Published in final edited form as:

Circ Res. 2016 March 18; 118(6): 920–922. doi:10.1161/CIRCRESAHA.116.308468.

Healing a Heart through Genetic Intervention

Jeanne James, MD and Jeffrey Robbins, PhD

Heart Institute, Department of Pediatrics, The Cincinnati Children's Hospital Medical Center, Cincinnati, OH 45229, USA

Abstract

A recent manuscript 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 (CM) population. Olson and colleagues utilized a CM-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 CM-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 CMs 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 less than 10 years ago.² Although there are at least three different CRISPR systems, it is the Type II CRISPR system that has, in less than five 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 Smithies and Capecchi groups,^{2,4} 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, two component system⁵ has truly revolutionized mammalian-based gene targeting and Olson and colleagues have now utilized the system to both prepare a generally useful reagent for the cardiovascular community and demonstrate how it can be quickly utilized for preparing CM-specific gene knockouts.

The Olson laboratory linked the *myh6* promoter to the CRISPR/Cas9 construct, and confirmed strong expression of Cas9 in the transgenic (TG) hearts. To test the efficacy of the *myh6*-driven Cas9, sgRNAs against exon 3 and exon 8 of *myh6* were individually

Correspondence to Jeffrey Robbins PhD, The Heart Institute, Division of Molecular Cardiovascular Biology, Cincinnati Children's Hospital Medical Center, MLC 7020, 240 Albert Sabin Way, Cincinnati, OH 45229-3039. Tel: (513) 636-8098; Fax: (513) 636-5958. jeff.robbs@cchmc.org.

inserted into AAV9 backbones, which also contained ZsGreen driven by a CMV-promoter, with the fluorescent protein allowing monitoring of transduction after AAV delivery via intraperitoneal injection. TG Myh6-Cas9 mice were injected at 10 days of age with the AAV9-Myh6 exon 3 sgRNA construct. After 5 weeks, CMs were isolated and examined for expression of the Cas9 fluorescent reporter and. GFP or TdTomato was expressed in essentially all CMs, suggesting robust expression of the transgene indicating effective transduction of AAV and robust expression of Cas9. At the whole organ level, TG mice injected with the AAV-Myh6 sgRNA construct showed cardiac hypertrophy compared to TG mice injected with saline control. To confirm that the hypertrophy was not a non-specific 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 ~30–40% 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 *myh7* 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 to 5–6 weeks after injection in TG 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 PCR analyses showing large genomic deletions between the guide sites.

Myh6 knockdown has been accomplished in the past using HR in mouse embryonic stem cells,⁶ allowing phenotypic comparison between the two approaches. Jones *et al* found that homozygous null animals died between ED 11–12, while heterozygous animals were viable and fertile. Compared to 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 to control, but no data were reported regarding Myh7 protein. While there are significant methodological differences in transcript analysis (radiolabeled oligonucleotide “dot blot” hybridization versus quantitative PCR) it appears unlikely that the variance in *myh7* expression between approaches is due solely to improved sensitivity provided by qPCR. Thus similar decreases in *myh6* expression do not have similar effects on *myh7* expression. Likewise, at the histologic level, the heterozygous knockout appears 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 two models due to significantly different evaluation techniques as a working heart preparation was used by Jones *et al* and non-

invasive 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 appear to be approach-related, with the CRISPR/Cas 9 technique as executed by Carroll *et al* possibly related to nonhomologous end joining (NHEJ) 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 paper 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 paper 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 very useful and powerful tool: a mouse with CM-directed expression of Cas9.

With the Cas9 protein being transgenically expressed in the CMs, delivery of sgRNAs targeting the *myh6* locus illustrated the functional efficacy of AAV-9 systemic delivery to a single cell type, resulting in CM 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 carried out 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 knock down 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.⁷ We believe that it is only a matter of time before almost any identified mutation in any gene of interest that is expressed in CMs 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 due to 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 TG protein and its effects, if any, at the molecular, biochemical, cellular, whole organ and whole mouse levels over the animal's lifespan. We know that even innocuous proteins, when expressed at high levels in the CM, can have deleterious effects over the short or long term but this is both dose and protein dependent.⁸ As expression from the *my6* promoter is both copy number dependent and position independent^{9–11} and both attenuated and inducible forms have been made, this should not present a serious obstacle to the widespread use of

the Cas9 mouse as 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 TG 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 Carroll's study Cas9 is driven by *myh6* promoter sequences derived from an FVB/N line.¹¹ 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 TG 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 non-homologous end joining compared to 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 using CRISPR/Cas9 and AAV strategies.¹²⁻¹⁴ The heart is a particularly attractive target for a proactive approach with this disease as heart disease overtly presents later in life,¹⁵ and indeed with improved respiratory care heart failure is now the most common cause of death in DMD.¹⁶ 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.¹⁷ Virus efficacy, titer production and safety would all need to be assessed over a prolonged period, particularly before sanctioned use in the pediatric population.

Despite these caveats, this manuscript 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, a sgRNA) that can be delivered at any stage, or even prophylactically before symptoms present if safety can be confirmed. The future looks bright.

Acknowledgments

Sources of Funding

This work was supported by NIH grants P01HL69779, P01HL059408, and R01HL105924 and The Transatlantic Network of Excellence Program grant from Le Fondation Leducq (to J.R.).

References

1. Ishino Y, Shinagawa H, Makino K, Amemura M, Nakata A. Nucleotide sequence of the iap gene, responsible for alkaline phosphatase isozyme conversion in escherichia coli, and identification of the gene product. *J Bacteriol.* 1987; 169:5429–5433. [PubMed: 3316184]
2. Barrangou R, Fremaux C, Deveau H, et al. Crispr provides acquired resistance against viruses in prokaryotes. *Science.* 2007; 315:1709–1712. [PubMed: 17379808]
3. Maeder ML, Gersbach CA. Genome editing technologies for gene and cell therapy. *Mol Ther.* 2016
4. Thomas KR, Capecchi MR. Site-directed mutagenesis by gene targeting in mouse embryo-derived stem cells. *Cell.* 1987; 51:503–512. [PubMed: 2822260]
5. Jinek M, Chylinski K, Fonfara I, Hauer M, Doudna JA, Charpentier E. A programmable dual-rna-guided DNA endonuclease in adaptive bacterial immunity. *Science.* 2012; 337:816–821. [PubMed: 22745249]
6. Jones WK, Grupp IL, Doetschman T, et al. Ablation of the murine alpha myosin heavy chain gene leads to dosage effects and functional deficits in the heart. *J Clin Invest.* 1996; 98:1906–1917. [PubMed: 8878443]
7. Merkle FT, Neuhausser WM, Santos D, et al. Efficient crispr-cas9-mediated generation of knockin human pluripotent stem cells lacking undesired mutations at the targeted locus. *Cell Rep.* 2015; 11:875–883. [PubMed: 25937281]
8. James J, Osinska H, Hewett TE, et al. Transgenic over-expression of a motor protein at high levels results in severe cardiac pathology. *Transgenic Res.* 1999; 8:9–22. [PubMed: 10399364]
9. Rindt H, Subramaniam A, Robbins J. An in vivo analysis of transcriptional elements in the mouse alpha-myosin heavy chain gene promoter. *Transgenic Res.* 1995; 4:397–405. [PubMed: 7581520]
10. Knotts S, Rindt H, Robbins J. Position independent expression and developmental regulation is directed by the beta myosin heavy chain gene's 5' upstream region in transgenic mice. *Nucleic Acids Res.* 1995; 23:3301–3309. [PubMed: 7667107]
11. Robbins J, Palermo J, Rindt H. In vivo definition of a cardiac specific promoter and its potential utility in remodeling the heart. *Ann N Y Acad Sci.* 1995; 752:492–505. [PubMed: 7755294]
12. Long C, Amoasii L, Mireault AA, et al. Postnatal genome editing partially restores dystrophin expression in a mouse model of muscular dystrophy. *Science.* 2015
13. Nelson CE, Hakim CH, Ousterout DG, et al. In vivo genome editing improves muscle function in a mouse model of duchenne muscular dystrophy. *Science.* 2015
14. Tabeordbar M, Zhu K, Cheng JK, et al. In vivo gene editing in dystrophic mouse muscle and muscle stem cells. *Science.* 2015
15. Villa CR, Czonek RJ, Ahmed H, et al. Ambulatory monitoring and arrhythmic outcomes in pediatric and adolescent patients with duchenne muscular dystrophy. *J Am Heart Assoc.* 2015; 5
16. Birnkrant DJ, Ararat E, Mhanna MJ. Cardiac phenotype determines survival in duchenne muscular dystrophy. *Pediatr Pulmonol.* 2016; 51:70–76. [PubMed: 26097149]
17. Arnett AL, Konieczny P, Ramos JN, et al. Adeno-associated viral (aav) vectors do not efficiently target muscle satellite cells. *Mol Ther Methods Clin Dev.* 2014; 1