Healing a Heart Through Genetic Intervention

Jeanne James, Jeffrey Robbins

A mouse model for adult cardiac-specific gene deletion with CRISPR/Cas9
Carroll et al
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A recent article from Eric Olson's group outlines the development of an important new tool that should catalyze the cardiovascular community's ability to create new animal models containing genetically engineered genes and proteins specifically in the cardiomyocyte population with cardiomyocyte. Olson and colleagues used a cardiomyocyte-specific promoter to express a critical component of the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)–associated (Cas)9 genomic editing system. The α-myosin heavy chain gene (*myh6*) promoter was used to drive high levels of cardiomyocyte-specific Cas9 expression. Subsequently, adeno-associated virus (AAV) was used to deliver single-guide RNA (sgRNA) against the *myh6* locus, and they were able to show efficient gene editing at the locus, establishing proof of principle for what the authors term "cardioediting," a strategy for revising a specific locus in the cardiomyocytes at any time post birth.

CRISPR and Cas genes are essential for adaptive immunity in certain bacteria and archaea, enabling these primitive cells to eliminate invading genetic material. The system was originally described in bacteria almost 30 years ago,[1] and its function confirmed <10 years ago.[2] Although there are at least 3 different CRISPR systems, it is the type II CRISPR system that has, in <5 years, revolutionized our ability to make targeted insertions, deletions, and modification in a large variety of organismal genomes, including the human's.[3] Although targeted mutation of mammalian genomes and the creation of stable mouse models has been feasible since targeted homologous recombination (HR) was reported by the Bronson and Smithies[4] and Thomas and Capecchi[5] groups, and has grown more precise in that we are now able to make precise mutations in selected cell types at directed times during development, gene targeting via HR has been and remains a time consuming, expensive and low throughput experimental technique that can often take years to complete and verify. The use of CRISPR–Cas9 for gene targeting using a simple, 2 component system[6] has truly revolutionized mammalian-based gene targeting, and Olson and colleagues have now used the system to both prepare a generally useful reagent for the cardiovascular community and demonstrate how it can be quickly used for preparing cardiomyocyte-specific gene knockouts.

The Olson laboratory linked the *myh6* promoter to the CRISPR/Cas9 construct, and confirmed strong expression of Cas9 in the transgenic hearts. To test the efficacy of the *myh6*-driven Cas9, sgRNAs against exon 3 and exon 8 of *myh6* were individually inserted into AAV9 backbones, which also contained ZsGreen driven by a cytomegalovirus promoter, with the fluorescent protein allowing monitoring of transduction after AAV delivery via intraperitoneal injection. Transgenic Myh6-Cas9 mice were injected at 10 days of age with the AAV9-Mhy6 exon 3 sgRNA construct. After 5 weeks, cardiomyocytes were isolated and examined for expression of the Cas9 fluorescent reporter. GFP or TdTomato was expressed in essentially all cardiomyocytes, suggesting robust expression of the transgene, indicating effective transduction of AAV and robust expression of Cas9. At the whole-organ level, transgenic mice injected with the AAV-Myh6 sgRNA construct showed cardiac hypertrophy compared with transgenic mice injected with saline control. To confirm that the hypertrophy was not a nonspecific consequence of AAV or AAV/sgRNA toxicity, the investigators also injected an AAV/sgRNA targeted for luciferase and observed no change in cardiac structure.

Histologically, the cardioedited mice showed massive dilation of the atria, thinning of the ventricular walls and modest interstitial fibrosis. At the transcript level, *myh6* expression was ≈30% to 40% of control and relative *myh7* expression ≈40-fold higher. Expression of natriuretic peptides A and B (*nppa* and *nppb*, respectively) was also increased, and taken together the data present a molecular signature of cardiac dysfunction. Echocardiography confirmed decreased systolic function in the Cas9+AAV/Myh6 sgRNA mice. To determine the effects of a double knockdown of *myh6*, AAV–sgRNAs against both exon 3 and exon 8 were injected at 10 days of age. *Myh6* expression was decreased to a similar amount, with *mhy7* expression increased ≈20-fold and similar alterations in both *nppa* and *nppb* expression. Significant cardiac compromise was noted as early as 3 weeks after injection in these so-called "double guide" animals, compared with 5 to 6 weeks after injection in transgenic mice injected with only exon 3 AAV–sgRNA. T7 endonuclease I assays in double guide animals confirmed mutations in both exon 3 and exon 8, with polymerase chain reaction analyses showing large genomic deletions between the guide sites.

Myh6 knockdown has been accomplished in the past using HR in mouse embryonic stem cells,[7] allowing phenotypic

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comparison between the 2 approaches. Jones et al[7] found that homozygous null animals died between ED 11 and 12, whereas heterozygous animals were viable and fertile. Compared with wild-type controls, *myh6* expression in HR heterozygous animals was decreased by ≈50% and Western blot analysis showed Myh6 protein to be decreased ≈75%. Compensatory upregulation of *myh7* was minimal (≈7%) in the HR-directed *myh6* knockdown, and there was no detectable accumulation of Myh7 protein. In stark contrast, Carroll et al found *myh7* expression was ≈40-fold increased in the cardioedited mice compared with control, but no data were reported about Myh7 protein. Although there are significant methodological differences in transcript analysis (radiolabeled oligonucleotide dot blot hybridization versus quantitative polymerase chain reaction), it seems unlikely that the variance in *myh7* expression between approaches is due solely to improved sensitivity provided by quantitative polymerase chain reaction. Thus, similar decreases in *myh6* expression do not have similar effects on *myh7* expression. Likewise, at the histological level, the heterozygous knockout seems to have had more significant foci of fibrosis and likely more severe hypertrophy, although comparable images of the cardioedited mice were not reported. At the functional level, it is difficult to compare the degree of impairment in the 2 models because of significantly different evaluation techniques as a working heart preparation was used by Jones et al[7] and noninvasive echocardiography by Carroll et al. Regardless, significant systolic dysfunction is present in both approaches.

Taken together, the models of *myh6* knockout or knockdown have important similarities, but also significant differences that seem to be approach related, with the CRISPR/Cas9 technique as executed by Carroll et al possibly related to nonhomologous end joining with resultant variations in breakpoint insertions and deletions. This complication could be avoided in future use of this technology by adopting a homology-directed repair approach, which has the advantage of defined gene editing that allows as precise as single-nucleotide changes.

The article has significant implications for the immediate future of reductionist cardiovascular research, in which gene function and protein structure–function relationships are explored on an individual gene or protein domain basis. The proof-of-concept established by the article promises to dramatically increase the speed and lower the cost of directed gene ablation and presumably directed mutation of targeted genes as well. The authors have presented the research community with a useful and powerful tool: a mouse with cardiomyocyte-directed expression of Cas9.

With the Cas9 protein being transgenically expressed in the cardiomyocytes, delivery of sgRNAs targeting the *myh6* locus illustrated the functional efficacy of AAV-9 systemic delivery to a single cell type, resulting in cardiomyocyte-specific gene ablation. Thus, an ablation that might have taken months or even years is within reach of laboratories without a substantial infrastructure in gene targeting technology, and targeting can be achieved in weeks rather than months or even years. The excitement engendered by using induced pluripotent stem cells as models for testing individual patient mutations could easily be extended to a mouse model if the experiment can be performed in a matter of weeks, allowing the phenotype to be observed not in an isolated cell system, but in the intact organ in the context of the whole animal. Although CRISPR/Cas9 is now used almost exclusively for knockdown experiments at the present, improvements and modifications are being reported almost weekly, which improve specificity and decrease off-target effects to the point where more precise knockins are certainly on the horizon.[8] We think that it is only a matter of time before almost any identified mutation in any gene of interest that is expressed in cardiomyocytes will become experimentally accessible in a reasonable time frame with minimal resources.

There are several important questions that investigators will need to keep in mind as they adopt cardioediting. A preliminary analysis of the animal did not reveal any ill effects because of chronic expression of presumably high amounts of the nuclease but this will need to be followed up with detailed, longitudinal data that delineate the absolute amount of the transgenic protein and its effects, if any, at the molecular, biochemical, cellular, whole-organ and whole-mouse levels during the animal's lifespan. We know that even innocuous proteins, when expressed at high levels in the cardiomyocyte, can have deleterious effects during the short- or long-term but this is both dose and protein dependent.[9] As expression from the *myh6* promoter is both copy number dependent and position independent[10–12] and both attenuated and inducible forms have been made, this should not present a serious obstacle to the widespread use of the Cas9 mouse because different *myh6* promoters are available to fine tune expression of Cas9 should the standard *myh6* promoter prove unsuitable for some long-term studies.

Another caveat to keep in mind in terms of the widespread use of the Cas9 transgenic mouse is the potential effects of breeding it into a mixed background or different mouse strain. The mouse strain used in these studies was not specified, but in the study by Carroll Cas9 is driven by *myh6* promoter sequences derived from an FVB/N line.[12] We know the promoter can exhibit different behaviors in terms of its overall activity (but not specificity) when crossed into different strains. Therefore, caution will need to be taken with respect to characterizing the control animals, Cas9 transgenic animals in the absence of systemic AAV9–sgRNA injection, at least initially, until both Cas9 activity and lack of toxicity are confirmed in a variety of widely used strains.

Finally, given the phenotypic differences between HR and Cas9 knockdown of *myh6*, careful consideration needs to be given to the choice of gene-editing approach as nonhomologous end joining compared with homology-directed repair may result in a variety of sequence differences that could affect transcriptional and translational results and thus phenotypic results as well.

Postnatal Genome Editing

What next? Clearly, we have gained an invaluable tool for basic research and the concept will undoubtedly be scaled, first to other genes important in cardiovascular disease and second to other cell and organ types, as numerous effective,

cell type–specific promoters exist. The ability to effectively deliver a systemic signal that triggers precise postnatal genetic modification has already been applied to mouse models of Duchenne muscular dystrophy (DMD) using CRISPR/Cas9 and AAV strtategies.[13–15] The heart is a particularly attractive target for a proactive approach with this disease because heart disease overtly presents later in life,[16] and indeed with improved respiratory care, heart failure is now the most common cause of death in DMD.[17] To effectively treat the skeletal muscle disease, it will probably be necessary to target the mouse satellite cells or muscle stem cells, which may be more refractory to viral-mediated interventions.[18] Virus efficacy, titer production, and safety would all need to be assessed during a prolonged period, particularly before sanctioned use in the pediatric population.

Despite these caveats, this article should generate tremendous optimism. We have been given a potent new reagent to achieve effective postnatal genome editing on a scale and level of precision undreamed of just 5 years ago. Basic research should thrive as a result and the general principle holds open the promise of relatively rapid translation into at least some aspects of clinical medicine. Many cardiovascular diseases have comparatively extended windows for effective therapeutic treatments as the disease course can take months or even years to reach a level of compromise sufficient to produce symptoms. This provides an opportunity to first model the particular disease, test the most effective treatments in an animal model, and then personalize a treatment (eg, an sgRNA) that can be delivered at any stage, or even prophylactically before symptoms present if safety can be confirmed. The future looks bright.

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