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Recent advances in genome editing for cardiovascular disease

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Abstract

Purpose of review—This review highlights recent progress in applying genome editing to the study and treatment of cardiovascular disease (CVD).

Recent findings—Recent work has shown that genome editing can be used to determine the pathogenicity of variants of unknown significance in patient-derived induced pluripotent stem cells. These cells can also be used to test therapeutic genome editing approaches in a personalized manner. Somatic genome editing holds great promise for the treatment of CVD, and important proof of concept experiments have already been performed in animal models. Here we briefly review recent progress in patient-derived cells, as well as the development of somatic genome editing therapies for CVD, with a particular focus on liver and heart.

Summary—Translating this technology into the clinic will require precise editing enzymes, efficient delivery systems, and mitigation of off-target events and immune responses.

Further development of these technologies will improve diagnostics and enable permanent correction of some of the most severe forms of CVD.

Keywords

CRISPR/Cas9; Adeno-Associated Virus; genome editing; cardiovascular disease

Introduction

Cardiovascular disease (CVD) research is undergoing a major transformation with the application of CRISPR/Cas9 and other genome engineering systems. The clustered regularly interspaced short palindromic repeats / CRISPR-associated protein 9 (CRISPR/Cas9) system is currently the most widely used genome editing platform. It consists of an RNA-guided nuclease (Cas9) that produces double-stranded breaks in DNA at specific locations based on the sequence of a short guide RNA engineered by the user[1]. Generating double-stranded breaks with CRISPR/Cas9 can introduce short insertion and deletion mutations (indels), delete large fragments of DNA, or improve the efficiency of homology directed repair (HDR). CRISPR/Cas systems have been cloned from a wide range of bacteria, providing a diversity of targeting options[2–5]. In addition, Cas9 has been fused with other protein

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moeities, allowing the user to direct enzymatic activities to specific sites in the genome. These include fluorescent proteins, transcriptional activators or repressors, chromatin modifiers, deaminases, and reverse transcriptases. The development and basic principles of CRISPR/Cas9 genome editing have been covered well in recent literature[6–8]. This minireview will focus on the application of these emerging technologies to CVD. Specifically, we will highlight genome engineering of patient-derived induced pluripotent stem cells (iPSC), and somatic genome editing approaches targeting the liver and the heart (Figure 1).

Disease modeling in patient-derived cells

Rare variants in multiple genes are known to cause cardiomyopathies. However, even for a known gene, it can be difficult to predict whether a given variant is pathogenic. These "variants of uncertain significance" (VUS) present a difficult problem for clinicians and patients. Determination of pathogenicity by traditional means can be difficult, and should ideally be assessed in the genomic context of that particular patient to account for gene-gene and gene- environment interactions. The use of patient-derived pluripotent stem cells (iPSC) makes this possible. These cells can be edited and differentiated into a variety of cell types including cardiomyocytes, macrophages, and hepatocyte-like cells. In a recent example, Ma *et al.* isolated iPSCs from a healthy individual carrying a VUS in the *MYL3* gene (NM_000258.2:c.170C>A, NP_000249.1:p.Ala57Asp) which encodes myosin light chain 3, an important sarcomeric protein[9]. Other mutations in *MYL3* are associated with hypertrophic cardiomyopathy (HCM), and this variant was predicted by *in silico* algorithms to be damaging[10]. CRISPR/Cas9 editing was used to generate iPSC lines from this individual, including clones homozygous for either the 170C>A allele or corrected to wild type, which were then differentiated into cardiomyocytes (iPSC-CM)[9]. The heterozygous and homozygous iPSC-CMs for the 170C>A variant had no discernible phenotype in terms of gene expression, cell size, sarcomere organization, contractility, or calcium handling[9]. In contrast, conversion of these patient-derived iPSCs to a known pathogenic variant at the same nucleotide (170C>G) did display changes in the β-/α-myosin ratio, faster contraction and relaxation velocities, and proarrhythmic alterations in calcium handling[11]. This work supports a benign classification for the 170C>A allele[9], and demonstrates that genome-edited iPSCs have potential utility for assessing the impact of VUS in a patient-specific manner. This approach has also been used to assess pathogenicity of VUS in *KCNH2* as a cause of long QT syndrome, where the T983I variant was deemed pathogenic[12].

CRISPR/Cas9 editing of iPSC-CMs can also be used to study gene-gene interactions. Deacon *et al.* found that the interaction between the sarcomeric gene for tropomyosin1 (*TPM1*) and the cosarcomeric gene for vinculin (*VCL*) influenced severity in a family with a dilated cardiomyopathy (DCM) history[13]. To determine the pathogenicity of the mutations found in *TPM1* and *VCL,* they derived cardiomyocytes from human iPSCs and CRISPR/Cas9 genome-edited H9 human embryonic stem cells (hESCs) with the same variants found in the patients[13]. *TPM1* and *VCL* variants acted synergistically to impact cardiomyocyte contractility and sarcomeric organization[13]. Genome editing in iPSC-CMs will also prove useful in testing new therapeutic approaches in a variant- or patient-specific manner. Long *et al.* recently generated iPSC-CMs from patients with Duchenne muscular dystrophy (DMD),

a lethal muscle disease with severe cardiac manifestations[14]. CRISPR/Cas9 targeting of conserved splice acceptor or donor sites produced *DMD* alleles with in-frame deletions, restoring dystrophin protein expression and contractile function[14]. While these are exciting advances, there are important practical considerations to the use of genome-edited iPSC-CMs. Incomplete differentiation to cardiomyocytes *in situ* is a limitation for assessing functional outcomes in a bulk cell population. Thus far, most applications have focused on sarcomeric organization or expression of key proteins that can be assayed in individual cells through microscopy. Likewise, genome editing of iPSC clones has its own challenges, including the enrichment and selection of edited cells, as well as avoiding unintended indel mutations when performing HDR. Despite these challenges, it is likely that iPSCs will become an important preclinical testing ground for genome editing therapies in the coming years.

Genome editing of the liver

The liver plays a central role in lipid metabolism and is a major target organ for genome editing to treat CVD. Initial proof of concept studies have involved disruption of the *Ldlr, Pcsk9,* and *Angptl3* genes. The low density lipoprotein receptor (LDLR) binds to ApoB-containing lipoproteins and mediates their clearance from the circulation[15]. Loss-of-function mutations in *LDLR* cause Familial Hypercholesterolemia, a severe genetic disorder characterized by xanthomas, excessively high plasma cholesterol, and premature atherosclerotic CVD[16]. Jarrett *et al.* showed that an all-in-one Adeno-Associated Virus (AAV) vector packaged with *Staphylococcus aureus* Cas9 (SaCas9) and an *Ldlr*-targeting gRNA efficiently knocks out *Ldlr* in mouse liver and causes atherosclerosis in just 20 weeks[17]. This study underscores the importance of hepatic *LDLR* expression in clearance of LDL cholesterol, and paves the way for AAV-CRISPR correction of mutations in *LDLR.* One drawback to gene editing of *LDLR* is the sheer number of mutations that have been linked to elevated circulating cholesterol - over 1200[18]. Ultimately, a successful gene editing therapy would need to be generalizable, and would most likely involve integration of a full-length LDLR coding sequence. There is currently a gene therapy trial in progress to correct Familial Hypercholesterolemia through AAV delivery of an *LDLR* transgene to the liver[19]. The success of this therapy remains to be determined, and it is possible that genome editing approaches may also be worth pursuing.

PCSK9 is a liver-secreted protein that promotes lysosomal degradation of LDLR[20]. Loss of function mutations in PCSK9 are associated with low levels of LDL-C and confer protection against CVD[21–25]. *PCSK9* is a major target for LDL lowering in CVD for patients who are resistant to statin therapy, and there are several highly successful monoclonal antibodies currently in use[26]. In 2014, Ding *et al.* demonstrated *in vivo* editing of the *Pcsk9* gene with the CRISPR/Cas9 system using adenovirus to target mouse liver[27]. *Pcsk9* was also disrupted by Ran *et al.* who identified a Cas9 ortholog from *Staphylococcus aureus* that is small enough for delivery with AAV vectors[3]. Since then, *Pcsk9* has been a frequent target for proof of concept studies using different CRISPR/Cas editors and delivery systems. For example, base editors that can convert cytosine bases to thymine have been used in multiple reports to edit *Pcsk9* in the liver[28–30]. Chadwick *et al.* used a base editing strategy to inactivate mouse *Pcsk9*[28]. The base editor gRNAs were designed to alter codons for

glutamine, arginine, or tryptophan, leading to a premature stop codon and PCSK9 truncation[28]. Wang *et al.* tested the feasibility of targeting human hepatocytes using CRISPR/Cas9 in a chimeric liver-humanized mouse model[30]. They demonstrated successful gene editing of human *PCSK9*, reduced blood levels of human PCSK9 protein, and a lack of detectable off-target mutagenesis at predicted sites[30]. More recently, Carreras *et al.* presented the use of base editors to correct hypercholesterolemia in a human *PCSK9* overexpression mouse model[29].

Angiopoietin-like proteins are a family of secreted proteins linked with regulation of various physiological and pathophysiological processes, including triglyceride-rich lipoprotein metabolism and CVD[31]. Chadwick *et al.* targeted hepatic *Angptl3* in 5 week old mice with a CRISPR/Cas base editor 3 (BE3) delivered via adenovirus[31]. They found that BE3-*Angptl3*-treated mice had lower circulating ANGPTL3, triglycerides, and cholesterol compared to control mice[31]. In *Ldlr* KO mice, targeting *Angptl3* substantially reduced plasma triglyceride and cholesterol levels (56% and 51%, respectively) at 14 days post treatment, whereas *Pcsk9* knockout did not[31]. Hypertriglyceridemia-induced CVD has proven a difficult target for drug development, but these studies have implicated Angptl3 as a promising candidate- either for knockdown, inhibition, or genome editing.

Viral delivery systems have been very effective for genome editing in animal models. However, these vectors have limitations for clinical translation. Adenoviral vectors elicit potent innate as well as adaptive immune responses[32]. Although these vectors have certain niche applications (i.e. oncolytic Adenovirus), serious safety concerns preclude the use of high doses of Adenoviral vectors for tissue-directed gene therapy in humans[33]. In the case of AAV, limitations include a high prevalence of neutralizing antibodies, and persistent expression of Cas9 which could increase the risk of off-target mutagenesis or immune responses. Therefore, there is a compelling need to develop complementary and alternative delivery systems. Recently, other groups have produced lipid nanoparticles to deliver CRISPR/Cas. Zhang *et al.* used gold nanoclusters modified with cationic HIV-1-transactivating transcriptor peptide complexed with Cas9 and a gRNA to disrupt *Pcsk9*[34]. The entire structure was further encapsulated by a galactose-modified lipid layer, which targets the gold nanoclusters to the liver[34]. They showed *Pcsk9* editing efficiency of 60%, and a 30% reduction in plasma LDL-C in mice[34]. Liu *et al.* developed a bioreducible nanoparticle that packages the Cas9 mRNA and gRNA together, and delivered this into mice intravenously[35]. They showed a 20% reduction in plasma PCSK9 as compared to controls[35]. While these studies show that lipid nanoparticles can deliver CRISPR/Cas9 and effectively target *Pcsk9* in the liver, further studies are required to analyze off-target mutagenesis and nanoparticle safety.

Genome editing of the heart

The heart is also an attractive target for genome editing. Carroll *et al.* were the first to report cardiac-specific Cas9 transgenic mice as a means of ablating gene expression in the heart[36]. The authors used AAV9 delivery of a gRNA to disrupt the *Mhy6* gene, which encodes cardiac alpha myosin heavy chain[36]. CRISPR/Cas9-deletion of *Myh6* at postnatal day 10 resulted in atria and ventricle dilation, ventricle wall thinning, cardiac dilatation and heart

failure, which was later reproduced by Johansen *et al*[37]. Johansen *et al.* also targeted *Sav1* and *Tbx20* using AAV delivery of gRNA[37]. Even with efficient editing there was no significant effect on protein levels with one gRNA, but using two gRNAs targeting *Sav1* resulted in a modest increase in heart size and hypertrophic markers[37]. Guo *et al.* used AAV9 to deliver gRNAs targeting junctaphilin 2 (*Jph2*), which tethers the T-tubule membrane to the sarcoplasmic reticulum (SR)[38]. High dose delivery of AAV-gRNA to *Jph2* in neonatal mice (P1) resulted in ventricular dilatation, cardiomyocyte hypertrophy, heart failure, and death[38]. However, lower doses produced mosaic removal of *Jph2*, allowing the authors to study its role in T-tubule formation in isolated cardiomyocytes[38]. The authors then used this "CASAAV" approach to screen 8 candidate genes, and uncovered a cell autonomous role for the SR calcium release channel (*Ryr2*) in this process[38]. These studies all observed effective but mosaic editing, and demonstrate the utility of this approach to study cardiac physiology.

Significant progress has been made towards therapeutic genome editing for the heart. In particular, autosomal dominant diseases present a unique opportunity for allele-specific gene disruption. Patients with familial Wolff-Parkinson-White (WPW) syndrome suffer from a conduction system abnormality that often manifests as paroxysmal supraventricular tachycardia. If not corrected with surgical ablation, certain cases of WPW can result in progressive heart failure or sudden cardiac death from ventricular tachyarrhythmia[39]. A knock-in mouse model for familial WPW was generated harboring the (c.1589A>G;H530R) mutation in the *PRKAG2* gene[39]. These mice were successfully rescued by delivery of AAV9-Cas9 and gRNA targeting the 1589A>G sequence as neonates, revealing a role for H530R *PRKAG2* allele in WPW syndrome[39]. Pan *et al.* used a similar approach to correct catecholaminergic polymorphic ventricular tachycardia in heterozygous animals harboring the a R176Q mutation in the *Ryr2* gene[40]. Targeted mice displayed reduced total *Ryr2* mRNA and protein levels, normalized calcium handling, and were protected from pacing-induced arrhythmias[40].

In addition to allele-specific disruption, others have explored targeted activation of disease-modifier genes that are too large for conventional gene therapy. Kemaladewi *et al.* used AAV9, to deliver a catalytically inactive version of Cas9 (dCas) fused to a VP64 transactivation domain, and a gRNA targeting the promoter for *Lama1* in a mouse model of muscular dystrophy type 1A (MDC1A)[41]. Treated mice showed *Lama1* upregulation in skeletal muscle and peripheral nerves, preventing muscle fibrosis and paralysis[41]. Disease progression was halted and reversed, showing for the first time a mutation-independent approach to treat MDC1A[41]. These studies show that it is possible to correct severe genetic diseases through somatic genome editing in the heart. In the case of autosomal dominant diseases, disruption of the disease-causing allele is now feasible in cardiomyocytes, but care must be taken to ensure that haploinsufficiency does not create new pathology. Likewise, off-target editing and unintended editing events at the on-target site (i.e. large insertions and deletions) will need to be avoided. The report of Kemaladewi et al. demonstrates the power of CRISPR/Cas9 transcriptional activation to upregulate expression of modifier genes, and possibly other targets that are too large for gene therapy vectors. However, this approach involves persistent expression of the Cas9 fusion proteins in the heart, where the long-term

efficacy and safety could be limited by pre-existing or acquired immunity to the Cas9 protein.

Landmark papers in *Science* in 2016 showed it is possible to correct DMD in the *mdx* mouse model through in-frame deletion of exon 23 with AAV9 delivery of CRISPR/Cas9[42–44]. In addition to severe muscle weakness and degeneration, DMD patients also suffer from dilated cardiomyopathy, and heart failure is the major cause of death. Refaey *et al.* used a similar editing strategy in the *mdx/Utr+/−* mice[45]. Mice that received high doses of AAV-CRISPR showed 23±5.1% restored dystrophin protein relative to WT by densitometry analysis, along with improved heart muscle fiber architecture and contractile strength[45]. Recently, the long-term safety and side effects of AAV-CRISPR editing for DMD has been examined by several groups[46–48]. Hakim *et al.* followed *mdx* mice for 18 months, and reported selective depletion of the AAV-gRNA vector[47]. In the same study, substantially higher doses succeeded in partially restoring dystrophin protein, reduced fibrosis, and improved hemodynamics[47]. Xu *et al.* used an AAVrh.74 vector to deliver CRISPR/Cas9 to the *mdx* mouse at age P3, and followed the animals for 19 months[48]. Genome editing was efficient and cardiac function improved without serious adverse events[48]. Nelson *et al.* reported humoral and cellular immune responses when AAV-CRISPR vectors were administered to adult mice, which was not seen with delivery to neonates[46]. Unintended on-target editing events were also found, including a high frequency of AAV vector insertions[46], as previously described in liver[21,49], and heart[40]. Efficacy has also been assessed in a canine model of DMD, where AAV9 was used to deliver SpCas9 and a gRNA targeting a region close to the exon 51 splice acceptor site[50]. Off-target analysis showed no unwanted gene editing, although only three predicted off-target sites were analyzed by targeted deep sequencing. Remarkably, dystrophin protein expression was restored to 92% of normal levels along with improved muscle histology[50]. These studies highlight the potential of CRISPR/Cas9 to achieve long-term editing for otherwise untreatable genetic diseases, but also raise concerns about host immune responses and unintended genetic alterations.

Conclusion

Genome editing is beginning to change the landscape of cardiovascular research and therapy. It is now possible to introduce specific variants in patient-derived cells, and determine their effects in an isogenic setting. Cellular phenotyping of genome-edited iPSC derived cardiomyocytes and other cell types will be an invaluable platform for assessing the safety and efficacy of gene-editing therapeutics. In parallel with these advances, delivery with viral vectors and nanoparticles can edit genes with high efficiency in the liver and heart. Somatic genome editing has been used in animal models to correct several disorders including hypercholesterolemia, hypertriglyceridemia, Wolff-Parkinson-White syndrome, Catecholaminergic Polymorphic Ventricular Tachycardia, and Duchenne muscular dystrophy. While these early successes are very promising, they have also identified important challenges. Off-target editing with CRISPR/Cas systems will vary depending on the specific editing enzyme, design, target cell type, and delivery method. Efficient delivery to somatic tissues is achievable in mice, but not all delivery systems will scale effectively to humans. Unintended editing events at the on-target site, such as large deletions and insertions, require further study and risk assessment. Likewise, pre-existing immunity and

subsequent development of immune response to the bacterially-derived Cas9 protein will need special attention. Despite these concerns, CRISPR/Cas therapeutic potential is expansive, and is expected to dramatically change the future of research and patient care.

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Key Points

- Genome editing of patient-derived induced pluripotent stem cells can be used to determine the pathogenicity of variants as well as to study gene-gene and gene-environment interactions.
- Liver-directed gene editing shows promise for correcting familial hypercholesterolemia and hypertriglyceridemia.
- Somatic gene editing in the heart is a useful approach for studying cardiac physiology in the whole heart as well as isolated cardiomyocytes *ex vivo*.
- Therapeutic editing strategies are in development to treat Wolff-Parkinson White syndrome, Catecholaminergic Polymorphic Ventricular Tachycardia, and Duchenne muscular dystrophy.

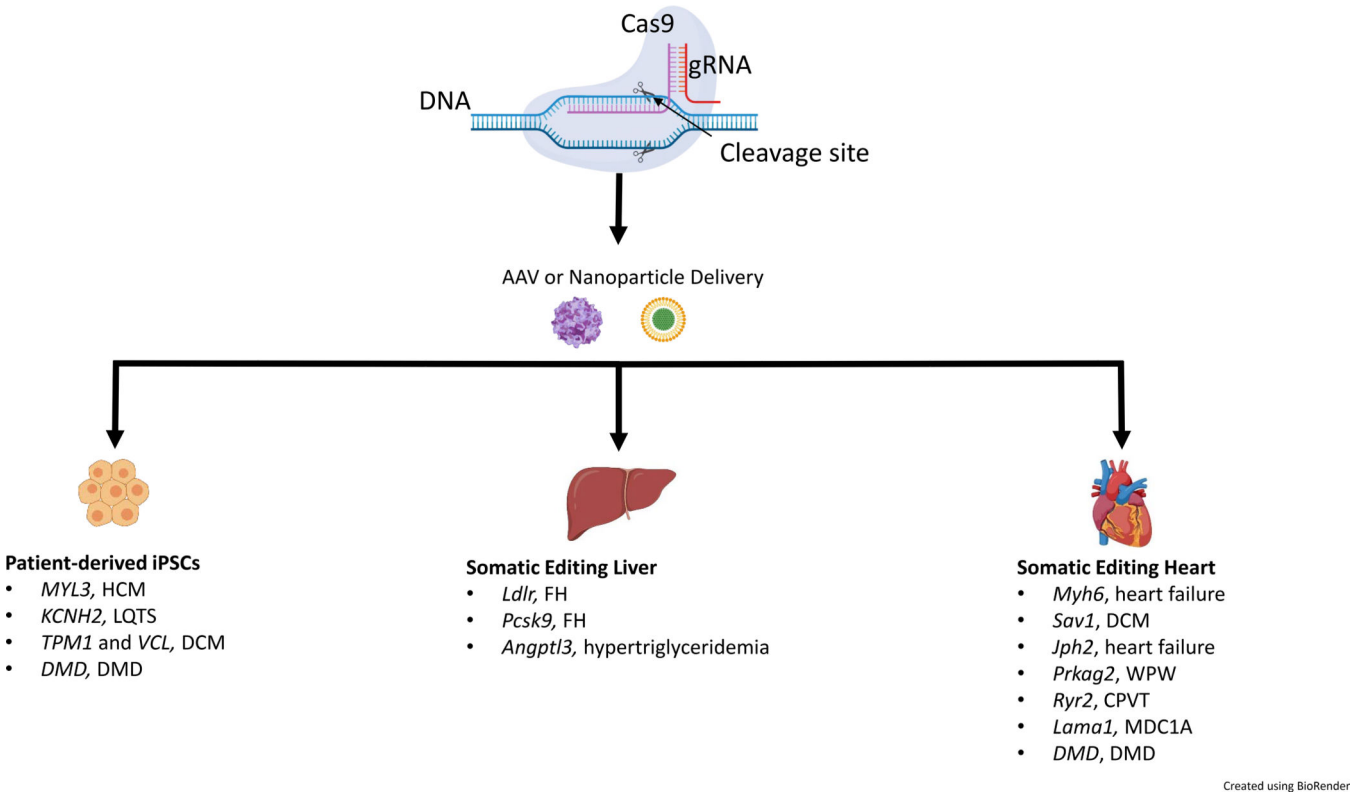

FIGURE 1:

Gene editing for cardiovascular disease. Adeno-associated viruses (AAV) and nanoparticles have been used to deliver CRISPR/Cas9 (clustered regularly interspaced short palindromic repeats/CRISPR-associated protein 9) components both in vitro and in vivo to edit genes involved in cardiovascular disease (CVD). Patient-derived induced pluripotent stem cells (iPSCs) have been edited with CRISPR/Cas9 to study variants associated with hypertrophic cardiomyopathy (HCM), long QT syndrome (LQTS), dilated cardiomyopathy (DCM), and Duchenne muscular dystrophy (DMD). Somatic genome editing in the liver and heart has been used to target genes that lead to familial hypercholesterolemia (FH), hypertriglyceridemia, heart failure, DCM, Wolff--Parkinson--White syndrome (WPW), catecholaminergic polymorphic ventricular tachycardia (CPVT), muscular dystrophy type 1A (MDC1A), and DMD. Although these studies have shown the CRISPR/Cas9 system's versatility for treating CVD, significant challenges remain – including delivery efficiency, off-target editing, and host-immune responses.