Published in final edited form as:

J Mol Cell Cardiol. 2015 September ; 86: 136–137. doi:10.1016/j.yjmcc.2015.07.007.

Giving credence to controls: Avoiding the false phenotype

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Cardiovascular science has undergone a revolution in the past 25 years with the application of genetic engineering to the heart and creation of relevant animal models in worms, flies, mice, rats and pigs [1]. A series of evermore-powerful tools have catalyzed the creation of hearts that can express, over-express or not express targeted genes and in some cases, control the developmental timing of the manipulation. Using animal models has allowed the cardiovascular community to establish causality, confirm proof-of-principal for therapeutic approaches, and helped us to understand the relevant pathways and their most important intersections for normal and abnormal cardiac function [1]. The mouse has been the model of choice for the largest number of investigators using these tools as the advantages of dealing with a mammalian, four chambered heart in terms of the data's application to human cardiovascular function and disease remain compelling. The definition of reagents such as the α - and β -myosin heavy chain promoters (MyHC) [2,3] that allowed investigators to drive cardiomyocyte-specific expression at various developmental times and in a chamber-specific manner, cemented the mouse as an important, genetically amenable model for cardiovascular disease. The community's ability to rigorously characterize the resultant phenotype and cardiac physiology using techniques that were first developed for larger animal models, followed quickly [4,5]. The promoters were used to drive expression of a large number of normal and mutated proteins in the heart with the use of the α -MyHC promoter predominating, as it drives cardiomyocyte-specific expression in the atria during fetal development and in all four chambers starting a couple of days before birth, as thyroid hormone production begins and activates transcription from the *myc6* locus [3].

Transgenesis, which involves the injection of naked DNA containing the gene sequence of choice into the nucleus of a fertilized 1 cell embryo, has its drawbacks [6] with random insertion of the transgene in varying copy numbers occurring. Although this can be in some cases an advantage, the lack of control and precision can also be a significant drawback [1,6]. Thus cardiac-specific transgenesis has been complemented and in many cases supplanted by the more precise tools of gene targeting, which have been applied with great

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Disclosures

None.

effectiveness to the cardiovascular system. While gene targeting was first restricted to systemic gene ablations, reagents became available that allowed the investigator to not only target an ablation or gene modification to a particular tissue or cell type, but allowed one to time the event as well. Cardiac-specific targeting strategies use cardiomyocyte-specific expression of a recombinase, Cre, which can recognize short sequences consisting of two 13 bp inverted repeats separated by an 8 bp asymmetric spacer region, termed a *loxP* site. During the targeting event, endogenous DNA is replaced by a construct containing the targeted locus flanked by *loxP* sites. Cre activity then excises the gene fragment, creating the targeted allele. Conditional gene deletion is usually worth the extra effort and, if one makes the promoter driving *cre* expression inducible [7], the gene targeting event can be controlled temporally in the cardiomyocyte population.

Schneider's group created the first cardiomyocyte specific *cre* construct by linking the gene to the α -MyHC promoter [8]. Although almost 20 years have passed and other lines have been created [9], it remains the most widely used mouse for cardiac specific *cre*-based experimentation. One of the strengths of the α -MyHC promoter is also a weakness: it can drive very high levels of expression, and high levels of any protein, let alone a recombinase, can cause cardiotoxicity [10]. However, Cre toxicity has never been explored in a systemic, rigorous manner and this gap has now been filled by the paper "Prolonged Cre expression driven by the α -myosin heavy chain promoter can be cardiotoxic" recently published by Pugach et al. in The Journal of Molecular and Cellular Cardiology [11].

The genesis for these experiments is grounded in the technical necessities imposed by cell type specific genetic manipulation: to obtain Cre-mediated excision, it is normally necessary to breed the Cre-expressing mouse into a mouse line carrying the *loxP*-flanked targeting construct. The resultant double transgenic animal will express Cre in cardiomyocytes such that the locus will be targeted. But even after the targeting event, Cre will continue to be expressed, and expressed from a strong promoter such that appreciable levels of the recombinase are present at steady state. It is well known that Cre can have off-target effects and these authors posed the very obvious and entirely legitimate question of whether chronic Cre expression results in a detectable phenotype or altered cardiac function over a 6 months period. What makes this question even more important is that the majority of investigators fail to include the relevant control cohort of mice in their experiments. That is, they do not report data on the Cre-expressing mice only, holding open the distinct possibility that some or all of an observed phenotype might be due to off target effects of the recombinase.

The study by Pugach et al. is thus particularly welcome as they carefully address the possible issue of α MyHC-Cre-induced cardiotoxicity in mice that lack engineered *loxP* sites. The authors carefully assess the short-term and prolonged effects of the α MyHC-cardiomyocyterestricted Cre expression using a combination of functional, molecular and bioinformatic analyses, characterizing the effects at 3 and 6 months in the different sexes. While the changes are subtle, they are statistically significant, with selected molecular markers indicative of hypertrophy or cardiac stress presenting statistically significant variation at 3 months and functional differences detectable at 6 months. Decreased cardiac function and significant increases in fetal gene expression, including the natriuretic peptides, as well as activation of potentially pathologic p38 signaling were documented. The authors

conclude that Cre expression can evoke cardiac toxicity, and these responses increase as expression continues and the animals age. They also observed small increases in a few proteins associated with the DNA damage response and at 6 months TUNEL staining was increased 3-fold over the levels observed in nontransgenic hearts.

Reasoning that the mild cardiac pathology might result from and/or trigger pro-inflammatory and fibrotic processes, they measured the relevant molecular markers and noted statistically significant increases. Consistent with those data, careful quantitation of the degree of fibrosis revealed a two-fold increase in the left ventricles. They also observed increased levels of inflammatory cells in the myocardium, as well as increased pro-fibrotic gene expression in Cre positive mice compared with age-matched wild-type mice. As Cre toxicity was previously noted in the absence of *loxP* target sites and resulted in growth arrest, chromosomal abnormalities and apoptosis [12], the authors go on to suggest that the toxic effects of Cre expression might be tied to Cre-mediated recombination of genomic DNA at degenerate *loxP* sites. Indeed, such sites exist in the mouse and human genomes and can serve as substrates for the Cre-mediated recombinase [13]. Pugach et al. used a bioinformatics approach to identify mouse genes that are both expressed in the heart and contain degenerate *loxP* sites. Testing 27 of these genes by looking at transcript levels in the α MyHC-Cre hearts, they found that approximately 26% showed significantly altered expression. The authors speculate that these genomic sites that form degenerate *loxP* sites may be targeted during prolonged expression of Cre at high levels and suggest that genomic sequencing of the α MyHC-Cre cardiomyocytes is necessary to assess the effects of off-target Cre recombination on genomic integrity. Consistent with this hypothesis, they observed a dose dependency of Cre induced cardiotoxicity, comparing the commonly used α MyHC-driven Cre line with another α MyHC-Cre with 30 fold less Cre protein expression [9]. A concomitant decrease in “cardiotoxicity” was observed.

A central concern for the investigator using these types of approaches is whether to change their approach along the lines suggested by the authors: either use a lower expressing line [9], which did not show the phenotypic or transcriptional changes, or carry a cohort of α -MyHC promoter-driven Cre mice as controls, carefully using that line as a baseline comparison in addition to nontransgenic animals. Why do most investigators omit such a control in the first place? After all, it is an obvious one to consider and include, as data bearing on this point did exist before Pugach et al. but in somewhat fragmentary and incomplete forms [10,14]. There are probably a number of factors that investigators have used to rationalize omission of the α -MyHC Cre-only cohort. First, although rigorously documented and statistically significant, the changes observed by Pugach et al. are modest and an investigator, if interested in an aspect of cardiac structure/function far removed from the parameters determined in this and other studies defining the consequences of Cre expression, might well decide that the possibility of its expression affecting the relevant parameter(s) is sufficiently remote such that it can be safely omitted. One might not agree with the judgment but, operationally in the real world, resources are limited, mice are expensive and the analyses are time consuming and therefore also expensive in terms of personnel. In this era of rate limiting resources, these factors dictate rigorous control of the animal census, keeping the colonies to an absolute minimum.

A key point raised by the authors is the potential for off-target recombination due to the chronically high levels of Cre protein. Although the data presented in Pugach et al. are consistent with this possibility, the authors did not actually show that such off-target recombination occurred and so this remains to be determined, presumably by sequencing the potential targets selected on the basis of *in silico* and transcriptional analyses. However, it is doubtful that this occurring as frequently as the transcriptional changes imply. Indeed, the changes observed may very well be due to secondary effects on the transcriptional activities of the loci as a result of the modest but real changes in cardiac function, fibrosis and elements of an inflammatory response. Indeed, while “cardiac” transcripts were selected for the analyses, it is unclear for a number of the potential candidates listed in Pugach et al. whether the cardiomyocyte is the primary cell type in the heart transcribing the locus.

Despite this limitation, the authors present a good argument for including α -MyHC-Cre mice as an essential control. The data are compelling and while some investigators might be skeptical about their significance for their particular study, the sin of omission could be a powerful factor in the argument against acceptance of the resulting manuscript. Considering the time and effort involved in bringing a study using gene targeted animals to submission for publication, and the rigor of current reviewers, any author runs a significant risk in omitting this control cohort in their overall experimental design and execution.

Acknowledgments

Supported in part by NIH grants P01HL69779, P01HL059408, and R01HL105924 (JR) and the Transatlantic Network of Excellence Program grant from Le Fondation Leducq (to JR).

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