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Therapeutic Genome Editing in Cardiovascular Diseases

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Abstract

During the past decade, developments in genome editing technology have fundamentally transformed biomedical research. In particular, the CRISPR/Cas9 system has been extensively applied because of its simplicity and ability to alter genomic sequences within living organisms, and an ever increasing number of CRISPR/Cas9-based molecular tools are being developed for a wide variety of applications. While genome editing tools have been used for many aspects of biological research, they also have enormous potential to be used for genome editing therapy to treat a broad range of diseases. For some hematopoietic diseases, clinical trials of therapeutic genome editing with CRISPR/Cas9 are already starting phase I. In the cardiovascular field, genome editing tools have been utilized to understand the mechanisms of diseases such as cardiomyopathy, arrythmia, and lipid metabolism, which now open the door to therapeutic genome editing. Currently, therapeutic genome editing in the cardiovascular field is centered on liver-targeting strategies to reduce cardiovascular risks. Targeting the heart is more challenging. In this review, we discuss the potential applications, recent advances, and current limitations of therapeutic genome editing in the cardiovascular field.

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1. Introduction

The development of genome editing technology was one of the biggest advances in biology in the last decade, and it has revolutionized biomedical research [1-4]. Compared to methods using conventional homologous recombination, which also can modify genomic sequences and are commonly used to generate genetic models, the new genome editing tools have made it much easier and faster to alter genomic DNA sequences within the genome of living organisms. Basically, the genome editing tools harness the endogenous repair process of DNA double-strand breaks (DSBs) generated by programmable nucleases such as zinc finger nucleases (ZFNs) [5],

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transcription activator-like effector nucleases (TALENs) [6, 7], and clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated 9 (Cas9) [2-4] nucleases. In particular, the key initial technological breakthrough was the application of CRISPR/Cas9 system, which was originally discovered as a part of the immune system within bacteria, to eukaryotic genome editing [8]. Because of their simplicity and efficiency, CRISPR/Cas9 genome editing tools have been widely adopted in various scientific fields.

There are mainly two ways of applying genome editing technology to biomedical research [2-4]. One is to apply genome editing as a research tool to study biological mechanisms and disease pathophysiology. The other is to apply genome editing as a therapeutic tool to treat or prevent diseases [1, 9-11] (Figure 1).

First, genome editing technology has served as a research tool for a wide variety of purposes [2-4]. For example, it dramatically accelerated the generation of genetic animal models and cellular models such as knockout and knock-in mouse models. The traditional methods to make knockout mice typically involve homologous recombination in mouse embryonic stem cells (ESCs), requiring colony selection in ESCs, injection of modified ESCs to mouse embryos, and breeding of chimeric mice. This process is time consuming, taking months to years to obtain knockout mice. By contrast, CRISPR/Cas9 genome editors can be injected directly to single-cell mouse embryos to disrupt a gene, taking only several weeks to yield knockout mice [12, 13]. Furthermore, CRISPR/Cas9 tools make it possible to generate genetic models of other species, including non-human primates, which had not been possible before [14-16]. Another example is the creation of isogenic cell lines from patient-derived induced pluripotent stem cells (iPSCs) [17-23]. Human iPSCs are an attractive platform to study genetic diseases because iPSCs can mimic the genetics of the patients of origin and be differentiated to any desired cell type in

principle. A number of studies have shown that patient-derived iPSCs can recapitulate disease phenotypes on a dish [24-48]. However, unlike model organisms such as mice and rats, patient-derived iPSCs have a wide variety of genetic background. The process of generating iPSCs from patients' somatic cells also results in variation among iPSC lines affecting epigenetics, pluripotency, and differentiation capability [49]. Thus, a simple comparison between disease iPSCs and healthy iPSCs may not be ideal to study the impact of a particular genetic locus on the disease phonotypes. To eliminate these complications caused by genetic variation in iPSCs, generating isogenic iPSC lines that differ only at the locus of interest from the original line would be ideal. Other examples in this category include generation of reporter cell lines, imaging of genetic loci, unbiased pooled genetic screening, *in vivo* lineage tracing, etc [4, 50-53].

Second, the development of genome editing tools raises the possibility of therapeutic genome editing in diseased cells or tissues that aims to remove or correct harmful mutations or to introduce protective modifications to patients' genome [1-4, 9-11]. The candidate diseases of therapeutic genome editing include both rare monogenic diseases and common diseases. To treat monogenic diseases such as hemophilia, sickle cell disease, and Duchenne muscular dystrophy (DMD), pathogenic mutations in the disease-causing genes (e.g., F9 for hemophilia, HBB for sickle cell disease, and dystrophin for DMD) would need to be removed or corrected via gene knockout or knock-in. To treat common multifactorial diseases such as dyslipidemia and HIV infection, beneficial variants or protective modifications would be introduced to non-causal genes of the diseases (e.g., PCSK9 for dyslipidemia, and CCR5 for HIV). Theoretically, there are several ways to use CRISPR/Cas9 genome editing for patients, including *in vivo* genome editing, *ex vivo* genome editing, and germline genome editing [1, 4, 9]. However, genome editing in germline cells or embryos has considerable ethical issues regarding hereditability even though it

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would be a powerful approach for severe congenital diseases [1, 54]. More feasible from an ethical viewpoint are *in vivo* and *ex vivo* genome editing of somatic cells. The *ex vivo* strategy of editing and re-implanting is an easier way to apply genome editing to patients than *in vivo* genome editing. This strategy has been studied especially in blood cells because *ex vivo* culture of blood cells is similar to *in vitro* cell culture, and gene delivery to blood cells is easier than delivery to solid tissues. In *ex vivo* editing therapy of blood cells, hematopoietic stem cells (HSCs) are collected from a patient, edited and then re-engrafted to the patient of origin because HSCs can survive outside of the body and home back to the bone marrow after transplantation [1, 55]. For a handful of diseases, clinical trials of therapeutic genome editing with CRISPR/Cas9 tools are already entering phase I. The first example is *ex vivo* genome editing for β-thalassemia and sickle cell disease using *Streptococcus pyogenes* Cas9 (SpCas9) [56]. Another example is *in vivo* genome editing in the retina for Type 10 Leber congenital amaurosis using *Staphylococcus aureus* Cas9 (SaCas9) delivered by adeno-associated virus (AAV) [57]. In addition, several clinical trials of chimeric antigen receptor T-cell (CAR-T) therapy combined with *ex vivo* CRISPR/Cas9 genome editing (e.g., knockout of checkpoint inhibitor PD-1) are also underway [58].

In the cardiovascular field, genome editing tools have been used both as a research tool and a therapeutic tool, but more intensively as a research tool [9, 10]. To study the pathophysiology of genetic diseases, various models have been generated. For example, patient-derived iPSCs and their isogenic control lines have served as a powerful platform to study familial hypertrophic cardiomyopathy (HCM), familial dilated cardiomyopathy (DCM), long QT syndrome (LQTS), laminopathy, and others [26, 29, 32, 34, 35, 37, 38, 41, 42, 44-48]. There are also many examples of genetic animal models generated with genome editing tools, including

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cardiomyopathy, hypertension, electrophysiology, and dyslipidemia models [59-63]. By contrast, the studies of therapeutic genome editing in cardiovascular field have been basically limited to those of liver, especially those targeting Proprotein convertase subtilisin–kexin type 9 (PCSK9) for dyslipidemia [64-69]. Although there are many cardiac diseases that could be cured in principle by editing disease-causing mutations as shown in iPSC disease modeling studies, currently targeting the heart is still challenging even in experimental models. In this review, we focus on therapeutic genome editing in the cardiovascular field, mainly that with CRISPR/Cas9-based tools. We discuss potential applications of genome editing for therapeutic purposes, advances in therapeutic genome editing, and current hurdles to realize genome editing in this field.

2. Potential target diseases of genome editing in the cardiovascular field

There are two types of cardiovascular diseases that can be treated or prevented by genome editing [1, 9]. The first type is those caused by responsible genomic regions in the heart or vessels. Many inherited or *de novo* genetic disorders in cardiovascular tissues are included in this type. Examples of heart diseases in this type are HCM, DCM, LQTS, and muscular dystrophies that cause cardiac dysfunction. Examples of vascular diseases are Marfan syndrome and familial pulmonary hypertension (Figure 2). Although these diseases are potential candidates of therapeutic genome editing in principle, it is not technically easy to target primary cardiovascular tissues by current genome editing technology because of the inefficiency of *in vivo* editing and lack of efficient delivery methods, as we will discuss later. Germline editing might be more helpful for these diseases than *in vivo* editing, but it has ethical issues to be solved. Another type is those related to cardiovascular risk factors such as dyslipidemia. For this type of diseases, non-

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cardiovascular tissues such as liver or blood cells are targets of genome editing. Currently, the liver is the organ that has been most intensively studied for therapeutic genome editing of cardiovascular diseases because of the feasibility [64-71]. For blood cells, an *ex vivo* strategy might be applicable to immune cells to improve chronic inflammation for the prevention of atherosclerosis [72, 73].

3. Molecular mechanisms and strategies of genome editing to treat or prevent diseases

3.1 Non-homologous end-joining (NHEJ) and homology-directed repair (HDR)

Most genome editing tools, including ZFN, TALENs, CRISPR/Cas9 and others, depend on endogenous DNA repair processes [1-4, 74]. Once an initial DSB is introduced by genome editors, the host cell activates endogenous DNA repair pathways to fix the damage. There are two main types of repair processes, non-homologous end-joining (NHEJ) and homology-directed repair (HDR) (Figure 3).

In the absence of a repair DNA template, DSBs are repaired by the NHEJ process [74]. In NHEJ, the ends of DSBs are directly ligated by endogenous repair machinery. However, NHEJ is error-prone and frequently results in random insertion/deletion (indel) mutations at the site of junction. If an indel occurs within the coding sequence of a gene, it can induce a frameshift or make a premature stop codon to result in gene knockout. NHEJ is a predominant repair process in most known mammalian cell types which is active throughout the cell cycle. Thus, NHEJ genome editing can be utilized to induce gene knockout in most cell types [1-4, 74].

In the presence of a repair DNA template, DSBs can be fixed through the HDR process [74]. Although HDR occurs at lower frequencies than NHEJ, it can generate precise modifications that can be defined by an exogenously introduced repair template. The repair template can be either

in the form of double-stranded DNA with homology arms flanking the insertion sequence like conventional homologous recombination methods, or single-stranded DNA oligonucleotides (ssODNs). In terms of cell cycle phases, HDR is active only in the S and G2 phases, unlike NHEJ, and its efficiency depends on the cell type and state. Thus, HDR can be utilized mainly in dividing cells to induce knock-in of a specific DNA sequence, whereas HDR generally occurs at a low frequency in non-dividing cells such as cardiomyocytes, which is often outcompeted by alternative repair pathways including NHEJ [1-4, 74].

Based on these two primary types of repair process, there are three main strategies to alter genomic DNA sequences to prevent or treat a disease: disruption of a gene, deletion of a specific genomic region, and correction of a gene [1-4, 9].

3.2 Disruption of a gene

The simplest way to prevent or treat a disease by genome editing is to disrupt a gene that is harmful or whose inhibition is protective (Figure 3). For this purpose, NHEJ can be used to induce disruption within a gene. A promising example of this approach is to reduce blood cholesterol levels by targeting PCSK9. Based on the observation that loss-of-function mutations in PCSK9 were associated with 15-28% reduction of low-density lipoprotein (LDL) cholesterol level and 47-88% reduction in the risk of coronary heart diseases, PCSK9-targetting therapies have been developed to lower LDL cholesterol level in patients with persistently high LDL cholesterol levels even on statin [75]. Recently approved monoclonal antibodies against PCSK9 that can produce substantial reductions in LDL cholesterol when administered alone or with a statin require periodic administration. Thus, permanent disruption of PCSK9 by genome editing would be a promising approach because loss-of-function mutations of PCSK9 do not cause any apparent adverse phenotypes. Several studies show that mouse PCSK9 can be targeted by NHEJ-

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mediated disruption to reduce LDL cholesterol using CRISPR/Cas9 that is virally delivered to the liver with high efficiency [64-69].

3.3 Deletion of a genomic region

The deletion of a specific genomic region can be also induced by NHEJ-mediated genome editing using two guide RNAs (gRNAs) flanking the locus to be deleted (Figure 3). In case a disease-causing mutation generates a frameshift, a premature stop codon, or an ectopic splicing site, deletion of the genomic regions around the mutation may restore the function of the gene at the protein level. A good example of this approach is the "exon skipping" strategy for Duchenne muscular dystrophy (DMD) [76]. DMD is an X-linked recessive muscle-wasting disease that is one of the most prevalent fatal genetic diseases (1:5000 male births). The responsible gene for DMD is dystrophin, which is a large cytoskeletal structural protein essential for muscle membrane stability. The mutations are often deletions of one or more exons that disrupt the reading frame of the dystrophin gene and result in a complete loss of functional dystrophin expression. Patients affected by DMD are usually diagnosed in childhood and abnormal cardiac function is detected in early teens. Patients usually die in their 20s from heart failure or respiratory failure. Although gene therapy is a promising option to treat DMD, gene delivery of intact dystrophin is challenging because of the large size of dystrophin coding sequence. Fortunately, the exon-skipping strategy against truncated dystrophin has been shown to restore most of the functional protein by fixing the reading frame [76-78]. It is reported that exon skipping could be a therapeutic option for 75% of DMD patients [76-78]. For the most common type of DMD with exon 51 mutation, oligonucleotide-mediated exon-skipping drugs are already approved by FDA. Thus, permanent exon-skipping by genome editing could be a promising therapy for severe DMD patients because genome editing can target any exons theoretically.

Several studies have shown that *in vivo* genome editing in a mouse model restored functional dystrophin protein by exon skipping [79-85]. They used an AAV virus to deliver CRISPR/Cas9 to the skeletal muscle of *mdx* mice that have premature stop codon in exon 23. Although the editing efficiency was only 2-3% in skeletal muscle, they showed functional recovery in muscle power after genome editing. The same approach was shown to work in a canine model [86]. Nevertheless, the exact efficacy of genome editing in the heart in these models is still uncertain. Whether exon skipping by genome editing can prevent heart failure, the most common cause of death in DMD patients, remains to be demonstrated.

3.4 Gene correction

Gene correction by HDR has a huge potential for therapeutic genome editing because there are many genetic diseases in the cardiovascular field [21] (Figure 2 and 3). However, it is currently difficult to apply this approach to *in vivo* models because of the low efficiency of HDR in non-dividing somatic cells, particularly cardiomyocytes. No *in vivo* studies have been reported using this approach in the cardiovascular field so far.

Currently, gene correction by HDR are used mostly in *in vitro* platforms in which successfully edited cells can be purified and amplified [1, 4]. Studies using patient-derived iPSCs have shown that phenotypes of monogenic diseases can be rescued after gene correction [87]. Because these disease-modeling studies with iPSCs use genome editing in the stem cell stage, and pick up edited iPSC clones and then differentiate them to cell types responsible for the disease (cardiomyocytes, endothelial cells, etc), this approach cannot be applied as it is to *in vivo* genome editing. However, these studies suggest that gene correction is a potential therapeutic option in the future if it can be achieved with high efficiency in somatic cells and 3D organs. Considering the low efficiency of HDR in current CRISPR/Cas9 technology, feasible targets

include diseases in which even a low percentage of corrected cells can at least partially rescue the function or diseases that can be treated by *ex vivo* editing whether in somatic cells or iPSCs.

3.5 Other approaches with newer CRISPR/Cas9-based tools

Since the discovery of CRISPR/Cas9 systems, there has been a growing number of novel CRISPR/Cas9-based tools whose application is not limited to genome editing, but include applications in gene regulation, epigenome editing, genomic imaging, etc [50-52, 88]. These non-conventional tools do not follow the rules of NHEJ or HDR discussed above. Here we briefly summarize several examples that have potential therapeutic applications.

One method with potential for cardiac genome editing is the homology-independent targeted integration (HITI) strategy [89]. As the gene correction strategy by HDR is inefficient in non-dividing cells, this novel tool has been developed to achieve gene correction without HDR. HITI enables DNA knock-in even in non-dividing cells. Instead of HDR, HITI utilizes modified NHEJ for gene correction because NHEJ is active both in dividing and non-dividing cells. Importantly, this method was shown to be able to knock-in a reporter gene into mouse heart using AAVs. The same group has recently developed a new method called SATI (intercellular linearized Single homology Arm donor mediated intron-Targeting Integration). SATI is a combination of single homology arm mediated HDR with HITI, enabling broader target flexibility [90].

Base editors may be also a useful alternative of HDR-mediated gene correction [91-94]. Because base editing relies on recruitment of cytidine deaminases rather than DSBs, they can offer potential improvements in efficiency while limiting damage by DSBs. The precise conversion of C to T has a huge potential for therapeutic repair of point mutation. It has been estimated that 3,000 genetic variants in ClinVar could be corrected by C>T substitution, although targetable mutations are limited by the PAM sequences of base editing tools [93, 94].

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Several studies demonstrated that base editors can be used for therapeutic gene correction in mouse models [95-97]. However, as with the NHEJ gene disruption strategy, research of base-editing therapy for cardiovascular diseases has been limited to PCSK9 in the liver and base-editing of the heart has not been reported yet [95]. Although there are many candidate diseases that can be treated by C>T substitution (e.g., familial cardiomyopathy, congenital heart diseases, LQTS, etc), delivery of base editors to the heart is still challenging because base editors are large fusion proteins that exceed the packaging capacity of AAVs.

Very recently a novel powerful tool called "prime editing" has been reported. It uses Cas9 H840A nickase fused to a reverse transcriptase. The prime editing guide RNAs (pegRNAs) contain sequences that would be reverse-transcribed and introduced to the desired locus as well as sequences to specify the target loci. The prime editing enables targeted insertions, deletions, and all 12 possible base-to-base conversions without requiring DSBs or donor DNA templates. This new system may have great promise for therapeutic genome editing because it could correct up to 89% of known genetic variants associated with human diseases in principle [98].

Besides genome editing tools, CRISPR/Cas9-based gene regulation tools would be also useful for therapeutic purposes [52, 38, 99]. To regulate gene expression instead of editing genomic DNA, CRISPR interference (CRISPRi) and CRISPR activation (CRISPRa) systems have been developed using nuclease-deactivated Cas9 (dCas9). dCas9 maintains its ability to bind gRNAs and targeted DNA, but it lacks nuclease activity, allowing it to serve as a unique platform to recruit protein and RNA factors to a targeted DNA site without cleaving genomic DNA. To achieve gene repression and activation, transcriptional repressors (e.g., KRAB) and activators (e.g., VP64) are fused to dCas9 for CRISPRi and CRISPRa, respectively. In addition to CRISPRi/a, several epigenome editors have been developed using dCas9 including histone

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acetylation (e.g., H3K27Ac using p300) [100, 101], histone demethylation (e.g., H3K9me3 using KRAB) [102], DNA methylation (using DNMT3A/L) [103, 104], and DNA demethylation (using TET1) [105]. In terms of therapeutic application, thus far no studies have been reported about *in vivo* gene therapy using CRISPRi/a or other epigenome editors. While a CRISPRi system can be theoretically applied to diseases that are targets of antibody drugs, CRISPRa may be more attractive because gene activation is difficult using current molecular targeted therapy.

4. Current hurdles for therapeutic genome editing

4.1 Flexibility and versatility of target sequence

The flexibility of target sites depends on how genome editors recognize the target site [1-4, 9]. Each genome editing tool has its specific DNA sequence to bind. Compared to ZFN or TALEN, CRISPR/Cas9-based systems have a simpler and more flexible rule for target-site selection. Basically, CRISPR/Cas9 genome editors bind to a 20-bp protospacer sequence defined by a gRNA, and the protospacer sequence needs to be followed by a Protospacer Adjacent Motif (PAM) sequence. Each Cas9 variant has a unique PAM sequence. For example, the PAM sequences for SpCas9 and SaCas9 are 5'-NGG and 5'-NNGRRT, respectively. For Cas9 variants with more complex PAM sequences, the target site flexibility is more limited. However, this limitation can be solved soon by the growing number of Cas9 variants that are becoming available rapidly (Figure 4).

In some cases, non-unique genomic sequences around the target sites limit their availability. If a target site is within repeat sequences or if it has no nearby sequences that are unique compared to the rest of the genome, designing appropriate gRNAs to deliver Cas9 to the target

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site can be difficult. Thus, genes in these sites would not be good targets for genome editing therapy.

4.2 On-target editing efficiency

For therapeutic genome editing, it is necessary to achieve high editing efficiency at a desired genomic site in a desired tissue. Although there are a number of new methods that can improve the efficiency of on-target editing, it is still challenging to achieve highly efficient *in vivo* editing in many types of solid tissues including the heart [106, 107] (Figure 4).

Because local chromatin structure and epigenetic status affect the accessibility to the target site by genome editors, the editing efficiency varies depending on different loci, phases of the cell cycle, cell types, and tissue types [106, 108-110]. In particular, the variation of editing efficiency in different tissues is an important factor for *in vivo* genome editing. In the cardiovascular field, several groups succeeded in *in vivo* genome editing of PCSK9 in mouse liver by NHEJ-mediated gene disruption as a potential target of dyslipidemia [64-69]. The editing efficiency in mouse liver reported in these studies is high enough to obtain the functional phenotype. Viral vectors were used to deliver CRISPR/Cas9 to mouse liver and more than 50% of cells were knocked-out, resulting in a lower LDL cholesterol level. By comparison, the efficiency of *in vivo* genome editing in the heart is much lower [79, 81-84, 86, 107]. The efficiency in cardiomyocytes was only 2-10% after systemic administration of viral vectors to postnatal mice. Although the editing efficiency depends on the efficiency of delivery systems, the tissue (or cell) type seems to be another important factor that affects the activity of genome editors. One interesting observation is that the editing efficiency in the heart was still low even in a Cas9-transgenic mouse model, in which the delivery of the large Cas9 protein would not be a problem [60]. Another important observation is that the editing efficiency of the heart was higher

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when genome editing was performed in neonatal mice [79, 83]. A study showed that approximately 9% of gene modification was detected in the heart up to one year after genome editing when AAV8 was systemically administered to neonatal (P2) *mdx* mice [83]. The discrepancies in editing efficiency between liver and heart, and between neonatal heart and adult heart, are probably due to the differences in epigenetic status including chromatin accessibility, activity of DNA repair machinery, and phases of the cell cycle. Because of the low editing efficiency of the adult heart, genome editing in the liver has been studied more extensively than genome editing in the heart in the cardiovascular field. Currently, targeting the liver with NHEJ for risk reduction of atherosclerosis is a more feasible strategy than targeting the heart to treat genetic cardiac diseases.

As to gene correction, although the cardiovascular field has many genetic diseases for which gene correction of the heart could be in theory a potential therapeutic option (e.g., HCM, DCM, LQTS, etc), the low editing efficiency in the heart would be a big obstacle [9-11]. Gene correction by HDR is even more challenging than NHEJ-mediated gene disruption or deletion as discussed above. To realize *in vivo* genome editing of the heart as a treatment of genetic heart diseases, methods that improve the efficiency of HDR need to be developed. Potential solutions are to inhibit NHEJ, activate HDR, or modulate cell cycle status [106, 111-114]. Another approach is to develop methods that do not depend on HDR for gene correction such as HITI or prime editing [89, 98].

4.3 Off-target mutagenesis

Although the on-target efficiency of genome editing has been improved, safety is the most critical issue to realize the potential of genome-editing therapy [115]. In addition to risks that are shared with other gene therapies (e.g., toxicity of delivery reagents, immune response, etc.), all

types of therapeutic genome editing have a potential risk of off-target mutagenesis; new mutations may be introduced to the sites in the genome other than the desired on-target site (Figure 4). The off-target mutations in the target cells or tissues may result in undesired functional phenotypes such as oncogenesis [1-4]. In the cardiovascular field, off-target mutagenesis makes targeting the heart more challenging than other tissues because even rare off-target mutations may cause cardiac arrhythmias that could be fatal to the patient.

An important point regarding off-target mutagenesis is that off-target mutations differ among individuals because each patient has a unique genetic background that cannot be reproduced using model organisms [1, 116, 117]. Thus, it is necessary to evaluate the off-target mutations in each patient prior to clinical application. For *ex vivo* genome editing, off-target mutations can be tested before implanting the edited cells or tissues to the original patients. However, prior evaluation of off-target effects for *in vivo* genome editing would be more challenging. Primary cells from the patients or their iPSC-derived cells may be useful to test genome editing and predict patient-specific off-target effects.

A number of methods to detect off-target mutations have been developed, but they are still in development and no standardized methods exist to allow therapeutic genome editing in patients [118]. There are three types of assays to assess off-target mutations. First, many *in silico* methods to predict the off-target sites have been developed and are commonly used to design gRNAs. These tools are based on the concept that the off-target potential of a particular locus depends on the sequence similarity to the on-target site [115, 119, 120]. Although these tools are helpful and widely used because of their simplicity, recent studies showed that the off-target editing can occur at sites other than those predicted by sequence similarity. Second, there are newer cell-based methods that can be used to assess off-target editing in a genome-wide

unbiased manner, including GUIDE-Seq, BLISS, and DISCOVER-seq [121-123]. For example, GUIDE-seq uses small oligonucleotides to tag DSBs generated by genome editors [123]. The tagged genomic sites are then amplified by PCR and sequenced to map the locus in the genome. GUIDE-seq has been commonly used in cultured cells, but it would be challenging to apply this method to *in vivo* genome editing because it requires transfection of oligonucleotides. No matter what cell-based methods in this category are used, appropriate platforms to test genome editing in patient cells are needed. Third, cell-free *in vitro* unbiased assays such as Digenome-seq, SITE-seq, and CIRCLE-seq have been developed recently [124-126]. The assays in this category use cell-free genomic DNA extracted from the target cells or tissues to detect genome-wide activity of CRISPR/Cas9. Because genome editing is performed in a cell-free *in vitro* system using extracted genomic DNA in these assays, they can avoid limitations associated with culture conditions and manipulation of living cells. Compared to cell-based methods, the sensitivity of cell-free methods is so much higher that they can detect even rare off-target sites. This advantage is important for clinical application of therapeutic genome editing, in which rare off-target mutations may result in fatal phenotypes. By combining cell-free CIRCLE-seq with amplicon sequencing, one study showed that almost all the off-target sites by *in vivo* genome editing can be detected [68]. This approach consists of two steps: CIRCLE-seq was performed using extracted genomic DNA to identify off-target candidates (*in vitro* discovery step) and then targeted amplicon sequencing against these candidates was performed in mouse liver samples after *in vivo* genome editing (*in vivo* confirmation step). This strategy would be applicable to therapeutic genome editing in patients because it is a noninvasive method that can reflect each patient's genetic background.

In terms of the efforts to reduce off-target effects, using well-designed gRNAs has been shown to be critical to achieve low off-target effects. For example, a study showed that 19 off-target mutations were detected in mouse liver when a "promiscuous" gRNA was used against *Pcsk9*, while no off-target mutation was detected when a well-designed gRNA was used [68]. Other than the sequences of gRNAs, a number of attempts have been made to improve the editing specificity, including methods that use shorter gRNAs [115, 119, 120], chemically-modified gRNAs [127], nickase variants of Cas9 to induce two single-strand breaks instead of DSBs [128, 129], and engineered new Cas9 variants [130-134]. Although some of these non-conventional methods may be promising, many higher fidelity Cas9 variants exhibit a lower editing efficiency and delivery methods need to be optimized for *in vivo* therapeutic genome editing.

4.4 Efficient and tissue-specific delivery of genome editors

Aside from the hurdles within genome editors, another major challenge for clinical application is the delivery of genome editors to the target tissues and cells [1, 52, 88]. As with on-target editing and off-target mutagenesis, the efficient and specific delivery of genome editors to the target tissues is required for therapeutic genome editing (Figure 4). Currently, for most gene therapies, the *in vivo* delivery methods mainly rely on AAV vectors, which have been approved by FDA [1]. Although current AAV systems still have limitations such as cargo size and immunogenicity, they have high efficiency of delivery to a variety of tissue types, including the eye, brain, liver and muscle [135, 136]. In the cardiovascular field, the viral delivery method with AAV9 has been already used in clinical trials of cardiac gene therapies [137].

For CRISPR/Cas9-based genome editing, basically both Cas9 and gRNAs need to be delivered, whether separately or together, to the target tissues (Figure 5A). For HDR-mediated

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gene correction, a repair template is needed additionally. Choosing an appropriate expression system and delivery method is critically important to achieve the desired genome editing. The common methods in early studies were those that deliver the coding sequence of Cas9 and gRNAs as plasmids or virus [1-3, 88]. Although these DNA-delivering methods have been used in many studies whether *in vitro* or *in vivo*, there are some potential complications that must be considered: high off-target effects due to prolonged Cas9 expression, requirement of promoters active in the target cells, risk of DNA incorporation to the genome, etc (Figure 5B). To avoid these complications, methods that deliver Cas9 as mRNA or protein to the target cells have been developed [52, 87, 88]. In particular, a ribonucleoprotein (RNP), Cas9 protein in complex with a gRNA, is preferably used in recent studies [138]. In principle, RNPs can work immediately after delivery because they do not require transcription of Cas9 and have lower off-target effects because they lack prolonged Cas9 expression. RNPs can be delivered to target cells by lipofection or electroporation *in vitro*.

For *ex vivo* therapeutic applications, such as editing of blood cells, most of the expression systems and delivery methods developed for *in vitro* platforms can be used [1, 52, 88]. Among currently available methods, RNPs with electroporation is ideal to achieve transient expression of genome editors. RNPs can be also delivered with engineered cell-penetrating peptides or chemical conjugation. However, this type of application in the cardiovascular field is limited.

For *in vivo* applications, the delivery options are currently limited to viral vectors [1, 52, 88]. In particular, AAV vectors are preferably used because of their low immunogenic potential, reduced genome integration, and broad range of serotype specificity [65, 135, 136]. Each AAV serotype has high efficacy in a variety of tissues such as eye, brain, liver, and muscle. In the cardiovascular field, AAV9 has been already used in clinical trials of cardiac gene therapies

[137]. However, there are several limitations of AAV vectors. First, the packaging capacity is relatively small (up to 4.5 kb excluding the inverted terminal repeats), making it challenging to deliver Cas9 and gRNAs in a single vector. The size of SpCas9, the most commonly used Cas9 variant is 4.2 kb and has little room left for gRNAs and expression control elements. Smaller Cas9 orthologs (SaCas9, CjCas9, and NmeCas9) would be better for AAV [65, 139-142]. There are ongoing efforts to identify natural Cas proteins or engineer Cas variants that are smaller in size and effective in function. For example, a newly identified RNA-guided nuclease, CasX is smaller (<1000 aa) than these Cas9 and may provide more room for AAV-mediated delivery [143]. Second, AAV-mediated delivery may result in constitutive expression of Cas9, which could induce genomic instability and DNA damage [135, 136]. Third, one-shot treatment is required for AAV delivery because the patient may develop immunity against the AAV serotype [135, 136]. Furthermore, a significant proportion of patients may have been naturally exposed to AAVs and already immunized prior to the initial administration of AAV vectors [135].

Although nano methods to deliver Cas9 mRNA or RNPs for *in vivo* application have not been studied intensively compared to viral vectors, several studies showed the potential of nanoparticle-based delivery methods [144, 145]. As with viral vectors, it is still important to achieve tissue-type or cell-type specificity, reduce the toxicity of nanoparticle components, and avoid immunogenicity of Cas9 proteins (50-80% are reported to be immunized to SpCas9 and SaCas9) [146].

4.5 Ethical issues

Despite genome editing’s tremendous potential for treating diseases, its ease of use and powerful effects raise considerable and growing ethical concerns [1-4, 50]. Notably, a major controversy involving the world's first “CRISPRed babies” in November 2018 publicized widely

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some of the serious ethical issues concerning the future use of human genome editing [147, 148]. Because the ethical issues regarding human genome editing are complex and debatable, comprehensive discussion is difficult in this review. We briefly summarize a few important points here.

One of the biggest concerns about genome editing is the heritability of editing [1-4, 50]. In theory, genome editing in embryos or in germline cells to treat familial genetic diseases would be more powerful than *in vivo* genome editing after birth. This approach could eliminate genetic disorders permanently even in the offspring. In mouse models, genome editing in embryos for the treatment of genetic defects has been shown to generate offspring with the edited gene [81, 149]. Similarly, genome editing in human embryos has been already tested and shown to be feasible [150, 151]. However, little agreement or consensus has been reached on implications from artificially introduced genetic changes that can be passed on to future generations by genome editing in embryos and germline cells.

Another important issue is what kind of diseases or traits should be approved as targets of genome editing [1-4, 50]. Treating rare genetic mutations which cause severe fatal diseases would be broadly acceptable. Preventing diseases by gene editing in those who have high risk factors (e.g., targeting PCSK9 for familial hyperlipidemia) would likewise find few objections. However, genome editing to prevent very common diseases or genome editing for "enhancement" of a trait (e.g., height, memory, learning, physical attractiveness, etc.) may invite controversy. The ethical acceptability of genome editing in a particular case depends on the societal norms and personal background of each individual patient, and these issues are beyond scientific discussion. Applicable international ethical guidelines and rules are needed to govern these issues [148].

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5. Conclusions

Genome editing tools, especially CRISPR/Cas9 systems, have emerged as a powerful and promising technology to achieve genomic manipulation both *in vitro* and *in vivo*. In the cardiovascular field, although genome editing has been often used as a research tool to investigate disease pathogenesis, actual clinical application of therapeutic genome editing is still limited. Genome editing in the liver for risk reduction of cardiovascular diseases has been studied intensively, and PCSK9 is one of the most promising targets for therapeutic genome editing. Nevertheless, treating the heart is still challenging because of the inefficiency of editing in the heart using current genome editing tools and delivery systems. Because therapeutic genome editing is still in its infancy but is progressing rapidly, we can expect that a continuing influx of novel editing tools seeking wider application. Efficient and safe delivery methods will need to be developed to apply those novel tools for therapeutic purposes.

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Figure Legends

Figure 1. Applications of genome editing technology. Genome editing tools have been used as a research tool to discover novel biological mechanisms and study pathophysiology of diseases. Genome editing tools can be also used as a therapeutic tool to treat or prevent diseases. The ways of therapeutic genome editing are mainly divided to *in vivo* genome editing and *ex vivo* genome editing. Target diseases include both rare monogenic diseases and common diseases.

Figure 2. Potential candidate diseases of therapeutic genome editing in the cardiovascular field. In principle, many monogenic diseases in cardiovascular tissues (heart and arteries) can be candidates of therapeutic genome editing. However, these diseases are challenging targets because most of them require gene correction by HDR. Non-cardiovascular tissues may be targeted to treat or prevent common diseases, especially dyslipidemia and atherosclerosis. Currently, genome editing therapy of PCSK9 in the liver is the most promising candidate in the cardiovascular field.

Figure 3. Three strategies based on non-homologous end-joining (NHEJ) and homology-directed repair (HDR). Genome editing tools utilize the endogenous DNA repair processes within the host cells. When DSBs are generated by genome editors, the DSBs are repaired by the cells using either non-homologous end-joining (NHEJ) or homology-directed repair (HDR). In the absence of a repair template, the DSBs are repaired by NHEJ, which is active throughout the cell cycle in most cell types but is also error-prone, resulting in insertions or deletions (indels) at the DSB site. In the presence of a homologous repair template, DSBs are corrected to the sequence of the template by HDR, which is active only in the S and G2 phases thus not active in

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non-dividing cells. For disruption of a gene, indels made by NHEJ can disrupt the coding sequence of the target gene. For deletion of a genomic region, two DSBs flanking region are required and the edges of two DSB sites are ligated by NHEJ. For correction of a gene, disease-causing mutations are corrected to the template sequence by HDR.

Figure 4. Current hurdles for therapeutic genome editing. There are a number of hurdles to realize therapeutic genome editing in clinics, including capability of genome editors, lack of ideal delivery systems, and ethical issues. In terms of genome editors, flexibility of target sequences, editing efficiency, and editing specificity may limit the applications. These limitations may be overcome by the development of novel genome editors in the future. In terms of delivery, current *in vivo* delivery methods mainly rely on AAV systems. Nanoparticle-based delivery methods in particular may prove useful for efficient and tissue-specific delivery in the future. Ethical issues also need to be considered. Genome editing in germline cells or embryos is complicated by the issue of heritability despite its biological effectiveness. No ethical consensus on the acceptability of using genomic editing for very common diseases and enhancement has been reached.

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Figure 5. Delivery methods of CRISPR/Cas9-based tools. For CRISPR/Cas9-based genome editing, both Cas9 and gRNA need to be delivered and expressed in the target cells or tissues. For HDR, repair DNA templates need to be delivered additionally. Early studies used plasmids or virus vectors to deliver Cas9 and gRNAs. For *in vitro* (or *ex vivo*) applications, recent studies now rely on methods that deliver Cas9 mRNA or Cas9 protein. In particular, ribonucleoprotein (RNP) is preferably used because of its efficiency and low off-target effects. On the other hand,

in vivo delivery still relies on AAV. The relatively small cargo size (up to 4.5 kb) is the biggest limitation of current AAV delivery system. Delivery of RNP by nanoparticles may prove to be an ideal option in the future.

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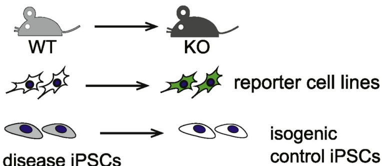
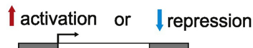
Genome editing as a research tool

Genetic models



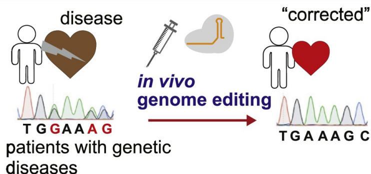
Gene expression control



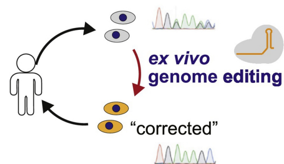
Pooled screening

etc

Therapeutic genome editing



patients with genetic diseases



Disease prevention or treatment
(rare/monogenic diseases)
e.g., Huntington disease
Myotonic Dystrophy
Cystic fibrosis
Sickle cell anemia
Duchenne Muscular Dystrophy

Modifying disease risk
(common diseases)
e.g., HIV (CCR5)
Atherosclerosis (PCSK9)
Alzheimer disease (APP)
Cancer (BRCA1/2)

Figure 1

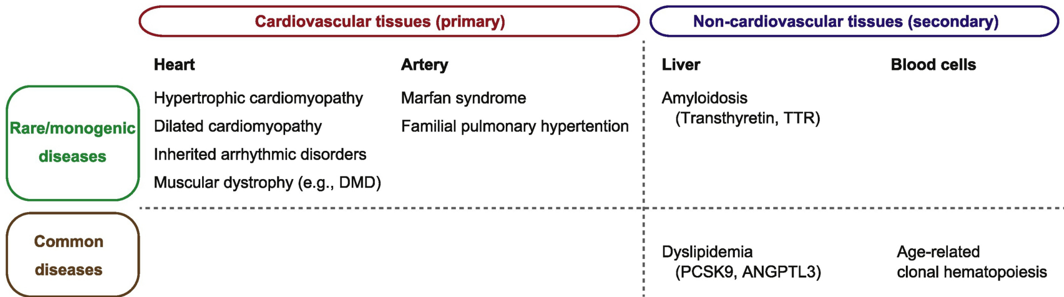
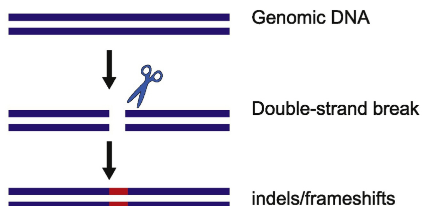
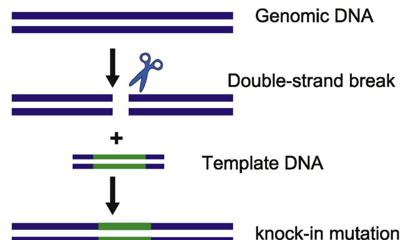


Figure 2

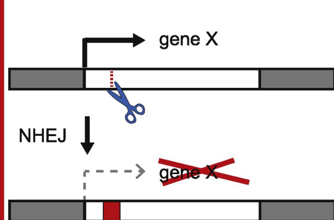
Non-homologous end-joining (NHEJ)



Homology-directed repair (HDR)



Disruption

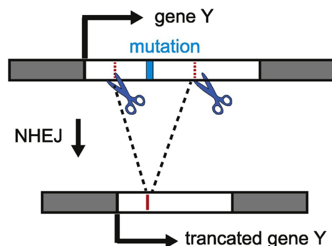


Potential target diseases/genes

PCSK9

Amiloidosis

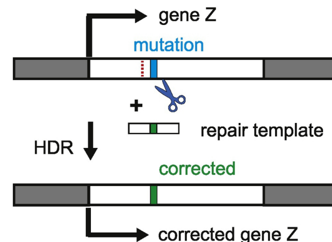
Deletion



Potential target diseases/genes

DMD (exon skipping)

Repair



Potential target diseases/genes

Familial HCM or DCM

Long QT syndrome

Laminopathy

Other rare diseases

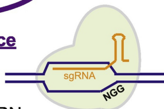
Figure 3

Hurdles for therapeutic genome editing

Genome editors

Flexibility of target sequence

- Limited by PAM sequence
  - * spCas9: NGG
  - * saCas9: NGRRT or NGRRN

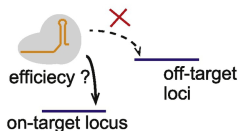


On-target efficiency

- Affected by local chromatin structure (chromatin accessibility)
- Depends on tissue types

Off-target mutagenesis

- Methods to assess off-target mutations are limited.
- Affected by SNPs
- Difficult to predict the risk in each individual



Delivery

(see Figure 5)

Delivery methods

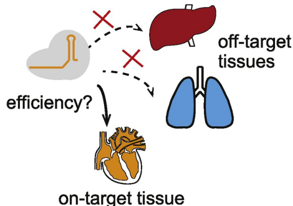
- Local or systemic delivery
  - viruses (AAV, Adenovirus, etc)
  - nanoparticles

Efficiency of delivery

- Some organs are difficult to target.

Tissue specificity

- Other tissues may be edited.



Ethical issues

Heritability of editing

- Germline genome editing
- *In utero* genome editing

Genome editing for common diseases

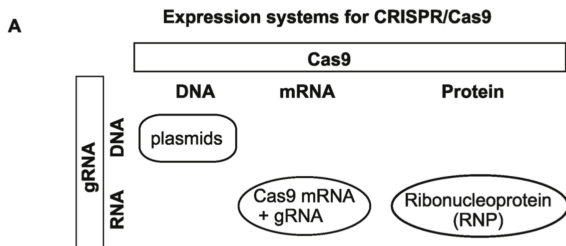
e.g., diabetes, hypertention, myocardial infarction, etc

Genome editing for "enhancement"

e.g., height, skin color, learning, memory, etc

Figure 4

A



Delivery methods for CRISPR/Cas9

- **Lipofection** } Typically used *in vitro*
- **Electroporation** } Typically used *in vitro*
- **Virus** (AAV, Adenovirus, lentivirus, etc)
- **Nanoparticles**

B

	Expression systems: DNA (plasmids)	Expression systems: Cas9 mRNA + gRNA	Expression systems: RNP	*in vitro* or *ex vivo*	*in vivo*
Delivery methods: Lipofection	✔	✔	✔	○	×
Delivery methods: Electroporation	✔	✔	✔	○	×
Delivery methods: Virus	✔			○	○
Delivery methods: Nanoparticles	✔	✔	✔	○	△
off-target	high	low			
Cas9 expression	delayed	immediate			
DNA incorporation risk	+	-			
Promoter optimization	necessary	not necessary			

AAV is currently the most common delivery method for *in vivo*.

Limitation
- Cargo size (up to 4.5 kb)
- Immune response to virus

Nanoparticles with RNP may become a future option.

Limitation
- Tissue specificity
- Toxicity of nanoparticles
- Immune response to Cas9 protein

Figure 5