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With great power comes great responsibility: using mouse genetics to study cardiac hypertrophy and failure

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Abstract

Over the past 20 years generation and subsequent characterization of genetically modified mouse models has revolutionized our understanding of disease-gene relationships and suggested numerous therapeutic targets for human disease. Cardiac biology has perhaps benefited more than most fields from the advent of modern genetic approaches in the mouse by providing a 3-dimensional integrated platform for phenotypic dissection of single gene function, largely replacing the unitary relationships derived from 2-dimensional cell culture-based platforms. Indeed, cardiac hypertrophy and end-stage heart failure are whole organ phenomena that occur within a dynamic neuroendocrine milieu, a backdrop that cannot be adequately modeled in cultured myocytes. Here we advocate the use of genetically modified mouse models for studying cardiac biology and show how, if employed properly, these models will continue to provide highly reliable data sets that suggest disease-gene relationships and novel therapeutic targets. In addition to a discussion of proper technique and controls, we will highlight examples of genetic approaches in the mouse that suggest novel disease relationships and therapeutic treatments for human heart failure, insights not possible with other experimental systems.

1. Introduction

The advent of genetic engineering in the mouse has ushered in a new era of tremendous investigative power whereby single disease-gene relationships can be unequivocally ascertained, but as Peter Parker reminisced in *Spider-Man* the movie; “with great power comes great responsibility”, and likewise the use of genetically modified mouse models requires fastidious technique and design. The power of mouse genetics has not gone un-noticed across all biological fields of study, and it has gradually become the mainstay technique for mechanistic assessment of gene function *in vivo*. Indeed, the 2007 Nobel Prize in Medicine was awarded for gene-targeting technologies, clearly indicating the revolution that has transpired and its on-going impact on modern medical research. Cardiac biology has arguably benefited the most from this revolution in mouse genetics given the generation of reliable and highly tissue-specific promoters, the restricted number of cell types in the heart, and the relative lack of cellular turnover or proliferation. Moreover, the progression of many cardiac diseases are characterized by structural remodeling that can only be investigated *in vivo* within the

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Counter-Point: In the preceding review/editorial by Cook, Clerk and Sugden, a number of strong arguments are made detailing the potential short comings associated with genetic approaches in the mouse as a means of unraveling cardiac disease mechanisms. We take very little issue with these arguments *per se*, although here we attempt to put these shortcomings into a greater context that extends beyond a single experimental setting, as well as to carefully construct a counterpoint that delineates the advantages of genetic approaches in the mouse compared with any other system currently in use in cardiovascular biology.

context of the whole organ, essentially precluding cell-based assays. Finally, the heart dynamically responds to neurohumoral status present within the local heart microenvironment (paracrine factors) as well as within the greater circulation (endocrine), which together dramatically affect maladaptive disease processes leading to or influencing heart failure. Hence, annotation of individual genes that underlie cardiac hypertrophy and heart failure initiation and progression is really only accurately modeled within an animal, and preferably a mammal to increase the relevance to human cardiac physiology and disease. Alternative systems, such as culturing of primary or immortalized cell lines, simply lack the “great power” and disease relevance associated with genetic engineering in the mouse.

2. Cell culture versus mouse genetics for dissection of cardiac disease states

Before mouse genetics entered the mainstream of cardiac experimental work, investigators largely relied on cultures of primary neonatal cardiac myocytes derived from newborn rats or inadequate “myocyte-like” cell lines, although *ex vivo* tissue slices in physiological baths could be used to study some basic physiological parameters. With respect to phenotyping of heart disease mechanisms, these earlier *ex vivo* and culture-based approaches provided some limited information on cellular growth and sarcomeric organization within a largely 2-dimensional environment. However, the most significant power of neonatal myocyte cultures is reductionist; a single molecular process can be investigated in a largely isolated system and temporal relationships established. For this reason culture-based approaches will always be necessary, although with questionable phenotyping capabilities. For example, activation of extracellular signal-regulated kinases 1/2 (ERK1/2) is easily assessed in neonatal myocytes at different time points of hypertrophic agonist stimulation. But extending these correlations to a greater causal understanding of hypertrophic processes becomes problematic. First, neonatal myocyte growth assays in culture are performed under serum free conditions for short periods of time, which does not even crudely approximate the *in vivo* situation. Second, growth of myocytes in culture is largely 2-dimensional given their flattened stellate shape, while *in vivo* myocytes are 3-dimensional and rod shaped. Third, myocytes in culture often lack the appropriate substratum and complex 3-dimensional extracellular matrix (ECM) that completely surrounds the cells *in vivo*. Fourth, myocytes in culture lack a proper beating synchrony compared with the rate of rhythmic contractile cycling observed *in vivo*, which could alter the growth response. Fifth, myocytes *in vitro* lack a functional load or stretch that could also impact the growth response. Sixth, growth assays in culture are highly sensitive to plating densities, which raises the question of physiological relevance and results in significant intra- and inter-laboratory variation. Seventh, growth assays in culture are sensitive to levels of contaminating fibroblasts, further enhancing intra- and inter-laboratory variability. In addition to these issues, heart failure simply cannot be modeled in any manner whatsoever in culture. Thus, while cultured neonatal cardiac myocytes will always be of value for the reductionist dissection of signaling and regulatory pathways in a highly controlled setting, they are of limited applicability to understanding organ disease events.

Genetic approaches in the mouse provide a powerful window into understanding whole organ cardiac disease mechanisms in the context of a single gene manipulation. However, a complex series of technical considerations underpin the success or failure of any experimental approach, mouse genetic-based or otherwise. The “great responsibility” referred to in the title of this essay is to fully understand the strengths, weakness, and pitfalls associated with gene-targeting and transgenic approaches in the mouse, so that disease-gene relationships are correctly annotated. Improper application of these techniques, poor experimental design, or inadequate controls will compromise the validity of any experimental approach. Apart from these caveats, genetic manipulation in the mouse remains the foremost experimental approach in cardiac biology for directly examining the molecular underpinnings of cardiomyopathic disease states with an emphasis directed towards identifying therapeutic opportunities.

3. Proper design and use of transgenic and gene-targeted mice

The mouse has rapidly moved to the forefront of cardiac biology as the premier model organism for disease-gene relationship investigation. Accordingly, nearly all technology that underlies the physiologic testing which define human cardiac mechanics and hemodynamics to measure cardiac performance, rhythm, and structural integrity, have been miniaturized and adapted to the mouse. While most basic cardiovascular physiology is conserved between mice and humans, certain limitations are important to understand. For example, the basal heart rate in the mouse is 7–9 fold higher than in humans, which is associated with significant differences in ion handling. The basic motor protein that underlies the cardiac pump, the myosin heavy chain, is represented in the mouse heart by the α -isoform while in the human ventricle the β -isoform predominates. More importantly, the mouse heart may also differ in disease susceptibility compared with the human heart on some levels, such as contractility. For example, deletion of the *phospholamban* gene in the mouse, which dramatically augments cardiac contractility, appears to be beneficial and to protect the mouse heart from certain disease causing insults [1]. However, recently described human families with a presumed null mutation in *phospholamban* develop lethal cardiomyopathy at a relatively young age [1]. These and many other physiological/disease susceptibility differences between mice and man must be factored into any proposed murine genetic experiment when the data are interpreted.

Genetic approaches in the mouse have been criticized on many levels, but often the method is disparaged under the auspices of broader issues that should be applied to any experimental system. Poorly controlled or just plain bad experiments have been, can and will be carried out with even the most impeccably precise tools. A sharp scalpel in the hands of an unskilled surgeon can do a tremendous amount of damage and by analogy, even the most precisely controlled genetic engineering experiment when poorly controlled or over-interpreted, can lead to faulty conclusions or the accumulation of discordant data.

As directed towards the heart, the differences between pronuclear injection of purified DNA, which is often referred to as transgenesis, and gene targeting, in which endogenous DNA is replaced or disrupted by exogenous sequences by homologous recombination, have been reviewed recently [2]. Although there are inherent pitfalls in transgenesis, many of these can be circumvented by creating additional transgenic lines that can be used as controls to check dosage or epigenetic sequelae. The factors that can potentially confound data interpretation of a pronuclear, transgenic experiment and ways of circumventing them are listed in Table 1.

A number of difficulties in interpreting a cardiac transgenic experiment can arise from the promoter that drives the transgene itself. For example, although the α -myosin heavy chain (α -MHC) promoter is often thought of as driving ventricular expression in the adult, its actual expression pattern is considerably more nuanced, with transient expression in the embryonic heart tube and atrial expression throughout development [3,4]. If the experimenter is attempting to isolate events that occur as a result of expression only in the adult, more precise manipulation of transgene expression may be necessary to generate interpretable data. Inducible transgene expression allows precise and reversible expression of a normal or mutated protein that can be directed to a particular cell type at a particular developmental time. A number of drug-inducible systems have been described but the tetracycline-based system is the most effective and widely used [5-7]. We have developed a cardiomyocyte-specific system which, when carefully used, can define a protein's effect(s) in the heart in a temporally restricted manner [8]. However, like most tools, it must be used carefully as the tetracycline activator can, when expressed at high levels or for long periods of time, be cardiotoxic. Despite this concern, transactivator lines have been developed that show no cardiotoxicity for at least 6 months and so these experimental limitations can be easily circumvented [8].

Another critical issue concerning transgenesis and protein overexpression in the heart is carefully considering the intended goal of the experiment from the onset. The discerning point here is whether or not the true physiologic function of a protein is to be interrogated, or just some greater aspect of protein function that is independent of absolute expression levels. Take phospholamban as an example. Controlled overexpression of this protein by transgenesis has a stoichiometric effect on sarcoplasmic reticulum calcium loading and contractility [9]. In this later case, carefully documenting transgene expression levels amongst individual founder lines produces a dose response relationship that further suggests the true physiologic function of phospholamban *in vivo*. However, overexpression of activated MKK1 in the heart by transgenesis, as will be discussed later, was performed from the onset to produce the non-physiologic effect of constitutively activating ERK1/2. When the endpoint of a transgenic experiment is carefully considered, functional effects can be obtained that are either intended to carefully elucidate the physiologic function of a protein *in vivo*, or just a greater mechanistic understanding of its potential consequences.

Gene targeting has often been heralded as being more precise and useful than transgenesis [10,11]. When coupled with tissue or cell type specific methodologies via Cre-lox technology, gene targeting offers a precise way of engineering in specific mutations that will only be expressed in a defined cell type at a particular developmental time. No other experimental avenue provides a more precise way of isolating the results of a protein's expression, or lack thereof, in a fully operational physiological system. However, as noted by Cook et al. discordant data have been reported (accompanying article). This is not due to any intrinsic deficiency in the approach however, and the discrepancies between groups can almost always be explained by subtle differences in the constructs employed. For example, gene targeting is typically performed by modifying the activity (i.e. deletion) of one particular gene in the genome. However, a number of potential problems that can confound data interpretation can arise. These could be largely avoided by a rigorous assessment of the targeting event at both the transcript and protein levels. It is not the basic approach that is flawed, it is the often cavalier application of the technology and incomplete definition of the outcome. The surrounding genetic context can be affected as well by the targeting event, resulting in altered transcriptional patterns of neighboring genes, hence adversely affecting the resultant phenotype and confounding interpretation [12-14]. In most, if not all cases, the pitfalls can be navigated by carefully assessing all the relevant experimental parameters in the modified animal.

As is the case for transgenesis, the more precisely the targeting event can be manipulated, the more straightforward the data interpretation. In the heart, this is accomplished by rendering the targeting event cardiomyocyte specific via controlled Cre expression using a cardiomyocyte-specific promoter or even making cardiomyocyte-specific Cre expression inducible [15]. However, we, and others have noted some cardiac toxicity due to high levels of Cre expression with the α -MHC promoter [16], especially in later adulthood. In fact, in our hands the α -MHC-Cre line generated by Schneider and colleagues leads to a lethal cardiomyopathy between 8–12 months of age (unpublished observations). Consequently, one simply designs the experiment such that the data are obtained before cardiac function and biochemistry are affected, circumventing any negative effect of Cre expression. More importantly, employing Cre-only transgenic mice in the same genetic background as part of the overall experimental design is absolutely necessary for proper data interpretation and to ascertain any potential effect of Cre alone. Alternatively, different cardiac Cre transgenes may be used. For example, β -MHC-Cre produces exceedingly efficient developmental gene deletion in cardiomyocytes throughout the entire developing heart, but transgene expression is lost during postnatal development so that the adult heart is Cre-free, hence no ongoing toxicity [17], but a caveat here is that the β -MHC-Cre transgene is also expressed within slow fibers of skeletal muscles (although the mouse is overwhelmingly fast fibers). Finally, tamoxifen administration only in the presence of the α -MHC-MerCreMer transgene produces

a temporary reduction in cardiac function with some ventricular dilation, although this phenotype resolves in 7–14 days after tamoxifen administration (unpublished observations). Thus, if the MerCreMer transgene is used to inducibly delete a gene from the heart, controls that only contain this transgene with tamoxifen are critical. In conclusion, if all potential details are carefully and rigorously assessed, the “great power” of transgenic approaches in the mouse can yield data of tremendous value for dissecting disease-gene relationships, which is simply not possible in any other experimental system at the current time.

4. A detailed case for mouse genetics over cell culture-based approaches: The MKK1-ERK1/2 pathway

Perhaps the best way to exemplify the revolutionary power of mouse genetic engineering in cardiac biology is to review one scientific area of investigation in depth. The MKK1-ERK1/2 signaling pathway is ideal for this purpose given the numerous culture- and mouse genetic-based studies catalogued over the past 15 years. Mitogen-activated protein kinase (MAPK) signaling pathways consist of a sequence of successively acting kinases that culminate in the dual phosphorylation and activation of terminal kinases such as p38, c-Jun N-terminal kinases (JNKs), and ERKs [18]. MAPK signaling is initiated in cardiac myocytes by G-protein coupled receptors, receptor tyrosine kinases, and by stress stimuli [19]. ERK1 and ERK2 are directly regulated and phosphorylated by two MAPK kinases, MKK1 and MKK2. Upstream of MKK1 and MKK2, Raf-1 functions as a MAPKKK that is activated at the plasma membrane by the low molecular weight G-protein Ras.

Numerous studies in neonatal rat cardiomyocyte cultures have measured ERK1/2 activation in association with growth inducing agonists or stress stimuli, suggesting the relatively straightforward hypothesis that ERK1/2 direct the hypertrophic response. In support of this, the use of dominant negative MKK1 or Raf-1, antisense oligonucleotides against ERK1/2, and pharmacologic inhibitors of MKK1/2 provided convincing data indicating that MKK1-ERK1/2 is both necessary and sufficient for cardiac hypertrophy [20–26]. However, a number of similarly designed culture-based studies have disputed this conclusion with those data suggesting that MKK1-ERK1/2 do not regulate cardiac hypertrophy [27–33]: one study even suggested that ERK1/2 activation is anti-hypertrophic [34].

As with genetic experiments in the mouse, the likely reasons for the discordant data sets are a combination of conditions employed, specific molecular effectors used, and possibly even bad technique and improper controls. More importantly, phenotypic characterization of cultured neonatal myocytes is influenced by cell density, media composition, number of contaminating fibroblasts, the dosage and time course of both the pharmacologic antagonist and the hypertrophic agonist employed. But underlying the difficulty in assessing the physiological relevance of the *in vitro* data is that cardiac hypertrophy is a 3-dimensional process associated with ventricular remodeling and ECM changes, neuroendocrine milieu alterations, and changes in loading; processes that cannot be modeled accurately in culture.

Cardiac hypertrophy is best studied within the context of an intact animal, and analysis of MKK1-ERK1/2 signaling in transgenic and gene-targeted mice has provided important and consistent insights into the function of this pathway in cardiac hypertrophy and disease. To investigate the ability of MKK1-ERK1/2 signaling to induce cardiac hypertrophy *in vivo*, transgenic mice expressing activated MKK1 under the transcriptional control of the α -MHC promoter were generated [35]. Nine separate lines of MKK1 transgenic mice were made and regardless of overexpression level, each showed highly specific and constitutive activation of ERK1/2 in the heart, without affecting other stress kinases, resulting in a phenotype of stable concentric hypertrophy [35] (Figure 1B). The results of this experiment also highlight another important consideration of transgenic approaches, that multiple independent lines with varying

levels of overexpression produce a logical phenotype. As another important control, high levels of ERK2 overexpression in the heart from 2 independent transgenic lines with the same α -MHC promoter had no effect whatsoever, indicating that simple overexpression of a signaling protein need not be detrimental or induce hypertrophy (Figure 1A, B). However, MKK1 transgenic mice crossed with ERK2 transgenic mice showed synergistic hypertrophy, indicating that ERK2 was fully functional, but exquisitely silenced without a signal from MKK1/2 (Figure 1B). Ras overexpressing transgenic mice also show cardiac hypertrophy, although Ras activates more than MKK1-ERK1/2, possibly explaining why these hearts are also cardiomyopathic, while MKK1 mice simply show a stable concentric hypertrophic response that is not pathologic [36]. Taken together these results clearly indicate that enhanced and constitutive MKK1-ERK1/2 activity within the heart promotes a stable hypertrophic phenotype in transgenic mice.

Loss-of-function studies in transgenic and gene-targeted mice suggest that ERK1/2 signaling may not be required to mediate cardiac growth *in vivo*, although it is required for cardioprotection [37,38]. Perhaps the best way to address the necessity of ERK1/2 signaling in mediating cardiac hypertrophy is simply to delete the genes encoding *Erk1* and *Erk2*. However, *Erk2* nulls are embryonic lethal, and it has been exceedingly difficult to even generate *Erk1*^{-/-} *Erk2*^{+/-} mice (3 of 4 alleles deleted), so alternate strategies were employed [38]. To this end, the ERK1/2 specific dual-specificity phosphatase 6 (DUSP6) was selected for regulated overexpression in the heart using the tetracycline inducible system described above [38]. This bitransgenic, cardiac myocyte-specific system showed nearly complete inactivation of all cardiac ERK1/2 at baseline and after multiple stimuli (pressure overload and neuroendocrine agonist infusion), with no effect on other MAPK effectors [38]. However, complete inhibition of ERK1/2 associated with DUSP6 overexpression did not reduce the ability of the heart to hypertrophy following pressure overload stimulation, neuroendocrine agonist infusion, or physiologic exercise stimulation. *Erk1*^{-/-} mice and *Erk2*^{+/-} mice also hypertrophied normally following stimulation [38]. However, DUSP6 transgenic mice and *Erk2*^{+/-} mice were more likely to fail following pressure overload stimulation [38] and *Erk2*^{+/-} showed greater cell death in the heart after ischemia-reperfusion injury [37]. These results suggest that MKK1-ERK1/2 signaling are not necessary for cardiac hypertrophy *in vivo*, but that this pathway protects myocytes from cell death inducing stimuli.

We previously generated transgenic mice with constitutive DUSP1 expression in the heart, which reduced signaling from all three major MAPK branches, and in this context partially reduced the growth response [39]. When compared with DUSP6 overexpression, which did not reduce the hypertrophic response (ERK1/2 specific), this later report emphasizes the importance of fully understanding the details associated with a specific molecular effector used in transgenesis, as DUSP1 overexpression also prominently inhibited p38 and JNK [39]. Another example is the phenotype of dominant negative Raf-1 transgenic mice, which showed attenuated hypertrophy following pressure overload stimulation, suggesting that the Ras-Raf-1-MKK1-ERK1/2 pathway was required for pathologic growth [40]. However, this interpretation may not be entirely correct because Raf-1 can have effects on many other signaling effectors and is not specific for MKK1-ERK1/2 activation [41,42]. Thus, the current literature in genetically modified mouse models with altered MAPK signaling is complicated, but when specific details are considered and stringent criteria are applied, a uniform picture emerges. The data at present suggest that MKK1-ERK1/2 signaling are sufficient to induce cardiac hypertrophy, but in their absence hypertrophy proceeds normally, likely through other pathways.

5. Three examples of genetically modified mouse models suggesting novel treatment strategies for human heart failure

Genetic approaches in the mouse are absolutely irreplaceable for understanding the mechanistic basis of heart failure. While heart failure can be modeled in other non-genetic mammalian systems, often with greater human applicability (such as large mammals), mouse models of heart failure have become the mainstay given our ability to manipulate single gene function and understand the consequences with respect to disease susceptibility and progression. Here we will highlight three examples of how mouse genetic approaches have uncovered novel molecular targets that specifically attenuate heart failure, suggesting novel treatment strategies in humans if properly designed drugs become available.

5.1. PKC α

The protein kinase C (PKC) family of Ca²⁺ and/or lipid-activated serine/threonine kinases function downstream of most membrane-associated signal transduction pathways [43,44]. Ten different isozymes comprise the PKC family, which are broadly classified as conventional isozymes (α , β I, β II, and γ), novel isozymes (ϵ , θ , η , and δ) and atypical isozymes (ζ , and λ) [43,44]. PKC α is the predominant PKC isoform expressed in the mouse, human, and rabbit heart [45-47], and PKC α expression and activity is increased in cardiac hypertrophy, dilated cardiomyopathy, ischemic injury, or mitogen stimulation [43,44]. We and others have shown that PKC α functions as a novel regulator of cardiac contractility through effects on Ca²⁺ handling and myofilament proteins [48-51]. For example, *Prkca* (PKC $\alpha^{-/-}$) gene-deleted mice are hypercontractile, while transgenic mice overexpressing PKC α in the heart are hypocontractile. Enhancement in cardiac contractility associated with *Prkca* deletion protected against pressure overload-induced heart failure and dilated cardiomyopathy associated with deletion of the *muscle lim protein (MLP)* gene (*Csrp3*) in the mouse [49]. These results suggested a translational vantage point if a properly designed pharmacologic inhibitor of PKC α were available. Indeed, the PKC inhibitors Ro-32-0432 or Ro-31-8220 each augmented cardiac contractility *in vivo* and in an isolated work performing heart preparation in wildtype mice, but not in *Prkca* deficient mice [45]. These compounds also improved cardiac function in failing *Csrp3* null mice, suggesting a potential treatment for heart failure [45]. More recently, cardiac-specific transgenic mice were generated with the tetracycline-inducible system to express a dominant negative (dn) PKC α protein in the heart. Induction of dnPKC α in the adult heart augmented baseline contractility, and protected against myocardial infarction induced heart failure [52]. Thus, use of both *Prkca* gene-deleted mice and inducible transgenic mice expressing dnPKC α suggest PKC α as a novel molecular target for treating human heart failure, provided that proper pharmacologic agents are developed. This exciting new lead would not have been possible without the use of genetically modified mouse models.

5.2. GRK2

Precedent for another molecular heart failure target that arose solely from studies in genetically modified mice is that of G-protein-coupled receptor kinase 2 (GRK2). GRKs are protein kinases that directly phosphorylate G-protein-coupled receptors (GPCRs) leading to desensitization, internalization and inactivation. GRK2 plays a prominent role in the heart where it desensitizes β -adrenergic receptors, down-regulating inotropy and functional reserve of the myocardium. Koch and colleagues showed that transgenic mice with GRK2 overexpression had reduced cardiac contractility, while transgenic mice expressing a decoy piece of GRK2 (also known as β ARKct) that inhibits GRK2 activity for GPCRs, augmented baseline contractility of the heart [53]. Importantly, *Grk2* heterozygous gene-targeted mice, which have a 50% reduction in total GRK2 protein levels, also show enhanced cardiac contractility [54]. Inhibition of cardiac GRK2 activity and the associated increase in cardiac

contractility was shown to significantly rescue or prevent heart failure in three different mouse models of disease. In a dilated cardiomyopathic mouse model of heart failure due to deletion of the *Csrp3* gene, expression of the β ARKct transgene resulted in complete rescue [55]. Similar data were obtained when β ARKct transgenic mice were crossed with transgenic mice that normally experienced heart failure due to cardiomyocyte-specific expression of either calsequestrin [56] or a mutant MHC [57]. Thus, inhibition of GRK2 enhances inotropic reserve and function of the heart by maintaining effective β -adrenergic receptor signaling, antagonizing the initiation and progression of heart failure in multiple mouse models of this disease. These results suggest that pharmacologic inhibitors of GRK2 would be powerful therapeutics for treatment of human heart failure.

5.3. CaMKII

Analysis of cardiac phenotypes associated with Ca^{2+} /calmodulin (CaM)-dependent protein kinase activation or inhibition has suggested a prominent role in disease and heart failure. For example, increased expression of CaMKII δ c in the hearts of transgenic mice caused a dilated cardiomyopathy with decreased contractile function [58]. Myocytes from these hearts were shown to have dramatically attenuated Ca^{2+} cycling and lower sarcoplasmic reticulum (SR) Ca^{2+} levels and greater SR leak [59]. Overexpression of CaMKII δ c in transgenic mice models a known increase in CaMKII proteins levels in pressure loaded mouse hearts, suggesting disease relevance [60]. Similarly, even ectopic expression of CaMKIV in the mouse heart by transgenesis induced a cardiomyopathic phenotype, collectively suggesting that increased CaMK activity in the heart is promyopathic [61]. Indeed, CaMKII activation *in vivo*, such as through an oxidation sensitive mechanism, is known to induce myocyte apoptosis and to reduce myocyte function [62,63]. More importantly, transgene-mediated inhibition of CaMKII in the heart prevented β -adrenergic and myocardial infarction-induced negative remodeling and maintained cardiac function better and reduced apoptosis [64,65]. Collectively, the results obtained in genetically modified mouse models suggest that CaMKII would be an ideal target for pharmacologic inhibition in treating heart failure or hypertrophic disease.

While we have outlined 3 exciting lines of research from genetically modified mouse models that suggest new treatment strategies for human heart failure, it should be noted that none of these have yet led to a clinically approved treatment in humans, nor to our knowledge has any currently employed heart failure treatment strategy resulted from genetic studies in the mouse. However, it is not uncommon for a drug to take over 20 years from inception to clinical application. Given that genetically modified mouse models have only recently become a mainstay approach, it may take many more years before approaches based on this technology make their way to the clinic.

6. Future perspectives

Science is a progressive undertaking that builds upon the observations of earlier generations and more simplified experimental systems. The use of genetically modified mouse models represents a current pinnacle approach for biologic investigation at the current time with many of the genes selected for *in vivo* manipulation first being implicated through studies in cultured cells. While studies in cultured myocytes will continue to have a place in our cardiovascular scientific arsenals, fully embracing and expanding the use of genetically modified mouse models in carefully designed and executed experiments is and will remain invaluable in defining new molecular effectors with the therapeutic potential to impact on human heart disease.

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Figure 1.

Transgenic analysis of MKK1-ERK1/2 signaling in the heart. (A) Western blot with ERK1/2 antibody from heart extracts taken from Wt mice and two different lines of ERK2 overexpressing cardiac-specific transgenic mice. The ERK2 protein is epitope tagged so it runs slightly slower than endogenous ERK2. Line 72 expresses significantly higher than line 80. (B) Analysis of heart weight normalized to body weight in 2 month-old mice that are transgenic for ERK2 overexpression (either line 80 or line 72) or activated MKK1 expression, or crossed to contain activated MKK1 and ERK2 transgenes. ERK2 transgenic mice show no hypertrophy, but when crossed with MKK1 they show synergistic increase in heart weights. * $P < 0.05$ versus Wt; # $P < 0.05$ versus MKK1.

Table 1
Considerations for carrying out a transgenic experiment

Experimental Disadvantage	Potential Solution
Mosaicism	Check for transgene expression in multiple tissues; only use F1's in which the transgene has been transmitted through the germline
Molecular torture: high levels of transgene expression lead to a non specific phenotype.	Test multiple lines with varying levels of expression and use the line with the lowest level of expression that gives a phenotype. If a mutation's effect is being tested, create lines in parallel that express normal protein at levels at or greater than the mutant line.
Animal husbandry and phenotypic drift: a phenotype changes over a period of months or years as the colony is bred	Animals should be kept in barrier conditions if possible to avoid selection of mice carrying an unknown pathogen that can influence morbidity/mortality. Only young mice should be used for breeding and only for 2–3 months in order to avoid any positive selection from occurring.
Sex linked transmission: no transgene positive females are detected in the first or second generation offspring	An insertion into the X chromosome may be difficult to interpret if the transgene is silenced by x-linked inactivation in which some cells are expressing an active transgene while other cells are not. Y chromosome integration is also possible. These lines should be discarded.
Insertional mutagenesis: genomic deletions may occur at the integration site. The deleted DNA may range from a few bases to a kilobase or more	Maintain mice as heterozygotes; the deletion as the mutation is only rarely dominant and one intact allele will remain present. Routinely characterizing at least 2 lines will also alleviate this problem
Illicit splicing	The transgenic mRNA should always be sequenced
Lack of a phenotype	<p>Possibilities:</p> <ol style="list-style-type: none"> 1. A suitable screening procedure has not been used. Additional assays should be employed 2. The transgenic protein is inactive. <ol style="list-style-type: none"> a) The protein may not be trafficked correctly within the cell. All appropriate endogenous trafficking signals should be included in the construct, unless a specific ectopic location is desired. b) The transgene is mutated, giving a truncated protein product or no protein at all. The construct should be sequenced completely at all steps of development in order to confirm that it is correct.
Strain dependent phenotypes: Strain variations are significant and must be considered as they can have a major impact on phenotype presentation. [66]	Most journals will ask the authors to list which strains were used and the percentage of each if the strain is not pure. Some journals demand that experiments be conducted in a pure, defined strain. Although strain variation remains a confounding factor, it can be used to advantage in that modifying genes can be potentially defined, and humans are out bred and have varying admixtures of modifying genes (better physiologic relevance in the spectrum of disease penetration)
Artifacts due to transgenic protein toxicity	The epitope tag or reporter sequences should always be tested in isolation in the tissue and normal heart function, anatomy and biochemistry confirmed. If toxicity is detected, the reporter should not be used but in most cases, suitable reporters or lines that express the toxic protein at sub-toxic levels can be found or developed.
Transgene silencing by epigenetic modification	Use endogenous sequences from the same strain for all elements of transgene construction
Promoter activity is affected by the ensuing pathology	Transgene activity should be monitored at all stages in the disease process. Although the most commonly used cardiomyocyte-specific promoter is derived from the α -MHC gene, which is down-regulated during hypertrophy, in practice the degree of transgene down-regulation during the onset and development of cardiovascular disease is relatively modest (15–45%) and steady state levels of transgenic protein are rarely affected to any great degree. However, this should be confirmed in all models.