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Developing genetically engineered mouse models to study tumor suppression

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Abstract

Since the late 1980s, the tools to generate mice with deletions of tumor suppressors have made it possible to study such deletions in the context of a whole animal. Deletion of some tumor suppressors results in viable mice while deletion of others yield embryo lethal phenotypes cementing the concept that genes that often go awry in cancer are also of developmental importance. More sophisticated mouse models were subsequently developed to delete a gene in a specific cell type at a specific time point. Additionally, incorporation of point mutations in a specific gene as observed in human tumors has also revealed their contributions to tumorigenesis. On the other hand, some models never develop cancer unless combined with other deletions suggesting a modifying role in tumorigenesis. This review will describe the technical aspects of generating these mice and provide examples of the outcomes obtained from alterations of different tumor suppressors.

Introduction

Cancer arises after a multitude of genetic changes disrupt the balance between cell proliferation and cell death, leading to excess proliferation. Many of the genetic changes, however, are passenger mutations that do not contribute to tumor initiation, progression, or maintenance (Stratton et al., 2009). To identify the mutations relevant to oncogenesis, we and others have relied on mouse models. This model has proved to be an invaluable tool in the effort to identify the functional significance of tumor-derived mutations.

In the last 25 years, the model has been so well developed that the genome of a mouse can be manipulated to make almost any kind of alteration ranging from a single base pair change to deletions and even translocations. This powerful tool allows us to study the changes that occur in human cancers and how those changes contribute to the initiation, progression, and maintenance of tumor phenotypes. By using this model to study tumorigenesis, we can study the functional effects of a mutation in the context of the whole mouse with an intact immune system and physiological stromal environment and hormonal milieu.

Two major technologies have allowed us to alter the genomes of mice (Doetschman et al., 1987; Gordon et al., 1980; Palmiter and Brinster, 1986) and, more recently, rats (Cui et al., 2011; Tesson et al., 2005; Tong et al., 2011). The first is targeted mutagenesis, whereby a specific normal gene is deleted to generate a knockout model or the normal gene is replaced with a mutant copy that mimics the genetic disease state to be studied (a knockin model) (Capecchi, 1989; te Riele et al., 1992; Thomas and Capecchi, 1987). The second is pronuclear injection, whereby an exogenous gene is added to the rodent genome (a transgenic model). Using these methods, we have been able to produce mutant mice whose pathologies simulate human diseases such as cancer.

To generate a mouse model of a particular cancer, the first step is to identify the genes that are altered in a naturally occurring human tumor and to decide how best to mimic that change in the mouse. Deletions are the most straightforward to create and often the first choice for probing a gene that is lost in tumors. However, some deletions are lethal to mouse development. Therefore, conditional alleles in which the gene of interest is normally expressed but can be deleted at any time were developed to bypass developmental phenotypes. In addition, these conditional alleles allow deletions in a single cell type and narrow the range of tumors that develop in the mouse. The generation of mice with more subtle mutations such as a point mutation that alters a specific amino acid is warranted when that alteration is commonly observed in human cancers. Thus, understanding the biology of the tumor and designing the genomic changes so that they recapitulate the human disease are the most important steps of generating a tumor mouse model. Once these steps are completed, the investigator is faced with the technical challenges of producing a model. In this review, we will first describe the steps to generate mice with a gene deletion and then give some examples of the effects of deletions of tumor suppressors. Subsequent sections will describe the conditional and knockin approaches to generate more sophisticated models.

Gene deletion via Knockout targeting strategies

The two essential components of designing a targeting construct to delete a gene in mice are sequences homologous to the gene of interest and a positive selection marker. Homologous recombination occurs when DNA from an engineered construct recombines with the genomic sequence (Hasty et al., 1992). The homologous sequences are stretches of DNA (often referred to as arms) on either side of the gene to be deleted (Figure 1). Homologous recombination requires pairing between the introduced DNA construct and the genomic DNA and is most efficient when perfectly matched to the DNA of the embryonic stem (ES) cells (Joyner, 2001; Nagy, 2003). This is generally achieved by cloning DNA sequences directly from the ES cells to be used. The length of the homology also affects recombination (Hasty et al., 1991; Zhang et al., 1994). For optimal recombination, the total match between the two arms should reach approximately 8–10,000 base pairs, and neither arm should be shorter than 1000 base pairs. The shorter the homologous sequences the lower the efficiency of recombination, and arms shorter than 500 base pairs will generally not recombine efficiently.

Another critical component of the targeting vector is a positive selection marker that codes for antibiotic resistance. Positive selection is used to select for ES cells that have incorporated the DNA. The most commonly used positive selection gene is that for G418 (neomycin) resistance (Yenofsky et al., 1990). Genes that code for hygromycin or puromycin resistance are often used as a second selection when the cells already contain the neomycin resistance gene.

The simplest construct with which to generate a knockout animal includes a 5' arm of homology of 5000 bp, the neomycin resistance gene replacing genomic sequences (a critical exon for gene function, or the exon that codes for the translational ATG start site), and a 3' arm of homology of an additional 5000 bp (Figure 1).

More sophisticated modifications can be incorporated into the DNA at the construction phase. These additional modifications are designed to increase the efficiency of ES cell selection. For example, the addition of a negative selection marker at one end of the DNA construct (Figure 1) enriches for the selection of homologous recombinant clones by a factor of more than 10^3 (Mansour et al., 1988). Since the DNA construct is incorporated into the mouse genome either randomly or at the homologous site, and because any cell that has taken up the construct DNA will survive positive drug selection, it is helpful to decrease the

numbers of clones that have random integrations by using negative selection. When clones recombine in the homologous locus, non-homologous DNA at the ends of the construct will be lost, and the clone will survive. However, when the construct DNA inserts randomly, the negative selection marker is often retained, and the selection screen will eliminate such cells. The *thymidine kinase* (*tk*) gene from herpes simplex virus 1 (HSV) is the most commonly used negative selection gene. Cells retaining the *HSV-tk* gene can be killed by addition of gancyclovir or 5-iodo-2'-fluoro-2'-deoxy-1- β -D-arabino-furanosyl-uracil (FIAU) to the medium (Salomon et al., 1995).

Once the construct has been designed and generated, it is purified and electroporated into ES cells. ES cells are generated from a mouse embryo at 3.5 days after fertilization, i.e. the blastocyst stage. When a cluster of cells in the blastocyst, the inner cell mass, is isolated and maintained in cell culture, they can give rise to ES cells. These pluripotent cells can be manipulated to produce a mouse (Koller et al., 1989). The ES cells can be expanded and then frozen for subsequent use in gene targeting experiments. For targeting, the cells grown in culture are mixed with the DNA construct of interest and then electroporated to allow for entry of the DNA. After entering the cell, the DNA integrates randomly (most of the time) or into the specific gene of interest by homologous recombination (rarely). Positive and negative selection methods are employed to isolate those cells in which the DNA likely integrates into the correct gene. Subsequently, the surviving ES cell colonies with homologous recombination at the locus of interest are identified.

Two general methods of testing ES cell clones for correct homologous recombinants are Southern blot and PCR analyses. While PCR testing may be faster, it often produces results that are difficult to interpret, and it produces false positives and false negatives. For those reasons, we also recommend testing by Southern analysis. Genomic Southern analyses are more accurate because the analysis interrogates the locus, and both endogenous and targeted bands can be visualized. Restriction enzymes that cut the DNA outside of the homologous region present in the targeting construct are used (for example, *EcoR1* in Figure 1). The hybridization probes should also match sequences outside those of the targeting construct. PCR is often used to genotype mice after Southern analysis. It can be used to determine the presence or absence of the targeted allele in future generations after the initial Southern blotting has established that the targeting is correct.

Next, 7–15 correctly targeted ES cells are injected into each of 30–50 blastocysts of another mouse strain that has a different coat color. These surviving blastocysts are then surgically placed into the uteri of pseudopregnant females. During embryo development, the ES cells are mixed with the cells of the inner cell mass present in the host blastocyst to create a mouse that is a mixture of both host and manipulated ES cells (called a chimera).

In general, the greater the contribution of the ES cells to the chimera, the more likely the targeted allele will be transmitted. A common method used to determine the contribution of the ES cells to the chimera is to use coat color variation as a marker (Nagy, 2003; Papaioannou, 2001). If ES cells contain a dominant coat color marker and blastocysts have a different, recessive marker, the mix of coat color in the chimera will indicate how much the ES cell contributes to the chimera. These chimeras are then bred with wild type to obtain germline transmission of the targeted gene.

For a more thorough analysis, the reader is directed to detailed discussions that are available in several regularly updated publications: 1) Nagy A, Gertsenstein M, Vintersten K, Behringer R. (Eds.) 2003. *Manipulating the Mouse Embryo: A Laboratory Manual*. Cold Spring Harbor NY: Cold Spring Harbor Laboratory Press; 2) Joyner AL (Ed.) 2001. *Gene Targeting: A Practical Approach*. Oxford University Press, New York; 3) Pease S and

Saunders T (Eds.) 2011. Advanced Protocols for Animal Transgenesis. Springer, Dordrecht, The Netherlands.

Characterizing tumor models

A cohort of heterozygous and homozygous (if viable) mice should be generated to study a tumor phenotype. These mice should be monitored daily and those in which palpable tumors appear are sacrificed. Because large tumors impede normal function, mice should be sacrificed before the tumors exceeded 3 cm³. Additionally, mice that appear moribund, even if they lacked obvious tumors, should be sacrificed and carefully dissected and examined pathologically. Dissection of mice should be coordinated with a veterinary pathologist. Institutional Animal Care and Use Committee (IACUC) guidelines should be followed closely. Tumor samples should be divided into two parts for the future analysis: a portion for paraffin embedding and histology, and a portion frozen for RNA, DNA, or protein analyses.

Tumor phenotypes of mice should also be analyzed by generating Kaplan Meier curves for either survival (i.e. inclusion of all mice regardless of cause of death) or tumor-free survival. These curves can also be used to determine how many mice should be monitored for tumorigenesis in order to achieve statistical significance. Typically, GraphPad Prism software is used for Kaplan-Meier analysis.

Power analysis is used to determine the number of mice needed to achieve statistical significance in tumor onset and survival analyses. This assessment typically relies on the use of past data sets. As an example, when determining the number of *p53* knockin mice (these mice express a mutant p53 R172H protein) needed for a statistical significance as compared to wild-type mice, a comparison to the *p53*-null data set was a good reference. However, when a novel tumor suppressor has been deleted in a mouse model for the first time, accurate power analysis is difficult. In these cases, it may be necessary to empirically determine the number of mice needed for statistical significance.

Generation of mice with deletions of tumor suppressors

The first studies that defined the function of *Trp53* (shortened to *p53*) as a tumor suppressor were done using the deletion mouse models. *p53* encodes a transcription factor that binds a specific DNA sequence and activates cell cycle arrest, senescence, and apoptotic programs that contribute to tumor suppression. The *p53* gene is deleted or mutated in more than half of human tumors (<http://www-p53.iarc.fr/>). In the first mouse models generated to examine the role of p53 as a tumor suppressor, the *p53* gene had been deleted (Clarke et al., 1993; Donehower et al., 1992; Jacks et al., 1994; Tsukada et al., 1993). The two *p53*-null mouse models most often used lack a *p53* region encompassing part of intron 4 and exon 5 as the result of the insertion of a *neo* cassette (Donehower et al., 1992) or deletion of exons 2–6 (Jacks et al., 1994). In both models part of the p53 DNA binding domain had been deleted. The first mouse contained a *neo* cassette that lacked a polyadenylation signal and was thus dependent on a polyA signal within the integration site (Figure 1). This modification enriched the recombination efficiency by 50 fold. The targeting construct also contained a *tk* gene for negative selection, which made it possible to enrich the targeting efficiency by 3 fold. Both knockout strategies resulted in an allele that resulted in mice that lacked the p53 protein.

In several studies published using deletion mutants, *p53* heterozygous mice developed spontaneous tumors, and the survival rate in these mice was 50% at 18 months. Three major tumor types developed: lymphomas, sarcomas, and carcinomas. Thus, haploinsufficiency, or loss of a single *p53* allele, was sufficient for tumor development, and the studies clearly defined *p53* as a tumor suppressor. *p53* null mice also developed tumors but much faster

than the heterozygous mice; the 50% survival mark occurs at about 5 months. Approximately 70% of these animals developed thymic lymphomas and about 40% developed sarcomas (Donehower et al., 1992; Jacks et al., 1994). Some mice had multiple tumors.

There were also mouse strain dependent differences in tumorigenesis. The *p53* null and *p53* heterozygous mice on a 129/Sv background developed tumors sooner on average than on the C57BL/6 background. Moreover, *p53* null mice in the C57BL/6 background developed predominantly lymphomas, whereas in the 129/Sv strain, testicular teratomas were as common as lymphomas (Donehower et al., 1995). In the BALB/c background, *p53* heterozygous mice develop breast carcinomas which are rare in C57BL/6 mice (Koch et al., 2007). These data thus provided the first direct evidence that *p53* was a tumor suppressor and its loss contributed to development of multiple kinds of tumors.

Developmental phenotypes have been observed for some tumor suppressors. To determine whether a specific allele results in embryonic lethality, the Mendelian ratio of the offspring from the heterozygous crosses must first be determined. Developmental problems are likely when the Mendelian ratio is skewed. As an example, in one study, the yield of *p53*-null offspring from heterozygous crosses was 16.6%, which was significantly lower than the expected 25% (Jacks et al., 1994). A significant proportion of *p53*-null mice, predominately female, died of exencephaly, a defect in neural tube closure (Armstrong et al., 1995; Sah et al., 1995). *p53* null female mice also had fertility defects and produced a significantly smaller litter size. In this study, *p53* was shown to regulate maternal reproduction of leukemia inhibitory factor (LIF), which is important for implantation of embryos (Hu et al., 2007).

Retinoblastoma is the most common eye tumor in children, which affects the retina of one or both eyes. The disease is highly associated with inherited or somatic mutation of retinoblastoma susceptibility gene (*RB*) (Knudson, 1971; Knudson et al., 1976). This *RB* gene was cloned 25 years ago (Friend et al., 1986; Lee et al., 1987). *RB* is also mutated in a high percentage of other cancers (Harbour et al., 1988; Hensel et al., 1990; Lee et al., 1988; T'Ang et al., 1988).

Mouse models with deletions of the *Rb* gene have also been generated. At least three *RB* knockout mice were made. *RB^{x3t}* was generated by introducing a stop codon in exon 3 of the *RB* gene, which resulted in early termination of the protein product (Jacks et al., 1992). *RB^{Δ20}* was generated by inserting the *pGK-neo* cassette into exon 20, which resulted in an unstable truncated protein (Lee et al., 1992). *RB^{t19neo}* was generated by inserting the *pGK-neo* cassette into exon 19, creating a stop codon in all three possible reading frames (Clarke et al., 1992).

In contrast to *p53*, *RB* loss results in embryo lethality of all mice. Analysis of embryogenesis at different stages in *RB^{-/-}* mice showed that the embryos died in utero between 13 and 15 days of gestation (Jacks et al., 1992; Lee et al., 1992). Originally, the phenotypes of *RB^{-/-}* embryos were believed to be altered erythropoiesis and massive cell death in the central nervous system. However, further study indicated that the most apparent effect of the lack of *RB* is an abnormal placenta, with excessive proliferation of trophoblast cells and a severe disruption of its normal labyrinth architecture (Wu et al., 2003). Thus, the phenotypes of knockout mice may be complex and identification of direct versus secondary phenotypes is important.

Most *RB^{+/-}* heterozygous mice die at approximately 11 months of age. Heterozygous humans develop retinoblastoma, but *RB^{+/-}* mice develop primarily thyroid carcinoma and pituitary adenocarcinoma (Hu et al., 1994; Jacks et al., 1992; Vooijs and Berns, 1999;

Williams et al., 1994). To generate retinoblastoma in mice, investigators deleted *RB* and a related gene *p107*, or *RB* and another related gene *p130* in the retina (Chen et al., 2004; MacPherson et al., 2004). The variations in phenotype resulting from *RB* deletion in mouse models and humans highlights the importance of recognizing that mouse models do not always recapitulate the human disease perfectly.

Another important tumor suppressor is *PTEN*, which is mutated or lost in many human tumors, including glioma (Bigner et al., 1988) and endometrial (Tashiro et al., 1997), prostate (Cairns et al., 1997), bladder (Cairns et al., 1998), and thyroid cancers (Halachmi et al., 1998). The *PTEN* gene has been cloned (Li and Sun, 1997; Li et al., 1997; Steck et al., 1997) and three different laboratories generated *PTEN*-null mice (Di Cristofano et al., 1998; Podsypanina et al., 1999; Suzuki et al., 1998). All three models replaced the phosphatase domain of *PTEN* with a neomycin resistance cassette.

Similar to *RB*^{-/-} mice, loss of *PTEN* results in embryonic lethality, at day 9.5 of gestation. To identify the mechanism of embryonic lethality, the embryos were dissected from heterozygous crosses at various gestational stages. In *PTEN* null mice, the embryos showed pattern defects in cephalic and caudal regions of the brain (Suzuki et al., 1998). *PTEN*^{+/-} mice have a high tumor incidence. Fifteen to 20% of all mice develop thymic and peripheral lymphomas with infiltration into multiple tissues and organs (Lee et al., 1987; Podsypanina et al., 1999). Half of *PTEN*^{+/-} female mice develop breast tumors, and 20% develop endometrial cancer. Hamartomatous tumors of the gastrointestinal tract, as well as prostate and adrenal neoplasia, are also often observed (Stambolic et al., 2000).

In summary, the knockout mouse models of these three important tumor suppressor genes demonstrate that they are drivers of tumorigenesis. Importantly, loss of one allele of any of these genes in the mouse is sufficient to induce tumorigenesis, indicating haploinsufficiency of these tumor suppressors. Another common observation for these tumor suppressor genes is loss of heterozygosity (LOH). In the *p53* heterozygous mouse, about half of the *p53* heterozygous tumors exhibit LOH. Tumors that develop late in *p53*^{+/-} mice rarely show LOH, suggesting accumulation of other defects in the *p53* pathway that obviate the need for LOH (Jacks et al., 1994; Venkatachalam et al., 1998). Similarly, *PTEN* heterozygous mice develop lymphomas associated with LOH of the wild-type *PTEN* allele (Suzuki et al., 1998). *RB* heterozygous mice develop pituitary adenoma with LOH (Williams et al., 1994). These data also suggest that loss of both alleles of tumor suppressor genes provides an advantage over the effect of losing only one allele for tumorigenesis.

Generating conditional loss-of-function alleles

The embryo lethal phenotypes described above for loss of *RB* and *PTEN* preclude examination of the effects of deletion of the tumor suppressor in adult tissues. Additionally, as is the case for *p53* null mice, the development of multiple spontaneous tumor types, makes it difficult to obtain sufficient mice to study a single type of tumor. The alternative is to produce tissue-specific knockouts, which allow gene deletion at precise time points post development and limit tumorigenesis to a single tissue.

Producing tissue-specific knockouts requires the use of site-specific recombinases. Using this strategy, the gene remains intact but is primed for deletion by recombination in the context of the site-specific recombinase (Figure 2). The most common system for this type of mutagenesis is the Cre-*loxP* system. Cre recombinase is the product of the P1 bacteriophage (Sauer and Henderson, 1988). It recognizes a specific 34 base-pair sequence called a *loxP* site (5' ATAAGTTCGATAGcatacatTATACGAAGTTAT 3') (Lee and Saito, 1998). *LoxP* sites are introduced into either side of the DNA to be deleted. Upon recognition

of the sites, the Cre recombinase recombines the sites to generate one *loxP* site and deletes the DNA sequences in between.

Another commonly used site-specific recombinase system is the FLP/FRT recombination system (Rodriguez et al., 2000; Schaft et al., 2001). The mode of action for the FLP/FRT system is equivalent to that for the Cre/*loxP* system. However, FLP recombinase is a product of *Saccharomyces cerevisiae* that has a 34-bp recognition sequence different from that of the Cre recombinase (5' GAAGTTCCTATACtttctagaGAATAGGAAGTTC 3'). These sequences are called FRT sites.

DNA constructs to generate conditional knockout mice often take advantage of both of these recombination systems. First, the positive selection marker is flanked with FRT sites, so that it can be removed from the locus, thus minimizing potential effects on gene expression. Sometimes even the positive selection marker present in an intronic region affects gene expression. For example, the *p53-neo* allele shows much less activity than wild-type when the *neo* cassette is present in intron 4 (Wang et al., 2011). Second, *loxP* sites flank the gene of interest, so it can be deleted by Cre recombinase expressed in a tissue-specific or time-dependent manner. Many more transgenic mice express the Cre recombinase than the FLP recombinase (JAX Cre Repository). Therefore, FRT sites usually flank the positive selection marker, whereas *loxP* sites normally flank the exons of the gene to be deleted (Figure 2).

After the conditional knockout mouse is generated, Cre-mediated excision of the floxed DNA will occur throughout the entire animal if the mouse is crossed with a mouse that has ubiquitous expression of the Cre recombinase (CMV-Cre, for example) (Meyers et al., 1998) or, if the mouse expresses a tissue-specific recombinase, in specific tissues (Utomo et al., 1999). The timing of expression of the recombinase can also be manipulated by using an inducible system, such as one consisting of tamoxifen-inducible Cre (Danielian et al., 1998; Guo et al., 2002). CreER is a fusion protein of Cre recombinase and a mutated version of the ligand binding domain of estrogen receptor that binds the antiestrogen, 4-hydroxyl-tamoxifen, but not estrogen. The fusion protein is inactive unless bound to tamoxifen, which allows for its translocation to the nucleus (Danielian et al., 1998).

To examine the efficiency and specificity of Cre recombinase transgenes, several reporter mice have been generated; the most commonly used line is ROSA26-*lacZ*. When Cre recombinase activity is present, the stop cassette in front of *lacZ* gene will be deleted, allowing *lacZ* expression (Soriano, 1999). Since the gene targeted at the *Rosa26* locus is ubiquitously expressed, it ensures the reporter can be used as a marker for all Cre recombinases.

Conditional loss-of-function alleles for tumor suppressors bypass lethality and expand tumor phenotypes

Conditional alleles of tumor suppressor genes *p53*, *RB*, and *PTEN* have been generated and thus these genes can be deleted in a spatial and temporal manner. For example, the *p53* conditional allele was generated to delete exons 2–10, which resulted in loss of 95% of the coding region (Figure 2) (Jonkers et al., 2001). The *p53* conditional allele has been used to investigate the roles of *p53* in tumorigenesis in different types of tissues, including prostate (Lei et al., 2006) and breast cancer (Derksen et al., 2006; Jonkers et al., 2001; Liu et al., 2007) by crossing to mice expressing a tissue specific Cre recombinase.

p53 heterozygous mice develop spontaneous osteosarcoma approximately 30% of the time with a long latency, and 70% of *p53*-null mice develop thymic lymphoma instead of osteosarcoma (Jacks et al., 1994). To develop a mouse model for osteosarcoma with shorter

latency and higher incidence, Rodda *et al.*, generated mice with deletion of *p53* in osteoblasts by crossing the *p53* conditional allele to *Osterix-Cre* transgenic mice (*Osx-Cre*) with Cre expression limited to committed osteoblast progenitors (Rodda and McMahon, 2006). These mice developed osteosarcomas with 60% penetrance and a latency of less than one year (Lengner et al., 2006; Walkley et al., 2008). These kinds of studies can thus also be used to identify the cell of origin of a specific tumor type.

Sometimes the deletion of a tumor suppressor using conditional loss-of-function alleles in a specific cell type has no phenotype. In these cases, combination with other tumor suppressors is examined. For example, deletion of *BRCA2* (a breast cancer susceptibility gene) specifically in the mammary gland epithelium using the K14 promoter-driven Cre recombinase does not yield tumors. But mice with conditional loss of both *BRCA2* and *p53* in the mammary epithelium develop breast tumors frequently, demonstrating that disruption of *p53* is critical in *BRCA2* associated breast cancer (Jonkers et al., 2001). Conditional knockout of *BRCA1*, another breast cancer susceptibility gene, and *p53* in basal epithelial cells results in breast cancer that is reminiscent of human basal-like breast cancer with the features of highly proliferative, poorly differentiated estrogen receptor-negative mammary carcinomas. Such tumor models will also be valuable for therapeutic research (Liu et al., 2007).

Generating Knockin alleles

Many of the same steps involved in making a knockout mouse are also needed to make knockin models. The targeting construct contains positive and negative selection markers. However, the knockin mouse model is generated by introducing a mutant gene or a reporter into the endogenous locus.

The first *p53* knockin *p53R172HΔg* mouse model was generated by using a two-step selection protocol (Wu et al., 1994). In the first step, the *pGK-neo-NTR-tkpolyA* cassette was introduced into the endogenous *p53* locus (Figure 3). G418 was used to select for positive clones. In the second step, negative selection was used to screen for a second recombination event that evicts the *pGK-neo-NTR-tkpolyA* cassette and introduces *p53R172H* point mutation. This process is called double replacement and generates a perfect knockin locus with only one nucleotide difference (Liu et al., 2000). Unfortunately in this example, the targeting construct contained an additional deletion of a G nucleotide at a splice acceptor site, so it produced low levels of mutant *p53* protein. Only one plasmid in the collection contained this additional alteration. Consequently, in constructing knockin models, we recommend sequencing of the introduced DNA construct to ensure that there are no other deleterious mutations. The advantage of the double-replacement protocol is that no *LoxP* site is left behind that might interfere with expression. However, a major disadvantage is that two recombination steps are required, which increases the length of time the ES cells are in culture, thus lowering their chances of contributing to the germline.

Subsequent knockin alleles of the *p53* locus were generated using a *neo* cassette flanked by *LoxP* sites (termed floxed) and a point mutation (Figure 4). The presence of the *neo* cassette in these mice greatly reduced expression of *p53*, emphasizing the need to delete the *neo* gene (Wang et al., 2011). Cre-mediated recombination deleted the *neo* cassette (Lang et al., 2004). Other *p53* knockin models were generated using a *Lox-stop-Lox* cassette, which uses puromycin selection and a stop cassette to introduce the point mutation. This strategy inhibits protein expression unless the stop cassette is deleted and is often used when expression of the mutant proteins is deleterious to ES cell survival (Iwakuma and Lozano, 2007; Olive et al., 2004). Since the *Lox-stop-Lox* cassette blocks the expression of *p53R172H*, this allele can be used as a conditional knockin allele, which switches the locus

from *p53*-null to *p53*^{R172H}. Such a model may be useful in determining the differences between *p53* null and mutant alleles in the genesis of a specific cancer. A model in which the surrounding stroma is wild-type for *p53* would make it possible to distinguish contributions to tumor evolution of the tumor cells and the stroma.

The Trp53ER^{TAM} allele is another conditional knockin allele that contains the hormone-binding domain of the modified estrogen receptor, ER^{TAM} inserted in-frame at the 3'-end of the coding sequence of endogenous *p53*. This fusion protein is mostly inactive since ER^{TAM} binds to heat shock protein 90 and stays in the cytosol. When p53ER^{TAM} binds to its ligand (4 hydroxyl-tamoxifen), the fusion protein dissociates from the heat shock protein and relocates to the nucleus to become active (Christophorou et al., 2005). This knockin mouse model makes it possible to specifically and temporally switch *p53* from wild-type to null and vice versa.

Translocations

Translocations such as the *c-Myc* translocation to the immunoglobulin locus occur in some kinds of tumors. Cre-*loxP* site-specific recombination can also be used to reproduce these translocations in mice. First, *loxP* sites are introduced into different chromosomes in the mouse genome. Transient expression of Cre recombinase is then used to mediate chromosomal rearrangements. Several translocations in mouse models have been generated using this method including a reciprocal translocation between the *c-Myc* and immunoglobulin heavy chain genes on chromosomes 15 and 12 (Smith et al., 1995) (Figure 5). Similarly, a site-specific translocation between the *Dek* gene on chromosome 13 and the *Can* gene on chromosome 2 in mouse ES cells was performed to generate an acute myeloid leukemia-associated fusion protein (Van Deursen et al., 1995). The estimated frequency of Cre-mediated translocation between non-homologous mouse chromosomes in this study was approximately 1 in 1200–2400 ES cells (Van Deursen et al., 1995).

In addition to inducing the translocations in the germline, tissue-specific translocations can also be achieved by crossing the two *loxP* alleles to a tissue-specific Cre transgene. The fusion protein AML1/ETO mimics the translocation that occurs in human acute myeloid leukemia and is recreated in a tissue-specific manner by crossing AML^X and EMO^X alleles to *Nes-Cre1* or infecting mice with a retrovirus-expressing Cre recombinase (Collins et al., 2000).

Transgenic Mice

The first transgenic mouse model was successfully created in the 1980s. Pronuclear injection allows the random integration of DNA into the genome, rather than specific targeting into a homologous locus. During pronuclear injection, linear fragments of cloned DNA are injected directly into fertilized oocytes. Once injected, the oocytes are then re-implanted into a surrogate mother. Once born, the genome of the manipulated mouse can easily be assayed for incorporation of the newly introduced genetic material, the transgene. The transgenic mice are bred to establish transmission through the germline and their offspring are then examined for phenotypes. Because the procedure involves simply adding DNA to the nucleus of an oocyte, the DNA often integrates with multiple copies at the same site of the chromosome in a head-to-tail array (Brinster et al., 1981; Brinster et al., 1982; Costantini and Lacy, 1981).

Because of the random nature of DNA insertion in the pronuclear injection process, the inserted genes are prone to position effects at the site of insertion and can also generate insertion mutations that will affect the ability to recover homozygous offspring (Shawlot et al., 1989; Woychik and Alagramam, 1998; Woychik et al., 1985). The initial mice identified

as transgenic are considered independent founders (F0), and separate lines of progeny need to be established and assayed for phenotype for each F0. Because of the possibility of position effects, it is generally recommended to recover at least three founder lines to show that the phenotype observed is not affected by the position of transgene insertion. Methods to ameliorate position effects include use of very large pieces of DNA as in bacterial artificial chromosomes (BACs) or yeast artificial chromosomes (YACs) (Giraldo and Montoliu, 2001) or by including an insulator cassette in the transgene. A commonly used insulator is chicken hypersensitive site 4 (cHS4) derived from the chicken β -globin locus (Arumugam et al., 2009; Ciavatta et al., 2006; Walters et al., 1999). This insulator enhances transgene expression and decreases the variation between transgenic lines (Rincon-Arango et al., 2007; Yao et al., 2003).

While pronuclear injection is generally used to provide exogenous gene expression, in many cases when and/or where this expression takes place needs to be controlled. This need can be met using inducible genes (Saunders, 2011). For example, when transgenes are detrimental to embryonic development, expression during the developmental stage can be blocked using a stop cassette, such as *lox-stop-lox* described earlier (Lobe et al., 1999). Once the gene is combined with Cre recombinase, the stop sequence is deleted and the gene is expressed (Nagy, 2003).

Another commonly used dose-dependent inducible system in mouse models is based on the tet operon/repressor bi-transgenic system. This is a two-gene system that includes an activator gene (called tTA or rtTA) that produces the TA protein that is regulated by doxycycline, and a responder gene (Gossen et al., 1995; Kistner et al., 1996). TA binding activates the responder gene. In the tet-on system, the rtTA gene produces a TA protein product that requires doxycycline for activation. In the absence of doxycycline, the tet-on system is inactive. In the tet-off system, TA protein is inactivated in the presence of doxycycline. In this system, the responder gene is always on. In the presence of doxycycline, the product of the tTA gene is inactivated. As previously mentioned, conditional knockout of *p53* in osteoblasts uses the OSX-Cre transgene, which is a tet-off Cre system. Cre expression is repressed when doxycycline is administered (Rodda and McMahon, 2006). When mutant animals are maintained on doxycycline from conception until four weeks of age to delay *p53* and *Rb* gene inactivation, of osteosarcoma development is delayed (Walkley et al., 2008).

Transgenic mice generated by pronuclear injection have random insertion of the transgene DNA, which can confound results. Thus, new technologies have been developed to introduce the transgene into a specific known site (Beard et al., 2006). These technologies are known as site-specific transgenesis. They use a docking site in the genome (for example, the *ColA1* gene) and a site-specific recombinase to introduce the transgene DNA in ES cells. This strategy results in single-copy insertion into a known locus. Once the correctly targeted cells are identified, they are injected into blastocysts to produce chimeras in the same manner as for standard knockouts. The mice inheriting the transgene are identical and can be compared with each other.

Mdm2 and Mdm4 transgenic mice to study the p53 tumor suppressor pathway

The studies described above outline the process of deleting or mutating tumor suppressors. However, a clear understanding of the pathways involved might also entail generating transgenic mice. For example, the activity of the p53 tumor suppressor is inhibited by two proteins, Mdm2 and Mdm4. Mdm2 is an E3 ubiquitin ligase that complexes with Mdm4 to target p53 for degradation (Marine et al., 2006). More importantly, Mdm2 and Mdm4 are

often present at high levels in human cancers and many of these tumors retain a wild-type *p53* allele (Evans et al., 2001). Thus, high levels of Mdm2 and/or Mdm4 obviate the need for deletion or mutation of *p53* to produce tumors.

In experiments designed to directly test whether overexpression of Mdm2 or Mdm4 could contribute to tumorigenesis, transgenic mice were made by introducing a BAC that expresses the entire gene into ES cells (Jones et al., 1998). The ES cells subsequently contributed to the germline of mice. *Mdm2* transgenic mice developed tumors, although the tumor spectrum is very different than that of mice with *p53* deletion, suggesting the Mdm2 might have additional activities independent of p53. Mdm4 transgenic mice have also been made. Similar to Mdm2, overexpression of Mdm4 driven by a strong promoter induces tumors in mice (Xiong et al., 2010). Importantly, heterozygosity for *p53* yielded more tumors and shorter survival curves, suggesting that Mdm2 overexpression did not completely inactivate p53. However, in these mice, LOH was rarely observed, indicating that Mdm2 dampened p53 activity sufficiently to promote tumor phenotypes. Thus, tumors could disrupt p53 activity via multiple mechanisms, which included deletion or mutation of *p53* and alterations such as Mdm2 and Mdm4 over production. The transgenic models have made it possible to directly test the importance of Mdm2 and Mdm4 in tumor development.

Allele Preservation

Lastly, this overview would not be complete without a description of the process of allele preservation, as each genetically modified animal is unique and all are valuable. Genetically modified mice are sometimes fragile, exhibit poor breeding behavior or a deleterious phenotype (such as a being tumor-prone), and are expensive to produce and maintain. For these reasons, the investigator should archive any genetically engineered rodent strains.

Two methods of cryopreserving embryos are widely used. One method is the use of vitrification to “flash freeze” embryos (Nakagata, 1995; Nakao et al., 1998). In this method, the embryos are suspended in a glass-like state within the cryopreservation media. This procedure is fast, and requires little special equipment. However, this method is exacting, and embryos can easily be lost if established procedures are not precisely followed. Another method of cryopreservation is to slow freeze embryos (Leibo, 1986; Whittingham et al., 1972). In this procedure, embryos are exposed to a cryoprotective medium that dehydrates them prior to freezing. By reducing or eliminating the water inside of the embryo, freeze/thaw expansion and contraction of the water is eliminated, and thus the embryos are not killed during the process. This method tolerates some variations in procedure; however, it requires more expensive equipment. When the model is needed at a future date, the embryos are recovered and implanted into a surrogate mother for gestation. Typically 200–500 embryos are cryopreserved for each line. The Jackson Laboratory has reported the successful recovery of embryos frozen since the early 1970's (forty years after cryopreservation)!

Recent developments have made the cryopreservation and recovery of sperm a routine procedure (Ostermeier et al., 2008; Szein et al., 2000). A major advantage of sperm cryopreservation is that as few as 1 or 2 males can be used to archive a line. However, in contrast to freezing embryos, sperm represent only half of the germline. Once thawed, the sperm must fertilize oocytes from wild-type females using in vitro fertilization to reconstitute the line. Since only half of the germline is present, sperm freezing is not a recommended method of archiving models that depend on the presence of multiple genes. After in vitro fertilization, all mutations will be heterozygous, and multiple genes may not segregate with each other. Thus, additional crosses may be needed to recover a model that depends on more than one mutation.

In summary, the ability to manipulate the mouse genome allows the development of models that are representative of human diseases such as cancer. The models developed thus far have shed light on the function of genes as tumor suppressors, identification of cooperating events in tumorigenesis, and the roles and interactions of members of a tumor suppressor pathway. Also studies have shown the importance of LOH in some but not all cases. Deletion of some tumor suppressors has also revealed embryo lethal phenotypes, highlighting the importance of these genes in development and cancer. Conditional alleles are necessary to understand the tissue specific roles of these tumor suppressors. For example, the *p53* conditional allele allowed an examination of p53 in osteosarcoma and breast cancer tumorigenesis. Knockin alleles make it possible to explore how the mutations that occur in human tumors contribute to tumorigenesis or tumor progression *in vivo*, via gain-of-function mechanisms. Thus, the many kinds of mouse models that have been generated over the years have made it possible to understand basic concepts in tumor cell biology.

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Figure 1.

A *p53* null allele. This diagram represents a *p53* null allele that was generated by deletion of part of intron 4 and exon 5, with insertion of *neo*. The *neo* gene does not contain a polyadenylation (poly A) site and is forced to use an endogenous poly A signal upon integration limiting the number of random insertions. *MC1-tk* encodes for the thymidine kinase. FIAU: 5-iodo-2'-fluoro-2'-deoxy-1- β -D-arabino-furanosyl-uracil. Numbers above boxes denote *p53* exons.

Figure 2.

A *p53* conditional loss-of-function allele. The initial step introduces three *loxP* sites (red ovals) and a *neo-tk* gene by homologous recombination into the *p53* locus. Cre mediated deletion of *neo* and not deletion of the entire *p53* gene is possible when Cre is weakly expressed. The *Trp53F2-10* allele then contains two *loxP* sites which upon recombination delete *p53* exons 2–10 in a tissue specific manner depending on Cre expression. Numbers above the boxes denote *p53* exons.

Figure 3.

Double replacement strategy to generate a point mutation in *p53*. In the first recombination step, *PGKneoNTRtkpA* which transcribes both *neo* and *tk* genes replaces *p53* exons 2–6. G418 selection is used to narrow the number of ES cell colonies examined. In the second step, a correct *p53* gene containing a single point mutation in exon 5 (asterisk) replaces *PGKneoNTRtkpA* and reconstructs the locus. This targeting construct also contained an unwanted deletion of a G nucleotide at a splice acceptor site. Numbered boxes denote *p53* exons.

Figure 4.

A knockin *p53* allele. To generate a *p53* knockin allele with a single point mutation, the targeting construct contains the *PGKneo* cassette flanked by *loxP* sites (red ovals) and the point mutation in exon 5. Cre-mediated recombination deletes the *PGKneo* cassette and results in the knockin allele with a point mutation and a single residual *loxP* site in intron 4. Numbers above the boxes denote *p53* exons.

Figure 5.

Chromosomal translocations. To generate chromosomal translocations, two alleles were generated each containing half of the *Hprt* gene, a resistance marker neomycin (neo) or hygromycin (hygro) and a *loxP* site. Addition of Cre recombinase brings the IgH enhancer region upstream of the coding exons (2 and 3) for *c-Myc* causing inappropriate over expression of *c-Myc*. The 5' and 3' ends of *Hprt* gene also come together and allow for selection by *hprt*.