Methodological Reviews discuss methods that are of broad interest to the community of cardiovascular investigators and that enable a better understanding of cardiovascular biology, particularly recent technologies in which the methods are still in flux and/or not widely known. It is hoped that these articles, written by recognized experts, will be useful to all investigators, but especially to early-career investigators.

Lost in Transgenesis
A User’s Guide for Genetically Manipulating the Mouse in Cardiac Research

Jennifer Davis, Marjorie Maillet, Joseph M. Miano, Jeffery D. Molkentin

Abstract: The advent of modern mouse genetics has benefited many fields of diseased-based research over the past 20 years, none perhaps more profoundly than cardiac biology. Indeed, the heart is now arguably one of the easiest tissues to genetically manipulate, given the availability of an ever-growing tool chest of molecular reagents/promoters and “facilitator” mouse lines. It is now possible to modify the expression of essentially any gene or partial gene product in the mouse heart at any time, either gain or loss of function. This review is designed as a handbook for the nonmouse geneticist and/or junior investigator to permit the successful manipulation of any gene or RNA product in the heart, while avoiding artifacts. In the present review, guidelines, pitfalls, and limitations are presented so that rigorous and appropriate examination of cardiac genotype-phenotype relationships can be performed. This review uses examples from the field to illustrate the vast spectrum of experimental and design details that must be considered when using genetically modified mouse models to study cardiac biology. (Circ Res. 2012;111:761-777.)

Key Words: genetics ■ mouse heart development ■ transgene ■ transgenic mice

Genetic engineering in the mouse has revolutionized many fields of disease-based research because it permits the isolation of a single gene to examine its function within the context of an integrated physiological system that shares the mammalia class with humans. In fact, the tremendous impact that gene-targeting technology has had on modern investigative disease-based research was recognized by awarding the Nobel Prize in Medicine in 2007. Prior to modern genetic engineering in the mouse, only lower phylogenetic organisms, such as Caenorhabditis elegans, Drosophila melanogaster, and Xenopus laevis, permitted routine interrogation of single gene function from a systems biological and developmental standpoint, although these organisms are particularly inadequate for addressing more complex physiological processes that often underlie heart disease in humans.

Apart from model organisms, interrogation of single gene function also heavily relied on cultured cells grown in 2-dimensions in artificial medias with artificial substrates under highly contrived conditions, which obviously lacked physiological integration with other systems (tissues, cells, etc) and the correct neurohumoral milieu.

Cardiac hypertrophy and end-stage heart failure are whole-organ phenomena that are affected by mechanical stress and strain and occur within a complex neuroendocrine environment, a backdrop that cannot be adequately modeled in cultured myocytes or even in lower model organisms. In addition, genetic manipulation of disease-gene relationships in the mouse heart is now routinely possible given the characterization of reliable and tissue-specific promoters, as well as a large cadre of mouse lines that permit heart-specific
These DNA regions are typically linked to a promoter to drive tissue-specific expression. As a technical note, it is critical that the bacterial-derived sequence used to propagate the plasmid DNA in Escherichia coli be excised from the desired injection fragment, otherwise these bacterial sequences can inactivate the transgene by methylation and chromatin condensation. With insertional transgenesis, the transgene randomly integrates primarily within one site of the genome as either a single copy or more commonly, as a random number of copies that concatemerize (2 copies to upward of 50 copies are possible). If the α-myosin heavy chain (α-MHC) promoter is used, the greater the number of inserted copies, the greater the level of expression that is observed.\(^5\)

By contrast, gene targeting harnesses an embryonic stem (ES) cell’s natural propensity for homologous recombination, hijacked to replace an endogenous gene using a properly designed targeting vector. The basics of gene targeting involve the use of a DNA targeting vector (constructed within a plasmid) that contains a selectable marker and 2 regions of DNA sequence homology designed to flank an area of genomic sequence that will be deleted once homologous recombination occurs in ES cells. The targeting construct is hence designed so that if homologous recombination occurs properly, the endogenous gene will be replaced with the targeting construct and the selectable maker in 1 of the 2 alleles, typically also causing deletion of 1 to several exons of the gene of interest. This technology can also be used to insert a mutation (knock-in [KI]) or insert another cDNA or exon region. The most vital steps in creating a targeted KO or KI mouse are the design of the targeting vector and strategy for genotyping. Careful preplanning at this stage can save an investigator time and resources. Because there are so many considerations when creating a gene-targeted mouse, we have included a set of detailed guidelines for developing a knock-out mouse in the Online Data Supplement.

There are resources such as the knockout mouse project (KOMP, http://www.komp.org/) or the international gene trap consortium (IGTC, http://www.genetrap.org) that have repositories of targeted mouse ES cells or gene trap ES cells available for public use. The international knockout mouse consortium (http://www.knockoutmouse.org/) also provides an excellent database of all of the available targeting vectors, confirmed targeted ES cells, and gene trap ES cells available for a given gene. Investigators should be cautious when using gene trap ES cells because these mutant alleles are not precisely targeted and can sometimes result in ES cells positive for the selection marker but with a gene that is either not affected whatsoever or is hypomorphic in expression.\(^6–8\)

**Designing Your Genetic Approach to Circumvent Known Limitations**

The number-1 consideration for choosing which genetic technology to use when making a mouse model is the experimental goal itself. In transgenesis, the transgene’s expression is typically superimposed over the endogenous gene’s expression as a gain-of-function approach. However, transgenesis can also be used as a loss-of-function approach when the expressed cDNA encodes a dominant negative (dn) mutant protein, a partial protein, an antisense RNA, mi-
croRNA, or shRNA. For example, an inhibitory transgenic strategy was effectively used in the heart to investigate the requirement of calcineurin (calcium-activated protein phosphatase that underlies cardiac hypertrophy) in pathological cardiac growth. In these studies, calcineurin function was inhibited through the overexpression of naturally occurring inhibitory proteins Cain/Cabin-1 (the calcineurin inhibitory domain),9 AKAP79,9 or RCAN110,11 or through the overexpression of a dn-calcineurin.12 The phenotypes observed in these calcineurin inhibitory transgenic lines, which showed inhibition of pathological cardiac hypertrophy, closely matched the antihypertrophic phenotype of calcineurin Aβ (Ppp3cb) gene-deleted mice, which were designed later. This comparison suggests that the transgenic approach, despite involving gross overexpression, was effective at uncovering the phenotype of calcineurin loss of function in the heart.13 Indeed, the more traditional usage of cardiac transgenesis, in which an activated mutant form of calcineurin was overexpressed in the heart with the α-MHC promoter, showed massive cardiac hypertrophy.14

Gene targeting is most often used to create deleted alleles for loss-of-function experimental strategies; however, KI models can be made in which a mutant version or selection cassette replaces the endogenous gene that in some instances can produce a gain-of-function–like effect.15 Replacement by gene targeting also uses the endogenous regulatory elements of the targeted loci, so that if a KI mutation is used, expression occurs at physiological levels. For example, a gene-targeted KI strategy was used to create a mouse model of inherited hypertrophic cardiomyopathy (HCM) by replacing a single amino acid in the endogenous α-MHC gene locus, R403Q.16 This mouse was instrumental in better understanding the molecular-genetic basis for the HCM clinical phenotype.17–19 By comparison, Leinwand and colleagues generated a transgenic mouse model of HCM by overexpressing a mutant α-MHC protein in the heart using a rat α-MHC promoter,20 which revealed a disease phenotype consistent with the R403Q mutant KI mouse.16,21 These results again suggest that transgenesis can be just as informative as gene targeting if properly designed, and typically many times faster with considerably less cost. Despite these considerations, the gene-targeting–based approach (such as a KI mutation) is often more elegant and can be more physiological.

Gene targeting technology can also be used to “knock-in” a reporter gene (ie, β-galactosidase) into a locus of interest to identify temporal and spatial patterns of gene expression during cardiac development or in response to stress. For instance, to study the role and expression of islet1 (Is1l) in murine heart development, a nuclear β-galactosidase construct was “knocked-into” the Is1l locus.22 This mouse was then used to visualize myocyte lineage subdomains that express β-galactosidase as a surrogate for Is1l during development.

Although both transgenesis and gene-targeting are powerful experimental approaches, there are pitfalls associated with each that should factor into an investigator’s decision-making. These pitfalls are listed in Table 1 and more thoroughly reviewed below. Perhaps the main drawback of choosing gene targeting is the considerable amount of time and money it takes to generate the mouse, assuming that one will be successful in targeting the desired locus in the first place (not a guarantee). Another issue with gene targeting is that many genes play vital roles during embryonic or early postnatal development, so that traditional somatic targeting in the mouse (absent from zygote stage onwards) might produce lethality, precluding an analysis of an adult heart disease-related phenotype. Indeed, we were unable to ascertain the adult cardiac hypertrophy-associated phenotype of ERK1/2 in the heart using traditional gene targeting because Erk2 null mice die early in embryonic development.23 We later bypassed this developmental lethality issue using Cre-LoxP technology to generate heart-specific deletion of Erk2 in the Erk1<sup>−/−</sup> background.24

In addition to embryonic lethality, another problem is that the adult cardiac phenotype observed in a traditionally targeted KO mouse could be influenced by loss of gene function in other organs or tissues. Finally, replacement of the endogenous gene at a given locus can alter transcriptional patterns of neighboring genes due to the deletion of their critical regulatory elements even with a precise targeting construct or from interference with the neomycin cassette and its promoter. Knowing as much as possible about the genomic locus of interest through comparative genomic programs can guard against this situation and is further discussed in the Online Data Supplement. A classic example of this was identified when 3 different labs independently deleted the muscle regulatory factor MRF4. Interestingly, 2 of the 3 MRF4 KO models were lethal during development, whereas the other was not. In the 2 nonviable models, expression from the neighboring Myf5 gene was also absent despite the Myf5 locus being intact.25,27

Transgenesis also has various technical challenges because the transgene randomly inserts into the genome such that expression levels are typically higher and can be considered “nonphysiologic” (Table 1). However, we have generated transgenic lines in which expression almost exactly approximates the known increase in expression of a disease-associated gene in the heart. For example, thrombospondin 4 is induced in the heart after many different disease-inducing stimuli, and this level of induction was approximated by transgenesis with the α-MHC promoter, accurately revealing its disease modifying phenotype.28

Another issue that can seriously compromise a genetic based experiment in the mouse is if more than 1 gene or transgene to be simultaneously manipulated resides on the same chromosome. The easiest way to understand the issue is by example from our own laboratory. We were attempting to develop double null mice between the δ-sarcoglycan (Sgcd) and the JNK2 (Mapk9) deleted loci to determine if loss of JNK2 protein might impact muscular dystrophy pathogenesis in mice lacking δ-sarcoglycan. We crossed the mice, but their offspring were never genotyped as double homoyzygous. Only double heterozygotes were identified. Checking the genomic database, we easily surmised that the reason was because both genes reside on mouse chromosome 11. This same issue can befall a transgene if one is trying to cross it into a homozygous null gene–deleted background. Moreover, transgenes
Table 1. Experimental Considerations for Genetically Modified Mice

<table>
<thead>
<tr>
<th>Potential Pitfalls</th>
<th>Transgenesis</th>
<th>Considerations/Solution(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Integration effects</strong></td>
<td>1. Only study lines with germline transmission.</td>
<td></td>
</tr>
<tr>
<td>– Insertional mutagenesis (an endogenous gene has been altered)</td>
<td>2. Study multiple lines of variable dosages to ensure the phenotypes are logical and possibly dose-dependent.</td>
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<tr>
<td>– Epigenetic silencing (transcriptional repression due to chromatin structure, methylation)</td>
<td>3. Use promoters with insulator sequences to protect against insertion site-dependent repression (such as α-MHC).</td>
<td></td>
</tr>
<tr>
<td>– Mosaicism</td>
<td>4. Study protein and transcript expression over multiple generations and in multiple tissues.</td>
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<tr>
<td>– Multisite integration</td>
<td>5. Avoid studying founders in which the transgene is on a sex chromosome.</td>
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</tr>
<tr>
<td>– Sex chromosome integration (X-inactivation, Y-chromosome)</td>
<td>6. Do not make transgenic lines homozygous for a given insertion.</td>
<td></td>
</tr>
<tr>
<td><strong>Overexpression produces nonphysiologic data</strong></td>
<td>1. Study multiple lines of variable dosages.</td>
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<tr>
<td></td>
<td>2. Study the line with the lowest level of expression that still has a phenotype or that approximates expression of known induction with disease.</td>
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<tr>
<td></td>
<td>3. Use an attenuated promoter to study regulatory proteins so that levels are more “physiological.”</td>
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<tr>
<td><strong>Protein artifacts</strong></td>
<td>1. Check protein expression and sequence the mRNA transcripts produced to look for truncated or misspliced transcripts.</td>
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<tr>
<td></td>
<td>2. Select a new cDNA from another species and start over if aberrant splicing is observed.</td>
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<tr>
<td></td>
<td>3. Make sure that any epitope tags that are used do not cause disease or a problem with the protein.</td>
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</tr>
<tr>
<td><strong>Promoter silencing or downregulation</strong></td>
<td>1. Monitor transgene expression at all stages of disease progression.</td>
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<tr>
<td></td>
<td>2. Avoid using a nonvertebrate cDNA for overexpression (Cre and GFP being the exception).</td>
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<tr>
<td></td>
<td>3. The α-MHC promoter is downregulated with heart disease, so any disease affect of such a transgene could be reduced.</td>
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<tr>
<td><strong>Developmental effects</strong></td>
<td>1. Chose a promoter that is more active in the adult heart.</td>
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<td></td>
<td>2. Utilize inducible expression systems (ie, Tet-O).</td>
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<tr>
<td></td>
<td>3. Analyze multiple independent lines to identify one that may have lower developmental expression/effects.</td>
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<tr>
<td><strong>Mouse strain effects</strong></td>
<td>1. Select a strain to use based on known course of investigation, and maintain strain purity during breeding.</td>
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<tr>
<td></td>
<td>2. If complex crosses are made between mice of different strains use the correct mix background nontransgenic littermate controls as the basis for comparison.</td>
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<tr>
<td><strong>Genetic drift</strong></td>
<td>1. Closely monitor phenotype and gene expression throughout the maintenance of a line and only breed mice that maintain the effect of the expressed cDNA as first characterized.</td>
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</tr>
<tr>
<td></td>
<td>2. Use younger mice for breeding.</td>
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<tr>
<td></td>
<td>3. Refresh the breeders every 5 generations.</td>
<td></td>
</tr>
<tr>
<td><strong>Same chromosome with crosses</strong></td>
<td>1. If one is not able to obtain a cross between a transgene and a homozygous-deleted loci, they may be on the same chromosome, then extensive breeding can give cross-overs.</td>
<td></td>
</tr>
</tbody>
</table>

Global gene targeting

<table>
<thead>
<tr>
<th>Potential Pitfalls</th>
<th>Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Developmental lethality</td>
<td>1. Use a conditional approach with the appropriate Cre.</td>
</tr>
<tr>
<td>Functional genetic redundancy between related genes or compensation</td>
<td>1. Cross with KOs of the other gene family members to create double or triple KO lines.</td>
</tr>
<tr>
<td>Distinguishing between heart specific effects and secondary effects from other organ systems</td>
<td>1. Identify other tissues that express the targeted gene and study the phenotype of those tissues.</td>
</tr>
<tr>
<td></td>
<td>2. Use a tissue-specific approach with the appropriate Cre line.</td>
</tr>
<tr>
<td>Same chromosome when double nulls are being attempted</td>
<td>1. If one is not able to obtain double homozygous deleted mice, it may be a lethal cross or the genes may be on the same chromosome, then extensive breeding can give cross-overs.</td>
</tr>
</tbody>
</table>

Cre-loxP technology

| Appropriate temporal and tissue specific excision | 1. Clone the Cre downstream of the promoter that permits the most precise expression of the transgene. |
| | 2. Consider inducible systems such as a MerCreMer fusion protein for temporal precision in studying gene deletion effects. |
| Variable excision efficiency | 1. Identify the promoter-Cre line that gives the highest degree of excision but still retains the tissue specificity and developmental timing required for the experiment. |

(Continued)
Choosing the right promoter is a vital component of your construct’s design as it determines the level, cellular, and tissue specificity, as well as temporal/spatial pattern of transgene expression. Although the overall design of a construct is relatively simple and one’s choice of promoter would seemingly rule the day in directing expression as desired, the transgene’s expression can be influenced by regulatory elements within the random locus of integration. There are a number of promoters listed in Table 2 and schematically illustrated in Figure 1 that can be used to gain robust expression of a transgene in cardiac muscle (Figure 1A). Some important considerations when choosing a promoter are (1) in what cell type is the promoter active? (2) Where in the heart or other tissues does it express? (3) When does the promoter drive expression—developmentally or in the adult heart? As illustrated in Table 2, each promoter turns on or off at different developmental time points and in different regions of the heart or other tissues, which must be considered when selecting the correct promoter as well as when analyzing the phenotype of the mouse.

Although this review will not detail all of the cardiac promoters in Table 2, it will cover the most highly used promoters for expressing a transgene in the postnatal/adult myocardium. The α-MHC promoter provides robust expression in ventricular myocytes that “appears” just a few days after birth (Table 2). However, this promoter is also transiently active for a brief window during primitive heart tube development and for all of development in the atrial region; thus, developmental problems could still arise due to this transient early expression. The α-MHC promoter is perhaps the most commonly used of the cardiac promoters because it is highly specific to the cardiac myocyte without “leaky” expression in other cell types. Even the so-called “ectopic” expression from this promoter observed in limited regions of the lung occurs within cardiac myocytes of the pulmonary myocardium. A 5.5-kb region of the α-MHC promoter, which contains the entire promoter region, was developed by Jeffrey Robbins and James Gulick and has become the mainstay construct in our field. Importantly, this 5.5-kb region contains uncharacterized insulator elements that protect it from the influence of the surrounding locus in which the transgene integrates; therefore ectopic expression is rarely observed, and almost every line shows good expression in the heart. These insulator elements also produce a rather linear relationship with copy number–dependent expression level (more copies integrated gives higher expression levels). The robustness of insulation in this construct also means that only 2 to 4 founder lines are needed to obtain at least 2 satisfactory lines for full analysis. A minor drawback to the α-MHC promoter is that it is downregulated by disease stimuli that induce hypertrophy or during heart failure (β-MHC promoter activity is induced) or if thyroid hormone levels drop, necessitating reevaluation of one’s data if these disease states are also part of the phenotype because expression can decrease as the disease evolves.

There are several other promoters listed in Table 2 that have reasonable cardiac muscle specificity and have been used for transgenesis, such as the β-MHC promoter, the ventricular myosin light chain 2 (MLC2v) promoter, and cardiac troponin T (cTnT) promoter. Although useful, these 3 other promoters often show lower transcriptional activity in the adult heart compared with the α-MHC promoter, and, more importantly, these 3 other promoters are not as well insulated, so several lines must be carefully examined to ensure expression in the heart without significant expression in other tissues. An additional consideration with both the MLC2v and β-MHC promoters is that they drive expression in slow skeletal muscle. Moreover, all 3 of these alternate promoters have robust developmental expression in the early heart tube, which is often why they are used in the
first place for studying cardiac development. With respect to insulation, whereas ectopic expression outside of the heart could make a given line of less utility, sometimes low-level transgene expression in the heart, as driven by these 3 alternate promoters, may be desirable. For instance many signaling proteins are better analyzed at lower levels of expression to guard against loss of endogenous regulation. In fact, most physiological increases in gene expression are only severalfold above endogenous levels. As a case in point for how expression levels are a serious consideration in interpreting results of a transgenic experiment, one merely must consider work from the Dorn laboratory. Overexpression of an inhibitor peptide (δV1) of the δ isoform of protein kinase C (PKC) with the standard α-MHC promoter caused early morbidity due to heart failure. To circumvent this early lethality and obtain viable δV1 mice to study, the authors created an attenuated α-MHC promoter that retained cardiac muscle specificity but dramatically reduced expression levels (Table 2). This was achieved by ablating either the first or second thyroid response element (TRE) in the α-MHC promoter. Hence, one would have initially interpreted that inhibition of PKCδ was pathological to the heart (in contrast

### Table 2. Cardiac-Specific Promoters and Cre Lines

<table>
<thead>
<tr>
<th>Promoter</th>
<th>Where and When Expressed</th>
<th>Is There a Cre/Level of Excision?</th>
<th>Inducible Versions</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-MHC3412</td>
<td>Developing atrium and throughout adulthood (α7.5 p.c.)</td>
<td>α-MHC-Creα77</td>
<td>α-MHC-Tetβ2</td>
</tr>
<tr>
<td>α-MHC-Creα77</td>
<td>α-MHC promoter</td>
<td>70% to 80% excision by 3 wk of age</td>
<td>Binary system requiring 2 transgenes</td>
</tr>
<tr>
<td>α-MHC–CrePR185</td>
<td>70% to 80% excision by early adulthood</td>
<td>Dox-inducible</td>
<td></td>
</tr>
<tr>
<td>α-MHC–MerCreMer56</td>
<td>α-MHC-MerCreMer</td>
<td>Tam- or Raloxifen-inducible</td>
<td></td>
</tr>
<tr>
<td>α-MHC–Tet52</td>
<td>α-MHC-Tet</td>
<td>RU486-inducible</td>
<td></td>
</tr>
<tr>
<td>β-MHC24,35</td>
<td>Developing ventricle and atria (starts expressing in the heart tube and most abundant at day 17 p.c.)</td>
<td>β-MHC–Creβ76,109</td>
<td>β-MHC-Creβ76,109</td>
</tr>
<tr>
<td>β-MHC-Creβ76,109</td>
<td>β-MHC promoter</td>
<td>70% excision by 17.5 p.c.</td>
<td>Recombination begins ~e7.5</td>
</tr>
<tr>
<td>β-MHC-Creβ76,109</td>
<td>85% by 8 wk of age</td>
<td>90% excision by 3 wk of age111</td>
<td></td>
</tr>
<tr>
<td>cTnT40,41</td>
<td>Developing heart and throughout adulthood (~e7.5 p.c.)</td>
<td>cTnT-Cre110</td>
<td>cTnT-Cre110</td>
</tr>
<tr>
<td>cTnT-Cre110</td>
<td>Low levels in developing skeletal muscle (13.5 p.c. to day 14 after birth)</td>
<td>Tmnt2-TTA117</td>
<td>Tmnt2-TTA117</td>
</tr>
<tr>
<td>cTnT40,41</td>
<td>Tongue (13.5 to 16.5 p.c.)</td>
<td>Tmnt2-TTA117</td>
<td>Tmnt2-TTA117</td>
</tr>
<tr>
<td>cTnT40,41</td>
<td>Developing bladder (16.5 p.c.) and is lost by adulthood</td>
<td>Tmnt2-TTA117</td>
<td>Tmnt2-TTA117</td>
</tr>
<tr>
<td>cTnT40,41</td>
<td>Cardiac-specific enhancer gives expression in the heart tube, ventricles, and outflow tract</td>
<td>Nkx2.5-Cre113</td>
<td>Nkx2.5-Cre113</td>
</tr>
<tr>
<td>Nkx2.5-Cre113</td>
<td>Expression in the developing heart beginning at day 7.5 p.c. through adulthood</td>
<td>Nkx2.5-Cre113</td>
<td>Nkx2.5-Cre113</td>
</tr>
<tr>
<td>Nkx2.5-Cre113</td>
<td>May get expression in the developing thyroid, stomach, spleen, and tongue (enhancer-dependent)</td>
<td>Transgenic</td>
<td>Nkx2.5-Cre113</td>
</tr>
<tr>
<td>Nkx2.5-Cre113</td>
<td>Recombination is detected ~e8–8.5 in the developing heart</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nkx2.5-Cre113</td>
<td>Anterior heart field at day 7.5 p.c.</td>
<td>Me2C-AHF-Cre117</td>
<td>Me2C-AHF-Cre117</td>
</tr>
<tr>
<td>Me2C-AHF116,117</td>
<td>Developing outflow tract and right ventricle and conduction system</td>
<td>Me2C-AHF-Cre117</td>
<td></td>
</tr>
<tr>
<td>Me2C-AHF116,117</td>
<td>Developing pharyngeal mesoderm</td>
<td>Me2C-AHF-Cre117</td>
<td></td>
</tr>
<tr>
<td>MLC2V20–24</td>
<td>Expression during heart tube development that persists and becomes</td>
<td>MLC2V-Creα18</td>
<td>MLC2V-Creα18</td>
</tr>
<tr>
<td>MLC2V-Creα18</td>
<td>stable in adults</td>
<td>80% ventricular excision by E8.3</td>
<td></td>
</tr>
<tr>
<td>MLC2V-Creα18</td>
<td>High levels of expression in the right and left ventricle by day 8 after birth</td>
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</tr>
<tr>
<td>MLC2V-Creα18</td>
<td>Low level of expression in the atria</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MLC2V-Creα18</td>
<td>Low level of expression in the soleus</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MLC2V-Creα18</td>
<td>Not well insulated, many lines needed</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Is11119</td>
<td>~8.5 to 10.5 expression is detected in the developing second heart field, outflow tract, right ventricle, atria, and a few cells in the left ventricle</td>
<td>Is1-Cre120,121</td>
<td>Is1-Cre120,121</td>
</tr>
<tr>
<td>Is1-Cre120,121</td>
<td>Developing motor neurons and dorsal root ganglia (12.5 to 14.5 p.c.)</td>
<td>Is1-Cre120,121</td>
<td>Is1-Cre120,121</td>
</tr>
<tr>
<td>Is1-Cre120,121</td>
<td>Turns on before Me2C-AHF</td>
<td>Is1-Cre120,121</td>
<td>Is1-Cre120,121</td>
</tr>
<tr>
<td>SM22103,124</td>
<td>Developing heart tube at days E8–13.5</td>
<td>SM22-Creα123,124</td>
<td>SM22-Creα123,124</td>
</tr>
<tr>
<td>SM22-Creα123,124</td>
<td>At E9, expression begins in the aorta, arterial smooth muscle, and vessels of the trunk and head. Expression becomes restricted to the vessels by E13.5</td>
<td>SM22-Creα123,124</td>
<td>SM22-Creα123,124</td>
</tr>
<tr>
<td>SM22-Creα123,124</td>
<td>Possible expression in developing skeletal muscle at days E9.5 to 12.5 p.c.</td>
<td>SM22-Creα123,124</td>
<td>SM22-Creα123,124</td>
</tr>
<tr>
<td>SM22-Creα123,124</td>
<td>Little expression in venous or visceral smooth muscle cells</td>
<td>SM22-Creα123,124</td>
<td>SM22-Creα123,124</td>
</tr>
</tbody>
</table>
to prkcd null mice that are viable without a major cardiac baseline phenotype, but, if the attenuated promoter was used, one would have interpreted the opposite because lower levels of overexpression of the \( \delta \)V1 inhibitory peptide protected from cardiac ischemia-reperfusion injury.

In other areas of cardiac biological investigation, high levels of transcript overexpression are absolutely required to achieve the desired effect, such as when studying sarcomeric proteins and the effects of mutations. For such experiments, the amount of transgenic transcript must outcompete or dilute the endogenous transcript so that the desired myofilament protein is effectively replaced. The \( \alpha \)-MHC promoter has been used extensively for studying sarcomeric proteins because transgenic mice can be obtained, with a range of replacement levels that can approach nearly 100%.

Artificial Chromosome-Based Transgenesis

An alternative to the traditional transgene design in which a gene or cDNA of interest is linked to an exogenous cardiac specific promoter is to use artificial chromosome vectors to direct transgene expression. The 3 categories of these vectors are yeast (YAC), phage (PAC), and bacterial (BAC) artificial chromosomes that are designed to accommodate large genomic inserts of 100 to 300 kb or larger in the case of YACs. Artificial chromosome vectors can be used for the same applications as a traditional transgene, and they integrate into the genome randomly like a transgene (usually just 1 copy), but their real experimental power is producing expression patterns and levels that highly mimic the endogenous gene. These artificial chromosomes can contain the entire gene of interest in its natural intron-exon arrangement with its “full” complement of regulatory elements, or one can insert a cDNA into a gene locus within the artificial chromosome in the hopes of obtaining expression of that cDNA in a pattern and level that exactly mimics the locus into which it was placed.

Inducible Transgenesis

For some experiments, it is highly desirable to induce transgene expression for the first time in the adult heart, hence bypassing early postnatal developmental expression, or to turn off expression in the adult heart once disease has been induced. Such control is critical for discerning the acute
versus compensatory effects of a genetic manipulation in vivo as well as the disease-specific effects of a given gene’s function. There are several inducible transgene systems to regulate expression in a temporally restrictive manner, but the most effective strategy is based on the tetracycline (Tet) regulatory system. Here, controlled induction of your gene of interest requires 2 transgenes: (1) a Tet transactivator (tTA) cDNA under the control of a tissue specific promoter (ie, α-MHC) and (2) a conditional transgene that is cloned downstream of a minimal cytomegalovirus (CMV) promoter coupled to multiple copies of the Tet-operator DNA elements (Tet-O). The Tet transactivator functions as a transcription factor that initiates expression of any gene downstream of its putative promoter element, the Tet-operator. In the “Tet-off” system tetracycline or its analog doxycycline are given to block the interaction between tTA and the Tet-O, which in turn silences the transcription of your transgene. The “Tet-on” system uses a mutant tTA to essentially cause the reverse of the Tet-off system such that doxycycline is required to activate transgene transcription.

The conventional Tet-off system based on the CMV promoter for inducible transgenesis in the heart was not successful and only produced low levels of unregulated expression. However, the Tet-Off system was re-engineered for the heart by Jeff Robbins’ laboratory such that the 5.5-kb mouse α-MHC promoter was modified to contain the Tet-O, along with deletion of key transcriptional enhancer binding sites to attenuate the greater promoter. The Robbins laboratory also generated a very low expressing tTA mouse line that was nontoxic using the “standard” 5.5-kb mouse α-MHC promoter (Figure 1B). Others have shown that higher levels of tTA, such as with the rat 2.9-kb α-MHC promoter, induced hypertrophy and cardiomyopathy on its own. The reengineered system by the Robbins laboratory is nonleaky, fully inducible, and completely cardiac-specific.

Another benefit of this inducible system is that the α-MHC-Tet-O promoter construct can be used singularly, without the α-MHC-tTA transgene, as an attenuated promoter that gives lower and more “physiological” levels of overexpression for baseline analysis. However, cDNAs driven from the attenuated α-MHC-Tet-O promoter construct still afford the opportunity for inducible expression at a later time if one then crosses in the α-MHC-tTA transgene (unpublished observations). As a final consideration, Sanbe et al demonstrated that 6 months of chronic tTA expression in mice is innocuous; thus, studies using the Tet-O system should probably be completed within 6 months to avoid any complications from tTA toxicity, although a control group of α-MHC-tTA only transgenic mice should always be used, especially if experiments are planned past 6 months. An example of using an adult-specific inducible transgenic approach was recently published by Hill and colleagues, where the ability of calcineurin to induce hypertrophy in the adult mouse heart was examined. It was previously shown that activated calcineurin cDNA was induced for the first time in the adult heart, validated the ability of this signaling pathway to mediate adult cardiac hypertrophy without a developmental complication.

Another means of achieving inducible expression of a cDNA in the heart involves using loxP-stop-inactivation cassettes contained within a traditional transgene, so that the Cre-loxP system can be used to temporally regulate a transgene (discussed more under gene targeting) (Figure 1C). A complete understanding of this system is best described with the following example. Wang and colleagues generated an α-MHC promoter construct that contained an upstream green fluorescent protein (GFP)-loxP flanked cassette, followed by the cDNA of interest. They selected an activated mitogen-activated protein kinase kinase 7 (MKK7) mutant protein to force c-Jun N-terminal kinase (JNK) activation in the heart, after Cre-mediated excision of the GFP cassette, which revealed a role for JNK as a mediator of cardiomyopathy.

This system is beneficial as it allows generation of founder lines that are inactivated and hence not subjected to lethality if the cDNA of interest causes such. The recombination of the loxP-dependent cassette in the transgene is permanent once it is crossed with a Cre-expressing line, after which expression persists for the cDNA of interest. When used with the α-MHC–MerCreMer, tamoxifen-inducible line (Figure 1D), one can achieve temporally regulated and cardiac-specific gene activation in the heart. A similar strategy can also be employed, using any ubiquitous promoter that has the loxP-dependent stop cassette, but only when used in conjunction with a cardiac-specific Cre. For example, Yutzey and colleagues used a CAG-CAT ubiquitous promoter (CMV enhancer-fused to chicken β-actin promoter) with a chloramphenicol acetyl transferase (CAT)-loxP dependent stop cassette to permit conditional transgene expression in the developing heart of a downstream Tbx20 cDNA, when crossed with β-MHC–Cre transgenic mice (Figure 1C). The benefit of the CAG-CAT transgene is that one is not limited to the heart, as any tissue where Cre is expressed can be subjected to tissue specific gene/cDNA induction, and if a drug inducible Cre line is used, recombination of the recipient loxP-dependent transgene can be temporally controlled as well.

**Pitfalls and Complications Inherent With Cardiac Transgenesis**

**Exclusive Use of the Founder Line**

One pitfall to navigate is the exclusive use of the founder transgenic line for phenotypic analysis. The founding mouse (F0) for a given line should only be used for breeding due to the potential for mosaicism. All transgenic founder mice should be backcrossed with wild-type mice of their same native strain (typically C57BL/6 or FVB/N) to propagate the transgene and obtain germline transmission (F1 generation). Founder transgenic lines (F0) are the direct result of the pronuclear injection of newly fertilized oocyte, which can generate mosaicism if the DNA injection fragment integrates into the genome after the first zygote cell division to the 2- or even 4-cell stage, meaning that only half or a quarter of the cells in the generated founder mouse contains the transgene. F1 progeny from these founders, when positive for the
transgene, are now germline, and hence all cells are positive for the transgene. These germline mice should then be used for all subsequent breeding and experiments (Table 1).

**Transgene Insertion Characteristics**

Another issue to consider is the number of copies that insert and the number of potential independent integrations that occur in a given line (Table 1). Conventional thinking might predict that a transgene integrates into the mouse genome at one site, as a single copy event. Although it is usually true that only a single integration site is observed per founder line, most often this event, as discussed earlier, contains multiple concatemeric copies of the transgene (5–20 copies seem typical). Given this characteristic, one can easily mix 2 different transgene fragments together before oocyte injection to generate matched expression levels between 2 proteins. For example, this technique permitted coexpression of dn cDNA versions of the stress-induced signaling proteins JNK1 and JNK2 in the heart at roughly the same levels from a single insertion site.58

**More Than 1 Integration Site**

On rare occasions, more than 1 integration site can occur in a single founder mouse (meaning that a single founder mouse [F0] can give more than 1 line; Table 1).59 For example, we generated transgenic mice expressing constitutively active MKK1 in the mouse heart under the control of the α-MHC promoter,60 and 1 of the F0 mice gave litters that were nearly 100% transgenic, when the predicted mendelian frequency should be 50% from a single integration site when bred with a nontransgenic mouse. When this MKK1 F0 mouse was bred into the second (F1) and third (F2) generations, we were able to account for 3 separate genomic integrations on 3 different chromosomes, each with its own unique expression level and each representing an independent line (unpublished observations).

**Making a Transgenic Line Homozygous**

Another issue to consider is whether to keep the transgene hemizygous or to interbreed the same line onto itself with the hopes of obtaining homozygous mice to increase expression levels. However, we discourage attempting to generate and hopes of obtaining homozygous mice to increase expression levels. However, we discourage attempting to generate and then these fragments are sequenced and referenced against the mouse genome.

**Transgene Inactivation Over Time**

Another issue to consider in cardiac transgenesis is the loss of expression over time due to loci inactivation associated with methylation (Table 1). The actual chromosomal location of the transgene can play a role in determining the level and pattern of transgene expression, and, if the transgene integrates into a transcriptionally inactive or silenced genomic region, it may be more susceptible to silencing due to methylation and irreversible chromatin condensation.63 Not all transgene constructs have the same susceptibility to this transcriptional silencing, and the α-MHC promoter is probably the least likely to be silenced over time, given its strong insulation. The nature of the cDNA insert can also have a dramatic effect, especially if it is prokaryotic or from certain species that have high GC content in their transcripts.63,64 The best rule of thumb is to use a mouse cDNA with a mouse-derived promoter, when possible, to guard against transgene silencing. A related issue is that once a transgenic silences in a given mouse within one’s colony, all its progeny will have the transgene silenced, but the exact same transgenic line could still express in other parallel or older mice from one’s colony. Hence, one should never maintain a transgenic line with only 1 breeder, and expression should be periodically verified when maintaining a line long-term.

**Genetic Drift of a Transgenic Phenotype**

The initial phenotype one identifies with a newly generated transgenic line can change over time. As a line is continuously bred within one’s colony, modifying mutations arise over the years that give a selective advantage toward reducing a disease phenotype. For example, we originally generated transgenic mice expressing activated calcineurin in the heart more than 14 years ago, which induced massive HCM that was lethal within 3 to 5 months.14 However, some 14 years, later mice from this same line now routinely live well over 1.5 years in our colony, despite strong overexpression as originally characterized with massive cardiac hypertrophy (unpublished observations). To prevent issues with genetic drift, the Jackson Laboratory recommends refreshing breeders on a regular basis, and, after 5 generations, a new foundation stock should be developed from cryopreserved pedigreed embryos.

**Sex Chromosome Integration**

A transgene can also insert into a sex chromosome, which can be problematic for 2 reasons (Table 1). First, if the transgene integrates into the X chromosome, it can be subjected to X-linked inactivation in females, leading to mosaicism in expression. Also, transgenic males from such a line will never give rise to transgenic males when bred with wild-type females, so only females can be analyzed from such an intercross. The second concern is if the transgene integrates into the Y chromosome, because it will never be possible to assess the effect of the transgene in females. Thus, it is generally a good rule to avoid any lines in which the transgene is incorporated on either the X or Y chromosomes.
Developmental Effects
Another issue to consider is that essentially all standard (noninducible) promoters used for generating a cardiac-restricted transgene have some or a great deal of expression in the developing mouse heart (Table 2), which, if the lines survive into adulthood, could still affect the heart due to a prior developmental effect. For example, Dorn and colleagues showed that overexpression of the heterotrimeric protein Gq in the heart with the “standard” α-MHC promoter caused disease in adulthood characterized by hypertrophy and myocyte dropout.65 However, this same Gq gene protein product, when overexpressed at the same levels but for the first time in the young adult mouse heart using an inducible system, produced no disease, suggesting that the early developmental or postnatal expression of Gq was the responsible factor in producing or sensitizing to adult disease.66 Hence, it may be desirable to use the inducible α-MHC–Tet-O system as a means of isolating the function of a gene product to the adult heart. However, as described earlier, use of this inducible system to overexpress activated calcineurin in the heart, in contrast to results observed with Gq, still showed that disease could be driven exclusive to the adult heart without secondary influences from earlier developmental expression.66

Atrial Effects of the α-MHC Promoter
Another issue to consider when using any of the α-MHC-based promoters is that of atrial enlargement due to myocyte hypertrophy, proliferation, fibrosis, and thrombi formation. The α-MHC promoter is continuously expressed in the atria of the heart throughout all of embryonic development, as well as at much higher levels in the atria of the postnatal and adult heart compared with the ventricles. Thus, even wild-type control proteins, such as the myosin light chains (MLC), when overexpressed at high enough levels, can lead to atrial disease that could eventually impact the ventricles.67 Thus, we often avoid any sort of atrial analysis in our transgenic experiments because they can show artifactual results, based only on the extreme level of persistent overexpression in this region of the heart. Thus, if one intends to study atrial biology or SA node function in the heart, the α-MHC promoter may not be the best choice. As an aside, we have noted that the α-MHC promoter drives expression in the SA and AV nodes, as well as the rest of the conducting system of the heart (unpublished observations).

Additional Factors to Consider
There are a number of additional factors listed in Table 1 that can complicate the phenotypic interpretation of transgenic experiments. Some of these factors include (1) “molecular torture,” in which high levels of protein expression cause a nonspecific phenotype or aggregation of a protein of interest; (2) protein toxicity effects, in which epitope tags or reporter constructs (ie, eGFP) cause nonspecific phenotypes or lethality; and (3) poison peptide or partial protein effects in which aberrant splicing events in the transgene generates dominant negatives that give an unanticipated phenotype, or again, protein aggregation. One can circumvent these complications with carefully designed experiments such that the total level of overexpression is carefully controlled, appropriate controls are used to rule out effects of epitope tags or bacterial protein fusions, or, if aberrant splicing is observed, one can select a new cDNA from a related species for overexpression (Table 1 and Figure 2). In addition, the Jackson Laboratory, through the mouse genome informatics website (http://www.informatics.jax.org or mgi-list@lists.informatics.jax.org), offers an online forum that discusses a wide range of topics that can be extremely helpful for designing experiments and circumventing these pitfalls.

Another good practice is to always analyze more than 1 transgenic line, and more importantly, to analyze various levels of overexpression across 3 independent lines. For example, the role of the calcium regulatory protein phospholamban (PLN) was carefully examined by transgenesis. For each experiment, nontransgenic and transgenic littermates from different lines with various degrees of overexpression were studied to develop a gene dose-response relationship.68 One should also routinely determine if the protein that is overexpressed is still properly localized to the desired intracellular compartment, that it is not degraded and absent (this sometimes happens with truncated proteins or peptides), that a signaling or regulatory protein of interest gives more activity in higher expressing lines, and that expression is maintained over time at the expected levels in the ventricles.

Gene Targeting Considerations in the Heart
Although gene targeting (KOs and KIs) is considered a more precise strategy for manipulating the murine genome when compared with insertional transgenesis, there are a number of theoretical and technical pitfalls to consider (Table 1). It is always important to carefully assess other tissues that might be affected in total somatic KO mice, which could secondarily affect the heart. However, there can be exceptions to this concern. For example, deletion of the gene encoding phospholamban (Pln) was engineered with a traditional, global gene targeting strategy, and, despite the lack of tissue specificity, its function in the heart was still readily characterized without secondary effects despite its deletion from skeletal muscle and smooth muscle where it is also appreciably expressed.69 Indeed, whereas slow skeletal muscles from Pln KO mice did demonstrate a significant change in contractile function,70 it did not impact the cardiac phenotype, although this may not be the case for other genes that are deleted globally. Hence, it is always important to carefully assess other tissues that might be affected in total somatic KO mice that could secondarily affect the heart. As will be discussed below, the only definitive means of establishing the myocyte autonomous function for a given gene through gene targeting is to use a cardiac-specific approach.

Using Tissue-Specific Gene Targeting
Whereas gene targeting typically offers a definitive means of extinguishing gene function, the traditional strategy that results in the total somatic (global) gene deletion can be subject to secondary and compensatory effects that mires interpretation of cardiac function/effects. First, total somatic deletion of a gene can result in embryonic lethality due to its necessity, sometimes even for proper heart maturation and function. Second, total somatic deletion of a gene is more
we use the example of crossing a transgenic mouse overexpressing Cib1 (Ca²⁺/ and integrin binding protein 1) with Ppp3cb⁻/⁻ (calcineurin Aβ) KO mice. The experimental group consists of mice that are genotype-positive for both the Cib1 transgene and null for calcineurin Aβ (Ppp3cb⁻/⁻). The control groups consist of the 3 following littermates: (1) Cib1 transgenic but wild-type for calcineurin Aβ (Ppp3cb⁺/⁺), (2) no Cib1 transgene and null for calcineurin Aβ (Ppp3cb⁻/⁻), and (3) no Cib1 transgene and wild-type for calcineurin Aβ (Ppp3cb⁺/⁺). Experiments in which a transgenic reporter mouse (NFAT-luciferase) is crossed with a different KO lines to create a double KO require 4 different KO lines to create a double KO require 4 groups for comparison. In this matrix, we use the example of crossing Crsp3 KO mice (muscle LIM protein, MLP⁻/⁻) to phospholamban KO mice (Pln⁻/⁻) to get double null mice with both MLP and PLN deleted (MLP⁻/⁻, Pln⁻/⁻) for the experimental group. The control groups in this case consist of the following littermates: (1) loss of both MLP alleles but wild-type for PLN (MLP⁻/⁻, Pln⁺/⁺), (2) loss of both PLN alleles but wild-type for MLP (MLP⁺/⁺, Pln⁻/⁻), and (3) wild-type for both MLP and PLN (MLP⁺/⁺, Pln⁺/⁺). Combinatorial heterozygotes were not included but could be analyzed as well for gene dosage effects. Experiments in which a transgenic model is crossed with a KO model requires 4 groups for comparison. In this matrix, it was later determined that global deletion of Mef2c also produced embryos without an organized vasculature, which can lead to embryonic death, with a secondary defect in heart maturation.

To circumvent these 3 main limitations plaguing total somatic gene targeting strategies in the mouse, tissue-specific approaches have become widely adopted by the research community. Cre-loxP technology has become the preferred means of generating tissue-specific gene deletion in the mouse. This approach uses the DNA site-specific Cre recombinase (Cre, 38 kDa), which is typically driven by a tissue-specific transgene or even an endogenous gene loci if Cre was likely to uncover and induce compensatory changes in other genes as a means of bypassing lethality or altered homeostasis during development. Also, such putative compensatory changes in other genes might not normally occur under any other conditions, thus obscuring a proper assessment of one’s gene of interest. Third, as discussed above, total somatic deletion of a gene can alter the heart through secondary effects associated with loss of said gene in other organs or tissues. For example, deletion of the transcription factor Mef2c in the mouse produced embryos with poorly developed hearts, resulting in early lethality, at first presumed to be solely due to its loss in the myocytes of the heart.
inserted by gene targeting. Once expressed, Cre recombinase recognizes a 34-bp DNA sequence element referred to as a loxP (fl) site, which is engineered into the desired gene loci for future manipulation. Placement of 2 loxP sites flanking exons of interest in a target gene permits subsequent deletion of those exons when Cre is introduced at a later time. Placement of the small loxP sites by traditional gene targeting is typically innocuous to the selected locus, although it is good to verify that expression of the targeted locus was not altered by introduction of these small sequence elements (after the antibiotic selection cassette has been removed). Heterozygote loxP-targeted mice can also give an intermediate phenotype compared with homozygotes and can be included in one’s experimental group. However, oftentimes, heterozygosity of a loxP-targeted allele has no discernable effect. Hence, in our laboratory we rarely analyze heterozygote loxP-targeted mice for purposes of economy, unless we suspect that a gene-dosage effect is in play.

There are several Cre transgenic lines available for cardiac expression that are listed in Table 2. The temporal and spatial pattern of subsequent gene deletion is regulated by the promoter or endogenous locus that drives expression of Cre. For example, the Nkx2.5-Cre KI mouse line expresses Cre in the developing heart beginning around day E7.5.75 By 1 day after birth, nearly 100% excision of the loxP flanked DNA region has been reported with this KI line, making it one of the strongest alleles for Cre-mediated excision in the heart (Table 2). However, the Nkx2.5-Cre KI allele is not absolutely specific to cardiomyocytes, so deletion can occur in other sites of the embryo/adult mouse. Also to consider, the KI approach deletes 1 functional copy of the Nkx2.5 gene, which can render a phenotype, as shown previously.74,75

Other Cre-expressing lines are also available, though most are driven by a transgene with various promoter options that produce variable excision efficiency in the heart (Table 2). Both the α-MHC–Cre and β-MHC–Cre offer excellent excision efficiency in which 70% to 90% of gene expression is lost.76 Using the β-MHC–Cre transgene gives strong DNA excision during early cardiac development (E7.5 onward)76 and then ceases shortly after birth, thus limiting any potential toxicity associated with chronic Cre expression in the adult heart. As a word of caution, the β-MHC–Cre will also induce gene excision in slow skeletal muscle of the mouse, so these effects must be considered. The β-MHC–Cre transgene will also be induced and reexpressed in the adult heart with disease stimulation, which could produce further levels of gene deletion that was not anticipated (the β-MHC gene is induced in the mouse heart by hypertrophic stimuli).

The α-MHC–Cre transgene can have transient expression during early embryonic development in the heart, so it could still produce developmental effects and even lethality, depending on the gene that is being deleted. This transgene is then mostly shut down in mid to late embryonic development of the ventricles (persists the entire time in the atria), only to be robustly induced in the postnatal ventricles of the heart, where it is maintained at high levels into adulthood. However, this persistent expression of Cre recombinase in the adult heart is not without potential concerns.

For example, the widely used mouse Cre line developed by Schneider and colleagues, under control of the α-MHC promoter,77 causes toxicity leading to cardiomyopathy by 8 to 12 months of age (unpublished observations). Hence, we attempted to develop our own α-MHC–Cre–expressing transgenic line. Figure 3 illustrates several α-MHC–Cre lines with high, medium, and low expression of Cre protein in the heart (Figure 3A). By 6 months of age, both the highest (line 10.10) and lowest (line 12.10) expressing lines presented with reduced cardiac function and cardiomyopathy (Figure 3B and 3C), with increased heart weight-to-body weight ratios (data not shown). The lower expressing line (12.10) might be cardiomyopathic because of a combined effect of transgene integration and Cre expression, especially since the other low/medium expressing Cre lines (10.6, 10.14, and 11.8) exhibited no change in cardiac growth or function (Figure 3). These results underscore how transgenesis in general can lead to cardiac disease, in this case due to a toxic effect of Cre that is probably related to its ability to cause genomic instability and chromosomal rearrangements.78 However, this initiative did result in the identification of a stable α-MHC–Cre line that we now routinely use,76 which does not predispose to cardiomyopathy as characteristic of the α-MHC–Cre line developed by Schneider and colleagues (Table 2). Our results also underscore how important it is to have a Cre-only transgenic group as a control (Figure 2). These control groups will aid in differentiating the effects of the Cre transgene versus the excised targeted DNA region. In fact, studies may need to be terminated early so as to avoid Cre-dependent cardiotoxicity if the α-MHC–Cre generated by Schneider and colleagues is used.77 Alternatively, Cre could be delivered...
using β-MHC–Cre transgenic line, which no longer shows appreciable expression in the adult heart, or other alternative Cre lines could be used (Table 2).

Using Drug-Inducible Gene Targeting for Temporal Control

An adjunct to tissue-specific gene targeting is to use one of the few drug-inducible Cre expression lines based on the α-MHC promoter (Table 2 and Figure 1D). These systems not only limit Cre expression to the heart, hence maintaining tissue-selectivity, but also provide temporal control over gene excision. In drug-inducible constructs, Cre recombinase is fused to a mutated version of the ligand binding domain of the estrogen receptor (MER), the most efficient version of which has a double fusion that is termed MerCreMer.79 The mutated version renders the ligand binding domain insensitive to the endogenous ligand estradiol but sensitive to a synthetic analog, tamoxifen, which is given to activate the Cre so it can induce recombination.80 Sohal et al linked a cDNA encoding this MerCreMer fusion protein to the α-MHC promoter for temporally regulated gene deletion approaches in the heart.56 When crossed with the ROSA26–Stop–β-galactosidase reporter line, this α-MHC–MerCreMer mouse line showed almost no leakiness in the adult heart at baseline but efficientloxP-site–dependent recombination with administration of 5 to 7 days of tamoxifen.56 The optimal tamoxifen dose and route of delivery can vary, depending on the characteristics of the genomic locus that is targeted with LoxP sites and the half-life of the protein encoded by the gene of interest. Most publications deliver tamoxifen at 20 to 80 mg/kg daily over 4 to 5 days via intraperitoneal injection. Tamoxifen food is also commercially available and can successfully induce recombination by feeding an estimate of 80 to 100 mg/kg daily dosage over 4 to 7 days. For example, in mice that have both floxed SERCA2A alleles and transgenic expression of α-MHC–MerCreMer, SERCA mRNA was reduced to less than 5% of that in controls after 4 days of either daily tamoxifen injections (80 mg/kg) or food (80–100 mg/kg).81

As a cautionary note, tamoxifen administration to MerCreMer transgenic mice alone can induce an acute reduction in cardiac function with some associated remodeling, but this typically resolves within 7 to 14 days after cessation of treatment82,83 (unpublished observations). Interestingly, MerCreMer-negative littermates receiving tamoxifen were unaffected, as were vehicle-injected. MerCreMer-positive littermates, suggesting that neither the MerCreMer transgene nor tamoxifen alone affected cardiac function but rather it was the interaction between the two that was detrimental. To navigate this issue, an oral route of tamoxifen administration was shown to be just as effective as an injection in promoting recombination,84 but without the transient cardiomyopathy (unpublished observations). Alternatively, one can give raloxifene, a weaker estrogen analog that typically does not produce as robust recombination as tamoxifen, but also does not appear to induce transient cardiomyopathy.83 One could also simply wait 2 to 3 weeks after tamoxifen treatment before assessment of the cardiac phenotype is attempted, so that any issues related to the tamoxifen-MerCreMer combination have resolved. Clearly, a tamoxifen-treated, MerCreMer-only transgenic group must be used as an important control in all such experiments using this line (Figure 2). Finally, there is an alternative α-MHC–driven, inducible Cre (CrePR1) that uses the progesterone receptor ligand–binding domain as the basis for the Cre fusion protein, which is induced with RU486 (Table 2). However, the α-MHC–CrePR1 showed unacceptably high baseline leakiness of recombination in the heart without RU48685; hence, the field has not adopted it.

Finally, tetracycline/doxycycline-dependent, Cre-based transgenic systems have also been defined for cardiac-specific recombination. One of these systems utilizes a CMV promoter Tet-O transgene to drive Cre expression in any potential tissue, but, if used with an α-MHC–reverse tTA (rTA) transgene, it produces heart-specific deletion when tetracycline is given.86 The other system involves the troponin T promoter to drive rTA in the heart with the same CMV–Tet-O–Cre responder line (Table 2).87 These 2 lines of rTA mice give another means of driving Cre expression specifically in the heart at any desired time point with the addition of a tetracycline, especially if tamoxifen is determined to be detrimental when the α-MHC–MerCreMer system is used.

An elegant example of how conditional gene targeting can provide “definitive” answers to longstanding controversial issues in cardiac biology was recently published. Lee and colleagues addressed the issue of ongoing myocyte replenishment and regeneration within the heart, using a genetic “pulse-chase” (fate-mapping) system. This system labels all adult myocytes in the heart at time 0 with the α-MHC–MerCreMer transgene and a ubiquitously expressed ZEG88 casette (β-galactosidase/eGFP) in the ROSA26 locus.89 Loss of properly recombined myocytes over time or after injury presumably reflects the rates of new myocyte generation in the heart from nonmyocyte sources. The authors found that after myocardial infarction–induced injury or pressure overload, the percentage of baseline-labeled cardiac myocytes had significantly decreased, indicating that new myocytes were formed from precursor cells. By contrast, normal aging showed no reduction in recombination-marked cardiac myocytes, indicating a lack of contribution from stem/precursor cell populations. As a whole, this study provided an elegant example of how modern genetic approaches in the mouse can provide definitive answers in controversial areas of cardiac biology.

Other Significant Considerations for Using Genetically Altered Mice

Strain Effects

Mouse strain can dramatically affect the resulting phenotype of a genetic manipulation (Table 1). Each transgenic and ES cell core has their preferred mouse strain. Most conventional transgenesis is performed in FVB/N or C57BL/6 strains,90 whereas most gene targeting is performed in SV129 ES cells, which typically generates targeted mouse lines with a mix of SV129 and C57BL/6 backgrounds.91,92 These are important considerations because strain-dependent differences in baseline cardiac function have been reported in several studies.93–96 The effects of strain differences can add a considerable source of variability that could partially ex-
plain differences between transgenic mouse models with similar genetic manipulations. For example, an E180G tropomyosin replacement transgenic mouse made on the C57BL/6 background exhibited no cardiac remodeling and only mild diastolic dysfunction. By contrast, the E180G tropomyosin mutation within the FVBN background resulted in massive cardiac hypertrophy and heart failure. Additional strain-dependent differences have been identified when measuring responses to ischemia reperfusion, and pressure overload. Our laboratory has observed that FVBN mice are generally resistant to pressure overload–induced heart failure, even past 16 weeks of stimuli, whereas C57BL/6 mice show signs of heart failure within 2 to 4 weeks of pressure overload stimulation (Liu et al and unpublished observations). Hence, strain effects must be considered when comparing models or crossing transgenic mice with mixed backgrounds, although one can always backcross at least 6 generations to possibly obtain a more homogenous effect in the desired background.

Generalization to Human Physiology

Because genetically engineered mice have rapidly become the premier reagent for studying cardiovascular biology, an investigator must keep in mind that while genetically manipulated mouse models provide a very precise means of studying genotype-phenotype relationships, the extrapolation to human disease may not be straightforward. In the context of cardiovascular biology, rudimentary physiological function is fairly conserved between the mouse and human, but there are important differences that need to be considered. First, heart rate and systolic pressure derivatives (dP/dt) are considerably different between mice and humans. Average resting murine heart rates range from 550 to 650 bpm versus 70 bpm in humans, and shortening rates are 17 000 mm Hg/s in mice versus 1885 mm Hg/s in humans. In fact, sympathetic tone overall appears to be higher in a mouse and therefore may affect cardiac reserve and in vivo function. Second, Ca\(^{2+}\) handling is different in the mouse and rat relative to higher mammals such as rabbits and humans. The primary difference is that murine ventricles utilize higher sarcoplasmic reticulum Ca\(^{2+}\) ATPase (SERCA) activity to remove Ca\(^{2+}\) from the cytosol during excitation-contraction coupling, which is reflected in the compressed time scale of the rodent cardiac action potential. In rodents, SERCA contributes to 92% of the Ca\(^{2+}\) removal during excitation-contraction coupling with the sodium-calcium exchanger (NCX) extruding 7% and the rest outside the sarclemma. By contrast, NCX plays a much larger role in Ca\(^{2+}\) extrusion in humans, contributing 28% to Ca\(^{2+}\) removal while SERCA sequesters the remaining 70%. Third, the primary contractile protein in the mouse heart is the kinetically faster α-MHC, whereas humans express the slower β-MHC isoform. Fourth, the mouse heart also presents a pretreatment for cardiovascular research for the foreseeable future, as no other system allows for the spatial and temporal manipulation of any gene or RNA product, with the goal of linking it as protective or causative in heart disease or other biological processes of significance in cardiac biology.

Complex Crosses

Transgenic and gene-targeted mouse models are frequently intercrossed to address the mechanistic function of a particular gene in the heart. Although such crosses have become commonplace, it is not always clear how to prioritize among all the potential control groups available when crossing multiple models. Figure 2 illustrates several examples of experimental matrices of the recommended control groups for some of the models discussed in this review (Figure 2). The number of control groups becomes more substantial with added genetic complexity, such as with a double transgenic system used for Tet-O-inducible expression or Cre-loxP technology. Of course, crossing multiple transgenic/gene targeted models will require single-gene transgenic and nontransgenic littermate controls on the appropriate genetic background. When using the α-MHC–MerCreMer transgene, other control groups must be included, such as tamoxifen treatment to α-MHC–MerCreMer mice that are not crossed with the loxP allele–containing targeted line.

Conclusions and Future Perspectives

Although there are nongenetic manipulations that can be used to create heart disease mimetics in larger animals that may be more generalizable to human heart disease, it is the mouse that has propelled the field of cardiovascular biology forward into a mechanistic assessment of single gene function for eventual human translational endeavors. However, properly using mouse genetic techniques, especially as the toolbox of lines and molecular manipulations continues to expand, requires all investigators to step back and reflect. We must ensure that these tools are properly used and that younger scientists are correctly trained in their usage, and we must keep an ongoing dialog concerning the limitations and pitfalls underlying any potential technology or approach. Control groups also must be fastidiously considered well in advance, prior to breeding, which can save 6 or more months in a research project. Genetic manipulation in the mouse represents a premiere approach for cardiovascular research for the foreseeable future, as no other system allows for the spatial and temporal manipulation of any gene or RNA product, with the goal of linking it as protective or causative in heart disease or other biological processes of significance in cardiac biology.

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Disclosures

None.


Lost in Transgenesis: A User's Guide for Genetically Manipulating the Mouse in Cardiac Research

Jennifer Davis, Marjorie Maillet, Joseph M. Miano and Jeffery D. Molkentin

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Supplemental Information

Creating a Knock-out Mouse

One of the most important steps in creating a knock-out (KO) mouse are the design of the targeting vector and the genotyping strategy. Failure to carefully deliberate and address potential problems revolving around the critical issues discussed below could result in lost time and expense. The steps below are intended to alert a KO designer to critical issues that should be considered before embarking on generating a targeting construct and submitting it for electroporation into embryonic stem cells. These days, targeting strategies can also involve insertion of loxP sequences ("floxing") around key exonic or promoter regions. This allows one to breed a "floxed" mouse with another mouse expressing Cre recombinase to induce global or tissue specific gene inactivation. This approach safeguards against potential embryonic lethality with a conventional gene replacement strategy, which is often difficult to predict in advance. Where genes have a single 5’ promoter and a coding first exon, it may be best to flox the promoter region and first exon. Note, internal promoters and/or non-coding first exons complicate the strategy of placing loxP sequences around the promoter and first coding exon so it is essential to have some insight into whether an internal promoter exists. The steps below provide key guidance in the design of a floxed gene with a bias for floxing the promoter/first exon. A figure outlining the positive/negative selection of ES cells targeted with the floxed allele is shown below in Supplement Figure I.

Annotation and Planning

1. Find your mouse gene of interest with the UCSC Genome Browser (http://genome.ucsc.edu).
2. Examine the gene structure paying close attention to neighboring or overlapping genes as well as microRNAs and the emerging class of long non-coding RNAs. The presence of overlapping genes or internal non-coding RNAs should be viewed cautiously in the design of a targeting vector since removal of these could confound interpretation of your KO.
3. Create a VISTA plot (http://genome.lbl.gov/vista/index.shtml) using mouse versus homologous human sequences to further assist in the identification of potentially important internal sequences that could represent non-coding RNAs including microRNAs, non-annotated single exon genes, or regulatory elements controlling your gene of interest or a distal gene.
4. Is your gene of interest a single exon gene? If so, it may be possible to flox the entire exon and create a true null allele; however, be aware that floxing a large genomic segment of DNA can result in a stable "epiosome" with expression of your gene. More likely, your gene of interest will have many exons and the challenge becomes deciding which exons should be bracketed with loxP sequences. Thus, it is important to have some understanding of the functional domains of a protein. First, create a map of your gene and superimpose the functional protein domains over each exon in your map. An easy way to find known functional domains of a protein is copying the primary amino acid sequence from the UCSC genome browser into the PROSITE algorithm (http://www.expasy.ch/prosite/). Importantly, not all functions of a protein are known and removal of select internal exons could result in truncated proteins that result in unknown functions or partial loss of function.
5. Having a reliable antibody is highly desirable, especially one whose epitopes are known. One should obtain several commercial antibodies and test each in a Western blot of both cells and tissues in order to identify the most reliable antibody for later validation of protein expression both in homozygous floxed and homozygous floxed/Cre+ mice. The antibody should also be tested on sections of mouse organs where the protein is most highly expressed.
6. If the first exon is coding and if its removal would create an out-of-frame amino acid sequence, it may be desirable to flox the first exon and even some promoter sequence (see step 7 below). This would likely result in a true null allele as long as there is no alternate downstream ATG sequence. In this context, one should examine the primary amino acid sequence for
potential downstream ATGs that could serve as internal start sites of translation and schematize what such a protein would look like in terms of remaining functional domains. The Kozak consensus sequence for a methionine suitable for translation initiation is, GCCA/GCCATGG.

7. If there are reliable information on the transcription start site and promoter activity of your gene of interest, it would be ideal to place a loxP sequence upstream of the known promoter region. First, define transcription start sites using the database of transcription start sites (http://dbtss.hgc.jp/). It may also be necessary to do 5’RACE using a reverse primer to a downstream exon and sequence at least 20 independent clones following TA-cloning of your 5’RACE library to further support the presence of a single promoter upstream of exon 1. Then, evaluate a long (~1 kb) and short promoter in a luciferase assay based upon regulatory elements defined through rVISTA or other regulatory element discovery tools such as TESS, http://www.cbil.upenn.edu/cgi-bin/tess/tess. We often test promoters in vivo using a lacZ reporter as well. Close attention to sequence homologies between mouse and human in the 5’ promoter is critical since placement of the 5’ loxP site in the promoter region MUST NOT alter normal promoter activity. We therefore test in vitro the effects of inserting the 5’ loxP and flanking sequences within the 5’ promoter region comparing it to an identical promoter without the loxP sequence. This may require clever mutagenesis to create a novel restriction site for insertion of the 5’ loxP sequence. If there is no change in promoter activity, it may be safe to insert the loxP sequence in the 5’ promoter of your targeting vector (typically in an island of non-homologous sequences based on the VISTA in step 3 above).
   a. Placement of the 3’ loxP site should be at least 50 bp downstream from the splice donor site and, using VISTA or other comparative genomic algorithms as a guide, away from any islands of sequence homology that could effect functionally important sequences by placement of the loxP site.

8. A detailed restriction map with exons and protein domains as well as 5’ promoter and introns should be constructed along with the approximate position of each loxP sequence and the neomycin expression cassette. Annotation of primers should also be illustrated as should any other features of the locus such as regulatory elements, microRNAs, etc. The map should be as detailed as possible and should extend at least 10-kb in either direction of where your targeting will occur. This map is also critical for you to carefully define placement and testing of probes for Southern blot analysis of your targeted ES cells as well as delineating the homology arms that are critical for homologous recombination.

9. During the labor-intensive analysis of your gene locus, genomic DNA or a BAC containing your gene of interest should be obtained; BACs can be obtained from the Children’s Hospital Oakland Research Institute (http://bacpac.chori.org/). The BAC and genomic DNA should be isogenic to the embryonic stem cell line that is to be electroporated with your targeting construct. Typically, the strain of ES cells used is 129SvEv (129S6), but success has been achieved using a hybrid line (SV129/C57BL/6); the latter is ideal because the PCR homology arms can be made with C57BL/6 mice genomic DNA (the mouse reference sequence is based on C57BL/6). It is easiest to obtain genomic DNA from the C57BL/6 strain of mouse. If, however, the ES cell is SV129, then you will need to obtain genomic DNA from this strain, or obtain ES cells that are C57BL/6. At the same time, you should get genomic DNA from the strain of mouse wherein 129S6 ES cells will be injected via blastocyst or by morula aggregation (typically C57BL/6). Both ES cell-derived (129S6) and recipient mouse-derived (C57BL/6) genomic DNA will be needed to test Southern probes for facile genotyping if the ES cell used is straight 129SvEv (see step 11 below).

Genotyping and Targeting Vector Construction
10. Design PCR primers to amplify the two homology arms and the KO sequence (that which will be “floxed out” following Cre-mediated excision) that will be cloned adjacent to the loxP
sequences. Typically, the homology arms represent short (2-3 kb) and long (3-5 kb) stretches of DNA while the KO sequence is smaller (1-2 kb). One must be judicious in selecting restriction sites for cloning as this will become an important factor when using Southern blot for screening ES cells (see point 11 below). For example, if there is a distal 5' XhoI site with a 3' XhoI site located a considerable distance away (eg, >10 kb), it may be advantageous to include an XhoI site in a PCR primer so that a closer XhoI site will be generated thus allowing for easy discrimination of the WT (bigger XhoI digest) versus the KO (smaller XhoI digest) allele. There is a strict order of operations in terms of building the targeting vector that can only be determined following exhaustive genomic sequence analysis as specified above. For example, one would not clone a homology arm that contains a restriction site that is needed for a subsequent cloning step! As a final note, one must also maintain at least 1 unique restriction site (8 base cutters are great for this) that can be used to linearize the targeting vector for electroporation.

11. As the targeting vector is assembled, PCR amplify several contiguous stretches of 200-500bp DNA regions, outside of the homology arms, for use as probes in Southern blotting. The probes (at least 3 each outside the 5' and 3' homology arm) should be at least 200 bp in length but not more than 500 bp. There should be no repeat DNA in the probes as this will likely lead to non-specific hybridization. Radiolabel the probes and test each on genomic DNA from step 10 above digested with the appropriate enzyme. Selection of the enzyme is based on the targeting vector versus endogenous WT sequence. Great care should be taken in choosing the best enzyme that will discriminate well between the WT and KO sequence from targeted ES cells. Ideally, both 5' and 3' Southern probes should yield a single unambiguous band for future use in screening the ES cell clones following electroporation.

   a. In the event that Southern probes do not work on ES cells or chimeric mouse DNA, it will be necessary to perform LA-PCR (Long and Accurate PCR) with forward primers that, like the Southern DNA probes, lie outside the 5' and 3' homology arms and reverse primers to the Neo cassette. In practice these types of long PCR amplification are unreliable and may even require a nested PCR. However, Southern blotting is much more reliable if at least one probe works effectively.

12. Design PCR primers flanking genomic DNA where one of the loxP sites will be present. There should be clear presence of 1 band (typically should be around 200 bp). These primers will detect a WT PCR product that is smaller than the floxed allele where a loxP sequence (along with some flanking sequences) will be present. These primers will be critical in routine genotyping of mouse DNA during breeding of chimeras and, ultimately, the intercross to generate homozygous floxed mice.

13. During each step of the PCR cloning process, great care must be taken to ensure that sequences at each junction are correct. Moreover, in the final vector, sequencing MUST be done across the loxP sites to ensure fidelity in loxP sequences and that both are in the same orientation.

14. A large-scale preparation of DNA should be made (>100 ug) for delivery to your Transgenic/KO Core.

   a. The Core will electroporate DNA into ES cells, plate ES cells at limiting dilution and then provide samples (usually >100) for the investigator to test by Southern blotting or LA-PCR as determined in Step 11 above.

15. Two independent ES cell clones that are correctly targeted, as determined by Southern blotting and/or LA-PCR, should be kept for long term cryopreservation and used to inject into the blastocoel of e3.5 day fertilized embryos (or for morula aggregation). Injecting two independent clones will control for any epigenetic changes that may occur in ES cells during in vitro propagation. Before the clones are injected by the core, it is advisable to test them one last time by LA-PCR or Southern blotting in case there was contamination with a WT ES clone that
may have propagated in the expansion phase (maybe grew even better than the targeted clone).

**Obtaining KO Mice and Analysis**

16. If the type of ES cells used permit it, versus the host blastocyst strain, chimeric mice will have differential coat color that can suggest degree of ES cell contribution. The greater the chimerism from the ES cells (typically agouti from SV129), the more likely the targeted ES cell is represented in the germ cell lineage in that chimeric mouse.

17. The next step is sometimes tricky. Several chimeric mice must be bred in order to pass the targeted allele through the germline to the F1 generation. Only following germline transmission can one begin to interbreed heterozygous mice with confidence for the generation of homozygous floxed mice. The PCR primers generated in Step 12 above should be used here although it is possible to also use Neo primers as well.

   a. Set up several harems (one male chimera and two female C57BL/6) and breed continuously (requires IACUC approval).
   b. Remove females and then rotate fresh females into male cage to increase chances of obtaining germline transmission.

18. Once germline transmission occurs, the heterozygous mice can be interbred to generate homozygous floxed mice. If homozygous mice cannot be generated, it may indicate a problem with the Neo cassette which can be easily removed by breeding mice to a Flp recombinase mouse (if your neomycin cassette was flanked by FRT sites) and then PCR genotyping/sequencing offspring to prove the Neo cassette is removed. Notice that this removal of NEO should always be done whether it is a heterofloxed or homofloxed mouse, as NEO can interfere with expression of your gene that is floxed, even if the mice are viable and the locus is mostly expressed correctly.

19. Once homozygous floxed mice are generated, they need to be bred to a Cre mouse in order to generate heterozygous floxed/Cre positive mice. These mice should be males and are then bred to homozygous floxed females to generate the KO. Initially, homozygous floxed mice should be bred to heterofloxed mice carrying a CMV-Cre driver mouse to simulate a pan-KO, particularly if the gene has not been previously analyzed in a KO setting. If mice are born with expected Mendelian inheritance, then it is likely that the gene of interest is not crucial for development. Under the typical breeding schema above (ie, homofloxed female x heterofloxed/Cre positive male), there should be 25% yields of pups with the following genotypes: homofloxed/Cre negative; homofloxed/Cre positive (the KO!); heterofloxed/Cre negative; and heterofloxed/Cre positive. The latter mouse should be the initial control mouse for study as it best models the situation under normal KO conditions where traditional heterozygous KO mice are examined as controls.

   a. Initial KO mice should be genotyped at as soon after birth as possible to ascertain whether there is embryonic lethality (this may require an addendum to an IACUC protocol)

20. If homozygous flox mice carrying CMV-Cre are not born, then the gene of interest is critical for development and two things should be done

   a. Perform timed matings between heterofloxed/Cre positive males and homofloxed females and analyze embryos at early (e9.5 days), mid-gestation (e12.5-e13.5 days) and late gestation (e18.5 days) time points. Genotype yolk sacs to try and pinpoint when during development embryos are being lost.
   b. Cross homofloxed mice with a cell-specific Cre (inducible or non-inducible) to circumvent the developmental lethal phenotype.

21. The next step should be the generation of RT-PCR primers that amplify 5’ and 3’ terminal exons of the floxed gene to interrogate expression of mRNA transcripts following Cre-mediated excision. This assay is particularly critical if no antibody is available to suggest if you have a
KO mouse. It is important to evaluate both the 5' and 3' ends of the transcript to document that the KO results in no detectable transcripts or only partial transcripts that cannot code for meaningful protein. Sometimes, splicing around a floxed exon (exon skipping) could result in the presence of truncated transcripts that may have unknown functions, which would then represent a potential hypomorphic allele rather than a true KO.

22. Histological analysis of tissues should be done and phenotypes may not be manifest unless the mouse is “stressed” in some manner (e.g., ligation injury to vessel wall).

23. If the mouse is very useful, consider having it deposited in JAX labs for future use. There is a submission form for novel mouse strains at http://www.jax.org/grc/strain-donation.php
Figure I. Schematic of how a targeting vector is used to delete a desired gene in mouse ES cells. (a) A replacement targeting vector is shown that is introduced into mouse ES cells to permit either homologous recombination (desired) or non-specific nonhomologous recombination that does not target or mutate the gene of interest. If homologous recombination occurs, the desired locus is rearranged and then (b) with then ES cells are selected for G414 resistance (growth) because they have the neomycin cassette, as well as lacking the thymidine kinase cassette that would be lost with homologous recombination, hence the ES cells are not killed by ganciclovir or FIAU. This allows both positive and negative selection of ES cells so that properly targeted clones can be isolated and expanded for Southern blot analysis. The figure was obtained with permission from the University of Michigan’s Transgenic Core Website. This website also has other useful protocols and additional information: http://www.med.umich.edu/tamc/
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