To Cre or Not To Cre
The Next Generation of Mouse Models of Human Cardiac Diseases
Kenneth R. Chien

Connecting genes and molecular pathways with in vivo physiological endpoints has never been a simple task. Dissecting the effects of the causal from the phenom- enal, the environmental from the inherited, the developmental from the postnatal, the primary from the secondary, and the polygenic from the monogenic has been confounding at best. For those of us interested in complex human cardiovascular diseases, the situation has been even more difficult, given the multifactorial and integrative nature of the most important human diseases and biological processes, such as angiogenesis, atherogenesis, obesity, hypertension, and heart failure, to name a few. Over the last few years, a movement to capitalize on the availability of genetically modified mouse models has led to a resurgence in integrative physiology that has been spurred on by the development of a host of new technology for assaying in vivo phenotypes in the living mouse that now encompasses conscious blood pressure recording, hemodynamic catheterization, microdigitized angiography, noninvasive imaging via echocardiography, new MRI technology, programmed stimulation, long-term Holter monitoring, microsurgical implementation of pressure overload in both the right and left ventricles, in utero myocyte implantation, and many other new techniques. In short, physiologists have reinvented themselves by pushing the limits of miniaturization in genetically modified mouse model systems.

In parallel, molecular biologists have expanded the toolbox for generating genetically modified animals, which now encompasses the activation of a given mutation in specific cell types and at specific times using cre-lox technology for conditional mutagenesis. In this regard, the study in this issue of Circulation Research by Minamino et al makes a valuable contribution by suggesting the utility of a single transgene to allow ligand-dependent activation of the genetic modification of interest, which in this case is the expression of a LacZ reporter gene. The work underscores the feasibility of jumping over embryonic lethality of a given mutation via engineering temporal and spatial control of the onset of the mutation of interest. Given the time, expense, and labor-intensive nature of creating and breeding genetically modified mouse models, this work highlights the question: to cre or not to cre? There is a daunting spectrum of existing experimental strategies to modify the mouse genome, each of which can be implemented with a diverse set of promoters, targeting constructs, and ligand activators. The pace of work in the field suggests that many new approaches are on the immediate horizon. To provide a framework for answering this query, a brief summary of the past, present, and next generation of technologies for modifying the mouse genome has been provided below, using cardiovascular disease as a prototypic example (see the Table).

Generation Past

In the beginning, there was transgenesis. Although the first transgenes resulted in the ubiquitous overexpression of a gene of interest, the cloning of a variety of cell type–specific promoters ultimately led to the identification of regulatory sequences that were capable of in vivo targeting of the expression of a given transgene to specific tissues. For example, the availability of well-characterized cardiomyocyte–specific promoters, particularly the α-myosin heavy chain promoter (MHC), has led to the generation of several informative transgenic mice, resulting in the identification of intrinsic cardiac myocyte pathways that regulate hypertrophy and contractility. However, the high level of expression of a large subset of these transgenes has resulted in the development of cardiac dysfunction and cardiomyopathy that can often be attributed to a generalized toxic effect of the transgenes, which are expressed at the level of sarcomeric proteins. As a result, the phenotype that reflects a primary effect of the transgene can be obscured by the nonspecific effects of cardiomyopathy that arise from the secondary effect of disrupting the normal stoichiometric relationship of cardiac signaling or structural proteins. In addition, the mosaic nature of the transgene expression, line-to-line variations in the level of expression, effects of the genomic site of integration, and wide differences in the copy number of the transgene can make discrimination of primary versus secondary effects quite challenging. Finally, gain-of-function studies do not necessarily indicate an endogenous role for a given candidate gene in the naturally occurring biological pathway, whereas the overexpression of dominant negatives are confounded by their nonspecific inhibitory effects. Thus far, the most informative cardiac transgenic genes have been those that display a gain of physiological function, where the observed phenotype cannot be ascribed to a toxic effect of the transgene per se, such as the cardiac-specific expression of an inhibitory peptide of the β-adrenergic receptor kinase that results in enhanced cardiac contractility. In short, transgenics have been valuable as a starting point for generating genetically modified mouse models of human disease, but the added value of gene ablation has become increasingly clear in identifying the role of endogenous genes in complex cardiac physiological endpoints.

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From the Institute of Molecular Medicine, University of California San Diego, School of Medicine, La Jolla, Calif.

Correspondence to Kenneth R. Chien MD, PhD, 0613C Basic Science Building, UCSD School of Medicine, La Jolla, CA 92093. E-mail kchien@ucsd.edu

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In this regard, gene targeting has been especially valuable in identifying genes that are involved in critical aspects of cardiogenesis, including NKX 2.5,8 GATA-4,9,10 MEF-2,11 the HAND genes,12–14 RXRα,15 and several others.16 Because the ablation occurs via a site-specific recombination event, issues of copy number, site of integration, and mosaicism are moot. A subset of the most informative gene-targeted mouse models has been based on the knockout of genes that are expressed in a cardiac muscle–restricted fashion, including mutations in cytoskeletal17–19 and calcium-cycling genes.20,21 However, gene targeting carries with it the risks of gene redundancy (particularly an issue for highly conserved transcription factors and signaling molecules that have several closely related family members that share overlapping expression patterns), inherent costs of time and labor, and the difficulties of interpreting whether any adult phenotype that arises actually reflects an earlier developmental effect. Because many of the most intriguing candidate genes are widely expressed, it can also be unclear as to whether these genes are impacting cardiac physiology within cardiac myocytes or via secondary effects in neighboring cell types or integrative signals from other organ systems. Finally, and perhaps most importantly, early embryonic lethality in conventional gene-targeted animals can prevent an examination of the role of the gene of interest in the physiology of the postnatal heart. These last two considerations formed the impetus for the development of new strategies to control the onset of the gene ablation in time and space, ie, conditional gene targeting.

### Generation Present

The latest wave of mouse models has advanced beyond generalized gene knockouts to develop new strategies for the precision engineering of endogenous genes within specific cell types. The most successful approach has been based on the generation of mice that harbor floxed alleles, which contain LoxP recognition sequences that flank a critical exon that is required for the expression or function of the gene of interest2 (see the Figure). These mice are generated by homologous recombination of targeting vectors in embryonic stem cells that bring in the LoxP sites into the germ line. The floxed allele mice express the normal gene product, because the LoxP sites are located within the intron sequences that are spliced out during RNA processing. However, the intervening sequences between the LoxP sites can be excised by the expression of CRE recombinase, which is brought into the genetic background of the floxed allele mice via interbreeding. By controlling the expression of CRE recombinase to a specific tissue, for example to the ventricular chamber, it is possible to generate mice that harbor a ventricular-restricted mutation in a gene that is widely expressed, thereby allowing a direct examination of the role of a given gene within cardiac muscle (Figure). The ability of the cre mouse to drive high-level, uniform expression of the cre recombinase and to maintain strict tissue specificity is critical, and several promoters have been developed that will allow the generation of cardiac-specific mutations using cre-lox strategies.22–24

The most sought after prize has been the development of approaches to control both the spatial and temporal onset of the gene

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MLC indicates myosin light chain; MHC, myosin heavy chain; MLP, muscle LIM protein; ALP, actinin-associated LIM domain protein; PLB, phospholamban; BARK, β-adrenergic receptor kinase; and VEGF, vascular endothelial growth factor.

*For a more complete list, see Reference 30.
†For a more complete list, see References 16 and 34.
modification of interest. Earlier studies have documented the ability of triggering the gene ablation with specific ligands that control the expression of a particular cre transgene that is introduced into the background of a given floxed allele. Previous studies have generated systems where the mutations are triggered by the administration of tetracycline, interferon, and ecdysone to name a few. However, the challenge has been in combining both tissue specificity and inducibility into the paradigm for conditional gene modification. The first-generation strategies required the introduction of 2 transgenes (one designed to control temporal activation and the other to control tissue specificity) into the background of a given floxed allele background. Whether this reflects the feasibility of modifying the ligand activation domain such that endogenous ligands are capable of activating the cre recombinase, thereby allowing a tight regulation of expression with little or no leakiness in the basal state, a critical consideration for gene modifications that would be expected to be embryonic lethal. Finally, the recent observation that nonspecific cardiac injury and cardiomyopathies can accompany the α-MHC driven overexpression of green fluorescent protein and several other genes raises the question as to whether progesterone CRE fusion protein will result in cardiac injury and dysfunction at baseline. It should be noted that previous studies with animals overexpressing α-MHC CRE have indeed documented cardiomyopathy in a subset of lines, additionally emphasizing this point. Nevertheless, the present study bodes well for those interested in conditional mutations that extend from presently established cre lines for achieving high-efficiency cardiac-restricted conditional gene mutations.

**Generation Next**

To cre or not to cre? Ironically, the answer may ultimately lie not in technology but in biology, ie, the nature of the question of interest. As noted above, there are a growing number of tools to modify the mouse genome, with the promise of many more on the horizon. The “generation next” of mutant mouse models is likely to include new approaches for somatic and germ-line gene rescue of conventional knockout animals, including new strategies where the knockout and rescue are encoded in a single targeting construct and triggered by a single switch. Double knockout mice have already been effectively used for true genetic complementation studies to identify new targets for heart failure progression. New smart vectors that will allow high-efficiency, tissue-tropic, and long-term somatic gene transfer of CRE or downstream genes that rescue the knockout phenotype are also in the pipeline. New mutant mouse models are appearing from chemical mutagenesis screens, and libraries of targeted embryonic stem cell lines and a host of floxed allele mice are becoming available from numerous laboratories. Ironically, the astounding results of the human and mouse genome projects underscore the remarkable conservation of their respective genomes, and new candidate genes are arising regularly in studies of other model organisms, including zebrafish, Drosophila, and Caenorhabditis elegans. Given the difficulty of establishing cause-and-effect relationships between given complex genotypes and in vivo physiological phenotypes in human populations, it is becoming increasingly likely that the molecular pathways will initially be identified in the mouse and subsequently corroborated in candidate gene studies in human populations. If such is the case, perhaps the well-bred mouse may eventually become man’s best friend.
References

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