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PART I The Biology and Pathogenesis of Cancer

1 The Etiology of Cancer

SECTION A: THE GENETIC BASIS OF CANCER

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Cancer is a powerful and fearsome word describing a group of diseases that have recently surpassed cardiovascular disease as the most common cause of death for humans in 12 European countries[1] and in 22 states in the United States.[2] Cancer is also believed to be the most common cause of disease-related death in companion and working dogs in the developed world.[3–6] The fear of cancer, however, is rooted in misunderstanding, misconception, and mysticism. Thus the goals of this chapter are to clarify why cancer happens and highlight advances have been made that allow many human and animal cancer patients to lead full and productive lives after diagnosis.

The seminal work of Nowell and Hungerford in the early 1960s describing the nonrandom translocation between two chromosomes in chronic myelogenous leukemia represents the first time a genetic event (the translocation) could be linked to a specific cancer.[7,8] These observations can be considered the start of the modern era of cancer genetics, and they still pose one of the best arguments for why a strong foundation in contemporary genetics is necessary to understand the etiology of cancer. This chapter provides context for the genetic basis of cancer, updating recent data from domestic animals, especially dogs, that highlights how the judicious application of comparative oncology studies can improve our understanding of cancer risk, progression, and therapy.

Cancer Risk

Cancer is neither a single nor simple disease. Rather, the term *cancer* describes a large number of diseases for which the only common feature is uncontrolled cell growth and proliferation. These only critical requirement for the manifestation of cancer is multicellularity, and neoplastic diseases have been described in representative species from every group in the animal kingdom and also in plants.[9]

An important concept that is now universally accepted is that cancer is a genetic disease, although it is not always heritable. Tumors arise from the accumulation of mutations that eliminate normal constraints of proliferation and genetic integrity in a somatic cell, promoting immortalization and the capacity to modify and maintain a supportive niche for survival and expansion. Among other causes, mutations can arise after exposure to environmental mutagens, such as cigarette smoke and ultraviolet irradiation. Changes in the cancer incidence in humans over the course of the 20th century underscore the significant influence the environment can exert on the genetic makeup of individuals. Some environmental effects reflect behavior patterns (e.g., lung cancer in smokers), infectious diseases (e.g., stomach cancer in people infected with *Helicobacter pylori*), or exposure to cultural factors, such as urbanization or changes in diet (e.g., increasing breast cancer rates in the second and subsequent generations of Asian American women). Nevertheless, it would be incorrect to assume that the environment is wholly responsible for most tumors, especially because the increased risk of cancer upon exposure to potential environmental carcinogens is relatively small, except for tobacco products, ultraviolet or gamma irradiation, and a small group of chemical mutagens.

Another important intrinsic "mutagen" is the inherent error rate of enzymes that control DNA replication, which introduces 1 in 10 million to 1 in 1 million mutations for each base that is replicated during each round of cell division. Mammalian genomes comprise approximately 2 billion to 3 billion (10^9) base pairs; therefore every time a cell divides, each daughter cell is likely to carry a few hundred to a few thousand mutations in its DNA. Most mutations, whether caused by extrinsic or intrinsic factors, are silent and do not hinder the cell's ability to function; however, others can disable tumor suppressor genes or activate proto-oncogenes, which respectively inhibit or promote cell division and survival. Thus it can be said that simply being alive is the single largest risk factor for cancer.

The concept of intrinsic mutagenicity describes cancer risk as a function of the number of stem cell divisions required to maintain structure and function for a given tissue; it also suggests that more than two-thirds of human cancers originate from mutations caused by errors in DNA replication and are stochastic (random) in nature.[10–12] Furthermore, this concept suggests that more than half of the mutational load present in cancer cells occurs before tumors ever form. This is actually a "good news" scenario, in that it makes it possible to envision the development of strategies for early cancer detection using genomic tools.

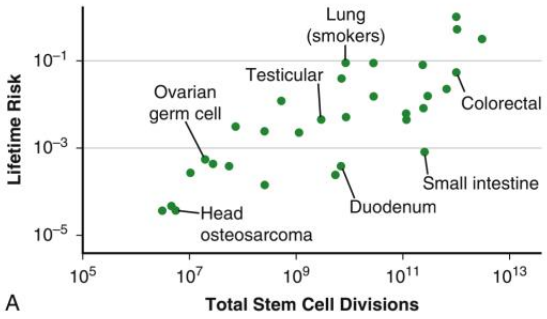
Normal tissues and organs contain different numbers of stem cells that maintain homeostasis. These stem cells self-renew and live longer than other tissue resident cells, which are replaced to

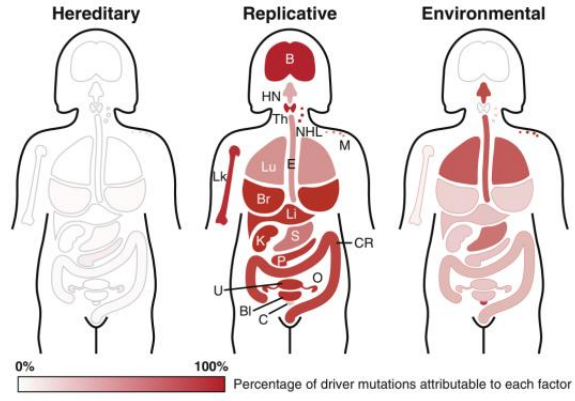
maintain normal organ structure and function. Recent studies by Tomasetti and Vogelstein identified a strong correlation (r = 0.81) between the total number of stem cell divisions and the lifetime risk of cancer arising from 31 distinct tissues (Fig. 1.1A).[11] In contrast, no significant correlations were seen between the risk for these cancers and heritable or environmental and geographic factors.[10]

The take-home message from this work is that DNA replication (R) represents a major risk factor for cancer, joining heritable risk (H) and environmental risk (E) as the major causes of mutations that can lead to cancer (see Fig. 1.1B). Heritable risk arises from mutations in the germline, such as those in the genes *BRCA1* and *BRCA2, TP53,* and *CDKN2A* that are associated, respectively, with breast cancer risk, with Li-Fraumeni syndrome and susceptibility to many cancers, and with susceptibility to melanoma and pancreatic carcinoma. Only one heritable cancer syndrome has been identified in domestic dogs. A germline mutation of the *BHD* gene encoding folliculin was identified in a family of German shepherd dogs that showed susceptibility to a syndrome of renal cystadenoma and nodular dermatobibrosis[13]; however, controlled breeding practices make it difficult for heritable cancers associated with single gene mutations to be perpetuated in domestic animals outside a laboratory environment.

In animals, and specifically in dogs, in which the lifetime risk of cancer seems to be approximately equivalent to that of humans, the apparent increased prevalence of certain tumor types in certain breeds (closed gene pools) suggests that incompletely penetrant, heritable factors might contribute to cancer causation. Indeed, even relatively minor traits that do not considerably alter the phenotypic appearance have been found to be associated with risk. Perhaps this is most easily appreciated in greyhounds; registered racing greyhounds are at higher risk of developing osteosarcoma (OSA) than American Kennel Club (AKC) "show" greyhounds.[14] This suggests that different components of risk could have become established in the founders of the racing and show greyhound lineages. In the racing greyhounds, however, part of the risk could be due to concussive forces during training and performance racing, which have the potential to create microfractures and activate chronic repair processes.

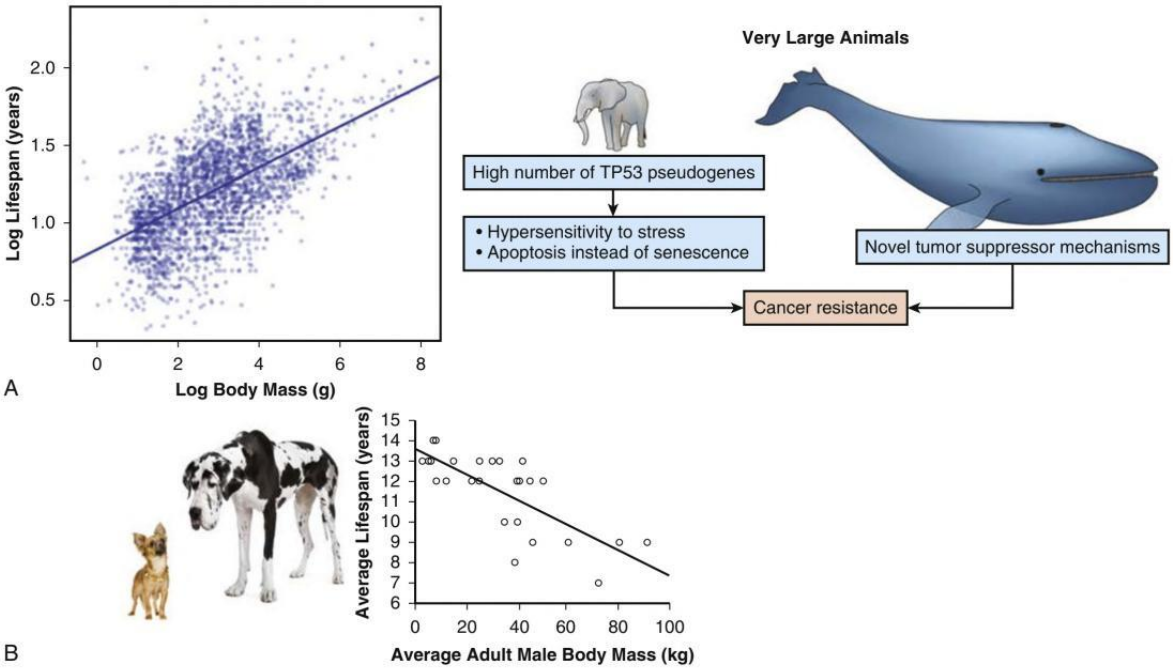
Environmental risk factors include chronic exposures to genotoxic agents, including habitual use of tobacco products; high-energy radiation from the sun or from occupational hazards, such as uranium mining; workplace chemicals, such as vinyl chloride; and agents that promote chronic inflammation and activate tissue repair processes unremittingly, such as asbestos. Tobacco use and sun exposure account for a large portion of human cancers worldwide. The risk of cancer from these exposures has been compounded in the past century by the increased longevity of human populations, which allows more time for mutations to accumulate.[10] In animals, strong associations have been established between exposure to ultraviolet radiation from the sun and some skin cancers in dogs, cats, cows, and horses; however, these account for a small proportion of cancer in these populations. Likewise, exposure to environmental tobacco smoke seems to increase the risk for a small proportion of cancers of dogs (nasal carcinoma) and cats (possibly lymphoma). The association between risk for cancer and exposure to other chemicals in dogs, cats, and other domestic animals is a topic of controversy; such exposure is likely to account for a small fraction of cancers seen in these species, although, as in humans, their effects might be compounded by the increased life span provided by modern veterinary care.





• **Fig. 1.1** (A) The relationship between the number of stem cell divisions in the lifetime of a given tissue and the lifetime risk of cancer in that tissue. (B) Etiology of driver gene mutations in women with cancer. For each of 18 representative cancer types, the schematic depicts the proportion of mutations that are inherited due to environmental factors or due to errors in DNA replication (i.e., not attributable either to heredity or to environment). The sum of these three proportions is 100%. The color codes for hereditary, replicative, and environmental factors are identical and span white (0%) to brightest red (100%). *B,* brain; *Bl,* bladder; *Br,* breast; *C,* cervical; *CR,* colorectal; *E,* esophagus; *HN,* head and neck; *K,* kidney; *Li,* liver; *Lk,* leukemia; *Lu,* lung; *M,* melanoma; *NHL,* non–Hodgkin lymphoma; *O,* ovarian; *P,* pancreas; *S,* stomach; *Th,* thyroid; *U,* uterus. (Reproduced with permission. (A) From Couzin-Frankel J. Biomedicine: the bad luck of cancer. *Science*. 2015 Jan 2;347(6217):12. doi: 10.1126/science.347.6217.12.; Tomasetti C, Vogelstein B. Variation in cancer risk among tissues can be explained by the number of stem cell divisions. *Science*. 2015 Jan 2;347(6217):78-81. https://doi.org/10.1126/science.1260825. Fig. 1.1. (B) Illustration by Corinne Sandone © 2017 Johns Hopkins University. Used with permission.)

Replicative risk of cancer is ever present, and it increases inexorably with age. The stochastic nature of replicative risk is reflected in the molecular heterogeneity observed in histologically similar tumors, and the strong correlation between cancer and advanced age in dogs suggests that the replication-associated R factor likely is responsible for an even greater proportion of cancers in this species than it is in humans.



• **Fig. 1.2** Body size and life span. (A) Relationship between body mass (g) and life span (years) among 2556 vertebrates. In the left panel, the *blue line* shows the linear regression between log (body mass) and log (life span), R2 = 0.32. The right panel illustrates potential anticancer mechanisms in the largest mammals: elephants and whales. Elephants have evolved multiple copies of the *TP53* gene (pseudogenes) that are associated with an increased apoptotic response. Anticancer mechanisms in the largest mammals, whales, are not yet known, but they do not involve *TP53* duplications. (B) The relationship between body mass and life span across 32 different dog breeds. The diversity of size and life span among dog breeds is remarkable, but it is also well known that larger breeds tend to be short-lived relative to small breeds. (Reproduced with permission. (A) Redrawn from Sulak M, Fong L, Mika K, et al. *TP53* copy number expansion is associated with the evolution of increased body size and an enhanced DNA damage response in elephants. *eLife*. 2016 Sep 19;5:pii:e11994. https://doi.org/10.7554/eLife.11994. Fig. 1.1A. (B) Redrawn from Selman C, Nussey DH, Monaghan P. Ageing: it's a dog's life. *Curr Biol*. 2013 May 20;23(10):R451-R453. https://doi.org/10.1016/j.cub.2013.04.005. Fig. 1.1.) Photo of dogs © iStockphoto.com.

It should be noted that infectious agents can also be causally linked to cancer, by acting as direct mutagens or by increasing inflammation, replication, and repair, and consequently cancer risk.

Body Size and Cancer

The notion that mutations that accumulate stochastically during normal cell replication drive malignant transformation raises an important question: Why aren't bigger animals that have more cells more vulnerable to cancer? This question, originally posed by Sir Richard Peto,[15] is commonly referred to as Peto's paradox (at the species level, the incidence of cancer does not appear to correlate with the number of cells in an organism). The answer involves evolution and natural selection. Several cancer-protective mechanisms have been identified in mammals at the extremes of size. Elephants are the largest living land mammals; in the elephant lineage, cancer protection seems to be associated with enhanced activity of the *TP53* tumor suppressor gene, which underwent multiple duplication events after the split from a common ancestor that gave rise to hyraxes and manatees but that preceded diversification into mastodons, mammoths, and modern elephants.[16,17] Whales include the largest living animals, and in the bowhead whale lineage, for example, variants or alterations of multiple genes seem to confer protection from cancer and aging.[18] However, in the common minke whale, an alternative adaptation has evolved that resulted in fewer microsatellites in genomic regions near proto-oncogenes and tumor suppressor genes, where cumulative mutations could lead to an increased cancer risk.[19] As these examples illustrate, adaptive solutions that enable large size and longevity likely are unique and specific to the evolutionary history of each species (Fig. 1.2A).

The norm for mammalian evolution is that large size is correlated with longevity. This is consistent with selective pressures that otherwise would disfavor the energy expenditure required to achieve large size. However, this trend is reversed in domestic dogs,[20] in which large body size is associated with shorter life spans and possibly with a higher rate of certain diseases, including cancer (see Fig. 1.2B). The precise reasons for this remain unclear, but dogs present a unique natural model to study

associations between age, body mass, and disease risk under conditions in which artificial selection has superseded natural selection. Specifically, natural selective pressures in dogs were replaced by artificial selection since the initial domestication events approximately 10,000 to 25,000 years ago.[21] Changes in demand for form, instead of function, drove the creation of more than 400 breeds in the past 300 to 400 years. This artificial selection, usually for a single or a few phenotypic traits, gives little chance for adaptation across the rest of the genome. Consequently, the risks of mutation associated with normal processes of cell replication during development, growth, and maintenance into adulthood are enhanced in large dogs (more cells), making it possible to explain the disproportionate risk of certain cancers, such as appendicular OSA, by manipulation of their genomic plasticity with extreme selection for size. This is also consistent with the fact that the overall risk for axial OSA in dogs is similar to that observed in other species, accounting for the effects of size and functional/mechanical stresses on bone. The greater risk for large and giant dogs to develop appendicular OSA can be explained, at least in part, by the fact that more cell divisions are needed to create and maintain large bones, especially as bone tissues undergo continuous remodeling. Each round of replication for an osteoblast, in turn, contributes to its mutational burden and potential transformation. The late age of onset in dogs is consistent with chronic selection for cells that accumulate a critical complement of mutations. In humans, OSA is among the cancers in which the R factor can explain virtually all of the risk; also, an association has been recognized that shows OSA is more common in children in the higher percentiles of size for their age.[22–24]

Accounting for the risk of appendicular OSA (or other cancer types) stochastically as a function of replication risk leaves another important question unanswered: Why does the risk for some dog breeds seem to be higher (or lower) than expected based on their overall size? Partial answers to this question are available, and again, solid data is available for OSA. In terms of breed-specific risk, multiple heritable factors seem to influence the risk for OSA. Data from a genome-wide association study (GWAS) in three high-risk dog breeds indicates that the patterns of heritable risk for OSA are complex and incompletely penetrant.[14] However, selective breeding, especially for large size, seems to have enriched risk alleles that are now fixed in certain populations.[14,25,26] Fixed alleles associated with risk are not unique to OSA; they have also been associated with breed-specific risk for canine mammary cancer,[27] canine digital squamous cell carcinoma,[28] and other cancer types (discussed later in the chapter).

In humans a GWAS identified two loci associated with the presence of OSA[29] and a single, distinct locus in the *NFIB* gene associated with the presence of metastasis at diagnosis.[30] The risk alleles for humans and dogs are not located in orthologous regions of the genome. It appears, then, that achieving a large size relatively rapidly, and not breed-specific (or individual) traits in the germline, is the overwhelming contributor to OSA risk. It is thus reasonable to conclude that Peto's paradox arises as a result of barriers of natural selection, and that when such barriers are removed and the natural life span of an organism is extended, as is the case for dogs, the paradox disappears, revealing the overwhelming influence of DNA replication errors on individual cancer risk.

The examples provided underscore that cancer risk and progression have both shared and unique traits across species in the animal kingdom. This creates opportunities to study the natural history of cancer in a spontaneous setting. However, it is important to avoid the significant pitfalls that arise from assumptions of equivalence where none exists. The next section of this chapter reviews the hallmarks of cancer that are shared by virtually every cancer from every species. The final section of the chapter focuses on recent studies that elevate companion dogs as models for understanding the complex genetics of cancer through the use of contemporary technology and cautious, deliberate interpretation of data.

The Hallmarks of Cancer

Forty years of research culminated in an insightful and a thorough review paper by Douglas Hanahan and Robert Weinberg in 2011 that synthesized knowledge about cancer into 10 essential, acquired characteristics.[31]

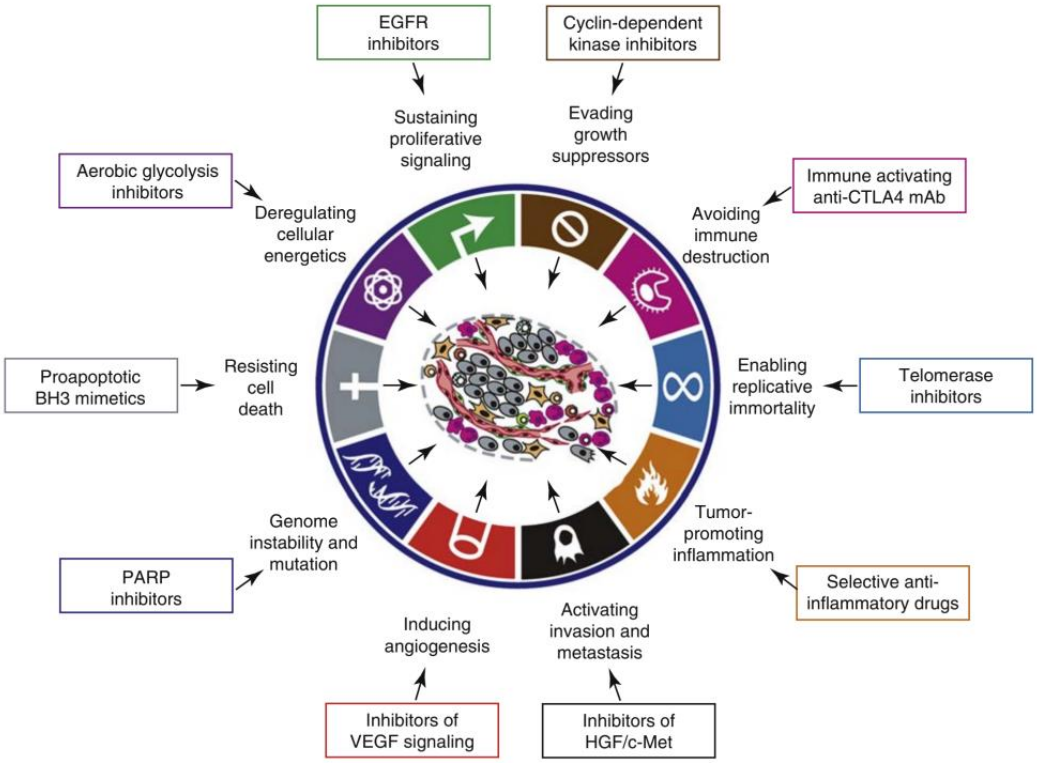
In 2000 the same authors had described six characteristics necessary for cellular transformation, which comprised the abilities to (1) sustain proliferative signaling, (2) evade growth suppressors, (3) resist cell death, (4) enable replicative immortality, (5) induce angiogenesis, and (6) activate invasion and metastasis.[32] In this initial paper describing the hallmarks of cancer, Hanahan and Weinberg created a paradigm shift by providing the first ever comprehensive synthesis of the molecular events leading to cancer. The important concepts that were clarified included these: no single gene is universally responsible for transformation; five or six critical (driver) mutations are the minimum theoretical number required to endow the cancer phenotype (an observation that has since been confirmed experimentally)[33]; each step in the path toward transformation and cancer progression is regulated by multiple interactive biochemical pathways,[34] and thus, mutations of different genes along a pathway can result in equivalent phenotypes and, conversely, mutations of the same gene can result in different cancers with distinct biology; tumors behave as tissues; and the interactions between the tumor and its microenvironment are major drivers of cancer behavior.

The updated hallmarks of cancer added two "enabling" characteristics, (7) genome instability and mutation and (8) tumor-promoting inflammation, and two "emerging" hallmarks, (9) deregulating cellular energetics and (10) avoiding immune destruction.

The effect of this unifying conceptualization of cancer genetics and this level of understanding are clearly evident when one considers how they have influenced the design, development, implementation, and success of new cancer therapies (Fig. 1.3). A summary of the information with added refinements is provided later in the chapter.

Sustaining Proliferative Signaling

Arguably the most important event in neoplastic transformation is the capability of cells to proliferate in perpetuity. Under normal conditions, cells communicate with each other and integrate environmental signals by sensing cues and gradients. For example, migration, metabolism, and proliferation of mature hematopoietic cells are regulated in autocrine and paracrine fashions by locally secreted cytokines. The same cytokines may act systemically in an endocrine fashion. With the notable exception of steroid hormones that bind to intracellular receptors, growth-promoting cytokines work by binding transmembrane receptors, which in turn initiate signaling cascades that culminate in transcriptional changes. These transcriptional responses, in turn, allow cells to adapt their behavior to match



• **Fig. 1.3** Therapeutic targeting of the hallmarks of cancer. Drugs that interfere with each of the acquired capabilities necessary for tumor growth and progression have been developed and are in clinical trials, or in some cases have been approved for clinical use in treating certain forms of human cancer. Additionally, the investigational drugs are being developed to target each of the enabling characteristics and emerging hallmarks, which also hold promise as cancer therapeutics. The drugs listed are examples; a deep pipeline of candidate drugs with different molecular targets and modes of action is in development for most of these hallmarks. (Reproduced with permission from Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell*. 2011 Mar 4;144(5):646-674. https://doi.org/10.1016/j.cell.2011.02.013. Fig. 1.6.)

the environmental signals. The activity of cytokines, their receptors, and the corresponding signaling molecules are finely tuned. The system can be shut down when the concentration of the cytokine falls below a threshold that can stably bind the receptor, when the receptor ceases to be expressed, or when signaling molecules are downregulated or otherwise inactivated; however, mutations in even one of the molecules involved in regulating these pathways can provide sustained growth signals in the absence of the initiating cytokine. Among many examples is a translocation between chromosome 2 and chromosome 5 (t(2;5)) that is present in almost half of human anaplastic lymphomas. The translocation creates a fusion protein between the nucleophosmin gene *(NPM1)* and the anaplastic lymphoma kinase gene *(ALK);* this aberrantly activates the Jak2/STAT5 signaling pathway,[35] which normally is responsive to various interleukins (IL), including IL-2, IL-3, and IL-6. The genes that encode the normal growth-promoting proteins (e.g., ALK, Jak2, and STAT5) are called *proto-oncogenes;* the mutated versions that allow cells to gain self-sufficiency from the environmental signals are called *oncogenes*. It is important to note that not all growth-promoting genes have the capacity to become oncogenes and that the outcomes of oncogenic activation are most commonly senescence or apoptosis, unless additional events promote stable transformation and survival.

Evading Growth Suppressors

In addition to the hallmark capability of inducing and sustaining positively acting growth-stimulatory signals, cancer cells must also circumvent powerful programs that negatively regulate cell proliferation; many of these programs depend on the actions of tumor suppressor genes. To maintain homeostasis, cells also must integrate antigrowth signals from the environment. Quiescence in nonhematopoietic cells is enforced by signals delivered by contact inhibition.[36] Hematopoietic cells, on the other hand, use cell-cell contacts to maintain interactions within the niche and to regulate the timing and intensity of hematopoiesis, inflammation, and immunity.[37]

"Stop" signals usually are delivered and integrated by the products of tumor suppressor genes, which derive their name largely from the observation that their inactivation facilitates tumor formation. Tumor suppressor genes balance the activity of

growth-promoting proto-oncogenes and tend to act in tandem with these in most biochemical pathways. Loss of function of one or more tumor suppressor genes occurs in virtually every cancer; inactivation of *TP53, RB1, PTEN,* or *CDKN2A* is seen in more than 50% of all tumors. Inactivation of these pathways seems to contribute to the pathogenesis of companion animal tumors, and their dysfunction also may be predictive for outcomes in some of them (see for example refs.[38–42]).

Resisting Cell Death

Apoptosis, or programmed cell death, is the imprinted outcome for every cell in multicellular organisms. Survival requires support from extrinsic (environmental) factors, in addition to a precise balance of cellular energetics and metabolism. Bone marrow–derived cells (BMDCs) normally undergo apoptosis when concentrations of survival factors (e.g., stem cell factor, IL-3, IL-7) or nutrients are limiting or when cellular bioenergetics is severely disrupted.[43]

Evasion of apoptosis is an essential acquired feature of all cancers, and it can result from loss of proapoptotic tumor suppressor genes, such as *TP53* or *PTEN,* or by gain of function of antiapoptotic genes, such as *BCL2.* Gain of function of *BCL2* in humans generally is associated with indolent, follicular lymphomas that carry t(14:18) translocations that juxtapose *BCL2* and the immunoglobulin heavy enhancer locus *(IGH).* These tumors rarely are seen in domestic animals, but evasion of apoptosis may be an important mechanism in the pathogenesis of other indolent tumors seen more commonly in these species.

A more recent concept in the cell death field is autophagy—a process that tumor cells have efficiently co-opted as a means to survive under adverse conditions.[44] As part of the autophagy program, intracellular vesicles called *autophagosomes* surround intracellular organelles and fuse with lysosomes. There, the organelles are broken down and then channeled to form new molecules that support the energy-producing machinery of the cell, allowing it to survive in the stressed, nutrient-limited environment that defines most cancers.

Tumor cells also must avoid death by anoikis, or loss of integral cell-to-cell or cell-to-matrix contacts.[36] Absent these physiologic death pathways, the body often reacts to the anatomic and physiologic disruptions caused by cancer cells by targeting these cells for destruction through inflammatory pathways, leading to necrosis. The process of necrosis might also be regulated genetically, providing another mechanism that favors survival of the whole (organism or tumor) over survival of the one. New findings that lend further nuance to the perception of how evasion (or incitation) of these cell death mechanisms contributes to neoplastic transformation and tumor progression continue to be published almost daily, and readers are encouraged not to limit their investigation to this summary, but rather to seek recent updates to the literature in this field.

Enabling Replicative Immortality

Immortalization is another essential feature of cancer. The genetic program limits the number of times a cell is able to replicate (the so-called Hayflick limit), and when this limit is reached, replicative senescence is induced. Induction of replicative senescence does not induce death; cells maintain energetic homeostasis and remain functional, but they undergo significant genetic changes characterized by telomere erosion. Cells that are able to replicate must maintain the integrity of telomeres, which are "caps" made of repetitive DNA sequence that protect chromosomes from destruction. Solid tumors acquire immortalization predominantly by activation of the telomerase enzyme system and the consequent maintenance of telomere integrity. In hematopoietic cells, telomerase activity seems to be retained longer than in other somatic cells, so this may facilitate immortalization in lymphoma and leukemia.[45] The role of immortalization and the importance of telomerase (both to maintain telomere length and to maintain other biochemical functions that are essential for cell survival) are well established; however, the role of replicative senescence has been questioned recently because improved technology has allowed researchers to circumvent this process in normal cells.[31] Mouse models complicate the story because of significant differences in telomere length between rodents and humans; therefore this is an area in which other models, such as companion animals, might provide clarity in the future.[46]

Inducing Angiogenesis

Folkman proposed a role for angiogenesis in cancer more than 30 years ago,[47,48] but this idea took time to gain traction in the scientific community. It is now apparent that angiogenesis not only is an important pathogenetic mechanism during tumor progression, but also a potential target for therapeutic intervention.

Angiogenesis is a complex, tightly regulated process that requires the coordinated action of a variety of growth factors and cell adhesion molecules in endothelial and stromal cells. So far, vascular endothelial growth factor (VEGF-A) and its receptors comprise the best-characterized signaling pathway in tumor angiogenesis.[49] VEGF binds several receptor tyrosine kinases, including VEGF receptor-1 (VEGFR-1 [also known as Flt-1]) and VEGFR-2 (KDR or Flk-1). Genetic polymorphisms of *VEGF* or of *FLT1* or *KDR* genes are associated with increased angiogenesis, and mutations of *KDR* are reported in human vascular tumors.[50,51] VEGF expression also is upregulated by hypoxia and inflammation. The transcription factor hypoxia-inducible factor-1α (HIF), which is part of a pathway that also includes regulation by the von Hippel-Lindau (VHL) tumor suppressor gene, is a major regulator of VEGF expression. Under conditions of normal oxygen tension, the VHL protein targets HIF for degradation; under low oxygen conditions, HIF increases as VHL-mediated degradation is reduced, allowing for upregulation of VEGF.

Other signaling molecules also contribute to angiogenesis, including platelet-derived growth factor-β (PDGF-β) and its receptor (PDGFR), and the angiopoietins Ang-1 and Ang-2 and their receptors Tie-1 and Tie-2. PDGF-β is required for recruitment of pericytes and maturation of new capillaries. Recent studies also document the importance of tumor-derived PDGF in the recruitment of stroma that produces VEGF and other angiogenic factors.

Tumors use multiple mechanisms to resist antiangiogenic therapy. For example, tumor cells cooperate with niche cells, such as endothelial cells, BMDCs, cancer-associated fibroblasts (CAFs), and pericytes, to create a microenvironment that abolishes the therapeutic benefits of VEGF blockade.[52] But overall, it is apparent that antiangiogenic therapies can benefit cancer patients by promoting vascular normalization, at least partially restoring the balance among blood vessel–forming and stromal cells, including pericytes, myeloid-derived cells, endothelial progenitors, and fibroblasts. This, in turn, can reverse the anatomic and hemodynamic dysfunction created by the tumor microenvironment,

disabling some of the intrinsic advantages this dysfunction provides for cancer cells and allowing better penetration of drugs. (Chapter 15, Section C, provides additional information about antiangiogenic therapies.)

Angiogenesis takes center stage in malignant vascular tumors such as hemangiosarcoma (HSA), which occurs commonly in dogs.[53] Malformed, disorganized vascular structures composed of a mixture of malignant and nonmalignant cells are the defining feature of canine HSA and human angiosarcomas. Among other proangiogenic drivers, canine HSAs show elevated production of VEGF,[54–56] IL-8,[57] and sphingosine-1 phosphate (S1P) and its receptor, S1P1[58]. Furthermore, the magnitude of the angiogenic drive is associated with somatic mutations of angiopoietin, VEGF, and PI3K signaling pathways, in addition to the biologic behavior of the tumors.[42,59] This data suggests that canine HSA may provide a powerful, spontaneous model for unraveling critical events that control tumor angiogenesis.

Activating Invasion and Metastasis

The role of genetic events in invasion and metastasis is still incompletely understood. The classic model of metastasis proposed by Fidler suggests a stepwise acquisition of assets that enables cells to leave the primary tumor site, travel through the blood or lymph, invade stroma in favorable locations, and thus become reestablished at distant sites.[60] Other research suggests that most tumors have the ability to dislodge cells that travel to distant sites, and the ability of such cells to survive in capillary beds may be the most important step in the metastatic process.[61–65] A systematic assessment of metastasis reveals that it is a complicated process partly controlled by tumor heterogeneity and in which genetically distinct cells contribute to the dissemination of tumor cells from primary sites to metastatic sites (Fig. 1.4).[66]

BMDCs have intrinsic properties that allow them to travel throughout the body, traffic through all major organs, and home to areas of inflammation. Thus bone marrow–derived tumors are inherently metastatic. Nevertheless, hematopoietic tumors that are cytologically indistinguishable can have distinct and preferential tissue distribution. The events that make leukemic cells stay in the peripheral circulation are not yet fully understood, even though cells from corresponding lymphomas or myeloid sarcomas, with virtually identical molecular signatures, stay confined to lymphoid or visceral organs.

In epithelial neoplasms that account for most tumors in humans, the epithelial-to-mesenchymal transition (EMT) has received increasing attention for its role in metastasis. It remains unclear whether EMT is equally important in the sarcomas more commonly seen in domestic animals, in which the cells of origin seem to retain EMT capabilities to a greater extent. Increasing evidence indicates that interactions between cancer cells, including both the "initiating" population in the tumor (colloquially referred to as cancer stem cells, or CSCs), and the remainder (bulk) of tumor cells and other cells in the tumor microenvironment, including mesenchymal stem cells (MSCs), CAFs, inflammatory cells, and angiogenic cells, may be responsible for cancers' invasive behaviors and for their ability to survive in hostile environments at distant (metastatic) sites. One example is signaling through the CXCR4-CXCL12 axis, which contributes to the metastatic process through interactions between tumor cells and the tumor-permissive niche.[67] CXCR4 is upregulated recurrently in canine HSA and OSA,[68–70] where it is presumed to promote invasion and migration upon binding CXCL12. Thus dogs with HSA and OSA provide an opportunity to test therapeutic inhibition of the CXCR4-CXCL12 axis as a means to delay or prevent metastasis. (Chapter 2 presents additional information about basic mechanisms and treatments to manage cancer metastasis.)

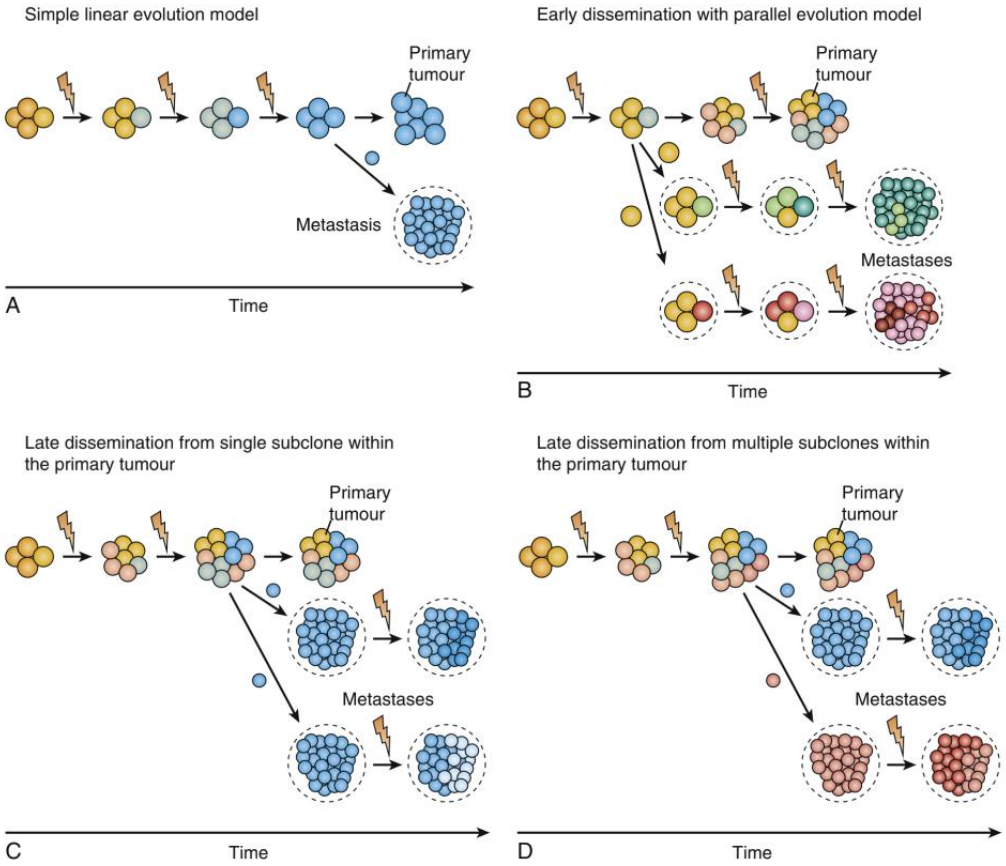
Genomic Instability and Mutation

The concept of genomic instability is not new, but it was incorporated as an "enabling hallmark" into the updated Hanahan and Weinberg model.[31] Traditionally, stepwise clonal evolution provided a satisfactory explanation of tumor progression because it could be correlated with discrete pathologic changes. This is especially true for epithelial tumors, in which such progression can be appreciated in lesions that go through stages of hyperplasia, atypical hyperplasia (dysplasia), adenoma, carcinoma in situ, invasive carcinoma, and metastatic carcinoma. However, analysis of tumor genomes, even in early stages, usually shows aneuploidy (an abnormal DNA copy number), in addition to chaotic changes indicative of multiple numeric and structural DNA abnormalities. Similar abnormalities, first noticed by Boveri more than 100 years ago in studies of sea urchin cells, led him to formulate the "aneuploidy theory" of cancer.[71] Aneuploidy now is known to be especially evident in solid tumors; based on this, Loeb proposed the existence of the "mutator phenotype," in which cells are predisposed to undergo multiple mutations, some of which inevitably lead to cancer.[72] Some tenets of his hypothesis appear to be correct, although perhaps through different mechanisms than those envisioned by Loeb, as they might relate to increased activity of polymerases with low fidelity under conditions in which the rate of DNA damage (and consequently mutations) is higher than the expected background from normal DNA replication (e.g., in lung epithelial cells from heavy smokers). However, direct measurements of mutation rates of sporadic tumors are much lower than those predicted if a "mutator phenotype" was operative in these tumors.[73] Indeed, the minimum number of "critical" or driver mutations required for the clinical onset of cancer in solid tumors, based on sequencing of solid tumor genomes, probably is on the order of 15 to 25.[74] However, this may apply mainly to tumors with chaotic karyotypes, because the number of mutations identified in a cytogenetically stable leukemia was significantly smaller.[33]

Still, genetic instability is a hallmark of most tumors, and although it can be partly explained by increased errors in DNA replication and chromosomal segregation in cells that are rapidly dividing, other mechanisms are clearly operative, involving telomeres and telomerase.[73,75–79] Although many of these changes are not "recurrent" and appear to be random products of instability, some may in fact contribute to a proliferative crisis.[80] This is consistent with Tomasetti and Vogelstein's observation that initiation events for many tumors occur early in life, during highly proliferative stages of tissue growth and remodeling, but they become evident later in life when one or a few critical mutations allow the transformed cell to reach this crisis stage.

Tumor-Promoting Inflammation

The role of inflammation in cancer has received considerable attention in the past 20 years. Although our understanding of this phenomenon remains incomplete, it clearly met the criteria for inclusion as an "enabling hallmark" in the updated Hanahan and Weinberg model.[31] The importance of inflammation was



• **Fig. 1.4** Models of metastasis evolution and implications of genetic heterogeneity. (A) The classic simple linear model, in which clones sequentially arise that dominate the primary tumor owing to survival and proliferative advantages. (B) The early dissemination and parallel evolution model, in which tumor cells begin to disseminate early in the primary tumor life span and continue to evolve somatically in parallel with the primary tumor during clinical dormancy until they acquire metastatic capacity and proliferate into a clinically relevant lesion. (C) The late dissemination model, in which tumors evolve over time until a late-arising subclone is able to seed multiple metastases successfully. (D) Late dissemination from multiple metastatically competent subclones within the primary tumor. Metastases seeded by this mechanism share all the somatic events acquired by the tumor preceding the divergence of the different metastatically competent primary tumor subclones. The blizzard symbol indicates somatic genetic alterations. (Reproduced with permission from Hunter KW, Amin R, Deasy S, et al. Genetic insights into the morass of metastatic heterogeneity. *Nat Rev Cancer*. 2018 April;18(4):211-223. https://doi.org/10.1038/nrc.2017.126. Fig. 1.1.)

inferred from the earliest microscopic studies of cancer, but it was a seminal paper by Dvorak in 1986, in which he described tumors as "wounds that never heal,"[81] that provided synthesis for the recurrent observation that tumors often were infiltrated by inflammatory cells of the innate immune system (granulocytes, histiocytes, and macrophages) and the adaptive immune system (lymphocytes). Mechanistic distinctions between inflammation that favors tumor growth and inflammation that retards growth or eliminates the tumor remain to be defined[82–84]; however, it can be concluded confidently that inflammation contributes to tumor growth and survival by supplying factors that sustain proliferation; factors that limit cell death; proangiogenic factors; extracellular matrix-modifying enzymes that facilitate angiogenesis, invasion, and metastasis; and other signals that lead to activation of EMT and other hallmark-facilitating programs.[31] Inflammatory cells also release reactive oxygen species that are actively mutagenic for nearby cancer cells, accelerating their genetic evolution toward states of heightened malignancy.[85]

Deregulating Cellular Energetics

In the early years of the 20th century, Otto Warburg observed that cancer cells preferentially used glycolytic (anaerobic) rather than oxidative (aerobic) pathways to generate, energy even under conditions of normal or high oxygen. This metabolic peculiarity of cancer cells, called the *Warburg effect*, seems to be driven by

activated oncogenes and/or by loss of tumor suppressor genes, providing cancer cells with selective growth and survival advantages by conferring the hallmark capabilities of cell proliferation, avoidance of cytostatic controls, and attenuation of apoptosis. The reliance of cancer cells on glycolysis can be further accentuated under hypoxic conditions. In fact, Warburg-like metabolism seems to be present in rapidly dividing embryonic tissues, suggesting a role in supporting large-scale biosynthetic programs that are required for active cell proliferation.

Cancer cells do not seem to enable the Warburg effect universally. Rather, much like other cells with high energetic demands, they seem to sort out into lactate-secreting (Warburg) and lactate-consuming cells, providing an efficient, albeit homeostatically disturbed, energy environment. Furthermore, it seems that oxygenation is not static in tumors, but instead fluctuates temporally and regionally as a result of the instability and chaotic organization of tumor-associated neovasculature. Altered energy metabolism is proving to be as widespread in cancer cells as in many of the other cancer-associated traits that have been accepted as hallmarks of cancer. This realization raises the question of whether deregulating cellular energy metabolism is a core hallmark capability of cancer cells that is as fundamental as the six well-established core hallmarks. In fact, the redirection of energy metabolism is largely orchestrated by proteins that are involved in one way or another in programming the core hallmarks of cancer. When viewed in this way, aerobic glycolysis is simply another phenotype that is programmed by proliferation-inducing oncogenes, and the designation of reprogrammed energy metabolism as an emerging hallmark seems most appropriate.

It is worth noting that this characteristic of tumor cells provides at least one important diagnostic advantage. Upregulation of the major glucose transporter, GLUT-1, is seen in virtually all tumors, making the cells efficient glucose scavengers. This can be exploited to image tumor cells precisely and noninvasively by visualizing glucose uptake using positron emission tomography (PET) with a radiolabeled analog of glucose (^{18}F-fluorodeoxyglucose, or ^{18}F-FDG) as a reporter. The combination of PET with computed tomography (PET-CT) now is one of the most robust means to evaluate composition of tumors, minimal residual disease, and tumor-specific objective responses in patients receiving conventional and experimental therapies, and it increasingly is being applied to improve the diagnosis and staging of dogs with cancer.[86–88]

In addition, evidence is accumulating that deregulated cellular energetics contribute to tumor progression through immunomodulation. Our group has proposed a model in which cancer cells' self-renewal is causally related to reprogramming of fatty acid metabolism and immune signaling[89]; this supports the notion that fate decisions of tumor-initiating or stem cells rely on cellular metabolism and immunomodulation in the tumor microenvironment.

Avoiding Immune Destruction

Burnet and Thomas proposed the concept that the immune system can recognize and destroy incipient tumors (cancer immunosurveillance) in the 1950s.[90] Their hypothesis was far ahead of its time, and technologic obstacles impeded proof, so the theory fell into disfavor. In recent years the immunosurveillance theory has gained traction anew because data strongly suggest that the immune system helps to keep tumors at bay, and thus tumors must evade the immune response to survive. In its recent incarnation, the theory has been refined to incorporate the concept of immunoediting, in which the immune system destroys strongly antigenic tumor cells, providing weakly antigenic cells a survival advantage.[90] Experimental evidence for this concept includes differences between tumors grown in immunocompetent mice (only weakly antigenic tumors survive) and immunocompromised mice (no selection against strongly antigenic tumors is observed), but evidence only now is emerging that will allow us to understand the importance of immunoediting in spontaneous cancers.

That the tumor microenvironment forms and maintains an immunosuppressive barrier provides more compelling evidence for the role of the immune system in limiting tumor growth and metastasis.[91,92] This immunosuppressive barrier includes cellular factors, such as regulatory T cells (T_{regs}), myeloid-derived suppressor cells (MDSCs), and MSCs. Soluble factors, including transforming growth factor-β (TGF-β) and immunoglobulins, also contribute to the immunosuppressive barrier directly and indirectly.[93] This is an active area of basic and clinical research in which companion animal oncology has been at the forefront; for example, through the generation and approval of the first active gene-based therapeutic cancer vaccine for canine melanoma.[94]

A transformational advance in cancer therapy has been the ability to block immune checkpoints that are engaged by a number of tumors—particularly those that have unstable genomes and tend to generate greater numbers of neoantigens (Fig. 1.5).[95,96] Antibodies against cytotoxic T lymphocyte-associated protein 4 (CTLA-4), and against programmed cell death protein 1 (PD-1) and its corresponding ligand, programmed death-ligand 1 (PD-L1), aim to reactivate tumor-specific T cells and cause a robust antitumor immune response.[97,98] The remarkable responses observed in patients receiving immune checkpoint blockade as adjunctive or first-line therapy have made this class of compounds part of the standard of care for several types of lung cancer, malignant melanoma, renal cell carcinoma, Hodgkin lymphoma, head and neck squamous cell carcinoma, urothelial carcinoma, certain colon cancers, and certain liver cancers.

Checkpoint inhibitors currently are being tested in canine clinical trials[99]; expression of canine PD-L1 has been detected in a number of canine tumor types, including mastocytoma, melanoma, renal cell carcinoma, and several others.[100] Canine CTLA-4 has been identified and cloned,[101] and although canine anti–CTLA-4 has not yet been developed, an agonistic recombinant canine CTLA-4 molecule has been successfully used to induce tolerance in a transplant model.[102] (Chapter 14 presents additional information about cancer immunotherapy.)

Adaptive Evolution and the Tumor Microenvironment

A bidirectional flow of information occurs between the tumor and the microenvironment, with each helping to mold the other into functional growing tissue that can evade or withstand attack by the host.[103] The previous reference to a "selective growth advantage" that is reminiscent of darwinian selection is not accidental. The clonal evolution theory[104] addresses the significance of sequential genetic changes providing growth and survival advantages; however, to this must be added the fact that, in addition to these self-sufficient events that influence growth and survival, tumor cells must also evade "predators" (e.g., inflammation and the immune system).[90,105] In essence, the interaction of the tumor