Genome Editing Technology: A New Frontier for the Treatment and Prevention of Cardiovascular Diseases

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Abstract: Over the past 2 decades, genome-editing technique has proven to be a robust editing method that revolutionizes the field of biomedicine. At the genetic level, it can be efficiently utilized to generate various disease-resistance models to elucidate the mechanism of human diseases. It also develops an outstanding tool and enables the generation of genetically modified organisms for the treatment and prevention

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of various diseases. The versatile and novel clustered regularly interspaced short palindromic repeats (CRISPR/Cas9) system mitigates the challenges of various genome editing techniques such as zinc-finger nucleases, and transcription activator-like effector nucleases. For this reason, it has become a groundbreaking technology potentially employed to manipulate the desired gene of interest. Interestingly, this system has been broadly utilized due to its tremendous applications for treating and preventing tumors and various rare disorders; however, its applications for treating cardiovascular diseases (CVDs) remain in infancy. More recently, 2 newly developed genome editing techniques, such as base editing and prime editing, have further broadened the accuracy range to treat CVDs under consideration. Furthermore, recently emerged CRISPR tools have been potentially applied in vivo and in vitro to treat CVDs. To the best of our knowledge, we strongly enlightened the applications of the CRISPR/Cas9 system that opened a new window in the field of cardiovascular research and, in detail, discussed the challenges and limitations of CVDs. (Curr Probl Cardiol 2023;48:101692.)

Introduction

Cardiovascular diseases (CVDs) have become a serious health problem, with an increasing prevalence of common and noncommon causes of cardiovascular mortality and morbidity rate annually. These remain challenging to acquire a more profound understanding and cause substantial global economic loss.[1] In 2019, it was reported that CVDs showed a high mortality rate of about 18.6 million people worldwide.[2] CVDs comprise various medical consequences, including heart and blood vessels. These are principally categorized as cerebrovascular disease (stroke), congenital heart disease, coronary artery disease (CAD), rheumatic heart disease, hypertensive heart disease, peripheral artery disease, cardiomyopathies, and arrhythmias.[3] Over the past 40 years, the opportunity to achieve specified disease-causing mutations and gene therapy has been efficiently developed.[4] Moreover, the first clinical experiment based on cardiovascular gene therapy was the integration of a gene responsible for a VEGF into infected patients

suffering from peripheral arterial disease. Still, it did not become a potential treatment as various patients showed lesions. The first clinical trial of cardiovascular gene therapy was the delivery of the gene encoding for efficiency in successfully delivering the desired gene into the targeted site.[5]

Recently, the development of genome editing has revolutionized a powerful technique that catch-all definitions and elucidates various genome editing techniques (GETs) of targeting site-specific DNA alterations of a desired site in a living cell. In the late 1970s and 1980s, the first report of the targeted genomic changes emerged.[6,7] At first, the achievement in this area was mainly conceivable through significant outcomes related to cellular mechanisms involved in genome plasticity achieved by homologous recombination (HR) that permit the delivery of exogenous DNA into the desired genomic locus.[8] After a short time, it became feasible to subsequently improve the initiation of double-strand break (DSB) within the host genomic locus,[9] which is the fundamental idea in advanced GETs.[10] To generate, various endonuclease strategies have been efficiently developed that can direct a targeted genomic locus and cleaves a desired cut at a high-precision rate. When DSB is generated the cut is repaired by non-homologous end-joining (NHEJ) and homologous recombination repair (HDR) mechanisms.[11] In this review, we comprehensively survey the newly emerged clustered regularly interspaced short palindromic repeats (CRISPR/Cas9) technique and enlighten how this system can be efficiently recolonized in various medical research and disease control mechanisms from animal and human models linked for the treatment and therapeutic applications in the cardiovascular system.

CRISPR/Cas9 System as an Emerging Editing Tool

A crucial innovation in the genome editing system focuses on discovering CRISPR/Cas9 protein system in bacteria, which acquired the adaptive immune system from *Streptococcus pyogenes* against various bacteriophagic infections.[12] Cas9 facilitates antiphage activity with the combination of CRISPR loci due to its simplicity and adaptability. These genomic loci consist of short, repetitive sequences containing 30-40 bp interacted on the spacer sequence in the sgRNA, followed by a shorter sequence known as PAM.[13] In the current burgeoning era of genome editing, it plays a multifaceted role in various editing functions, such as in vivo and in vitro, for its wide variety of applications, including disease modeling, clinical trials of new therapeutic techniques, and identification of gene function.[14] Various newly emergent CRISPR/Cas tools, mainly

known as (Cas9 and Cas12a), are identified widely based on the presence of effectors proteins, serotypes, and subtypes; beyond these, Cas9 is the most widely and efficiently utilized protein fused with the CRISPR system.[15] This system depends on Watson-Crick base pairing models with an engineered RNA and the desired locus. First, this system recruits CRISPR RNA (crRNA) to bind and form a complex with Cas9 protein due to the availability of a conserved PAM sequence.[12,16] Secondly, this protospacer sequence is introduced into the 5′ end at the targeted site of the spacer sequence. When the foreign invaders link with the host genome, the host CRISPR transcription will occur, transcribing the precursor RNA and tracrRNA. After that, tracrRNA directly recruits the maturation of crRNAs through the activity of endogenous CRISPR/Cas9 and RNase III.[17] The hybridization of tracrRNA and crRNA integrated into the targeted sequences to form crRNA/tracrRNA complex, also known as sgRNA. The sgRNA signals the Cas9, which recruits split the large multifunctional protein into 2 domains, namely HNH and RuvC-like, to recognize the PAM sequence, efficiently target the gene of interest, and perform the cleavage action at the desired genomic locus.[18] Adjacent to a PAM site, recognition of the target sequence and cleavage events will occur, which mainly varies due to the presence of the Cas enzyme used. For example, *Streptococcus pyogenes* Cas9 (SpCas9) recognizes the 3′NGG sequence, while *Staphylococcus aureus* Cas9 (SaCas9) recognizes either 3′NNGRRT or NNGRR(N).[19] After Cas9 catalytically catalyzes, DSB can be repaired by HDR or NHEJ, which is mostly the same for all cell lines. The mechanism of NHEJ can generate error-prone deletion or insertion (indel) at the cleavage site, disrupting the translational initiation mechanism. On the contrary, HDR efficiently generates specified point mutations or the insertion of a targeted gene with the help of a donor DNA template.[20] as shown in Figure 1. Whereas the flow chart of the CRISPR/Cas9 system is depicted in Figure 2.

Generation of Newly Developed GETs

Base Editing (BE) System. BEs have been developed are categorized into 2 classes such as CBEs and ABEs, to target point mutation, which has the potential to catalyze C > T, T > C, A > G, and G > A transitions in the PAM sequence,[21] without the initiation of DSB or require exogenous DNA template. Owing to greater precision editing efficiency and low off-target effects, both BEs considered fascinating GETs and can

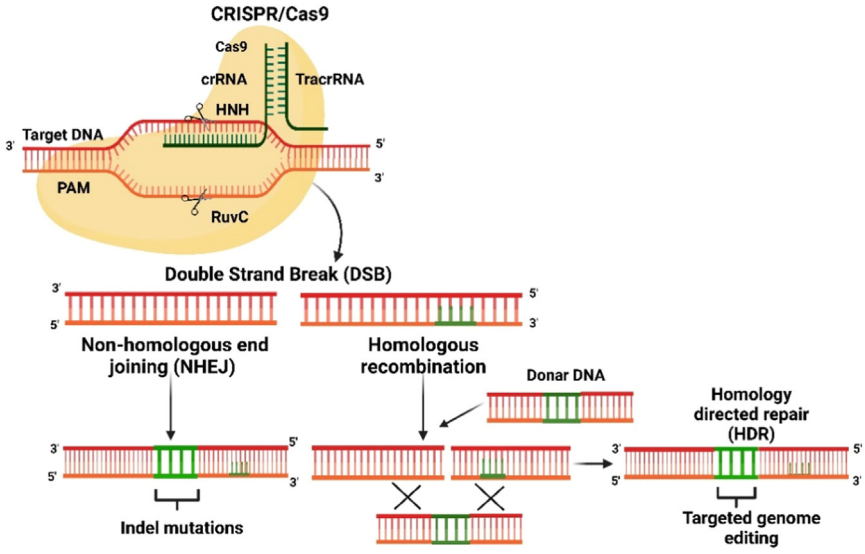

FIG 1. Mechanism of CRISPR/Cas9 by genome editing system. (Color version of figure is available online.)

proofread the single-nucleotide modifications considering various pathogenic conditions for humans.[22,23]

Mechanistically, CBE comprises a macromolecular complex in which catalytic impaired CRISPR/Cas9 directly fused uracil glycosylase

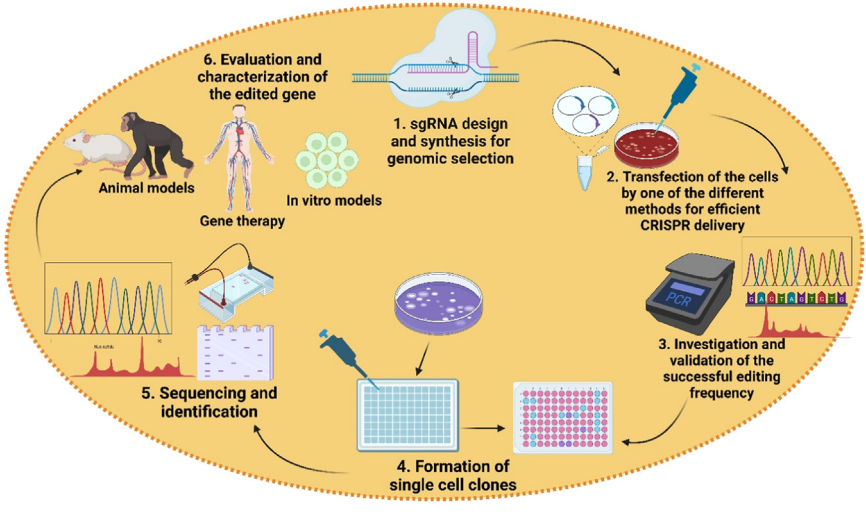

FIG 2. Diagrammatic representation of different steps of the CRISPR/Cas9 system. (Color version of figure is available online.)

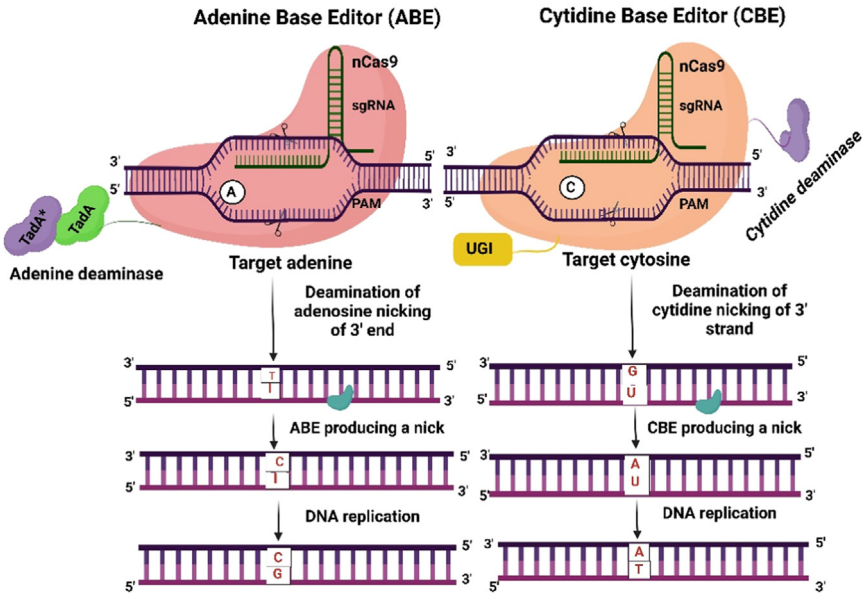

FIG 3. Mechanism of the base editing system. (Left) Adenine base editor, (Right) cytosine base editor. (Color version of figure is available online.)

inhibitor protein and an APOBEC1 deaminase enzyme. In CBEs, cytosine deaminases actively convert cytosines within the R-loop to uracils which, after the proofreading of DNA polymerase, read as thymines. In ABEs, TadA within the R-loop converts adenosines to inosines, which read as guanines after polymerase proofreading[24] as illustrated in Figure 3. Owing to the potential mechanisms, BEs can induce point mutations with low chances of undesired byproducts in contrast to CRISPR/Cas editing system.[22]

Prime Editing (PE) System. PE is the newest type of GET that allow the introduction of (12 base-pair transitions and transversions) point mutations, small insertions, and deletions without a DNA donor template or the initiation of DSBs.[25] PEs are the fusion proteins of Cas9 nickase that direct with reverse transcriptase and peg RNAs, containing a target sequence to recognize the binding site and a primer binding site (PBS). Recruitment and binding of DNA-pegRNA PBS and reverse transcriptase enzyme generate a low off-target ratio in the desired genome editing. To date, 3 generations of PE have been developed. First-generation PE (PE1) uses the model M-MLV RT, an RNA-dependent DNA polymerase, hybridizes to the C-terminal of an endonuclease Cas9 nickase (H840A).

When transversion mutations were integrated, the editing efficiency of PE1 improved from 0.7% to 5.5%, respectively.[23] The editing efficiency was further enhanced for second-generation PE (PE2), displaying 1.6-5.1-fold enhancement compared to PE1 by stimulating the desired genomic sequence.[25] For the PE3 editing system, all 12 types of point mutations (transition and transversions) improved the editing efficiency by 33% (7.9%), respectively. Prime editing system inhibited the alteration of introns/promoter regions more efficiently, thus permitting the replacement of alleles at the desired DNA site more efficiently. Interestingly, it is escalated that the editing efficiency of the PE system is much similar to the BE system; however, the efficiency and specificity were much higher compared to previously developed genome GETs.[26]

Therapeutic Applications of CRISPR/Cas9-based Genome Editing Systems for the Treatment of CVDs Models

CVDs are the primary cause of mortality death globally[27]; therefore, a deeper consideration is urgently required to mitigate the risk of severe diseases and to generate therapeutic strategies. Mechanistically, genome editing is a versatile approach for initiating or deleting site-specific point mutation to treat CVDs in vivo and in vitro, as shown in Figure 4. Consequently, the HDR editing system is constrained to the G2 and S phases of the cell cycle and post-mitotic division in different tissues, such as the heart. HDR is characteristically inefficient and poses a more significant bottleneck for CRISPR/Cas9 genome editing system.[28] Currently, Limptikul and his coworker's generation a mutation in Calmodulin 2 (*CALM2*) gene in the human-induced iPSC-CM cells, therefore reducing cardiac arrhythmias occurs due to the onset of long QT syndrome (LQTS).[29] Due to the increased risk of calmodulin-related diseases (calmodulinopathies) related to LQTS, mutational frequencies in *CALM1*, *CALM2*, and *CALM3* genes occurred. Similarly, under the dominance of malignant calmodulinopathic, LQTS is present in only 1 coded allele out of 6 alleles of calmodulin. Newly CRISPR developed tool such as the CRISPR intervention (CRISPRi) approach was utilized to edit the mutated allele. The CRISPRi technique used the nuclease-deficient Cas9 protein to stimulate the transcriptional and gene expression level. The hybridization of dCas9-sgRNA fused to the desired site of the targeted gene in the coded region restricts the transcriptional and elongational process and RNA polymerization activity.[30] Notably, this approach is attractive; the editing efficiencies of GETs for disease treatment are based on the versatility of the potential delivery approach of CRISPR/Cas9 into the infected

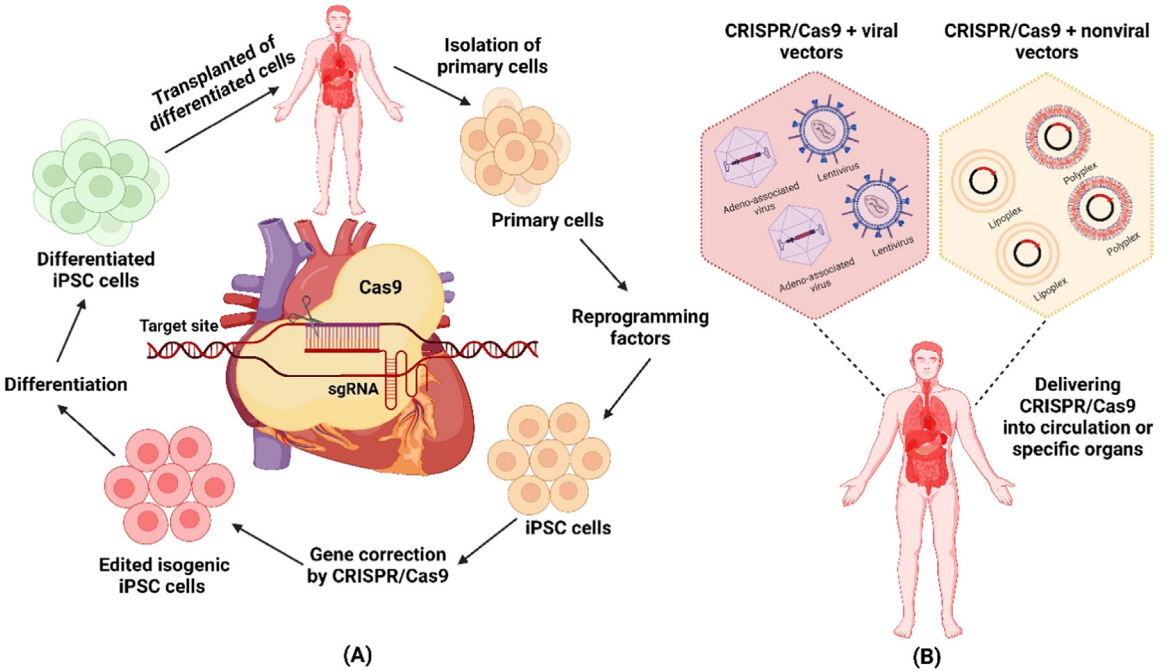

FIG 4. Therapeutic applications based on 2 genome editing approaches in CVDs. (A) Isolation of somatic cells from infected patients and then reprogrammed in iPSCs. The extracted iPSCs can be engineered in vitro, and after genome editing, they can be differentiated and transplanted back into the patient. (B) Site-specific point mutations can be edited in vivo to deliver the CRISPR/Cas complex directed at the targeted site of the specific cells or tissues. (Color version of figure is available online.)

patients. This could be accomplished either in vivo model by direct integration and binding CRISPR/Cas9 system into the targeted patient or in vitro by following the "patient-to-patient" strategy by engineering autologous cells and then transplanting them into the infected patient.[31]

This ground-breaking CRISPR/Cas9 technique can hybridize the targeted tissue and organs; however, it is challenging to hinder it to an edited targeted site potentially. Furthermore, various in vitro findings introduce CRISPR/Cas9 tools by utilizing AAVs, but the packaging capacity of AAVs is hindered to multiple organs.[32]

In Vitro Model for CRISPR/Cas9 System

Recently, tremendous advancements in the technique have been made to generate hiPSC-CM from patients, straightening out a new opportunity to treat CVDs. In vitro, experimental findings are widely utilized owing to comparatively easy, high precision rate, greater adaptability, species specificity, and high specificity.[33] For further functional analysis, these cells are differentiated into various cells. More information on the dominance of cardiomyopathy, such as Barth syndrome caused by (mitochondrial dysfunction) occurring by the tafazzin (*TAZ*) gene mutations, has been characterized through zinc-finger nucleases, transcription activator-like effector nucleases, or CRISPR/Cas9 system.[31,34] Moreover, Zhang and his correspondence observed CRISPR/Cas9 attrition of microRNAs unrevealed their editing efficiencies during mouse embryonic stem cells. Similarly, miRNA106a, miR17, and miR93 hybridize the suppressor *Fog2* gene, a multi-zinc finger protein linked with a transcription factor GATA-4, as GATA-4 is essential for the normal functioning of the heart and hypertrophic responses in cardiac functions.[35] Furthermore, CRISPR/Cas9 approach was feasible to edit mutations in the *DMD* gene by deleting exons through the exon skipping process. Further considerations to prove this concept, myoediting was achieved in iPSC cells from various infected patients with point mutations, deletions, and duplications with the *DMD* gene's targeted sequence, potentially introducing dystrophin protein expression in cardiomyocyte derivatives. In 3D engineering heart muscle (EHM), mutations in the *DMD* gene modified the expression of dystrophin and its contraction force during myoediting. Researchers investigated that introducing RNA splicing donor/acceptor binding sites and the direction of splicing control to delete exogenous exons by myoediting permitted the improvement of *DMD*-related CVDs.[36] The efficient delivery of transfection components through CRISPR/Cas9 cargo to targeted hiPSCs may also be a bottleneck; up to date, nucleofection

demonstrated one of the most robust approaches efficiently utilized in delivery method. Other useful approaches also showed the efficiency of single-cell cloning method. Indeed, the low editing efficiency in targeting hiPSCs is mainly because of the less cell viability after manipulation. Through nucleofection, hiPSCs are placed at a single-cell density by decreasing single-cell sorting or dilution, which primarily governs reducing a portion of transfected cells, decreasing the frequency of recognizing edited clones. Until recently, the above-discussed circumstances have been standardized and thus influencing the potential use of the CRISPR/Cas9 system to generate hiPSC isogenic cell lines. Mechanistically, these strategies, single or combined, govern the characterization and identification of robust features related to dilated cardiomyopathy, hypertrophic cardiomyopathy, arrhythmogenic cardiomyopathy, Noonan syndrome, PRKAG2 cardiac syndrome, and many other CVDs.[37-41]

Furthermore, CRISPR/Cas9 has been widely utilized to develop in vitro allele-specific knockouts of CVDs, including channelopathy long QT syndrome (LQTS).[42] CVDs are frequently linked with hiPSCCMs and electrical abnormalities to handicap these modifications.[43] Likewise, CVDs are strongly connected to mechanical improvements. The recently developed methodologies to identify and measure mechanical force, such as optical cell stretcher,[44] optical flow-based displacement analysis,[45] as well as atomic force microscopic analysis,[46] in association with GETs for the successful generation of robust disease moieties may facilitate to acquire of deeper apprehension of the phenotypes analyzed in vitro, highlighting on the stress-strain interrelation and many other beneficial disciplines. Controversially, the recent newest GETs, such as base editing and prime editing effectively utilized to deliver phenotypic changes in vitro for the treatment of cardiac diseases. Chemello and his teammates experimentally examined the editing efficiencies of both BEs and PEs to rejuvenate the dystrophin protein expression in iPSC-CMs by enhancing exon skipping for the deletion of 51 exonic mutations in the *DMD* gene. Deleting exon mainly forms a premature stop codon in 52 exon and displays a non-functional truncated dystrophin protein. Following the iPSC-CM model, this finding developed a hiPSC isogenic line by deleting 51 exon in the *DMD* gene through CRISPR/Cas9.[47]

In addition, the PE system was effectively displayed as a strand-in correction strategy for successful genome editing. This system was handy to employ through nucleofection to reexamine the correct open reading frame (ORF) of 52 exonic regions and also observed the potential correction of dystrophin expression in altered hiPSC-CMs lines.[47]

In Vivo Model for CRISPR/Cas9 System

GETs have been robustly utilized to study in vivo strategies to generate disease model organisms and to testify to the medical consequences of the risk of severe diseases such as CVDs. Until now, recently CRISPR/Cas9 is a versatile genome editing technique that is efficiently utilized to generate in vivo models for treating CVDs. In the meantime, BE and PE systems are still in their earlier developmental stages; for some in vivo studies, their editing efficiencies are frequently higher than reported in some previously reported literature.[48,49] CRISPR/Cas9 genome editing tool has been successfully employed in different model organisms such as rats, mice, zebrafish, and large animals like pigs to check and accelerate its editing efficiency. Some site-specific mutations lead to caused cardiomyopathies such as long-QT syndrome, Duchenne muscular dystrophy (DMD), Barth syndrome, and hypertrophic cardiomyopathy (HCM) have been successfully engineered through GETs in patients specified iPSC-derived cardiomyocytes.[50-52]

The in vivo mechanism requires the correction of different diseases to isolate primary cells. Recently, an endonuclease Cas9 has been reported that derives CRISPR/Cas9 through an adeno-associated viral vector.[53] Moreover, CRISPR/Cas9 is employed to correct the mutation that occurred through large deletions in vivo, including Duchenne muscular dystrophy (DMD). El Refaey and his team mates targeted exon 23 of the *DMD* gene through SaCas9 owing to its smaller size and greater packaging capacity for sgRNA into AAVrh74 and actively integrated into the body of neonatal mice. These findings facilitated a partial rehabilitation due to the expression of dystrophin protein in the structural restoration of cardiac myofiber and cardiomyocytes with a reduced effect on the fibrotic region in the heart. The outcomes exhibited the potential in vivo therapeutic application by CRISPR/Cas9 and structural and mechanical rehabilitation of cardiomyopathy linked with DMD.[54] Furthermore, drug resistance remains a significant bottleneck that circumvents the treatment of proprotein convertase subtilisin/kexin type 9 (*PCSK9*) 2-overexpressed low the efficiency of low-density lipoprotein. Through CRISPR/Cas9, Ding and his coauthors observed a loss of gene function for the *PCSK9* gene in mice's liver, reducing cholesterol levels by 40%.[55] Another finding inhibiting the function of various genes such as low-density lipoprotein receptor, apolipoprotein E (ApoE), leptin receptor, ryanodine receptor type 2 (RyR2), and a cluster of differentiation 36 (CD36) through the action of endonuclease Cas9.[56] In mice, a DMD improved skeletal muscle

function 4 weeks after integrating IMadeno-associated virus9 (AAV9)/Cas9.[57]

The CRISPR/Cas9 tool can be effectively utilized for in vivo editing in somatic cells. Suppression of the *PCSK9* gene through the CRISPR/Cas9 technique showed decreased blood cholesterol levels and lowered risk of coronary heart disease (CHD).[58]

Zebrafish have become popular compared to mice as a model organism to test the onset of heart disease due to their significant ability to carry genetic analysis. Introducing an excess amount of oxygen during the 1-week interval of its developmental stage is not a constraint in zebrafish as it belongs to other model organisms; thus, it allows the detailed study of cardiovascular abnormalities that would lead to fetal death. Zebrafish heart possesses strong regeneration capability not observed in other organisms, such as rodents.[59] Many scientists reported the successful editing efficiency of various CRISPR/Cas9 systems in zebrafish.[60]

More recently, BE system has also been reported as an excellent GET for treating DMD in mice. Xu and his correspondence successfully edited the correction in DMD with an altered ABE (iABE-NGA), hybridized by a modified NG PAM-linking domain that involves the availability of a PAM site near the targeted mutation site, in addition to exhibiting on-target increased DNA binding specificity and activity. The researchers monitoring a complete dystrophin restoration ranged from (95.9±1.6%), respectively, in the treated mdx4cv mice heart at ten months of age, which is displayed and achieved the T-to-C editing frequencies 84.6% ± 2.6%, respectively.[61] An excellent report recently represented the potential utilizing of BE system for the target site correction of disease-causing mutations in utero, inhibiting the risk of various pathological diseases. In utero, the AAV9-mediated intravascular delivery of ABE potentially corrected nonsense in mutation in the *Idua* gene in a mouse of mucopolysaccharidosis type I (MPS-IH), characterized as lysosomal storage that affects various organs, more specifically, heart. In utero-treated mice displayed an improved IDUA activity and echocardiographic function as well as decreased cardiac lysosomal accumulation of glycosaminoglycans as contrasted with controlling and found editing efficiency ranged from (13.9% ± 0.8%), respectively in mice.[62]

Another study demonstrated by Koblan and his coworkers evaluated the in vivo BE system by using ABE to target and delete the point mutation in the lamin A (*LMNA*) gene related to Hutchinson-Gilford progeria syndrome (HGPS). The targeted mutation initiates the mis-splicing of RNA, facilitating progerin synthesis. This highly toxic protein speeds up aging in children and causes death early, mainly followed by CVD.

The ABE system was efficiently extracted from children suffering from this disease and in mice with a mutation in the human *LMNA* gene. AAV9 viral vector with dual activity was utilized to convert ABE into progeria-relevant tissues, such as heart and muscle cells in mice, eventually bringing human *LMNA* gene mutation. The editing frequency was achieved up to 20%-60%, giving back to the normal splicing condition and inhibiting the activity of progerin protein, and significantly enhancing the onset of vascular disease, improving the lifecycle of ABE-engineered mice by 2.4-fold as contrasted to the control group.[63]

Current Challenges and Limitations of Genome Editors (GEs)

Protection is the prime challenge for the applications of genome editing therapy in humans.[64,65] The antagonistic effects shared with different gene therapies include immune response, drug-delivery components, toxicity, and GEs therapies. This brings the editing off-target effects and produces *de novo* mutations at the untargeted genomic site. Even though the newly emerged CRISPR techniques that do not introduce DSBs mitigate the chances of off-target mutations, for this BEs have opened a new window that generates off-target modifications without the availability of Cas protein as compared to the utility of conventional Cas9 endonucleases.[66-68] Consequently, the off-target mutation is still a significant problem due to the generation of undesirable phenotypic changes. In the heart, off-target effects can lead to severe arrhythmic problems; even if the mutation is small in cardiac cells, governing GEs for cardiac cells and tissues remains a significant bottleneck.

Recently, there has been no profound strategy to envision off-target mutagenesis before editing genomic sequences in patients. Distinct from model organisms like rats and mice, every patient has a diverse genetic makeup and potential off-target editing sites. Off-target editing also relies on cell and type types because epigenetic changes alter genome editors' availability to chromatin modeling. Furthermore, off-target effects generated from CRISPR-based tools could significantly change from those generated from Cas9 endonuclease.[66-68]

Another bottleneck to circumvent the therapeutic effect in GE is the unavailability of efficient and robust delivery cargos. Viral vectors, specifically AAV, are recently the source of delivering GEs to the targeted heart tissues. Eleven serotypes are present, and more than >100 AAV variants with various amino acid sequences are inside the capsid.[69] The significant challenge AAV vectors pose is their limited packaging

capacity of up to (4.7 kb). For Cas9, the coding sequence is 4.2 kb, whereas various Cas endonucleases are characterized owing to the exact size and limited space to package sgRNAs or other packaging components. One option to hinder this bottleneck is to divide Cas9 into 2 AAV vectors.[61,70,71] Engineered Cas9 endonucleases such as (CjCas9, Cas13bt, NmeCas9, Cas12b, and Casx) that are feasible for packaging AAV vectors are under consideration.[19,72-75]

Conclusions

Recently, structurally and mechanically reported pieces of literature on the emergence of CRISPR/Cas9 GETs and their potential and functional applications in vivo, in vitro, and humans have opened a new way for therapeutic applications in treating and preventing CVDs. To date, GE has become a ground-breaking technology for engineering cells and organisms and examining the biological and pathophysiological changes due to the onset of various genetic disorders. Moreover, another challenge is presented by the ethical alarm about using the CRISPR/Cas9 technique in human lines. Rapid progression in the field of GE permits us better to consider various diseases' pathogenicity and development behavior and prevent and cure CVDs. The emergence of modern GETs is handy to encounter the routine practice of this tool for effective therapy in CVDs. Clinical challenges of these GETs include the incapability to envisage off-target mutations and inadequate delivery methods, specifically for the targeted cells, tissues, vessels, and endothelial linings of heart muscles.

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