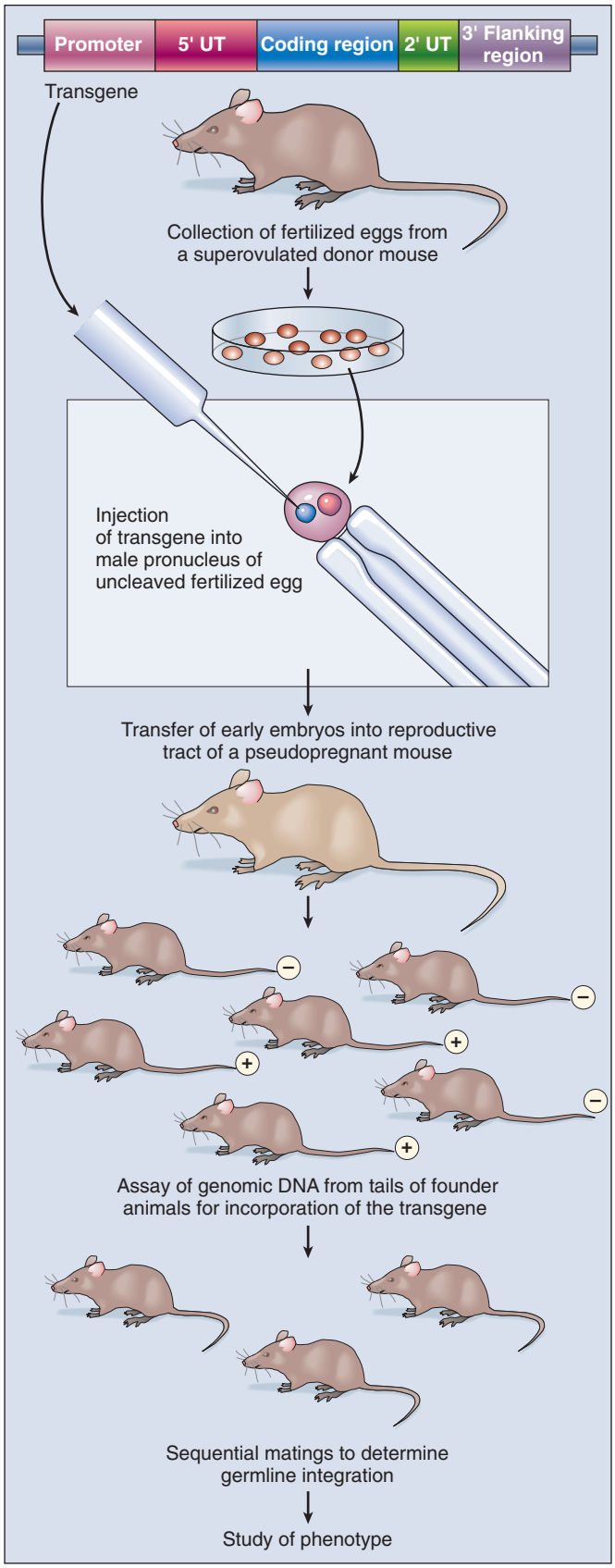

Figure 1.17 • Generation of transgenic mice. The transgene containing the DNA sequences necessary for the expression of a functional protein is injected into the male (larger) pronucleus of uncleaved fertilized eggs through a micropipette. The early embryos are then transferred into the reproductive tract of a mouse rendered "pseudopregnant" by hormonal therapy. The resulting pups (founders) are tested for incorporation of the transgene by assaying genomic DNA from their tails. Founder animals that have incorporated the transgene (+) are mated with nontransgenic mice, and their offspring are mated with each other to confirm germline integration and to establish a line of homozygous transgenic mice. Several transgenic lines that have incorporated different numbers of transgenes at different integration sites (and thus express various amounts of the protein of interest) are usually studied. *UT,* Untranslated. (From Schuldiner AR. Transgenic animals. *N Engl J Med.* 1996;334:653–655.)

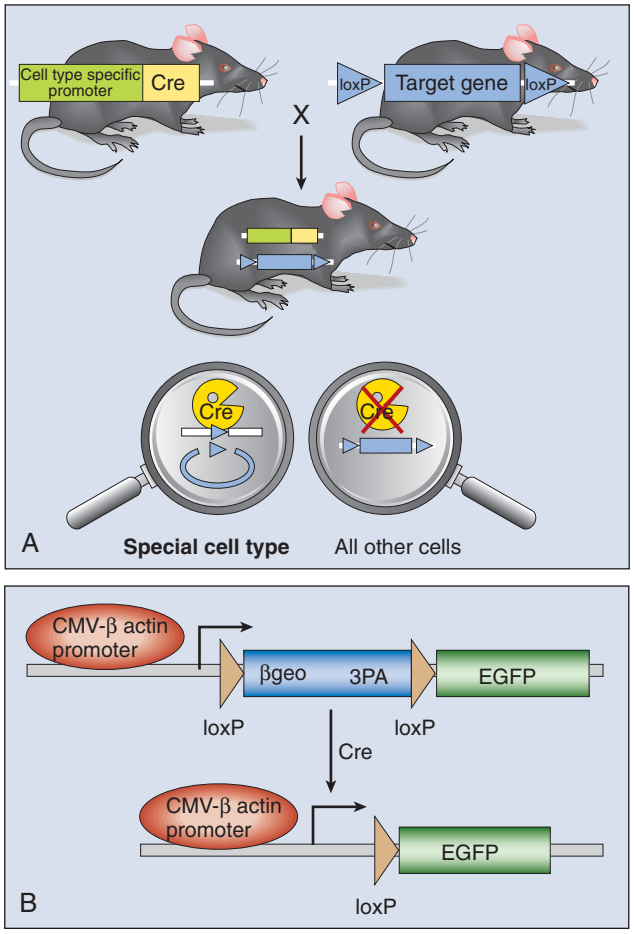

Figure 1.18 • Conditional mutagenesis schemes. (A) Two mouse lines are required for conditional gene deletion: first, a conventional transgenic mouse line with Cre targeted to a specific tissue or cell type; and second, a mouse strain that embodies a target gene (endogenous gene or transgene) flanked by two loxP sites in a direct orientation ("floxed gene"). Recombination (excision and consequently inactivation of the target gene) occurs only in those cells expressing Cre recombinase. Hence, the target gene remains active in all cells and tissues that do not express the Cre recombinase. (B) The Z/EG double reporter system. These transgenic mice constitutively express lacZ under the control of the cytomegalovirus enhancer/chicken actin promoter. Expression is widespread, with notable exceptions being liver and lung tissue. Expression is observed throughout all embryonic and adult stages. When crossed with a Cre recombinase-expressing strain, lacZ expression is replaced with enhanced green fluorescent protein expression in tissues expressing Cre. This double reporter system makes it possible to distinguish a lack of reporter expression from a lack of Cre recombinase expression while providing a means to assess Cre excision activity in live animals and cells. (A Courtesy Kay-Uwe Wagner, National Institutes of Health; B from Novak A, Guo C, Yang W, Nagy A, Lobe CG. Z/EG, a double reporter mouse line that expresses enhanced green fluorescent protein upon Cre-mediated excision. *Genesis.* 2000;28:147–155.)

combining a bacterial tet repressor and a viral transactivation domain drives expression of the target transgene by binding to upstream tet operator sequences flanking the transgene transcription start site. In the presence of the antibiotic inducer, the fusion protein is dissociated from the operator sequences, inactivating the transgene. In a complementary design, called reverse tetracycline-controlled transactivator (*rtTA* or tet-on), structural modification of the tet repressor makes the antibiotic an active requirement for binding of the fusion protein to the operator sequences, such that its administration activates transgene expression at any time during the life span of the mouse, whereas withdrawal results in downregulation of the gene. It is important that the transgene integrate into a genomic locus that permits proper tTA or rtTA regulation so that the system exhibits minimal "intrinsic leakiness" and good antibiotic responsiveness.

Conditional expression systems have already been developed to generate hematopoietic, leukemogenic, and lymphomagenic mutations in the mouse, as well as solid tumors. These inducible cancer models can be exploited to identify oncogenic signals that influence host-tumor interactions, to establish the role of a given oncogenic lesion in advanced tumors, and to evaluate therapies targeted toward cancer-causing mutations. Potential clinical application of inducible systems include targeting virally delivered transgene expression to malignant tissues by the use of specific inducible regulatory elements, restricting the expression of transgenes exclusively to affected tissues, and increasing the therapeutic index of the vectors, particularly in the context of solid tumors. In all cases, a basic knowledge of the specific mutations involved in the molecular genetics of malignancies is required because it is often unclear that the causal mutation underlying the genesis of neoplasia continues to play a central role in the progression to the fully transformed state. This is particularly important in modeling cancers characterized by genetic plasticity, wherein drug resistance can arise subsequent to primary tumor formation.

MODELS OF RECESSIVE GENE MUTATIONS IN CANCER

In contrast to dominantly acting oncogenes, recessive genetic disorders, such as loss-of-function mutations in tumor suppressor genes, require both copies (alleles) of a gene to be inactivated. The methods needed to produce animal models of recessive genetic disease differ from those used in studying dominant traits. Gene knockout technology has been developed to generate mice wherein one allele of an endogenous gene is removed or altered in a heritable pattern (Fig. 1.19). Gene disruption or replacement is first engineered in pluripotential cells, termed embryonic stem cells (ESCs), which are genetically altered by introduction of a replacement gene that is inactive or mutant.

To reduce random integration of the foreign DNA, the replacement gene is embedded into a long stretch DNA from its native locus in the mouse, which targets the recombination event to the homologous position in the ESC genome. Inclusion of selectable markers along with the replacement gene allows selection of the cells in which homologous recombination has taken place. Site-specific recombinase systems combined with gene targeting techniques in ESCs can also be used to induce recessive single point mutations or site-specific chromosomal rearrangements in a tissue- and time-restricted pattern. In a variation on this theme called knockin, a foreign gene, such as one encoding a marker or a mutated gene, can be placed in the locus of an endogenous gene. The engineered ESCs are then microinjected into the cavity of an intact mouse blastocyst sufficiently early in gestation that they can, in principle, populate all the tissues of the developing chimeric embryo. This is rarely the case, so contribution of ESCs to the resulting animal is most often assessed with use of ESCs and blastocysts whose genes for coat color differ.

If the ESCs contribute to the germ cells of the founder mouse, their entire haploid genome can be passed on to subsequent generations. Through mating together of subsequent progeny of the founder mouse, both alleles of the mutated gene can be passed to a single animal. Overlapping genetic functions can also be defined by crossbreeding mice with mutations in different genes. In this way it is possible to study the combinatorial effects of oncogene and tumor suppressor gene mutations.

Several caveats are important in considering the use of knockout technology in modeling cancer. Most knockouts generate loss-of-function (null) germline mutations. Inactivation of widely expressed genes with multiple functions may have complex phenotypes. Conversely, if the functions of two genes overlap, a mutation in one of the genes may not produce an abnormal phenotype, owing to compensation by the unaltered partner.

Perhaps the greatest drawback of conventional knockout technology derives from the disruption of gene function at the earliest stage of its expression. If the gene has a vital developmental role, the identification of functions later in development can be occluded. Therefore, although the generation of a null mutation is an excellent starting point for analysis, it is far from being functionally exhaustive. For these reasons, conditional mutagenesis is the emerging method of choice for the elucidation of the gene functions that exert pleiotropic effects in a variety of cell types and tissues throughout the life of the animal, which is particularly relevant for the generation of mouse models of adult-onset diseases such as cancer.

Use of recombinase-mediated gene mutation as described earlier for conditional transgenesis, conditional knockout mutations can be designed to disrupt the function of a target gene in a specific tissue (spatial control) and/or life stage (temporal control). Depending on the design of the experiment, recombinase action can delete an entire gene, remove blocking sequences to induce gene expression, or rearrange chromosomal segments. With the advent of recent internationally coordinated systematic mutagenesis programs aiming to place a conditional inactivating mutation in each of the 20,000 genes in the mouse genome, the possibilities for modeling cancer are limited only by a researcher's choice of the gene loci to test. The constantly evolving techniques for gene manipulation in vivo constitute a major advance in cancer research.

Genetically modified mice are of great value in dissecting the pathogenesis of many tumor types. In some knockout studies, the phenotype of the mutated gene is anticipated by prior knowledge of the gene's function. However, unexpected mutant phenotypes may help clarify the mechanism of the underlying neoplasia. Pharmacologic manipulation of transgenic, knockout, diversified animal models of cancer will prove useful in screening therapeutic agents with potential for study in clinical trials. Therapy involving gene or cell replacement can be also tested in genetically engineered disease models.

EXPLOITING MOUSE DIVERSITY FOR CANCER RESEARCH

A novel in vivo tool has emerged that aids in understanding the etiology of cancers, by more accurately reflecting the broad genetic variability in the human population. Cancer research performed with mice has largely focused on a few individual highly inbred strains with limited genetic diversity, which would equate to single individuals in the population. Yet drugs designed to treat one individual are often not effective in other patients. The Collaborative Cross (CC) was created to provide mouse models that better represent the diversity seen in natural human populations while still retaining the broad power of genetic analysis seen in mice. The CC resource is a large panel of recombinant inbred (RI) strains generated by randomly mixing the genetic diversity of eight extant inbred mouse lines, and can be used to test the impact of treatments in a diverse genetic pool akin to the human population (Fig. 1.20). A related resource, the Diversity Outcross (DO), offers higher mapping resolution by randomized outcrossing of partially inbred CC strains, which segregates the same allelic variants but embeds them in a distinct population architecture in which each