Genome Editing for Cardiovascular Diseases—A Brief Review for Cardiologists

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The recent technical advances in genome engineering have accelerated our understanding of the molecular mechanisms of human diseases and are leading to increased clinical applications of gene-targeting therapies. The field of cardiovascular medicine, rich in knowledge of molecular level disease mechanisms, is particularly well positioned to receive significant benefits from this technology. Specifically, a new generation of genome editing tools capable of introducing targeted sequence modifications at high frequencies initiated by induced DNA double-strand breaks has been developed. Of note is the RNA-guided genome editing system, clustered regularly interspaced short palindromic repeats (CRISPR)-CRISPR associated protein 9 (Cas9), which has provided researchers and clinicians a malleable gene-targeting platform with high specificity. Recent reports have robustly demonstrated proof-of-concept in using CRISPR-Cas9 based gene therapy for treating common cardiovascular diseases and are testaments that the new genome editing technology holds promise for treating patients with cardiovascular ailments in the clinic in the near future. In light of this trend, a basic understanding of genome editing technology is becoming more relevant to clinical cardiologists. To this end, a concise explanation of terms and the biological basis of genome editing, on-going research, and clinical trials highly relevant to clinical application are presented. In conclusion, the aim of this short review is to introduce clinicians to the core concepts of current genome editing technology. **(Am J Cardiol 2019;123:1002−1006)**

Rapid advancements in genome editing technologies are moving the therapeutic gene correction of cardiovascular diseases from a theoretical possibility to a clinical reality. Cardiovascular medicine has been one of the leading forces in applying genomics-based research to further clinical therapies as exemplified by the recent development of proprotein convertase subtilisin/kexin type 9 (PCSK9) inhibitors.[1] Because the majority of hereditary cardiovascular diseases such as familial hypertrophic cardiomyopathy are caused by dominant mutations (1 pathogenic mutation overriding the remaining wild type [normal] gene),[2] if the pathogenic mutation is corrected, the disease manifestation could be ameliorated in an adult patient. If the correction is done at the stage of fertilization, as demonstrated by the recent proof-of-concept report,[3] the individual developing from that embryo would be free of the disease. Below, the recent genome editing technologies applied to the development of clinical therapies are discussed.

A Brief History of Genome Editing: Honing Genome Editing Tools Toward Gene Therapy

A breakthrough was made in the 1980s which paved the way to the development of gene targeting. It was the proof that mammalian cells are capable of mediating homologous recombination between externally introduced DNA and chromosomal DNA.[4] This meant that the intrinsic cellular machinery could be harnessed to introduce a specific sequence alteration in a chosen genome sequence. This original discovery was followed by a series of technical enhancements to improve the accuracy, frequency, and detection of homologous recombination targeted to a specific gene in cultured mammalian cells. Following this initial development, the use of mouse embryonic stem cells was incorporated, extending the gene-targeting approach to the whole organism, thereby enabling production of the knockout-mouse (sequence disruption) and knock-in mouse (sequence replacement),[4] all-important tools in genetic research. Gene targeting in mouse embryonic stem cells is now a standard approach, and its impact on biomedical research cannot be overstated because the biological functions of numerous genes critical to human health have been uncovered through this technology.

Homologous recombination-based gene-targeting technology, although significant as a research tool, has not been utilized in the development of clinical therapies mainly because of the complexity of gene-targeting design and the low efficiency of gene-targeting events. To resolve these issues and pave the way for clinical use of gene therapy, a new generation of genome editing tools, zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs), was developed in the mid-1990s and early 2000s.[5] ZFNs and TALENs are genetically engineered proteins capable of 2 functions: (1) recognizing a specific sequence and (2) introducing a double-strand break (DSB) at a defined target sequence. These systems are versatile because the sequence for gene targeting is determined by

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the amino acid sequence of ZF and TALE proteins which are engineered and programmable. Also introducing a DSB in chromosomal DNA improved the frequency of homologous recombination by several orders of magnitude.[5] This breakthrough allowed gene targeting to become a viable therapeutic option and led to several clinical trials which are yielding promising results for the treatment of cancer and infectious disease.[6] However, engineering a protein capable of precisely recognizing a specific DNA sequence remained a formidable challenge. This technical difficulty led to the development of the latest genome editing tool, the CRISPR-Cas9 system which was first tested on human cells in 2013.[7,8,9] Rather than a sequence-specific DNA binding protein—as is the case with the ZF and TALE proteins, the targeting specificity of the CRISPR-Cas9 system is achieved by engineering a short RNA sequence complementary to the DNA sequence of interest. As a result, this genome editing system is technically far simpler and less laborious for obtaining a desired sequence alteration at the target location.[10,11] Because of its relative technical ease, the CRISPR-Cas9 system has rapidly become the choice of genome editing for a wide range of applications, including the development of gene therapies, many of which are now in the early stage of clinical trials (Table 1).

The CRISPR-Cas9 Based Genome Editing: The Basic Components of the System and DNA Repair Pathways for Genome Editing

The CRISPR-Cas system was originally identified in bacteria as an adaptive immune system against viral infection.[12] Its mechanism of defense is to search for specific sequences in the invading pathogen's genome sequence using complementary RNA sequences (CRISPR RNAs) and to destroy the pathogen's DNA by introducing a DSB using the bacterial endonuclease Cas protein.[13] Because of the high targeting specificity and malleability of the system, the basic scheme of the bacterial CRISPR-Cas immune system has been exploited to develop RNA-guided programmable genome editing tools which are now utilized in a wide variety of cell types and organisms.[10] Importantly, in mammalian cells including humans, introduction of a DSB by the Cas protein induces DNA repair, and it is through this process that genome editing derives its practical use.

Table 1
Registered clinical trials involving treatment using CRISPR-Cas9 gene editing

Disease conditions	Targeted genes and intended outcome	Treatment/cell types	Phase	Identifier number
HIV-1 infection	CCR5; immunity to HIV infection	ex vivo, T helper cells	NA	NCT03164135
Human Papillomavirus (HPV)-related malignant neoplasm	HPV E6/E7; disruption of the HPV DNA in infected cervical epithelial cells	in vivo, external application of gel containing the CRISPR-Cas9 targeting construct	1	NCT03057912
β-thalassemia	BCL11A; re-expression of the fetal β-globin gene	ex vivo, hematopoietic stem cells	1/2	NCT03655678
	HBB; correction of β-thalassemia mutations	ex vivo, hematopoietic stem cells	1	NCT03728322
Sickle cell disease	BCL11A; re-expression of the fetal β-globin gene	ex vivo, hematopoietic stem cells	1/2	NTC03745287
Multiple myeloma, Melanoma, Synovial sarcoma, Myxoid/round cell liposarcoma	TCRα, TCRβ, PD-1; enhanced anti-tumor immune response	ex vivo, T cells targeting tumor antigen NY-ESO-1 (NYCE T cells)	1	NCT03399448
Solid tumor	PD-1; enhanced anti-tumor immune response	ex vivo, chimeric antigen receptor (CAR)-T cells	1	NCT03545815
	PD-1; enhanced anti-tumor immune response	ex vivo, CAR-T cells	1	NCT03747965
B cell leukemia/lymphoma	TCR, B2M; minimize immunogenicity of non-autologous CAR-T cells	ex vivo, universal CAR-T cells	1/2	NCT03166878
		ex vivo, universal CAR-T cells	1/2	NCT03398967
T cell leukemia/lymphoma	CD7; provide CAR-T cells immunity to CD7 antibodies used in therapy	ex vivo, CAR-T cells	1	NCT03690011
Esophageal cancer	PD-1; enhanced anti-tumor immune response	ex vivo, T cells	2	NCT03081715
Metastatic non-small cell lung cancer	PD-1; enhanced anti-tumor immune response	ex vivo, T cells	1	NCT02793856
Bladder cancer	PD-1; enhanced anti-tumor immune response	ex vivo, T cells	1	NCT02863913
Hormone refractory prostate cancer	PD-1; enhanced anti-tumor immune response	ex vivo, T cells	1	NCT02867345
Metastatic renal cell carcinoma	PD-1; enhanced anti-tumor immune response	ex vivo, T cells	1	NCT02867332
Epstein-Barr virus (EBV) positive advanced malignancies	PD-1; enhanced anti-tumor immune response	ex vivo, EBV-specific cytotoxic T-cells	1/2	NCT03044743

clinicaltrials.gov was accessed December 3, 2018. NYCE (NY-ESO-1 redirected CRISPR Edited) T cells and CAR-T cells are genetically engineered T cells to recognize specific cancer cell surface antigens.

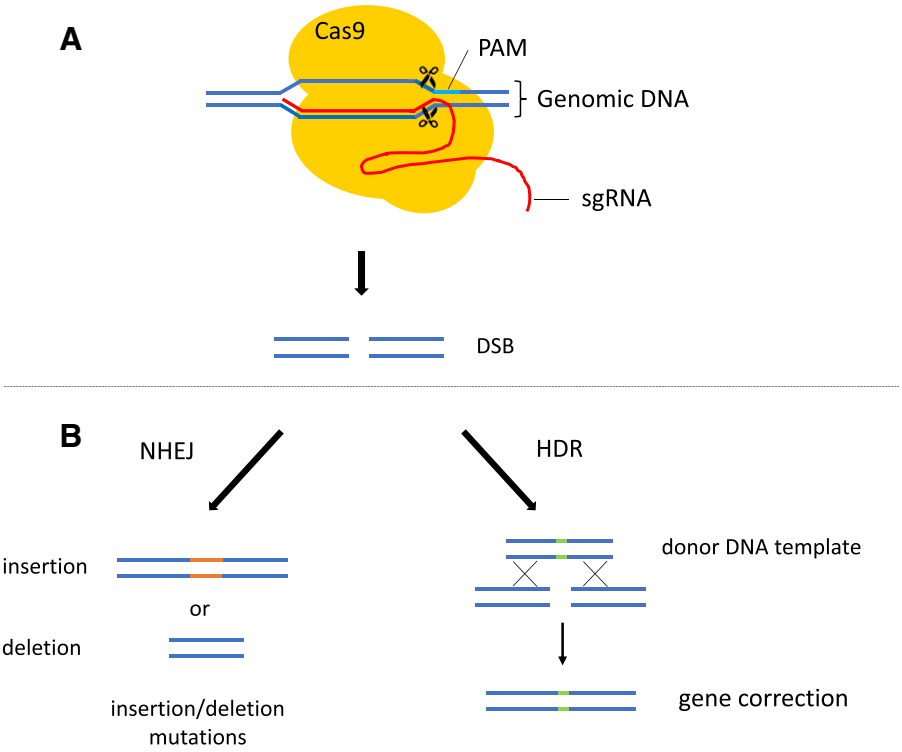

Figure 1. Schematic diagram of the CRISPR-Cas9 genome editing system. *(A)* Representative components of CRISPR-Cas9 with sgRNA. The Cas9 protein (yellow) generates a DSB in DNA (dark blue) at the target sequence (indicated by scissors). Recruitment of the Cas9 protein to the target site is achieved by a sgRNA (red). To initiate DNA cleavage at the target sequence, a short stretch of DNA sequence termed protospacer adjacent motif (PAM) (indicated in light blue) needs to exist immediately downstream to the target sequence. The PAM sequence is not part of the sgRNA but needs to be present in the genomic DNA which is being targeted. The PAM sequence is very short and present in the genome frequently enough to not pose serious technical challenges. Cas proteins derived from different bacterial strains have different PAM sequence requirements. In the case of *Streptococcus pyogenes* Cas9 (the most frequently used Cas9), PAM sequence requirement is NGG (N—any 1 of 4 nucleotides, adenine, guanine, thymidine, and cytosine, followed by 2 guanines). *(B)* Two repair mechanisms of a DNA double-strand break exploited by genome editing. NHEJ repair functions in the absence of a template and frequently introduces insertions or deletions as a result of repair. HDR functions in the presence of a repair template, copying the template sequence, and thus is highly accurate.

The most commonly used CRISPR-Cas genome editing system today is based on the CRISPR-Cas9 derived from *Streptococcus pyogenes*.[11] The currently available CRISPR-Cas9 system is streamlined to consist of 2 components: (1) a short RNA molecule to specify the target sequence (single guide RNA [sgRNA]) and (2) the Cas9 nuclease protein to introduce a DSB at the target sequence[10] (Figure 1A). The targeting specificity of genome editing can be readily managed by designing a short RNA sequence complementary to the target in the sgRNA, which then guides the Cas9 nuclease to the target DNA. Because of its flexibility in designing targets, the CRISPR-Cas9 system has become the primary choice of genome editing tools in recent years.[10]

Introduction of a DSB at the target sequence induced by CRISPR-Cas9 triggers DNA repair response within the cell. The outcome of genome editing—for example, generation of a mutation or correction of the existing pathogenic mutation—depends on the type of cellular repair mechanism subsequently employed by the cell to repair the broken DNA strand. Genome editing is, therefore, an event governed by probability, and a concerted effort has been made to increase the odds of generating the desired genome editing. Two types of DSB repair pathways are routinely exploited by the CRISPR-Cas9 system: the nonhomologous end joining (NHEJ) repair pathway and the homology-directed repair (HDR) pathway.[11] These 2 repair pathways produce very different outcomes and therefore are applied in different circumstances. NHEJ is best suited when gene knockout is the intended outcome. It is highly error-prone and often introduces small insertions, deletions, or substitutions of nucleotides at the break-point (Figure 1B). Therefore, it is ideal for gene knockout. In contrast to NHEJ, HDR repairs the broken DNA strand with high fidelity. This is because HDR fixes the disjointed DNA ends using a template and repairs the broken DNA sequence by faithfully replicating the template sequence (Figure 1B). Because of this fidelity, HDR is the desired repair mechanism when a precise sequence change is the intended outcome (e.g., correction of a deleterious mutation). One major limitation associated with this method is that the activity of HDR is limited to dividing cells[14]; therefore, this strategy is not readily applicable to fully differentiated cells in adult patients at this point in time.

Therapeutic Applications of CRISPR-Cas9 Genome Editing Technologies for Cardiovascular Diseases

Currently, therapeutic application of genome editing for adult somatic cells is rapidly advancing. Somatic genome editing aims at ameliorating the patient's pathogenic condition by genetically modifying a specific lineage of cells.

This type of genome editing can be performed indirectly, as ex vivo, or administered directly as in vivo. In the case of ex vivo genome editing, cells with regenerating capacity are derived from a patient, whose genome sequence is then modified in a culture dish. Once the desired genome sequence alteration is achieved, the genetically modified cells are expanded and transplanted back to the patient.[15] In the case of in vivo genome editing, components of the genome editing tools are delivered directly into the patient's system, usually by injection, and the somatic genome sequence is modified in situ at the targeted tissue site. For both protocols, the genome editing tools are most frequently delivered to the target cells using genetically engineered virus-based delivery vehicles.[16] Currently, the majority of clinical trials using CRISPR-Cas9 based treatment apply the ex vivo approach (Table 1).

In the case of cardiovascular disease, in vivo genome editing holds promises for developing therapies for dyslipidemias such as hypercholesterolemia.[17] As previously reported, naturally arising loss-of-function mutations of *PCSK9* in humans result in the reduction of blood low-density lipoprotein cholesterol levels, thus providing protection against coronary heart disease.[18] Therefore, if *PCSK9* is successfully disrupted in the liver, it should mimic the effect of these beneficial mutations. Several proof-of-concept studies have been conducted to test this premise using animal models. In earlier studies, *Pcsk9* in the adult mouse liver was targeted by CRISPR-Cas9 using virus-based vehicles delivered via injection.[19,20] In these mice highly efficient NHEJ-mediated *Pcsk9* knockout was observed, reducing serum levels of PCSK9 by ~90% and total cholesterol by ~40%. Subsequently, in an effort to increase the specificity of sequence editing, a Cas9 protein engineered to catalyze base pair substitution with no DSB (base editing) was used to target *Pcsk9* in the adult mouse liver, reducing serum levels of PCSK9 by >50% and total cholesterol by ~30%.[21] More recently, this base editing strategy was applied to target *Pcsk9* in the embryonic mouse liver in utero, also achieving significant reductions in postnatal serum levels of PCSK9 and total cholesterol.[22] Of note, *PCSK9* has been also successfully disrupted by NHEJ-mediated gene knockout in the adult rhesus macaque liver, achieving up to 60% reduction in serum low-density lipoprotein cholesterol levels.[23] Together, these reports provide a therapeutic basis for applying in vivo genome editing to treat hypercholesterolemia.

Genome editing is also applicable to the early stage embryo, specifically at the stage of zygote (1-cell stage embryo). The goal of embryonic genome editing is correction of deleterious mutations to avert pathogenic conditions from manifesting in life. Because the majority of hereditary cardiovascular diseases is caused by dominant mutations,[2] a single gene correction could prevent development of the disease. Hereditary cardiovascular diseases, therefore, provide an attractive model for testing the feasibility of gene therapy based on embryonic genome editing. In light of this concept, the efficacy of CRISPR-Cas9 based gene correction in human embryos was assessed using a myosin binding protein c3 (MYBPC3) mutation causing familial hypertrophic cardiomyopathy.[3] In this study, the pathogenic mutation was corrected in >40% of targeted embryos. Notably, the authors significantly reduced the formation of mosaic embryos containing both a corrected MYBPC3 gene and a mutant MYBPC3 gene, improving the therapeutic applicability of genome editing.[3] This report demonstrates the technical feasibility of germline genome editing of human embryos in a research setting; however, further discussion on ethical and safety issues will be necessary before its clinical realization.[24]

Hurdles for Genome Editing Technologies: Toward Clinical Application

One of the critical issues hindering the clinical application of CRISPR-Cas9 is its safety. It is a multilayered issue including undesired sequence changes in the patient's DNA,[25] immunological reaction to the virus-based delivery vehicles,[16] and unpredictable long-term effects of permanent genome editing. Extensive efforts have been made to overcome these issues. For example, to reduce unintended off-target sequence editing, the specificity of the CRISPR-Cas9 system components has been significantly refined by approaches such as genetically engineering the Cas9 protein[26,27] and optimizing sgRNA design for targeting.[28]

Another challenge is to utilize the HDR mediated mutation correction in nondividing cells, where the HDR repair pathway is inactive.[14] Currently, albeit limited, select sets of precise base substitutions have become possible in nondividing cells using a specially engineered Cas9 enzyme capable of base editing.[29] In addition, a CRISPR-Cas system which edits RNA sequences instead of DNA sequences (CRISPR-Cas13) has recently been developed.[30] The RNA editing CRISPR-Cas system not only can function in nondividing cells but also can circumvent long-term effects of permanent genomic DNA editing, because the presence of RNA is transient in nature. Taken together, the fidelity of CRISPR-Cas genome editing systems has improved significantly in recent years and their clinical application in a wide range of human pathogenic conditions is progressing rapidly.

Conclusions

The advancement of genome editing technology in the past 3 decades has led to the emergence of ZFNs, TALENs, and CRISPR-Cas systems setting the stage for gene targeting to become a realistic therapeutic strategy. Particularly, CRISPR-Cas9 is widely applied to develop gene therapies because of its streamlined targeting strategy. In cardiovascular medicine, there are robust presentations of proof-of-concept in using CRISPR-Cas9 to disrupt or correct the sequence of specific genes for disease treatment. The ultimate goal for the use of genome editing in medicine is to correct pathogenic mutations in humans to ameliorate or cure the diseases caused by genetic alterations. Considering the rate at which the technical hurdles confronting genome editing tools are being surmounted, this goal appears on the horizon.

Disclosures

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