Chapter 3
Transgenic Animal Models in Lung Research

Chi F. Hung and William A. Altemeier

Introduction

Advances in molecular biology have enabled the use of genetically modified mammalian vertebrates in human disease research. Due to their physiological similarity to humans, relative cost-effectiveness, and ease of husbandry, rodents have become the most widely used transgenic animals in modeling human disease. The goal of this chapter is not to undertake an exhaustive review of the history of transgenic animals, but rather to highlight recent advances in animal models used in pulmonary research and to offer practical guidance in the use of these transgenic animals. Over the past decade, the development of Cre-loxP technology has enabled the development of sophisticated transgenic animal models to address complex questions about development and function. While this technology is a powerful tool, many considerations need to be made when using these transgenic models. This chapter will give a brief overview of this technology as well as practical considerations in the use of these transgenic animals.

Transgenic Mice in Lung Injury Studies

Ever since the ability to introduce genetic modifications into mammalian embryonic stem cells was discovered in the 1980s, transgenic mice have assumed an integral role in the laboratory study of human disease [1]. Manipulations that result in

C.F. Hung (✉) · W.A. Altemeier
Division of Pulmonary and Critical Care Medicine, Department of Medicine, Center for Lung Biology, University of Washington, Seattle, WA, USA
e-mail: cfhung@uw.edu

W.A. Altemeier
e-mail: billa@uw.edu

L.M. Schnapp and C. Feghali-Bostwick (eds.), *Acute Lung Injury and Repair*, Respiratory Medicine, DOI 10.1007/978-3-319-46527-2_3

gain-of-function and loss-of-function animal models have been used extensively over the last three decades to study biological pathways in lung pathogenesis. Heritable gene knock-ins and knockouts have been traditionally used to model human disease. Baron et al. [2] summarized genetically modified strains in their review of transgenic animals in lung biology research. For example, genetic knockouts of surfactant protein D and tissue inhibitor of metalloproteinases-3 lead to spontaneous histological changes compatible with chronic obstructive pulmonary disease [3, 4]. Models in which genetic manipulation does not lead to an overt phenotype but confers susceptibility to or protection against an injurious agent have also been widely used. For example, metalloproteinase-12 knockout mice have no observable lung phenotype during embryogenesis or in adulthood, however they are protected against emphysema with cigarette exposure [5]. These studies suggest metalloproteinases play a complex but important biological role airway inflammation, remodeling, and development of emphysematous changes.

Use of transgenic mice with global gene disruption has its limitations. Global knockout or overexpression of a gene may disrupt development and lead to embryonic or postnatal lethality. Conversely, biological pathways in pathogenesis may differ between species. Genetic manipulation in mice may not adequately recapitulate human disease. For example, attempts to generate a mouse model of alpha-1 antitrypsin (A1T1) deficiency have been unsuccessful to date. Depending on the mouse genetic background, serine protease expression in mice is controlled by multiple genes in the serpin family (Serpina1) whereas in humans, A1T1 expression is limited to one gene. Attempts to generate a knockout in one of the serine protease genes in mice (Serpina1a) have not been successful to date, with the unexpected discovery that Serpina1a-/- leads to embryonic lethality [6]. While human A1T1 expression is only controlled by one gene, Serpina1 null genotypes have been reported in humans suggesting A1T1 is not essential in human embryogenesis in contrast to mice. Given the important limitations seen in the generation of global knockout transgenic animals, other genetic manipulation strategies are required to study individual biological pathways in animal models. Recent advances in technologies that allow temporal and spatial control of genomic manipulation in mouse models are paving way to elegant transgenic models that address some of the limitations encountered in global knockout models.

Cre recombinase

Genomic manipulation using site-specific recombinases has become increasingly popular in transgenic mouse models. Recombinases are enzymes that serve a host of vital biological function in viruses and yeast. They recognize specific DNA sequences on the genome and mediate rearrangement of DNA with high efficiency, enabling molecular phenomena such as viral genomic integration and excision. The Cre recombinase derives from bacteriophage P1 and mediates recombination at specific 34 base pair sequences termed loxP sites [7, 8]. Of the many recombinases

known, Cre recombinase requires no additional accessory proteins or specific substrate topology to mediate recombination efficiently at loxP sites [9, 10]. For these reasons, the Cre-loxP system has been widely used in transgenic mouse models to modify gene expression. Another well-studied recombinase with applications in transgenic animal studies includes the yeast recombinase Flp from *Saccharomyces cerevisiae* [11]. Application of these recombinases has enabled a host of novel studies in lung research including developmental studies, cell-fate mapping, and spatial or temporal gene disruption duties.

The 34 bp loxP sequence is directional. When two loxP sites are inserted into the host genome or linearized DNA in a head-to-tail fashion, Cre will mediate recombination between the loxP sites leading to the excision of the DNA between the loxP sites as a circular DNA plasmid [12]. In transgenic models, loxP sites may be introduced into targeted coding sequences in a specific gene (called a "floxed" gene). When crossed with a transgenic animal that expresses Cre recombinase under the control of a specific gene promoter, the bitransgenic progeny will undergo deletions of the floxed coding sequences where Cre recombinase is expressed. Such deletions may result in frameshift mutations or truncations during mRNA transcription, leading to null phenotypes.

Applications of Cre-loxP System in Animal Models

Studies in lung development and regeneration. The ability to manipulate the mouse genome using Cre-loxP transgenes was first demonstrated by Lewandoski and Martin [13] in 1997. The introduction of Cre-loxP technology in transgenic models has enabled a number of exciting discoveries and refinements in our understanding of cardiopulmonary development and regeneration. In these studies, transgenic animals expressing Cre under the control of a developmental gene promoter are crossed to transgenic animals carrying a reporter construct under the control of a ubiquitous promoter such as *Rosa26*. These constructs contain a STOP codon flanked by loxP sequences, and a chemical or fluorescent reporter downstream such as LacZ or any one of the fluorescent proteins (e.g., green fluorescent protein, tdTomato, yellow fluorescent protein) [14]. The result is constitutive silencing of the reporter until the floxed STOP codon is excised in the presence of Cre recombinase. In the presence of Cre recombinase, cells and their daughter cells constitutively and heritably express the reporter construct. Bitransgenic animals expressing Cre under a specific gene promoter (e.g., a gene of interest expressed only during embryonic development in a specific progenitor cell type) and the Rosa26-STOP$^{flox/flox}$-reporter transgene allow investigators to map the fate of early progenitor cells by visualizing labeled cells and their daughter cells in the developing lung. The ability to identify the temporal and spatial fate of lung progenitor cells allows for more detailed examination of the pathways essential to the proper differentiation of the many lung cell types and improved understanding of disease states. Similarly, whereas the fully developed adult lung remains fairly quiescent,

adult progenitor populations are present and may reenter cell cycle to repair and/or regenerate damaged cells following experimental injury stimuli. While the composition of and pathways utilized by these putative adult progenitor cells remain unclear, Cre-loxP technology has enabled a number of studies directed at identification of the pools of progenitor cells in the adult lung. For example, labeling of differentiated type II alveolar epithelial cells has shown that they are capable of acting as stem cells to repair damaged alveolar epithelium following lung injury [15]. Furthermore, this technology has been used to identify the progenitors of lung myofibroblasts following experimental lung injury and to expand on the field's understanding of the key cellular effectors in fibrosis and lung repair [16, 17].

Spatial deletions. Another powerful application of Cre-loxP technology is its use in cell-specific deletion of genes. By carefully selecting cell type-specific promoters, investigators are able to engineer transgenic mice with targeted gene deletions in restricted populations of lung cells. These studies have led to a more refined understanding of the functional heterogeneity of different cell types in lung injury and repair. For example, an area of intense debate within the lung fibrosis field has been whether bone-marrow-derived, circulating progenitor cells termed "fibrocytes" directly contribute to collagen I deposition in lung fibrosis. These cells are CD45+ and display collagen *IαI* gene and protein expression. Furthermore, they localized to areas of fibrosis in experimental models of lung injury by immunohistochemistry [18] However, conflicting data have shown no evidence of CD45+ cells in areas of fibrosis by lineage-tracing studies [16, 17], calling into question the importance of fibrocytes in lung fibrosis. Kleaveland et al. further elaborated on these findings and examined collagen I production by fibrocytes in experimental lung injury. In their study, transgenic mice expressing Cre under a hematopoietic cell lineage promoter, Vav-Cre, crossed to a *collagen IαI* floxed transgenic animal were used to disrupt *collagen IαI* expression specifically in fibrocytes, without affecting fibrogenic cell types of non-hematopoietic lineages. Despite the inability of fibrocytes to transcribe the *collagen IαI* gene, collagen I deposition was unaffected in experimentally induced lung fibrosis. Therefore, the authors concluded fibrocytes did not functionally contribute to collagen I deposition [19]. Without the ability to restrict the deletion of the *collagen IαI* gene in fibrocytes, this type of study would not be possible. Another advantage of spatial deletion in gene disruption studies is that global knockout of a gene of interest sometimes leads to an embryonic lethal phenotype, rendering the model unsuitable for studies requiring adult animals.

Temporal Cre activation and deletions. A related application of the Cre-LoxP system involves the conditional activation of Cre in a temporally controlled manner. To achieve this, various constructs of Cre recombinase fused to ligand-binding domains have been generated, resulting in Cre recombinase (tethered to a ligand-binding domain) that is sequestered in the cytosolic compartment and unable to mediate genomic recombination at target loxP sites. Upon ligand exposure and binding to the ligand-binding domain (typically exogenously administered at the desired time point), Cre recombinase translocates to the nucleus to mediate genomic recombination. The most commonly used conditionally activated Cre recombinases

involve mutated estrogen receptors and the administration of tamoxifen to liberate the Cre recombinases. Various widely available constructs include CreER, CreER^{T2}, and MerCreMer. The ability to temporally control Cre activation enables studies in which timed recombination of the target, floxed gene is desired. For example, conditional knockout of a gene critical in development can be studied in adults using the CreER technology by administering tamoxifen to adults, thus bypassing the developmental stage where the gene's function is vital.

Inducible Cre beyond tamoxifen. The doxycycline system was developed to reversibly suppress or activate gene expression in transgenic mice. This transgenic model consists of two transgenic lines bred to each other: (1) an activator line expressing either the tetracycline activator (TA, or tet-off) or the reverse tetracycline responsive transactivator (rtTA, or tet-on) and (2) an operator line with the desired transgene under the control of the tetO operator. In bitransgenic animals containing the activator and operator transgenes, doxycycline administration allows tetracycline activator to bind to tetO, suppressing transgene expression downstream (tet-off), or rtTA to bind to tetO, activating transgene expression (tet-on). This model was developed separately from the Cre/lox model to study gene expression in mouse models. Combining the Cre/lox system with tetO system has allowed for sophisticated methodolgies in doxycycline-dependent, cre-mediated gene manipulations. The generation of triple transgenic mice with rtTA expression under a cell-specific promoter, TetO-Cre, and loxP sites flanking a gene of interest result in animals that will have deletion of a gene of interest in a cell-specific manner when they are exposed to a diet with doxycycline. Perl et al. used this strategy to generate a triple transgenic animal combining rtTA expression under the SP-C promoter for epithelial tissue specificity, tetO-Cre, and CMV-loxP-LacZ-loxP-AP (ZAP) or CMV-loxP-LacZ-loxP-GFP (ZEG) reporter construct [20]. Following doxycycline exposure, these mice demonstrated reporter expression (alkaline phosphatase or GFP) in distal lung epithelial cells.

In addition to doxycycline, other inducers have been described. The use of a Cre fused to a progesterone receptor ligand-binding domain was first described by Kellendonk et al. [21]. In these transgenic mice, Cre activity is induced by the administration of RU-486. However, transgenic mice using this fusion Cre showed variable "leaky Cre" activity in which Cre activity was observed in unexposed transgenic animals (i.e., no RU-486) [22, 23]. Partly due to the leaky Cre phenomenon, inducible Cre transgenic lines using progesterone receptor ligand-binding domain fusion are less widely available compared to the tamoxifen-inducible CreER or CreER^{T2} counterparts.

Novel inducible Cre lines are under development to harness advances in pharmacologic stabilization of destabilized proteins. This technology utilizes mutant destabilizing proteins from humans or bacteria fused to a protein of interest. When the protein of interest is fused to the destabilizing protein, the complex is usually degraded by proteosomes. However, this degradation can be blocked by administration of a pharmacologic stabilizing agent. For example, Cre recombinase has been fused with the destabilizing dihydrofolate reductase (DHFR) from *Escherichia coli* to generate an inducible Cre transgenic animal that targets genes specifically in

neurons [24]. In this model, the administration of trimethoprim (TMX) stabilizes the Cre-DHFR fusion protein driven by a neuron-specific promoter and leads to efficient Cre-mediated recombination in neurons. The advantage of TMX-inducible system includes ease of administration in animal models, high penetrance in most tissues, and lack of endogenous targets in mammals. This may prove to be a highly attractive model in lung research, but specific transgenic lines have yet to be developed and characterized for lung biology.

Lung-specific Cre Mouse Models

Murine lung derives from the endoderm foregut and develops by branching morphogenesis. Differentiation of various lung cell types appears during lung development. A number of transgenic models using lung-specific and nonspecific promoters to drive Cre expression have been developed to examine the fate and function of various progenitors and differentiated lung cell populations. Lung bud from the foregut appears around E9.5 in mice while the trachea and esophagus separate from the foregut between E10.0 and E11.5. The choice of transgenic model and timing of inducible Cre activity will influence the lung specificity of the model.

Lung epithelium. Models for epithelial progenitors, type II alveolar epithelial cells (ATII), type I alveolar epithelial cells (ATI), Clara cells, basal cells, and ciliated cells have been developed. The human surfactant associated protein C (Sftpc) promoter has been used to generate transgenic strains that specifically target the lung epithelium. In the developed, adult lung, Sftpc promoter expression is restricted to ATII and some cuboidal bronchiolar cells. During embryogenesis, however, the promoter is active once the endoderm is committed to lung development and drives recombination throughout the lung epithelium [25]. Another transgenic line Sftpc-CreERt2-rtTA has been developed that contains a knock-in of the CreERt2 and rtTA cassettes in the endogenous mouse Sftpc promoter [26]. Expression of CreERt2 and rtTA in this line is restricted to ATII cells in the adult lung and may present a very useful model to examine conditional gene disruption in ATII cells.

Still other Cre transgenic mice are available to study lung epithelial cells though they are not lung specific. Transgenic models that utilize promoters active in epithelial precursor cells are active in the endoderm before lung bud formation, and thus may drive expression in extrapulmonary tissues following embryogenesis. Sonic hedgehog (Shh) knock-in Cre-GFP is active in the ventral foregut endoderm by E9.5 before lung and trachea-esophagus separation. Islet1-Cre is a knock-in Cre line with expression in pharyngeal endoderm by E9.5. While it labels the lung epithelial precursor cells widely, it is not specific to the lung epithelium. Small populations of mesenchyme are also labeled by this strain. The Id2-CreERt2 is a CreERt2 knock-in mice that shows tamoxifen-dependent activity in distal epithelial tips and a subset of mesenchymal cells. Nkx2-5 Cre is another knock-in transgenic

line that has been used to disrupt lung epithelial genes, however Nkx2-5 is also active throughout the foregut endoderm and the surrounding mesoderm before lung budding, and thus has activity in other developing organs such as the myocardium as well [27].

While transgenic mice using the Sftpc promoter to label ATII cells are fairly specific to the lungs, promoters for ATI cells are less specific. Aquaporin 5 (Aqp5) is expressed in ATI but not ATII cells in rats and humans. Mice express Aqp5 predominantly in ATI cells and in a small minority of ATII cells. Cre transgenic mice using the Aqp5 promoter have been developed but extrapulmonary expression (e.g., salivary glands and stomach) has also been observed [28]. To date, no transgenic line exists with Cre activity exclusively in ATI cells.

Lung stromal cells. Stromal cells in the lung have traditionally been defined by negatives: they are non-epithelial, non-endothelial, and non-immune. These interstitial cells are histologically and functionally heterogeneous with potentially very different roles in lung pathobiology. Contractile smooth muscles that surround larger airways and arterioles constitute one type of stromal cell. Perivascular stromal cells at the capillary level (also termed pericytes) constitute a histologically and functionally distinct subtype of stromal cell. Finally, interstitial fibroblasts in alveoli may constitute yet another functionally distinct group of stromal cells. Within the group of fibroblasts, there are further functional and histological distinctions such as lipofibroblasts and matrix-producing fibroblasts. Classification of stromal subpopulations remains controversial and there is no consensus on markers that uniquely identify each of these stromal cell subtypes. Exactly how these cellular subtypes contribute to lung repair remains under active investigation. For example, recent studies using lineage-tracing mouse models have shown that multiple stromal populations including resident fibroblasts and pericytes contribute to scar-forming cells in experimental lung fibrosis [16, 17]. To understand the function and lineages of the diverse populations of stromal cells, investigators have developed transgenic Cre models that trace the lineage of various mesenchymal cell types, though none of the models is specific to the lung. We have used a mouse model originally developed to fate-trace pericyte progenitors in the kidney and examined its suitability as a pericyte lineage-tracing model in the adult mouse lung [29]. FoxD1 is a forkhead transcription factor expressed only during embryonic development. We observed that FoxD1-derived cells are enriched for platelet-derived growth factor receptor beta positive stromal cells in the adult mouse lung and are histologically compatible with pericytes [16]. The FoxD1-Cre model thus represents a useful tool for examining the function of pericytes in the lung. To study smooth muscle cells, various transgenic Cre models utilizing promoters SM22α, smooth muscle actin α (SMA), and smooth muscle myosin heavy chain (SMMHC) have been developed [30]. Their utility in the adult mouse lung is limited by the fact that Cre activity is not inducible in these lines. Inducible Cre lines using the SMA and SMMHC promoters have been developed and these lines may prove to be useful tools in studying smooth muscle and myofibroblast function in the adult mouse lung [31, 32].

Lung endothelial cells. To date, there is no lung-specific endothelial transgenic mouse line. The most widely used endothelial lineage-tracing model utilizes the murine Tek promoter (commonly referred to as Tie2) [33]. Tie2-Cre mice labels endothelial cells in many organs and have been a useful tool in vascular biology research. The utility of this model in lung biology is limited by its widespread expression throughout the mouse vasculature and caution must be made when using this line as nonspecific activity, presumably transmitted through germline activation of Cre, has been reported [34]. Individuals with an interest in lung-specific Cre transgenic lines are encouraged to read a comprehensive review on this topic by Rawlins and Perl [35].

Protocols

Tamoxifen preparation. Numerous resources and protocols are available online for the preparation of tamoxifen. Tamoxifen is a hydrophobic compound and thus dissolves poorly in aqueous solutions. Most commonly, tamoxifen is supplied in powder form and needs to be reconstituted fresh prior to each administration. Tamoxifen is soluble in ethanol, methanol, 2-propanol, and propylene glycol. Common preparations of injectable tamoxifen involve dissolving tamoxifen powder (Sigma T5648) in ethanol (commonly to a final concentration of 50 mg/ml). Preheating the ethanol to 37 °C aids in solubilizing the tamoxifen powder. Aliquots of tamoxifen prepared in this manner may be stored in −20 °C for several months but should be kept from light exposure. Prior to injection, aliquots are further diluted in 4× volume of corn oil (Sigma C8267) and then injected into the peritoneum of animals. Concentrations of tamoxifen greater than 10–20 mg/ml in corn oil may result in tamoxifen becoming insoluble and precipitating out of solution. A typical preparation includes dissolving tamoxifen powder in 100 % ethanol to a stock concentration of 50 mg/ml. Prior to injection, the stock solution is further diluted in 4× volume of corn oil to a concentration of 10 mg/ml. Injection of 100 μl of tamoxifen in corn oil (10 mg/ml, or 1 mg per injection) may be adequate to activate the recombinase. Users are advised to consult with IACUC at their institutions to determine an appropriate injection schedule and the maximum allowable dosage. One disadvantage we observed with this method is that mice (C57bl6 background) may have poor absorption of the corn oil solution. At necropsy 4–5 weeks beyond the last administration of tamoxifen, oil droplets may continue to be present in the peritoneum. Furthermore, studding of the diaphragm and peritoneum with white plaques has been observed by many investigators which may represent insoluble tamoxifen deposits or from the corn oil. To address these concerns, alternative vehicles to emulsify poorly water-soluble tamoxifen have been tested. Kolliphor (previously Cremophor) is an amphiphilic polyethoxylated castor oil derivative that is commonly used to solubilize poorly water-soluble pharmacologic agents. It has been used to solubilize tamoxifen for intraperitoneal as well as intravenous injection of tamoxifen [36]:

- Add prewarmed 100 % ethanol (37 °C) to dissolve tamoxifen powder (conc. 50 mg/ml)
- Vortex to dissolve tamoxifen completely (usually 5–10 min is adequate)
- Add equal volume of Kolliphor (Sigma C5135) to tamoxifen/ethanol mix (tamoxifen conc. 25 mg/ml)
- Vortex and mix well. Aliquot and store in −20 °C protected from light
- To use: add 4× volume of PBS to Kolliphor/tamoxifen/ethanol mix and vortex well to ensure complete emulsification (final tamoxifen conc. 5 mg/ml). For 1 mg tamoxifen administrations, use 200 µl.

Tamoxifen administration. Tamoxifen may be administered by various routes. The most common route of administration is by intraperitoneal injection. Commonly, injection schedules of one to five times once daily are performed depending on the efficiency of Cre-mediated recombination. Intravenous administration using the Kolliphor preparation emulsified in sterile saline has also been reported [36].

For schedules that require multiple daily injections, the labor required presents a major disadvantage. Moreover, multiple injections increase the stress experienced by animals and the risk of organ injury during injections. A less labor-intensive alternative that is equally effective is by feeding tamoxifen-impregnated pellet food. A number of vendors have made these pellet feeds widely commercially available but care must be taken to ensure the animals are accepting the pellet food when it is first introduced. Various strategies to help the animals acclimate to the tamoxifen chow exist and they include: addition of sucrose to the pellet (can be done by the vendor), wetting the food in the initial stages, and introducing the tamoxifen chow mixed with regular chow in the beginning. Consultation with veterinary services responsible for care of animals at the research facility prior to initiation of tamoxifen feed is essential to develop monitoring and action plans for the period when mice are placed on special diet.

Special Considerations (Troubleshooting)

Tissue-dependent efficiency. The efficiencies of Cre recombinase expression as well as Cre-mediated recombination at loxP sites are dependent on the promoter driving the Cre expression, the level of Cre recombinase activity in the cell, the location and distance of loxP sites, and the tissue in which recombination occurs [37–39]. A number of observations from published reports suggest Cre expression may not be uniform between organs despite the use of a universal promoter. For instance, the ubiquitous strain CAGGCreER^{TM} has a widespread expression of CreER and administration of tamoxifen to either pregnant dams or adult mice induces widespread Cre-mediated recombination [40]. However, multiple observations show that penetrance of Cre activity is dose dependent, and importantly, some organs show incomplete Cre activity despite high doses of tamoxifen [40]. To ensure the proper

expression of Cre, investigators must characterize the specificity of Cre activity in the organ or cell type of interest with a reporter line before developing Cre/lox animals in gene deletion studies.

Cre toxicity. The presence of Cre recombinase alone may lead to cellular toxicity. Observations in Cre transgenic animals have shown that Cre recombinase may have various cytotoxic effects. First, sites that mimic loxP sequences exist in the mammalian genome and they may serve as unintended targets of Cre recombinase in transgenic animals even in the absence of loxP sites [41, 42]. Such off-target activity may lead to unintended DNA damage and/or rearrangement, a phenomenon that has been observed in transgenic mice [43, 44]. Second, Cre recombinase itself may act as a transcriptional repressor. As the recombination product between two loxP sites leaves one loxP site on the genomic DNA and one loxP site on the excised fragment, continued production of Cre recombinase in the cell may lead to Cre recombinase occupying the recombined loxP sequence on the genomic DNA. This event has the potential to inhibit transcription downstream from the occupied loxP sequence [45]. Third, lessons from cardiovascular research suggest phenotypic alterations may result in transgenic animals due solely to the presence of Cre recombinase. Use of α-myosin heavy chain (aMHC) gene as a cardiomyocyte-specific promoter for Cre recombinase has been extensively studied in cardiovascular literature. The tamoxifen-sensitive aMHC-MerCreMer transgenic mice have been observed to develop cardiac dysfunction independent of side effects from tamoxifen exposure or intended gene disruption in aMHC-MerCreMer myocytes. Phenotypes ranging from cardiac fibrosis to dilated cardiomyopathy have been observed in these transgenic mice [46–48]. Inclusion of Cre transgenic animals without the loxP transgene as controls is thus recommended in studies where the experimental endpoint may be affected by Cre toxicity.

Parental Cre transmission. Another factor to consider when breeding Cre transgenic animals for lineage or gene disruption studies is the choice of parents carrying the Cre transgene. Whether Cre is maternally or paternally transmitted may have a significant impact on the phenotype of the offspring. It has been observed that Cre activity may be more active in the offspring depending on whether the Cre transgene is transmitted through the mother or the father. For example, the EIIa-Cre transgenic mouse displays widespread Cre activity in all tissues when the EIIa-Cre transgene is transmitted maternally. In contrast, the Ella-Cre transgene activity is spatially mosaic and sparse when transmitted paternally [49]. This difference could be explained by the post-zygotic persistence of Cre activity expressed in the maternal germline. Cam2a-Cre, which is designed to restrict Cre expression to the hippocampus, demonstrates Cre expression in the male germ line cells (http://cre.jax.org/Camk2a/Camk2a-creNano.html) as well. In our own experience with the FoxD1-Cre line, we have found the parental transmission to be an integral part of experimental design. FoxD1 encodes a forkhead transcription factor that is active during embryonic development and is subsequently silenced in the adult mouse. The lineage of mesenchymal cells that express FoxD1 during development labels perivascular stromal cells (or pericytes) in the adult kidney and lung [16]. When a FoxD1-Cre;Rosa26-floxSTOP-tdTomato male breeder is used, we found in certain

offspring widespread expression of the tdTomato reporter, even in the absence of the Cre transgene. This was likely a result of Cre activity in the male germline that passed on the recombined reporter transgene to the offspring. Thus, we adjusted the breeding strategy when using this line such that the breeder female carries the FoxD1-Cre trangene to minimize the effect of male germline Cre activity. Therefore, depending on the promoter driving Cre expression, consideration for parental transmission must be made and generation of reporter animals to examine tissue/cell-type specificity of Cre activity in the offspring is often a necessary first step prior to carrying out further experiments.

Leaky inducible Cre. For certain genes, generation of knockout transgenic mice may lead to undesired or unexpected phenotypes, such as embryonic lethality or aberrant development. The ability to temporally control the gene deletion or modification through conditional knockouts models is thus an essential part of working with transgenic animals. The advent of inducible Cre lines has become a powerful tool in this regard. While inducible Cre constructs are designed to exclude Cre recombinase from the nucleus until ligand binding (e.g., tamoxifen administration in Cre-ERT transgenic mice), there are reports of Cre activity in the absence of ligand administration. This phenomenon has been termed "leaky Cre." One example of leaky Cre is seen in the RIP-CreER transgenic line. RIP is a rat insulin promoter expressed in insulin-producing pancreatic beta islet cells. This transgenic line has been used to study the origin of beta cells in mice. However, RIP-CreER transgenic mice can display Cre recombinase activity as early as 2 months of age even in the absence of tamoxifen exposure [50]. The phenomenon highlights the importance of validating the inducible Cre transgenic line by crossing with a reporter such as Rosa26-lacZ or Rosa26-tdTomato to look for evidence of autonomous Cre activity in the absence of inducer.

Cre mosaicism and incomplete recombination. Different tissues and different genes allow varying efficiencies in Cre-mediated recombination. Ubiquitous promoters that drive conditional CreER expression may display varying degrees of Cre recombinase activity depending on the tissue. Examples of this can be seen at the Jackson Laboratory website with the chicken beta-actin promoter (http://cre.jax.org/Cag-creERT/Cag-creESR1.html) or the human ubiquitin C promoter (http://cre.jax.org/UBC-creERT/UBC-creERT.html). In addition to tissue dependence, efficiency of recombination is also dependent on the gene being targeted. Voojis et al. showed that inducible Cre recombinase activity varies not only between tissues but also between different genes using a Rosa26-CreERT transgenic animal with Rb flox, p53 flox, and Brca2 flox transgenes [39]. When using Cre or inducible Cre to delete genes, it is therefore important to ensure the gene of interest does in fact undergo efficient recombination in the organ of interest.

Controls. Given the numerous unintended consequences that may be introduced with Cre transgene expression, it is important to design animal experiments with the appropriate controls. Littermates without Cre expression are standardly used as controls alongside experimental animals with Cre expression. Mice with Cre expression but without the floxed genes may serve as additional controls if there is evidence that Cre expression under the specific promoter leads to phenotypic

alteration in animals, as is the case with aMHC-MerCreMer mice discussed previously. Confirmation that the intended gene product is deleted at the protein level (preferred) or by genomic DNA analysis in experimental animals is important to ensure valid interpretation of experimental results.

References

1. Manis JP. Knock out, knock in, knock down–genetically manipulated mice and the Nobel Prize. N Engl J Med. 2007;357(24):2426–9.
2. Baron RM, Choi AJ, Owen CA, Choi AM. Genetically manipulated mouse models of lung disease: potential and pitfalls. Am J Physiol Lung Cell Mol Physiol. 2012;302(6):L485–97.
3. Yoshida M, Korfhagen TR, Whitsett JA. Surfactant protein D regulates NF-kappa B and matrix metalloproteinase production in alveolar macrophages via oxidant-sensitive pathways. J Immunol. 2001;166(12):7514–9.
4. Leco KJ, Waterhouse P, Sanchez OH, Gowing KL, Poole AR, Wakeham A, Mak TW, Khokha R. Spontaneous air space enlargement in the lungs of mice lacking tissue inhibitor of metalloproteinases-3 (TIMP-3). J Clin Investig. 2001;108(6):817–29.
5. Hautamaki RD, Kobayashi DK, Senior RM, Shapiro SD. Requirement for macrophage elastase for cigarette smoke-induced emphysema in mice. Science. 1997;277(5334):2002–4.
6. Wang D, Wang W, Dawkins P, Paterson T, Kalsheker N, Sallenave JM, Houghton AM. Deletion of Serpina1a, a murine alpha1-antitrypsin ortholog, results in embryonic lethality. Exp Lung Res. 2011;37(5):291–300.
7. Sternberg N, Hamilton D. Bacteriophage P1 site-specific recombination. I. Recombination between loxP sites. J Mol Biol. 1981;150(4):467–86.
8. Sternberg N, Hamilton D, Hoess R. Bacteriophage P1 site-specific recombination. II. Recombination between loxP and the bacterial chromosome. J Mol Biol. 1981;150 (4):487–507.
9. Abremski K, Hoess R. Phage P1 Cre-loxP site-specific recombination. Effects of DNA supercoiling on catenation and knotting of recombinant products. J Mol Biol. 1985;184 (2):211–20.
10. Abremski K, Wierzbicki A, Frommer B, Hoess RH. Bacteriophage P1 Cre-loxP site-specific recombination. Site-specific DNA topoisomerase activity of the Cre recombination protein. J Biol Chem. 1986;261(1):391–6.
11. O'Gorman S, Fox DT, Wahl GM. Recombinase-mediated gene activation and site-specific integration in mammalian cells. Science. 1991;251(4999):1351–5.
12. Ghosh K, Van Duyne GD. Cre-loxP biochemistry. Methods. 2002;28(3):374–83.
13. Lewandoski M, Martin GR. Cre-mediated chromosome loss in mice. Nat Genet. 1997;17 (2):223–5.
14. Soriano P. Generalized lacZ expression with the ROSA26 Cre reporter strain. Nat Genet. 1999;21(1):70–1.
15. Barkauskas CE, Cronce MJ, Rackley CR, Bowie EJ, Keene DR, Stripp BR, Randell SH, Noble PW, Hogan BL. Type 2 alveolar cells are stem cells in adult lung. J Clin Investig. 2013;123(7):3025–36.
16. Hung C, Linn G, Chow YH, Kobayashi A, Mittelsteadt K, Altemeier WA, Gharib SA, Schnapp LM, Duffield JS. Role of lung pericytes and resident fibroblasts in the pathogenesis of pulmonary fibrosis. Am J Respir Crit Care Med. 2013;188(7):820–30.
17. Rock JR, Barkauskas CE, Cronce MJ, Xue Y, Harris JR, Liang J, Noble PW, Hogan BL. Multiple stromal populations contribute to pulmonary fibrosis without evidence for epithelial to mesenchymal transition. Proc Natl Acad Sci USA. 2011;108(52):E1475–83.

18. Phillips RJ, Burdick MD, Hong K, Lutz MA, Murray LA, Xue YY, Belperio JA, Keane MP, Strieter RM. Circulating fibrocytes traffic to the lungs in response to CXCL12 and mediate fibrosis. J Clin Investig. 2004;114(3):438–46.
19. Kleaveland KR, Velikoff M, Yang J, Agarwal M, Rippe RA, Moore BB, Kim KK. Fibrocytes are not an essential source of type I collagen during lung fibrosis. J Immunol. 2014;193 (10):5229–39.
20. Perl AK, Wert SE, Nagy A, Lobe CG, Whitsett JA. Early restriction of peripheral and proximal cell lineages during formation of the lung. Proc Natl Acad Sci USA. 2002;99 (16):10482–7.
21. Kellendonk C, Tronche F, Monaghan AP, Angrand PO, Stewart F, Schutz G. Regulation of Cre recombinase activity by the synthetic steroid RU 486. Nucleic Acids Res. 1996;24 (8):1404–11.
22. Kellendonk C, Tronche F, Casanova E, Anlag K, Opherk C, Schutz G. Inducible site-specific recombination in the brain. J Mol Biol. 1999;285(1):175–82.
23. Minamino T, Gaussin V, DeMayo FJ, Schneider MD. Inducible gene targeting in postnatal myocardium by cardiac-specific expression of a hormone-activated Cre fusion protein. Circ Res. 2001;88(6):587–92.
24. Sando R 3rd, Baumgaertel K, Pieraut S, Torabi-Rander N, Wandless TJ, Mayford M, Maximov A. Inducible control of gene expression with destabilized Cre. Nat Methods. 2013;10(11):1085–8.
25. Okubo T, Knoepfler PS, Eisenman RN, Hogan BL. Nmyc plays an essential role during lung development as a dosage-sensitive regulator of progenitor cell proliferation and differentiation. Development. 2005;132(6):1363–74.
26. Chapman HA, Li X, Alexander JP, Brumwell A, Lorizio W, Tan K, Sonnenberg A, Wei Y, Vu TH. Integrin alpha6beta4 identifies an adult distal lung epithelial population with regenerative potential in mice. J Clin Investig. 2011;121(7):2855–62.
27. Que J, Luo X, Schwartz RJ, Hogan BL. Multiple roles for Sox2 in the developing and adult mouse trachea. Development. 2009;136(11):1899–907.
28. Flodby P, Borok Z, Banfalvi A, Zhou B, Gao D, Minoo P, Ann DK, Morrisey EE, Crandall ED. Directed expression of Cre in alveolar epithelial type 1 cells. Am J Respir Cell Mol Biol. 2010;43(2):173–8.
29. Humphreys BD, Lin SL, Kobayashi A, Hudson TE, Nowlin BT, Bonventre JV, Valerius MT, McMahon AP, Duffield JS. Fate tracing reveals the pericyte and not epithelial origin of myofibroblasts in kidney fibrosis. Am J Pathol. 2010;176(1):85–97.
30. Wamhoff BR, Sinha S, Owens GK. Conditional mouse models to study developmental and pathophysiological gene function in muscle. Handb Exp Pharmacol. 2007;178:441–68.
31. Wendling O, Bornert JM, Chambon P, Metzger D. Efficient temporally-controlled targeted mutagenesis in smooth muscle cells of the adult mouse. Genesis. 2009;47(1):14–8.
32. Wirth A, Benyo Z, Lukasova M, Leutgeb B, Wettschureck N, Gorbey S, Orsy P, Horvath B, Maser-Gluth C, Greiner E, et al. G12-G13-LARG-mediated signaling in vascular smooth muscle is required for salt-induced hypertension. Nat Med. 2008;14(1):64–8.
33. Kisanuki YY, Hammer RE, Miyazaki J, Williams SC, Richardson JA, Yanagisawa M. Tie2-Cre transgenic mice: a new model for endothelial cell-lineage analysis in vivo. Dev Biol. 2001;230(2):230–42.
34. de Lange WJ, Halabi CM, Beyer AM, Sigmund CD. Germ line activation of the Tie2 and SMMHC promoters causes noncell-specific deletion of floxed alleles. Physiol Genomics. 2008;35(1):1–4.
35. Rawlins EL, Perl AK. The a"MAZE"ing world of lung-specific transgenic mice. Am J Respir Cell Mol Biol. 2012;46(3):269–82.
36. Chevalier C, Nicolas JF, Petit AC. Preparation and delivery of 4-hydroxy-tamoxifen for clonal and polyclonal labeling of cells of the surface ectoderm, skin, and hair follicle. Methods Mol Biol. 2014;1195:239–45.

37. Comai G, Sambasivan R, Gopalakrishnan S, Tajbakhsh S. Variations in the efficiency of lineage marking and ablation confound distinctions between myogenic cell populations. Dev Cell. 2014;31(5):654–67.
38. Lewandoski M. Conditional control of gene expression in the mouse. Nat Rev Genet. 2001;2 (10):743–55.
39. Vooijs M, Jonkers J, Berns A. A highly efficient ligand-regulated Cre recombinase mouse line shows that LoxP recombination is position dependent. EMBO Rep. 2001;2(4):292–7.
40. Hayashi S, McMahon AP. Efficient recombination in diverse tissues by a tamoxifen-inducible form of Cre: a tool for temporally regulated gene activation/inactivation in the mouse. Dev Biol. 2002;244(2):305–18.
41. Thyagarajan B, Guimaraes MJ, Groth AC, Calos MP. Mammalian genomes contain active recombinase recognition sites. Gene. 2000;244(1–2):47–54.
42. Ito M, Yamanouchi K, Naito K, Calos MP, Tojo H. Site-specific integration of transgene targeting an endogenous lox-like site in early mouse embryos. J Appl Genet. 2011;52 (1):89–94.
43. Janbandhu VC, Moik D, Fassler R. Cre recombinase induces DNA damage and tetraploidy in the absence of loxP sites. Cell Cycle. 2014;13(3):462–70.
44. Schmidt EE, Taylor DS, Prigge JR, Barnett S, Capecchi MR. Illegitimate Cre-dependent chromosome rearrangements in transgenic mouse spermatids. Proc Natl Acad Sci USA. 2000;97(25):13702–7.
45. Iovino N, Denti MA, Bozzoni I, Cortese R. A loxP-containing pol II promoter for RNA interference is reversibly regulated by Cre recombinase. RNA Biol. 2005;2(3):86–92.
46. Lexow J, Poggioli T, Sarathchandra P, Santini MP, Rosenthal N. Cardiac fibrosis in mice expressing an inducible myocardial-specific Cre driver. Dis Model Mech. 2013;6(6):1470–6.
47. Buerger A, Rozhitskaya O, Sherwood MC, Dorfman AL, Bisping E, Abel ED, Pu WT, Izumo S, Jay PY. Dilated cardiomyopathy resulting from high-level myocardial expression of Cre-recombinase. J Card Fail. 2006;12(5):392–8.
48. Koitabashi N, Bedja D, Zaiman AL, Pinto YM, Zhang M, Gabrielson KL, Takimoto E, Kass DA. Avoidance of transient cardiomyopathy in cardiomyocyte-targeted tamoxifen-induced MerCreMer gene deletion models. Circ Res. 2009;105(1):12–5.
49. Heffner CS, Herbert Pratt C, Babiuk RP, Sharma Y, Rockwood SF, Donahue LR, Eppig JT, Murray SA. Supporting conditional mouse mutagenesis with a comprehensive cre characterization resource. Nat Commun. 2012;3:1218.
50. Liu Y, Suckale J, Masjkur J, Magro MG, Steffen A, Anastassiadis K, Solimena M. Tamoxifen-Independent Recombination in the RIP-CreER Mouse. PLoS ONE. 2010;5(10): e13533.