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Genetically Engineered Mouse Models in Cancer Research

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Abstract

Mouse models of human cancer have played a vital role in understanding tumorigenesis and answering experimental questions that other systems cannot address. Advances continue to be made that allow better understanding of the mechanisms of tumor development, and therefore the identification of better therapeutic and diagnostic strategies. We review major advances that have been made in modeling cancer in the mouse and specific areas of research that have been explored with mouse models. For example, although there are differences between mice and humans, new models are able to more accurately model sporadic human cancers by specifically controlling timing and location of mutations, even within single cells. As hypotheses are developed in human and cell culture systems, engineered mice provide the most tractable and accurate test of their validity *in vivo*. For example, largely through the use of these models, the microenvironment has been established to play a critical role in tumorigenesis, since tumor development and the interaction with surrounding stroma can be studied as both evolve. These mouse models have specifically fueled our understanding of cancer initiation, immune system roles, tumor angiogenesis, invasion, and metastasis, and the relevance of molecular diversity observed among human cancers. Currently, these models are being designed to facilitate *in vivo* imaging to track both primary and metastatic tumor development from much earlier stages than previously possible. Finally, the approaches developed in this field to achieve basic understanding are emerging as effective tools to guide much needed development of treatment strategies, diagnostic strategies, and patient stratification strategies in clinical research.

I. INTRODUCTION

In 2008, for the first time in 10 years of reporting, the incidence and death rates from cancer declined significantly (Jemal *et al.*, 2008). Although encouraging, there were still just under 1.5 million new cases of cancer in the United States, and just over 500,000 deaths per year. The decrease is attributed to a reduction in the most common cancers, lung, prostate, colorectal, and breast, likely due to better screening for early detection of cancer and reduced tobacco use (Jemal *et al.*, 2008). Among the remaining cancers, rates remained stable or increased, highlighting the need for continued research to develop better prevention, detection, and treatment of cancer.

Although improvements in prevention and early detection are leading to a drop in cancer incidence and death, the ability to treat established cancers is still quite limited. Relatively new therapies, such as Gleevec, bevacizumab, and herceptin, are beginning to make a difference to patients with particular molecular subtypes of cancer, demonstrating the power of correctly targeted molecular-based therapy (Normanno *et al.*, 2009; Soverini *et al.*, 2008; Tan *et al.*, 2008), but also highlighting the limitation in our understanding of the molecular pathways critical to cancer and how they vary between individuals. Cancer is a complex disease in which normal cellular pathways are altered to give rise to the properties leading to cancer, such as inappropriate growth, survival, and invasion. While the study of human tumors has yielded many insights into the molecular changes present in cancers, more rigorous testing of hypotheses through experimental manipulation is necessary to better

understand which changes are causative, and therefore targetable, and which are secondary. Mouse model systems provide an experimentally tractable mammalian system to test the hypotheses generated from the observation and study of human tumors, and also provide opportunities to identify novel mechanisms to be confirmed in human tumors. Cross-species comparison has proven to be powerful in improving the understanding of a wide variety of human diseases, including cancer (e.g., see Brown *et al.*, 2008; Kim *et al.*, 2005; Wang and Paigen, 2005; Wang *et al.*, 2005). We describe in this chapter the engineering tools and methods currently available to model cancer in the mouse, and highlight how some key challenges have been and are being addressed using these sophisticated approaches.

Humans are a highly heterogeneous population with variation in genetic background, diet, and environmental exposures. Any of these variables can affect how cancer manifests itself, and thus can become confounding issues in the analysis of human tumors. Studies in mice allow researchers to control these variables to simplify experiments and ask well-structured questions. For example, genetic background can be held constant while testing the effects of diet or carcinogens on cancer, and diet/environment can be held constant while examining the effects of genetic changes on tumorigenesis. As mammals, mice share many anatomical, cellular, and molecular traits with humans that are known to have critical functions in cancer, such as an immune system, maternal effects in utero, imprinting of genes, and alternative splicing. Thus, mice provide an experimentally tractable model system with a wealth of developed research tools to understand basic mechanisms of cancer.

One of the difficulties and frustrations in the development of cancer therapies is that preclinical studies have historically had limited predictive value for the efficacy of drugs in patients. Preclinical studies have been relatively predictive of toxicity in humans, such that many drugs that enter Phase I clinical trials are taken forward to Phase II clinical trials due to tolerable toxicity profiles. In contrast, most drugs (95%) are dropped in Phase II and Phase III clinical trials because of limited efficacy against cancer (e.g., see Sharpless and Depinho, 2006). There are several potential explanations for this. It is clear that some drugs are only effective in a subset of patients (Haas-Kogan *et al.*, 2005; Mellinghoff *et al.*, 2005), but because current preclinical testing strategies do not model the relevant molecular and cellular milieu, drug specificity for subtypes can go unappreciated. Indeed, preclinical vetting of drugs is largely performed on xenografts of human tumor cell lines grown subcutaneously in immune-compromised mice. It is now clear, however, that cancer stroma is distinct from normal stroma and provides a supportive microenvironment for tumor growth. Human cancer xenografts lack the appropriate cancer stroma support when grown over relatively short periods of time in immune-compromised mice. As a result, they may be more vulnerable to drug treatment than cancers that have coevolved with their stroma (Olive *et al.*, 2009). Similarly, inflammatory responses play an integral role in cancer development (de Visser and Coussens, 2006; Tlsty and Coussens, 2006), but are not effectively modeled in xenografts. Moreover, immune-compromised mice could mount a limited immune attack on engrafted cells (Quintana *et al.*, 2008), making them more susceptible to treatment.

Mice engineered with cell-specific human cancer-associated aberrations can address many of these issues, as tumors arise *de novo* in the context of a normal immune system and coevolve with surrounding stroma. Furthermore, more than 100 years of genetic research in the mouse has provided powerful strategies for assessing the complex genetics of disease susceptibility, therapeutic responses, and associated toxicities that are so diverse among the human population. Identifying these parameters is key to the development of personalized medicine; while current technologies have accelerated the pace of human epidemiological association studies, there is no doubt that discovery can be accelerated by cross-species comparisons. Finally, several initiatives are under way to use the *de novo* mouse cancer models in preclinical guidance for human clinical trials. While challenging due to the

complexity of the human diseases and the difficulty of modeling them accurately in mice, early studies indicate that this approach offers great promise, opening up a new era of translational research using engineered mouse models (Becher and Holland, 2006; Carver and Pandolfi, 2006; Frese and Tuveson, 2007; Gutmann *et al.*, 2006; Huse and Holland, 2009; Pritchard *et al.*, 2003; Sharpless and Depinho, 2006).

While subcutaneous xenograft models have not been predictive for targeted cancer therapies in humans, they continue to provide a simple testing ground for triage and pharmacodynamic studies prior to analysis in more complex models. However, of significance, xenograft approaches refined to better model the tumor microenvironment may hold significant promise in predicting some human therapeutic outcomes and are currently under study. These methods include the transplantation of human primary tumors into immunocompromised mice at the orthotopic site and into mice that have been “humanized” with transplanted human hematopoietic and other organ systems (reviewed in Bibby, 2004; Legrand *et al.*, 2009; Richmond and Su, 2008; Sharpless and Depinho, 2006). In the end, it is likely that a series of integrated model systems will hold the most power in discovery and prediction of effective human disease management. The uses of xenograft models have been reviewed elsewhere (Lee *et al.*, 2007; Pegram and Ngo, 2006; Sausville and Burger, 2006; Teicher, 2009; Troiani *et al.*, 2008), and we focus in this chapter on genetically engineered mouse models of cancer. We describe here the state of the art techniques for modeling cancer in mice both for basic mechanistic research and for preclinical applied studies. We also review the issues in cancer research for which these mouse models are particularly useful due to the preservation of temporal disease development, including coevolution of cancer and stromal compartments.

II. GENERATION OF GENETICALLY ENGINEERED MOUSE MODELS

With the availability of the complete sequence of the mouse genome, technology to manipulate the mouse genome, and well-defined inbred strains as well as extensive information on the polymorphisms among strains, the ability to engineer mice to test hypotheses of tumorigenesis is impressive. Experiments can now be easily undertaken to assess the outcome when the function of a gene is lost, mutated, underexpressed, or overexpressed in the appropriate cell types *in vivo*. In addition to individual research efforts, several organized initiatives have been launched using a variety of approaches to systematically target every gene in the genome (Adams *et al.*, 2004; Friedel *et al.*, 2007; Hansen *et al.*, 2008; Schnutgen *et al.*, 2005; Skarnes *et al.*, 2004; To *et al.*, 2004). Additionally, much can be learned about the natural history of cancer through the use of reporters to image molecular, cellular, and anatomical changes during tumorigenesis. Finally, mutagenesis studies in the mouse have identified new cancer-causing mutations that can then be confirmed in studies of human cancers.

A. Studying Loss of Gene Function in Mouse Models

1. MOUSE GENE KNOCKOUTS—Studying the loss of function of genes provides insight into understanding the biological functions for which the protein product is required. Loss-of-function studies most commonly use “knockout” strategies to remove the gene of interest by engineering constitutive or conditional deletions in the gene. For genes that span large genomic regions, deletion of the first few exons encoding the start codon is often sufficient to block transcription or translation into a functional protein product. However, sometimes an alternative start codon or alternative splicing can lead to a truncated protein product with partial function that can mask the significance of the gene of interest in biological loss-of-function studies. Thus, careful molecular characterization of genetically engineered alleles is important to verify that the function of the gene is truly lost and also that additional inadvertent gene rearrangements or deletions are not present.

In translational cancer research, loss-of-function studies provide a powerful approach to assessing the potential validity of targeted therapies, since the target can be specifically inactivated in the context of a developing or developed tumor. In this approach, accurate interpretation requires understanding of any functional redundancy differences between mouse and human. For example, if one species can utilize alternative gene products in a cancer pathway, but the other cannot, results will not be concordant. Fortunately, careful study using existing technologies, along with the available extensive genomic information, allows for this assessment and provides additional information on which to base further therapeutic design.

In basic research studies, the use of knockout strategies have been critical in understanding cause and effect relationships in cancer development, and can be applied to the assessment of many gene classes, including oncogenes, tumor suppressor genes, and metabolic (“housekeeping”) genes. The classification of a gene as a tumor suppressor depends on the demonstration that impaired function can facilitate tumor development, and that can only be achieved in the context of a developing tumor within the host. Germline loss-of-function (homozygous deletion) studies often lead to embryonic lethality precluding assessment for adult diseases, making conclusive determination of tumor suppressor genes more difficult. In many cancer models, however, animals heterozygous for a tumor suppressor knockout allele are susceptible to tumor formation either due to haploinsufficiency (impaired function from insufficient levels) or by somatic loss of the wild type allele (Cichowski *et al.*, 1999; Kost-Alimova and Imreh, 2007; Macleod and Jacks, 1999; Reilly *et al.*, 2000; Zheng and Lee, 2002). Nonetheless, a more valid and versatile approach is to conditionally induce a somatic mutation, as discussed below. Use of loss-of-function mouse models to study cancer has been extensively reviewed elsewhere (Maddison and Clarke, 2005; Van Dyke and Jacks, 2002). Knockout gene targeting strategies and techniques have also been reviewed extensively (Capecchi, 2005; LePage and Conlon, 2006; Mikkola and Orkin, 2005; Porret *et al.*, 2006) and will not be discussed in detail here.

2. MOUSE CONDITIONAL GENE MUTATIONS—With conventional knockouts, loss of a vital gene can often lead to embryonic lethality, severe developmental abnormalities, or adult sterility, making it impossible to study the gene in the desired disease context. For example, one cannot study the role of genes such as BRCA1 and BRCA2 in breast cancer if the animals die before birth or adulthood (Evers and Jonkers, 2006). In addition, ablation of the gene of interest in the entire body does not mimic spontaneous tumorigenesis in humans, where tumors evolve in a wild-type environment, and the timing of gene loss may be a critical factor in disease development.

To circumvent conventional knockout limitations, sophisticated conditional genetic engineering technology has been developed to create systems where genetic events can be tightly controlled spatially and temporally. Bacterial Cre and yeast FLP enzymes are site-specific recombinases that catalyze specific recombination between defined 34 bp *loxP* and *FRT* sites, respectively (Branda and Dymecki, 2004). Therefore, in the presence of Cre or FLP protein expression, homologous recombination is induced between *loxP* or *FRT* sites that flank the gene of interest and are oriented in the same direction, thus recombining out the flanked genetic sequence and deleting the gene of interest. By temporally and spatially controlling expression of the recombinase, it is then possible to temporally and spatially control deletion of the gene of interest, overcoming interference from developmental abnormalities and lethality (Branda and Dymecki, 2004). Mice carrying the Cre or FLP recombinase under control of a tissue-specific promoter are crossed with mice carrying the gene of interest flanked by *loxP* or *FRT* sites to conditionally knockout the gene in a specific tissue or cell type, such as progenitor cells, or at specific times during development.

Multiple types of Cre delivery systems have been developed to temporally and spatially control Cre expression. These include promoter-driven cell or tissue-specific (discussed above), viral, somatically introduced, and temporally inducible systems. Viral Cre, such as adenoviral or lentiviral Cre, in which the *Cre* gene is packaged into viral particles, can be locally delivered topically or by injection to infect cells and create a regional or clonal knockout of cells within a given area (Jackson *et al.*, 2001; Marumoto *et al.*, 2009). Both adeno- and lentiviral vectors have advantages and disadvantages that should be considered in the context of the experiment (DuPage *et al.*, 2009). For example, adenoviral vectors typically induce a significant inflammatory response that can confound interpretations of causation. However, adenoviral infection is transient and does not result in viral genome insertion. Lentiviruses are suitable for infecting nondividing somatic cells while adenovirus requires dividing cells. Lentiviral genome integration can also present a confounding variable due to insertion site mutation, although there is evidence that this may be less of an issue with lentivirus than with other insertional viruses (Gonzalez-Murillo *et al.*, 2008; Montini *et al.*, 2009). Nonetheless, the ability to mark a clone by a specific insertion site or long-term expression of a viral gene product also offers a strategy to track tumorigenesis (De Palma *et al.*, 2003; Hosoda *et al.*, 2009).

Conditional Cre systems that temporally control Cre expression (e.g., using the tet promoter or ER fusion systems described below) are continuing to be developed and offer the greatest amount of control over gene removal. Two of the most commonly used systems are the tetracycline-inducible system (Gossen and Bujard, 1992) and the tamoxifen-inducible system (Metzger and Chambon, 2001) (see also Bockamp *et al.*, 2008, and references therein). The tetracycline-repressor-based system is composed of a transactivator and an effector. The DNA-binding domain of the *Escherichia coli tetR* gene fused to the transactivation domain of the herpes simplex virion protein 16 (VP16) gene (tetR/VP16) makes up the tetracycline-controlled transactivator (tTA) that can then be driven by tissue-specific promoters (Baron *et al.*, 1997). The tTA binds to the tetracycline operator (tetO) that controls the activity of the human cytomegalovirus promoter driving conditional gene expression, including Cre to generate conditional knockouts. In this Tet-Off system, tTA is bound by tetracycline, or its more stable analogue doxycycline, inhibiting association with the tetO and blocking gene transcription. In the Tet-On system, the tetracycline-repressor has been mutated (rtTA) such that it is only in the proper conformation for association with tetO when it is bound to tetracycline or doxycycline, thus inducing expression of the gene in the presence of drug. The expression of tTA or rtTA can lead to cytotoxicity, likely due to overexpression of the VP16 transactivator domain and titration of transcription factor machinery (Berger *et al.*, 1990), which has spurred the development of next-generation tTAs (Bockamp *et al.*, 2008). Success using the tetracycline-inducible systems depends on screening transgenic mouse lines for tight repression, good induction, and appropriate levels of repressor expression to avoid secondary toxicity issues.

The tamoxifen-inducible system depends on fusion of the Cre recombinase gene to a mutated ligand-binding domain of the human estrogen receptor (Cre-ER(T)) that is specifically activated by tamoxifen. In the absence of tamoxifen, the ER fusion protein is excluded from the nucleus, but is transported to the nucleus upon binding to tamoxifen where Cre can then recombine DNA. Using these techniques, temporal expression of Cre can be manually controlled by simply delivering or withholding tamoxifen. Other, less commonly used inducible systems include the insect steroid molting hormone ecdysone (No *et al.*, 1996), the progesterone antagonist mifepristone (Ngan *et al.*, 2002), the Lac operator-repressor (Cronin *et al.*, 2001), and the GAL4/UAS system (Wang *et al.*, 1999). While all of these inducible systems have been shown to be functional in the mouse, they differ in their efficiency and leakiness that affects tight control of gene expression. Because recombinase-

mediated excision of the gene is irreversible, leaky Cre expression can have substantial and permanent effects that could compromise experimental outcomes.

3. MOUSE MODELS OF RNA INTERFERENCE—Alternatively, loss-of-function studies can use RNA interference (RNAi) to specifically knockdown the expression of target genes posttranscriptionally before the mRNA can be translated into protein (Meister and Tuschl, 2004). Target sequence-specific small interfering RNAs (siRNAs) are short antisense peptides 21–28 nucleotides long. The RNA-induced silencing complex (RISC) recognizes the double-stranded siRNA fragments and cleaves the endogenous complementary messenger RNA (mRNA) that is then rapidly degraded. Though siRNA has been widely used to knockdown expression of target genes, it is limited to transient transfection *in vitro*. Short hairpin RNA (shRNA) can be used to cause long-term gene knockdown, both *in vitro* and *in vivo*. The shRNAs are much longer, generally between 50 and 70 nucleotides in length, allowing them to be transcribed as stable messages *in vivo*. They are made as a single-strand molecule that then forms a short hairpin tertiary structure, folding in on itself to form a stem–loop structure *in vivo*. After transcription and folding, the enzyme Dicer cleaves off the loop leaving behind a double-stranded siRNA molecule that can then be recognized by RISC. The shRNA sequences can be cloned into viral vectors to be stably incorporated into the genome for sustained knockdown of target genes.

Several groups have used shRNA-expressing constructs to successfully create RNAi transgenic knockdown animals (Sandy *et al.*, 2005). The shRNA expressing mice can be generated by inserting the promoter-shRNA construct into the mouse genome using normal knockout and transgenic techniques, including embryonic stem cell electroporation as well as lentiviral shRNA transgene injection into the pronucleus of fertilized eggs or the perivitelline space of single-cell mouse embryos. Knockin shRNA animals can also be made using homologous recombination to target the insertion of the shRNA transgene to a specific site, such as the ubiquitously expressed *Rosa26* locus. Since shRNAs are promoter-driven, they can be constructed using inducible, reversible, or tissue-specific promoters (Dickins *et al.*, 2007), or with lox-STOP-lox (LSL)(described in Section II.B.3), or other Cre-lox-regulated control elements (Tiscornia *et al.*, 2004; Ventura *et al.*, 2004). Recently, Cre-regulated RNAi was achieved by combining a Cre-regulated shRNA transgene within an FLP expression cassette such that the RNAi transgene and a green fluorescent protein (GFP) reporter are inverted upon recombination to induce expression (Stern *et al.*, 2008). Although these methods have shown promise, they do have limitations, especially regarding the incomplete penetrance of the silencing, possibly due to varying expression of shRNAs from retroviral constructs (reviewed in Westbrook *et al.*, 2005).

As the details of RNAi in mammalian cells became better understood, new shRNAs have been developed that take advantage of natural miRNA biogenesis (Silva *et al.*, 2005; Westbrook *et al.*, 2005). Artificial vectors are designed that contain the shRNA sequence within miR30 (Silva *et al.*, 2005), a naturally occurring, well-characterized microRNA transcript (Cullen, 2004; Zeng *et al.*, 2002). The expression of mature shRNA and subsequent knockdown of the target gene are greater when expressed from the endogenous miR30 promoter than from exogenous promoters, due to more efficient microRNA biogenesis (Westbrook *et al.*, 2005). Current studies are focusing on utilizing lentiviral vectors to deliver shRNA-miRs and generate *in vivo* mRNA knockdown (Singer and Verma, 2008; Stegmeier *et al.*, 2005).

Since RNAi results in a knockdown of gene expression rather than complete inhibition, RNAi transgenic animals are likely to be hypomorphs as opposed to null phenocopies. RNAi is also susceptible to nonspecific effects and off-target repression of other genes. Nevertheless, RNAi animal models are a powerful approach to studying gene knockdown,

because targeting constructs can be rapidly designed and tested, genes that give a lethal phenotype when knocked out can be studied without needing the complex genetic crosses involved in conditional knockouts, and model organisms can be studied for which knockout technology has not been developed, such as the worm and rat.

4. MOUSE SINGLE-CELL KNOCKOUTS—Human cancers are spontaneous diseases caused by accumulation of somatic mutations that arise from single mutant cells. Sporadic tumors developing in a wild-type or heterozygous microenvironment can develop differently compared to those developing in a homogeneous genetically mutant environment. Therefore, conventional knockout, knockin, and transgenic GEM tumor models, even tissue-specific or cell-specific models, in which the deletion or mutation occurs in a whole organ or animal, are limited in that they cannot reproduce the clonal nature of human cancer (Fig. 1). Tumors from heterozygous mouse models, such as *p53*^{+/-} mice, arise from clonal expansion of cells that have sporadically lost the wild-type allele of the tumor suppressor. While these loss of heterozygosity-dependent models more closely recapitulate the clonal nature of human tumors, the incidence and spectrum of spontaneous tumor development is dependent on variables that affect the stochastic nature of the loss of heterozygosity event (Attardi and Donehower, 2005). Single-cell knockout approaches can be used to control tumor-initiating events by driving genetic events in individual cells, generating homozygous mutant cells on a heterozygous mutant background at very low frequency to create *in vivo* mosaics (reviewed in Lozano and Behringer, 2007). The clonal nature of single-cell knockouts can be used to more clearly elucidate the mechanisms of cellular processes such as cell cycle control and tumor formation in the context of a more normal microenvironment, potentially redefining the roles of genes previously studied in other GEM models.

Sporadic single-cell knockout mouse models have been created using genetic techniques derived from *Drosophila* systems. Recombinase enzymes can be used under tissue specific and temporal controls to induce rare G2 mitotic interchromosomal recombination events between *FRT* or *loxP* sites residing on homologous mouse chromosomes, yielding a wild-type and a mutant daughter cell amidst a population of heterozygous cells. During the G2 phase of the cell cycle, homologous chromosomes align in preparation for mitosis. While aligned, the Cre or FLP recombinase can recombine the homologues such that regions of the chromosome are homozygosed in the daughter cells. For example, Wang *et al.* (2007) used a ubiquitously expressed recombinase to induce rare interchromosomal recombination events between *FRT* or *loxP* sites centromeric to *p53* and generate single cell *p53*^{-/-} clones (Wang *et al.*, 2007) within a *p53*^{+/-} environment. The sporadic *p53* knockout phenotype more closely resembles human Li-Fraumeni syndrome, in which the human *p53* gene is mutated, than previous models. This system can be used to target loss of heterozygosity events to particular cells or tissues using different Cre or FLP recombinase mouse lines.

The mosaic analysis with double markers (MADM) approach described by Muzumdar *et al.* (2007) uses a tissue-specific Cre/*loxP* system to induce sporadic mitotic interchromosomal recombination and knockout target genes while simultaneously labeling resultant daughter cells (Muzumdar *et al.*, 2007). Homozygous knockout and wild-type daughter cells are identified with red and green markers and further distinguished from neighboring yellow heterozygous cells. Thus, MADM allows progeny from single loss of heterozygosity events to be tracked and distinguished from normal cells within the microenvironment.

B. Studying Gain of Gene Function in Mouse Models

1. MOUSE CONSTITUTIVE TRANSGENIC MODELS—Gain-of-function studies are often used to study oncogenes in mouse models. Transgenic or knockin animals constitutively overexpressing an oncogene can be used to study how the oncogene drives

tumorigenesis *in vivo*. Transgenic animals have been very useful in studying many oncogenes and for creating Cre-expressing mice and other inducible systems. Transgenic animals are created by the pronuclear injection of transgenes directly into fertilized oocytes, followed by implantation into pseudopregnant females (Macleod and Jacks, 1999; Porret *et al.*, 2006). The transgene is randomly incorporated into the genome and thus can incorporate into a gene necessary for development or fertility, causing deleterious effects and limiting the usefulness of the transgenic model. Furthermore, the epigenetic regulation of gene expression in the region surrounding the transgene integration can affect transgene expression levels and often result in silencing. Therefore, multiple founders must be screened to confirm adequate and specific expression of the transgene.

In addition to insertion site effects, transgenes often do not show the expression patterns of the endogenous gene promoter, due to the lack of regulatory sequences that can be located several kilobases from the coding region of the gene of interest. Bacterial artificial chromosomes (BAC) contain large fragments of genomic DNA (150–300 kb), and therefore can accommodate entire genes, including the cis-regulatory sequences, allowing for increased fidelity of gene expression (Shizuya *et al.*, 1992). Under control of the gene's regulatory elements, the gene of interest will likely show the endogenous expression pattern (Swing and Sharan, 2004). Due to their large size, BACs are more likely to insert at a lower copy number, increasing the chances of single copy transgenics, which will be expressed at endogenous levels due to the regulatory sequences. Although the effects of additional genes on the BAC must be considered, molecular analysis of the BAC can be used to eliminate the possibility of other known genes being present, as well as confirm the presence of the entire coding sequence of the gene of interest (Swing and Sharan, 2004). With the development of recombineering technology (Sharan *et al.*, 2009), large BACs can be rapidly modified and used to generate transgenic mice to compare the phenotypic effects of precise changes in a gene (Chang *et al.*, 2009) or regulatory sequence.

2. MOUSE GENE KNOCKIN MODELS—To circumvent transgenic limitations associated with random insertion, knockin mice are created by inserting a gene of interest into a specific region of the genome using homologous recombination techniques, much like those used when creating knockouts. The *Rosa26* locus is commonly used as an insertion site for knockin animals because it is devoid of essential genes and allows for good expression of the transgene (Friedrich and Soriano, 1991). While transgenics have the potential for multiple insertions of the transgene, knockin animals carry only one copy of the transgene. Knockin approaches can also be used to replace a normal gene copy with a mutated version of the gene to examine specific mutational events in the context of normal control of the gene (e.g., Johnson *et al.*, 2001; Lang *et al.*, 2004).

3. MOUSE CONDITIONAL OVEREXPRESSION MODELS—Transgenic and knockin expression of deleterious genes may lead to lethality, sterility, and developmental defects that impede study of the gene of interest in cancer, as seen with many conventional knockouts. Therefore, spatial and temporal control of transgene and knockin expression may be necessary to circumvent these limitations. Conditional transgenics and conditional knockins can be created using tissue-specific promoters to constitutively drive expression or created by inserting a strong translational and transcriptional termination (STOP) sequence flanked by *loxP* or *FRT* sites in between the promoter sequence and the gene of interest (Lakso *et al.*, 1992). Examples of commonly used STOP cassettes are the lox-STOP-lox (LSL) in which multiple STOP sequences are arrayed between *loxP* sites (Jackson *et al.*, 2001) and the NEO-STOP cassette in which the neomycin resistance gene and a STOP sequence is inserted between *loxP* sites (Dragatsis and Zeitlin, 2001). The presence of the STOP sequence blocks transcription of the gene of interest. However, in the presence of Cre or FLP recombinase, the STOP cassette is removed, allowing expression. Since gene

expression is dependent on excision of the STOP cassette and recombinase expression, gene expression can be spatially, temporally, and inducibly controlled with the myriad of Cre systems. STOP cassettes can be leaky and thus must be assessed empirically. STOP technology has greatly expanded the opportunities for controlling gene expression in gain-of-function studies. For example, initial *K-rasG12D* transgenic models of lung carcinoma showed a large variance in tumor penetrance, as well as death due to respiratory failure prior to tumor progression (Johnson *et al.*, 2001). Treatment of the conditional *LSL-K-ras G12D* strain with virally-infected allowed control of the timing, location, and number of tumors, therefore allowing the study of initiation and early-stage pulmonary adenocarcinoma (Jackson *et al.*, 2001).

C. Modeling Chromosomal Translocations in Mice

1. TRANSLOCATOR MICE—Human cancers, such as leukemia, often involve chromosomal translocations that lead to fusion proteins with new functions that play a role in tumorigenesis. Mouse models have been developed to study *de novo* protein functions as a result of chromosomal translocations. Basic approaches such as transgenic or knockin insertion of fusion genes resulting from human chromosomal translocations can lead to embryonic lethality or nonauthentic phenotypes (Prosser and Bradley, 2003). While this can be circumvented with conditional transgenic or conditional knockin approaches, these approaches still suffer from the limitation that they produce a population of cells expressing the fusion gene within the specified tissue compartment and do not recapitulate the clonal onset of the disease in humans. Translocation- derived cancers develop from a single cell that undergoes chromosomal translocation and then clonally expands in an environment that is initially composed of normal cells. Therefore, a more physiological approach to mimic chromosomal translocations in human disease is through induction of the chromosomal translocation event in mice (Forster *et al.*, 2003, 2005a; Prosser and Bradley, 2003). This can be accomplished by inserting *loxP* sites in trans at chromosomal breakpoints observed in human cancers, resulting in Cre-dependent interchromosomal reciprocal translocation (Forster *et al.*, 2003; van der Weyden *et al.*, 2009) (Fig. 2). This is similar to the approach used for single-cell knockouts, described above, except that the *loxP* sites are on different chromosomes, rather than homologous chromosomes. As with single-cell knockouts, the interchromosomal recombination is a rare event and as such more closely mimics the situation seen in human cancers. If the chromosomal translocation results in a fusion protein with *de novo* function animals may develop cancers similar to those in humans. For example in a mouse model of the MLL-ENL translocation fusion protein, the animals can develop a leukemia very similar to humans, with high penetrance and most likely from a clonal origin (Forster *et al.*, 2003). Since not all mouse models using this approach successfully develop cancer (Buchholz *et al.*, 2000; Collins *et al.*, 2000), it is very likely that cancer development resulting from Cre-driven interchromosomal reciprocal translocations are dependent on the expression level and distribution of the Cre transgene expression (Forster *et al.*, 2003). This could reflect the importance of the translocations occurring in the proper cell type, compartment and/or fusion gene sequence for cancer development. The success of this approach also depends on the compatibility of the transcriptional orientation of the two mouse chromosome genes.

2. INVERTOR MICE—Since not all fusion genes arising from chromosomal translocations in human cancers are compatible with the Cre-driven interchromosomal translocation approach, a conditional inverter approach has been used to study the *de novo* function of fusion genes in mice (Forster *et al.*, 2005b; Lobato *et al.*, 2008). Chromosomal inversions occur when two breaks occur within the same chromosome and the genetic material in between the breaks is inverted. This is achieved using *loxP* sites orientated in opposite directions at the breakpoint sites resulting in inversion of the flanked DNA sequence

containing the second half of the fusion protein (Fig. 2). Thus, after inversion, the sequence for the entire fusion protein is in frame allowing for expression of the *de novo* protein (Forster *et al.*, 2005b).

Since recombination products between inverted and wild-type chromosomes do not produce viable gametes, inverted regions suppress recombination. Therefore, inversions can also be used as genetic tools to maintain the linkage of mutant loci on chromosomes. Inverted regions carrying phenotypic markers, such as the dominant *K14 Agouti* coat-color gene, are known as balancer chromosomes and can be used for large scale mutagenesis screens, similar to experiments in *Drosophila* (van der Weyden *et al.*, 2009).

D. Studying the Mechanisms and Timing of Tumorigenesis

1. MOLECULAR-GENETIC IMAGING—Molecular-genetic imaging utilizes reporter transgenes to visualize proteomic, metabolic, cellular, or genetic events *in vivo*. The three major methods of animal imaging are optical, magnetic resonance imaging, and nuclear medicine (for review see Kang and Chung, 2008; Lyons, 2005). Although conventional magnetic resonance imaging, computed tomography, ultra-sound, and radiography imaging modalities have the advantage of not being dependent on genetically engineered reporter mice, they are limited to visualization of anatomic morphological changes. Optical imaging using genetically engineered reporters can be used to detect rapid molecular and genetic changes, including those that occur in response to drug treatment or during early stages of carcinogenesis before the onset of anatomical change. Furthermore, fluorescent and bioluminescent optical imaging modalities are sensitive, less expensive, less time consuming, and more convenient and user friendly than other imaging techniques.

Reporter transgenes can be made to express reporter genes under the control of specific promoter/enhancer elements to assess promoter activation *in vivo* during tumorigenesis or drug treatment. For example, transgenic reporter mice expressing luciferase under control of the estrogen receptor enhancer have been used to study the dynamics of estrogen receptor activity in response to various ligands and drug treatments (Maggi *et al.*, 2004). Furthermore, transgenic reporter mice can be crossed onto mouse models of human cancer to visualize tumor growth *in vivo*. For example, transgenic mice expressing firefly luciferase under control of the human E2F1 promoter, which is active during proliferation, can be crossed with mouse cancer models to characterize tumor cell proliferation *in vivo* (Momota and Holland, 2005).

Firefly luciferase is the most commonly used reporter gene for bioluminescence in mice, although renilla luciferase has some advantages in that it does not require ATP, and so may be less dependent on changes in the metabolic state of cells. Bioluminescence can be detected within 10 min of intraperitoneal injection of the luciferase substrate luciferin and multiple animals can be imaged together leading to a relatively quick turn-around time. Although bioluminescence has the advantage of extremely low background signal and high sensitivity, photon emissions are attenuated by hair color and the amount of tissue between the tumor and the detector, making it difficult to accurately compare tumors at varying depths and in different coat color backgrounds.

Green fluorescent protein (GFP) and red fluorescent protein (RFP) are commonly used as reporters for *in vivo* imaging (Hoffman, 2009). Since there are five different fluorescent proteins with unique emission wavelengths between 450 and 650 nm, simultaneous imaging of multiple fluorescent reporters in one animal is possible. Since fluorescence requires excitation, tissue scattering and absorption by endogenous fluorochromes can limit the sensitivity of *in vivo* fluorescence imaging.

2. RECOMBINATION REPORTERS AND LINEAGE TRACING—Cre- and FLP-dependent reporters are useful not only for determining where site-specific recombination of conditional knockout and transgenes occur (Soriano, 1999) but also for lineage tracing studies (Chai *et al.*, 2000; Jiang *et al.*, 2000). A ubiquitous promoter drives a conditional reporter transgene, such as a *lacZ* or GFP reporter, and is interrupted by the presence of a STOP cassette flanked by *loxP* or *FRT* sites. When conditional reporter transgenic mice are crossed with site-specific Cre or FLP mice driven by cell-type specific promoters, the STOP cassette of the conditional transgene is removed only in Cre- or FLP-expressing cells and the reporter continues to be expressed in the progeny of the recombined cells. For example, by using mice carrying a transgene for Cre or FLP expression in progenitor cells, the reporter expression is turned on in the progenitor cells and inherited by all subsequent progeny.

Yamamoto *et al.* (2009) have recently described a sophisticated dual reporter $R26^{NZG}$ mouse line for Cre- and FLP-dependent lineage analysis. The $R26^{NZG}$ reporter mice were constructed by knocking the NZG reporter cassette into the *Rosa26* locus. The NZG reporter cassette consists of the CAG promoter followed by the neo-STOP cassette, the *lacZ* reporter gene flanked by *FRT* sites, and finally the enhanced GFP (EGFP) gene. In the absence of Cre and FLP recombinases, neither reporter is expressed. In the presence of Cre recombinase, the *PGK-neo* cassette is removed and *lacZ* expression is turned on; however, EGFP expression is still turned off due to a STOP codon in the preceding *lacZ* sequence. In the subsequent presence of FLP recombinase, the *lacZ* sequence is removed and EGFP is expressed. Thus, *lacZ* expression is turned on following Cre-expression and EGFP expression is only turned on after expression of both Cre and FLP. Although there is an increasing breadth of Cre-expressing lines becoming available, the advantages of the dual reporter technology are currently limited by the smaller availability and lower efficiency of FLP-expressing lines. Nevertheless, the $R26^{NZG}$ mouse line in conjunction with mouse cancer models is likely to be a powerful tool in future lineage-tracing studies of tumor initiating cells.

3. GENOMIC INSTABILITY—Human cancers often show abnormal karyotypes that include, most prevalently, aneuploidy and multiple chromosomal translocations. Genomic instability is believed to contribute to human cancer by increasing the number and rate of genetic changes seen in a tumor, thus accelerating cancer development. However, the identification of the “driver” mutations necessary for tumorigenesis is confounded by the presence of many “passenger” alterations that do not contribute to tumorigenesis. Because mouse models of cancer are predisposed to tumorigenesis by engineered mutations, they can result in tumors with far fewer chromosomal aberrations, giving a means to separate driver and passenger mutations through cross-species comparisons (Peeper and Berns, 2006). For example, by comparing copy number aberrations found in mouse and human melanomas and liver tumors, new oncogenes associated with chromosomal aberrations were identified (Kim *et al.*, 2006; Zender *et al.*, 2006). Between mouse models, differences in genome stability can correlate to differences in tumor subtype, as shown in mammary tumor models with either *p53* loss or *p53* and *Brca1* loss (Liu *et al.*, 2007). Both tumor models develop mammary tumors; however, the *p53;Brca1* null tumors show greatly increased genomic instability and more closely resemble human *BRCA1* mutant tumors molecularly, whereas the *p53* null tumors show very few chromosomal alterations and more closely resemble human estrogen receptor mutant breast cancer.

While there are advantages to the reduced mutation number seen in many mouse tumors, the mutations driving mouse tumors serve to limit the range of mutations that develop. In this way, specific mouse models may filter out human “driver” mutations that are important in a different genetic context, in addition to filtering out the “passenger” mutations in cross-species comparisons. To ensure a more unbiased approach, mice with increased genomic

instability may be better models for comparative oncogenomics (Maser *et al.*, 2007). Multiple approaches have been developed to drive chromosomal instability in mouse models and better mimic the structural abnormalities seen in human tumors.

In humans, loss of telomeres as a part of aging is believed to contribute to cancer incidence due to genomic changes and chromosomal fusions. Mouse chromosomes have much longer telomeres than humans and sustained telomerase expression. This may explain the reduced number of chromosomal abnormalities in mouse tumors, as well as the difference in tumor types seen, as epithelial cancers are believed to require more aberrations than the mesenchymal tumors that are more commonly seen in mice. In order to examine the importance of telomeres in cancer development, telomerase knockout mice were generated (Lee *et al.*, 1998). Early generations of telomerase-deficient mice show initial resistance to tumorigenesis, due to apoptosis or cell-cycle arrest (Chin *et al.*, 1999; Karlseder *et al.*, 1999). As mice are bred over successive generations, the long mouse telomeres erode in the absence of telomerase, and in late generations tumorigenesis occurs through increased chromosome instability. Interestingly, mice with both telomere deficiency and loss of p53 show both an increased rate of tumorigenesis and a shift in tumor spectrum toward the epithelial cancers seen in humans (Artandi *et al.*, 2000). Additionally, the chromosomal changes seen in these mouse epithelial cancers mimic those often seen in human tumors, suggesting that increasing genomic instability contributes toward a more accurate model of human tumor development (O'Hagan *et al.*, 2002).

Another system to investigate genomic instability is the mouse model of Bloom syndrome, a genetic disorder involving mutation of the *BLM* helicase gene. Bloom syndrome is associated with both genomic instability and increased risk of a wide variety of malignancies (German, 1993). A conditional mouse model of *Blm* results in mice with an increased rate of mitotic recombination, leading to an increased loss of heterozygosity (Luo *et al.*, 2000) resulting in increased tumorigenicity (Goss *et al.*, 2002; Luo *et al.*, 2000). The increased genomic instability of the *Blm* mutant mouse and resulting loss of heterozygosity is being used to identify novel tumor suppressor genes in mice (discussed below) (Suzuki *et al.*, 2006). In addition to the mouse model of Bloom syndrome, many models have been developed targeting specific steps in the DNA damage response and DNA repair, allowing researchers to study how DNA instability/damage alters tumor phenotypes (e.g., see Wei *et al.* (2002) for models of DNA mismatch repair genes; see Hande (2004) for DNA damage signaling genes; see Barlow *et al.* (1996) and Westphal *et al.* (1997) for *Atm* mutant models).

In order to assay *in vivo* for genetic instability caused by toxins and carcinogens, many groups make use of the commercially available Big Blue[®] mouse and Muta[™] Mouse animal models (Stratagene, La Jolla, CA). Both models harbor multiple copies of a mutational target within a recombinant phage that can be extracted from the genomic DNA of mouse tissues. For a review of animal mutation models, see Nohmi *et al.* (2000). In the Big Blue[®] Transgenic Mouse Mutagenesis Assay System, 30–40 copies of the *lacI* repressor gene have been stably integrated in tandem into the mouse genome (Kohler *et al.*, 1991). Big Blue[®] mice are either treated with mutagenic compounds or crossed with genetically engineered mouse models and used to determine changes in chromosomal stability by measuring mutagenesis of the *lacI* gene (Fig. 3). Mutation of *lacI* disables its ability to repress *lacZ* expression when the extracted phage is infected into host bacteria. *LacZ* is measured in bacteria by growing on substrate that turns blue in the presence of enzymatic activity of the product of the *LacZ* gene. The ratio of blue to colorless plaques is used as a measure of mutagenicity. The Muta[™] Mouse uses a similar system, but relies on negative selection in measuring mutagenesis of *lacZ*, rather than *lacI* (Gossen *et al.*, 1989). While these systems rely on colorimetric differentiation to identify mutations, addition of *cII* selection measures

cell survival using bacteriophage lambda lytic and lysogenic multiplication cycles. Using the *cII* system in conjunction with the Big Blue® or Muta™Mouse can lead to better quantification of mutation frequencies, reduction of false positives, and reduced cost.

E. Discovery of Novel Tumor Genes

1. INSERTIONAL MUTAGENESIS—Forward genetic screens can identify cancer-causing genes by unbiased, whole genome mutation screening in mouse tumors. Chemical mutagenesis has been used to produce a high frequency of mutations in the mouse. Phenotypic screens of these mutant mice identify cancer-related phenotypes, but gene identification is very difficult. Alternatively, insertional mutagenesis allows for identification of mutation sites using the integrant as a molecular tag that marks the region of the genome important for tumorigenesis.

Retroviruses have been used as insertional mutagens to identify cancer-causing genes since it was determined that certain strains of mice develop leukemia while other strains develop mammary tumors due to ecotropic retroviral infection (Gross, 1978; van Lohuizen and Berns, 1990). The cancers in these mouse strains are not due to acute transforming retroviruses that express viral oncogenes such as *v-abl* or *v-myb* (reviewed in Lipsick and Wang, 1999; Shore *et al.*, 2002). Rather, these slow-transforming retroviruses, such as murine leukemia virus (MuLV) and mouse mammary tumor virus (MMTV), lead to transformation by acting as insertional mutagens that integrate into the genome and disrupt the normal expression of oncogenes and tumor suppressor genes.

Retroviral integration is regulated by the long terminal repeats (LTRs) that contain enhancer and promoter regions, as well as start and stop sites for transcription of retroviral genes. Retroviruses disrupt gene expression by four basic mechanisms, dependent on the viral location and orientation relative to the gene (Jonkers and Berns, 1996). Viral integrations can cause gene mutations by promoter insertion, disruption of RNA or protein destabilization motifs, viral enhancement of transcription, or premature termination of genes (Jonkers and Berns, 1996, reviewed in Uren *et al.*, 2005) (Fig. 4). For example, proviral insertion in the gene promoter region (Fig. 4A) places the gene under the control of the LTR promoter region and has been found to upregulate the oncogenes *LCK*, *N-Ras*, and *E2a* in MuLV-induced leukemia, as well the novel candidate oncogene *Evi-1* (Morishita *et al.*, 1988, reviewed in Hirai *et al.*, 2001). Disruption of 3' UTR sequences due to proviral integration causes activation of *N-myc* in MuLV-induced leukemia (van Lohuizen *et al.*, 1989) (Fig. 4B). *Wnt1* and *Fgf3* are commonly overexpressed in MMTV-induced mammary tumors, due to activation by enhancer sequences of the integrated MMTV provirus, often at a distance up to 25 kb 5' or 3' of the gene (Fig. 4C). Although tumor suppressor inactivation is less common, mice infected with Friend leukemia virus have inactivating viral integrations within the *p53* gene (Ben David *et al.*, 1988; Mowat *et al.*, 1985), and *Nf1* is often inactivated in MuLV leukemia (Cho *et al.*, 1995; Largaespada *et al.*, 1995) (Fig. 4D). By utilizing the virus as a molecular marker, many groups have cloned sites of integration to identify genes involved in specific types of cancer. High-throughput methods of cloning using PCR-based methods have allowed the identification of a large number of sites, compiled in the Retroviral Tagged Cancer Gene Database (<http://rtcgdb.ncifcrf.gov/>) (Akagi *et al.*, 2004). Common sites of integration, sites that are disrupted in multiple independently derived tumors, are more likely to play a causal role in tumor progression pathways.

One drawback of retroviral insertion is that the majority of tumors are caused by gene activation of proto-oncogenes, suggesting that this model is inefficient at identifying tumor suppressor genes. However, as technologies improve and greater numbers of insertion sites are rapidly and completely identified (reviewed in Kool and Berns, 2009), the increased number of insertions sites per tumor will improve the frequency of identifying tumor

suppressor genes (Uren *et al.*, 2008), especially those with haploinsufficient tumor suppressor functions. Additionally, in order to increase loss of function by viral insertion, screening can be combined with the *Blm* mutant mouse (Suzuki *et al.*, 2006) that has a higher rate of mitotic recombination and increased rate of loss of heterozygosity (discussed above) (Luo *et al.*, 2000). When the *Blm* mutation was crossed onto mouse strains prone to retroviral insertion-mediated B-cell lymphomas, cancer latency was reduced, and loss of heterozygosity was found in multiple known and novel tumor suppressor genes (Suzuki *et al.*, 2006). The increased efficiency of retroviral oncogene activation compared to tumor suppressor gene inactivation may be due to the preference of retroviruses to integrate in the 5' end of genes (Johnson *et al.*, 2001). This promoter region preference may also indicate that only specific regions of the genome are accessible to viral integration, suggesting that whole genome coverage by viral insertional mutagenesis may be impossible. Although retroviral integration has been useful in identifying candidate cancer genes, these models generally only develop hematopoietic and mammary tumors.

2. MUTAGENESIS BY TRANSPOSONS—In an attempt to utilize somatic mutagenesis beyond hematopoietic and mammary tumorigenesis, and perhaps to more fully cover the genome with mutagenic events, DNA transposons are now being used as a mobile genetic tag in the mouse. Transposable elements have been used in multiple organisms for years, but the first active transposon in the mouse is *Sleeping Beauty*, a member of the *Tc1/mariner* family, which was reconstructed by directed mutagenesis from a dormant element (Ivics *et al.*, 1997). More recently, *piggyBac* has been demonstrated to be an efficient transposable element in mammalian cells *in vitro* and *in vivo* (Ding *et al.*, 2005; Wilson *et al.*, 2007). Both systems consist of two elements: the transposon (the mobile DNA sequence) and transposase (the enzyme that mobilizes it) (Fig. 5). The T2/onc (Collier *et al.*, 2005) and T2/ onc2 (Collier *et al.*, 2005; Dupuy *et al.*, 2005) *Sleeping Beauty* transposons are currently the most commonly used for somatic mutagenesis in cancer gene identification experiments. These transposons contain splice acceptors followed by polyadenylation sequences in both orientations in order to generate loss-of-function mutations, as well as retroviral LTR enhancer/promoter elements to drive overexpression of nearby genes. Two groups have shown the ability of *Sleeping Beauty* to mobilize in somatic cells, leading to tumor formation (Collier *et al.*, 2005; Dupuy *et al.*, 2005). Although the transposons are similar, the transgenic lines vary greatly in copy number. The lines contain multicopy arrays of the transposons at the initial site of insertion; T2/onc lines contain approximately 25 copies (Collier *et al.*, 2005), whereas T2/onc2 transgenic lines contain 150–350 copies of the transposon (Dupuy *et al.*, 2005). The differences in copy number are believed to contribute to the phenotype seen in the mice (Collier *et al.*, 2005; Dupuy *et al.*, 2005). The T2/onc2 lines cause tumorigenesis in an otherwise wild-type background; however, these mice develop aggressive hematopoietic tumors that kill the mice prior to the development of any solid tumors (Dupuy *et al.*, 2005). Although the T2/onc line does not cause tumorigenesis in wild-type mice, a wide variety of tumors form when crossed onto a *p19^{Arf}* deficient background, including osteosarcomas, soft tissue sarcomas, lymphomas, malignant meningiomas, myeloid leukemia, and pulmonary adenocarcinoma (Collier *et al.*, 2005), similar to the tumor spectrum seen in *p19^{Arf}*^{-/-} mice on the C57BL/6 genetic background (Kamijo *et al.*, 1999). Crossing different transposon and transposase transgenic lines allowed the system to be refined to generate high-penetrance hematopoietic tumors without secondary effects of embryonic lethality (Collier *et al.*, 2009). Furthermore, by combining a Cre-inducible *Sleeping Beauty* transposase with a third generation *Sleeping Beauty* transposon line (T2/Onc3), Dupuy *et al.* (2009) were able to successfully control the extent of hematopoietic tumors to generate a wide variety of mouse solid tumors by insertional mutagenesis. Because *Sleeping Beauty* shows preferences for local integration near the original transposon site, the system may have some limitations for saturating the genome

with mutations. However, the differences in accessibility of the genome to mutation by DNA transposons and mouse retroviruses may allow saturating mutagenesis through a combination of the two techniques, particularly as mouse panels are developed with DNA transposon arrays integrated systematically onto the different mouse chromosomes.

In order to more accurately model specific tumor types, a conditional *Sleeping Beauty* system has been developed by knocking a *lox-STOP-lox SB11* transposase allele into the *Rosa26* locus (Dupuy *et al.*, 2009; Keng *et al.*, 2009). Expression of the *Sleeping Beauty* transposase can therefore be activated using a tissue-specific Cre to drive transposition in the tissue and developmental stage of interest. A model of hepatocellular carcinomas was developed by crossing the *Sleeping Beauty* line to a hepatocyte-specific albumin-Cre (Keng *et al.*, 2009), and a colorectal cancer mouse model was developed by crossing to a Villin-Cre transgenic mouse line that expresses Cre only in the epithelial cells of the gastrointestinal tract (Starr *et al.*, 2009). Analysis of the insertion sites in both models identified multiple common insertion sites near genes known to be mutated in hepatocellular carcinoma and colorectal cancer, as well as genes not previously associated with these cancers (Keng *et al.*, 2009; Starr *et al.*, 2009). These models demonstrate the ability of transposons to identify relevant cancer-causing genes, in a tissue-specific manner in solid tumors.

3. GENETIC MODIFIER SCREENS—Many of the genetic mutations that have been identified as cancer causing are rare mutations that have highly penetrant effects on tumor susceptibility. Studies have shown that more common variants with subtle effects, such as polymorphisms seen in different mouse strains, also contribute to tumorigenesis. Different inbred strains show large differences in susceptibility to different tumor types, confirming the ability of low-penetrance polymorphisms to modify cancer risk (reviewed in Dragani, 2003). Similarly, the analysis of multiple human studies has shown that the majority of cancers occur in individuals with a genetic predisposition (reviewed in Demant, 2003). These studies suggest that the combined contributions of low-penetrance genes have a larger effect on cancer morbidity and mortality than the rare mutations that have been identified (reviewed in Demant, 2003). Modifier genes have been seen to affect a variety of tumorigenic phenotypes including overall resistance, metastatic ability, angiogenic potential, multiplicity, size, and survival.

As with quantitative trait loci (QTL) screens in mouse models of many human diseases (Hunter and Crawford, 2008; Peters *et al.*, 2007), modifier screens identify cancer susceptibility loci in the mouse by taking advantage of the known phenotypic differences between strains. The sequencing of the genome and the identification of genetic markers, such as simple sequence length polymorphisms, microsatellite markers, and single nucleotide polymorphisms (SNPs), have increased the ability to identify these low-penetrance modifier genes. Generally, modifier screening is based on crossing two parental strains, one resistant and one highly susceptible to a specific tumor, then backcrossing or intercrossing the F1 progeny to identify those genetic regions which affect the tumorigenic phenotype in a statistically significant way (Fig. 6A).

Even with the increase of genetic markers, many modifier loci have been found but few specific genes have been identified due to the difficulty of narrowing down a genetic locus to a single gene via positional cloning. Recent advances are being utilized to increase the ability to identify the specific genes that underlie QTL, including cancer modifiers. These include both genomic and animal resources, as well as new techniques and analytical tools (reviewed in Flint *et al.*, 2005). Some current studies have taken advantage of recombinant inbred strains (Fig. 6B), panels generated from an initial intercross of two inbred strains, followed by repeated brother–sister matings, which result in each recombinant inbred strain containing unique combinations of the progenitor genomes. These strains have been a useful

resource for mapping by contrasting phenotypes and genotypes between individual recombinant inbred strains, but their use in establishing linkage to low penetrance genes is limited by both the small set size of available recombinant inbred panels, and the small number of different recombinant inbred panels that have been generated (Demant, 2003; Flint *et al.*, 2005). A more recent development has been the use of chromosome substitution strains (Fig. 6B), in which one chromosome comes from a different strain background than the other 20. This contributes to increased QTL detection by reducing potential epistatic interactions between the modifier of interest and modifiers on other chromosomes, and allowing initial studies of modifier function (Flint *et al.*, 2005; Nadeau *et al.*, 2000). Additionally, backcrossing the chromosome substitution strains can further increase resolution of modifier mapping (Hunter and Crawford, 2008).

A current proposal to further ease the difficulty in cloning modifier genes is an expansion of the idea of the recombinant inbred strain. This Collaborative Cross resource, suggested by the Complex Trait Consortium (Churchill *et al.*, 2004) is currently being generated through the collaboration of multiple groups (Chesler *et al.*, 2008; Iraqi *et al.*, 2008; Morahan *et al.*, 2008). The Collaborative Cross is a panel of an estimated 1000 recombinant inbred lines being generated, following a specific breeding scheme, from eight divergent founder strains A/J, C57BL/6J, 129S1/SvImJ, NOD/LtJ, NZO/HiLtJ, CAST/Ei, PWK/PhJ, and WSB/EiJ (Chesler *et al.*, 2008; Iraqi *et al.*, 2008; Morahan *et al.*, 2008). Each new recombinant inbred line will be genotyped initially, resulting in a large number of genetically defined strains to be used in comparative studies (Fig. 6B) (Churchill *et al.*, 2004). The Collaborative Cross, when fully inbred, will have a QTL mapping resolution of approximately 1 MB (Broman, 2005; Valdar *et al.*, 2006), much higher than is currently possible. Because many inbred strains are closely related and only differ in a subset of their genome, comparison of any two inbred strains (as is the case with backcrossing or intercrossing (Fig. 6A), and chromosome substitution strains or recombinant inbred strains (Fig. 6B)) leads to “blind spots” in the genome where the strains are identical by descent. The Collaborative Cross takes advantage of more diverse strains, increasing the amount of genetic diversity between the strains, and thus the extent of the genome available to be screened (Churchill *et al.*, 2004). In conjunction with the Collaborative Cross, the Jackson Laboratory is generating the Diversity Outbred Mouse Population to further improve resources for gene mapping. The diversity outcross is a heterogeneous stock population that will be maintained as an outbred stock, approximating the heterogeneous human population. Diversity Outbred mice will be generated from 160 breeding lines of the Collaborative Cross, allowing the early recombination events to be maintained while avoiding inbreeding (Lambert, 2009).

As mentioned above, although many modifier loci have been mapped, few genes have been cloned (reviewed in Flint *et al.*, 2005), with the number involved in cancer being even smaller. The first locus to be mapped as a cancer modifier in the mouse was the *Mom1* locus on chromosome 4 (Dietrich *et al.*, 1993), found to modify the *Apc* gene mutation, *Min* (*Apc^{min}*), an inducer of intestinal cancer (Moser *et al.*, 1992). Additional studies showed that *Mom1* modified not only intestinal tumor number, the phenotype for which it was identified, but also tumor size (Gould *et al.*, 1996). Further analysis identified secretory phospholipase A2 (*Plag2g2a*) as a candidate gene for the *Mom1* locus (MacPhee *et al.*, 1995); *Plag2g2a* was later shown to functionally modify the *Apc* mutation (Cormier *et al.*, 1997). Interestingly, studies in humans suggested that *PLA2G2A* does not play a major modifying role in human colorectal cancers, due to a lack of functional polymorphic differences in the human gene (Riggins *et al.*, 1995; Tomlinson *et al.*, 1996). Although this highlights the differences between mouse and human, additional *Modifier of Min* (*Mom*) genes have been mapped, and the genes are currently being identified and tested for their role in human cancer.

In contrast, the gene *Sipa1*, originally identified as the gene underlying the metastasis modifier loci *Mtes1*, is associated with breast cancer tumorigenesis in both mouse and humans. The MMTV-polyoma middle T (MMTV-PyMT) transgenic mouse model develops mammary tumors as well as extensive metastases, with metastatic growth being influenced by strain background (Lifsted *et al.*, 1998). QTL mapping identified *Mtes1* as a metastasis modifier locus on mouse chromosome 19 (Hunter *et al.*, 2001), and the region was further narrowed down by taking advantage of haplotype analysis (Park *et al.*, 2003b). A combination of sequence and functional analysis originally identified *Sipa1* as a polymorphic candidate for the *Mtes1* locus (Park *et al.*, 2005). SNP analysis of human breast tumors found that germline polymorphisms of the *Sipa1* gene are associated with metastatic potential and poor prognosis (Crawford *et al.*, 2006), demonstrating that genes identified by modifier screens in the mouse can play a role in human cancer.

Another example of the complexities of modifier interactions in cancer is the *nerve sheath tumor resistance* locus (*Nstr1*), a modifier locus on mouse chromosome 19 found to affect incidence of peripheral nerve sheath tumors in an *Nf1*^{-/+}; *p53*^{-/+} cis mouse model of neurofibromatosis type 1 (Reilly *et al.*, 2006). Interestingly, the *Nstr1*-modifying effect is dependent on epigenetic factors on other chromosomes, with susceptibility differences only being seen if the *Nf1*; *p53* mutant chromosome 11 was passed on to the progeny from the father. More recent studies used a chromosome substitution strain with a C57BL/6J background and A/J chromosome 19 to show that a modifier on chromosome 19 affects both the incidence and latency of peripheral nerve sheath tumors, as well as astrocytoma, another tumor type seen in the *Nf1*^{-/+} *p53*^{-/+} cis mouse model but not previously believed to be modified by a locus on chromosome 19. Additionally, the effects of the A/J chromosome 19 vary based not only on inheritance of the *Nf1*; *p53* mutant chromosome as seen previously but also on the sex of the progeny (Walrath *et al.*, 2009). These findings demonstrate not only the complex interactions between genetic background and tumorigenesis but also stress the importance of interpreting the role of genes in tumorigenesis in the context of the genetic background being studied.

III. CANCER PARADIGMS AND LESSONS FROM THE MOUSE

Although much has been learned over the past three decades about the cellular and genetic changes that occur within the cancer cell, there is a growing appreciation that the environmental context in which the cancer cell develops is critical for how cancer forms. This highlights the importance of performing cancer cell experiments in more physiologically relevant contexts to recapitulate the environments experienced by the cancer cell in the patient. Mouse model systems can provide experimentally tractable systems to study the role of development and environment in cancer. We describe here ongoing research into the distinction between different cell types within a tumor, with a focus on the cancer stem cell (CSC), the role of the microenvironment, with special emphasis on the vasculature and immune system, and metastasis, in which cancer cells leave their current environment and adapt to survive at distant sites from the tumor. We discuss how mouse models can help to better understand these non-cell-autonomous aspects of cancer.

A. The Cancer Stem Cell and Initiating Cell

In order to eradicate tumors and prevent recurrence, it is important to understand tumor growth dynamics and the cellular subpopulations driving tumor growth. A comparison of the current hypotheses of tumor growth mechanisms is reviewed by Adams and Strasser (2008). According to the CSC model (Fig. 7), tumor growth is driven by a rare population of CSCs that maintains the ability to self-renew and gives rise to more differentiated cell types (reviewed in Tan *et al.*, 2006). This theory is based on evidence from mouse and human studies showing that malignant tumors, such as acute myeloid leukemia, contain a

subpopulation of cells that have stem cell-like self-renewal and differentiation characteristics, express stem cell markers, and can engraft into immune-compromised NOD-SCID mice. The clonal evolution model postulates that tumor growth can be maintained by a substantial portion of the tumor cells that are proliferating rapidly and that may be at various stages of the differentiation pathway rather than a specific CSC. A third mixed model provides that a clone with CSC properties arises late as a dominant subclone after selection within the growing tumor. Given the broad diversity of cancer, it may be impossible to generalize across all tumor types and it is likely that multiple models accurately describe tumors from different origins (Gupta *et al.*, 2009). Therefore, the predominant growth model for each tumor type must be determined experimentally.

In contrast to the CSCs that can maintain tumor growth within established tumors, the tumor-initiating cell is the cell that first undergoes mutation in the progression to cancer. There is much ongoing debate as to whether tumors must initiate in a normal stem cell compartment, in a cell with ongoing proliferative capacity, or whether normal, differentiated cells arrested in the G₀ phase of the cell cycle can give rise to tumors. Identification of the tumor-initiating cell will provide insight into the process of tumorigenesis that may lead to new ideas in cancer prevention and treatment. While the idea that CSCs are present in many tumors is widely accepted, how and where they come from is still under debate. One possibility is that CSCs arise from stem/progenitor cells that have lost the ability to senesce or differentiate properly, as has been shown for acute myeloid leukemia and promyelocytic leukemia. Another possibility is that more differentiated cells have acquired stem cell-like characteristics (Hambardzumyan *et al.*, 2008), as has been suggested by work on astroglia (Moon *et al.*, 2008; Steindler and Laywell, 2003), oligodendroglia (Grinspan *et al.*, 1996), myocytes (Odelberg, 2002), adult mouse fibroblasts, and human somatic cells (Takahashi and Yamanaka, 2006). Whether the tumor-initiating cell that gives rise to the CSC comes from a stem/progenitor cell gone awry or a more differentiated cell with acquired stemness, the identity of the tumor-initiating cell is likely to be a major determinant of tumor characteristics.

Conditional knockout mice with tissue-specific Cre expression have been used to identify stem/progenitor cell types that can serve as tumor-initiating cells. Conditional loss of *Nf1* in GFAP⁺ progenitor cells of the subventricular zone is sufficient to drive astrocytoma formation in mice, suggesting that stem/progenitor cells can be tumor-initiating cells for astrocytoma (Zhu *et al.*, 2005). Conditional loss of *Nf1* in fetal neural crest stem/progenitor cells of the Schwann cell lineage leads to development of abnormal differentiated Schwann cells, which then serve as neurofibroma tumor-initiating cells (Zheng *et al.*, 2008). This suggests that the neural crest stem/progenitor cell is not the direct tumor-initiating cell, but that deregulation at the stem/progenitor level can develop into an abnormal cell type that initiates tumors. Although conditional genetically engineered mouse models facilitate elegant studies that indicate which cell types are sufficient to initiate tumorigenesis, induction of genetic changes in specific cell types does not test whether tumorigenesis must initiate in the tested cell population, or whether alternate pathways to tumorigenesis are possible. Thus, the true identity of the tumor-initiating cell in spontaneous tumors is still unclear for most tumor types. The development of additional mouse models to identify tumor-initiating cells in spontaneous tumors would help address this question. Unfortunately, the identification of tumor-initiating cells has been largely dependent on the definition of stem/progenitor cell lineages using markers of cell differentiation. These markers help define the cells being studied and are used to specifically drive Cre expression in cell populations to test hypotheses of tumor initiation. Whereas cell lineage and characteristic markers of the different stages of differentiation are well defined in the hematopoietic system, other organ systems are not as clearly understood, making it difficult to study in which cells tumors arise. Furthermore, as more stem/progenitor cell markers are

identified and more differentiation steps are defined, the developmental steps from a stem cell to more committed progenitor cells and ultimately differentiated cell types may expand. Therefore, the cell identities capable of initiating tumors are likely to be refined as more stem/progenitor cell types along differentiation pathways are identified.

The progression of stem cells from totipotent to pluripotent to developmentally restricted progenitors is controlled by changes in expression of transcription factors through chromatin remodeling and modification, generally referred to as epigenetic changes (Niwa, 2007; see reviews by Zardo *et al.* (2008) and Jones and Baylin (2007) for more information). Alterations in epigenetic control, such as changes in chromatin regulators and chromatin modifications, may alter the expression of lineage-specific genes in stem cells during development, thereby inhibiting proper differentiation into more committed progenitors or differentiated cells and leading to expansion of cells with CSC potential. For example, overexpression of the chromatin regulator, *Bmi1*, in hematopoietic stem cells of transgenic mice results in increased stem cell self-renewal and leukemia (van Lohuizen *et al.*, 1991), while loss of *Bmi1* blocks leukemic proliferation and transplantation (Lessard and Sauvageau, 2003; Park *et al.*, 2003a).

Mouse models have been used to study the importance of regulation of epigenetic determinants, such as DNA methylation. The control of gene transcription and epigenetics is tightly regulated by DNA methyltransferases (DNMTs). Changes in DNA methylation profiles reflect changes in epigenetic states and cell fate decisions, including stemness (Bibikova *et al.*, 2006) and tumorigenesis (Lee *et al.*, 2008; Martinez *et al.*, 2009), as well as playing a critical role in genome stability (Eden *et al.*, 2003; Gaudet *et al.*, 2003). Studies that compare the role of *de novo* DNMTs, *Dnmt3a* and *Dnmt3b* (Okano *et al.*, 1999), to the maintenance DNMT, *Dnmt1* (Hirasawa *et al.*, 2008; Lei *et al.*, 1996), have helped to delineate the role of DNMTs in development and cell differentiation. Embryonic stem cells lacking DNMTs are severely hypomethylated and fail to differentiate. In stem cells with reduced levels of methylation, *Dnmt1* is critical for stem cell differentiation, whereas embryonic stem cells in which the *de novo* DNMTs are mutated can still differentiate, despite similar levels of DNA methylation in the two situations. These data emphasize that the regulation of stem cell epigenetics and differentiation capacity is dependent not on the methylation levels *per se*, but on the mechanism by which methylation is maintained (Jackson *et al.*, 2004). Given that tumors are often globally hypomethylated and locally hypermethylated and that upregulation of *DNMT1* correlates with poorer prognosis (Kanai, 2008), it will be important to fully understand the relationship between normal epigenetic control of stem cells and the role of epigenetic changes in cancer, both in terms of gene expression changes and genome stability. Genetically engineered mice with altered expression and/or function of epigenetic regulators, such as *Bmi1* and *Dnmt*, are powerful tools to study the epigenetics of stem cells and how deregulation of stem cell epigenetics can lead to cancer.

B. The Tumor Microenvironment

Cancer research has long focused on the mutated neoplastic cells and as a result early mouse models were designed to identify the effects of cell-autonomous mutations on tumorigenesis. Over the past decade, tumors have been found to consist of many nonneoplastic cell types, including fibroblasts, endothelial cells that form the vasculature and lymphatic system, and innate and adaptive immune cells, all of which interact to influence tumorigenesis (reviewed in Weinberg, 2008). There is some debate over whether stromal cells evolve and mutate to make them more capable of supporting tumor growth. Recent studies indicate that large DNA alterations are not present in fibroblasts, epithelial cells, or other neighboring stromal cells of human breast and ovarian cancers (Allinen *et al.*, 2004; Qiu *et al.*, 2008, reviewed in Weinberg, 2008), although expression changes occur

suggesting that epigenetic changes may play a role in evolving tumor stroma. In contrast, studies in mouse models support the hypothesis that mutations in the microenvironment are important for tumorigenesis (Hill *et al.*, 2005; Zhu *et al.*, 2002). Additional experiments are needed to determine the role of mutations outside the neoplastic cells, as well as to determine how epigenetic changes in stroma contribute to tumorigenesis. Meanwhile, research suggests that the interaction between a tumor and the microenvironment causes a disruption in normal tissue homeostasis, resulting in an environment more conducive to tumorigenesis (Fig. 8).

1. INFLAMMATION—Chronic inflammation is one common mode of microenvironment disruption that contributes to a tumorigenic phenotype and has been found to affect tumor development, both in the clinic and in mouse models of tumorigenesis (reviewed in de Visser and Coussens, 2006; Tlsty and Coussens, 2006). Two different models of colon cancer, the *T-cell-receptor- β /Trp53* double knockout and the *Rag2/TGF β* -deficient double knockout, do not develop tumors when maintained in germ-free housing, demonstrating a requirement for inflammation in tumorigenesis (Engle *et al.*, 2002; Kado *et al.*, 2001). Inflammation has also been implicated in a transgenic model of epithelial cancer in which the early genes of human papillomavirus type 16 are expressed under control of the human keratin 14 promoter (K14-HPV16) (Arbeit *et al.*, 1994; Coussens *et al.*, 1996, reviewed in de Visser and Coussens, 2006; de Visser *et al.*, 2006). In this model, the premalignant skin is infiltrated by innate immune cells, primarily mast cells and granulocytes (Coussens *et al.*, 1999; de Visser *et al.*, 2005). In a mast cell-deficient background, the K14-HPV16 mice show attenuated tumor development, confirming the functional contribution of the immune cells to cancer development (Coussens *et al.*, 1999). Interestingly, a similar model in which HPV16 infection occurs in the cervical epithelium also shows an influx of immune cells, but in contrast to the skin model the immune cells in the cervix are infiltrating macrophages (Giraud *et al.*, 2004) rather than mast cells. This demonstrates the specificity of changes in the microenvironment in different tumor types, even when initiated by the same oncogene. The importance of mast cells in tumor initiation has also been illustrated in a study of neurofibroma development. Although Schwann cells are the tumor-initiating cells in an *Nf1* conditional knockout, *Nf1*^{+/-} mast cells are required for the development of neurofibromas, and infiltrate the area around the peripheral nerve prior to tumorigenesis (Yang *et al.*, 2008; Zhu *et al.*, 2002).

Inflammation has been found to affect not only premalignant progression and tumor initiation but also tumor growth and metastasis. In the polyoma-middle-T-antigen (PyMT) transgenic mouse model of mammary tumors, PyMT mice lacking the cytokine CSF-1 had reduced macrophage recruitment to neoplastic tissue, but early neoplastic development was unaffected (Lin *et al.*, 2001). However, lack of CSF-1 significantly delayed the development of invasive carcinoma, and pulmonary metastases were reduced. This metastatic ability was restored with transgenic CSF-1 expression in the mammary epithelium (Lin *et al.*, 2001).

Inflammatory cells modulate tumor development by both direct and indirect effects on neoplastic cells. Cytokines and chemokines, as well as other inflammatory cell signaling molecules, promote tumor progression by several mechanisms (reviewed in Kundu and Surh, 2008). Two independent mouse models show that the signaling molecule nuclear factor κ B (NF- κ B), a proinflammatory transcription factor, may provide a link between inflammatory cells and signal pathway activation within neoplastic cells (reviewed in Balkwill and Coussens, 2004). In the *Mdr2*-knockout mouse model of inflammation-associated hepatocellular carcinoma, Pikarsky *et al.* (2004) demonstrate that hepatocyte NF- κ B activation is controlled by inflammatory cell upregulation of tumor-necrosis factor α (TNF- α). In a colitis-associated colorectal cancer, IKK β , an upstream positive regulator of NF- κ B, has been shown to play a role in both the tumor cell and the inflammatory cell.

When IKK β is deleted in colonic enterocytes, the putative tumor-initiating cell, tumor incidence is decreased without affecting inflammation pathways. Myeloid-specific IKK β deletion decreases tumor size through decreased production of cancer-promoting factors and a reduction in enterocyte proliferation (Greten *et al.*, 2004). In combination, these studies suggest that inflammatory cells directly impact neoplastic cells by promoting proliferation through secretion of growth factors into the tumor microenvironment. Immune cells are also known to have an indirect role on tumor growth by impacting the cellular microenvironment through tissue remodeling and angiogenesis. Mouse models of inflammation and immunomodulation are reviewed by de Visser *et al.* (de Visser and Coussens, 2006; de Visser *et al.*, 2006).

It is clear that inflammation can have a tumor-promoting effect through the stimulating effect of cytokines on tumor cells and the ability of inflammatory cells to remodel the microenvironment. However, it should be noted that certain types of innate immune cells, particularly type 1 natural killer T cells, have an antitumorigenic response, as do some cytokines (reviewed in Fujii, 2008; Terabe and Berzofsky, 2007, 2008). Genetically engineered mouse models lacking certain cell types of the immune system, or certain cytokines, have been critical in understanding the role of immune cells on tumor immunity. For example, the mice mutant for V α 14J α 18 (J α 18 mice) lack type 1 natural killer T-cells, whereas CD1d mutant mice lack both type 1 and type 2 natural killer cells. Comparison of tumor growth in these two mouse models has led to the understanding that type 1 natural killer T cells promote tumor immunosurveillance, whereas type 2 natural killer T cells suppress this immunosurveillance (reviewed in Terabe and Berzofsky, 2007). This suggests that inflammation can have both a positive and negative effect on tumor growth, stressing the importance of understanding the balance between normal and abnormal regulation of the immune response.

2. ANGIOGENESIS—It was originally proposed by Folkman (1972) that cancers need to be vascularized to grow beyond a certain size and become malignant, and this theory has been supported over the years. The induction of vasculature growth to provide sufficient nutrients to the tumor has been termed the angiogenic switch, and occurs at varying stages of tumor progression, dependent on tumor type and environment. It was originally believed that tumor cells themselves were driving tumor angiogenesis, but recent data suggests that inflammatory cells play an important role by impacting tissue remodeling and activation of angiogenesis. The angiogenic switch is regulated by the balance between pro- and antiangiogenic factors. Normal angiogenesis is tightly regulated, whereas tumors lose appropriate control, such as failure of endothelial cells to become quiescent, allowing for constant growth of tumor blood vessels. Two classic mouse models of tumor angiogenesis are the K14-HPV16 mouse, described above, and the RIPTag mouse model of pancreatic islet carcinoma (Hager and Hanahan, 1999; Parangi *et al.*, 1996). Neovascularization occurs early in dysplasia in these models, and is required for tumor formation. The tumors develop in temporally and histologically distinct stages that can be characterized by angiogenic status: normal cells, hyperplasia, angiogenic dysplasia, and last, highly vascularized invasive tumors.

The angiogenic factor vascular endothelial growth factor A (VEGF-A) has been confirmed to play a role in tumor development in multiple mouse models, including the RIPTag model mentioned above (reviewed in Crawford and Ferrara, 2009). In the APC^{min} mouse discussed above, studies suggested that VEGF was upregulated in adenoma epithelial and stromal cells. Treatment with the VEGF inhibitor, Mab G6-31, was found to arrest tumor growth but not prevent incidence, supporting the hypothesis that upregulated VEGF was contributing to tumor progression, but may not play a role in initiation (Korsisaari *et al.*, 2007). Interestingly, the effectiveness of VEGF inhibition was found to be time dependent, such

that when mice are treated earlier, the tumor number is reduced (Goodlad *et al.*, 2006), whereas at later times only reduction in tumor size is seen (Goodlad *et al.*, 2006; Korsisaari *et al.*, 2007). In the MEN1 mouse model of multiple endocrine neoplasia (Crabtree *et al.*, 2001), anti-VEGF treatment has been found to reduce vascular density and tumor size in both pituitary and pancreatic tumors, suggesting blocking of VEGF may be effective in treating multiple tumors within the same individual (Korsisaari *et al.*, 2008). In the RIP-Tag2 model of pancreatic islet carcinogenesis, angiogenesis is involved in the progression of tumorigenesis. Although VEGF-A expression is not significantly upregulated in tumors, VEGF deletion in the B cells of the Rip-Tag mice reduces angiogenesis and attenuates tumor growth, supporting the necessity for VEGF in tumor growth. Matrix metalloproteinase 9(MMP-9) promotes angiogenesis in the RIP-Tag model and it also increases the bioavailability of VEGF, providing a potential explanation for VEGF dependence without mRNA upregulation (Bergers *et al.*, 2000). Certain antiangiogenic therapies, such as the therapeutic antibody bevacizumab, specifically inhibit only human VEGF (Yu *et al.*, 2008), making it difficult to test the effects of these drugs in mouse models. A knockin mouse has been developed that replaces 10 of the 19 differing amino acids between mouse and human VEGF, under the control of the endogenous murine promoter, minimizing developmental abnormalities. The biological activity of “humanized” VEGF is similar to human VEGF, as is its interaction with VEGF-blocking antibodies (Gerber *et al.*, 2007) (reviewed in Crawford and Ferrara, 2009).

The many cell types and cellular interactions in the microenvironment have made it difficult to determine which changes within the tumor are truly causal. Mouse models with activation and deactivation of specific oncogenic mutations *in vivo* have been created to follow the chain of tumorigenic events that occurs following oncogene mutation. In a Myc model of pancreatic cancer, MycER^{TAM}, a Myc fusion protein with a modified estrogen receptor-binding domain is activated in the presence of 4-hydroxytamoxifen (Lawlor *et al.*, 2006). Removing 4-hydroxytamoxifen stops Myc activity and allows analysis of both the Myc requirement and the timeline of events to tumorigenesis. Activation of Myc in islet cells triggers apoptosis, leading to islet involution. However, when apoptosis is blocked by coexpression of Bcl-x_L, Myc activation triggers pancreatic β cell expansion, leading to highly vascularized carcinoma (Pelengaris *et al.*, 2002). Myc overexpression in the islet cell compartments leads to the activation of angiogenic factors, such as VEGFA, that recruit endothelial cells and drive angiogenesis. Additional studies with this model suggest that VEGFA is released from the extracellular matrix via MMPs, such as MMP-9 (Shchors *et al.*, 2006) (reviewed in Shchors and Evan, 2007), and that secretion of interleukin-1 β (IL-1 β) from the β islet cells (Maedler *et al.*, 2002) is also important for the release of VEGFA and tumor angiogenesis.

There are exceptions to the angiogenic requirement for tumor growth. For example, astrocytomas are initially able to co-opt the blood supply from normal brain blood vessels without initiation of angiogenesis. Typically, astrocytomas develop along blood vessels without a tumor capsule, allowing growth and causing invasion into the brain. At higher grades, astrocytomas and glioblastomas become hypoxic and necrotic due to increased proliferation, tumor size, and ANG2-activated vessel regression (D'Angelo *et al.*, 2000). Hypoxia then induces VEGF, promoting vascular remodeling and vessel sprouting, defining the progression from grade III astrocytoma to grade IV glioblastoma (Bergers and Benjamin, 2003; Theurillat *et al.*, 1999). In this case where tumor cells are highly invasive from the early stages of tumorigenesis, angiogenesis is only required at late stages of progression when the tumor bulk overwhelms the normal vasculature system.

Different tumors respond varying to antiangiogenic therapy, making treatment less reliable than originally hoped (Folkman, 1972). The efficacy of antiangiogenic treatments is

dependent on tumor stage. The angiogenesis inhibitor endostatin, as well as MMP and VEGF inhibitors, have been most successful in treating early-stage disease in the RIP-Tag mouse model, both by blocking growth and preventing the angiogenic switch. Other inhibitors are able to block proliferation and migration in end-stage tumors, but have no effect on early-stage disease prevention, suggesting that there may be qualitative differences in angiogenic vasculature or its regulation at different stages (reviewed in Bergers and Benjamin, 2003).

3. METASTASIS—Metastasis occurs when tumor cells leave the primary tumor and colonize another organ. For metastasis to occur, tumor cells disassociate from the primary tumor, invade and break down the extracellular matrix to enter the blood or lymphatic system, and disseminate to a new site, requiring survival in order to proliferate and colonize the new tissue (Chambers *et al.*, 2000; Husemann and Klein, 2009; Weinberg, 2007). However, many questions remain, including what factors trigger metastatic spread, the genetic and epigenetic changes required, and the importance of selection or adaptation at the new site (Husemann and Klein, 2009).

Modeling metastasis has been difficult in the mouse, although mouse models have played an important role in studying the role of genetic background in metastasis, as discussed above (Section II.E.3). Spontaneous metastasis is rare in the mouse, and those tumors that do metastasize have long latency periods. Even for genetically engineered mouse models of metastasis, penetrance is variable, often much lower than primary tumor incidence, and the primary tumor may need to be surgically removed to allow the study of metastases. In the majority of models, latency is still over 3 months (summarized in Khanna and Hunter, 2005). However, mouse models of metastasis will be able to address specific questions that cannot be answered in cell culture or in human studies, such as what are the specific interactions that occur between the tumor and microenvironment that facilitate specific mechanisms of metastasis.

Recently, mouse models have provided important information regarding the metastatic potential of tumors, and the timing of acquiring this potential. It had been thought that metastatic capability develops as a late step in tumor progression, due to continuing accumulation of somatic mutations in the primary tumor and selection for metastatic ability in a small subset of cells (Fearon and Vogelstein, 1990; Fidler and Kripke, 1977). However, recent data suggests that tumors capable of metastasis actually show a specific and predictive gene expression fingerprint very early in tumorigenesis (Ramaswamy *et al.*, 2003; van 't Veer *et al.*, 2002). Similar findings have also been made in the mouse, as shown by Qiu *et al.* (2004). They demonstrate that a gene expression signature can distinguish between MMTV-PyMT mammary tumors in a highly metastatic strain and tumors in a low metastasis strain. Importantly, when looking specifically at orthologs of the 17 differentially expressed genes in the human fingerprint (Ramaswamy *et al.*, 2003), 16 of the 17 genes show the same directional expression differences in the mouse correlating to a calculated metastatic potential (Qiu *et al.*, 2004). These findings support the idea that a small number of metastasis-associated genes may be predictive in both humans and mouse. Interestingly, because these arrays were done comparing the signatures of primary tumors, these findings suggest that the metastatic predictive signature is not a rare phenomenon seen only in a small group of cells. Similarly, as the tumors in this experiment were all generated by the same oncogenic event, this suggests that the differences are most likely due to genetic background rather than oncogenic mutations. Therefore, qPCR was performed on normal mammary tissue from strains with high and low metastatic potential, using 10 of the 16 metastatic predictive genes, and it was found that nine of these signature genes show differential expression in normal tissues as well (Yang *et al.*, 2005). These data suggest that gene expression patterns that predict metastatic ability may be influenced by hereditary

polymorphisms, rather than just genetic mutations. It also suggests that certain subgroups of humans may be more susceptible to metastases than others, and that nontumor tissue can be used to predict risk (Qiu *et al.*, 2004).

Other studies in the mouse have also supported the idea that metastasis is not a late step in tumor development. Another study of mammary tumors in MMTV rat *HER-2/neu* transgenic BALB/c mice found that HER-2+ mammary cells were detected in the bone marrow as early as 4–9 weeks, during a period of atypical ductal hyperplasia, but prior to carcinoma development (Husemann *et al.*, 2008). Similarly, MMTV-PyMT mice had tumor cells present in the bone marrow at 4–6 weeks, with lung micrometastases found beginning at 14 weeks (Husemann *et al.*, 2008). Electron microscopy confirmed that individual cells could be seen breaking through the basement membrane at early time points (Husemann *et al.*, 2008).

As mentioned previously, genetically engineered mouse models are useful for imaging, as techniques for early detection become more accurate, and tumor cells can be labeled to follow cell growth. The ability to detect metastatic cell clusters and fully formed metastases by imaging can be used to improve the understanding of how metastases target specific organs. Recent studies have been done following the fate of single cells in mouse cancer models, and have given insight into interactions with the microenvironment, as well as the efficiency of cell survival in ectopic tissues (Chambers *et al.*, 2000; Khanna *et al.*, 2004).

4. PRECLINICAL TESTING OF THERAPEUTICS IN MOUSE MODELS—Though our understanding of cancer has expanded greatly in the past 40 years, our ability to translate that knowledge into patient treatment and therapy has been much more limited. Although genetically engineered mouse models have now been widely used to study and understand the basic science and molecular mechanisms of cancer, applying these models to preclinical studies of candidate therapeutics has been slower to develop. Most preclinical testing has been done in cell culture systems *in vitro* and xenografts *in vivo* due to the ability to control timing of tumorigenesis and generate large numbers of synchronized test subjects. Unfortunately, while these model systems have been successful at predicting toxicity issues in humans, they have been less successful at predicting efficacy of antitumor compounds. Genetically engineered mouse models are more difficult and costly to use for preclinical testing due to the number of animals that need to be bred to generate a test cohort; however, these models have certain advantages in modeling human disease that may make them more predictive of efficacy in humans. Because of the expense of clinical trials and the limited population of patients to be entered into clinical trials, the increased cost of using genetically engineered models may be offset by the increased ability to predict responses in humans, reducing the number of human trials that need to be performed. Genetically engineered mouse models of cancer have the advantages that the initiating mutation is known, which is particularly important for the testing of molecularly targeted therapies; that tumors develop spontaneously in the normal tissue for that tumor type, with a coevolving microenvironment; and that the immune system is intact (Becher and Holland, 2006). The advances in mouse models discussed in this chapter may provide the tools to better predict drug efficacy prior to Phase II/III drug trials.

Improvements in drug discovery and screening will require numerous steps to which improved mouse models may be able to contribute. These include the identification of cancer targets, including the context in which they are required for tumor maintenance, the determinations of most effective and least toxic compounds on these targets, and the identification of biomarkers (reviewed in Gutmann *et al.*, 2006; Sharpless and Depinho, 2006). Genetically engineered mouse models can play an important role in target validation, because specific genetic events in human cancer are used to guide the development of the

mouse model. Many genetically engineered tumor models have been generated to mimic the genetic, molecular, and phenotypic traits of the specific human cancer (Becher and Holland, 2006).

In order to make genetically engineered models effective for preclinical drug screenings, models with short tumor latency and high penetrance will help to keep maintenance manageable and costs reduced. However, an increase in mutations in order to decrease latency may skew tumor development, as well as reduce the requirement for gain of secondary or epigenetic mutations. A K-ras model of pancreatic cancer shows that in an *Ink4a/Arf* heterozygous background, the primary tumors have increased genomic complexity than those on an *Ink4a/Arf*^{+/−} background (Bardeesy *et al.*, 2006), suggesting that a less biased model leads to greater genetic instability, as seen in human cancers, and is therefore a more accurate representation of the human tumor. Additionally, fast-growing tumors may not accurately reflect the interaction between the tumor cells and the microenvironment.

Genetically engineered mouse models can be used for systematic screening of compounds, and have been used to test drugs for both therapeutic and cancer prevention properties, as reviewed in Carver and Pandolfi (2006), Gutmann *et al.* (2006), and Sharpless and Depinho (2006). Potential therapies can be screened and compared when tested on a well-defined model. Effective drug therapy can also be stopped to determine if the drug cures the cancer, or if recurrence or drug resistance occurs. Multiple drug combinations can also be tested for synergistic effects in the mouse. Because recent molecularly targeted therapies are showing very specific efficacy in a subset of human patients (e.g., see Haas-Kogan *et al.*, 2005; Mellinger *et al.*, 2005), compounds can be tested on modeled tumors with different engineered mutations or on different strain backgrounds to determine whether specific efficacy can be predicted in advance to allow better design of clinical trials.

IV. SUMMARY

As reviewed here, advances in mouse modeling techniques continue to allow the further understanding of the biology of tumor development and growth. Mouse models have shown some of the complexities of tumor cell interactions, including tumor interaction with nonneoplastic cells in the microenvironment and the effect of normal genetic polymorphisms on tumor susceptibility. Identifying cancer-causing genes, and most specifically targets that are responsive to therapeutics, continues to be a challenge. New modalities of genetic engineering have given us the ability to more accurately model human cancer in an effort to identify new targets and markers that will allow us better predictive and treatment abilities. Future therapies will utilize the advances that have been made in these mice to further understand and treat cancer.

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Fig. 1. Genetically engineered mouse models of cancer. Technology is now available to control the overexpression and loss of gene expression, shown in gray, (A) throughout the mouse, as is often the case with classic knockouts, constitutive transgenics, or in some cases knockins; (B) in a specific organ (e.g., the heart) or at a specific time in development, using conditional knockouts or conditional overexpression transgenics or knockins; and (C) in single-cell knockouts through sporadic loss events.

Fig. 2. Modeling chromosomal rearrangements in mouse models of cancer. (A) Interchromosomal rearrangements are generated in translocator mice by inserting single flox recombination sites on different chromosomes. Recombination between the sites exchanges pieces of the two chromosomes. (B) Intrachromosomal rearrangements are generated by inserting two flox recombination sites on the same chromosome oriented in opposite directions. By using flox sites with two different mutations, the resulting flox sites on the inverted chromosome cannot recombine and the inversion becomes stable in the presence of Cre enzyme.

Fig. 3.

Measuring mutagenesis levels using the Big Blue[®] mouse. Big Blue[®] mice have been engineered to carry many copies (30–40) of the bacterial *lacI* gene, encoding the LacI repressor protein. When Big Blue[®] mice are subjected to mutagens or crossed to mice with a mutator phenotype, the *lacI* gene is mutated. Genomic DNA is isolated from the mice and infected into *lacI*⁻ bacteria via lambda phage. Bacteria carrying unmutated *lacI* continue to repress the *lacZ* gene, whereas bacteria carrying mutant *lacI* expressed *lacZ* and form blue colonies on X-gal indicator plates. The ratio of blue to clear bacterial colonies is a measure of mutagenesis.

Fig. 4. Mechanisms of retroviral mutagenesis. Because of the presence of enhancer and promoter sequences in the LTR regions of the retrovirus, there are four basic mechanisms by which retroviruses disrupt gene function. (A) Retroviruses can insert into gene promoters, providing a stronger promoter signal from the LTR and increasing the level of gene expression. (B) Retroviruses can insert into the 3' UTR of a gene, changing mRNA stability thus altering the amount of protein translated. (C) Retroviruses can act as enhancers of gene transcription when inserted upstream, or downstream, of the gene. (D) Retroviruses can mutate genes by inserting into an exon and causing premature truncation of the protein.

Fig. 5.

Transposon insertional mutagenesis with *Sleeping Beauty*. Mice genetically engineered to carry arrays of transposon insertions are crossed to mice genetically engineered to express the transposase. (A) The *Sleeping Beauty* T2/Onc2 transposon structure is shown (Dupuy *et al.*, 2005) in which the transposon carries two splice acceptors (SA) in opposite directions, a bidirectional poly(A) tail (pA), and the MCSV LTR (MCSV) with a splice donor (SD) that can act as a promoter/enhancer of gene expression. These sequences allow for both gain-of-function and loss-of-function mutations when inserted into a gene locus. The transposon is carried by transgenic mice in arrays of 150–350 transposon copies. Transposon-carrying mice are crossed to mice expressing the *Sleeping Beauty* (SB) transposase from a variety of different promoters to induce mutagenesis in different tissues. (B) The progeny of

transposon and transposase mice express the transposase and cause both intrachromosomal and interchromosomal hopping of the transposon to mutate the genome.

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Fig. 6.

Genomic tools for mapping cancer modifiers in mouse. (A) Classic mapping approaches have used two generations of breeding to generate recombinants in the genome, either through F2 intercrosses or backcrosses. Individual mice are then genotyped at polymorphic loci between the resistant and susceptible strain to correlate the genotype to the modifier phenotype. (B) Reference strain panels, such as chromosome substitution strains, recombinant inbred lines, and the Collaborative Cross, provide stable, genotyped recombinants and can be compared for their cancer phenotype to directly map modifiers, or can be crossed to genetically engineered mouse models of cancer to map modifiers in the first generation.

Fig. 7. The cancer stem cell model. In this model, tumors contain rare populations of cells with the ability to repopulate the tumor (cancer stem cells; CSC). These CSCs divide asymmetrically to give rise to more differentiated cells (light gray) and additional CSCs (dark gray). Only the CSCs can give rise to tumors. More differentiated cells can proliferate, but are not sufficient to repopulate the tumor.

Fig. 8.

The complexity of the tumor microenvironment. This cartoon shows an example of the tumor cell types making up the tumor microenvironment, based on the current understanding of brain tumors. In addition to differentiated tumor cells that make up the bulk of the tumor, tumor stem cells reside in a specific niche along blood vessels. Normal cells, such as reactive astrocytes, can react to the changes in the local microenvironment to become altered in morphology or gene expression. Inflammatory cells, such as lymphocytes and microglia, invade the region of the tumor and can further change the local microenvironment.