CRISPR-Cas9 Applications in Cardiovascular Disease

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Abstract: The CRISPR-Cas9 system is an economical and accessible gene-editing technology first discovered as a naturally occurring bacterial immune system. Since its fairly recent discovery, CRISPR-Cas9 system's efficiency and simplicity have been successfully used to edit genomes of living organisms in many fields, working in vitro and in vivo in germline and somatic cells to knock-out harmful mutated genes or in some cases working to knock-in a beneficial gene. A current application of the gene-editing system works against specific mutations that cause certain cardiovascular diseases. However, there are current technical limitations as well as ethical dilemmas in introducing gene-editing to humans. Here, we explore highlights on the current state of research of the CRISPR-Cas9 system through the lens of cardiovascular disease and examine potential untouched applications of the system in the field of cardiology. (Curr Probl Cardiol 2021;46:100652.)

Background

CRISPR-Cas9 technology is derived from a naturally occurring adaptive bacterial defense system against virus or phage attack. The bacterium captures a small length of DNA of an invading virus and inserts it to its genome as spacers in between repeated sequences of its own DNA. This genome is then transcribed into RNA that is complementary to the invading phage's DNA. As similar phages try to

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attack the cell again, the bacterial cell recognizes the attack, and the CRISPR (clustered regularly interspaced palindromic repeats) loci, then transcribed into crRNA (CRISPR RNA) together with a Cas nuclease, will bind itself to the complementary sequence of the attacking phage DNA.[1] The Cas nuclease then cuts and destroys the invading material, stopping the effect of the phage attack.

This intrinsic bacterial system is modified to edit other genomes in the lab by using a guide RNA (gRNA) sequence that is complementary to a specific site in a genome and a Cas9 enzyme that will cut at this specific site. Cas9 is derived from *S. pyogenes* bacteria and is very prevalent in gene-editing lab technology.[2] A gRNA sequence will lead and target the Cas9 nuclease to the targeted site, and the Cas9 nuclease will make a double-stranded break in the genome. gRNA is composed of a short nucleotide sequence for the binding of the Cas9 enzyme and a protospacer, a 20-nucleotide sequence that defines the DNA target. The gRNA runs along the target genome DNA until it recognizes a protospacer adjacent motif (PAM), a three-nucleotide sequence, NGG, that directly neighbors the target gene sequence (N defined as any DNA nucleotide and G as a guanine nucleotide).[3] After binding the PAM and the protospacer, the Cas9 nuclease creates a straight double-stranded break in the genome which is repaired by one of two endogenous mechanisms. The more dominant mechanism is nonhomologous end joining (NHEJ), in which the break ends are directly ligated to fix the break. This mechanism is highly error prone, creating a random indel mutation (insertion-deletion) and is more beneficial in cases in which a deleterious mutated gene must be turned off or disabled, creating a "knockout."[4] The second and less understood mechanism is homology-directed repair (HDR), in which a template DNA strand is added and ligated in between the double stranded break. The mechanism is much less successful than NHEJ, but is useful in adding a beneficial or healthy gene to a genome, creating a "knock-in."[5]

Genome-editing techniques have been applied to developing embryos and adult organisms to produce loss-of-function mutations to deleterious genes and gain-of-function mutations to restore function of a protein in the cell. The CRISPR-Cas9 system is most often used in vitro, delivered to cells through traditional methods of electroporation or microinjection. However, for in vivo delivery of Cas9 and gRNA, viral vectors such as adeno-associated viruses and adenoviruses (AAV and AdV) and lentiviral vectors (LV) are most typically used.[6] The nature of most genome-editing technologies gives them potential to be effective in treating diseases that are caused by a single gene for which other medical treatments have been largely ineffective. The CRISPR-Cas9 system is generally used to explore

a specific disease-causing mutation in the human genome and alleviate the disease's effects by editing the deleterious mutated gene by either deleting it or by adding a functional portion of the gene to the genome.

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Coronary Heart Disease

Low-density lipoprotein cholesterol (LDL-C) has been established as a leading risk factor for worldwide cardiovascular disease mortality. Statin drugs have demonstrated effectiveness in reducing the risk of coronary heart disease by reducing LDL-C levels on the expense of common side effects often resulting in poor compliance. Normally functioning LDL receptors reduce cholesterol levels. *PCSK9*, a gene expressed in the liver and an antagonist to the LDL receptor, has emerged as a desired target for genome editing. Gain-of-function mutations in the gene have been shown to drive elevated LDL-C levels resulting in higher risk of hypercholesterolemia and coronary heart disease.[7] Studies on loss-of-function mutations in *PCSK9* show that a decreased amount of the gene caused no adverse clinical consequences but resulted in reduced LDL-C levels as well as a reduced risk for coronary heart disease.[8] This leads researchers to believe that gene therapy against *PCSK9* would serve to lower risk for cardiovascular disease.

Ding et al used adenovirus (AdV) to express Cas9 and gRNA targeting *PCSK9* in mouse liver to introduce a loss-of-function mutation in vivo. They successfully edited approximately 50% of *PCSK9* alleles with a range of 1-228 base pair indels with no significant off-target effects. Additionally, the gene therapy for the edited mice was able to reduce plasma *PCSK9* levels by approximately 90% and total plasma cholesterol levels approximately 35% to 40%.[9] Ran et al similarly targeted *PCSK9* in mouse liver using an AAV, a vector more suitable for human therapies, and found a successful knockout via NHEJ. This process was able to decrease blood PCSK9 levels by approximately 95% and decreased blood cholesterol by 40%.[10] These studies and others provide evidence that it is possible to lower risk of cardiovascular disease in patients by using somatic gene editing to decrease blood and plasma cholesterol levels.

Hypertrophic Cardiomyopathy

Autosomal dominant mutations on a single gene can express as late-onset adult disorders. A mutation in the *MYBPC3* gene as such accounts

for 40% of genetic defects causing hypertrophic cardiomyopathy, characterized by ventricular hypertrophy and abnormal myocardial relaxation leading to arrhythmias and diastolic heart failure.[11,12] Ma et al successfully corrected a heterozygous four base pair deletion in the *MYBCP3* gene using CRISPR-Cas9.[13] They microinjected recombinant Cas9 with gRNA from sperm from a male donor with a heterozygous *MYBCP3* mutation into healthy human embryos. By creating double-stranded breaks at the mutant paternal allele, they were able to use a homologous wild-type maternal gene to repair it. Of the 54 CRISPR-Cas9 injected embryos, 66.7% were uniformly homozygous for the normal wild-type allele while 9% showed to be uniformly heterozygous, and 24% revealed mosaicism (having blastomeres with both wildtype and mutant alleles). This data showed a higher proportion of homozygous healthy alleles over the 19 control embryos, of which 47.4% were homozygous and 52.6% were heterozygous. Mosaicism is attributed to the CRISPR system's failure to correct 100% of mutant genes after cell division. The researchers were further able to eliminate signs of mosaicism by injecting in M-phase oocytes (before the egg started dividing), with a higher proportion of homozygous embryos, 72.4%, and 27.6% uniformly heterozygous. Ma et al's research showed that the timing of injection of Cas9 significantly increases the efficiency of HDR (homology directed repair), which was previously thought to be highly unpredictable. This study further provides evidence that the CRISPR system through HDR can be used to correct cardiovascular disease-causing mutations in embryos or germline cells.

Wolff-Parkinson-White Syndrome

Mutations in the *PRKAG2* gene, which encodes the $\gamma 2$ regulatory subunit of AMP-activated protein kinase, results in an early-onset autosomal dominant inherited disease, PRKAG2 cardiac syndrome (PS). PS is characterized by cardiac hypertrophy, ventricular pre-excitation, and supraventricular arrhythmias.[14] Xie et al identified a H530R mutation in the *PRKAG2* gene in patients with familial Wolff-Parkinson-White syndrome and induced the mutation in transgenic mice, showing cardiac hypertrophy and abnormal glycogen storage, causally relating the mutation to PRKAG2 syndrome. They further combined an adeno-associated virus-9 with CRISPR-Cas9 to disrupt the mutated *PRKAG2* allele while leaving the wild-type allele untouched.[15] With a single systemic injection of the system in postnatal mice, they were able to restore normal cardiac morphology and function. Their results show drastic changes in morphology, including a significant reduction in heart size, decrease in the thickness of

the left ventricular wall, a ~70% decrease in myocardial glycogen content, and a reduction in the ejection fraction of the left ventricle. Furthermore, these changes led to a normalization of the shortened PR interval and broad QRS with the delta wave which are characteristic of Wolff-Parkinson-White syndrome, reducing the incidence of ventricular pre-excitation by ~40%. Their study shows how effective gene editing can be in treating arrhythmias linked to familial cardiovascular diseases.

Calmodulinopathic Long-QT Syndrome

Calmodulinopathies are a rare group of diseases caused by missense mutations in calmodulin protein, a Ca^{2+} sensor that is vital to cardiac function. Affected patients suffer from life-threatening arrhythmias associated with long QT syndrome (LQTS) including ventricular tachycardia and ventricular fibrillation. A heterozygous mutated *CALM1, CALM2,* or *CALM3* calmodulin gene accounts for all reported cases of LQTS-associated calmodulinopathies, meaning only a small amount of mutant protein causes the severe phenotype. The mutation causes drastic overexpression of the protein, causing the prolongation of the action potential. Limpitikul et al used CRISPRi, also known as CRISPR interference, to lower the levels of calmodulin protein overexpression. CRISPRi is a specific method of using the CRISPR system to alter gene expression with a dCas (dead Cas9) and a transcriptional suppressor or activator.[16] This system does not permanently edit the genome, but modifies gene expression with a Cas9 without endonuclease ability and the transcriptional modifier. Limpitikul et al derived induced pluripotent stem cells from a patient with a *CALM2* mutation, showing the cardiomyocytes exhibit LQTS phenotype. Using CRISPRi, they were able to significantly lower levels of calmodulin protein, thereby reducing duration of the action potential ($P < 0.001$ comparing treated versus untreated cells), diminishing the effect of LQTS.[17] Successful use of the CRISPRi system shows potential application to other cardiac diseases caused by overexpression or underexpression of certain genes.

Limitations

Despite great potential, concerns over safety and efficiency remain major obstacles in developing techniques for wider clinical use. First, the CRISPR system can create undesirable modifications to other areas of the genome that could increase risk of other diseases, namely cancer. Current advances in germline editing techniques ensure that unwanted changes are extremely rare. These off-target effects can also be detected prior to embryo

implantation. Second, CRISPR is still significantly more efficient in disabling rather than repairing genes i.e. it is better at knock-out (NHEJ) than knock-in (HDR). Knock-in editing efficiency of embryos, the proportion successfully altered, is variable. Currently improved efficiency minimizes the number of embryos that would need to be made available for targeting. This is especially important given that IVF produces a limited number of embryos and even fewer live births. Finally, mosaicism—a mixture of successfully edited cells and failed unedited cells—in the same embryo would fail to completely treat the disease and potentially cause undesired effects. However, Ma et al addresses all of these limitations in his research repairing *MYBCP3* mutations in embryos by essentially eliminating off-target effects, achieving a high rate of altered embryos (72.4%), and completely eliminating mosaicism by injecting embryos before they have a chance to replicate their DNA and divide.[13]

Conclusion

Overall, the CRISPR/Cas9 system shows promise in completely revolutionizing treatment of cardiovascular diseases and many other diseases. Current research offers potentially effective treatments using somatic and germline editing of disease-causing genes, however, clinical research does not go beyond applications in vitro and in small animal models. Largely due to ethical concerns, the CRISPR system has not been implemented legitimately in vivo in humans. For example, mosaicism is often a result of the use of CRISPR in embryos and cannot be risked in introducing to real human genomes. Germline genome editing has not been deemed to be safe and has not shown greater benefit over existing technologies, such as preimplantation genetic diagnosis. However, somatic genome editing provides a safe alternative because any edit made in the genome will not be passed to offspring. Furthermore, concerns are that genome editing will be used for non-therapeutic purposes in order to enhance certain traits in humans. The research could potentially be used to create babies, planned to the very gene, which creates ethical dilemmas on the essence of humanity. Reports of twin CRISPR babies developed by researcher He Jiankui has sent the scientific world and bioethicists in a scramble to determine morally acceptable boundaries. On the other hand, some bioethicists argue that it should be a moral imperative to use genome editing to cure disease, if it is shown to be safe. Public awareness and debate about gene editing must develop rapidly in order to make definitive decisions on the nature of this novel therapeutic approach and to launch medicine into the future.

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