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Junjie Xiao *Editor*

Genome Editing in Cardiovascular and Metabolic Diseases

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Genome Editing in Cardiovascular and Metabolic Diseases

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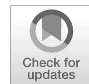
Part I

Overview



An Overview of Genome Editing in Cardiovascular and Metabolic Diseases

Kiran Musunuru

Abstract

This chapter summarizes the definition, classification, and function of genome editing and highlights the breakthroughs of genome editing in cardiovascular and metabolic diseases for disease modeling, diagnostics, and therapeutics, with a particular focus on clustered regularly interspaced short palindromic repeats (CRISPR)-CRISPR-associated 9 (Cas9) technology as applied to nuclease editing, base editing, and epigenome editing.

Keywords

Genome editing · Base editing · Epigenome editing · Nonhomologous end joining · Homology-directed repair · CRISPR · Cardiovascular disease · Disease modeling · Diagnostics · Therapeutics

1 Genome-Editing Technologies

Any extended discussion of genome editing invariably begins with an introduction to the panoply of genome-editing technologies that are now available to biomedical investigators. The first applications of genome editing involved an approach that prevailed for decades and remains in broad use—homologous recombination. In homologous recombination, a DNA sequence in the genome in a cell is replaced with a synthetic version of the same DNA sequence in which a desired alteration has been made [1, 2]. The synthetic DNA sequence is typically carried in a double-strand DNA vector that is introduced into the cell by transfection or another method. If there is sufficient matching between the cellular DNA sequence and the synthetic DNA sequence—within two homology arms flanking the altered sequence—a spontaneous swapping can occur, incorporating the alteration into the cell's genome. Homologous recombination has been exploited to generate thousands of genetically modified mouse models, as well as other animal models and cellular models.

Yet homologous recombination is extremely inefficient, with far below 1% of cells acquiring the alteration. The reason for this inefficiency is the need for an initiating event, namely, a spontaneous double-strand break in the chromosome within the target cellular DNA sequence, which is a rare event. Modern genome-editing tools emerged from efforts to deliberately introduce double-strand breaks into target sites in the genome, greatly boosting the efficiency of homologous recombination at those sites [3]. Perforce, these tools have two types of functionality, the ability to search out and specifically bind to a target site—typically a unique location within the entire genome—and the ability to generate a

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double-strand break at that site. Collectively, these tools are known as engineered nucleases. After the development of a variety of engineered nucleases, it became possible to separate the two types of functionality and pair the search-and-bind ability with any of a variety of different kinds of gene-modifying activity, including chemical modification of DNA bases (base editing), modification of gene expression (epigenome editing), and reverse transcription to introduce new DNA sequences from RNA templates (prime editing). Each of these genome-editing technologies is briefly described in the following sections. A more comprehensive discussion of genome-editing technologies and their applications can be found elsewhere [4].

1.1 Nuclease Editing

There are four major types of engineered nucleases in use for research and clinical applications: zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), meganucleases, and clustered regularly interspaced short palindromic repeats (CRISPR)-CRISPR-associated (Cas) systems. All have the ability to seek out specific genomic sites and to introduce double-strand breaks at those sites, although the mechanisms by which they carry out these tasks are quite distinct.

ZFNs use arrays of zinc fingers, which are DNA-binding motifs found in numerous proteins in species across the entire phylogenetic tree [5]. Each zinc finger typically recognizes and binds three consecutive DNA base pairs. Accordingly, zinc finger arrays will specify stretches of DNA with lengths that are multiples of three. A ZFN comprises a zinc finger array fused to a cleavage domain adapted from the bacterial restriction enzyme *Fok* I. The *Fok* I cleavage domain cannot make a double-strand break in DNA as a monomer, but only as a dimer. As such, ZFNs are typically used as pairs, wherein 1 ZFN binds a DNA sequence along 1 strand (typically 9–18 base pairs in length) and the other ZFN binds a separate DNA sequence along the other strand, with the binding sites juxtaposed such that the *Fok* I cleavage domains of the 2 ZFNs are in proximity, can dimerize, and can then produce a double-strand break between 2 ZFN binding sites.

TALENs work much the same way as ZFNs, except that they use an entirely different DNA-binding motif, the TAL repeat [6]. Unlike zinc fingers, TAL repeats naturally occur only in a group of plant pathogens, and each TAL repeat recognizes and binds a single DNA base pair. Arrays of TAL repeats can bind sequences as long as dozens of base pairs in length. Similar to ZFNs, each TALEN comprises a TAL-repeat array fused to the *Fok* I cleavage domain, and TALEN pairs are used to specify target sites and produce double-strand breaks there.

Meganucleases, despite their name, are actually quite small compared to the other engineered nucleases [7]. They are based on naturally occurring nucleases—I-*Cre*I being the prototypic one, identified in an algae species—that can specifically bind and cleave DNA sequences as long as dozens of base pairs in length. Altering key amino acids allows for the reprogramming of nucleases like I-*Cre*I to bind and cleave different DNA sequences.

ZFNs, TALENs, and meganucleases are all protein-based genome-editing tools. As such, changing the target specificity of any of these nucleases requires at least some degree of protein engineering, which can limit their use as research tools since such engineering lies beyond the practical reach of most academic laboratories. In contrast, the CRISPR-Cas systems of bacterial origin that are widely used for genome editing—CRISPR-Cas9 and CRISPR-Cas12—each have two components, a Cas protein and a guide RNA, which each has a distinct functionality [8]. The guide RNA provides the search-and-bind capacity, encoded within a stretch of nucleotides within the guide RNA itself, and the Cas protein has the inherent ability to produce a double-strand break, using either one or two cleavage domains to cut the two DNA strands.

Streptococcus pyogenes Cas9 (SpCas9) was the first CRISPR-Cas system to be adapted for use in genome editing of mammalian cells (Fig. 1) [9–13]. Its guide RNA, about

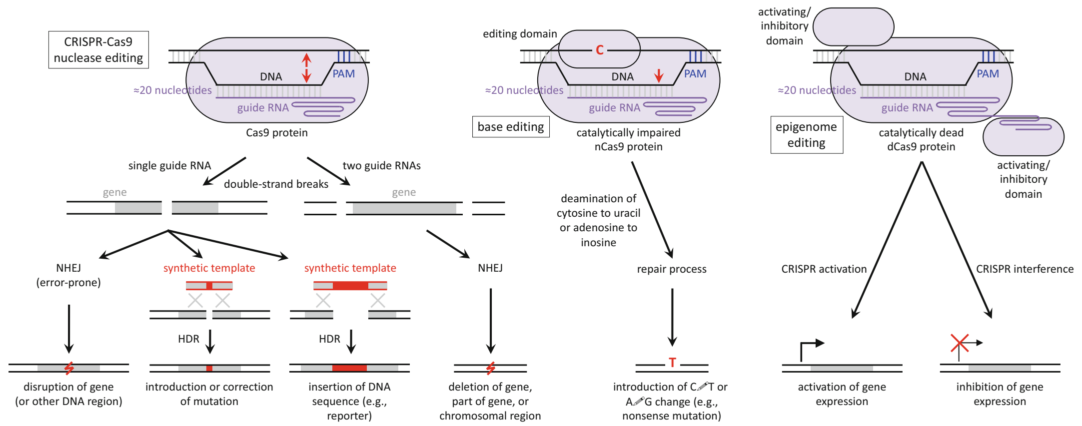

Fig. 1 Genome-editing technologies. *HDR* homology-directed repair, *NHEJ* nonhomologous end joining, *PAM* protospacer-adjacent motif

100 nucleotides in length, encodes the DNA targeting specificity in its first 20 nucleotides, known as the spacer. SpCas9 binds to the remaining portion of the guide RNA, and the protein-RNA complex scans along any double-strand DNA molecule into which it comes in contact—in the nucleus of a human cell, the entire human genome. SpCas9 unwinds DNA as it scans, searching for an NGG motif (N being any nucleotide), whereupon it will position the spacer portion of the guide RNA opposite the DNA strand that does not contain the NGG motif, called the target strand. If there is perfect (or, in some cases, near-perfect) complementarity of the spacer sequence and the target strand sequence, there will be extensive Watson–Crick base pairing between the RNA and DNA that will cause SpCas9 to make a double-strand break three positions upstream of the NGG motif. The DNA sequence on the non-target strand corresponding to the RNA spacer sequence is called the protospacer sequence, which is the 20-nucleotide sequence just upstream of the NGG motif, which itself is called the protospacer-adjacent motif (PAM).

Redirecting SpCas9 to a different sequence in the genome merely entails finding a protospacer/PAM sequence at the desired target site and changing the guide RNA's spacer sequence to match. Since it is very straightforward to change the spacer sequence, by simply altering the DNA template from which the guide RNA is transcribed—far easier than the protein engineering needed for ZFNs, TALENs, and meganucleases—SpCas9 was quickly and broadly adopted by laboratories after its introduction as a genome-editing tool in early 2013. Furthermore, SpCas9 generally shows higher rates of editing efficiency at target sites than other genome-editing tools [14], which has only increased its popularity.

Although SpCas9 is by far the most commonly used CRISPR-Cas system, Cas9 proteins adapted from other bacterial species are also employed for genome editing, most notably *Staphylococcus aureus* Cas9 (SaCas9), which has the advantage of being smaller than SpCas9; it recognizes a different PAM sequence, NNGRRT (R is either G or A), which gives it a different targeting range than SpCas9 [15]. Rational mutagenesis of SpCas9 and SaCas9 have yielded versions that recognize different PAM sequences [16, 17]. At least three other Cas proteins, all of the Cas12 family, have proven to be efficient genome editors—Cas12a (also known as Cpf1) [18], Cas12b (also known as C2c1) [19], and Cas12e (also known as CasX) [20]. Although their properties are somewhat different from Cas9 (different PAM sequences, different guide RNA configurations, etc.), they are similar in that they too are two-component, protein-RNA systems that offer ease of use.

Regardless of the genome-editing tool used, the result is (hopefully) a double-strand break at the desired target site. The final outcomes of nuclease editing depend on how the cell repairs the double-strand break and are largely unrelated to the tool used. The most common repair mechanism is nonhomologous end joining (NHEJ), in which the free ends are ligated together (Fig. 1) [3, 21]. Often, the original pre-cleavage sequence is restored, but occasionally an error occurs with NHEJ, typically a small insertion or deletion (indel mutation) of one or a few base pairs, though the indels can be much larger, in some cases dozens, hundreds, or even thousands of base pairs. Different cells will acquire different indels, since the mutations occur in semi-random (not fully random) fashion. If the objective is to simply disrupt a gene or a noncoding regulatory element, NHEJ serves the purpose well, since the exact nature of the indel is not important, and NHEJ editing can achieve up to 100% editing efficiency in some contexts.

If the objective is to make a precise change, such as the introduction or correction of a disease-causing mutation, or the insertion of a sequence, then one must rely on a second cellular repair mechanism, homology-directed repair (HDR) (Fig. 1) [3, 22]. Ordinarily, HDR uses a perfectly matched DNA repair template that is already present in the cell—a sister chromatid or sister chromosome—and mimics the process of homologous recombination to achieve accurate repair of the double-strand break. If a synthetic DNA repair template bearing a mutation or sequence insertion is introduced into the cell, HDR can use the synthetic template instead and thereby integrate the altered sequence into the genome. The synthetic template can be in the form of a double-strand DNA vector or a single-strand DNA oligonucleotide. Although precise, HDR editing has significant limitations that make it far less efficient than NHEJ editing (usually not exceeding a few percent); besides requiring an extra piece of DNA, HDR is active only in proliferating cells, and only during the S and G2 phases of the cell cycle in those cells. Fortunately, there are other genome-editing technologies that can make precise yet efficient changes, free of the limitations of HDR.

Nuclease editing can have undesirable consequences of two kinds, particularly in the context of therapeutic applications. First is unintended on-target editing: very large indel mutations, in some cases affecting entire chromosomal regions, and even chromosomal rearrangements can occur. Second is off-target editing: each nuclease has the potential to occasionally bind to sites that are an imperfect match to the target site specified by the nuclease, resulting in indel mutagenesis elsewhere in the genome. Though it is unlikely for any given off-target edit to be deleterious, there is a theoretical possibility that an off-target edit could occur in a tumor suppressor gene or oncogene, predisposing cells to tumorigenesis and conferring an increased risk of cancer. Alterations of the Cas9 protein or the guide RNA can reduce the risk of off-target editing, though usually at the cost of reduced on-target editing [23–25].

1.2 Base Editing

Base editing (and other types of editing described in subsequent sections) takes advantage of the fact that CRISPR-Cas9 can be directed to a desired site in the genome (via the spacer sequence of the guide RNA) independently of the ability to make double-strand DNA breaks (inherent in the Cas9 protein). If the two cleavage domains of Cas9 are mutated so that they can no longer cut the two DNA strands—known as catalytically dead Cas9, or dCas9—the protein-RNA complex will nonetheless search out and bind to the target genomic site. This phenomenon offers the opportunity to fuse additional domains to the Cas9 protein and add different types of functionality to the CRISPR-Cas9 system.

There are two types of base editors, cytosine base editors that can cause C on a DNA strand to be replaced by another base (typically T) [26] and adenine base editors that can cause A to be replaced by G [27]. The editing is achieved by a deaminase domain that directly chemically modifies DNA bases (Fig. 1). If CRISPR-Cas9 is fused to any of a variety of naturally occurring cytidine deaminase domains (e.g., from the APOBEC1 protein or the AID protein), the

deaminase has the potential to act upon any C within an editing window on the non-target DNA strand, which is accessible as a single strand due to hybridization of the target strand with the guide RNA. The deaminase converts C to U (uracil), which ordinarily would be repaired back to C; the U would be removed, and due to the presence of G in the complementary position in the other strand, the cell would replace the removed nucleotide with C. Two mechanisms typically used in cytosine base editors act to prevent this repair. First, an additional domain, an inhibitor of the enzyme responsible for uracil repair, is added to Cas9. Second, Cas9 is mutated so that it can cut, or "nick," the target DNA strand (nickase Cas9, or nCas9) but not the non-target strand. After the cytosine base editor moves on from the site, the cell's nick repair machinery acts by stripping away nucleotides around the site of the nick on the target strand and then replaces the nucleotides via complementarity to the non-target strand. For any U present in the non-target strand, an A is introduced into the complementary position in the target strand. After nick repair is completed, the cell eventually removes the U, but instead of replacing it with the original C, it replaces it with a T due to complementarity with the A now present in the target strand. Thus, a C-G base pair is edited to a T-A base pair.

Adenine base editing works in much the same way, except that an evolved adenosine deaminase domain (there is no naturally occurring adenosine deaminase that acts on single-strand DNA, hence the use of protein evolution to create a novel one in the laboratory) is fused to nCas9. Within the editing window on the non-target strand, A is converted to I (inosine), nick repair occurs on the target strand, a C is placed in the target strand opposite the I, and eventually the I is replaced with a G to complement the C in the target strand—and so a A-T base pair is edited to a G-C base pair.

The key to base editing is to ensure that the target C or A to be edited lies within the editing window of the non-target strand, with the window's range being set by the distance from the PAM sequence (e.g., 13–17 bases upstream of the PAM). However, any C or A within the window has the potential to be edited, even if such editing would be undesirable. These so-called bystander edits can be minimized by switching the Cas9 protein, the guide RNA, or the deaminase domain used in the editor.

Although cytosine and adenine base editors are limited with respect to the types of edits that can be made—single-nucleotide changes, largely transition mutations—if a desired edit is feasible with base editing, it can occur with very high efficiency, rivaling the efficiency of NHEJ editing while maintaining the precision of HDR editing. Indel mutations can occur at a low rate (typically $<1\%$) at the target site with a base editor, and off-target editing can occur as well, although off-target edits tend to be restricted to single-nucleotide changes consistent with the base-editing mechanism.

1.3 Epigenome Editing

If dCas9 is deployed to a site in the genome, with no extra domains fused to it, it has the potential to interfere with factors that ordinarily interact with that site via steric interference [28]. If the targeted site is regulatory in nature, e.g., in a promoter or transcriptional enhancer, then dCas9 can interfere with gene expression. This so-called CRISPR interference has been exploited to knock down the expression of specific genes, reminiscent of but quite distinct from RNA interference mediated by short hairpin RNAs. CRISPR interference is potentiated further if dCas9 is fused to a domain, such as the KRAB (Krüppel-associated box) domain, that actively represses gene expression via modulation of chromatin structure around the genomic site (Fig. 1) [29]. Because no change is made to the DNA sequence itself, the interference is reversible—once dCas9 is no longer present at the site, gene expression returns to normal. The opposite phenomenon, CRISPR activation, can be achieved by either fusing domains that enhance gene expression (such as the transcriptional activator VP16) to dCas9 or by extending the sequence of the guide RNA on its 3′

end with RNA aptamers that can recruit activator domains to the complex (Fig. 1) [29]. CRISPR interference and CRISPR activation have proven to be so generalizable across the genome that they can be used to do large-scale, genome-wide functional screens to identify individual genes that modulate a cellular phenotype of interest [30].

A different type of epigenome editing entails alteration of the methylation state of DNA sequences, particularly at cytosine bases in CpG dinucleotide sequences. Methylation around the transcription start site usually is linked to gene silencing, whereas non-methylation is linked to gene activation. Fusions of engineered nucleases to demethylase or methyltransferase domains can modulate gene expression in either direction [31, 32], though the permanence of the methylation changes introduced by these epigenome editors remains to be established.

1.4 Other Types of Editing

Like base editing and epigenome editing, prime editing takes advantage of the search-and-bind capacity of catalytically impaired Cas9 to bind a target site specified by a guide RNA. Prime editing was recently developed in an attempt to overcome the limitations in the types of changes that can be made by base editing, as well as the limitations in efficiency of HDR editing [33]. It entails two modifications to a CRISPR-Cas9 complex. The first is a fusion of nCas9 to a reverse transcriptase that can build a DNA strand complementary to a single-strand RNA substrate. The second is extension of the guide RNA on its 3′ end to add an RNA sequence that is complementary to the non-target DNA strand but also includes a desired mutation; this extended guide RNA is called the pegRNA. The prime editor's nCas9 nicks the non-target strand (unlike base editors, which nick the target strand), and the 3′ end of the pegRNA hybridizes with the non-target strand on one side of the nick (5′ direction), which creates an RNA-DNA duplex that serves as a template for reverse transcriptase, which builds a DNA sequence (including the mutation) on the middle portion of the pegRNA. When the reverse transcriptase completes its job, and the prime editor moves on from the site, there is a new, extra DNA strand that can replace part of the non-target strand, resulting in permanent incorporation of the mutation after DNA repair is complete. Although this process is complex, and the efficiency is not high (though typically higher than that of HDR), prime editing can precisely introduce a wide variety of mutations at the target site: single-nucleotide changes, whether transition or transversion mutations, and indel mutations of various sizes up to dozens of base pairs in length.

The final type of editing is RNA editing, which takes advantage of the Cas13 family of proteins [34]. As with Cas9 and Cas12, CRISPR-Cas13 systems comprise two-component, protein-RNA complexes, but they bind and act upon RNA molecules, not DNA molecules. RNA editors can be used to either degrade target RNAs [34] or to catalyze base edits (A-to-I edits or C-to-U edits) in target RNAs [35, 36]. Because RNA molecules are labile, unlike DNA, the persistence of the RNA effects depends on the continual presence of the RNA editor and its acting on any newly transcribed RNA molecules.

2 Disease Modeling and Diagnostics

The predominant use of genome-editing technology has been to generate animal and cellular models of disease. As recounted above, homologous recombination has been exploited to generate thousands of genetically modified mouse models, many of which have been useful in understanding the pathobiology of various cardiovascular and metabolic disorders. The development of modern genome-editing tools has only accelerated the pace of model-based discovery. Whereas homologous recombination involves a substantial amount of work over 1–2 years to develop mouse models—modification of mouse embryonic stem cells, addition of modified stem cells into mouse blastocysts, derivation of chimeric mice in which (hopefully) the genetic modification is present in germ cells, and breeding of

subsequent generations to eventually obtain knockout or knock-in mice with the desired genotypes—engineered nucleases, particularly CRISPR-Cas9, have made it possible to reduce the time needed to generate mouse models to a matter of weeks. The genome-editing tool is injected directly into single-cell mouse embryos of any genetic background—either as DNA vectors, RNA, or protein—with the result that any edits made in the zygote will be present in the entire animal following birth. CRISPR-Cas9 has proven especially potent in both knocking out genes and knocking in specific alterations [37, 38]. The technique has been applied across many species, including large animals that recapitulate human cardiovascular and metabolic diseases more faithfully than mice.

Genome editing has proven highly advantageous in facilitating the use of human pluripotent stem cells (hPSCs) for disease modeling. Early hPSC-based modeling studies used non-matched induced pluripotent stem cell (iPSC) lines from patients with disease and from healthy individuals, ignoring differences in sex, ethnicity, genetic backgrounds, epigenetics, iPSC derivation technique, cellular source, etc., meaning that any differences observed between somatic cells differentiated from the iPSC lines were subject to severe confounding. Genome editing permits the generation of matched, isogenic hPSC lines with and without a disease-associated mutation, making any observed differences much more reflective of disease pathogenesis. In the first study that used isogenic hPSC lines to dissect cardiovascular and metabolic traits, TALENs were used to edit knockout and knock-in mutations into hPSCs, which were then differentiated into somatic cell types like hepatocytes and adipocytes that were used to clarify the roles of the *SORT1*, *AKT2*, and *PLIN1* genes in lipoprotein metabolism, hypoinsulinemic hypoglycemia with hemihypertrophy, and lipodystrophy, respectively [39]. Since then, numerous studies of cardiovascular and metabolic traits with isogenic cell lines have been undertaken [40].

As an offshoot of disease modeling studies, isogenic iPSC lines are now being used to ascertain the pathogenicity of genetic variants of "uncertain significance" identified in patients suspected to have inherited cardiovascular diseases, like cardiomyopathies and rhythm disorders. These studies entail either (1) starting with an iPSC line derived from a healthy individual, following by editing in of the variant into the iPSC line, or (2) generating an iPSC line from the patient, and correction of the variant in the iPSC line. In one of the first demonstrations of this diagnostic approach, the process of generating iPSCs with and without a variant of uncertain significance identified in a patient with hypertrophic cardiomyopathy, followed by phenotyping of iPSC-cardiomyocytes, was completed in less than 3 months and found the variant to be benign, allowing for the patient to be informed of the results at her regularly scheduled follow-up clinic visit [41].

3 Therapeutic Genome Editing

There have been a number of exciting advances in therapeutic genome editing in the past decade. With respect to cardiovascular and metabolic diseases, virtually all therapeutic genome-editing applications that are currently envisioned would take place in vivo (within the bodies of living patients) rather than ex vivo (in cells taken from the body, treated outside of the body, and then transplanted back into the body). As it happens, many of the proof-of-concept studies on in vivo therapeutic genome editing have focused on a gene of outstanding significance for cardiovascular disease, *PCSK9*—due to the ease of assessing the pharmacodynamic effects of *PCSK9* editing, reduction of blood PCSK9 and cholesterol levels—and a review of just those *PCSK9* studies provides a comprehensive overview of the progress in the field.

PCSK9 is a key regulator of the metabolism of low-density lipoprotein cholesterol (LDL-C). People with naturally occurring *PCSK9* nonsense mutations have substantially reduced LDL-C levels as well as up to 88% reduction in risk of coronary heart disease [42], and a few individuals with complete knockout of *PCSK9* have been

identified [43, 44]. These observations have made *PCSK9* one of the most compelling therapeutic targets for the treatment and prevention of coronary heart disease, with a number of PCSK9-targeting drugs either approved for use in patients or being evaluated in clinical trials.

In the first demonstration of highly efficient in vivo mammalian genome editing, an adenoviral vector encoding SpCas9 and a guide RNA targeting exon 1 of the mouse *Pcsk9* gene were used to knock down *Pcsk9* in the liver by introducing loss-of-function mutations via NHEJ [45]. Adenoviral vectors are generally not used in patients due to the risk of severe and possibly fatal immune responses to the vectors; adeno-associated viral (AAV) vectors are better tolerated and preferred for clinical use. A disadvantage of AAV vectors is that they have a limited cargo capacity (<5 kilobases) that cannot accommodate most genome-editing tools in a single vector, e.g., SpCas9 (the gene alone being ≈4.2 kilobases) and a guide RNA expression cassette (≈500 base pairs). Adenoviral vectors have a much large cargo capacity that can easily fit any genome-editing tool. In this study, the investigators administered the CRISPR adenoviral vector or a control adenoviral vector to wild-type mice. After several days, the livers of mice receiving the CRISPR vector had >50% editing at the *PCSK9* target site. The most common edits were one-base pair or two-base pair deletions or insertions, with bigger edits as large as dozens of base pairs in size occurring much less frequently. The editing was accompanied by reductions of blood PCSK9 protein of ≈90% and blood cholesterol levels of 35–40%, almost as much as the 36–52% reduction of cholesterol observed in germline *Pcsk9* knockout mice [46]. This initial study showed no evidence of mutagenesis at a handful of candidate off-target sites.

In a later study by a different group of investigators, the same editing results were reproduced in mice treated with a similar adenoviral vector with SpCas9 and the same *Pcsk9* guide RNA [47]. The investigators rigorously assessed for off-target mutagenesis within the liver through a two-step approach. They first screened for potential off-target sites using a biochemical technique called CIRCLE-seq, in which circularized mouse genomic DNA fragments were exposed to SpCas9 protein and the *Pcsk9* guide RNA in vitro, followed by next-generation sequencing to identify linearized DNA fragments, yielding a list of 182 candidate sites. PCR amplification of the candidate sites and deep next-generation sequencing of the amplicons from liver genomic DNA samples from the CRISPR-treated mice found no evidence of off-target mutagenesis.

Despite the encouraging results suggesting that it was possible to perform genome editing in a manner that was both efficient and safe (with respect to off-target mutagenesis), they were not directly relevant to what might happen with *PCSK9* editing in human beings, due to three major differences between mice and humans. First, there are substantial differences between the mouse *Pcsk9* sequence and the human *PCSK9* sequence, making it almost impossible to identify an efficient guide RNA matching both species. Second, there are substantial differences between the mouse genome and the human genome, meaning that off-target profiling in the context of the mouse genome is not predictive of off-target editing in the human genome. Third, there are substantial physiological differences between mouse hepatocytes and human hepatocytes, and editing outcomes might differ significantly between the two cell types.

In order to better assess the efficacy and safety of a potential human *PCSK9*-editing therapy, a study was undertaken in chimeric liver-humanized mice, a model system in which a mouse's own hepatocytes have been replaced with transplanted primary human hepatocytes [48]. Liver-humanized mice were treated with an adenoviral vector encoding SpCas9 and a guide RNA targeting exon 1 of the human *PCSK9* gene. There was ≈50% NHEJ editing of the human *PCSK9* alleles present in the humanized liver, with no mutagenesis observed at a handful of candidate off-target sites. The editing was accompanied by ≈50% reduction of the amount of human PCSK9 protein in the blood. Although the adenoviral vector would not be appropriate for use in patients, the results of this study speak

to the possible efficacy and safety of *PCSK9*-editing therapy in humans.

The next set of advances entailed moving away from adenoviral vectors to delivery approaches more amenable to clinical translation. In the first study to use AAV to achieve highly efficient in vivo mammalian genome editing, SaCas9—which is significantly smaller than SpCas9—along with either of two guide RNAs targeting the mouse *Pcsk9* gene was encoded in a single AAV vector [15]. Either AAV vector, upon administration to wild-type mice, resulted in 40–50% editing of the *Pcsk9* gene in the liver, with corresponding reductions of blood PCSK9 protein of >90% and blood cholesterol levels of ≈40%, very similar to the results seen in the earlier studies with adenoviral delivery of SpCas9.

The successful use of AAV was followed by the demonstration of nonviral methods to deliver CRISPR-Cas9 into the liver in vivo. The best proven nonviral vehicle is the lipid nanoparticle (LNP). In the first study with CRISPR-Cas9 delivered into the liver solely via a nonviral method, LNPs, formulated either with the SpCas9 messenger RNA or with a synthesized guide RNA targeting *Pcsk9*, were serially injected into wild-type mice, resulting in moderate reduction of the liver PCSK9 protein level, by 40–50% [49]. In a subsequent study, LNPs formulated either with the SpCas9 messenger RNA or with two synthesized guide RNAs targeting *Pcsk9*, with chemical modifications to enhance stability of the RNAs in vivo, were administered together into wild-type mice as a one-shot therapy [50]. LNP treatment resulted in >80% editing of the gene in the liver—the very high editing rate resulting from NHEJ-mediated deletion between the sites targeted by the two guide RNAs (Fig. 1), which typically is more efficient than NHEJ editing at a single site—as well as an absence of PCSK9 protein in the blood and 35–40% reduction of blood cholesterol levels. No editing was observed in the lungs or spleen, suggesting that either the LNPs were taken up specifically by the liver or that the *Pcsk9* locus was accessible to SpCas9 only in liver cells.

After the various demonstrations of efficacy of in vivo genome editing using CRISPR-Cas9 nucleases, the next advance was the development of base editing as a therapeutic approach. Exploiting the ability of cytosine base editors to introduce nonsense mutations into genes via C-to-T changes or G-to-A changes (the latter resulting from C-to-T edits on the antisense strand) in specific codons, a proof-of-concept study used an adenoviral vector to deliver the cytosine base editor BE3 along with a guide RNA targeting *Pcsk9* into the livers of wild-type mice [51]. The guide RNA targeted tryptophan-159, with the codon TGG, for which editing of either or both guanines to adenines results in a stop codon. Mice treated with the adenoviral vector displayed ≈30% edited alleles, mostly the expected stop codons but with some bystander edits resulting in missense mutations, as well as indel mutations at a rate of 1–2%. There were corresponding reductions of blood PCSK9 protein of ≈60% and blood cholesterol levels of ≈30%. These were smaller reductions than observed in the previous mouse studies with CRISPR-Cas9 nucleases, though this reflects the use of early, non-optimized base-editing technology.

The same *Pcsk9* base-editing strategy was used in one of the first demonstrations of fetal genome editing in mice. Whereas with fetal surgery, life-threatening anatomical defects are treated while patients are still in the womb, fetal genome editing would be reserved for patients with severe genetic disorders already causing damage at the prenatal stage and resulting in high morbidity and mortality after birth. An adenoviral vector expressing the BE3 base editor targeting *Pcsk9* was administered to the livers of fetal mice via injection into the vitelline vein, the precursor to the portal vein [52]. This procedure performed before birth resulted in permanently reduced blood PCSK9 and cholesterol levels after birth. (Of note, hypercholesterolemia is not a condition that under any circumstances would require prenatal treatment, and the experiment was performed only as a proof of concept of fetal genome editing.)

Other types of genome-editing technologies have been employed to knock down *Pcsk9* in mice, although their prospects for long-term therapeutic use are unclear. In a demonstration of epigenome editing, catalytically dead SaCas9

was fused to a KRAB repressor domain; the editor and a guide RNA targeting the *Pcsk9* promoter were encoded in two separate AAV vectors [53]. The AAV vectors were co-administered to mice, resulting in ≈50% reduction in hepatic *Pcsk9* gene expression and ≈80% reduction in blood PCSK9 protein levels, along with a corresponding reduction in blood LDL-C levels. However, the therapeutic effects weakened over the course of a few months, suggesting that as expression of the epigenome editor waned, so too did the repression of *Pcsk9*. A different study successfully demonstrated the use of an RNA editor, specifically CasRx (Cas13d) delivered by an AAV vector, to knock down *Pcsk9* expression [54], though as with epigenome editing, the therapeutic effect would be expected to last only as long as the expression of the editor persisted. Both epigenome editing and RNA editing would likely require repeated administrations of the treatment in order to maintain a chronic therapeutic effect, unlike nuclease editing or base editing, which could provide "one-and-done" treatment options.

A key step toward translation of therapeutic genome editing to human patients is the demonstration of efficacy and safety in nonhuman primates. One of the first such studies used meganucleases rather than CRISPR-Cas9 to target the *PCSK9* gene [55]. The investigators used an AAV vector encoding a meganuclease specific for a sequence in exon 7 of *PCSK9* and expressed from a strong liver-specific promoter. When administered to rhesus macaques via intravenous injection at various doses, a very high dose of the AAV vector resulted in 46% editing of *PCSK9* in the liver, with corresponding reductions of blood PCSK9 protein of 85% and blood LDL cholesterol levels of 56%. (Lower AAV doses produced substantially lower editing rates.) In this ongoing study, the reductions have persisted to more than 3 years so far [56]. However, several serious shortcomings have emerged from this study. First, there was on-target editing of an unexpected nature. Although the intent was to disrupt *PCSK9* via NHEJ, there was substantial integration of AAV vector sequences at the site of the double-strand break, at such a high rate that in fact the most frequent editing event was viral sequence insertion—with unknown safety consequences. Second, there was significant off-target mutagenesis from the meganuclease at numerous genomic sites both in the monkeys and in human hepatocytes. Third, there were substantial T-cell immune responses against both the AAV vector and the meganuclease, resulting in surges in blood transaminase levels in all treated monkeys several weeks after treatment, consistent with immune-mediated hepatocyte death, though the surges spontaneously resolved over the course of several weeks to months without any apparent lasting effects. Despite these shortcomings, this study established the feasibility of "one-and-done" genome editing with therapeutic effects lasting for years and, likely, for the lifetimes of the treated animals.

In a more recent nonhuman primate study, the investigators used adenine base editing to knock down *PCSK9* in cynomolgus monkeys [57]. LNPs encapsulating both the adenine base editor messenger RNA and a synthetic guide RNA targeting *PCSK9* were used to deliver the editor into the liver, resulting in ≈66% editing of *PCSK9*, ≈90% reduction of blood PCSK9 levels, and ≈60% reduction of blood LDL-C levels persisting more than 8 months in an ongoing study. This study contrasted with the meganuclease study in several important ways. The editing rates were consistently and substantially higher with LNP-delivered adenine base editor compared to AAV-delivered meganuclease. Due to the LNP approach not using any DNA components, there was no risk of vector sequence integration into the genome, and the use of base editing resulted in a specific base pair change in *PCSK9*, in contrast to the semi-random indels from NHEJ induced by the meganuclease. With base editing, there was no discernible off-target editing at a large number of candidate sites in human hepatocytes, and low-level off-target editing at just a single candidate site in monkey liver, with the off-target editing being confined to single-base pair changes (rather than indels). Finally, the LNP treatment resulted in immediate, transient rises in blood transaminase levels that spontaneously resolved in 1–2 weeks, with no subsequent transaminitis or other signs of immune responses.

On the strength of all of the aforementioned studies, *PCSK9* editing appears to be poised to enter clinical trials for patients with hypercholesterolemia and coronary heart disease. The therapeutic potential for genome editing in cardiovascular and metabolic diseases, of course, extends beyond *PCSK9*. Preclinical studies have established the prospects for the treatment of homozygous familial hypercholesterolemia by targeting the *ANGPTL3* gene in the liver [58] and the treatment of Duchenne muscular dystrophy-associated cardiomyopathy by targeting the *DMD* gene in the heart [59]. A clinical trial in which genome editing is being used to treat transthyretin cardiac amyloidosis by targeting the *TTR* gene in the liver [60] is already underway, with patients dosed as of late 2020.

4 Outlook

Genome editing is already having a transformative effect on research on cardiovascular and metabolic diseases, and it has the potential to have a similar impact on the practice of cardiovascular medicine as therapeutic applications begin to reach the clinic. Remarkably, almost all of the work described in this chapter has unfolded in just the last decade, and we can undoubtedly expect the next decade to see just as extraordinary a rate of progress of development of genome-editing technologies.

Note Portions of this chapter were adapted from reference [61] in accordance with the terms of the Creative Commons Attribution 4.0 International License: https://creativecommons.org/licenses/by/4.0/.

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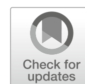
Part II

Bioinformatics



Online Databases of Genome Editing in Cardiovascular and Metabolic Diseases

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Abstract

Metabolic and cardiovascular diseases are world-concerning pathologies that affect an important percentage of the population. Nowadays, advances in the genetic background of these diseases allow new approaches to models and therapies, as well as different gene edition trials. Furthermore, technological improvements in gene editing go along with the development of new online and biocomputational tools that provide us alternative ways to explore pathologies. In this chapter, historical gene editing methods are discussed but focusing on CRISPR-Cas system in detail and also online resources available to perform these types of experiments. Here, the different strategies for gene editing and their online tools are gathered, putting the light on its application in the study and treatment of cardiovascular and metabolic diseases.

Keywords

Cardiovascular disease · Metabolic disease · Gene editing · CRISPR-Cas systems

1 Cardiovascular and Metabolic Disease Genetic Basis

New genomic techniques have transformed our knowledge of the sources of congenital heart disease and have permitted a more rigorous pathogenesis definition of congenital heart disease in all ages of patients and even prenatal stages of life [1]. Numerous genes are associated in the propensity to arrhythmogenic syndromes as well as cardiomyopathies [2]. Cardiac arrhythmias are

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common and hereditary cardiomyopathies; sodium voltage-gated channel alpha subunit 5 mutations are the main causes of inherited arrhythmia syndromes, affecting myocyte structural proteins [2].

Hypertrophic cardiomyopathy and dilated cardiomyopathy (DCM) are genetic disorders that lead to life-threatening arrhythmia and heart failure, ultimately needing cardiac device implantation or heart transplantation [3]. Alpha 1-3-galactosyltransferase and metallopeptidase with thrombospondin type 1 motif 7 genes and alpha 1-3-N-acetylgalactosaminyltransferase are associated with angiographically confirmed coronary atherosclerosis, while cyclin and CBS domain divalent metal cation transport mediator 2 and apolipoprotein A5 genes were associated with hypertension and hypertriglyceridemia [4].

Conduction defects, including atrioventricular, atrial, and left bundle branch blocks, ventricular arrhythmias, and supraventricular tachycardia, are frequently detected in patients with dilated cardiomyopathy produced by the lamin A/C (LMNA) mutations. These mutations in LMNA interrelate with the genome and affect the several gene expressions. Mutations in titin and desmosome genes are related to an elevated incidence of atrial and ventricular arrhythmias. Functional genetic variants in genes encoding protein constituents of ion channels might influence to develop patients with cardiac arrhythmias with hereditary cardiomyopathies [2].

DNA sequencing technology advances have discovered numerous causative genes for hereditary thoracic aortic aneurysms and dissections, including Marfan syndrome (MFS), a heritable disorder of the tissues that connect the skeletal, cardiovascular, ocular, and pulmonary organ systems, cardiovascular manifestations (aortic root aneurysm and/or dissection), and ectopia lentis and offered molecular genetic testing of fibrillin 1 (FBN1) [5]. Filamin C gene pathogenic variants were initially described in myofibrillar myopathy. Mutations in the ryanodine receptor 2 gene are connected with cardiac arrhythmias as well as cardiomyopathies. Mutations in the phospholamban (PLN) gene cause arrhythmogenic cardiomyopathy (ACM) and DCM. The phenotype is described by heart failure with refractory characteristic and a fairly elevated prevalence of ventricular arrhythmias [2]. Plakophilin 2 (PKP2) gene mutations are the primary bases of ACM. Among genes coding for the desmosome proteins, only the PKP2 gene has been related to develop cardiac arrhythmias, with independency of cardiomyopathy [2].

Cystic fibrosis (CF) is an autosomal recessive genetic disorder that is produced by CF transmembrane conductance regulator (CFTR) gene inherited mutations and is an important lung genetic disorder that primarily causes severe injury of normal lung functions [6]. The CFTR gene encodes a protein that mostly controls ion and fluid homeostasis across epithelial barriers and, when the gene mutates, transports chloride and bicarbonate in the epithelial tissues of the lung developing abnormal features [7], leading to reduced mucociliary clearance and distorted hydration of airway surface fluid. In addition to lungs, the CFTR gene is similarly expressed in the epithelium of many organs including the kidney, liver, and pancreas [8]. The main population of CF patients have a defect in the gene characterized by the absence of three nucleotides in the in-frame deletion of a phenylalanine residue at position 508 of the polypeptide chain (ΔF508) [7]. Chronic obstructive pulmonary disease (COPD) is a usual inflammatory disease with elevated global morbidity and mortality, and it is now expected to be the third largest cause of related death and chronic illness [7]. The main replicated and most important genome-wide association studies single nucleotide polymorphisms (SNPs) at the 17q12-21 locus are associated with asthma symptoms in early life [9].

Lung cancer is growing as one of the main malignancies with excessive mortality and morbidity worldwide. Lung cancer progression includes multiple gene mutations and signaling pathways [7]. Mutations of genes include B-Raf proto-oncogene, KRAS proto-oncogene (K-RAS), epidermal growth factor receptor, Ret proto-oncogene, and MET proto-oncogene that are related to lung cancer prognosis [10].

Proto-oncogenes related to lung cancer comprise insulin-like growth factor 1 receptor, catenin

delta 2, remodeling and spacing factor 1, SRC proto-oncogene, protein tyrosine kinase 2, CD38, ROS proto-oncogene 1, and Fos proto-oncogene, among others. The aforementioned genes might act as oncogenes to increase the metastatic and invasive ability of cancer cells and might also stimulate progression of lung cancer [7].

The Runx3 inactivation is a critical event in the lung adenocarcinoma development. Targeted Runx3 inactivation provokes lung adenomas and distinctly reduces the adenocarcinoma formation latency induced by oncogenic K-RAS. Abnormal RUNX3 inactivation is commonly discovered in lung cancer tissue and is also related to lowly prognosis in lung cancer patients [10]. The tumor suppressor gene inactivation also has an important role in the lung cancer progression. These genes include cyclin-dependent kinase inhibitor 2A and tumor protein P53, among others. The protein expression of these tumor suppressor genes can reduce cell proliferation and colony formation, avoid migration and cell invasion, and display tumor regression during tumorigenesis [7].

Huntington's disease (HD), a neurodegenerative disorder, is produced by CAG trinucleotide repeat expansion that encodes a polyglutamine (poly Q) tract in the huntingtin (HTT) gene [11]. The mutation is an extended CAG trinucleotide repeat in the gene encoding the HTT protein, which produces a polyglutamine stretch at the N-terminus of the protein [12]. Those who inherit 36–40 repeats in the gene have a less developed form of the disease; even they never become symptomatic or may progress symptoms of HD in older age [13].

Chorea is increasingly observed in patients with pathogenic mutations in genes linked to cerebellar ataxia. Different types of disease mechanisms can affect medium spiny neuron cells and clinically lead to chorea, including degenerative processes (e.g., HD and HD-like), developmental abnormalities (e.g., NKX2-1- and FOXG1-related choreas), and disrupted post-receptor intracellular signaling (ADCY5- and PDE10A-related choreas) [14].

2 Significance of Genome Editing in Cardiovascular and Metabolic Diseases

The possibility of DNA manipulating has permitted many advances in cardiovascular and metabolic diseases [15]. Genome editing technologies include transcription activator-like effector nuclease (TALEN), zinc finger nuclease (ZFN), and clustered regulatory interspaced short palindromic repeats. The clustered regulatory interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (CRISPR/Cas9) system has been starting up a new era of management throughout precision genome surgery [16].

Genome editing technology is developing and being useful into cardiovascular medicine and research to accelerate a greater understanding of the cardiovascular pathogenesis and the development of novel therapies. Genome editing-based therapy comprises the addition of therapeutic genes to specific sites in the genome, correction or inactivation of deleterious mutations that cause cardiovascular diseases, and the deletion of detrimental genes. Genome editing can be used for therapeutics in monogenic cardiovascular diseases (i.e., sudden cardiac death, familial hypercholesterolemia, and MFS) or prevention of polygenic cardiovascular diseases (i.e., hypertension) [4].

3 Lung Genetic Disorders and Therapeutic Options

CRISPR-Cas is evolving as an innovative technology for the management of various genetic diseases. Genome editing biomacromolecule delivery through viral or nonviral vectors has been suggested as new therapeutic options for CF and α-1 antitrypsin deficiency [17]. While traditional gene therapy pursues to transport a new functional gene into targeted cells to fix the genetic defect, genome editing can exactly remove and insert targeted genes to treat genetic defects in a site-specific manner [7]. CRISPR-Cas9-based genome editing approaches have

been projected for the therapy of lung cancers, including editing tumor suppressor genes, proto-oncogenes, or chemotherapy resistance-related genes [8]. CRISPR-Cas9 technology has edited some genes such as mitofusion 2, tumor suppressor miR1304, and Kelch-like ECH-associated protein 1 [7].

Genome editing by CRISPR-Cas9 in COPD and asthma denotes an auspicious method to reversal corticosteroid resistance. The disruption of corticosteroid resistance-related genes which are overexpressed or overactivated in the airways and alveolar sacs via CRISPR-Cas9 technology may deliver better therapeutic results [8]. HTT dropping by gene silencing is a methodology that has comprehended immense progresses in recent years. Mutant HTT is recognized to mainly source disease by a dominant toxic effect. Zinc finger proteins can be synthetically influenced to bind *HTT* DNA, fused to a functional domain such as a nuclease [13]. The future comes with delivery technologies for genome editing, nonviral delivery of genome editing systems, viral delivery of genome editing systems, nanoparticle-mediated delivery, delivery routes for genome editing of the lung genetic disorders, intratracheal/intranasal instillation, and inhalation delivery [8].

4 History and Genome Editing at Present

Traditionally, genetic studies were based on the discovery and analysis of spontaneous mutations and attempts to improve mutagenesis through the use of radiation or chemical treatment. Subsequently, the technique of inserting transposons was introduced in certain organisms; however, in the case of the aforementioned procedures, they originate in changes in random areas of the genome [18]. It is worth mentioning because of its huge importance that the first targeted genomic changes were in organisms such as yeast and mice in the 1970s and 1980s. To develop this technique, homologous recombination was used in the process of gene selection. Although this method, namely, gene targeting, was accurate in mouse cells, the same was not true when it was applied to other species [18].

Thanks to new genome editing techniques, targeted genetic manipulations can be performed in most organisms and cells. Highly efficient genome editing methods are based on making a directed DNA double-strand break (DSB) in the sequence of the pointed chromosome. There are also extremely specific nucleases capable of promoting homologous restoration in mammalian cells, among others, as has been demonstrated in numerous studies. This fact has enabled their use in programmable genome editing. Therefore, today, three main classes of programmable nucleases are used in genome editing to produce the aforementioned DSB, and these are TALENs, ZFNs, and CRISPR-Cas (this technique is the most widely used in research worldwide in recent years) [18, 19].

ZFNs are characterized by combining a DNA excision domain of a bacterial protein and a cluster of zinc fingers (discovered in sequence-specific eukaryotic transcription factors). TALEN, for its part, is similar to ZFN in that it uses the same bacterial excision domain; however, it is associated with DNA recognition zones through transcription factors originating from a specific type of bacteria, namely, phytopathogenic bacteria. On the other hand, CRISPR-Cas is defined as a prokaryotic system of acquired immunity to invading DNA or RNA [18].

CRISPR was discovered in the *Escherichia coli* genome as uncommon repeat segments. Subsequently, CRISPR was shown to have certain repeat spacer sequences from attacking bacteriophages. Also noteworthy is the presence of a cluster of *cas* genes within this set, which is of great importance for the excision of foreign genetic material. The type II CRISPR-Cas system from the bacterium *Streptococcus pyogenes* was suggested as a potent tool for gene editing of various organisms [19].

The enormous importance of CRISPR-Cas system use lies in the correction of genetic disorders such as Duchenne muscular dystrophy and beta-thalassemia. It can also be used in the inactivation of certain viruses such as hepatitis B virus, human papillomavirus, human

immunodeficiency virus 1, and virulent phages [19]. In summary, the CRISPR-Cas system has become an innovative technology that has transformed genome engineering as well as genome editing techniques [20].

5 How to Perform a Genome Editing Experiment Nowadays (CRISPR-Cas)

5.1 Molecular Mechanism and Components of the CRISPR-Cas System

CRISPR is a system displayed by most archaea and some bacteria that allows them to develop an antivirus response based on the distinction between self and nonself [21]. This system has spacers between the repeated sequences, which are DNA fragments derived from infecting viruses or plasmids that are processed by Cas nucleases. When there is another infection from this virus, the spacers act as transcriptional templates and are transcribed and processed to create a guide CRISPR-RNA (crRNA). The function of the crRNA is to guide Cas to cleave target DNA sequences of the attacking viruses [22]. CRISPR has transformed the field of gene edition and genome engineering, as it permits scientists to target and alter genes in a very specific way. It can also be used to regulate transcription and to develop gene therapy. However, it has some challenges to overcome, such as off-target mutations, protospacer adjacent motif (PAM) dependency, the fabrication of gRNA, and delivery methods [22].

The principal components of the CRISPR-Cas system are as follows:

- Cas protein, which has nuclease domains used to cleave determined sequences of the genome.
- Noncoding RNA: transactivating crRNA (tracrRNA), CRISPR-RNA (crRNA), and artificial fusion of both called single guide (gRNA). They target specific sequences and can include templates to add new sequences to the genome [23].

To accomplish genome edition, we need to create a DSB, so that the DNA repair process can follow and change the sequence. First, the gRNA binds the Cas, forming a ribonucleoprotein complex, and leads the nuclease to a selected spot of the genome [22]. The gRNA will recognize this site because it includes a sequence match to the crRNA part of this molecule and a PAM after the target sequence [24]. Then, Cas cleaves the single DNA strand upstream of the PAM and the opposite strand, in a way that forms a blunt end DNA DSB in the selected place [22]. This break will activate the DNA repair procedure, which can take place in two different processes: nonhomologous end joining (NHEJ) and homology-directed repair (HDR); and they will determine the type of alteration in the gene. The NHEJ pathway repairs the DSB but leads to short insertions or deletions close to the cutting site, so it is useful to knockout genes. HDR, which is a more difficult process, can insert specific sequences into the cutting place, when you add an exogenous template DNA in the system [24].

Customizable nucleases, like Cas for CRISPR-Cas system, open new doors to incorporate their use in other application, including fields beyond genome editing and gene and cell therapies, such as to change the epigenome, to perform genome imaging in live cells, or even to use them as a diagnostic method to detect nucleic acids [25]. It is also worth mentioning that to improve the characteristics of Cas nucleases, scientists are currently developing engineered variants with an expanded targeting scope and with higher DNA specificity [26].

6 How to Prepare a CRISPR-Cas Experiment

1. Choose the appropriate CRISPR system for the experiment aim, the cell line, the target species [27, 28], and the repair DBS (HDR for repair/insert genes and NHEJ for indels) [29].
2. Select single-guide RNA (sgRNA) target sequence of 120 nucleotides approximately and design the repair mechanism. This step

requires the identification of one PAM sequence in the targeted gene and his 20-nt upstream sequence to direct the nuclease activity. Furthermore, it is important to scan the genome of the species, looking for off-target effects nearly of the problem sequence [27, 30].
3. Produce sgRNA and transfect, cloning it in a plasmid-based system co-expressed with Cas protein or amplifying the sequence for PCR in vitro and, afterward, his insertion in the cell [27, 30].
4. Test the sgRNA to see if the sgRNA can create the DSB for the NHEJ successfully by PCR [27, 30].
5. Design donor RNA, one single- or double-strand oligonucleotide sequence of 100 bp approximately that contains the changes to repair the DNA or introduce the mutations in the target gene. This donor sequence has to have in its extremes cohesive ends homologous to the DSB (HA_L and HA_R), causing the adequate repair and orientation of the sequence [30].
6. Reduce off-target effects, doing the system precisely, efficiently, and permanently to gene editing [29].
7. Clonally select edited cells with markers that can be removed or with PCR-based strategies. Moreover, it's important to assure the gene modification and the changes in protein and transcript levels [28, 30].

7 Different Types of CRISPR-Cas-Based System

There exist two principal classes of CRISPR-Cas system in the function of the structure and number of Cas in the complex: Class I with a multi-protein Cas complex and II, with single multi-domain Cas protein [29, 31, 32]. Moreover, these classes have three subtypes: I, III, and IV for class I and II, V, and VI for class II.

- Type I: Six different Cas proteins take part in this system. They are encoded in one or more operons, forming a CRISPR-associated complex called cascade that makes large gaps in the complementary strand of target sequence [32].
- Type II: This system consists of monomeric Cas9 protein (with target PAM 5′-NGG-3′) [33], tracrRNA, and crRNA, both in a sgRNA. The nuclease activity of Cas9 can make cleavages in the same and complementary strand of the target sequence for the crRNA. The DBS leaves blunt ends, facilitating the HNH or NHEJ repair of the DNA [32]. Despite that, this system is more efficient in cells with low or without p53 [15].
- Type III: It seems like type I, but the difference is that this system can cleave DNA or RNA with Cas10 protein according to the complex subunits [34].
- Type V: The complex only has a nuclease (Cas12a in this case, called as Cpf1 too) and a crRNA and recognizes AT-rich PAM sequences. This endonuclease makes cohesive ends in the target RNA sequence, cutting both strands with Nuc and RuvC activities [32, 35, 36]. Besides that, it doesn't need the RNase III activity to process pre-crRNA. For all this, Cpf1 is more efficient than the CRISPR-Cas9 system [32, 36].
- Type VI: The system only needs a Cas13 protein and a crRNA molecule. Unlike the other Cas proteins, Cas13 has two higher eukaryotes and prokaryotes nucleotide-binding domains that cleave the target RNA [37].

Type II and V systems are less complex and, because of that, more popular in gene editing experiments. Although these systems are useful by themselves, a lot of modifications have been made in the Cas nucleases to improve their functions or implement others. For example, SNPs in one HNH/Nuc otrr RuvC nuclease sites in Cas result in a protein (nickase Ca or nCas) that only cuts one strand and forces the HDR repair [32]. On the other hand, the SNPs in both nuclease activity sites achieve the production of dead Cas9 (dCas). The mutated protein lost its nuclease activity, making possible her fusion with different

complements to modify the transcription activity of DNA target like CRISPRa (activation) system or CRISPRi (inhibition) system or even nucleotide/epigenetic conversion by fusion with other activity domains like deaminases [33, 32, 38] or KRAB or KRAB-MeCP2 fusion protein [15]. Also, modifications of Cas endonuclease with nuclear location signal are implemented to maximize the expression in mammals' single-cell embryos [30].

While the major CRISPR-Cas systems have as target DNA, development systems that are RNA-targeted are relevant nowadays. For example, the type III-based CRISPR-Cas system has an ribonucleoprotein complex with different nuclease and ribonuclease activities by distinct Cas proteins [37].

8 Off-Target Effect, Predictive Tools, and Strategies to Avoid Them

The specificity of the CRISPR-Cas9 method depends both on the segment of the target DNA within the gene to be disrupted and on the nuclease and guide RNA with the corresponding PAM sequence. This RNAg will produce the desired effects on the target sequence, called on-targets. However, the possible binding of the guide RNA to other genomic locations can produce off-target effects [39]. These off-target effects can lead to genomic modifications, unwanted mutations, and risk of genetic mosaicism [40, 41]. Therefore, one of the most important requirements of genomic editing experiments currently is that the number of off-targets should be kept to a minimum, as these effects are unacceptable in medicine [42].

The off-target effects vary greatly depending on where the mismatch is and the number of mismatches. On the one hand, if the mismatch is at the 5′ end of the target, with less than 8–12 base pairs upstream of the PAM sequence, this defect is tolerated. On the other hand, if there are three mismatches outside the 5′ end or in an amount of up to five nucleotides, these mutations can even match the modifications on the target [43]. In addition to off-target effects, they are not the only problem, as there may also be alterations (deletions or rearrangements) in the proximal or distal regions, unspecific polymorphisms, or deregulation of the tumor suppressor protein P53 [40, 41].

To assess off-target gRNA activity, we can divide the study into two main steps. First, it consists of a bioinformatic search for off-target sites. For this purpose, a wide range of tools is available, such as conventional alignment algorithms among them bowtie2, bowtie, TagScan, bwa, or CUSHAW. However, these programs have limitations, as they have a restricted number of mismatches and use a fixed MAP. Therefore, new custom prediction algorithms have been designed for CRISPR-Cas systems to predict off-target sites such as Cas-OFFinder, FlashFry, dsNickFury, CRISPOR, GUIDE-seq, DISCOVER-seq, or CRISPRdirect [44–46].

The algorithms mentioned above will predict mutations, and these predictions can be compared by the latest generation methods. That is, once the off-target sites are searched, scoring methods based on rankings and selections are used. Some of them are the MIT server, which estimates the off-target score by a formula that considers the quantity of mismatched nucleotides and the distance between them. Another is cutoff frequency determination used to calculate the off-target score by multiplying the occurrence of the bases at each position of the guide RNA spacer sequence. Others include CNN_Std, DeepCRISPR, CCTop, CROPIT, preCRISPR, CRISTA, and elevation. And some, such as CCTOP, focus more on the position of the mismatches at the target site, while CROPIT takes more into account the number of mismatches and uses the information of the chromatin state of the whole genome. In addition, modern genomic analysis platforms such as NGS or ddPCR can also identify off-target effects [44].

The use of these tools is intended to simplify the selection of guide RNAs with low off-target effects. But it should also be noted that the differences between off-target effects in certain cell types also depend on epigenomics and

chromatin structure [47]. Because of these obstacles produced by CRISPR technology, CRISPR-Cas9 design strategies are being designed to avoid the production of off-targets. Some of these include the Sniper Cas9 technique, which is a directed evolution method based on *E. coli* to obtain a Cas9 variant with optimized specificity and activity on the target [47]; others include modifying Cas9 to act as a nickase and only generate one cut of the DNA strands [48] or the fusion of Fok I nucleases to Cas9 enzymes forming the fCas9 complex, a dimerization that allows increasing fourfold the specificity of nickases and 140 plus the specificity of conventional Cas9 [49].

9 Guide RNA Designing and Available Online Tools and Databases

The design of the gRNA is a determinant factor for the outcomes of the experiment. Bacterial gRNA is composed of two kinds of RNA: crRNA, which recognizes the target sequence, and tracrRNA, which helps the first to bind the DNA in the correct orientation for the Cas protein [50]. Nevertheless, they can be synthesized together in a sgRNA that is fully functional for genome editing [23]. However, not all Cas-type nuclease requirements are the same; Cas12a protein does only need a single 40-nucleotide crRNA [51].

As it has been described before, it's clear that three-dimensional structure is determined. Formation of the R-loop once the DNA is bound to the gRNA is a crucial step that could disrupt the efficiency of the process and can be estimated [52]. Other chemical modifications can involve changes in CRISPR-Cas activity potentiating the strong interaction between the molecules involved [53, 54]. For instance, it has been reported an increased CRISPR-Cas system efficiency due to the addition of functional groups (i.e., methyl, fluor, or thiol) in the ribose molecule [55]. Other modifications in the phosphate group and the nucleic acid can lead to an improvement in stability, CRISPR-Cas activity, or immune response [54]. Length variation of gRNA also makes a quantitative difference. Thus, truncated gRNA can be as efficient as a non-truncated gRNA, although some cell lines like stem cells seem to be less potent [56]. Additionally, extending the length of the gRNA at 5′ also led to a good response to in vitro and in vivo gene editing [57]. The tertiary structure of the gRNA can also be modified to improve or implement protein recruitment in the catalytic zone, add enzymatic activities, or even carry the substrate for a catalytic reaction [54]. Finally, the gRNA can also be modified by conjugating the donor DNA leading to or facilitating an HDR-mediated gene repair [58].

Off-target effects are determinant in designing the experiment, but on-target efficiency can be also calculated. There are several gRNA designing online tools; some of the most popular are E-CRISP (http://www.e-crisp.org/E-CRISP/), CHOPCHOP (https://chopchop.cbu.uib.no/), and CRISPOR (http://crispor.tefor.net/). On-target efficiency can be measured by a wide variety of web resources, but the methods used differ between them [59]. The most extended ones are from Doench et al., Xu et al., and Ruleset2 [60–62]. All three studies share some conclusions and strategies predicting on-target efficiency. They assessed that certain nucleotides at specific positions of the target DNA sequence would increase on-target efficiency, so as in the gRNA, where GC content is determinant in this efficiency score [60–62]. Nevertheless, there are some limitations to these methods. The main drawback is the molecular context surrounding both the target sequence and the sgRNA. Local chromatin structure [60] or secondary structure of the gRNA [61] can decrease the predictive power of these models, preventing and detecting about 40% of inefficient gRNA. Other tools like Ruleset2 have implemented new parameters such as melting temperature to improve the predictive power [62]. Another miscalculation occurs when the target sequence belongs to a low-expressed exon. This setback can be corrected by using GUIDES (http://guides.sanjanalab.org/#/), which is connected to the tissue genetic expression database GTEx (https://www.gtexportal.org/home/).

Some of the online resources available use multiple of these methods; this is the case of CHOPCHOP (all three), CRISPOR (all three), and E-CRISP [59–62]. However, other tools only use one method: sgRNA Designer (https://portals.broadinstitute.org/gpp/public/analysis-tools/sgrna-design) only uses Ruleset2, whereas SSC (http://cistrome.org/SSC/) calculates the on-target efficiency using the method designed by Doench et al. [60].

There are other features to be considered when using these online tools. One of them is the input: you may need to include a list of target genes with their gene symbols like in CRISPRscan (https://www.crisprscan.org/), or you might need to input the target sequence in a text file like in Cas-OFFinder (http://www.rgenome.net/cas-offinder/). Another important consideration is the experiment type. As it has been described before, CRISPR-Cas system doesn't limit to gene editing, and there are some tools to design gRNA for these new applications such as CASPER (https://github.com/TrinhLab/CASPER), which could be very useful in epigenomic and genome imaging [31]. At last, many of these online resources let you work with multiple species, but some of them are specific in one, for example, fly (https://www.flyrnai.org/crispr/) and mosquito (https://www.flyrnai.org/tools/fly2mosquito/web/) tools.

Ultimately, the gRNA synthesis can be made by different methods; two main strategies can be distinguished: extracellular gRNA production, for example, amplifying by PCR and transfecting the RNA via delivery systems as liposomes, and gRNA manufactured by the machinery of the cell, based on gRNA cloned onto a vector such as a plasmid [63].

10 Transfection Mechanisms

To enable the access of the interest proteins (the system CRISPR itself) and Cas9, the host cell must be treated to accept these exogenous molecules. One of the most used methods is electroporation [64], consisting of the electrical stimulation of the cell to open the most carrier proteins as possible and even to open up pores [65]. Also, for producing the plasmid, electroporation is the method used, allowing the incorporation of gRNA or cassette [66]. Another mechanism for the introduction of the ribonucleoproteins inside the cell is the use of lipid vesicles [67]. These vesicles fuse with the membrane of the cell, introducing their content in the cell.

Depending on the CRISPR system that is being used, this process may not be necessary, since the introduction of constitutive expressing Cas9 protein is mediated by adenoviruses, making the hosting cell produce continually the protein (being only necessary the gRNA to activate the system). This mechanism has been proved to generate much cellular toxicity [68] requiring many molecular control mechanisms, most times, making this design inviable.

The use of inteins is being studied, since they are proteins (naturally found in *Archaea*) that are transcribed among their proteins, as normal introns but can auto-excite themselves and control the activity (even the splicing) of other proteins [68]. It has been proposed as a solution for the low capacity of adenoviruses, since protein Cas9 could be split in two, and the inteins could re-form this protein entirely [68]. The main problem of this method is the irreversibility and possible off-targets generated [68].

The Golden Gate assembly method [69] is one of the solutions for the variable simultaneous target gene limitation of the CRISPR-Cas9 system. It consists in the construction of plasmids, containing up to 30 different gRNA. To that the assembly is properly done in the plasmid [69]; the ampicillin resistance gene is used to only survive the plasmids with those cassettes. One of the drawbacks of this method is the low percentage of success in the entrance in the cell and transfection [69], around 30%, but this is the best option when we want to modify several genes at once. The Gibson Assembly or isothermal in vitro recombination is one of the other mechanisms used for the DNA ligation of diverse fragments

with overlapping ends [70]. Both showed promising plasmid activities.

When the inserted sequence is in the nucleus inside the cell, the gRNA leads the complex to the single-stranded DNA, where both PAM and gRNA sequences recognize their binding site [71]. Using the Cas' nuclease activity, the recognized sequence is cut, and, from this point on, the natural systems of DNA reparation act by reconstructing the complementary sequence of the gRNA and changing this way the cell's genome (https://www.chilebio.cl/edicion-de-genomas/crispr/).

There are sequences such as cis-regulatory elements [72], where the mechanism of the action itself is still exactly unknown but indications are showing that the PAM sequence is related to those elements [72]. The trans-regulatory elements (sequences encoding for transcription factors) may be highly affected, since the "new" inserted sequence could be recognized better, allowing better control of trans-regulatory elements [72].

The use of libraries is one of the most important tools, since they allow finding and using models of sequences, generating the highest efficiencies. Databases, such as BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi), provide trustful aligning sequences to compare with other groups. Libraries, like GeCKO and GeCKOv2 [73], Thermo Fisher Scientific (https://www.thermofisher.com/es/es/home/life-science/genome-editing/geneart-crispr.html?SID=fr-crispr-main), and Cultek (https://www.cultek.com/molecular/edicion-genica/librerias-crispr-cas9-crrna.html?___store=default&___from_store=default) provide base sequences and information for future developments of gRNA, PAM, or some other interesting sequences. Even some commercial houses have tools to design and improve sequences, using already existing oligonucleotides, showing the reading frame, mRNA, codons, etc. Table 1 summarizes the main online databases for gene editing in cardiovascular and metabolic diseases.

11 Cardiovascular and Metabolic Disease Genome Editing in the Field

Several models have been used in the genomic edition tools, leading to a variety of approaches varying on the target cell type, their differentiation state, the persistence of the modification, and the gene modification itself [15]. Up next, we describe some experimental studies that show the efficiency of different tools and systems in the context of genetic cardiopathies and metabolic diseases.

12 Experiments Using CRISPR-Cas9

12.1 PRKAG2 Cardiac Syndrome

PRKAG2 cardiac syndrome is produced by a dominant autosomal mutation in the H530 locus of the PRKAG2 gene, manifesting in arrhythmia, fast heartbeat, syncope, and chances of cardiac arrest [74]. A single dose of non-integrative gene therapy carrying AAV9-Cas9/sgRNA designed by Xie et al. [75] can effectively restore cardiac function in mice with a *PRKAG2* pathogenic variation. sgRNAs targeting the DNA sequence which leads to the H530R allele were designed using Benchling (https://www.benchling.com/crispr/), constructed in an AVV9-Cas9 carrier and administered on mice on a postnatal day (P) 4 and P42. Mice of different ages improved their cardiac function, suggesting the success of editing both splitting and non-splitting heart cells. The experimental model also considered off-targets of the candidate sgRNAs, analyzed by Jefferson's Computational Medicine Center's Off-Spotter (https://cm.jefferson.edu/Off-Spotter/). The off-target deep sequencing analysis demonstrated a low yield of off-targets by using AAV9-Cas9/sgRNA, suggesting that this non-integrative gene therapy can safely disrupt the H530R pathogenic variant without causing major side effects [75].

Table 1 Available online tools and database for gene editing

Available online tools	
Off-target effects	
Cas-OFFinder	https://www.rgenome.net/cas-offinder/
FlashFry	[44]
dsNickFury	[44]
CRISPOR	http://crispor.tefor.net/
GUIDE-Seq	[44]
DISCOVER-Seq	[45]
CRISPRdirect	[46]
Off-Spotter	https://cm.jefferson.edu/Off-Spotter/
Guide RNA designing	
CHOPCHOP	https://chopchop.cbu.uib.no/
E-CRISPR	http://www.e-crisp.org/E-CRISP/
CRISPOR	http://crispor.tefor.net/
GUIDES	http://guides.sanjanalab.org/#/
sgRNA Designer	https://portals.broadinstitute.org/gpp/public/analysis-tools/sgrna-design
SSC	http://cistrome.org/SSC/
CRISPRscan	https://www.crisprscan.org/
Cas-OFFinder	https://www.crisprscan.org/
CASPER	https://github.com/TrinhLab/CASPER
Benchling	https://www.benchling.com/
Sequence libraries	
BLAST	https://blast.ncbi.nlm.nih.gov/Blast.cgi
GeCKO	[73]
Thermo Fisher Scientific	https://www.thermofisher.com/es/es/home/life-science/genome-editing/geneart-crispr.html?SID=fr-crispr-main
Cultek	https://www.cultek.com/molecular/edicion-genica/librerias-crispr-cas9-crrna.html?___store=default&___from_store=default

12.2 MYH7 Dysfunction

MYH7 gene encodes a protein that mediates myosin-actin interaction, allowing cardiomyocytes to contract properly to perform heart functionality [76]. Mosqueira et al. [77] have used human pluripotent stem cell (PSC) to induce pathogenic variants on the MYH7 gene with a CRISPR/gRNA/Cas9-nickase system along with a flippase excision, generating nine different variants of the mutated MYH7 gene to study each polymorphism effect in cell metabolism and proper myosin-actin mediation.

Obtention of human PSC was assessed by somatic dedifferentiation of the dental pulp and skin punch biopsies via lentiviral transduction with Yamanaka factors Sox2, Oct3/4, Klf4, and c-Myc [78], while the CRISPR/gRNA/Cas9-nickase system was designed using pUC57 vectors and gRNA prediction tools from Zang Lab's Guide Design Resources (http://crispr.mit.edu). The result analysis yielded different cellular metabolic processes such as ROS increase, energy depletion, fetal gene expression, reduced filament contraction, and multinucleation in cardiomyocytes, interesting for drug repositioning [77].

13 Experiments Using TALENs

13.1 PLN-Associated Hereditary Heart Failure

Dilated cardiomyopathy triggered by the R14del variant in the PLN gene is characterized by arrhythmia and cardiac dysfunction due to an impairment in the Ca^{2+} influx between the

sarcoplasmic reticulum and the cytosol of myocardial cells [78]. In a study by Karakikes et al. [79], they have used induced PSC-derived cardiomyocytes of heterozygous patients, AAV6 vectors carrying miRNA targeting the PLN R14del variant, and a TALEN system in order to correct the R14del variant to a wild type non-pathologic allele, showing efficiency by Ca^{2+} influx recovery phenotype when the nucleases were administered.

TALEN was designed using TALEN Hit Software (Cellectis Bioresearch, http://talen-hit.cellectis-bioresearch.com/search) and delivered by electroporation, while AVV6-miRNAs were cocultured with cardiomyocytes after being fully differentiated from induced PSCs and miRNA selection using BLASTn.

13.2 Obesity and LepR

Chen et al. [80] successfully generated an obesity disease model by using a TALEN editing system that induced a premature stop codon in the leptin receptor (LepR) gene. Rat zygotes that were given the TALEN system in vitro showed shorter PCR products of LepR gene and lower mRNA expression and greater body weight. TALEN was designed with Cornell University's TALE-NT software (https://tale-nt.cac.cornell.edu/) and cloned into vectors described by Huang et al. [81], which comprise a pMD18T-simple vector and pCS2-TALEN expression vectors.

14 Experiments Using ZFN

14.1 FBN1 and Marfan Syndrome

Marfanism is an autosomal dominant disorder in which the skeletal and cardiovascular systems present abnormalities due to an unusual arrangement of extracellular microfibrils, composed of FBN1 protein. MFS patients are prone to fatal aortic rupture, being an interesting model for genome edition in cardiovascular pathologies of more complex disease onset. Fetal fibroblasts were electroporated with ZFNs targeting FBN1 exon 10, and pigs were cloned by somatic cell nuclear transfer of the former cells, creating piglets with connective tissue abnormalities. FBNIZFN05 nuclease was designed based on Sscrofa9 DNA database (http://feb2012.archive.ensembl.org/Sus_scrofa/Info/Index), recognizing a long 18-nucleotide motif to reduce off-target probability. Analysis of the cloned piglet's genome revealed no off-targets by the ZFN system.

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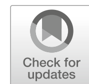
Part III

Genome Editing in Cardiovascular Disease



Genome Editing and Cardiac Regeneration

Rayhaan Bassawon, Kashif Khan, Ateeque Siddique, and Dominique Shum-Tim

Abstract

Although the field of cardiac regeneration is relatively young, it is progressing rapidly with technological advancements. Genome editing tools are allowing researchers to creatively influence signaling pathways to be able to shed light on them and are important for addressing certain issues and limitations associated with in vitro and in vivo aspects of cardiac regeneration, such as imaging and immune rejection. In this chapter, the pathways involved in cardiac regeneration will be highlighted, and the role of gene-editing tools in endogenous and exogenous approaches to regenerate injured myocardium is discussed.

Keywords

Ischemic heart disease · Cardiomyocytes · iPSCs · CRISPR/Cas9 · Adeno-associated virus

1 Background

Despite the development of sophisticated stenting techniques and arduous monitoring of door-to-balloon times, long-term effects of myocardial infarction (MI) are often unavoidable, and heart failure ensues, accounting for most hospitalizations in the United States and affecting more than 23 million people worldwide [1]. The condition is characterized by the inability of the heart to provide the necessary metabolites and nutrients to the rest of the body from the increased cardiac workload [2]. Of the several conditions that contribute to heart failure, ischemic heart disease (IHD) remains the most common cause globally. In an acute MI, the left ventricle is deprived of oxygen, causing irreversible loss of cardiomyocytes (CMs) and resulting in maladaptive left ventricular remodeling. Patients suffering

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from heart failure have a very poor prognosis, with only half surviving 5 years beyond the time of diagnosis [3].

Although its prevalence continues to rise, current medical therapies consist for the most part of damage control measures, with only renin-angiotensin system drugs and beta-blockers showing humble mortality benefits [1]. Mechanical assist devices also exist but are again limited in their contribution to tackle tissue remodeling [4]. The patient's ultimate hope lies in cardiac transplantation. Unfortunately, access to this is limited by the number of available donor hearts. To cater to the millions suffering from IHD, new therapeutic avenues are therefore required.

One such avenue is that of cardiac regeneration. The terminally differentiated adult CM was once thought to lack regenerative capabilities after injury; however this view has drastically changed over time [5, 6]. Stem cells in the heart, known as cardiac progenitor cells (CPCs), have been suggested to play a role in repair [7, 8]. CPCs were first discovered in 2003, but their characterization remains controversial [9]. Furthermore, the exact mechanism by which CPCs regenerate the myocardium after injury is not well understood, but is likely to occur through paracrine release of cytokines, chemokines, and regulatory factors that aid in anti-apoptosis, immunoregulation, neovascularization, and resident cell fusion [10].

Over the past three decades, the use of stem cells to induce cardiac regeneration has evolved from a theoretical consideration to a pragmatic player in the arena of IHD. Models have been developed to study the phenomenon, and different approaches have come about to reproduce this phenomenon in patients with the goal of mending the failing ventricle. Several of these techniques have been under clinical investigation; however only subtle improvements in cardiac function have been reported [11, 12]. This may be in part due to the lack of stem cell engraftment into the native myocardium, or failure to trans-differentiate into adult postmitotic CMs [13–15]. A promising strategy to circumvent these limitations is the use of genome editing of these exogenous stem cell sources, or of the residing cells in the peri-infarct region, to initiate a proliferative response that improves function in hopes of propelling the field toward clinical success.

2 Scope of Cardiac Regeneration

The field of cardiac regeneration is highly experimental and innovative, aiming to undo damage secondary to infarction. To that effect, research efforts follow a three-pronged approach. The first focus is on understanding the phenomenon of myocardial regeneration as it occurs in the natural world. The second objective is to influence the cellular response after injury in view of favoring myocardial restoration. Finally, studies assess the efficacy of these approaches on cardiac function of affected patients.

The adult human heart possesses little ability to spontaneously rehabilitate itself after an insult. Therefore, external interventions are necessary to make this process possible [16]. While the traditional paradigm has been to identify defective genes and edit them out in an attempt to restore physiological phenotypes [17], the field of cardiac regeneration has employed genome editing tools in creative ways. The development and manipulation of animal and cellular models using genome editing enabled us to highlight pathways involved in heart regeneration to further our comprehension of how the myocardium regenerates after injury [18, 19].

2.1 Understanding Cardiac Regeneration from Animal Models

Intrinsic, spontaneous restoration of the myocardium following an insult is well known to happen in discrete organisms. For instance, the zebrafish has been extensively studied for its ability to trigger cellular proliferation from existing CMs [20]. Interestingly, one of the early uses of gene-editing technologies in zebrafish models was to cause ventricular damage through an inducible recombineering approach, so-called genetic ablation [21]. This allowed for subsequent identification of the factors involved in the post-injury

response [22, 23]. More recently, CRISPR/Cas9 methodology has been applied to knock out genes from somatic zebrafish cells and highlight the role of macrophages in the fibrotic process [24, 25]. TALEN technology has also proved practical in finding proteins necessary for repair such as caveolin-1 [26]. Similarly, the neonatal mouse was found to possess myocardial regenerative capacity, specifically up to 7 days after birth [27]. Again, Cas9-engineered mice were a versatile tool in delineating regulators of rehabilitation in the mammalian heart through knock-in and knock-out protocols [28, 29].

2.2 Developing Approaches for Cardiac Regeneration

The general mechanism behind the failure of the infarcted myocardium is the death of CMs and their replacement by scar tissue. Two main methods have been explored to replenish the myocardium. The first approach is to trigger myocardial rejuvenation in vivo by causing viable CMs to reenter the cell cycle and divide, or by causing surrounding fibrotic tissue to transdifferentiate into functional myocardium [30]. The second method employs external sources of CMs or stem cells to be transplanted to the site of injury through various strategies including injections and application of patches [31]. Although these exogenous sources mostly consist of stem cells reprogrammed into CMs [32], the importance of supplying supportive vascular and structural tissue was also recognized, and concomitant injection of different cell types has been attempted [33, 34]. Furthermore, exogenous approaches to mending the myocardium have benefited from scaffolding, 3D printing, and various other bioengineering advancements which are beyond the scope of this chapter [35, 36].

3 Pathways and Regulators of Cardiac Regeneration

Several molecular pathways play a critical role in myocardial development, regulation of organ size and growth, and cell cycle re-entry. Of these mechanisms, the Hippo, Wingless (Wnt), and phosphoinositide 3-kinase-protein kinase B (PI3K-AKT) pathways are the most widely studied. Given their complexity, it comes as no surprise that these pathways communicate to control cardiac organ size and growth as depicted in Fig. 1. Genetic manipulation of these pathways is a promising tool for generating useful cardiac regeneration models in vitro and in vivo.

3.1 Hippo Signaling Pathway

Originally described in *Drosophila melanogaster*, the Hippo cell signaling pathway is a conserved cascade of kinases and effector proteins that regulate cell proliferation, tissue development, homeostasis, organ size, and tissue regeneration [37–40]. The pathway activation signals include G protein-coupled receptors, cell-cell interactions, and disruptions in cytoskeletal dynamics [41–45]. Mammalian sterile 20-like kinases 1/2 (Mst1/2) are the orthologs of Hippo kinase in *Drosophila*. When the signaling pathway is in the *on* state, Mst1/2 form a complex with the Salvador homolog 1 (Sav1) adaptor protein, and the resulting complex allows for the phosphorylation and activation of the C-terminal hydrophobic motif of the large tumor suppressor homolog kinase 1/2 (Lats1/2), as well as Mob1a/b kinase adaptors that coactivate Lats1/2. Following activation, Lats1/2 phosphorylate two transcription cofactors, Yes-associated protein (YAP) and tafazzin (TAZ), thereby preventing their translocation to the nucleus and leading to their degradation [46–48]. In the *off* state, inactive Mst1/2 and Lats1/2 allow for the nuclear localization of YAP and TAZ where they interact with TEA domain (TEAD1-4) transcription factors to promote the expression of genes involved in cell proliferation, inhibition of apoptosis, angiogenesis, and anti-fibrogenesis [49, 50].

In addition to its role in cardiac organogenesis, the Hippo pathway is also dysregulated post-MI. Patients with compromised cardiac function showed significantly increased phosphorylated Lats/Lats protein expression ratio in their left ventricles, while YAP/phosphorylated YAP

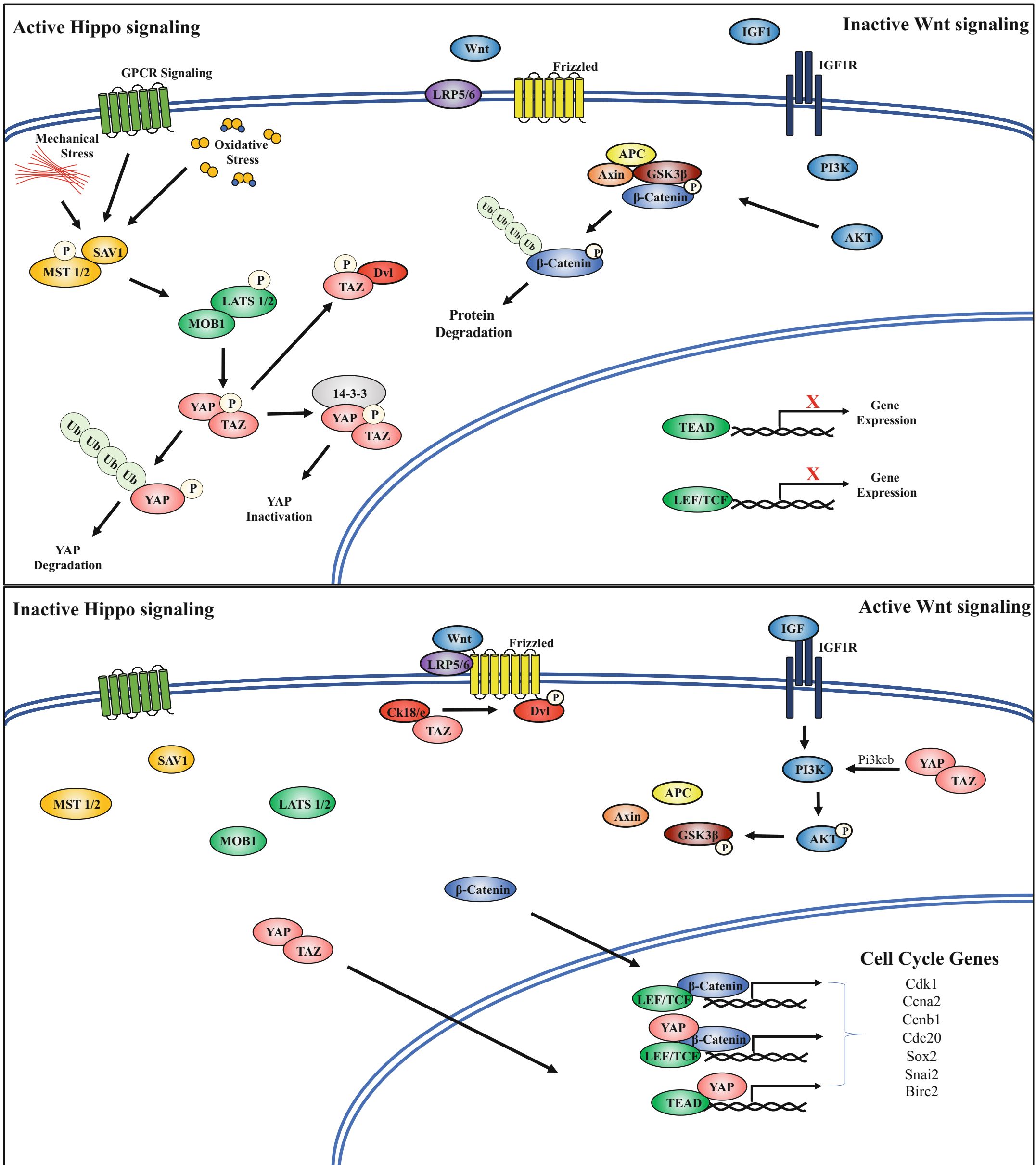

Fig. 1 Schematic of Hippo, Wnt and PI3K-AKT cross-talk in cardiomyocytes to initiate cell cycle re-entry and repair the injured myocardium

ratios were decreased, suggesting activation of the Hippo pathway and degradation of YAP [51]. This may in part be due to the role of YAP in modulating the inflammatory and immune response. For example, epicardial-specific deletion of YAP and TAZ in a mouse model showed increased inflammation and myocardial fibrosis with less T-regulatory cells in the infarcted myocardium [52]. Interestingly however, patients with hypertrophic cardiomyopathy were found to have decreased YAP expression, while Mst1 expression was increased [53]. These results are corroborated by CM-specific expression of YAP in a murine model of induced hypertrophy.

Therefore, Hippo dysregulation post-MI is highly dependent on the type of remodeling taking place. Careful understanding of these mechanisms will be important in determining whether targeting the Hippo pathway will prove beneficial for patients.

3.2 Wnt Signaling Pathway

The Wnt signaling pathway is essential for proper embryonic development, cell fate determination, and tissue homeostasis [54, 55]. At least 19 Wnt genes exist in humans, encoding for secreted glycoproteins that bind to 1 of 10 Frizzled (Fz) membrane receptor isoforms. Three main Wnt signaling branches are described: the canonical Wnt/β-catenin-dependent pathway, the noncanonical planar cell polarity (PCP) pathway, and the noncanonical Wnt/Ca^{2+} pathway. The canonical pathway is the most related to cardiac regeneration [56]. Canonical Wnt ligands bind to the Fz membrane receptor, activating the intracellular dishevelled protein and subsequently dissociating the β-catenin destruction complex made of glycogen synthase kinase 3β (GSK3β), Axin, and adenomatous polyposis coli (APC) [57]. When this complex is intact, β-catenin is targeted for ubiquitination and degradation. However, activation of this pathway allows β-catenin to localize to the nucleus and activate the T-cell factor/lymphoid enhancer factor (TCF/LEF) which triggers a pro-proliferation, migration, and differentiation gene expression profile. A study performed on human embryonic stem cells (hESCs) using CRISPR/Cas9 determined that YAP maintains hESC pluripotency by preventing the expression of WNT3 in response to activin, a TGF-β family ligand [58]. This demonstrates not only the interplay between the Hippo and Wnt pathways but also the utility of gene editing in unraveling these links. Furthermore, CRISPR/Cas9-mediated deletion of disabled homolog 2 (DAB2), a regulator of cardiac development, negatively regulates Wnt/β-catenin signaling and promotes differentiation in the developing heart [59].

Following an MI, there is an increased activation of Wnt signaling in CMs neighboring the border zone and several resident stem cell populations, as depicted by LacZ expression under control of the TCF/LEF promoter [60, 61]. Surviving endothelial cells also increase β-catenin expression to induce cell proliferation and neoangiogenesis [62, 63]. In addition, the release of the noncanonical Wnt-5a from CMs after MI promotes interleukin expression by mononuclear cells and differentiation of stem cells toward cardiac lineage [64]. Finally, increased expression of Wnt-1, β-catenin, and GSK3β was found in the rat myocardium during fibrosis following infarction [65]. Given the complexity of the Wnt signaling pathway and its role in multiple cell types within the myocardial tissue, there has been some discrepancy in its exact role in the pathogenesis of heart failure and regeneration.

3.3 PI3K-AKT Signaling Pathway

The PI3K-AKT pathway is a prominent intracellular signaling axis governing processes such as glucose regulation, energy metabolism, and cell cycle progression that has been shown to function abnormally in many human diseases [66, 67]. A variety of stimuli, such as growth factors binding to receptor tyrosine kinases at the cell surface, can initiate a downstream cascade to activate PI3K. Active PI3K phosphorylates phosphatidylinositol 4,5-bisphosphate (IP2) into phosphatidylinositol 3,4,5-trisphosphate (IP3) which recruits phosphoinositide-dependent kinase-1 (PDK-1) in order to phosphorylate AKT [68, 69]. Once active, AKT can then turn on other axes, including mTOR, FoxO, and p21/p27 signaling to induce cell growth, survival, and cell cycle progression, respectively [66]. Using targeted gene editing, the FoxO3 gene in human mesenchymal progenitor cells underwent S253A and S315A serine-to-alanine replacements, unable to be phosphorylated by AKT and thereby constitutively active. Mice transplanted with these cells after an induced MI exhibited significant cardiac repair [70]. AKT can have an inhibitory effect on pathways such as mitogen-activated protein kinase (MAPK) and c-Jun N-terminal kinase (JNK) to attenuate apoptosis [71]. Nevertheless,

the role of the PI3K-AKT pathway in cardiac regeneration is mainly through its ability to induce cell cycle re-entry and promote G2/M phase progression [72]. Moreover, it is involved in the migration of cardiac stem cells through vascular endothelial growth factor (VEGF) and regulates cardiac autophagy through microRNA (miRNA) cluster miR302-367 [73, 74]. After ischemia-reperfusion injury, rat hearts were shown to have reduced phosphorylated AKT/AKT ratio, which was reversed by insulin-like growth factor 1 (IGF1) administration further indicating the role of the pathway in injury and repair [75]. Another study noted that improvement in functional recovery was associated with increased AKT activation and reversed by the PI3K inhibitor, wortmannin [76]. AKT also plays a significant role in the phosphorylation and regulation of GSK3β, which is a strong molecular candidate for myocardial repair therapies [77]. Taken together, these results indicate a cardioprotective role for the PI3K-AKT pathway.

4 Approaches to Cardiac Regeneration

Current avenues to achieve cardiac regeneration include in situ genetic engineering of host cells to generate new CMs. Additionally, administration of stem cells manipulated ex vivo is under substantial investigation. These approaches summarized in Fig. 2 make use of genome editing technologies to varying degrees.

4.1 Genome Editing of Endogenous Cells to Initiate Cardiac Repair

Endogenous cardiac repair is currently being explored through two paradigms: promotion of cell cycle re-entry in host CMs and trans-differentiation of host fibroblasts into CMs.

4.1.1 In Situ Promotion of Proliferation and Cell Cycle Re-entry

Using Cre-Lox gene manipulation, it has been demonstrated that deletion of YAP impaired the regenerative ability of CMs in neonatal mice, whereas mice with constitutively overexpressed YAP have more CMs and smaller scars with improved cardiac function after infarction [39]. Furthermore, the inactivation of Sav1 and Lats1/2 in transgenic mice promoted cardiac regeneration in both postnatal and adult MI models [78]. Cardiac-specific activation of YAP using the adeno-associated virus 9 (AAV9) delivery vector stimulated adult mouse CM proliferation after MI using a doxycycline-inducible system [79]. Transcriptional profiling of the YAP-induced regenerating apices revealed enrichments of pathways involved in cell cycle re-entry and response to inflammation. These results are corroborated by a lentiviral infection model of YAP activation in human CMs, where YAP-infected cells exposed to ischemia-reperfusion injury showed significant attenuation of apoptosis, DNA damage, and cellular hypertrophy [80].

Genome editing has been employed to trigger repair through cell cycle re-entry by exploiting

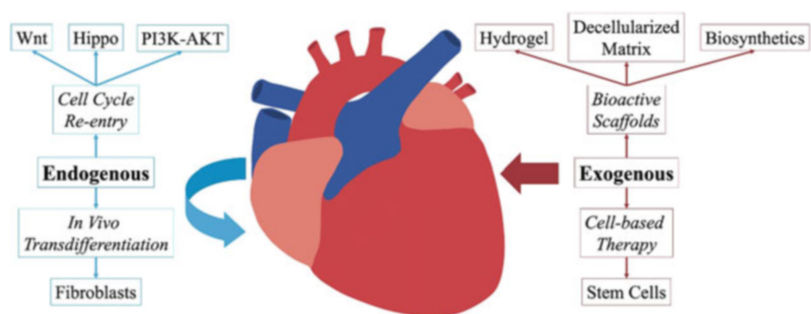

Fig. 2 Components of potential therapeutic approaches to cardiac regeneration

miRNA pathways, and there is evidence to suggest convergence with the Hippo pathway. A large-scale screening study revealed that 96 miRNAs are capable of increasing human-induced pluripotent stem cell-derived CM (iPSC-CM) proliferation, and 67 of them increased the ratio of nuclear to cytosolic YAP [81]. In mice, the postnatal expression of miR302-367 enabled CM cell cycle re-entry, which resulted in decreased scar tissue formation after MI simulation [82]. Similarly, miR19a/b were shown to be necessary and sufficient for in vitro proliferation of CMs isolated from embryonic, neonatal, and adult mouse hearts [83]. Furthermore, the intracardiac or systemic delivery of miR19a/b using AAV9 reduced cardiac injury following MI while preserving cardiac function [84].

With regard to the Wnt pathway, modulation of signaling to induce tissue regeneration remains controversial. Adenovirus-mediated overexpression of β-catenin in CMs resulted in anti-apoptosis, cellular hypertrophy, and reductions in infarct size [85]. In addition to this, YAP-overexpressing CMs increased β-catenin nuclear localization, resulting in synergistic improvements in cellular hypertrophy and apoptosis after ischemia-reperfusion injury [80]. However, other studies suggest that β-catenin plays the opposite role, where depletion of β-catenin improves cardiac function [86, 87]. In a mouse MI model, conditional knockout of GSK3β under tamoxifen control showed increased CM proliferation and attenuated cardiac remodelling after pressure overload [88]. Similar results were obtained in a following study, showing that CM-specific conditional GSK3β deletion preserves cardiac function post-MI, which may be in part due to activation of cyclin E1 and E2F1 [89]. Interestingly, cell cycle progression was also shown to occur spontaneously in CM-specific tamoxifen-inducible GSK3β knockout mouse model, along with significant DNA damage and apoptotic cell death [90]. Although promising, further investigation should proceed carefully, as targeting GSK3β can have unwanted side effects due to its role in other signaling cascades [91].

The PI3K-AKT pathway has also been investigated as a target to increase proliferation. Adenovirus-mediated induction of a constitutively active form of YAP increased CM proliferation in vitro, and this was associated with increased expression of IGF1 receptor, p-AKT, p-GSK3β, and active β-catenin. Furthermore, gene expression of YAPS112A (constitutively active form of YAP)-infected neonatal CMs showed increased expression of IGF-signaling genes including *IGF1*, *IGFBP2*, and *IGFBP3*. siRNA knockdown of either IGF1 or β-catenin resulted in an attenuation of the increased proliferation seen in YAPS112A-infected CMs [92]. In a later study by the same group, transgenic mice, with a CM-specific single nucleotide mutation of YAP that prevents its phosphorylation, underwent ligation of the left anterior descending artery to induce a MI and were found to have enhanced cardiac regeneration and improved fractional shortening. This was associated with increased protein expression of the IGF1 receptor and p-AKT [39]. The field was expanded by the identification of a strong relationship between YAP and PI3K in the context of cardiac regeneration [93]. First, a genetic screen of AAV9-mediated YAP-overexpressing mice identified 13 genes that were directly activated by YAP, one of which was phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit beta (*PI3KCB*). Second, AAV9-mediated gain of function mice showed significant activation of the PI3K-AKT pathway, including increased expression of *PI3KCB*. This was in part mediated by modulation of the cell cycle inhibitor p27, a target of AKT. Lastly, YAP-mediated activation of CM proliferation in vivo was mediated through Pi3kcb. This was corroborated by using AAV9 to deliver *PI3KCB* to attenuate the cardiac dysfunction seen in a CM-specific YAP conditional knockout model. Together, these results suggest a relationship between YAP and β-catenin

through a YAP-IGF1-PI3K-AKT-β-catenin signaling axis and can be taken advantage of using gene editing to promote cardiac repair.

4.1.2 In Vivo Trans-differentiation of Cardiac Fibroblasts

The advantages of converting fibroblasts to myocytes are clear. First, there would be no need to manipulate cells outside of their physiological milieu. Any change would be brought in situ through the delivery of factors currently under investigation. Second, the off-target effects of such a therapy would be minimal given the targeted delivery systems available nowadays. Despite the alluring thought of a pool of fibroblasts ready to serve as replacement for dead CMs at the administration of a simple injection, thought needs to be given to the consequence of depleting the myocardium of seemingly detrimental cells. Cardiac fibroblasts are by no means an unnecessary component of cardiac physiology. In fact, they are the most common non-CM cells in the healthy heart. Their function in extracellular matrix (ECM) synthesis is crucial. Besides providing structure to what would otherwise be entangled muscle sheets, the ECM serves as a nurturing environment for CMs. It is well known that absence of functional cardiac fibroblasts results in lethality [94].

Attempts to transform scar tissue into myocardial tissue go back to the late nineteenth century when cardiac fibroblasts were transduced with copies of the myogenic differentiation 1 gene (*MYOD1*) using adenoviruses [95]. Subsequently, viral delivery of three transcription factors (Gata4, Mef2c, Tbx5) was found to trigger genetic reprogramming of fibroblasts into cells possessing phenotypes and a genetic landscape characteristic of CMs [96]. This approach was later refined with additional transcription factors and showed promise in vivo [97]. However, the efficiency of reprogramming was consistently suboptimal with hardly 10% of fibroblasts showing CM-like traits [98]. Using a modified CRISPR/Cas9 approach, it was demonstrated that transcription factors were not enough to complete reprogramming and combining them with miRNA yielded substantially better maturation, although the percentage of successfully transformed cells was still inadequate [99]. This led to the exploration of "non-integrative" chemical reprogramming techniques. Delivery of small molecules to inhibit TGF-β resulted in more efficacious reprogramming and other studies followed using various compounds [100]. Regulators of molecular reprogramming such as DNA methyltransferase 1-associated protein 1 (Dmap1) have also been identified thanks to systematic CRISPR knockout strategies [29].

Whereas most of the aforementioned studies use viral transduction or transient transfection of genetic material to trigger the observed phenotypic changes, mainstream genome editing is partially being used for reprogramming purposes. Part of the TALEN system has been used to reprogram epiblast stem cells into iPSCs [101]. In a simpler way, deactivated Cas9 has been engineered into a transcriptional activator of endogenous *MYOD1* in murine embryonic fibroblasts, resulting in skeletal myocytes [102]. All in all, this approach is promising, but our understanding of lineage manipulation remains poor, and current attempts at direct reprogramming are insufficient to address myocardial degeneration after infarction.

4.2 Transplantation of Exogenous Cells

Somatic multipotent stem cells and pluripotent stem cells are promising approaches for cardiac regeneration via transplantation to the heart tissue damaged following an infarct [103–105]. While various cells with the potential for cardiac regeneration are available, most recent work has been focused on CMs derived from iPSCs and ESCs [106, 107]. HESCs treated with cytokines activin A and bone morphogenic protein 4 (BMP4) showed enhanced CM-directed differentiation, and rats receiving an intracardiac injection of these CMs post-MI display a lesser degree of ventricular dilation and a higher local and global cardiac contractile function relative to control rats [108]. IPSCs are a feat of genetic reprogramming,

a result of dedifferentiating mature somatic or blood cells into an embryonic-like pluripotent state via retroviral transfection of Yamanaka factors (Oct3/4, Sox2, Klf4, c-Myc) [109, 110]. Human iPSC-derived CMs, endothelial cells, and smooth muscle cells have been successfully integrated into infarcted porcine myocardium and have induced cardiac regeneration [111, 112]. Transplantation may be performed either by direct injection or through surgical implantation of bioactive scaffolds such as 3D fibrin-based patches, hydrogel matrices, decellularized/acellular extracellular matrices, or other biosynthetic materials [113–115].

Gene editing is being increasingly used to enhance cell visualization and in vivo tracking of transplanted cells' engraftment, migration, metabolic activity, and calcium dynamics. Transposon-mediated gene insertion has been used to induce green fluorescent protein (GFP) expression in cells to be transplanted post-MI. The cells showed organized sarcomere structure, improved left ventricular function, and reduced infarct size without any arrhythmias [112]. In contrast, a study using hESC-CMs transplanted into primates showed electromechanical coupling, but ventricular arrhythmias were an issue. This study utilized ZFN gene editing via the AAVS1 locus to insert a transgene for constitutive GCaMP3 expression, a genetically encoded fluorescent calcium indicator (GECI), for the visualization of calcium signaling in the cells [116]. CRISPR/Cas9 has been used to target the AAVS1 harbor locus in rhesus macaque iPSCs (RhiPSCs) for the stable knock-in of GCaMP6, a more recent version of GCaMP3 [117]. This allows for the cells to emit GFP-like fluorescence reflective of calcium transients associated with contraction and is useful in in vitro and ex vivo physiological assays and drug screening assays. Lentiviral vectors have also been used to produce firefly luciferase and GFP-expressing iPSC-CMs [118]. Mice transplanted with iPSC-CMs edited with TALEN and ZFN to insert enhanced GFP showed sustained expression for several weeks [119]. With the advent of multiphoton imaging techniques combined with such calcium indicators, intravital calcium imaging of the beating heart may be performed [120]. Similarly, monitoring of implanted cells can be done using radiotracers. For example, the sodium/iodide symporter (NIS) allows imaging with radiotracers detected with positron emission tomography (PET) or single-photon emission computed tomography (SPECT). The human NIS gene was successfully inserted into RhiPSCs via CRISPR/Cas9. NIS-RhiPSC-derived CMs transplanted into mice post-MI were followed with PET and SPECT injected with ^{18}F-tetrafluoroborate, a NIS-specific radiotracer, and could be safely detected until 8–10 weeks [121]. Likewise, a triple-fusion reporter gene consisting of monomeric red fluorescent protein, firefly luciferase, and herpes simplex virus thymidine kinase was integrated into the AAVS1 harbor locus of iPSCs via CRISPR/Cas9 technology, allowing for fluorescent, bioluminescence, and PET imaging, respectively [122].

Recently, the transplantation of human umbilical cord blood-derived mesenchymal stem cells (MSCs) with CRISPR/Cas9-inserted lymphoid enhancer-binding factor 1 gene (*LEF1* involved in the Wnt pathway) significantly improved the survival of rats following an MI, outlining its cardioprotective effect [123]. In another study, bone marrow-derived MSCs were transduced to retrovirally express AKT and injected at two sites along the infarct border. AKT-expressing MSCs decreased infarct size in rats and restored early cardiac function [124]. An analogous study in a porcine MI model determined that AKT-MSCs enhanced cardiac repair by increasing viability of transplanted cells through paracrine action of secreted frizzled-related protein 2 [125, 126].

Exosomes are a subset of cellular secretomes that are important for intercellular communication and have been recently proposed to be beneficial for mediating endogenous cardiac repair by regulating cellular processes such as proliferation, apoptosis, and angiogenesis [127, 128]. Using a TALEN gene-editing approach, researchers knocked out the *Rab27a* gene in mice, thereby halting exosome secretion. Bone marrow MSCs were then implanted into viable myocardium along the infarction border of normal and *Rab27a* knockout mice. It was found that the

viability of transplanted MSCs was increased in the knockout mice compared to the control, suggesting that exosomes from injured CMs accelerate the injury of transplanted MSCs [129].

In the context of enhancing stem cell engraftment and viability following transplantation, it is crucial to consider immune rejection. Although allogeneic umbilical MSCs are a convenient resource, transplanting these cells is an immunological challenge. To circumvent this, HLA class I light chain β2-microglobulin (*B2M*) in umbilical MSCs was knocked out with CRISPR/Cas9, and their transplantation post-MI did not induce a $CD8^+$ T-cell-mediated response as opposed to control cells. Furthermore, exosomes derived from *B2M* knockout MSCs were more effective at inhibiting fibrosis and restoring cardiac function [130]. TALEN-mediated insertion of the *IL10* gene in amniotic MSCs resulted in improved ventricular remodeling and reduced infarct size and pro-inflammatory markers [131]. While YAP overexpression in CMs leads to improved cardiac regeneration, YAP activation in macrophages enhances their pro-inflammatory response via IL-6 which impairs repair post-MI. Myeloid-specific deletion of YAP/TAZ in mice was found to impair the pro-inflammatory response and enhance the reparative response [132]. This is an important contrast between the cell types involved in cardiac regeneration, as the Hippo pathway also mediates the macrophage response in the infarcted area. Future studies using genetically edited elements of the signaling pathways involved in cardiac regeneration must differentially examine cell types to ensure a specific and desired response and reduce unwanted side effects.

5 Perspective

The use of CRISPR/Cas9 to edit CM biology in vivo is commonly utilized to address conditions with a single genetic defect like Duchenne muscular dystrophy, Wolff-Parkinson-White syndrome, and catecholaminergic polymorphic ventricular tachycardia [133]. The application of the CRISPR/Cas9 system in cardiac regeneration is limited by inefficiency in gene disruption within CMs using AAV9 [134]. Rather, this technology is more likely to be used to edit a cell source exogenously for delivery into the myocardial tissue, and we may see a rise in these studies in the near future.

While a plethora of clinical trials have been approved to study gene modifications in humans, there is currently no clinically approved therapy available for patients suffering from heart failure [135]. Given the specific tropism toward postmitotic CMs and indefinite gene expression, the use of AAVs remains the most promising gene delivery system in this field [136]. The main goal of this system is to deliver genes that initiate cell cycle re-entry in adult CMs, leading to proliferation and reduction of the myocardial scar size. Continued research will be necessary to ensure there are no side effects from this gene delivery system, such as arrhythmias, hyperproliferation, or continued dedifferentiation as seen with some AAV-delivered miRNAs [137]. Although still primitive at this point, genetically engineering the existing postmitotic CMs remains a promising avenue to induce cardiac regeneration and improve function. With the fundamental pathophysiology of heart failure caused by the lack of contractile CMs, the ongoing research and better understanding of genomic editing promises to improve the efficacy of myocardial regeneration and the treatment of heart failure.

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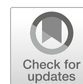

Genome Editing and Myocardial Development

Sifa Turan, J. Richard Chaillet, Margaret C. Stapleton, and Yijen L. Wu

Abstract

Congenital heart disease (CHD) has a strong genetic etiology, making it a likely candidate for therapeutic intervention using genetic editing. Complex genetics involving an orchestrated series of genetic events and over 400 genes are responsible for myocardial development. Cooperation is required from a vast series of genetic networks, and mutations in such can lead to CHD and cardiovascular abnormalities, affecting up to 1% of all live births. Genome editing technologies are becoming better studied and with time and improved logistics, CHD could be a prime therapeutic target. Syndromic, nonsyndromic, and cases of familial inheritance all involve identifiable causative mutations and thus have the potential for genome editing therapy. Mouse models are well-suited to study and predict clinical outcome. This review summarizes the anatomical and genetic timeline of myocardial development in both mice and humans, the potential of gene editing in typical CHD categories, as well as the use of mice thus far in reproducing models of human CHD and correcting the mutations that create them.

Keywords

Congenital heart disease (CHD) · Heart development · Cardiovascular disease

1 Overview

The heart is the first visceral organ to be formed during organogenesis [1]. Myocardial development involves orchestrated series of genetic networks turning on and off cooperatively to execute molecular, cellular, and morphogenetic events to form a normal heart [2, 3]. Mutations in these genes result in congenital heart disease (CHD) at birth or cardiovascular abnormalities later in life [1, 2]. CHD is the most common congenital abnormality, which occurs in about 1 in 100 live births [4] and in 10% of aborted

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fetuses [5]. More than 400 genes have been associated with CHD [6]. Oyen and associates [7, 8] investigated the overall CHD risks among family members of the proband and found that the risks for concordant [7, 8] and discordant [8, 9] defects among the first-degree relatives are 3–80 [7, 8] and 2 [8, 9], respectively. This is indicative of the genetic and genomic underpinning of CHD [2, 10] (Excellent recent reviews please see Williams et. al. [6, 11], Zaidi and Brueckner [6, 11]).

Should the enormous scientific, logistical, clinical, ethical, and regulatory issues regarding the use of therapeutic genome editing technologies be resolved, CHD would be prime targets. The syndromic CHDs are typically due to large chromosomal defects, such as microdeletions and microduplications, and associated with significant abnormalities apart from the cardiovascular system. Nonsyndromic CHD cases outnumber syndromic cases, and are mainly due to monogenic or digenic mutations, which are small (point mutations mostly), and vis-à-vis state-of-the-art genome editing, correctable.

Familial inheritance of most CHD cases favors the use of genome editing in treatment. The causative mutated gene is often known or can be easily identified, particularly as the list of genes associated with CHD increases (for comprehensive gene lists, see [6]). As well, phenotypic abnormalities of nonsyndromic cases are usually confined to the heart, their developmental onset and progression may be known, and the severity of the anomaly and thus the need for some form of intervention predictable. Lastly, the position of the heart in the vascular system may mean the delivery of editing tools and their effectiveness would be outstanding.

Mouse models of CHD would be essential to formulating a CHD gene editing plan of known efficacy and a strong prediction of a beneficial clinical outcome. Mice are a favorable model system as many of their genes, gene modifiers, and molecular pathways are conserved with those of humans. Biological processes, anatomy, and physiology are likewise conserved with humans, examples being four-chambered hearts, vascular systems, and visceral organs, which share similar structure and function [12–15].

Mouse models of specific human molecular variants can be easily constructed to determine the best developmental time and approach to intervene and correct the gene defect.

This review begins with a discussion of the anatomical development of the human and mouse hearts (Table 1 and Figs. 1 and 2), including a list of the genes (Table 2) and their roles at anatomical positions and gestational times. We follow this with a discussion of the potential roles of gene editing in different traditional categories of CHD. We complete our review with examples of how mice have been used so far, both in generating models of human CHD and in correcting mutations in orthologs of human CHD genes.

2 Cardiac Development

2.1 Early Development and Cardiac Crescent

Heart development begins [20] when cells in the anterior lateral mesoderm move from the primitive streak and give rise to cardiomyocytes, beginning the process that allows the heart to form and contract. These mesodermal cells gather in shape known as the cardiac crescent, made of the first and second heart fields. It is thought that this movement is facilitated by an influx of transcription factors and secreted molecules [10]. Cells in the first heart field go on to form the linear tube; meanwhile, cells in the second heart field, which is medial and dorsal to the first heart field, become the right ventricle, outflow tract, and parts of the atria [16].

2.2 Cardiac Looping

One of the first cells to be specified are cardiac muscle cells, which quickly find their way to the ventral midline of the embryo and form a beating heart tube. This tube has an endocardial and myocardial layer with a layer of extracellular

Table 1 Cardiac development in humans and mice

Cardiac development stage	Milestones	Human embryonic weeks	Mouse embryonic days
Establish left–right body axis	Breaking symmetry around the midline organizer, the node		E7
Establish cardiac fate	Perinodal signaling to the lateral plate mesoderm for cardiac differentiation		
Gastrulation	Cardiac progenitor cells migrate to the splanchnic mesoderm		
Cardiac crescent	Cardiac progenitor cells form first heart field (FHF) and second heart field (SHF)	2 weeks (Fig.1)	E7.5 (Fig.2)
Linear heart tube	Heart tube formation, first heartbeat, anterior–posterior segmental patterning for committing precursors for future aortic sac, conotruncus (outflow tract), atria, pulmonary and systemic ventricles.	3 weeks (Fig.1)	E8.0 (Fig.2)
Cardiac looping	Rightward looping, forming inflow track at the arterial pole and inflow track and the primitive atria at the venous pole	4 weeks (Fig.1)	E8.5–E10.5 (Fig.2)
Chamber formation	Common atrium has moved superior to the ventricles and is separated by atrio-ventricular canal, trabeculation, cushion formation, outflow track separation, and early conduction formation	4.5 weeks	E9.5 (Fig.2)
Cardiac neural crest cell migration	Cardiac neural crest cells from the dorsal neural tube migrate to the cardiac outflow track	4.5 weeks	E10.5 (Fig.2)
Node formation	Sino atrial and atrioventricular nodes detectable	5 weeks	E10.5–E11.5
Atrial septation	Growing two septa: the septum primum and septum secundum	6.8–8.0 weeks	E10.5–E13.5
Outflow track septation	The truncus arteriosus septation into two separate arterial channels.	7.1–7.7 weeks	E11.5–E13.5
Interventricular Foramen	The interventricular foramen changes from wide and open to become narrow with distinct opening.	7.4–8.8 weeks	E10.5–E11.5
Ventricular septation	Forming muscular, inlet, and outlet interventricular septa	7/4–9.1 weeks	E11.5–E13.5
Valve formation	Forming mitral valve, tricuspid valve, aortic valve, and pulmonary valve	8.0–9.4 weeks	E12.5–E13.5
Mature four-chamber heart	The mature heart formed.	8 weeks to birth (Fig. 1)	E15.5 (Fig.2) to birth

Information based on [2, 3, 6, 10, 11, 16–19]

matrix (ECM) in between. [2]. The following process to occur is known as cardiac looping and it takes place around embryonic day E9.5 and E10.5 in mice and weeks 6 4/7 and 7 5/7 in humans [17]. In the lateral mesoderm, uneven gene expression will cause the linear heart tube to loop to the right. This event is essential for heart chamber formation, and the proper alignment between heart chambers and vasculature [2]. Structurally on the left side, blood will go from, what at this point is made up of the atrial cavity, atrial ventricular junction, and what will be the left ventricle to the interventricular foramen. On the right side, blood will flow from the early right ventricle to the truncus arteriosus. In addition, the arterial ventricular junction is enveloped in endocardial cushion tissue [18]. During this time of development, the ventricular and atrial chambers grow in size and start to become distinct on the left and right. Along with this distinction, the interventricular foramen narrows as the ventricles grow, allowing the ventricles to

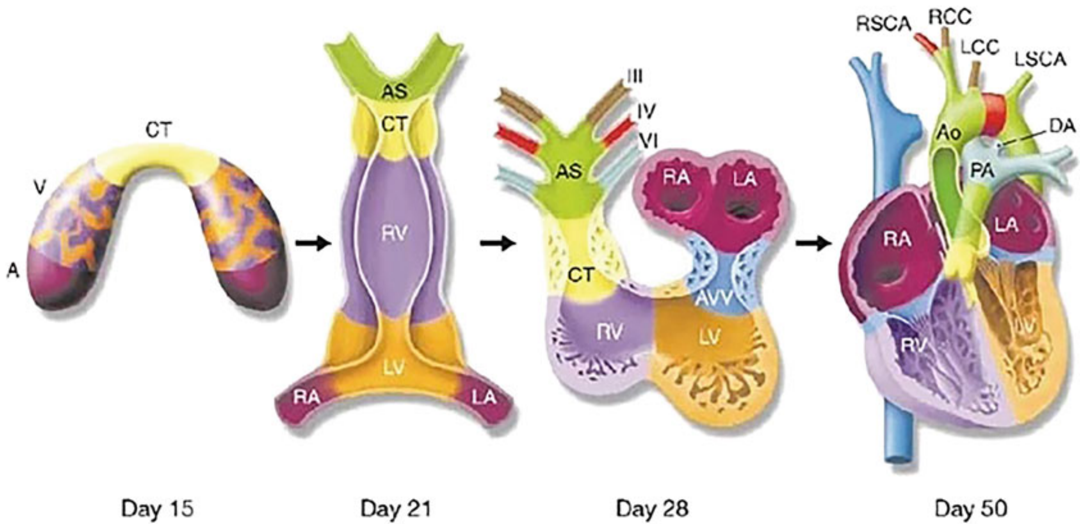

Fig. 1 Schematic of human cardiac morphogenesis. Illustrations depict cardiac development with color coding of morphologically related regions, seen from a ventral view. Cardiogenic precursors form a crescent (*left-most panel*) that is specified to form specific segments of the linear heart tube, which is patterned along the anterior–posterior axis to form the various regions and chambers of the looped and mature heart. Each cardiac chamber balloons out from the outer curvature of the looped heart tube in a segmental fashion. Neural crest cells populate the bilaterally symmetrical aortic arch arteries (*III, IV*, and *VI*) and aortic sac (*AS*) that together contribute to specific segments of the mature aortic arch, also color coded. Mesenchymal cells form the cardiac valves from the conotruncal (*CT*) and atrioventricular valve (*AVV*) segments. Corresponding days of human embryonic development are indicated. *A* atrium, *Ao* aorta, *DA* ductus arteriosus, *LA* left atrium, *LCC* left common carotid, *LSCA* left subclavian artery, *LV* left ventricle, *PA* pulmonary artery, *RA* right atrium, *RCC* right common carotid, *RSCA* right subclavian artery, *RV* right ventricle, *V* ventricle. (Re-use with copyright permission granted from [2])

continue communicating. Also, the atrial ventricular junction becomes denser [18].

2.3 Atrium, Sino Atrial Node, and Atrial Ventricular Node Development

Beginning in the primary heart tube, the atrial septum divides into the septum primum and the septum secundum and looks similar in both mice and humans. The process spans E10.5 to E13.5 in mice and days 48–56 in humans [17]. Starting at the posterior wall of the atrium, the septum primum extends out and eventually meets the endocardial cushions surrounding the atrial ventricular junction. This spine that extends from the wall of the atrium is made of mesenchymal cells that later become muscle cells. This process helps to close the interventricular foramen [18]. Later, the septum secundum formation begins in the dorsal wall of the atrium, when it in folds and grows to the right of where the pulmonary vein will develop [17].

In addition to septation, between E10.5 and E11.5 in mice and around week 5 in humans, some atrial mesenchymal cells will become the sinoatrial node. The comma-shaped node's characteristic head and tail regions develop independently, in order of functions. As it develops, the left-sided sinoatrial node joins the surrounding myocardium and begins to look like characteristic working myocardium. But unlike the left-sided atrial node, the right side does not look like its working myocardium surroundings. Instead, it

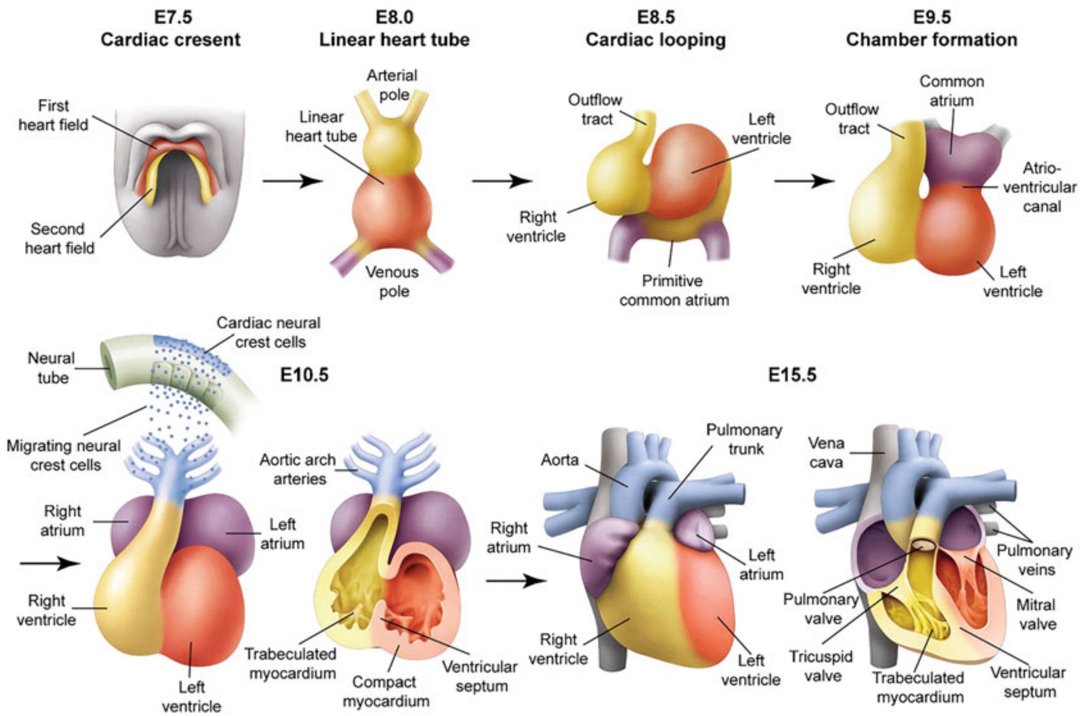

Fig. 2 Mouse cardiac development. The heart originates from mesodermal cells in the primitive streak. During gastrulation, cardiac progenitors migrate to the splanchnic mesoderm to form the cardiac crescent. At E7.5 in the mouse, the cardiac crescent can be divided into two heart field lineages based on differential gene expression and their respective contribution to heart, a first heart field (*red*) and a second heart field (*yellow*), which is located posteriorly and medially to the first heart field. At E8.0, the linear heart tube is present. At E8.5, the looping is associated with uneven growth of cardiac chambers. The outflow tract is at the arterial pole and the inflow tract and primitive atria are at the venous pole. By E9.5, the common atrium has moved superior to the ventricles and is separated by a distinct atrio-ventricular canal. By E10.5, cardiac neural crest cells from the dorsal neural tube migrate via the pharyngeal arches to the cardiac outflow tract. Further cardiac development involves a series of septation events and myocardial trabeculation that result in a mature four-chambered heart integrated with the circulatory system depicted at E15.5. (Re-use with copyright permission granted from [16])

keeps a more primitive function, enabling it to be tracked even in late development [19].

2.4 Ventricular Development

Like atrial development, ventricular development looks very similar between mice and humans. It begins on day E11.5 in mice and week 8 in humans, near the end of the looped heart stage [17]. During ventricular development, the ventricular septum protrudes from the ventral part of the ventricular chamber floor and grows toward the atria. Closing the ventricular septum is thought to be mediated by cells moving from the secondary heart field in the dorsal direction.

2.5 Atrioventricular Valve Development

Atrioventricular valve development spans over E10.5-E17.5 in mice and days 48–66 in humans. The valves' structure is complete by E12.5 and day 56, in mice and humans, respectively, but continues to develop into their polished versions until E17.5 and day 66 [17]. Endocardial cushions, or extracellular matrix areas, that line

Table 2 Genes involved in cardiac development and congenital heart disease

Name	Human phenotype/role
MYBPC3	ASD, PDA, VSD, MR
SCN5A	Long QT syndrome
HRAS	PS, ASD, VSD, PDA, other structural heart disease, hypertrophy, rhythm disturbances
TNNT1/ TNNT2	HCM
LDB3	LVNC
MYPN	RCM
LMNA	LVNC
CASQ2	CPVT
KCNH2/ KCNQ1	Long QT syndrome, short QT syndrome
TAZ	LVNC
Structural proteins	
MYH7	EA, LVNC, HCM, DCM
FBN1	Marfan syndrome
Receptors and ligands	
RyR2	CPVT
Transcription factors and co-factors	
GATA5	AVSD, DORV, LVNC, BAV, CoA
GATA4	Dextrocardia, AVSD, DORV, TOF, BAV, CoA, AR, PAPVR, PDA, PS, ASD, VSD,
GATA6	AVSD, TOF, PDA, PTA, PS, ASD, VSD, OFT defects
NKX2-5	ASD, AVSD, VASD, BAV, CoA, Dextrocardia, DORV, Ebstein's anomaly, HTX, HLHS, IAA, LVNC, Mitral valve anomalies, PA, PAPVR, PDA, PS, SVAS, TA, TAPVR, TGA, TOF, PTA, VSD
TBX5	AVSD, TOF, BAV, CoA, ASD, VSD, Holt-Oram syndrome, PDA
PBX1	CAKUTHED syndrome
Signaling	
JAG1	Aortic dextroposition, TOF, BAV, CoA, PS, VSD, Alagille syndrome, peripheral pulmonary hypoplasia
NOTCH2	AVSD, TOF, BAV, CoA, PS, Alagille syndrome, peripheral pulmonary hypoplasia
TGFB2	VSD, Loeys-Dietz syndrome
TGFB3	Loeys-Dietz syndrome
TGFBR1	BAV, Myxomatous mitral valve, TAAD, Loeys-Dietz syndrome, Marfan syndrome

Information taken from [2, 3, 6, 10, 11, 16, 19]

ASD atrial septal defect, *AVSD* atrioventricular septal defect, *BAV* bicuspid aortic valve, *CoA* Coarctation of the aorta, *CPVT* catecholaminergic polymorphic ventricular tachycardia, *DORV* double outlet right ventricle, *HCM* hypertrophic cardiomyopathy, *HLHS* hypoplastic left heart syndrome, *HTX* heterotaxy, *IAA* interrupted aortic arch, *LVNC* left ventricular noncompaction, *OFT* outflow tract, *PAPVR* partial anomalous pulmonary venous return, *PDA* patent ductus arteriosus, *PS* pulmonary stenosis, *PTA* persistent truncus arteriosus, *TA* tricuspid atresia, *TAAD* thoracic aortic aneurysm and dissection, *TAPVR* total anomalous pulmonary venous return, *TGA* transposition of the great arteries, *TOF* tetralogy of Fallot, *VSD* ventricular septal defect

the atrial ventricular canals divide this area into left and right, and create the atrial ventricular valves. There forms the mitral valve on the left and the tricuspid valve on the right, though at this stage they are thick. In the next 10 days of human gestation, these valves thin and are developed. In mice though, the tricuspid valve takes until E17.5 to develop, and even further development occurs postnatally [17].

2.6 Outflow Track Development

The truncus arteriosus is the site of many events in cardiac development. Cells from the second heart field interacting with neural crest cells will create a septum and become arteries [18]. In humans and mice, at days 50 and 11.5, respectively, neural crest cells facilitate the formation of two ridges. As the truncus arteriosus cushions

begin twisting, they separate into the aorta and pulmonary arteries. At least this is true distally. At this point, proximally the truncus arteriosus is still connected as one [18]. By day E12.5 in mice, the outflow tracts are separate proximally as well. In general, the development of the outflow tracts looks similar between humans and mice [17]. This process is delicate in that 30% of congenital heart defects are due to this neural crest cell process. ET-1, dHAND, and neurophilin-1 are known to regulate neural crest cell development [2]. Neural crest cells are proven to be needed to properly close the ductus, separate the outflow tract, form the aortic arch, and form the ventricular septation [10]. Neural crest cells are also thought to induce the development of the cardiac conduction system, though it is not known precisely how [19].

The semilunar valve is made in a process similar to that of the atrial ventricular valves, in that it comes from cushion tissue, this time truncal, and is thick to start. Semilunar valves then thin out over time. This process begins in mice at day E12.5 and week 8 in humans [17].

2.7 Conductance System

Development of accessory pathways, or accessory bundles of cardiomyocytes, are essential as the atria and ventricles develop, because they conduct action potentials in both the atrial ventricular direction and the ventricular-atrial direction. When the atrial ventricular junction forms, conduction between the atria and ventricles is one of the only connections the two areas have. They are located both endocardially and epicardially to start, and thus have different cellular morphologies. However, it is also imperative that these decrease in number and size as the heart, specifically the atrioventricular junction, develop, or else it can lead to cardiomyopathies later on in development [19].

When it comes to the cardiac conduction system, the posterior end of the heart field will give rise to the sinoatrial node. Some suspect it will also contribute to the formation of the atrioventricular node. However, it is still debated [19]. Furthermore, at E9.5 in mice, epicardial cells from the venous pole of the heart migrate over the developing heart to create the outer layer of the epicardium. Epicardium-derived cells go on to help form smooth muscle cells, coronary vasculature, the atrial ventricular valves, and the compact myocardium. Still, they are also thought to play a role in developing the peripheral conduction system through Purkinje fiber cells [19]. When it comes to the atrioventricular node, it starts to develop at week 5 in humans and day E11.5 in mice. The atrioventricular node develops from the myocardium and begins as an anterior and posterior node, the posterior node eventually playing the more significant role. After all, it is the node that connects to the His bundle. Ultimately, the anterior and posterior atrioventricular nodes fuse [19]. There are many theories as to which cells exactly give rise to the atrioventricular node, but for now, it is only agreed upon that it has multiple cell sources [19]. During early development, the atrioventricular canal conducts slowly, which is known as the atrioventricular delay. As the heart continues to develop, the annulus fibrosus forms and interrupts the myocardial continuity, which would interrupt conduction to the ventricles. As a result, the common bundle begins conducting the electrical impulse to the now working ventricular myocardium and, these electrical impulses speed up [19].

3 Genetic Archetypes in Cardiac Development

As a beginning step, the heart tube formation initiates with the help of the progenitor cells within the anterior lateral plate mesoderm, which becomes committed to a cardiogenic fate around embryonic day (E) 15 in humans. Specific signaling molecules such as bone morphogenetic proteins, fibroblast growth factors (Fgfs), and Wnts are responsible for this step [21–23]. Cardiac precursors bilaterally come together and fuse at the cephalic portion of the primitive streak and forms the cardiac tube. This straight heart tube contains an outer myocardium and an inner endocardium separated by an extracellular matrix

(ECM) known as the cardiac jelly. This process is shown to be under GATA transcription factors control [24]. The tubular heart initiates rhythmic contractions at approximately E23.0 in humans. The linear heart tube is segmentally shaped along the cranial (arterial pole) to caudal (venous pole) end into precursors of the aortic sac, conotruncus (outflow tracts), and primitive ventricle, primitive atria, and sinus venosus. The original upside-down heart tube lies in the cranial part of the embryo and needs to be curved. This critical development is called cardiac looping. In all vertebrates, the linear heart tube at first undergoes rightward, C shape looping and next S shape looping, which is essential for proper orientation of the pulmonary (right) and systemic (left) ventricles, and remodeling of the heart chambers with the vasculature [25]. With this looping process, the heart tube changes its orientation. From cranial to caudal direction, the structures lie as the aortic sac, primitive atrium to the primitive ventricle, and bulbus cordis.

The molecular mechanisms controlling the cardiac looping remain unknown but transforming growth factor-ß (TGF-ß) seems to be playing the role. The creation of a looped heart tube then enables the structure of four chambers and the arterial venous poles. After proper looping, the heart tube is ready to be divided into four chambers. This is followed by symmetrical atrial septation into the left and right atria, which governs NKX2.5 and TBX5 genes. The formation of heart valves and sequential ventricular septation into the left and right ventricles with a formation of the primitive interventricular septum between them, is mainly controlled by the TBX5 gene [2]. As we improve our understanding of cardiac development and the role of genetics in this process, the more underlying pathological processes depend on genetic abnormalities. We have enough evidence to assume that a significant portion of the CHD's originates from errors or disruption in heart development's genetic control.

The etiology of congenital heart defects is recently becoming a more interesting topic in the literature. When we look at the genetic determinants of CHD, we can identify almost 30% of the genetic abnormalities behind CHD's. The majority of the genetic determinants, nearly 70%, are still unrecognized [11]. However, as we improve our understanding of the genetic contribution to heart development and improve genetic technology, the undetermined portion will be less in the near future. Gene therapy is becoming a compelling treatment option when genetic etiology is apparent in many diseases. In this review, we will divide the genetic archetypes behind the CHD, and we will review the gene therapy options based on the genetic model of the CHD.

4 Genetic Archetypes for Syndromic Congenital Heart Defects

This subset of CHD cases has an exact genetic etiology, including various chromosome abnormalities, microdeletion/microduplication syndromes or, single-gene disorders, some of which are syndromic and some of which are nonsyndromic. Recurrence risk estimation is much easier for these cases with a clear genetic etiology; the magnitude of the risk depends on the specific cause. Except for the nonsyndromic single-gene causes of CHD, genetic causes of CHD often involve clinical or developmental features in addition to CHD. People with Down syndrome often have a higher than average number of abnormalities, such as intellectual disabilities, hypotonia, dysmorphic features, and other extracardiac symptoms [26]. Deletion 22q syndrome is commonly associated with oral clefting, velopharyngeal insufficiency, learning disabilities, calcium regulation issues, and thymus hypoplasia [27]. People with Holt–Oram syndrome due to mutations in TBX5 are often characterized by abnormalities in the limb and heart, such as atrial and ventricular septal defects [28]. Costello syndrome occurs due to HRAS mutations and can cause pulmonary stenosis and hypertrophic cardiomyopathy in the heart. People with Alagille syndrome typically have bile duct paucity, typical facies, and vertebral and heart anomalies. In this syndrome, the mutations are generally in JAG1and NOTCH2, and most

cardiac defects are pulmonary stenosis, hypoplasia, and Tetrology of Fallot [29]. Currently, prevention and treatment for this group have not been the focus of the current era. We are doing an excellent job counseling the recurrence risk and diagnosing them prenatally to inform the patients.

5 Genetic Archetypes of Nonsyndromic Isolated Congenital Heart Defects

In recent years, we have learned about several single genes that, when mutated, are associated with nonsyndromic familial CHD. Unlike syndromic CHD, where individuals often have various other medical and developmental concerns in addition to their cardiac problems, nonsyndromic familial CHD is associated with isolated heart defects. Many point mutations of NKX2.5 have been found in families with atrial septal defects and arrhythmias [30]. Sporadic mutations of the NKX2.5 can cause tetralogy of Fallot, an outflow tract alignment defect, and tricuspid valve defects. The exact mechanism of these mutations resulting in cardiac defect is not precise yet. The linkage of particular loss of function with mutations in some distinct abnormalities suggests that different aspects of NKX2.5 functions can be altered in other developmental portions of the heart. Typically, these conditions exhibit both reduced penetrance and variable expressivity. For example, some people in a family who inherit an NKX2.5 mutation will have completely normal hearts, some will have Tetralogy of Fallot, some may have atrial septal defects, etc., but everyone who inherits the mutation, regardless of their phenotype, can transmit that mutation to the next generation [31]. Mutations in GATA4 can cause atrial septal defects, atrioventricular septal defects, and great artery abnormalities, specifically pulmonary artery abnormalities. NOTCH1 mutations go with the bicuspid aortic valve, aortic stenosis, aortic coarctation, and hypoplastic left heart syndromes [32]. The currently known genes to cause nonsyndromic familial CHD's are listed in Table 2. These new developments demonstrate that single-gene defects can lead to isolated congenital heart disease and reveal more about molecular pathways important in cardiac morphogenesis.

6 Genetic Archetypes for Left–Right Patterning

The left-right asymmetry of the heart is required for proper oxygenation of the body, with the left side of the heart holding the responsibility of systemic circulation in order to provide oxygenated blood throughout the body. In contrast, the right side of the heart is responsible for pulmonary circulation to the lung for gas exchange. The abnormal left-right patterning, or laterality defect, is highly associated with CHD [33, 34] indicating the importance of left-right patterning in cardiac development.

This left-right asymmetry established with the rightward cardiac looping (the 4th week of human gestation and E8.5-E10.5 in mice) reflects the left–right body axis. The human body is highly asymmetric, with the body plan following the three axes (anteroposterior [A/P], dorsoventral [D/V], left-right [L/R]) that are established very early in embryonic development (human embryonic day E23 and mouse embryonic day E8.5) [35]. The major visceral organs are packed into the human body with a striking left-right asymmetry. The vast majority of the human population has developed this asymmetric thoracoabdominal organ arrangement, known as the normal situs, called *situs solitus* (SS). Comparative studies revealed that this directionality of the situs asymmetry is vertebrate-conserved, from fish, frog, mouse, to higher mammals, including humans [36]. However, when the asymmetry fails to develop correctly, it results in a pathogenic condition, *heterotaxy*, also known as *situs ambiguous*, which is generally associated with a spectrum of intra-cardiac defects, found in 1 of 10,000 births, and is associated with at least 3% of CHD cases [37]. Over the past few decades, it has been recognized that cilia, the highly conserved microtubule-based structures found in

almost all cell types, play central roles in left-right asymmetry in development. Ciliary abnormality, primary ciliary dyskinesia (PCD) [38], accounts for a host of human diseases such as cystic kidney disease, retinal degeneration, and Bardet-Biedl and Meckel-Gruber syndromes [39–46]. The left–right patterning [47] is established by nodal motile cilia rotating clockwise to generate a leftward flow of morphogens resulting in an asymmetrical gradient around the node, thus breaking the initial embryonic symmetry and establishing left and right asymmetry [41, 45]. Intra-ciliary calcium oscillation dynamics [41–43, 48] are identified as a key signaling pathway that initiates cascades of subsequent events in left–right development. Growing numbers of genetic analyses in both humans [37, 49–52] and mice [53–58] have uncovered arrays of PCD genes. Among all the PCD patients examined [49], about 48% developed SIT, 6% heterotaxy, and 46% had normal SS. How the bodily left–right axis established during nodal development affects the left–right patterning in the heart is not completely clear. A recent discovery of intrinsic cellular chirality [59] showed that the developing chick cardiomyocytes are intrinsically chiral and exhibit dominant clockwise rotation in vitro. Furthermore, the developing myocardium is chiral as evident by a rightward bias of cell alignment and a rightward polarization of the Golgi complex, correlating with the direction of cardiac looping. It is possible that the intrinsic cellular chirality regulates the left–right patterning from a cellular level, cardiac looping, to the overall body plan.

7 Genetic Archetypes of Inherited Arrhythmias

The most common inherited primary arrhythmia syndromes are Long QT syndromes, catecholaminergic polymorphic ventricular tachycardia, Brugada syndromes, and short QT syndromes. The inherited primary arrhythmia syndromes are mainly caused by cardiac channelopathies. Genetic mutations that cause inherited primary arrhythmia syndromes are mostly in genes encoding ion channels and associated regulatory proteins in the heart [60]. The inheritance pattern for primary arrhythmia syndromes is usually Mendelian. The onset of the disorders appears early in life. Epidemiological studies on the spectrum of etiologies of sudden cardiac death (SCD) indicated that the primary arrhythmia disorders are one major culprit of SCD among young and healthy individuals [61]. However, clinical features and phenotypical expression of the inherited primary arrhythmia syndromes resulting from cardiac channelopathies can be variable [62]. The complex interplay of the mutation characteristics, epigenetic, and environmental factors can influence the vulnerability to arrhythmias as well as the disease progression [63]. Many of these diseases exhibit overlapping symptoms. Therefore, precise genetic testing can be of merit in diagnosis, prognosis, guiding clinical management and more specific therapy [62].

7.1 Long QT Syndromes

The congenital long QT syndrome (LQTS) is one of the most common inherited arrhythmias in structurally normal hearts. It is usually diagnosed with prolongation of the QT interval on the electrocardiogram (ECG). Clinically it can cause syncope, seizures, polymorphic ventricular tachycardia (VT) (torsades de pointes), cardiac arrest, and sudden death.

To date, 16 genes have been associated with LQTS. The most common genes seen in 90% of all genotype-positive cases are KCNQ1 (LQTS1), KCNH2 (LQTS2), and SCN5A (LQTS3). The diagnostic yield of these mutations is high. It allows us to understand the penetrance type and determine the risk for the upcoming generations in the same family before being discovered by their clinical symptoms. As a treatment option, the type of mutation will help us start prophylactic treatments, which have already been proven to reduce cumulative mortality [64].

7.2 Catecholaminergic Polymorphic Ventricular Tachycardia (CPVT)

Catecholaminergic polymorphic ventricular tachycardia (CPVT) is an inherited arrhythmic disorder. It is characterized by adrenergic mediated polymorphic or bidirectional ventricular tachycardia (VT) that may degenerate into ventricular fibrillation (VF) which can cause cardiac arrest or sudden cardiac death in patients with structurally normal hearts. As indicated by Drs. Asatryan and Mederios-Domingo [65], for patients with clinical CPVT is suspected, the presence of pathogenic mutations of RyR2 or CASQ2 can be diagnosed by genetic testing, which can have almost 60% diagnostic yield. Besides confirming the diagnosis, a positive result is very useful to identify other affected family members at risk for sudden cardiac death [65, 66].

7.3 Brugada Syndromes (BrS)

Brugada syndrome (BrS) is characterized by a typical ECG pattern of coved-type ST-segment elevation with successive negative T waves in the right precordial leads with or without cardiac conduction delays. Ventricular tachyarrhythmias and sudden death in sleep are the most common clinical findings and manifest between 30 and 40 years of life. The prevalence is higher in males. Although it is rare compared to Long QT syndrome, it is a silent killer due to silent course and intermittent ECG patterns. Although the genetic test's diagnostic yield is around 30%, mutation-specific genetic testing is recommended for family members after identifying a causative mutation. It allows for presymptomatic diagnosis in relatives at risk who need further clinical follow-up, prophylactic treatment, etc. At least 20 genetic mutations have been found to account for 30–35% of BrS cases [65, 67], with loss-of-function mutations in SCN5A contributing to about 30% of said cases [65, 68, 69]. Therefore, global as well as specific SCN5A genetic testing is an expected course of action for any patient suspected of having BrS. It is generally recommended that family members of BrS patients have genetic testing as well in order to allow for early diagnosis and presymptomatic clinical and treatment plans.

7.4 Short QT Syndrome (SQTS)

While exceedingly rare, short QT syndrome (SQTS) is a heritable, grave, deadly cardiac channelopathy. ECG reveals short QT intervals in these patients, making them increasingly vulnerable to atrial and ventricular arrhythmias and sudden death [65, 70]. While some patients present first with these arrhythmias in the form of heart palpitations or syncope, 40% of cases present cardiac arrest as their first symptom, with any survivors showing a high rate of recurrence. KCNH2 (SQTS1) was the first gene discovered in relation to SQTS, with about an 80% penetrance, though data is limited. Therefore those with any clinical suspicion or family history of SQTS should undergo genetic screening for three major genes associated with SQTS, KCNH2, KCNQ1, and KCNJ2, the yield of which is around 40% [65, 71, 72].

8 Genetic Archetypes of Inherited Cardiomyopathy

Cardiomyopathy is a form of heart disease affecting the cardiac muscle and can cause major cardiac-related morbidity in almost all ages. A significant portion of them has a genetic origin. Advances in molecular genetics allowed us to identify multiple genes responsible for cardiomyopathies. Surprisingly, different mutations in the same gene can cause different types of cardiomyopathies. Cardiomyopathies can be classified as dilated, hypertrophic, arrhythmogenic right ventricular, restrictive, or left ventricular non-compaction cardiomyopathies. As a neuromuscular disorder, especially Duchenne and Becker muscular dystrophies, cardiomyopathy is also characterized by skeletal myopathy [73].

8.1 Dilated Cardiomyopathy (DCM)

Dilated cardiomyopathy (DCM) is characterized by left ventricular dilatation and abnormality in systolic function. DCM is the most common indication for cardiac transplant. Inheritance patterns are generally autosomal dominant in 30–50% of cases. Small percentages can be autosomal recessive, X-linked, and mitochondrial inheritance. More than 40 genes have been described in DCM. Defects with LMNA-encoded lamin mutations, myosin heavy chain beta mutations, ribonucleic acid-binding protein mutations, and many other complex molecular deficits have been implicated in the pathogenesis of DCM [74]. Most genetic mutations associated with DCM have extremely low prevalence and high heterogeneity. Therefore, it is often necessary to sequence large numbers of genes in order for effective genetic testing. If the DCM is together with conduction disease and/or arrhythmia and strong family history, then focused testing for LMNA, desmosomal, and SCN5A mutations may have a substantial clinical impact. Identification of a genetic mutation in the setting of family history allows early screening, appropriate monitoring, and prophylactic treatments [75].

8.2 Hypertrophic Cardiomyopathy (HCM)

The inheritance pattern of hypertrophic cardiomyopathy (HCM) is autosomal dominant, characterized by concentric hypertrophy of the left ventricle and the septum [76]. The genes that encode sarcomeric proteins are involved in the pathogenesis of HCM. The most common of them that accounts for 20–30% of the HCM is mutations in MYH7 (encoding the β-myosin heavy chain), MYBPC3 (encoding the cardiac myosin binding protein C), and cardiac troponin T (TNNT1and 2) [77]. These mutations, in general, cause decreased myocyte relaxation and increased myocyte growth with prominent involvement of the interventricular septum. Approximately 10% of the patients may carry multiple sarcomeric mutations, presenting with more severe diseases at younger ages. This indicates the need for detailed genetic evaluation of the family for early diagnosis and treatment. The genetic diagnosis can go up to 70% if there is a family history of HCM. The yield is lower when sporadic diseases are considered. For effective screening, it is important to know the pathogenicity of the mutation. Many mutations that cause HCM can be unique to the individual family; therefore, careful genetic counseling and family assessment are needed in this type of inherited cardiomyopathy [78].

8.3 Arrhythmogenic Right Ventricular Cardiomyopathy (ARVC)

The arrhythmogenic right ventricular cardiomyopathy (ARVC) is a form of heart disease characterized by fibrosis and fatty infiltration of the myocardium, mainly in the right ventricle. The inheritance pattern for ARVC is autosomal dominant with incomplete penetrance. Mutations in the genes cause encoding desmosomal proteins are the main etiology. These mutated genes disorganized desmosomal integrity, making muscle fibers more fragile, sensitive to tearing, fragmentation, and eventually cell death in the course of the cardiac cycle. As a result, the desmosomal function, the gap junction remodeling, sodium channel function, and electrocardiographic parameters in cardiomyocytes are also compromised. Besides, disturbance of desmosomal proteins promotes adipogenesis in mesodermal precursors by suppressing the Wnt/β-catenin signaling pathway. This particular pathway is known for its role in cardiac myogenesis [79].

As a consequence of this abnormal process, the fibro-fatty replacement of the ventricular myocardium becomes more prominent in the RV. Multiple variants in the mutation cause early presentation and more severe presentation of the disease. The presence of more than one variant was associated with a nearly fivefold increase in odds of penetrant disease. This

information is essential during the genetic evaluation, and recommendation is usually needed to sequence all five desmosomal genes [80].

8.4 Restrictive Cardiomyopathy (RCM)

Restrictive cardiomyopathy (RCM) is a form of heart disease in which the heart chambers gradually become stiff over time. The initial findings are increased ventricular stiffness that impairs ventricular filling without ventricular hypertrophy or systolic dysfunction [81]. The most common inheritance is autosomal dominant. Alterations in genes encoding for sarcomeric proteins (e.g., TNNT2), Z-disc proteins (e.g., MYPN), or transthyretin (TTR) have been identified in patients with RCM [82]. Familial RCM is increasingly recognized as a specific phenotype within the HCM spectrum and can be seen in those who share mutations expressed as classic hypertrophic cardiomyopathy in other family members.

8.5 Left Ventricular Non-compaction Cardiomyopathy

This is a heterogeneous disorder characterized by prominent trabeculae, a thin compacted layer, and deep intertrabecular recesses most evident in the left ventricle apex. Non-compaction may involve the right ventricle, presenting as either a biventricular or isolated right ventricular non-compaction phenotype. The genetic form is commonly inherited as an X-linked recessive or autosomal dominant condition [83]. Mutations that affect the compaction of the endomyocardial layer progress from the base to the apex of the heart during embryogenesis. The genes encoding for sarcomeric (e.g., MYH7), Z-disc (e.g., LDB3), nuclear envelope (e.g., LMNA), mitochondrial (e.g., TAZ), and ion channel proteins (e.g., SCN5A) are found to be responsible for this type of cardiomyopathy.

8.6 Cardiomyopathy in Other Disorders

Duchene muscular dystrophy (DMD), Beker's muscular dystrophy, Marfan syndrome, and Barth syndrome are the other disorders where different types of CMP can be observed. In DMD, three stages are present, usually starting with hypertrophic CMP and some diastolic dysfunction with no heart failure symptoms. Later the heart dilates and accumulates fibrosis and, as the last stage represents, the end-stage heart failure findings such as diastolic dysfunction and arrhythmias. The female carriers of DMD mutations may also manifest dilated cardiomyopathy. This has the potential to progress to heart failure in some cases; therefore, the appropriate genetic counseling and close monitoring of the carriers are also needed. Marfan syndrome is caused by mutations in the FBN1 gene that codes for fibrillin-1. The inheritance is autosomal dominant [84]. Fibrillin-1 is an extracellular protein that plays a role in microfibril formation and provides elastic properties to tissues [85]. Dilated cardiomyopathy is typically associated with Marfan syndrome. The Barth syndrome is an X-linked autosomal recessive disorder is caused by mutations in the tafazzin (TAZ) gene [86]. The loss of tafazzin and increased cardiolipin results in changes in energy stores decreased contractility, and increased heart damage. Barth syndrome cardiomyopathy is usually dilated cardiomyopathy, but cases of hypertrophic and left ventricle non-compaction cardiomyopathies have been described.

9 Genome Editing in Modeling Inheritable Heart Diseases in Model Organisms

Model organisms, such as mice, are indispensable tools for understanding the etiology of inheritable heart diseases and gene functions [87]. Clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated Cas9-based gene editing technology [88–90] allowing efficient

generation of mutant mice in one step [91], mutant mice can be systematically generated for interrogating and modeling human inheritable heart diseases, as well as for dissecting mechanisms for gene functions.

Pre-B cell leukemia factor 1 (PBX1) is a transcription factor essential for development and associated with CAKUTHED syndrome, characterized by multiple congenital defects including CHD. Alankarage and associates identified a de novo missense variant, PBX1: c.551G>C p.R184P, in a syndromic CHD patient with tetralogy of Fallot with absent pulmonary valve [92]. Using CRISPR-Cas9 gene editing to generate a mouse model with this mutation, Alankarage et al. [92] conducted functional and phenotypical analysis of Pbx1 in mice to show that p.R184P is disease-causal. Wnt/β-catenin signaling cascade [93–95] is important transcriptional regulation for morphogenesis. Combining zebrafish and mouse genetics, Cantù et al. [96] used CRISPR-Cas9 gene editing to demonstrate that tissue-selective perturbation of Bcl9 and Pygo as selective β-catenin cofactors in a subset of canonical Wnt responses caused severe CHD.

Sufficiently sizeable cohorts of probands when searching for causative genes in CHD can be challenging to assemble [8] because of large numbers of causative variants, low frequency of causative variants for individual genes, and diverse genetic backgrounds of the human population. Forward genetic screening with *N*-ethyl-*N*-nitrosourea (ENU) mutagenesis [97] in model organisms, such as inbred mouse strains, with very highly induced rates of mutation throughout the genome in the homogeneous genetic background, is therefore invaluable to uncover causative genes in CHD [53, 55, 98, 99]. However, ENU mutagenesis can cause point mutations in ~100 genetic loci per genome; consequently, it is imperative to rule out non-causative mutations for the CHD phenotypes. Gene editing to knock in or knock out specific gene mutations in the inbred wild-type (WT) background can authenticate the causality of the gene in question. Leveraging forward genetic screening with ENU mutagenesis, Liu et al. [98] have identified causative digenic mutations in *Sap130* and *Pcdha9* that can synergistically cause hypoplastic left heart syndrome (HLHS) in double homozygous mutants with incomplete penetrance and non-mendelian complex genetic inheritance [98, 99]. Gene editing using the CRISPR/Cas9 system to generate the same point mutations in these two genes in the WT background produced identical phenotypes with similar penetrance and substantiated that *Sap130* and *Pcdha9* are causative genes for HLHS.

Furthermore, the CRISPR/Cas9 system has shown tremendous potential to correct genetic defects in zygotes or postnatal mice [100]. Using adeno-associated virus (AAV9 or AAV8) to deliver CRISPR/Cas9-mediated gene editing components, in vivo somatic genome editing has been shown to correct the disease-causing gene mutation of Duchenne muscular dystrophy (DMD) in mice and improve phenotypical outcomes in postnatal mice [101, 102]. Sarcoplasmic reticulum Ca^{2+}-ATPase 2a (SERCA2a) and its inhibitory protein called phospholamban (PLN) are pivotal for Ca^{2+} handling in cardiomyocytes. Their expression levels and activities were changed in heart failure patients. Using the CRISPR-cas9 system, Kaneko et al. [103] showed that PLN inhibition could significantly improve cardiac function and survival in calsequestrin overexpressing mice, a severe heart failure mouse model, suggesting PLN deletion could be a promising approach to improve both mortality and cardiac function in the heart failure.

One challenge for using the gene editing approach to dissect functional mechanisms for cardiac development and CHD is the presence of functionally redundant genes in the genetic network for cardiac development. It is necessary to knock out multiple genes in the same functional network to exhibit phenotypes. Conventionally, mutant mice carrying multiple genetic mutations were generated by time-consuming intercrossing of mice with different single genetic mutations. Wang et al. [104] have shown the feasibility of multiplex gene editing with the CRISPR/cas-9 system. Coinjection of Cas9 mRNA and single-guide RNAs (sgRNAs) targeting both Tet1 and Tet2 genes into zygotes

generated mice with biallelic mutations in both genes with an efficiency of 80%. The CRISPR/Cas system allows the one-step generation of animals carrying mutations in multiple genes, an approach that will accelerate the in vivo study of functionally redundant genes and epistatic gene interactions [104].

With the availability of the Mouse Genome Database (MGD) [105, 106], large-scale efforts such as the Knockout Mouse Project (KOMP) [107] and the European Conditional Mouse Mutagenesis (EUCOMM) Program [108] are systematically generating knockout mice for dissecting mechanisms for gene functions. International collaborations combining European Mouse Mutant Archive (EMMA), Infrastructure for Phenotyping, Arching and Distribution of Mouse Diseases Models (IPAD-MD), and International Mouse Phenotyping Consortium (IMPC) [109, 110] are pursuing efforts for detailed phenotypic characterization to gain mechanistic insights into gene function. The challenges remain that over 30% of the genes in mice are essential for development and cause embryonic lethality or neonatal survivability when deleted [109], consequently, it is not feasible to analyze postnatal gene functions. Conditional knockouts [111] with Cre/loxP system can overcome the embryonic lethality by knocking out the gene later in life. Conditional knockouts with CRISPR/cas systems [112, 113] can facilitate efficient conditional knockouts for dissecting gene functions in viable adult animals.

10 Other Considerations

Although strong genetic underpinning regulates cardiac development and CHD, extreme locus heterogeneity, incomplete penetrance, and lack of a genotype-phenotype correlation [114, 115] indicate other non-genetic factors [116] can impede cardiogenesis and contribute to the development of CHD. The penetrance of CHD is incomplete and highly variable. Probands [8] and their relatives carrying the same genetic variants can exhibit different cardiac outcomes, ranging from nearly normal to complex CHD with different CHD lesions. Mechanical perturbation [115, 117–123] of ventricular preload pressure and shear stress, as well as exposure to alcohol [124] and environmental toxins [125] can cause CHD. Epidemiology studies have identified maternal risk factors associated with CHD, such as cardiometabolic disorders, stress, preeclampsia, obesity, and diabetes mellitus [126–129]. The risk of congenital anomalies in infants of diabetic mothers is estimated to be between 2.5 and 12%, with an over-representation of CHD [130]. Better understanding of the gene–environment interactions in myocardial development and the pathogenesis of CHD is needed to facilitate effective genome editing as a therapeutic intervention. Additionally, ethical concerns of germline genome editing to correct developmental diseases need to be addressed before the genome editing technologies can treat curable CHD and other cardiovascular diseases [131–133].

11 Conclusions

Reverse and forward genetics will continue to enhance and refine our models of heart development, associate mutated genes with abnormal phenotypes, be used to screen embryos, fetuses, parents, and family for mutations in these genes, and provide highly informed genetic counseling. If and when gene editing is available for treatment of inheritable disorders, the accumulated knowledge of heart development and disease will guide details on when, where, and how to apply gene editing.

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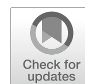

Genome Editing and Heart Failure

Daniele Masarone, Martina Caiazza, Federica Amodio, Enrico Melillo, Roberta Pacileo, Giuseppe Limongelli, and Giuseppe Pacileo

Abstract

Heart failure is a leading and growing cause of morbidity and mortality worldwide and clinically is defined by the presence of typical symptoms and signs due structural or functional cardiac abnormalities. In addition to family history of heart failure, genetic predisposition to cardiomyopathies and exposure to cardiotoxic agents, risk factors for heart failure with reduced ejection fraction are the same as for chronic coronary syndrome. Genome editing technologies can provide the tools to correct genetic defects responsible for various diseases, including cardiomyopathies. These technologies aim to reverse specific mutations. The same methods can also be applied to modulate and improve heart function. This chapter will briefly explain the pathophysiological and genetic aspects of heart failure and then discuss the clinical applications of genome editing in patients with heart failure.

Keywords

Heart failure · Genome editing · Disease models · Beneficial therapeutic effect

1 Introduction

Heart failure is a clinical syndrome characterised by typical symptoms (i.e. shortness of breath, ankle swelling and fatigue) and signs (i.e. elevated jugular venous pressure, pulmonary crackles and peripheral oedema) caused by structural or functional cardiac abnormalities. These anomalies can determine elevated intracardiac pressure, reduced cardiac output or both at rest or under stress [1]. Heart failure is a leading and growing cause of morbidity and mortality worldwide with a prevalence of approximately 2% in the general population [2]. Below the values of the ejection fraction, patients with heart failure can be classified into three groups (Fig. 1): patients with heart failure with reduced left ventricular ejection fraction (≤40%), patients with preserved left ventricular ejection fraction (≥50%) and patients with ejection fraction with intermediate values (>40% and < 50%) [3]. To date, almost all experimental drugs or devices show a beneficial therapeutic effect exclusively in patients with chronic heart failure with reduced ejection fraction. Conversely, few treatment options are available for patients with heart failure with preserved ejection fraction [4, 5].

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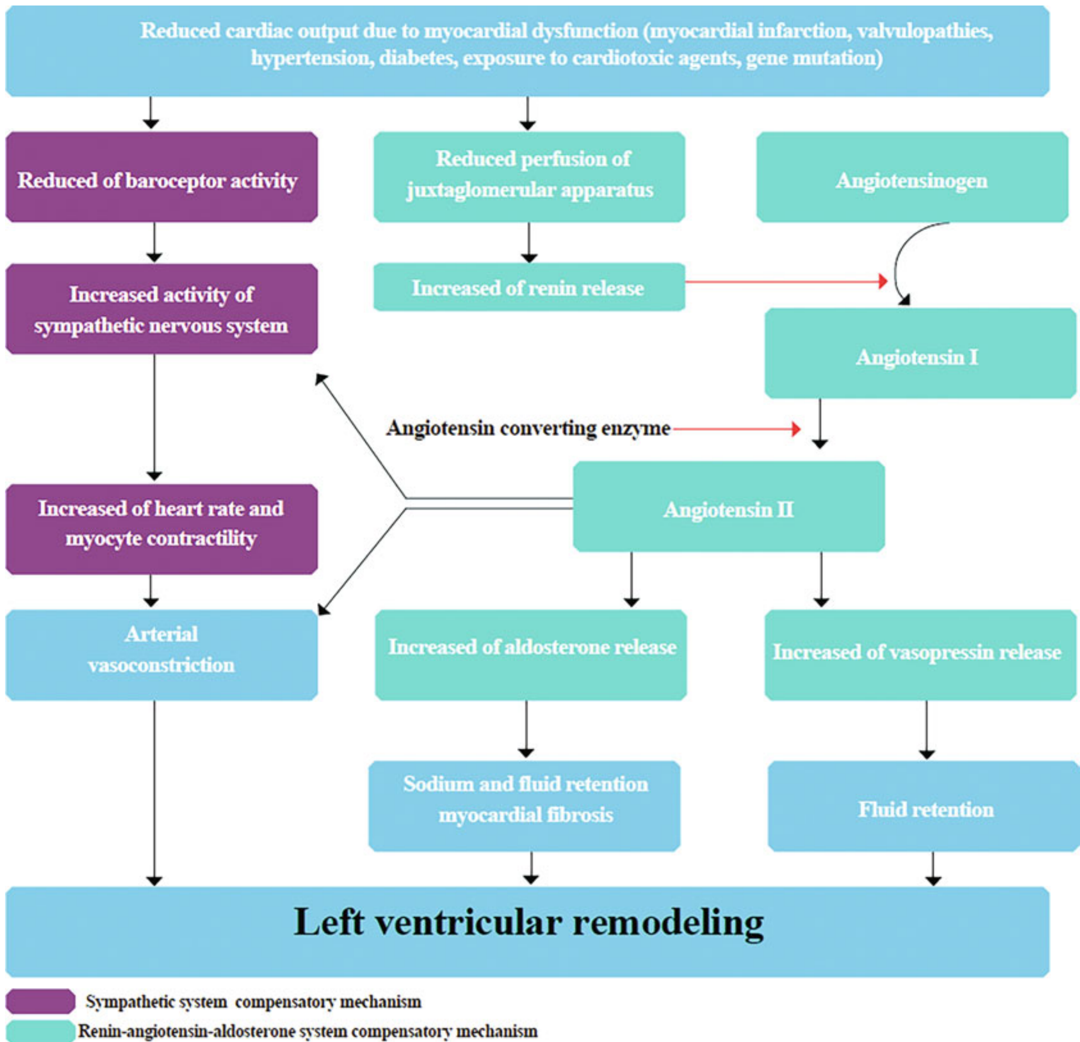

Fig. 1 The pathophysiology of heart failure with reduced ejection fraction

Advances in molecular genetics have made it possible to use genome editing in various fields of biomedical research, including cardiovascular science. Currently, zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) and clustered regularly interspaced short palindromic repeats (CRISPRs) whose use is also proposed for the treatment of patients with heart failure are the most used tools [6].

This chapter will briefly explain the pathophysiological and genetic aspects of heart failure and then discuss the clinical applications of genome editing in patients with heart failure.

2 Pathophysiology of Heart Failure

The pathophysiology of heart failure with reduced ejection fraction is characterised by disease progression. Risk factors lead to cardiac injury subsequently to initially asymptomatic myocardial dysfunction [7]. Symptoms worsen until they reach an advanced stage of the disease. In addition to family history of heart failure [8, 9], genetic predisposition to cardiomyopathies [10] and exposure to cardiotoxic agents (e.g. alcohol, amphetamines, radiation and anthracyclines)

[11], risk factors for heart failure with reduced ejection fraction are the same as for chronic coronary syndrome (hypertension, hypercholesterolemia, diabetes mellitus and obesity). Cardiac injury may also occur as a result of any cardiovascular disease. Such cardiac injury results in the loss of myocyte cells and an increase in stress on the residual myocytes with subsequent development of left ventricular eccentric hypertrophy [12], mediated by neurohormonal activation of the sympathetic system and the renin-angiotensin-aldosterone system [13]. This is followed by the progressive dilation of the left ventricle, development of fibrosis, change in the shape of the left ventricle (from elliptical to spherical) and often functional mitral insufficiency [14]. These changes are called left ventricular remodelling. They result in reduced myocardial contraction efficiency and increased myocardial oxygen consumption [15]. Besides, neurohormonal activation causes renal dysfunction resulting in sodium retention, fluid overload, oedema and reduced response to diuretics [16]. Although heart failure is a heterogeneous condition with multiple aetiologies, each of these results in the activation of common mechanisms that result in progression to a single endpoint, which will happen regardless of the initial pathophysiology. To date, the pathophysiology of heart failure with preserved ejection fraction is poorly understood [17]. The most reliable hypothesis is that comorbidities (e.g. obesity, chronic kidney disease, iron deficiency, hypertension, diabetes mellitus and chronic obstructive pulmonary disease) cause a systemic proinflammatory state. This determines the reduction of reactive oxygen species, the endothelial dysfunction, the reduction of nitric oxide production and the decrease in the activity of protein kinase G [18].

The proinflammatory state causes myocyte hypertrophy, titin hypophosphorylation, increased collagen production and consequent deposit in the extracellular matrix. Furthermore, a reduction in left ventricular compliance is observed (Fig. 1) [19].

3 Management of Heart Failure with a Reduced Ejection Fraction

Treatment of heart failure with reduced ejection fraction is mainly based on the use of diuretics (to relieve symptoms associated with congestion), 'disease-modifier' drugs and devices for the electrical therapy of heart failure [20–22]. Disease-modifying drugs work by antagonising the neurohormonal systems activated in heart failure and are β-blockers, angiotensin-converting enzyme (ACE) inhibitors, angiotensin receptor blockers, mineralocorticoid receptor antagonists [23] and the newer angiotensin receptor neprilysin inhibitors [24]. All of these drugs have been shown to improve symptoms, reduce hospitalisations and improve survival. Some of these drugs, such as β-blockers, mineralocorticoid receptor antagonists and angiotensin receptor neprilysin inhibitors, also result in a reduction in sudden cardiac death [25, 26].

A detailed discussion of cardiac resynchronisation therapy and modulation of cardiac contractility is beyond the objectives of this chapter. However, these treatments will increasingly be used in patients with chronic heart failure with reduced ejection fraction (Table 1) [27].

4 Management of Heart Failure with a Preserved Ejection Fraction

There is not therapy with a definitive and favourable effect in patients with heart failure with preserved ejection fraction. Thus, symptom improvement is the primary goal in these patients [28]. Diuretics are the mainstay of treatment for patients with signs and symptoms of congestion, but care must be taken to avoid an excessive decrease in left ventricular preload. Appropriate treatment of hypertension, diabetes mellitus, coronary artery disease and atrial fibrillation remains the best management strategy of these patients [29, 30].

Table 1 Type of drugs, devices and surgery to management of heart failure with a reduced ejection fraction

Drugs	Device	Surgery
β-Blockers	Cardiac resynchronisation therapy	Coronary artery bypass graft
Angiotensin-converting enzyme inhibitors	Cardiac contractility modulation	Left ventriculoplasty
Angiotensin receptor blockers		Valvular surgery
Mineralocorticoid receptor antagonists		Left ventricular assist device implantation
Sacubitril/valsartan		Heart transplant

5 The Genetic Architecture of Heart Failure

Genetic architecture refers to the genetic contribution to a given phenotype [31]. It includes the number of genetic variants that influence a phenotype, the size of their effects on the phenotype, the frequency of those variants in the population and their interactions with each other and the environment [32]. Variation in heart failure phenotype has been attributed to different aetiologies and varying severity of myocardial damage. However, there is evidence that myocardial damages of the same magnitude can lead to different outcomes. Other evidences involve important hereditary and environmental contributions to the development of heart failure [33].

Non-ischaemic heart failure is familial in 30–50% of cases, and more than 30 genetic loci are known to date, although few have been cloned [34]. In family members of patients with chronic heart failure who have asymptomatic left ventricular dysfunction, a genetic predisposition to ventricular remodelling is likely to be present in response to many different stimuli [35]. Recently, some authors reported that several genes (modifier genes) influence the risk of heart failure or modify the clinical course [36]. Most genes related to specific phenotypes (dilated, hypertrophic and restrictive) or polymorphisms in the genes encoding proteins of the renin-angiotensin system influence treatment effect [37, 38]. Furthermore, environmental variables contribute in determining the phenotypic pleiotropy observed in some monogenic disorders [39]. Finally, microRNAs and epigenetic factors have recently been recognised to play an important role in the pathophysiology of heart failure [40, 41].

6 Tools for Genome Editing

Genome editing is a type of genetic engineering in which DNA is inserted, deleted, modified or replaced in a living organism's genome. Unlike early genetic engineering techniques, which randomly insert genetic material into a host genome, genome editing aims at insertions in site-specific locations [42]. Genome editing was pioneered in the 1990s, but editing efficiency limited its use. Genome editing takes place through the use of engineered nucleases belonging to three enzymatic classes: ZFNs, TALENs and engineered meganucleases [43]. In 2015, another very efficient method was born: the CRISPRs (clustered regularly interspaced short palindromic repeats)/ Cas9 system [44]. In 2018, engineered nucleases were the most commonly used method. These create site-specific double-strand breaks repaired through nonhomologous end joining. In recent years, the emergence of new genome editing technologies has provided researchers with the ability to introduce sequence-specific modifications into the genomes of a broad spectrum of cell types. Genome editing using engineered nucleases will certainly contribute to many life sciences fields, boosting the study of gene functions in plants and animals and leading to future gene therapy in humans. The development of cellular transcriptomics, genome editing and new stem cell models may offer an alternative to functional genetics experiments no longer limited to animal models. Another significant step is

represented by the opportunity to perform genetics experiments directly in human samples. Using genome editing, it is possible to block or remove key genes to elucidate function in a human setting and, where possible, to cure monogenic diseases [45]. Gene therapy is the therapeutic release of nucleic acids into a patient's cells as if it were a drug to treat the disease. Clinical trials are currently underway using gene therapy as a treatment for some genetic diseases. These include diseases caused by recessive genetic mutations (cystic fibrosis, haemophilia, muscular dystrophy and sickle cell anaemia) and acquired genetic diseases (cancer and some viral infections). Genome editing and related approaches are already transforming cardiovascular research and the practice of cardiovascular medicine [46]. Very soon, a wide variety of cardiovascular diseases could be treated using genome editing technology, including those we now believe to be completely incurable. Genome editing could solve monogenic cardiovascular disorders for the offspring of affected individuals. Although technically easier, this approach remains ethically controversial [47].

7 Genome Editing in Heart Failure

Cardiomyopathy and heart failure may have a genetic basis. Genome editing technologies can provide the tools to correct genetic defects responsible for various diseases, including cardiomyopathies. These technologies aim to reverse specific mutations. The same methods can also be applied to modulate and improve heart function. Most genetic correction strategies are gene specific. These approaches require in-depth knowledge of the genetic mutations responsible for cardiomyopathies and heart failure, which underlines the importance of genetic diagnosis. The applications of gene editing to cure heart failure conditions are many and relevant.

Cardiomyopathies can be caused by genetic mutations that give rise to familial or inherited forms. Various genetic mutations, such as autosomal dominant (most frequent), autosomal recessive, X-linked recessive and mitochondrial inheritance mutations, can contribute to develop some form of cardiomyopathies [48–50].

The most common genetic mutations linked to cardiomyopathies are single nucleotide variants (SNVs) or small insertions/deletions. These mutations can produce in-frame or out-of-frame effects on the protein they encode. In-frame deletions can produce internal truncations, but the protein can maintain its function depending on the mutation's length. Out-of-frame deletions may reduce the amount of protein mRNA and the stability of amino-terminal domains. Similarly, the loss or addition of stop codons can affect the functioning of the residual protein. To evaluate the most suitable gene correction strategy for the treatment of cardiomyopathies, it is very important to analyse the precise mode of action of the gene. In addition, it is important to identify mutant genes to avoid introducing mutations into the wild-type gene copy. Gene editing of an autosomal dominant disease, like cardiomyopathies mutations, must correct the mutated copy and leave the healthy copy intact. Cardiomyopathies can be caused by SNVs alias mutations that often span only 1–2 bp. SNVs determine the difference between healthy and mutated gene. Therefore, extreme precision is required in corrective technology. It requires a homology-directed repair to correct this type of mutations. Possible genetic therapeutic correction strategies are directed on the disease mutations. For cardiomyopathies, we need to test and analyse several site-specific corrective strategies. In this complex situation, some researchers evaluated the possibility of broader genetic corrective pathways targeting normal genes to enhance cardiac function [51, 52]. It may be possible to use gene editing techniques to upregulate specific sarcomere proteins that increase actomyosin interactions. In this way, it would make the heart more energetically efficient [53].

8 Using Genome Editing to Create In Vitro and In Vivo Disease Models of Heart Failure

The reproduction of disease mutations through the use of genome editing is quick and simple and can be applied to cells or animal models. There are several systems for genome editing, but the CRISPR/Cas9 system is the most widely used for reproducing the disease model (in vivo and in vitro) [54]. The CRISPR method can be used to modify any aspect of gene regulation. Disease models can be generated in cell cultures or in animal models. It is possible to generate knock-in and knockout animal models using the CRISPR approach. Pathogenic variants can be restored or introduced in human-induced pluripotent stem cells. The CRISPR system enables one to fuse it to an effector protein, modulating gene function.

Currently, the application of CRISPR/Cas9 in the field of cardiac biology is still in its infancy. To specifically study the heart, researchers utilised cardiotropic adeno-associated virus (AAV) constructs for the in vivo CRISPR/Cas9 experiments. Several studies utilised a dual-vector approach with the Cas9 protein (derived from *Streptococcus pyogenes*) and single-stranded guide RNA (sgRNA) that are delivered to the heart by separate AAV constructs [55, 56]. Researchers have experimented genome editing techniques in cardiomyopathy models. With an intraperitoneal injection, they used the cardiotropic AAV9 to deliver a sgRNA targeting the Myh6 locus. They subsequently analysed the hearts 5–6 weeks later. The most important goal to achieve was the transduction efficiency, leading to a marked decrease in Myh6 expression. This was accompanied by severe cardiomyopathy and reduced cardiac function [57]. Other authors used similar CRISPR/Cas9 methods to study many genes specifically in cardiomyocytes to investigate their role in heart maturation and to assess specific phenotype in presence of mutations [58]. The use of CRISPR can also be beneficial for generating mouse models. Researchers created knock-in and knockout models with injections of DNA or modified embryonic stem cells into the blastocyst [59]. Kaneko et al. had performed genome editing with CRISPR/Cas9 to eliminate the PLN gene in mouse model of severe heart failure. Compared with heart failure control mice, PLN knockout mice survived longer and had improved cardiac size and function [60]. Most cardiac genome editing studies with CRISPR/Cas9 have been conducted in a murine model. CRISPR has also been used in other species such as rats [61], rabbits [62], pigs [63] and zebrafish [64].

With the application of CRISPR/Cas9 in vitro, pluripotent stem cells can be differentiated into any type of heart cell. Through this method, it is possible to introduce or correct mutations in the cells that determine the disease. These cells represent valuable models to define the pathogenic mechanisms underlying cardiac disease. Some authors have used CRISPR/Cas9 methods for pluripotent stem cells with a pathogenic mutation in MYH7 (responsible for hypertrophic cardiomyopathy). In the model, they observed all the main hallmarks of hypertrophic cardiomyopathy: multinucleation, sarcomeric disarray and hypertrophy. The mutant cells exhibited increased metabolic respiratory activity, impaired calcium handling and contraction force. The authors concluded that their findings supported the proposed energy depletion model to be involved in the progression of hypertrophic cardiomyopathy [65]. Although these studies indicate therapeutic potential, future research will have to prove whether these strategies are also applicable to other cardiac conditions.

9 Genome Editing for Therapy of Heart Failure

A wide variety of cardiovascular diseases can potentially be treated with CRISPR/Cas9. Multigenic diseases are too complex to hope to treat with today's gene editing strategies and are out of our reach. Fortunately, genome editing may potentially solve monogenic cardiovascular disorders, even with current technologies. Although technically easier and likely already

available for use in humans, this approach remains ethically controversial. Public debate and public policy decisions will have to proceed rapidly to allow clinical use of these therapies. Further technical issue will need to be resolved more comprehensively, including those related to long-term risks, off-target effects, mosaicism and applicability to a wider variety of cardiovascular mutations and conditions.

'Standard' pharmacological strategies against cardiomyopathies reduce symptoms and adverse outcomes, slowing the progression of the disease. Current knowledge on genetics and its molecular mechanisms underlying heart disease opens the way to new therapeutic approaches and possibly to the treatment of heart failure.

The development of human cardiomyocytes from iPSCs and the advancement of genome editing offer an innovative approach for the study of cardiomyopathies and their treatment. The human application of these technologies is still ethically discussed, especially from the point of view of eugenics, but the potential therapeutic applications in the cardiovascular field cannot be ignored.

Currently, there are no approved human gene therapies for heart failure. However, intense studies are underway on mouse models of the pathology. A single gene is known to be rarely involved in any disease. Not surprisingly, in most cases, cardiovascular diseases are the result of multiple genomic, epigenetic and environmental risk factors. Therefore, for the correct use of genome editing, it is necessary to fully understand the causes of the disease and the pathways involved.

In 2017, the CUPID study was born to develop a genome editing treatment of patients with heart failure with reduced left ventricular ejection fraction [66]. In contrast, there are currently no clinical trials of gene therapies for patients with heart failure characterised by preserved left ventricular ejection fraction. Underlying the CUPID study is the analysis of the sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase (SERCA2a) protein, which is a major cardiac calcium cycle protein in heart failure. Studies have reported that reduced SERCA2a activity can contribute to the development and progression of heart failure [67–70]. From these data, preliminary studies based on gene therapy were born. The gene transfer of SERCA2a in animal models resulted in cardiac improvement of contractile function, with almost no side effects [71]. In the first phase, CUPID study enrolled only few patients, obtaining promising results in cases with heart failure characterised by severely reduced ejection fraction. AAV1 was used as a vector to insert SERC2a by intracoronary injection. The excellent results obtained 3 years after the start of the study [72] were the basis for a second trial, CUPID2, which included a larger and more randomised population. Again, intracoronary release of AAV1/SERCA2a or placebo was used. However, the results contradicted the promises that emerged from the first study, forcing the researchers to cancel the trial. Nonetheless, the negative results should not be a source of abandonment of the study of the treatment of gene-edited cardiomyopathies, but a starting point for future studies. The trial authors themselves identified some possible causes of failure, proposing to conduct further research to improve the technique [73–75].

10 Conclusions

Heart failure is a leading and growing cause of morbidity and mortality worldwide, whether it results from structural or functional cardiac abnormalities. Today there are few drugs or experimental devices that have a real beneficial therapeutic effect in all heart failure. Therefore, a new type of therapy is needed. The answer to this problem could be genome editing technology, in particular the promising CRISPR/Cas9. Current therapies are based on the use of 'disease-modifying' drugs (beta blockers, ACE inhibitors, angiotensin receptor blockers, etc.), diuretics and devices for the electrical therapy of heart failure. Recently, especially in patients who have a family history of heart failure, it has been seen that there are modifier genes that influence the level risk. These modifier genes can be polymorphisms in the genes that code for the proteins of the renin-angiotensin system or related to specific

phenotypes such as cardiac dilatation or hypertrophy. However, at the molecular level, microRNAs and epigenetic factors involved in cardiac pathophysiology have been identified. It is above all at this level where it is possible to act with genome editing, which aims to insert or remove specific genes in a site-specific way. If this approach proves valid, the future of heart failure therapy will no longer be based on symptomatic treatment alone but on more in-depth biological analysis and genetic therapy.

In conclusion, we are getting closer and closer to personalised/precision cardiological therapy with the advancement of diagnostic (with the use of biomarkers) and therapeutic technologies. Thus, the genomic characteristics of each patient become part of the diagnosis and individualised treatments. Therefore, the in-depth study of cardiovascular diseases at the molecular and cellular level could in the future yield clinical benefits obtained from gene therapy.

However, there is no doubt that there is still a long way to go for the application of genome editing in the field of cardiology. However, this should not discourage researchers from persevering and continuing to seek a solution to this global scourge.

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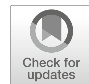

Genome Editing and Pathological Cardiac Hypertrophy

Takao Kato

Abstract

Three major genome editing tools, transcription activator-like effector nucleases (TALENs), zinc finger nucleases (ZFNs), and clustered regularly interspaced short palindromic repeat (CRISPR) systems, are increasingly important technologies used in the study and treatment of hereditary myocardial diseases. Germ cell genome editing and modification can permanently eliminate monogenic cardiovascular disease from the offspring of affected families and the next generation, although ethically controversial. Somatic genome editing may be a promising method for the treatment of hereditary cardiomyopathy various diseases for which gene knockout is favorable and can also treat people who are already ill, although there are currently some technical challenges. This chapter describes the application of genome editing in the experimental studies and treatment of hypertrophic cardiomyopathy as well as other cardiomyopathies.

Keywords

Genome editing; HCM · DMD · CRISPR/Cas

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1 Introduction

The knowledge of the genetic mechanism of cardiovascular diseases has exploded, and the understanding of monogenous cardiovascular diseases has improved. Advances in molecular genetics have made it possible to incorporate genome editing into cardiovascular science [1]. Zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs) are both engineering restriction enzymes based on *Fok*I restriction enzymes [2–4]. A genome editing tool based on the adaptive immune system of bacteria is the clustered regularly interspaced short palindromic repeats (CRISPR) [1, 5]. This method has made it possible to develop powerful treatment for cardiovascular diseases that currently do not have effective therapies.

The DNA-binding domain of ZFN is diverted from the classical zinc finger transcription factor and consists of 3–6 zinc finger repeats that recognize 9–18 bp [4]. The DNA-binding domain of TALEN is composed of 10–30 repeats consisting of 33–35 amino acids. This amino acid sequence is highly conserved except for two amino acids, allowing TALEN to recognize specific DNA sequences [6–8]. These DNA-binding domains allow ZFNs and TALENs to have target specificity. Binding of two independent nucleases to the contralateral DNA strand allows dimerization of the *Fok*I domain, followed by cleavage of the double-stranded DNA at the sticky end [2, 9].

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CRISPR has two major components: guide RNA (gRNA) and CRISPR-associated (Cas) protein [5, 10–13]. A gRNA is an RNA sequence approximately 100 nucleotides in length that directs the Cas protein to a specific location in the genome. The first 20 nucleotides (protospacers) of gRNAs are designed to hybridize to specific sites on DNA, which imparts sequence specificity to the CRISPR/Cas system. In addition, the Cas protein needs to bind to the species-specific PAM (protospacer-adjacent motif) sequence. When the gRNA protospacer and a complementary DNA sequence are hybridizing and the Cas protein binds to the PAM sequence, the Cas protein cuts both strands of the DNA, resulting in a double-stranded DNA break. This process applies to the most common version of CRISPR/Cas9, but other systems such as CRISPR/Cpf. CRISPR/Cpf1 can create double-stranded DNA breaks at the sticky end [14].

This review is intended to explain the genetics of cardiomyopathy (mainly hypertrophic cardiomyopathy) and the genome editing in the understanding of and potential therapy for cardiomyopathy [15–19]. However, as we will see later, CRISPR seems to be the most widely used of the three technologies, so we will focus on CRISPR in the following discussion.

2 Genetic Backgrounds of Cardiomyopathies

Primary cardiomyopathy is classified into major four groups: dilated cardiomyopathy (DCM), hypertrophic cardiomyopathy (HCM), restrictive cardiomyopathy, and arrhythmogenic right ventricular cardiomyopathy based on the function and morphology [19]. When analyzing molecular genetics, multiple genes are responsible for the development of cardiomyopathies [19–23]. However, mutations in the same gene can cause various clinical symptoms, resulting in various types of cardiomyopathy [20–23]. Genome editing can be a promising method for the treatment of monogenetic diseases such as hereditary HCM and Duchenne muscular dystrophy (DMD) [17]. Hereditary arrhythmia and transthyretin amyloidosis are other candidates for therapeutic application [17].

2.1 HCM and DCM

HCM is defined as a group of diseases characterized by hypertrophy of the myocardium of the left or right ventricle and decreased left ventricular diastolic function due to myocardial hypertrophy. Hypertrophy of cardiomyocytes, damage to cardiomyocytes and myofibrils, and increase in interstitial collagen cause left ventricular remodeling, which leads to changes in disease type and pathology, and cardiovascular events are observed according to the pathology [24]. About 60% of HCM is autosomal dominant, and about 40–60% of cases are caused by more than 1400 mutations, including genes encoding myocardial component proteins such as sarcomeres [19, 25]. At present, we do not have sufficient evidence to apply to the long-term prognostic management of individual cases, but the discovery of the responsible genes and the elucidation of the molecular mechanisms of pathogenesis using animal models will lead to early diagnosis and improved diagnostic methods. It may also lead to the development of mechanism-based therapies, and a number of genetically modified mice have been produced so far. Table 1 shows HCM model mice and their target genes.

DCM in humans is defined as “a group of diseases characterized by (1) diffuse contractile dysfunction of the left ventricle and (2) left ventricular hypertrophy” and is characterized by chronic heart failure symptoms that progress with repeated acute exacerbations. It is a disease with a poor prognosis. It has been reported that 15–27% of patients hospitalized for heart failure have dilated cardiomyopathy as an underlying disease [26–28], indicating that it is a group of diseases. Therefore, the role of dilated cardiomyopathy models is very important for the investigation of the cause of the disease and for its treatment. The classification of the disease can be broadly divided into familial (hereditary) and

Table 1 Mouse models of hypertrophic cardiomyopathy and other cardiomyopathies and their target genes

Gene	Phenotype in person	Model mouse [Ref]	Phenotype in mouse	Virulence
α-MHC	HCM	R403Q [98]	HCM	Myocyte disarray, fibrosis, atrial dilation
α-MHC	HCM	Arg403Gln [99]	HCM	Altered voltage-gated K channel
α-MHC-cre		CS-Cre [100]	DCM	Activated p38, JNK, and p53
Caveolin-3	HCM	Caveolin3 KO [101]	HCM, DCM, cardiac dysfunction	ERK1/2 activation, Src signaling
Caveolin3	HCM	P104L TG [102]	HCM, enhanced contractility, apoptosis	nNOS production, altered endoplasmic reticulum (ER) stress response
Tropomyosin	HCM	E180G TG [103]	HCM, fibrosis, and atrial enlargement	Increased myofilament sensitivity to Ca^{2+}
Tropomyosin	HCM	D175N KO [104]	HCM, contractility, and relaxation reduction	Thin filament enhanced Ca^{2+} sensitivity
Tropomyosin	DCM	KO [100]	Embryonic lethal	
Tropomyosin	DCM	TG-E54K [31]	DCM	Decrease in Ca^{2+} sensitivity and tension
Troponin T	CM	Myosin heavy chain TG [105]	HCM, reduced number of myocytes	Multiple cellular mechanisms
Troponin T	CM	R92Q TG [106]	Mitochondrial pathology, diastolic dysfunction	Induction of ANP and bMHC
Troponin I	HCM	R145G TG [107]	HCM, diastolic dysfunction, death	Increased Ca^{2+} sensitivity and hypercontractility
Troponin I	HCM	Troponin I KO [107]	Acute HF, shortened sarcomeres	Reduced Ca^{2+} sensitivity, elevated resting tension
Troponin I	RCM	TG R193H [108]	RCM	Increase in Ca^{2+} sensitivity
Troponin I	RCM	TG R145W [109]	Diastolic dysfunction	Prolonged force and Ca^{2+} transient
MyBPC	HCM	Myosin-binding protein C TG [110]	Sarcomere disorganization	Stable truncated protein
MyBPC		Myosin-binding protein C KO [111]	HCM, reduced myofilament stiffness	Abnormal sarcomere shortening
MyBPC	CM	cMyBPC KO [112]	HCM	Dysregulation of Xirp2 and Zbtb16
Myopalladin	DCM HCM	Y20C TG [113]	HCM and heart failure	Desmin, DPS, Cx43, and vinculin disruption
CARP	HCM DCM	α-MHC TG [114]	HCM in response to pressure overload stress	Reduced TGF-β, ERK1/2, MEK, and Smad3
Talin		Talin KO [115]	HCM, hypercontraction to pressure overload	Blunted ERK 1/2, p38, Akt, and Gsk3 after stress
SGLT1		SGLT1 KD TG [116]	HCM	HCM leading to heart failure

ANP atrial natriuretic peptide, *CARP* cardiac ankyrin repeat protein, *CM* cardiomyopathy, *CSRP3* cysteine- and glycine-rich protein 3, *Cx* connexin, *DCM* dilated cardiomyopathy, *DSP* desmoplakin, *ERBB* erythroblastic leukemia viral oncogene homolog, *ERK* extracellular signal-regulated kinase, *Gsk* glycogen synthase kinase, *HCM* hypertrophic cardiomyopathy, *HF* heart failure, *KO* knockout, *MHC* major histocompatibility complex, *MyBPC* myosin-binding protein C, *nNOS* neuronal nitric oxide synthase, *ROCK* rho kinase, *SGLT1* sodium glucose cotransporters 1, *TG* transgenic, *TGF* transforming growth factor, *TNNT2* cardiac troponin T

nonfamilial. In humans, at least 60 different gene mutations have been reported [28], and about 20–30% of dilated cardiomyopathy is familial. However, even in nonfamilial cases, genes that have not yet been identified are expected to be involved in the development of dilated cardiomyopathy [29]. Table 2 shows some of the gene mutations that are currently known. The genetic

Table 2 Genes and proteins involved in dilated cardiomyopathy

Genes	Protein
Sarcomere	
MYH6	α-Myosin heavy chain
MYH7	β-Myosin heavy chain
TPM1	α-Tropomyosin
ACTC1	α-Cardiac actin
TNNT2	Cardiac troponin T
TNNC1	Cardiac troponin C
TNNI3	Cardiac troponin I
MYBPC3	Myosin-binding protein C
TTN	Titin
TNNI3K	Troponin I-interacting kinase
Z-band	
ACTN2	α-Actinin 2
BAG3	BCL2-associated athanogene 3
CRYAB	α-B-crystallin
TCAP	Titin-cap/telethonin
CSRP3	Muscle LIM protein
ANKRD1	Cardiac ankyrin repeat protein
LDB3	Cypher/ZASP
NEBL	Nebulette
Dystrophin	
DMD	Dystrophin
DTNA	Dystrobrevin
SGCA	α-Sarcoglycan
SGCB	β-Sarcoglycan
SGCD	δ-Sarcoglycan
Cytoskeleton	
DES	Desmin
VCL	Metavinculin
FLNC	Filamin C
DSP	Desmoplakin
LAMA4	Laminin 4
Nucleus	
LMNA	Lamin A/C
EMD	Emerin
RBM20	RNA-binding protein 20
Ion channel and Ca handling	
PLN	Phospholamban
SCN5A	Type 5 voltage-gated cardiac Na channel
ABCC9	ATP-sensitive potassium channel
KCQN1	Potassium channel
Mitochondria	
DNAJC19	HSP40 homolog, C19
TAZ/G4.5	Tafazzin

mutations can be broadly divided into those within the sarcomere and those outside the sarcomere (Table 2). Looking at the frequency of occurrence, in familial dilated cardiomyopathy, titin is 25%, lamin A/C 6%, myosin heavy chain 7 7%, MYBPC3 (cardiac myosin-binding protein

C) 4%, troponin T type 2 (TNNT2) 3%, myosin heavy chain 6 3%, and sodium channel protein type 5 subunit alpha 3% [30, 31]. Furthermore, abnormalities in the titin gene are found in 18% of nonfamilial dilated cardiomyopathy and 3% of healthy individuals and are considered to be not rare [29]. The interaction of genetic and environmental factors may contribute to the disease manifestations in DCM patients.

2.2 Arrhythmogenic Right Ventricular Cardiomyopathy

The main feature of ARVC is myocardial fiber-fat infiltration, primarily in the right ventricle (RV), but LV may also be involved [32]. ARVC is autosomal dominant and incompletely permeable [2, 35, 36]. In ARVC, changes in the gene encoding desmosomal proteins have been identified as the cause of the disease [21, 33]. Mutations in these genes disrupt the integrity of desmosomes and can lead to muscle fiber tearing, fragmentation, and ultimately cell death during the cardiac cycle. Loss of desmosome function also affects cardiomyocyte gap junction remodeling, sodium channel function, and electrocardiographic parameters [21, 33]. In addition, perturbation of desmosome proteins promotes mesoderm precursor adipose formation by inhibiting the Wnt/β-catenin signaling pathway, which plays an important role in cardiomyopathy [34–36]. As a result, fiber-fat replacement of the ventricular myocardium occurs mainly in RV [37].

2.3 Restrictive Cardiomyopathy

RCM is the least common of cardiomyopathy and usually has increased ventricular sclerosis, which prevents ventricular filling, without ventricular hypertrophy [20, 38]. Most of the disease-causing mutations are autosomal dominant, but there are also forms of autosomal recessive, X-linked, and mitochondrial inheritance. In RCM patients, changes in genes encoding sarcomere proteins (TNNT2, etc.), *Z*-disk proteins (MYPN, etc.), and transthyretin (TTR) have been confirmed [39].

2.4 Left Ventricular Noncompaction Cardiomyopathy (LVNC)

LVNC is characterized by prominent corpus cavernosum, a thin compression layer, and the most prominent deep corpus cavernosum depression at the LV apex [37]. Uncompressed extends to RV and may exhibit a biventricular or isolated RV uncompressed phenotype. The genetic form of LVNC is generally inherited as X-linked recessive or autosomal dominant [37] but heterogeneous. It has been confirmed that mutations in multiple genes are involved in the development of LVNC. These genes include genes encoding sarcomeres (such as MYH7), *Z*-disks (such as LDB3), nuclear envelopes (such as LMNA), mitochondria (such as TAZ), and ion channel proteins (such as SCN5A) [33].

3 Genome Editing in Cell Models of Cardiac Disease

Genome editing allowed to create allogeneic cell lines that differ only in the locus of interest. To create human ESC-based models, TALEN has been used for studying cardiomyopathy. TALEN construct was used to knock out 88 genes associated with cardiomyopathy and congenital heart disease [40] and was validated to effectively disrupt target loci. Mutations in the TNNT2 gene are well associated with autosomal dominant HCM with disarray of sarcomere and abnormal intracellular Ca^{2+} cycling [40].

Unlike ESCs, iPSCs have no ethical issues and are the most suitable model for studying cardiomyogenesis and cardiomyocyte in human cells [41], where human in vitro models are lacking [42]. Because CRISPR/Cas9 tools can be used to relatively efficiently and easily create homogeneous cell lines that differ only in the DNA sequence of interest [43], the differences in genetic background and epigenetic memory can be eliminated [43, 44]. So far, the CRISPR/

Cas9 system is an effective and useful tool for investigating the role of various genes and proteins in the human cells, especially in iPSCs [10, 45].

In the studies for cardiomyopathies, the CRISPR/Cas9 system was used to assess the pathogenicity of titin gene mutations in dilated cardiomyopathy. Missense or frameshift titin mutations were introduced into iPSCs, and then contractile disorders were evaluated by iPSC-cardiomyocyte (iPSC-CM) [46]. The HCM phenotype was able to be reproduced, and mutations in the RAF1 gene were identified as a cause of HCM through activation of mitogen-activated protein kinase kinase 1/2 and extracellular signal-regulated kinase 5 in people with Noonan syndrome using iPSC-CM and CRISPR/Cas9 [47]. Seeger et al. used CRISPR/Cas9 and TALEN to investigate the underlying mechanism of HCM associated with mutations in the MYBPC3 gene that introduces premature stop codons via aberrant Ca^{2+} handling and other molecular dysregulations [48]. Genome editing is also used to evaluate variant of unknown significance from the perspective of gene screening for HCM [49]. Through the correction of genetic mutations in iPSC-related disease model [50, 51], genome editing has already been applied in the study for treating various heart diseases [52].

4 Genome Editing in Animal Models

Introduction of genome-specific mutations in model organisms laid the foundation for the understanding of physiology and pathophysiology of the heart [53, 54]. In classical approaches, the researchers induced homologous recombination in mouse ESCs, selected mutant ESCs by antibiotic resistance, and excised antibiotic cassettes, and injected ESCs into blastocyst recipient mice [55]. In contrast, the CRISPR/Cas9-based technique allows the production of mutant mice in a single step, consisting of simultaneous injection of Cas9 mRNA, different sgRNA, and DNA donors into the zygotes [56]. In a mouse model of heart failure caused by overexpressed calsequestrin, the knockout of phospholamban by the CRISPR/Cas9-based technique improved cardiac function [57]. Furthermore, in mdx mice, CRISPR/Cas9 genome editing could be applied to zygotes to correct the mutations that cause Duchenne muscular dystrophy and restore dystrophin expression in cardiac tissue as well as skeletal muscle [58].

5 Germline and Somatic Genome as a Therapeutic Implication and Ethical Problems

The significance in the treatment using genome editing is to better the treatment of monogenic cardiovascular diseases that is currently ineffective or has minimal efficacy. Hereditary cardiomyopathies such as HCM and DMD are potential candidates for clinical application of genome editing technology for germ cells. In these diseases, a single gene mutation is involved in the expression of the disease. Editing germ cells can permanently improve the disease in the offspring of the affected or those with the deleterious mutation. In germline gene editing, several ethical issues must be considered, as both intended and unintended changes can be transmitted subsequently [17]. Social debate and public policy decisions need to be fast-tracked to determine when and how these therapies can be used clinically [17]. Mosaicism is a major concern. Although Ma et al. showed mosaicism can be largely avoided if the introduction of CRISPR is prior to the start of cell division and no off-target effect was not observed [59], it is unclear whether the human fetus can always recognize and correct such off-target effects [60]. Developing new base editing technologies may allow more accurate correction of mutations in selective cases.

Somatic genome editing may eventually be applicable to the treatment for various diseases with the interaction of genetic and environmental factors in the development of the disease [17]. Partial or complete gene knockout is favorable in the treatment of atherosclerosis and hyperlipidemia. Somatic genome editing may also be applicable in the postnatal treatment of

monogenic diseases, as germline genome editing may be more ethically controversial than somatic genome editing [17]. There are technical challenges of introducing Cas9-gRNA (immune response to viral vectors, introduction of nonviral vectors, vector size) to be solved in large animals and humans.

6 Hypertrophic Cardiomyopathy and Genome Editing

About one-third of human HCM are due to the mutations in MYBPC3, and hereditary DCM are also involved with the mutations in MYBPC3 [61]. Ma et al. recently used CRISPR/Cas9 to correct mutations in MYBPC3 in human germ cells [59]. Recombinant Cas9 protein with gRNA and ssODN DNA were injected into human zygotes derived from healthy donor oocytes and sperm from a male donor heterozygous for MYBPC3 mutation. Although the two-third of embryos injected by this method showed homozygous wild-type genotype, one-fourth of embryos showed mosaicism, and about 9% of embryos showed heterozygous mutant genotypes [59]. This mosaicism could be attributed to the failure of CRISPR to correct all mutant genes after cell division. In addition, when Cas9 was injected into M-stage oocytes with sperm, about 70% of the embryos obtained showed homozygous wild-type genotype, with no mosaic or mutant genotypes [59]. The lack of mosaicism in the embryos was probably due to genetic correction during embryogenesis. The remaining 27% of embryos were uniformly heterozygous due to wild-type alleles and nonhomologous end joining (NHEJ)-mediated repair. Furthermore, examination of the germ genome sequence targeting CRISPR/Cas9 did not show a significant off-target effect [59]. This study demonstrates that CRISPR/Cas9 can be used to eliminate disease-causing mutations in human embryos for the first time and that modifying the timing of Cas9 injection during embryogenesis can greatly improve the efficiency of homology-directed repair (HDR) [59].

Even in HCM, somatic genome editing is considered possible because cardiac hypertrophy, myocardial fibrosis, and symptom development are generally slow processes. Mearini et al. used non-mutated Mybpc3 cDNA without CRISPR/Cas9 in AAV vectors to Mybpc3 knockout mice [62]. Functional cMyBP-C was increased to 60% of wild-type levels without cardiac hypertrophy and cardiac dysfunction in mice [62]. Viral delivery has the size problem to package SpCas9 enzymes, but other smaller Cas9 enzymes can be used [63]. However, it should be understood that HDR is uncommon in somatic cells and, therefore, there are hurdles to gene correction in human myocardium.

7 Duchenne Muscular Dystrophy and Genome Editing

Mutations in the DMD gene, which encodes dystrophin, a long gene with 79 exons caused DMD, a relatively common X-linked disease with progressive atrophy of the skeletal muscle and heart muscle. Since DMD is an inherited disease and there is no effective treatment, it is attracting attention as a candidate for germline genome editing [16].

When Cas9, gRNA targeting exon 23, and template ssODN DNA were injected into the zygotes of mice with a nonsense Dmd mutation, mosaicism in most animals was observed by sequencing of Dmd exon 23 in these modified mice [58]. Improved skeletal muscle function was observed in the majority of animals, even if functional dystrophin had been partially restored by either NHEJ or HDR [58]. Recently, attempts have been made to correct genetic mutations by introducing AAV-Cas9-gRNA. This approach can utilize two viral systems with appropriate Cas9 (less than 4.7 kB in size) or a split intein Cas9 equally distributed with gRNA in two AAVs. Both approaches have proven to be effective in large animal models [64, 65].

Many of the in vivo studies developing CRISPR therapies for DMD have been conducted over a short period of time. Assessments of therapeutic efficacy are typically performed at 3–14 weeks postinjection, which is not a long enough period to evaluate the long-term effects of treatment. It is also not surprising that these

studies failed to assess the ability of CRISPR therapy to improve cardiac function, given that the injections were initiated in mice of relatively young age (P1–11 weeks old) and mostly used mdx models. According to one study, signs of cardiac dysfunction are developed in mdx mice around 18 months of age [66]. This highlights the need for longer-term evaluation.

Recently, addressing the above limitations, the long-term effects in mdx mice of DMD CRISPR treatment have been published [67–69]. In all of them, exon 23 of the Dmd gene, which is intended for deletion, is injected alone or together with exons 21 and 22 to generate in-frame transcripts for dystrophin translation. Assessment of therapeutic effects was done between 12 and 19 months after injection. Recovery of dystrophin in the heart was sustained in three studies, with one study observing protein expression of dystrophin up to 20% of normal at 18 months posttreatment [67]. In addition, immunohistochemical studies showed the development of the dystrophin-positive fibers with significant improvement in cardiac function with CRISPR treatment [67, 68]. These indicate the promise of CRISPR for the treatment of DMD-related cardiomyopathy in patients.

In addition, various treatments are being used to re-express dystrophin. Ataluren, a small molecule compound, is being tested in DMD to achieve read-through of stop codons. In addition, eteplirsen has received FDA approval as a treatment for exon skipping using antisense oligonucleotides. Three types of microdystrophin encoded by AAV vectors are being tested in clinical trials by various companies, and future results are expected [70].

8 Transthyretin Cardiac Amyloidosis

To find the optimal way to deliver the CRISPR/Cas9 component to the cells of interest is important. Viral vectors are not the sole methods for this purpose. Transthyretin (TTR) myocardial amyloidosis is a myocardial invasive disease caused by abnormal prealbumin (transthyretin) protein deposition, either a hereditary TTR gene mutation or wild-type transthyretin accumulation. Most of the transthyretin is produced in the liver, which may make it an attractive candidate for somatic genome editing. Finn et al. administered lipid nanoparticles packaged with Cas9 mRNA and gRNA targeting the TTR gene to mice and found a significant reduction in serum TTR levels (97% or more) [71]. It is unclear whether this method can rescue or prevent the phenotype of the disease and further studies would be needed. Other nonviral vectors (such as microbubbles) that can selectively destroy the target tissue with ultrasound and locally introduce the vector are also interesting [72].

9 Long QT Syndrome

Long QT syndrome (LQTS) is an attractive candidate for genome editing. Limpitikul et al. used iPS-CM derived from a patient with a calmodulin 2 mutation, characterized the phenotype of LQTS [73], and treated with CRISPR interference to reduce calmodulin protein and action potential duration indicating that postnatal correction of this disease is possible. Further studies are needed to apply these results to different types of genetic mutations and apply to large animals to validate the corrected phenotype.

10 Future Directions in CRISPR and Cardiomyopathy Treatment

Because CRISPR is currently a mainstream technology and most research is directed toward improving this technology, the advances in CRISPR are focused. An ongoing area of research is how to increase the HDR rate of CRISPR in the heart; HDR only occurs in the cell cycle S and G2 phases [74]. Since the heart is basically composed of postmitotic cells, the main mode of DNA repair is NHEJ, which may be undesirable if the intended strategy is gene replacement or knock-in. There are also safety concerns as the unpredictable formation of NHEJ indels can cause

off-target effects. Several strategies have been devised to suppress NHEJ and promote HDR repair, for example, the use of small molecule compounds such as Src7 that inhibit DNA ligase IV [75, 76], shRNA knockdown of KU70 and KU80 [76], genome editing combined with cell cycle synchronization [77], and use of geminin-Cas9 fusion protein [78].

Homology-independent targeted integration (HITI), a new genome editing strategy developed by Suzuki et al. in 2016 [79], is another relatively recent advance. HITI works like a hybrid of NHEJ and HDR. The main advantage of HITI is that it is efficient in actively proliferating cells as well as in nondividing cells. In addition, intravenous administration of HITI-CRISPR/Cas9 has been shown to knock in cardiac genes at a much higher rate than HDR. It will be very interesting to see if HITI can be applied to the treatment of cardiomyopathy in the future.

Attempts to reduce the off-target effects associated with genome editing, especially CRISPR, are also underway. The main strategies can be divided into two, depending on which CRISPR component is modified: one approach focuses on the Cas enzyme. Besides engineering Cas9 itself, the group has also divided the enzyme in half so that it can only cleave double-stranded DNA when both are at the target site [80, 81]. These so-called paired nickases may be more efficient than the original single enzyme [82]. Self-repression mechanisms have also been devised to reduce Cas9 transcription and/or translation by simultaneous administration of gRNAs to the genome of delivery vectors or by the use of synthetic repression systems [83–85]. Another approach aims to enhance the design of gRNAs. gRNA sequences, lengths, and chemical properties are being optimized, and the development of bioinformatics tools is further aiding in the screening of gRNAs [86–90]. Guide-seq [91] and Digenome-seq [92] are more robust and comprehensive methods for assessing off-target effects in the genome, and they will further facilitate efforts to mitigate concerns related to the safety of genome editing for therapy.

11 Advances in Nonviral Delivery System

Although AAV9-based protease-activated AAVs have recently been demonstrated to specifically deliver transgenes to the heart of a mouse model of myocardial infarction [93], the current trend is to develop nonviral delivery methods for genome editing [16]. Immunogenicity, integration of undesirable viral genomes, and packaging are the major limitations in viral vectors, and nonviral delivery overcomes these problems. Examples of nonviral approaches are lipid nanoparticles, polymer-based particles, cell-infiltrating peptides, DNA nanoparticles, and inorganic nanoparticles (silicon or zinc-based) [94–96]. The use of gold nanoparticles for the delivery of CRISPR/Cas9 (CRISPR-Gold) has been applied to correct the Dmd point mutation in mdx mice via HDR [97]. It is still unclear whether CRISPR-Gold shows effective targeting to the heart. The application of such a nonviral delivery approach to the heart may improve the study of cardiomyopathy.

12 Conclusions

Genome editing is a powerful tool for modifying cell lines and organisms in order to study the biological and pathophysiological mechanisms as well as therapeutic implications of various genetic diseases [16–18]. Genome editing of germ cells is one of the most promising technologies. Genome editing of germ cells is expected to make monogenic cardiovascular disorders permanent in offspring and future generations. Although technically straightforward and likely to be introduced into humans in the future, germline genome editing is ethically controversial. Technical issues such as off-target effects, mosaicism, and long-term risks need to be more fully addressed. Somatic genome editing can be used for cardiovascular diseases in which gene knockout, if partial, is favorable. It also has several technical challenges to be resolved successfully applied in humans.

Conflicts of Interest None reported.

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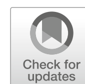

Genome Editing and Diabetic Cardiomyopathy

Tyler N. Kambis and Paras K. Mishra

Abstract

Differential gene expression is associated with diabetic cardiomyopathy (DMCM) and culminates in adverse remodeling in the diabetic heart. Genome editing is a technology utilized to alter endogenous genes. Genome editing also provides an option to induce cardioprotective genes or inhibit genes linked to adverse cardiac remodeling and thus has promise in ameliorating DMCM. Non-coding genes have emerged as novel regulators of cellular signaling and may serve as potential therapeutic targets for DMCM. Specifically, there is a widespread change in the gene expression of fetal cardiac genes and microRNAs, termed genetic reprogramming, that promotes pathological remodeling and contributes to heart failure in diabetes. This genetic reprogramming of both coding and non-coding genes varies with the progression and severity of DMCM. Thus, genetic editing provides a promising option to investigate the role of specific genes/non-coding RNAs in DMCM initiation and progression as well as developing therapeutics to mitigate cardiac remodeling and ameliorate DMCM. This chapter will summarize the research progress in genome editing and DMCM and provide future directions for utilizing genome editing as an approach to prevent and/or treat DMCM.

Keywords

Heart failure · MicroRNA · Therapeutics · Regulator

1 Introduction

Genes remain at the heart of cellular signaling and function, as well as the prevention and/or treatment of diseases. Differential expression of coding and non-coding genes play a pivotal role in the initiation and progression of several diseases, including diabetic cardiomyopathy (DMCM). DMCM is a cardiac muscle disorder caused by diabetes mellitus (DM), which develops independently from hypertension, valvular, or other vascular diseases. DMCM was initially described in 1972 by Rubler et al., during the evaluation of causes of heart failure in four DM patients who did not have hypertension or vascular disease [1]. How DMCM is initiated at the molecular level and which signaling pathways should be targeted to mitigate the risk of heart failure in DM remain an area of intensive research. However, empirical evidence suggests that the expression of several genes are altered in the DM heart. Among these genes are several non-coding RNAs, some of which are constitutively expressed, while the expression of others occur

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during adverse remodeling. Thus, incorporating non-coding RNAs when targeting gene expression may prove to be a promising therapeutic strategy to ameliorate DMCM. Additionally, categorizing differentially expressed genes based on tissue origin and temporal expression may be crucial for understanding the genetic causes of DMCM and developing therapeutic genomic editing approaches. Here, we discuss how the field of genomic editing has progressed and how this technology is useful for investigating molecular signaling and developing therapeutics for DMCM.

2 Diabetic Cardiomyopathy

DM is a highly prevalent, chronic disease with a complex and multifactorial etiology [2, 3]. Defined as a silent killer, it slowly promotes adverse cardiac remodeling leading to heart failure [3]. The heart is a highly sophisticated organ where subtle changes in molecular regulation may result in catastrophic effects. Through hyperglycemia, hyperlipidemia, and hyperinsulinemia, DM fosters an environment which causes both direct and indirect effects preceding DMCM. DM ultimately affects heart function by inducing cell death signaling, which causes the loss of cardiac cells and instigates adverse remodeling. This loss of cardiac cells, termed cardiomyocytes, impairs cardiac contractility leading to cardiac dysfunction and heart failure. DM directly affects the heart via metabolic remodeling, where a lack of insulin prevents glucose uptake leading to increased cardiac utilization of fatty acids. This induces mitochondrial stress and dysfunction due to the increased amount of adenosine triphosphate (ATP) needed to oxidize fatty acids. DM indirectly affects heart function by adversely affecting central mechanisms—signals from the brain to control heart function. Sympathetic and parasympathetic signals from the brain are critical in regulating cardiac contractility. Thus, DM causes neuronal dysfunction and thereby impairs sympathetic and parasympathetic drive leading to cardiac dysfunction [4, 5]. Additionally, DM causes renal (kidney) dysfunction that increases blood volume in venous returns contributing to volume overload in the heart [6, 7]. Altogether, DM has detrimental effects at multiple molecular and physiological levels to initiate and exacerbate DMCM.

3 Genetic Editing

Genomic editing is utilized to modify endogenous genes. Genes are broadly categorized into two types: (1) genes that encode for proteins and (2) genes that encode for non-coding, functional RNA. Endogenous changes in gene expression are affected either by an organism's developmental stage or maladaptive disease remodeling and are regulated by a variety of epigenetic modifications and upstream transcription factors. This change in gene expression is best observed during cardiac development, in which over 800 genes are differentially expressed while cells undergo terminal differentiation via hyperplasia—an increase in cell number [8]. During pathological remodeling, the adult heart compensates for terminal differentiation via hypertrophy—an increase in cell size. While both hyperplasia and hypertrophy are distinct phenomena, fetal genes typically expressed during cardiac development become re-expressed during heart failure [9]. During this event, termed fetal reprogramming, expression of half of the top 500 genes affected during heart failure is correlated with fetal development [10]. Additionally, the expression of approximately 20 microRNAs, non-coding genes that regulate cellular signaling via mRNA targeting, is affected during heart failure [11–13].

With respect to expression, genes can be categorized into two types: (1) constitutively expressed genes which are always transcribed and (2) inducible genes which are transcribed under specific conditions. Editing constitutively expressed genes requires caution, as these genes may regulate several signaling pathways. These genes can be attenuated (decreased in expression) or induced (increased in expression) depending on their expression in pathological condition to alleviate disease pathology. Contrary to this,

inducible genes can be targeted and deleted/suppressed as their expression may relate to disease condition. As genetic editing approaches evolve over time, genes can be deleted or overexpressed with increasing specificity. This includes the creation of transgenic models in all tissues (global) or specific tissue such as the heart, via the insertion of genes into tissue-specific promoter regions [14, 15]. If deleting a gene is embryonically lethal, the use of Cre-lox site-specific recombination systems allows for the induction of knockout mutations post-development [16]. Based on cardiac transcriptome profiling of the diabetic Akita mouse, our lab was able to identify 137 differentially expressed transcripts that potentially acted as inducible genes [17]. Genome editing technology can be used to ablate individual genes to determine their specific roles in the Akita heart.

4 Advancement in Genetic Editing Technology

Genome editing studies were initially performed in fruit flies, *Drosophila melanogaster*, where radiation and chemical treatment were used to enhance random mutagenesis [18, 19]. However, these mutations—change in gene sequence—lacked specificity in their genome targeting. In the late twentieth century, targeted genomic editing with homologous recombination was attempted in both yeast and mice, resulting in a genome editing technology that was more specific, but inefficient [20–24]. More recently, progress in genome editing has made a remarkable leap in the field of targeting and efficient genetic manipulation by utilizing the knowledge on DNA damage and repair and recombination between homologous DNA sequences during meiosis [25, 26]. Targeted DNA double-strand breaks can be induced by nucleases which are then rejoined by nonhomologous ends via inherent DNA repair mechanisms in the cell [27–31]. Recently, three important nucleases have been used to induce DNA double-strand break for targeted genome editing. These nucleases are zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and clustered regularly interspaced short palindromic repeats (CRISPR)-associated protein 9 (Cas9) or CRISPR-Cas9, each of which has gained popularity in targeted gene editing [26, 32–35] (Fig. 1).

ZFNs: This is the first targeted genome editing technique and has been used in a wide range of organisms and cell types to introduce genetic alterations such as point mutations, deletions, insertions, inversions, duplications, and translocations [36, 37]. Repetitive zinc-binding domain protein containing cysteine and histidine residues was initially observed in a transcription factor of oocytes of *Xenopus laevis* [38]. Later, two zinc-finger proteins along with the cleavage domain of the "Fok*I*" restriction endonuclease were utilized in the development to develop ZFNs, a novel artificial site-specific nuclease with high efficiency [39, 40]. Fok*I* mediates dimerization—functional activation—of ZFNs. Functional ZFNs with 3–4 zinc-finger DNA-binding domains bind to 9–18 bp of DNA and cleave DNA into 5–7-base pair spacer sequence [41, 42]. Each zinc-finger binding domain recognizes a distinct DNA triplet, and these domains can be assembled in a myriad of ways to target DNA sequences [43–57]. The details on how ZFNs are designed, characteristics of the Fok*I* catalytic domain, how ZFNs introduce DNA double-strand breaks, different endogenous genes modified by ZFNs, the use of ZFNs in different species, and therapeutic applications of ZFNs have been extensively reviewed by Philip D. Gregory's group [36].

TALENs: It was initially observed that some plant pathogenic bacteria could regulate host cell by targeting DNA base pairs [58]. This observation leads to the engineering of artificial nucleases by fusing a customizable sequence-specific DNA-binding domain to a nonspecific DNA-cleaving nuclease. TALENs quickly and efficiently bind to any target DNA sequence, induce double-strand breaks in the DNA binding site, and alter gene sequences through DNA repair mechanisms in different types of plant and animal cells [59–65]. An extensive review on customizing DNA-binding domain of

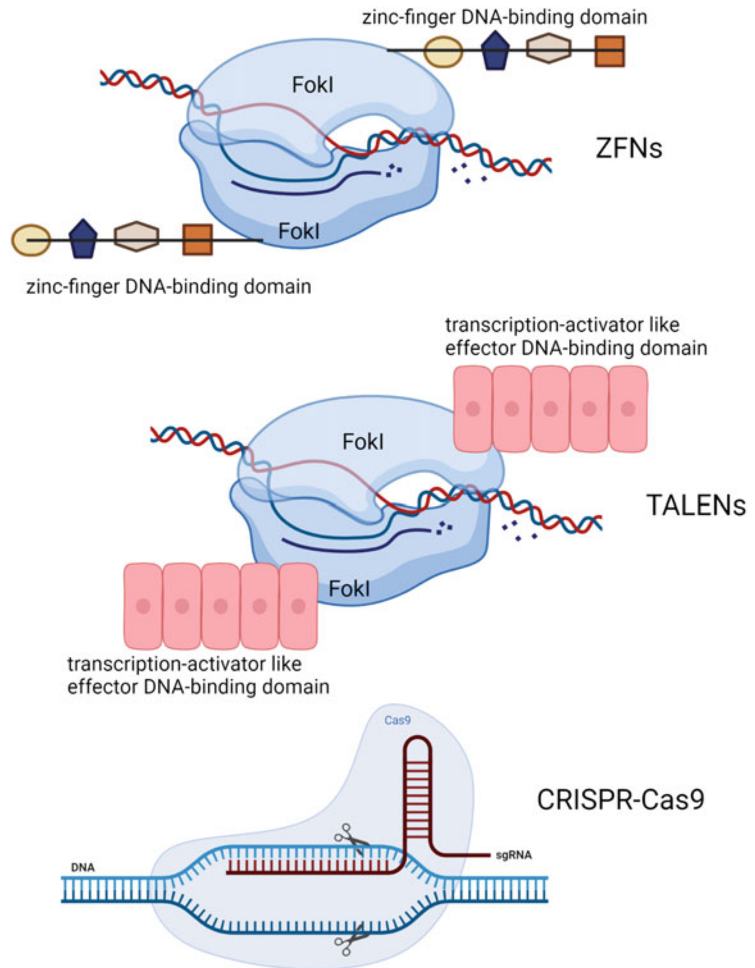

Fig. 1 Schematic showing the three major genomic editing technologies: zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and clustered regularly interspersed short palindromic repeats (CRISPR)-associated protein 9 (CRISPR-Cas9). *sgRNA* single guide RNA

TALENs; different genomic editing performed by TALENs; application of TALEN-mediated genetic alterations in model organisms, plants, and livestock; cell-based disease modeling; and therapeutics have been published by Joung and Sander [66]. TALENs have potential applications against murine cytomegalovirus infections, curing cytoplasmic male sterility via mitochondrial genome editing, and performing genetic alterations in plants, yeasts, insects, mammals, as well as T cells, and chemokines [67–76]. TALENs also show promise in gene targeting for cystic fibrosis, sickle cell disease, and heteroplasmic mitochondrial DNA mutation [77–79]. TALEN-mediated gene editing of interleukin 10-secreting mesenchymal stem cells mitigates adverse remodeling of left ventricle after myocardial infarction in the heart [80]. Considering the broad applicability of TALENs in genome editing in different animal models and cell types, and its therapeutic potential, this technique was named "Method of the Year" for 2011 [81].

Both ZFNs and TALENs employ the same bacterial cleavage domains, FOK*I*; however, they use different DNA-binding domains. ZFNs use a set of zinc-finger domains similar to eukaryotic transcription factors, whereas TALENs use transcription factors produced by plant pathogenic bacteria to recognize DNA sequences [82] (Fig. 1).

CRISPR-Cas9: This is the most advanced, efficient, and utilized genome editing approach [34, 83–85]. The CRISPR system is naturally

expressed in bacteria and plays an endogenous role in the immune response to invading viruses and plasmids through RNA-guided cleavage of DNA by Cas protein [86–88]. The CRISPR-Cas9's adaptive role in bacteria occurs via its ability to integrate short sequences of foreign DNA into CRISPR locus to make trans-activating CRISPR RNA that targets and degrades pathogenic DNA with the help of the Cas9 protein [89–91]. This technique has been used in *D. melanogaster* germ-line cells to generate heritable mutant alleles [92]. With the advancement of time, several modifications of the CRISPR-Cas9 technique have resulted in a more simplified version, where the Cas9 nuclease utilizes a single guide RNA to recognize target DNA. This guide RNA makes CRISPR-Cas9 the most flexible and user-friendly genome editing technology [93–95]. By binding to target DNA and forming a heteroduplex [96–98] (Fig. 1). Although CRISPR-Cas9 is the most accesible genome editing technology, inducing off-target mutations is the greatest limitation of this technology [84, 99, 100]. Several approaches have been adopted to reduce the off-target effects of CRISPR-Cas9 such as use of paired Cas9 nickases and truncated guide RNA, reducing doses of Cas9 or guide RNA, and using Cas9 variants with inducible Cas9 architecture or small-molecule-responsive intein domain [101–106].

CRISPR-Cas9 is the most promising technique for gene therapy [107–109]. It has high therapeutic potential in cancer, Duchenne muscular dystrophy, and sickle cell disease [110–113]. This technology has promising roles in editing heart cells and mitigating cardiovascular disease [114–120].

A simple schematic of the three genomic editing technologies—ZFNs, TALENs, and CRISPR-Cas9—is provided in Fig. 1 that shows similarities and differences in these three methods. ZFNs and TALENs share several similarities such as having common catalytic domain and to have a DNA-binding domain. However, CRISPR-Cas9 differs significantly from both ZFNs and TALENs in using both guide RNA and a Cas9 nuclease. All three technologies share similarity in binding and cleaving double-stranded DNA (Fig. 1).

5 Genetic Editing in Diabetic Cardiomyopathy

Although several coding and non-coding genes are associated with adverse cardiac remodeling during DM, we will focus on an example of how genetic editing of each may be utilized to prevent DMCM. The gene encoding for matrix metalloproteinase-9 (MMP9), which is latent in the healthy heart, is robustly expressed in the DM heart [121]. To determine the specific role of inhibition of MMP9 in the DM heart, we cross-bred MMP9KO (global) with DM Akita mice and developed Akita/MMP9KO mice [122]. These Akita/MMP9KO mice showed improved cardiomyocyte contractility when compared to the DM Akita mice [123]. Thus, genomic manipulation of MMP9 is beneficial to the DM heart.

We have also overexpressed miR-133a, a non-coding RNA, in the diabetic Akita heart. miR-133a is a muscle-specific miRNA which is transcribed primarily in cardiac and skeletal muscles [124]. Out of nearly 800 miRNAs, miR-133a the most abundant in the heart [125]. However, it is reduced in the DM heart [17, 126]. Similar to the development of the Akita/MMP9KO mice, we crossbred cardiac-specific miR-133a transgenic (Tg) with DM Akita mice to develop Akita/miR-133aTg mice [127]. These Akita/miR-133aTg mice displayed lower accumulation of lipids within the heart, and decreased cardiac fibrosis and hypertrophy, thus demonstrating protection against DM-induced cardiac remodeling [128].

In addition to the crossbreeding of genetically altered animal models, the transient delivery of genetic materials such as miR-133a mimic and siRNA offers another approach for genomic alteration [126, 129]. Genetic regulators may be delivered to the heart via circulation through a variety of nanoparticle packaging, primarily consisting of either lipopolysaccharides or polymers [130]. There are different methods to deliver nanoparticles with different therapeutic efficacies

[131]. The genetic materials—DNA and RNA—may also be delivered utilizing viral packaging [132, 133]. The progress and problems in viral delivery have been elaborated by the Mark A Kay group [134]. One of the limitations of gene delivery is package accumulation in non-targeted tissue, primarily in the liver. Adding tissue-specific ligands facilitates gene delivery to specific tissues, such as the heart genes can be directly delivered through intramuscular or intracoronary injection further increasing specificity of delivery.

In order to transiently express or inhibit a gene, the mimic or inhibitor of the specific gene is packaged into an adeno-associated virus (AAV) [135]. A Phase 2b trial—calcium upregulation by percutaneous administration of gene therapy in cardiac disease 2b (CUPID 2)—has used an AAV1 delivery approach to introduce the sarcoendoplasmic reticulum calcium ATPase-2A (SERCA 2A) gene through intracoronary infusion [136, 137]. For long-term gene expression, genes need to first be inserted into the host genome. Lentiviruses, which infect both dividing and nondividing cells with high efficiency, also integrate into the genome for long-term and stable expression of the transgene. In addition, lentivirus have low immunogenicity and are considered suitable vectors for gene delivery into the heart for cardiac diseases [138]. In preclinical studies using a rat myocardial infarction model, SERCA 2A gene delivery through lentivirus via intracoronary injection has demonstrated successful integration of SERCA 2A into the host genome, which had compensated for the reduced expression of SERCA 2A in the heart and prevented adverse cardiac remodeling [139]. However, the stage of heart failure depends on the conditions of cardiomyocytes—the contractile apparatus of the heart. Gene therapy is only considered suitable for the failing heart when cardiomyocytes are healthy, while cell therapy is considered a better approach for permanently lost cardiomyocytes [140]. After packaging miR-133a mimic into lentivirus and delivering it to the DM mouse heart, we observed that miR-133a overexpression mitigated adverse cardiac remodeling and improved cardiac function [126, 141]. Altogether, genetic editing shows high potential as a therapeutic technique in the amelioration of DMCM (Fig. 2).

6 Future Direction

Gene therapy shows huge promise for the treatment of cardiovascular disease [142]. With the

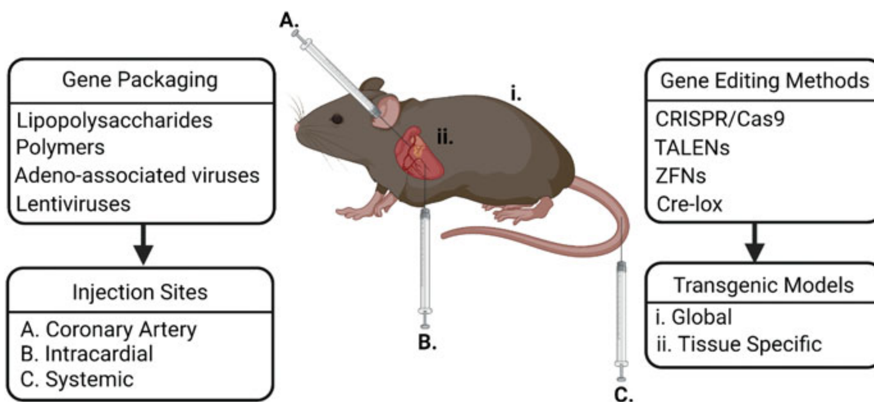

Fig. 2 Gene editing approaches to ameliorate diabetic cardiomyopathy. Both gene and non-coding RNA, such as miRNA, can be packaged into lipopolysaccharides, polymers, adeno-associated viruses, or lentiviruses. These packaged gene mimics/shRNAs/siRNAs can be delivered into the diabetic heart via (*A*) direct intracoronary, (*B*) intracardial (injecting into cardiac muscle), or (*C*) systemic (tail vein injection) injection. For gene editing, different methods are used, such as CRISPR/Cas9, TALENs, Cre-lox, and ZFNs. Animal models have been developed by gene editing that overexpress a specific gene (transgenic) either globally or in a tissue-specific manner

advancement of technologies in genetic editing, more options are available for genetic manipulation of the DM heart to mitigate DMCM. However, it is important to discern which technology is appropriate for each specific type of genetic editing in DMCM. There are several caveats that need to be considered while investigating the cause of initiation or progression of DMCM, or for developing therapeutics for DMCM. (1) It is important to evaluate the stage (prediabetic, DM, advanced DM) of DMCM because genetic profile alters with advancement of DMCM. (2) The duration of genetic editing is also important. Transient overexpression or inhibition of genes via AAV is considered better than the permanent lentivirus approach because gene expression alters when the heart is reverting toward healthy condition. Overt induction or inhibition of genes may have adverse effects on the prediabetic heart while reverting toward healthy condition or becoming healthy heart. (3) The gene delivery approach is also critical. Direct delivery is considered good because of target specificity. However, a drawback is its invasive nature. Injecting mimic or inhibitor into the circulation and adding cardiac-specific ligand to the liposome or nanodroplet are considered a less invasive approach for genetic editing in DMCM. Altogether, development of genetic editing tools and delivery approaches has pivotal role in understanding the causes of DMCM and developing novel therapeutic approaches for DMCM.

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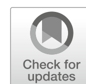

Genome Editing and Inherited Cardiac Arrhythmias

Laura Lalaguna, Laura Ramos-Hernández, Silvia G. Priori, and Enrique Lara-Pezzi

Abstract

Inherited arrhythmic disorders are a group of heterogeneous diseases predisposing to life-threatening arrhythmias and sudden cardiac death. Their diagnosis is not always simple due to incomplete penetrance and genetic heterogeneity. Furthermore, the available treatments are usually invasive and merely preventive. Genome editing and especially CRISPR/Cas9 technologies have the potential to correct the genetic arrhythmogenic substrate, thereby offering a cure for these fatal diseases. To date, genome editing has allowed reproducing cardiac arrhythmias in vitro, providing a robust platform for variant pathogenicity, mechanistic, and drug-testing studies. However, in vivo approaches still need profound research regarding safety, specificity, and efficiency of the methods.

Keywords

Sudden cardiac death · Primary arrhythmia · Channelopathy · Gene editing · CRISPR/Cas9 · LQTS · Brugada syndrome · CPVT · SQTS

1 Introduction

Sudden cardiac death (SCD) in young individuals with anatomically normal hearts has been reported for decades [1]. Due to inability to identify a causal relationship, this SCDs were initially termed as "idiopathic ventricular fibrillation" [2]. However, the discovery of the first long QT syndrome susceptibility genes in the late 1990s had transformative effects in their management, introducing what we now call primary arrhythmia syndromes [3–5]. These inherited arrhythmic disorders have a low prevalence, and patients suffer from increased predisposition to life-threatening arrhythmias, which arise spontaneously or upon a trigger in the absence of structural cardiac abnormalities. As most of the genes affected encode cardiac ion channels, they are also referred to as cardiac channelopathies. Their diagnosis is not always simple due to incomplete penetrance and genetic heterogeneity. The available treatments are merely preventive and involve

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life-long or invasive approaches like the implantable cardiac defibrillator (ICD) or left cardiac sympathetic denervation (LCSD) [6]. This is a big challenge for clinicians, whose decision may have life-altering consequences for the patients, especially for those with an inconclusive diagnosis. For these reasons, the lack of highly effective pharmacological treatment makes inherited cardiac arrhythmias the perfect candidate for genome editing-based approaches, potentially reversing the genetic substrate and offering a "cure" for the disease.

As the availability and use of genetic testing increases, so does the probability that rare variants of uncertain significance (VUS) are found. Regarding channelopathies, to date genome editing has mainly been used to either directly introduce the desired VUS mutation in in vitro models, particularly human-induced pluripotent stem cells (hiPSCs), or to generate the proper isogenic controls from patient-derived lines. Both approaches result in isogenic sets of cells, allowing the elimination of epigenetic differences and unknown genetic modifiers that may introduce phenotype variability (Fig. 1). Consequently, genome editing and especially CRISPR/Cas9 provide a robust platform to study genotype-phenotype correlations, being able to identify causality or association of the variant to the disease. Furthermore, this system allows molecular and mechanistic studies, identification of regulatory elements, and comparative studies of different mutations.

In summary, this chapter focuses on the knowledge that CRISPR/Cas9 technologies have helped acquire regarding the four major channelopathies: long QT syndrome (LQTS), Brugada syndrome (BrS), catecholaminergic polymorphic ventricular tachycardia (CPVT), and short QT syndrome (SQTS).

2 Long QT Syndrome

Long QT syndrome (LQTS) is the most frequent primary arrhythmia with a prevalence of up to 1: 2000. The inheritance is autosomal dominant, although some very rare and extremely severe recessive variants have also been described [7, 8]. LQTS comprises a heterogenous family of diseases characterized by a prolonged QT interval and T-wave abnormalities in the electrocardiogram (ECG) (Fig. 2b). Especially upon adrenergic stimulation, these patients are at a high risk of ventricular tachycardia, which can end in sudden cardiac death due to torsade de pointes.

To date, congenital LQTS has been classified based on mutations associated with up to 17 genes [9]. The QT prolongation arises mainly from loss of function mutations of the K^+ channels, which cause a decrease in repolarizing potassium current in phase 3 of the action potential, or gain of function mutations of Na^+ and Ca^{2+} channels, which cause a late inward entry of positive ions in the cardiomyocyte. LQT1-3 subtypes comprise about 75% of all the patients with LQTS and affect KCNQ1, KCNH2, and SCN5A genes, respectively [10]. KCNQ1 and KCNH2 both encode for the alpha subunits of K^+ channels conducting the slow and rapid delayed rectifying current, I_{ks} and I_{kr}. SCN5A, on the other hand, encodes for the alpha subunit of the cardiac sodium channel, conducting the depolarizing sodium inward current (I_{Na}) [11]. These major genes were first identified in 1995 [3–5], and, as 20% of LQTS remained genetically elusive, the past 25 years have experienced an exponential growth in publications reporting LQTS-associated genes and mutations, describing more than 600 genetic variants [10, 12].

In this race for genotype-phenotype association, the advances in hiPSC culture and differentiation together with CRISPR/Cas9 genome editing tools have undoubtedly provided an easy and rapid method to study the causality of genetic variants in a dish. For example, the missense mutation T983I in KCNH2 was initially classified as VUS due to very low population frequency and lack of prior clear phenotypic data. Isogenic sets of cells consisting of patient-derived and CRISPR/Cas9-corrected hiPSC-cardiomyocytes (hiPSC-CMs) allowed the reclassification of this variant to likely pathogenic. The mutant cells showed prolonged action potential (AP), reduced

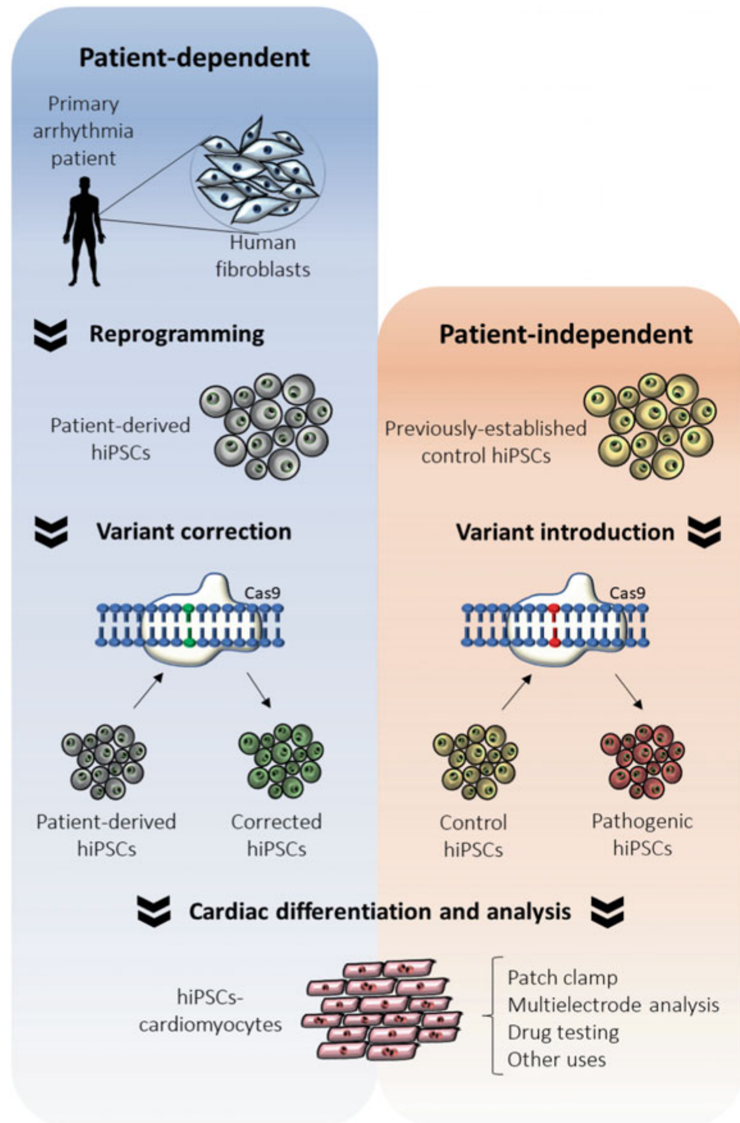

Fig. 1 Comparison of patient-dependent and patient-independent hiPSC models in primary arrhythmias. Patient-dependent approach (left) in which the hiPSC line is generated de novo from affected fibroblasts and CRISPR/Cas9 genome editing is used to generate the isogenic controls by correcting the variant. A much more rapid patient-independent approach (right) uses previously established control hiPSCs to introduce the mutation with CRISPR/Cas9. After cardiac differentiation, both models are ready for analysis

I_{Kr}, and a greater propensity to proarrhythmia upon high-risk torsadogenic drugs. On the other hand, correction of the mutation through genome editing restored the aberrant cellular phenotype. In a complementary set of experiments, the mutation was introduced in homozygosis in healthy hiPSCs, getting rid of the patient's genetic background, and hallmark features of the LQTS disorder were again recapitulated [13]. Missense mutations in KCNH2, also called hERG, usually have a dominant negative effect and result in inappropriate maturation of the potassium channel and reduced I_{Kr}. The dominant mechanism associated with the protein loss of function has been reported to be the generation of trafficking deficient channels [14]. To gain further insight, a missense mutation known to cause LQT2 was introduced in homozygosis in control hiPSC-CMs using CRISPR/Cas9 and compared to unedited cells and heterozygous patient-derived hiPSC-CMs [15]. hERG immunostaining showed similar intracellular presence of the channel for all the cells but reduced fluorescence intensity in the plasma membrane in both LQT2

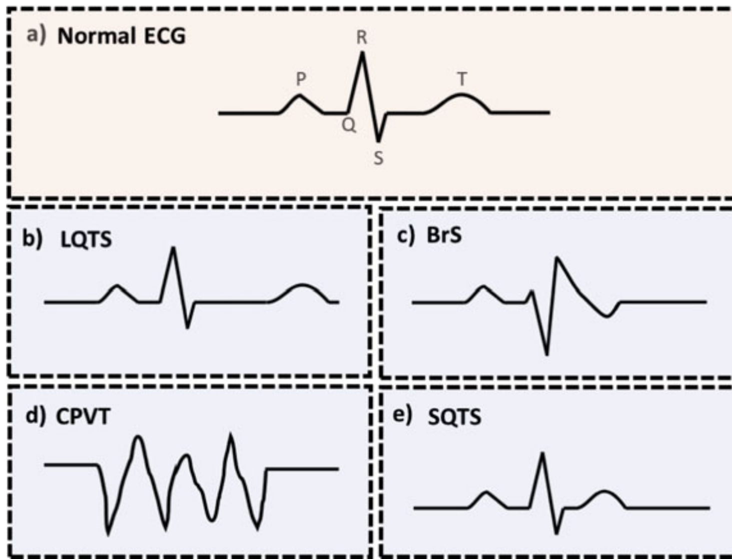

Fig. 2 Electrophysiological characteristics of the main cardiac channelopathies. (**a**) Normal ECG. (**b**) Prolonged QT interval in long QT syndrome (LQTS). (**c**) Coved-type ST segment elevation in Brugada syndrome (BrS). (**d**) Shortened QT interval in short QT syndrome (SQTS). (**e**) Polymorphic ventricular tachycardia in catecholaminergic polymorphic ventricular tachycardia (CPVT)

models, suggesting trafficking defects. This same mutation had been previously studied in heterologous systems, and its transient expression showed insufficient processing in the Golgi apparatus [14]. Therefore, the reduced I_{Kr} and prolongation of AP duration (APD) phenotype observed in the mutant hiPSC-CMs may be the result of a non-glycosylated hERG that fails to be transported to the plasma membrane [16]. The fact that the mutation in homozygosis aggravated the phenotype supports not just its pathogenic role but also the use of patient-independent models to confidently study disease pathogenesis.

VUS in less common LQTS-associated genes have also been studied with this same approach. The R518C mutation in the CACNA1C gene was reported to be the genetic substrate of cardiac-only Timothy syndrome (TS) [17, 18]. TS is a very rare and severe variant of LQT8 in which there is a coexistence of LQTS, cardiomyopathy, and extracardiac phenotypes. The affected gene encodes for the heart's voltage-gated L-type calcium channel, LTCC. The ion current studies of patient-derived hiPSC-CMs (CACNA1C-R518C) and its CRISPR/Cas9-corrected isogenic control showed that this gain of function mutation is sufficient to cause the patient's QT prolongation due to an increase in LTCC late current and delayed calcium transient resolution. This prolongs the plateau phase of the myocyte action potential, leading to delayed repolarization, and is the monogenetic substrate for the LQTS phenotype in the patient [19]. Furthermore, a patient-independent model in which another CACNA1C VUS was introduced into hiPSCs using CRISPR/Cas9 also showed prolonged AP due to reduced LTCC voltage-dependent inactivation [20]. Not only do these results support the pathogenicity of this specific variant but also, together with the previous report, support that CACNA1C is a susceptibility gene for LQTS.

Taking a step further, Yoshinaga et al. proposed the use of these patient-derived hiPSC-CMs together with their corresponding CRISPR/Cas9-corrected controls to develop a novel method for LQTS phenotype-based classification. It consists of specific currents blockade and their electrophysiological assessment using multielectrode array (MEA) systems, which allow multiple simultaneous recordings at once. Studying cells

from patients suffering from the three main subtypes of LQTS and their CRISPR/Cas9-corrected controls, they observed that LQT1-3 could be distinguished by I_{Ks}, I_{Kr}, and I_{Na} blockade, respectively [21]. This strategy reduces variability compared to traditional single-cell patch clamp recordings, allowing the detection of subtle electrophysiological differences. Therefore, it could potentially allow high-throughput screening, efficient recognition of pathogenic variants, and phenotype-based diagnosis of LQT subtypes.

Combination of the patient-independent platform together with MEA has also been used to observe intragenotype differences in disease severity attributable to the KCNH2 mutation site. Heterozygous missense mutations known to affect the pore-loop domain (KCNH2-A561T) or the cytoplasmic tail of hERG (KCNH2-N996I) were introduced into control hiPSCs using CRISPR/Cas9. Action potentials (APs) and field potentials (FPs) were recorded using both patch clamp and MEA in single cells and confluent monolayers of hiPSC-CMs, respectively. Furthermore, to mimic the triggering factors that induce arrhythmic events in LQTS, the researchers examined the behavior of the cells upon inhibition with the I_{Kr} blocker E-4031. In summary, the pore-loop mutation had longer APs and FPs and a higher risk of developing an arrhythmic cardiac event upon stimulation with a triggering factor [22]. These results are in line with the fact that pore mutations cause a more severe clinical course due to a dominant negative effect, while usually, C-term mutations cause haploinsufficiency and therefore less severe phenotypes [23]. Although a larger panel of KCNH2 mutations should be assessed to further evaluate this scoring system, it appears to be sufficiently sensitive to detect subtle intragenotype-phenotype mutational differences and could have clinical implications in diagnosis, prognosis, and risk stratification of LQTS patients [22].

In combination with next-generation exome sequencing, genome editing also enables the identification of plausible genetic causes for families with genotype-phenotype discordances. A large Cleveland family that was studied for 20 years showed a homogenous LQTS population carrying the LQT2 KCNH2-R752W mutation. Nevertheless, out of 26 mutation-positive members, only 6 had severely affected phenotypes, making it so variable that clinical analysis did not allow an accurate diagnosis of those individuals carrying these mutations [24]. Whole exome sequencing analysis identified a variant in the GTP-binding protein REM2, common for the severe phenotypes. REM2 encodes a member of the Ras superfamily, which are well-known modulators of voltage-gated calcium ion channels, suggesting it could be a promising modifier gene in LQTS [25]. Five patients were selected from this family, and as their hiPSC-CMs were able to reproduce phenotype discordances, Chai et al. used a CRISPR/Cas9 strategy based on homologous recombination to correct the REM2 variant in the cells from severely affected individuals. The hiPSC-CMs showed enhanced LTCC and prolonged action potentials that were successfully reversed upon genome editing. Therefore, they linked the REM2 gene variants to arrhythmias and concluded that the REM2-driven increased L-type Ca^{2+} current in combination with primary KCNH2 haploinsufficiency is the permutation that produces the full-blown disease phenotype [26]. A similar situation was studied recently in a family in which both father and son were carriers of the same Y111C missense mutation in KCNQ1 gene but presented opposite clinical phenotypes. The functional and molecular study of their hiPSC-CMs showed impaired trafficking and increased degradation of the mutant KCNQ1 protein in the symptomatic (S) patient. In contrast, for the asymptomatic (AS) patient, the degradation was reduced as a result of a reduced activity of Nedd4L, which is involved in channel protein degradation via the proteasome. Whole exome sequencing found two single nucleotide variants (SNVs) on a Nedd4L interactor gene, MTMR4, present in the AS patient and his two siblings, also AS carriers. Correction of the SNVs in AS cells using CRISPR/Cas9 unmasked the LQTS phenotype, showing reduced I_{Kr} density. Furthermore, they confirmed that their presence reduced MTMR4 dephosphorylation activity,

thus blunting the proteasomal degradation of KCNQ1 mediated by Nedd4L. In a separate cohort, they found that the same MTMR4 variants were present in 77% of AS Y111C mutation carriers, additionally supporting their protective effect and their role in the incomplete penetrance of Y111C-LQT1 [27].

As we mentioned before, dealing with incomplete penetrance is one of the major hindrances to effective clinical diagnosis. At the molecular level, multiple mechanisms may be responsible for the penetrance heterogeneity in LQTS. Introduction of a very low penetrance SCN5A mutation in hiPSCs through CRISPR/Cas9 showed prolonged action potentials and arrhythmogenic delayed afterdepolarizations. The LQT3 phenotype was reversed by using PIP_3, a known sodium late current modulator. This is consistent with the results obtained in heterologous expression systems, in which PIP_3 could also reverse the late current phenotype in this variant. However, a fully penetrant SCN5A mutation did not show sensitivity to PIP_3. Therefore, this penetrance differences from almost 0% to 100% may be the result of distinct molecular mechanisms, which need to be considered when interpreting the severity of a late current derived from sodium channel functional defects [28].

Although gene correction is the most appealing application of CRISPR/Cas9, knocking out or down genes is also possible. This approach is especially interesting in those diseases affecting redundantly expressed genes. That is the case for calmodulinopathies, since the human genome harbors three distinct genes encoding for an identical calmodulin protein (CALM1-3). This protein is a ubiquitous Ca^{2+} sensor that modulates several ion channels, including LTCCs, which inactivation is promoted by the formation of Ca^{2+}-CaM complexes. As calmodulin is also an LQTS susceptibility gene, in 2017, two groups used this approach to investigate CALM2-LQT15 mutations. On the one hand, Limpitikul et al. used a CRISPRi system in which a dead Cas9 is fused to a suppressor, allowing downregulation of the target gene and avoiding double-strand breaks that could permanently alter off-target or downstream elements in the genome. The CRISPRi suppressed patient-specific iPSCs, normalized the prolonged APD, and corrected fully the magnitude of LTCC's Ca^{2+}-CaM dependent inactivation. Furthermore, it provided additional evidence that mature cells like cardiomyocytes could potentially be targeted by this approach [29]. On the other hand, Yamamoto et al. leaned toward a mutant allele-specific ablation in another LQT15 model of patient-derived iPSCs. This approach used a Cas9 double nickase system to reduce off-target effects and premature stop codons. They achieved the rescue of the electrophysiological abnormalities of the LQT15-hiPSC-CMs, indicating that the mutant allele caused dominant negative suppression of LTCC inactivation, resulting in prolonged AP duration [30]. In contrast to the former strategy, this allele-specific approach does not affect the WT allele, and therefore can be used in any other dominant negative disease with no need for genetic redundancy. Both strategies hold great promise in the treatment and diagnosis of LQTS and other inherited diseases, whose management is moving into the realm of precision medicine.

3 Brugada Syndrome

Like LQTS, Brugada syndrome (BrS) belongs to the group of inherited primary arrhythmia syndromes, predisposing to ventricular fibrillation and sudden cardiac death in the absence of structural heart abnormalities. This channelopathy is characterized by a coved-type ST segment elevation in the right precordial leads of the ECG (Fig. 2c), occurring spontaneously or upon the intravenous administration of class I antiarrhythmic drugs [31]. The main gene associated to BrS is SCN5A, the alpha subunit of the voltage-gated Nav1.5 cardiac sodium channel responsible for phase 0 of the cardiac action potential. More than 350 rare variants have been identified in SCN5A, accounting for 30% of the diagnosed cases [32]. Although BrS remains to be classified as a monogenic disease, incomplete penetrance and variable expressivity suggest a complex mode of inheritance, and most of these genetic variants remain of questionable causality

[33]. More information about the pathophysiological mechanism of the disease is needed in order to develop BrS-specific treatments, for which the only proven therapeutic option is ICD [6].

BrS genotype-phenotype associations have been studied using genome editing. One of the SCN5A variants examined showed reduced inward sodium current (I_{Na}), abnormal Ca^{2+} transients, and increased triggered activity in patient-derived hiPSC-CMs, reproducing the single-cell phenotype features of BrS. When this variant was corrected to wild type with CRISPR/Cas9, the maximal upstroke velocity and inter-beat variability were ameliorated, resulting in an improvement of the proarrhythmic phenotype and the disturbances found in AP recordings and Ca^{2+} imaging [34]. In another SCN5A variant, a patient-independent approach was used to study causality of the mutation, irrespective of the patient's genetic background. The loss of function BrS A735V-SCN5A variant was introduced in homozygosis in control hiPSC-CMs using CRISPR/Cas9. Apart from observing strongly reduced upstroke velocities and abnormal APs associated to the mutation, they identified a shifted activation curve of Nav1.5 channels that represents a key mechanism underlying the pathology of the variant [35]. In short, both approaches found evidence to support the association of the mutations to the observed BrS phenotypes.

Furthermore, usage of isogenic pairs of cells has enabled the identification of new BrS susceptibility genes. Whole exome sequencing of a large pedigree with BrS and history of SCD identified a rare non-synonymous variant (R211H) in RRAD, a gene encoding the RAD GTPase, present in all the affected members of the family. Insertion of the variant in an extrafamilial control iPSC line with CRISPR/Cas9 technology recapitulated the same phenotype of patient-derived hiPSC-CMs, including persistent Na^{+} current and cytoskeleton disturbances. This confirms the involvement of the RRAD variant in the BrS phenotype, thus identifying a new BrS susceptibility gene [36].

As we have already observed, I_{Na} reductions are characteristic of BrS. Therefore, understanding the molecular mechanisms underlying this reduced current could be of help in the search for potential therapeutic options. Wnt/β-catenin signaling, which is active in heart disease, has been reported to potently inhibit Nav1.5 expression in both neonatal and adult rat cardiomyocytes [37]. Furthermore, chromatin immunoprecipitation showed that TCF4, a downstream effector of the pathway, had binding sites in the SCN5A promoter. Therefore, CRISPR/Cas9 genome editing has been used to induce mutations within these TCF4 binding sites in neonatal rat ventricular myocytes, showing attenuated Wnt inhibition of SCN5A and demonstrating that those sites were functionally important for Wnt regulation of SCN5A [38]. All in all, strategies to block this intracellular cascade would represent novel methods for cardiac-specific inhibition of the Wnt pathway to rescue I_{Na} and prevent SCD. Following the regulation of Nav1.5, a conserved regulatory cluster with super enhancer characteristics has been identified downstream of SCN5A. It drives localized cardiac expression and contains conduction velocity-associated variants, including BrS variants [39]. Deletion of its component regulatory elements using genome editing in the one cell stage of mouse embryos showed that the cluster and its individual components are selectively required for cardiac SCN5A expression, normal cardiac conduction, and normal embryonic development. These studies reveal physiological roles of an enhancer cluster in the SCN5A-SCN10A locus that controls chromatin architecture and SCN5A expression. Thus, alteration of its activity by genetic variants like the ones found in BrS may contribute to the disease phenotype [40].

3.1 Catecholaminergic Polymorphic Ventricular Tachycardia

Catecholaminergic polymorphic ventricular tachycardia (CPVT) is the third of the main primary arrhythmia syndromes, characterized by adrenergic-induced bidirectional and polymorphic ventricular tachycardias (Fig. 2d) in the absence of structural cardiac abnormalities [41]. Treatments for CPVT include beta-blockers,

flecainide, and ICD [6]. Two main types of CPVT have been described: an autosomal dominant disease affecting the RyR2 gene (CPVT1) [42] and a less common recessive form involving the CASQ2 gene (CPVT2) [43]. The RyR2 gene encodes the cardiac ryanodine receptor, which is the main intracellular calcium release channel. On the other hand, CASQ2 is involved in the regulation of the RyR2 activity. Mutations in these proteins therefore are associated with defects in Ca^{2+} handling by the sarcoplasmic reticulum (SR) and underlie the pathophysiology of the disease [44]. CPVT1 accounts for approximately 60% of the cases, while CPVT2, usually more severe, accounts for 10–15% [45]. The remaining CPVT cases are due to mutations of known or unknown origin. In this regard, other genes like triadin, calmodulin, and TECRL are also being studied as potential susceptibility genes [46–48].

As for the other channelopathies, genome editing tools have helped in generating robust models for studying CPVT in vitro. As such, a CPVT1 model generated with CRISPR/Cas9 exhibited aberrant Ca^{2+} signaling properties indistinguishable from those previously recorded in cells derived from patients carrying the same mutation [49, 50]. This supports the pathological effect of the variant as well as the feasibility of the patient-independent model.

With respect to the molecular mechanism underlying CPVT, RyR2 mutations result in an abnormal protein that is prone to spontaneous calcium release from the SR, drives depolarizing Na^{+}-Ca^{2+} exchange, and results in afterdepolarizations that can trigger subsequent action potentials, causing ventricular ectopy and arrhythmias [45]. Gene editing studies in CPVT have been centered into understanding these imbalances in calcium homeostasis, which are triggered upon catecholaminergic stimulation. Studies in mouse models have shown that CaMKII-mediated phosphorylation of RyR2 is able to promote ventricular arrhythmias and its inhibition has proven to be successful in preventing arrhythmogenesis in several CPVT1 mutations in vitro and in vivo [51–53]. The use of genome editing has further supported this hypothesis, identifying a serine (RYR2-S2814) that induces CPVT1. As clinical arrhythmias emerge from the collective behavior of cardiomyocytes assembled into myocardial tissue, researchers developed a bidimensional model using integrated muscular thin films (MTFs) from patient-derived and CRISPR/Cas9-introduced R4651-RyR2 hiPSCs. Together with optogenetics, this enabled simultaneous assessment of myocardial Ca^{2+} transient propagation and contraction. Both patient-derived and engineered MTFs reproduced the CPVT phenotype at the tissue level and implicated CaMKII as a key signaling molecule in the pathogenesis of CPVT. To further study the mechanism of reentry, they used genome editing to replace a critical target serine of CaMKII with alanine (S2814A) in RYR2 alleles, in both WT and R4651I background. By doing so, they blocked the phosphorylation event and observed normalized pacing- and isoproterenol-induced Ca^{2+} propagation speed heterogeneity and relative diastolic Ca^{2+} level, resulting in a substrate that is less vulnerable to reentry [54].

Comparative analysis of different disease-causing mutations can also be performed using genome editing. Recently, Zhang et al. explored three mutations introduced by CRISPR/Cas9 in different domains of the RyR2 to determine whether the molecular mechanism underlying their pathological effect is dependent on the specific RyR2 mutation site. The mutations were located at the N-terminus, C-terminus, and central domains of the protein. All three mutants exhibited CPVT phenotype with prolonged calcium releases. However, in the C-terminus and central domain mutations, the SR Ca^{2+} leak was significantly increased, and the SR Ca^{2+} content was reduced compared to control cells or the N-terminus mutant, which showed moderate leak and Ca^{2+} content. In the C-terminus domain, this might be explained by the higher fractional Ca^{2+} releases and calcium-induced calcium release (CICR) gains observed. Furthermore, dantrolene, reported to bind to RyR2 N-terminus domain, was more effective in suppressing the SR leak and aberrant Ca^{2+} releases in the C-terminus mutation. Although no other drug tested showed mutation-site specificity, these results suggest that

the treatment of CPVT1 should move toward personalized medicine, applying mutation-specific pharmacotherapy [55].

4 Short QT Syndrome

As opposed to LQTS, short QT syndrome is characterized by a shortened QT interval as a consequence of abbreviated ventricular repolarization (Fig. 2e). Pathogenic mutations have been identified in both potassium and calcium channel genes, and at least six subtypes of SQTS have been reported. Like the rest of the primary arrhythmia syndromes, it predisposes to life-threatening ventricular arrhythmias and sudden cardiac death. The treatments of choice for SQTS are the class Ia antiarrhythmic drug quinidine or ICD implantation [6].

SQTS is one of the rarest and less studied channelopathies; therefore models for this syndrome developed with genome editing are scarce. Nevertheless, as for the abovementioned diseases, it has been demonstrated that the phenotype of SQTS can be reproduced in vitro in single cells. Compared to its gene-corrected isogenic control, SQTS cells (KCNH2-T618I) showed shortened action potential duration and increased beat-beat interval variability. In addition, this particular missense mutation produced gain of function of KCNH2, with increased I_{Kr} and protein expression in the membrane [56].

However, more complex electrophysiological phenomena, such as conduction and reentrant arrhythmias, need to be studied in the whole tissue, rather than in individual cells. Cardiac cell sheets (CCSs) provide a bidimensional approach that can overcome this restraint [57]. CCSs from SQTS patient-derived and gene-corrected hiPSCs allow to study the mechanisms underlying SQT pathophysiology. This approach was used to investigate the most common mutation causing SQTS, KCNH2-N588K. It recapitulated the SQTS disease phenotype in both cells and tissues, including a shortened APD and wavelength, increased susceptibility for induction of reentrant arrhythmias, and increased arrhythmia complexity as observed by optical mapping in the CCSs. To validate this tissue model further, the effects of several potential SQTS therapies were screened. Interestingly, despite being able to prolong AP in both healthy and isogenic control hiPSC-CMs, sotalol did not show effects on CCSs [58]. This reinforces the importance of using tissue models over single cell ones while studying arrhythmogenic diseases, since sotalol also failed to produce QTc prolongation in SQTS patients [59].

5 Summary and Future Perspectives

For primary arrhythmias, the first report using genome editing dates back to 2014, when zinc finger nucleases were used to correct an LQTS mutation in vitro [60]. A couple years later, coinciding with CRISPR/Cas9 bursting applications, more groups interested in arrhythmias slowly started to consider the use of genome editing. Regarding in vitro models, we have seen that the generation of isogenic control cells with CRISPR/Cas9 has allowed the identification of new susceptibility genes and variants. Furthermore, the patient-independent approach in which the mutation is introduced by CRISPR/Cas9 in control hiPSCs is much cheaper and rapid than obtaining patient-derived cells for evaluating VUS. This approach does not require access to human samples, making feasible even postmortem studies.

Genetic heterogeneity is very common in channelopathies, and as a result, more and more mutations are being discovered and added to the potential list of variants susceptible for genetic testing. Although both patient-dependent and patient-independent in vitro approaches can support the pathogenicity of a variant, further robust scientific and statistical evidence of disease causation must be considered in order to include them in routinely used clinical screening [61, 62]. In addition, even though huge progress has been made into understanding the pathogenesis of inherited cardiac arrhythmias, the recommendations for therapeutic interventions

have barely changed in the last four decades [63], including beta-blockers, LCSD, or ICD [6]. Being able to introduce distinct disease-causing mutations while keeping the same genetic background has also allowed unbiased comparison of multiple variants. This comparative analysis showed that different mutations in the same gene might be the consequence of distinct molecular mechanisms, reinforcing the concept that the treatment of inherited arrhythmias needs to move into the realm of precision medicine and patient-specific approaches.

Another point worth mentioning is that despite the fact that missense variants are relatively easy to be corrected by CRISPR/Cas9, targeting complex mutations, such as double heterozygosity, may pose additional challenges that still need to be surpassed [64]. Additionally, CRISPR/Cas9 itself has several limitations that have delayed the application of genome editing in vivo to the treatment of cardiac arrhythmias. Correcting a mutation requires the activity of the homologous recombination cellular machinery, which is downregulated in terminally differentiated cells like cardiomyocytes, thereby reducing the chances of success. Furthermore, correction of only a small number of cells might trigger proarrhythmic events and even worsen the patients' clinical scenarios [65].

All in all, what we have learned from these recent studies of primary arrhythmia syndromes and genome editing is that these diseases can be successfully reproduced in a dish, showing defective ion currents and providing a useful platform for molecular, comparative, and drug-testing studies. Although very promising, this technology is still very young, and translating it from bench to bedside will need additional research to improve safety, efficiency, and specificity of the methods. Hopefully, in the upcoming years, more in vivo CRISPR/Cas9 research in cardiac channelopathies will help us see its potential to cure these diseases and make precision medicine a reality.

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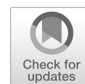

Genome Editing and Atrial Fibrillation

Michael Spartalis

Abstract

Atrial fibrillation (AF) is a frequent rhythm disturbance that raises the possibility of heart failure (HF) and stroke. AF is a multifactorial disorder combining genetic and environmental etiologies. Over the last decade, advancements have been made regarding the genetic base of this arrhythmia. We present the existing knowledge of genetic analysis and genome editing for atrial fibrillation, indicating the existing gaps and future directions. We aim to elucidate how genome editing could be utilized for patients with atrial fibrillation.

Keywords

Genome editing · Atrial fibrillation · Gene therapy · Genetics · Arrhythmia

1 Background

Interventional electrophysiology is a rather new subspecialty of cardiology. Over the last 50 years, novel ablation procedures and approaches have evolved [1]. Nevertheless, the therapy of rhythm disturbances remains substandard. Many cardiac rhythm disturbances like ventricular tachycardia (VT) and AF are arduous to treat with either interventional or antiarrhythmic drug treatment [2]. Cardiologists are continuously studying novel approaches such as genome editing to manage rhythm disturbances and address patients' increasing demands with arrhythmias [1, 2]. Genome editing has a great future and can be an exceptionally efficient patient-tailored therapy for rhythm disturbances [1, 2]. We will examine the present status and advancements in genome editing for AF.

2 Genome Editing and Atrial Fibrillation

The main issue with AF is that it enhances the patients' possibility of stroke, dementia, HF, and death [3, 4]. Several specific genetic loci linked with AF are found via genome-wide association investigations [5]. In addition, some loci were identified via familial linkage investigations [6]. The diverse genetic, electrical, and structural deviations causing AF are the most challenging aspects of AF therapy. Present AF management is based on reestablishing sinus rhythm (SR) but is hindered by substandard effectiveness and possible mortality and morbidity. Antiarrhythmic medication treatment is known to have severe side effects [7, 8]. AF ablation is progressively being utilized for symptomatic patients; however, its

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effectiveness in subjects with persistent AF is inadequate [9]. AF ablation is linked to increased mortality risk and increased number of deaths [10, 11]. There are several promising genome editing methods for AF prevention and treatment [1, 2]. Most AF studies are experimental, based on porcine and dog models [1, 2]. Because AF is mainly an atrial disorder, investigations in such designs display a huge translational edge over mouse ones [1, 2]. Various mechanistic AF investigations that show the aspect of anatomical and electric remodeling in the origin of AF were implemented in dog, sheep, and goat examinations [12, 13]. The framework of ion channel and atrial change of electrophysiological properties of canines was found similar to that observed in AF subjects [14]. The basis of electric remodeling seen in the atria has proven to be identical in genome editing porcine model investigations to that found in canines and several big animals [15, 16]. Table 1 summarizes the primary experimental studies regarding gene-focused aiming of AF substrate.

3 Atrial Fibrillation Substrate and Modifiable Electrical Targets

3.1 Ion Channels

A typical pathophysiological process of alterations in electrophysiological properties of AF is the decrease of the action potential duration (APD) [23]. This shortened duration causes the reentrant loops to be effortlessly induced and sustained [23]. In experimental studies, genome editing has been utilized to extend the APD via diminishing the expression of the delayed rectifier potassium channel IKr. This is accomplished through inhibiting the KCNH2 gene, a gene that codes for the alpha subunit of the IKr channel [23]. Furthermore, a porcine study found that potential duration can be lengthened by painting an adenovirus vector with an epicardial gene encoding a dominant-negative (DN) mutation of KCNH2 [24]. The authors demonstrated that halting the alpha subunit of the IKr channel resulted in an APD prolongation, inability of burst pacing induction of AF, and enhanced AF return to SR. [24]. The modifications were changed back after two weeks, associated with loss of gene expression [24]. Furthermore, Soucek et al. investigated the electroporation and epicardial injection in order to transfer the AdCERG-G627S transgene to hinder KCNH2 activity [17]. The authors showed that pigs after editing had significantly greater APD, and AF formation was impeded in an accelerated atrial pacing design [17]. It is essential to mention that the porcine hearts that acquired the transgene and formed chronic AF did not have reduced ejection fraction in comparison to the control arm [17]. However, these investigations did not evaluate the long-lasting outcomes of atrial IKr abolishment, because they were designed for limited period [17]. New investigations have revealed that reduced L-type calcium channel density (ICaL) is involved in the pathophysiological development of AF [25]. Genome editing focusing on enhancing a remarkably expressed gene replica, or through upregulation of specific channels, can be efficient in modifying this result [25].

Kv 1.5 ion channels can be an alternative option for AF genome editing [26–28]. Kv 1.5 ion channels are preferentially found in myocardial cells of the atrium and control the ultrarapid delayed rectifier current (IKur), leading to atrial repolarization following an action potential [26–28]. Hindrance of these channels is an attractive target of several molecule AF treatments due to the ability to cause selective lengthening of only the atrium's action potential [29]. Kv 1.5 knockout by CRISPR-mediated gene targeting or through an expressed siRNA or analogous oligonucleotide moieties can have the same therapeutic results without the necessity for continuous anti-arrhythmic drug therapy [29].

The TASK-1 potassium channel is an atrial distinct modulator of APD, making it an attractive option for anti-arrhythmic drug treatment [30]. Three distinct TASK-1 mutations were found to associate with AF generation [31]. Mathematical design and patch clamp techniques have shown that regulation of TASK-1 could modify

Table 1 Genome editing studies based on experimental models of AF [1]

AF prevention/cessation approach	Reference	Gene	Vector	Transfer techniques	Animal
Suppression of IKr current Lengthening of the atrial APD Suppression of IKr current	2005 [16] 2012 [17] 2013 [18]	KCNH2-G628S KCNH2-G627S KCNH2-G628S	AV AV AV	Epicardial gene painting Electroporation and gene injection Epicardial gene painting	Porcine Porcine Porcine
Enhanced expression of cardiac gap junction proteins CX43/CX40 to maintain more homogenous atrial conduction	2011 [19] 2012 [20]	CX43 CX40 CX43	AV AV	Electroporation and gene injection Epicardial gene painting	Porcine Porcine
Halting vagal signaling via dismantling Gi and/or Go proteins in LA	2009 [21]	Gi/Go terminal peptides	Plasmid	Electroporation and gene injection	Dog
Reducing LA fibrosis to homogenize atrial conduction	2016 [22]	TGR-B Type II receptor	Plasmid	Electroporation and gene injection	Dog

AF atrial fibrillation, *AV* adenovirus, *APD* action potential duration, *LA* left atrium

the action potential of cardiac muscle cells [32]. The specific expression methods of TASK-1 were examined in extensive studies with persistent AF, resulting in increased expression of TASK-1 in the atrium [33, 34]. While TASK-1 hindrance leads to an enhancement in the APD, which may have a positive effect on the AF substrate status, in vivo pig designs have demonstrated that in the frame of AF and HF pathophysiology, TASK-1 expression is downregulated [30]. The double impact obscures the TASK-1 narration, showing a need of a deeper understanding of TASK-1 prior to thoroughly labeling TASK-1 as an alternative AF strategy therapy [30].

3.2 Gap Junctions

Gap junctions are vital mediators of normal atrium conduction that control the electrical impulse transmission velocity linking with neighboring cytoplasms [35]. Connexins, which are transmembrane ion channels, control this relation [35]. These channels regulate the movement of small proteins and ions between adjacent cells, granting conduction and electrical coupling [35]. The atrium has two different connexin subtypes that develop gap junctions, CX40 and CX43 [35]. These proteins are critical regulators of a cell-to-cell coupling, and consequently, the altered expression of these molecules causes tremendous changes in the conduction [36]. Furthermore, altered connexin expression sequences could enhance atrial refractoriness discrepancy, leading to heterogeneous conduction sequences which may cause an arrhythmia [35]. Experimental models and studies with human atrial tissue have shown that reduced CX 40 and 43 contribute to AF-related structural alterations [37]. A porcine model study showed that CX43 expression could be repaired through electroporation and epicardial direct injection of an adenovirus encoding CX43 [19]. The authors concluded that these animals showed no arrhythmia episodes after 14 days of burst stimulation whereas everyone from the control group formed AF [19]. Igarashi et al. also showed that the epicardial painting repaired expression of connexins 40 and 43 in a rapid pacing porcine model and improved not only the electrical conduction but the gap junction concentration as well [20].

3.3 Parasympathetic Signaling

Aberrant autonomic parasympathetic signaling is an essential element that could lead to AF development [38]. Because of its denser innervation than different atrial segments, the posterior wall of the left atrium (LA) has more parasympathetic properties that can promptly generate AF [39]. Parasympathetic signaling begins with acetylcholine discharge from vagal nerve endings, which later stimulate muscarinic type 2 receptors that connect with heterotrimeric Gi proteins [40]. Gαi/o subunits of this G protein then hinder adenylate cyclase cyclic monophosphate (cAMP)—protein kinase action, delay SR, and atrioventricular nodal conduction while reducing refractoriness of the atrium [41]. Consequently, the reduction of refractoriness increases the possibility of reentry and contributes to new arrhythmia episodes [42]. Aistrup et al. demonstrated that hinder of Gαi proteins through adding Gαi2/3 C-terminal peptides could facilitate vagal-induced refractory period reduction and as a result cause a reduction in vagal-induced AF [21]. They showed that the C-terminal Gαi/o peptides expressed from a cytomegalovirus plasmid after in vivo electroporation can reduce vagal-induced arrhythmia in a dog study [1].

4 Atrial Fibrillation Substrate and Modifiable Structural Aspects

4.1 Fibrosis

It is well known in current literature that AF is linked to a proinflammatory pathophysiological process that contributes to increased atrial oxidative stress [1, 2]. The inflammation development and the oxidative stress cause disturbance of the balance of regulatory processes, causing enhanced apoptosis and cellular fibrosis [1, 2]. As fibrosis is linked to an increased expression of the transforming growth factor (TGF-b), this growth factor prompts the generation of extracellular matrix proteins, collagen, and reactive oxygen species (ROS) [18, 43, 44]. The myocardial segment of the posterior LA can sustain AF via an enhanced susceptibility to heterogeneous conduction and fibrosis [22]. The transduction of a transgene that interrupts TGF-b signaling in the posterior wall of the LA can alter the electrophysiological properties and framework of this segment [1]. Electroporation and direct injection are used to deliver a DN mutation of the TGF-b receptor to the posterior LA of 12 dogs [1]. The dogs presented a remarkable decline in atrial fibrosis [18, 43, 44]. Reverse remodeling of the posterior wall of the LA enhanced electrical impulse transmission, and decline in AF induction during pacing was observed after three weeks of burst stimulation [18, 43, 44]. A shift in the restitution slope was also noted, forming the plasmid inserted atrial tissue more impervious to AF. These electric alterations were associated with a decline in atrial fibrosis, showing a connection between AF substrate and conduction properties [18, 43, 44]. The results highlight the role of genome editing to alter atrial function and structure via downregulating the inflammation found in AF [18, 43, 44]. These observations pave the way for not only focusing on the treatment of AF but also the prevention, interrupting the structural changes that generate AF [1, 18, 43, 44].

4.2 Apoptosis

The enhancement of apoptosis is an alternative approach for genome editing. Superoxide dismutase-1(SOD1) enzyme is a key element in oxidative stress signaling and cellular apoptosis. Dysregulation of the SOD1 enzyme is found in canine AF models [45]. Zhang et al. showed that suppressing micro-RNA 206 can reduce AF susceptibility by reducing the SOD1 enzyme [45]. The authors transduced an anti-MRI 206 lentivirus into the superior left ganglionated plexi in dog models. The animals showed a decline in AF induction and an increased APD [45]. A different technique based on apoptosis is via inhibiting the function of caspase-3, an enzyme mediating

apoptosis which can be hindered with siRNA [46]. Adenovirus vector therapy involving siRNA targeting caspase-3 in a porcine model caused elimination of apoptosis and delay of persistent AF [46].

5 Oxidative Stress and Modifiable Structural and Electrical Aspects

AF is a multifactorial disease involving different structural and cellular factors. This complex interplay will reasonably demand various sites of adequate contact modulation. ROS produced by oxidative stress connect with biomolecules such as DNA, lipids, and protein [47, 48]. This increased reactivity connects a huge number of integrations with the AF substrate. ROS have many thoroughly established connections with a number of recognized AF rotors, and their modulation has a great possibility to tremendous positive results on the AF disorder status [49].

Increased ROS levels such as H2O2 and superoxide are correlated with the AF disorder status [50]. This correlation is upheld by secondary oxidative stress methods, identified in the plasma of AF subjects [50]. AF subjects have less degrees of nitric oxide bioavailability and greater oxidized/reduced rates of cysteine and glutathione in comparison to subjects without AF [51, 52]. Hydroxyl radicals and peroxynitrate, two of the most important ROS, can generate oxidative impairment to myofibrils, causing anatomical remodeling that defines AF [53]. ROS are associated with augmented TGF-B communication and AF fibrosis that is typical of anatomical restructure [54]. ROS can harm mitochondrial DNA, leading to cellular calcium burden and alterations of the electrophysiological properties that causes AF [55]. In addition, elevated ROS levels are linked to augmented oxidized CaMKII that is correlated with varied calcium handling and consequently atrial changes of the electrophysiological properties [49]. Current literature has validated ROS as a fascinating and compelling approach to alter course of AF [49–55].

5.1 ROS Generation and NADPH Oxidase

NADPH oxidases are membrane-bound proteins identified in various tissue categories [56]. NADPH oxidases are the catalysts of the transformation of oxygen to superoxide [56]. The Nox group of these proteins are the main ROS producers in the normal heart, and this role is augmented in AF setting [50, 57, 58]. The function of Nox group is raised in the fibrillating atrium [57]. Several studies have demonstrated that this effect is independent of Nox expression changes, showing that greater degrees of Nox stimulation, rather than expression in the fibrillating atrium, stimulate enhanced ROS generation [57]. As a result, there are two paths to abolish the number of ROS being generated in the myocardial cells, either by reducing Nox stimulation or by reducing the all of the Nox proteins [57]. There are two different gene-based translational methods to regulate Nox levels [57]. The first is via Nox hinder through transgene-regulated expression of inhibitory polypeptides [57]. The other one requires Nox knockdown via RNAi or CRISPR knockout [57].

5.2 Oxidized Calmodulin-Dependent Protein Kinase II (oxCAMKII)

CaMKII acts as a ROS sensor as well as a proarrhythmic indication in the fibrillating atrium [59]. CaMKII is prone to oxidation at methionines 281 and 282, resulting in a constitutively active design linked to enhanced phosphorylation of RYR2 channels [60]. The phosphorylation causes greater sarcoplasmic reticulum calcium leakage, delayed after depolarizations, triggered action potentials, and conclusively AF [61–63]. In addition, this profibrillatory effect was abolished in mice with an oxidation-resistant CaMKII type showing that oxCaMKII is a vital element of the ROS-induced profibrillatory path [61–63]. Genome editing focusing on oxCaMKII can be managed either by a CRISPR or RNAi knockdown or CaMKII

replacement with an oxidation resistant type of the particle to sustain regular function and diminishing protein's profibrillatory size.

6 Conclusions

AF is a multifactorial disorder with a pathophysiological process involving genetic basis and environmental elements. The use of genome editing to treat AF at the substrate level could provide a revolutionary treatment for a disorder that the present standard of care is inadequate. Our knowledge of the AF substrate has greatly been advanced recently, designing alternative management approaches that could specifically be identified and consequently targeted. Our knowledge of the disorder is the only limit to identify a perfect genome editing tool for AF. As our understanding of gene vectors and transfer techniques progress, a novel approach in AF therapy will arise, where cardiac muscles are modified to be impervious to AF, promoting patients' quality of living and lowering the pressure on the healthcare organizations from AF comorbidities. Further refinement of the genetic core of this arrhythmia will eventually identify novel treatment strategies and more accurate risk stratification.

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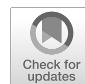

Genome Editing in Dyslipidemia and Atherosclerosis

Zhifen Chen, Constanze Lehertshuber, and Heribert Schunkert

Abstract

Despite successive advancement of genome editing technology with zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs), the recent breakthrough in the field has been related to clustered regularly interspaced short palindromic repeats/associated proteins (CRISPR/Cas). The high efficiency and convenience of CRIPSR/Cas systems dramatically accelerate pre- and clinical experimentations of dyslipidemia and atherosclerosis. In this chapter, we review the latest state of genome editing in translational research of dyslipidemia and atherosclerosis. We highlight recent progress in therapeutic development for familial dyslipidemia by genome editing. We point to the challenges in maximizing efficacy and minimizing safety issues related to the once-and-done therapy focusing on CRISPR/Cas systems. We give an outlook on the potential gene targets prioritized by large-scale genetic studies of cardiovascular diseases and genome editing in precision medicine of dyslipidemia and atherosclerosis.

Keywords

Dyslipidemia · Atherosclerosis · Genome editing · CRISPR/Cas · Familial hypercholesterolemia · Genome-wide association study · Precision medicine

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1 Dyslipidemia and Atherosclerosis

Atherosclerosis represents the major cause of coronary artery disease and thereby mortality worldwide [1]. The complex etiology of atherosclerosis is initiated by dysfunctional endothelial cells lining the arteries that are no longer capable of appropriately regulating vascular tone and permeability for molecules and cells [2]. Progressive infiltration of lipoprotein particles carrying cholesterol into the vessel wall triggers an inflammatory response mediated by cholesterol-loaded macrophages. Proliferation of smooth muscle cells causes vascular remodeling and ultimately leads to narrowing of the vessel and obstruction of blood flow. Dyslipidemia, a common and strong risk factor for atherosclerosis, describes elevated plasma levels of low-density lipoprotein cholesterol (LDL-C), lipoprotein(a) (Lp(a)), and/or triglyceride-rich lipoproteins (TRLs, VLDL, and IDL) [3] and/or decreased levels of

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high-density lipoprotein cholesterol (HDL-C) [4]. In addition to lifestyle and environmental influences, dyslipidemia is largely determined by genetic factors. Its extreme forms are manifested as familial dyslipidemias caused by gene mutations, including hypercholesterolemia (e.g., *LDLR*, *APOB*, *PCSK9*, *LPA*, and *ANGPTL3*) [5–7], hypertriglyceridemia (e.g., *LPL*, *APOC3*, *APOC2*, *APOA5*, *ANGTPL4*, *GPIHBP1*, and *LMF1*), dysbetalipoproteinemia (e.g., *APOE*), analphalipoproteinemia (e.g., *ABCA1*), LCAT deficiency [8], and combined hyperlipidemia (e.g., *USF1*) [9, 10]. Familial hypercholesterolemia (FH), the most common form of the overall rare dyslipidemias, occurs in 1 out of 200,000–250,000 people heterozygously and in 1 out of 160,000–320,000 people homozygously [11, 12]. To fulfill the pressing need of precision medicine, efforts have been increasingly committed to developing targeted therapies for dyslipidemia and atherosclerosis.

2 Current Therapies of Dyslipidemia and Atherosclerosis

2.1 From Traditional Pharmacology to Targeted Therapy

Pharmacological treatment of dyslipidemia and atherosclerosis predominantly focuses on cholesterol lowering [4]. For many years, statin (inhibiting cholesterol synthesis), ezetimibe (suppressing intestine uptake of cholesterol), and bile acid sequestrants have been the major treatments of the conditions [13, 14]. However, a significant proportion of patients do not achieve guideline-recommended cholesterol levels with these medications. Recently approved bempedoic acid further reduces LDL by about ~20% [15]. PCSK9 monoclonal antibodies [16], another new drug type, enable effective LDL reduction in addition to statin therapy but with high costs, hampering the general use. While small molecules targeting PCSK9 are under investigation to bring down the cost, drugs lowering other causal lipids and their inflammatory responses are on the way to treat the residual cardiovascular risk [17]. Revolutionary discoveries of human genetics in the past decade have been a nutritious ground for novel drug developments [18–20]. Genetic studies of atherosclerosis, coronary artery disease [1], and myocardial infarction (MI) not only nominated but also validated causal genes, pathways, and risk factors for the conditions. For instance, genetic studies supported Lp(a) and TGs as causal risk factors for atherosclerosis, which led to intensive investigations of related genes, such as *LPA*, *APOC3*, *ANGPTL3*, and *ANGPTL4* [3, 21–23]. Based on a better understanding of the affected mechanisms, these genes evolved as novel targets for biological drugs, monoclonal antibodies, and nucleic acid-based therapies [24].

2.2 Nucleic Acid-Based Therapy

Nucleic acid-based therapies were initially designed as replacement for dysfunctional genes by delivery of the correct coding sequence [25]. Recently, this concept has been expanded to include gene silencing by antisense oligonucleotides, or short interfering RNAs (siRNA), transcriptional modulation by microRNAs, and long noncoding RNAs (lncRNA), as well as modification of epigenetics and genome editing [25–27]. For instance, gene supplementation of *LDLR* is currently investigated in a phase 1/phase 2a first-in-man trial (NCT02651675) for homozygous FH due to function loss of the gene [28]. AON (antisense oligonucleotide)- and/or siRNA-based therapies targeting several dyslipidemia genes have been intensively tested in large-scale clinical trials for treating atherosclerotic CAD, such as *APOA*, *PCSK9*, *APOC3*, and *ANGPTL3* [29–34]. LncRNA BM450697 was reported to regulate LDLR via epigenetic-dependent mechanism, and siRNAs targeting the lncRNA enhanced hepatic cholesterol uptake [35]. These novel therapeutic strategies not only expand the druggable genome that previously was largely limited to enzymes, membrane proteins, and circulatory factors but also potentially have advantages of

specificity, efficacy, and safety. However, limited half-lives of nucleic acids, requirement of frequent injection, and medication compliance are general limitations. The limitations are not applicable for gene editing-based therapies that could introduce permanent therapeutic changes to specific gene targets. It is conceivable that in the future, a single administration of such drugs mediates durable cure of dyslipidemias and atherosclerosis.

3 Genome Editing

3.1 Evolution of Genome Editing Technology

Genome editing generally refers to the specific modification of nucleotide sequences (mainly DNA) by enzymic activities (e.g., nucleases and nickase) [36]. In a broader sense, it also includes RNA editing. Nucleases usually cut a nucleotide sequence and create damage (typically a double-strand break (DSB)), whereas nickases introduce single-strand breaks (SSB) [37]. Both DSB and SSB in turn trigger natural genetic repair mechanisms, such as nonhomologous end joining (NHEJ) and homology-directed repair (HDR) enabled by a homologous-armed template [38]. The cellular repairing machinery is hijacked to install precise nucleotide manipulations.

In the late 1970s, the first generation of gene editing tools was engineered based on hybrid proteins including zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs) [39–41]. Both types of nucleases rely on a recombinant recognition domain to bind the target DNA sequence. Target-specific ZFN and TALEN engineering involves in tedious designing and screening of the optimal recombinant protein with high binding accuracy and affinity. Of note, TALENs have reached clinical experimentation to generate universal allogeneic CAR T-cells for B-cell lymphoma [42, 43].

Ever since 2012, genome editing has become easier, faster, and more economic, due to the discovery and engineering of RNA-guided gene rewriting technology—the CRISPR/Cas system [44]. The new system holds promise to cure genetic diseases through (1) inactivating detrimental or aberrant gene expression, (2) amending disease-causing or associated mutations, or (3) targeted insertion of therapeutical DNA (Fig. 2a–c). CRISPR/Cas harnesses the marriage of two independent components, the small guide RNA (sgRNA) and a Cas protein. The allocation of two functions of the traditional recombinant nucleases into the nucleotide sequence recognition by a sgRNA and the enzymic cutting by a Cas nuclease dramatically simplified the design and construction of the editing tools. The classic CRISPR/Cas9 system creates DSB and relies on NHEJ for gene knockout and HDR for an error-free DNA retyping. For newer types of CRISPR tools, the nuclease activity of a Cas protein was either inactivated to only bring transcriptional activators or suppressors to the targeted genomic site [46–48] or transformed into nickase tandem to other enzymes, such as deaminases in base editors (BEs) [49]. By directly triggering chemical reaction (deamination) on DNA and converting C to T (CBE) or A to G (ABE), BEs allow gene knockout without DSB and individual nucleotide(s) rewriting independent of a template, which hold promise for therapeutic gene editing with minimum off-target effects. In fact, point mutations represent the most common genetic variations associated with human diseases [50]. Recently, more types of Cas proteins, such as Nme2Cas9 and Cas13, have been discovered, extending the coverage of editable genome and enabling RNA manipulation [51, 52].

3.2 In Vivo Delivery of Genome Editing Systems

Intracellular delivery of gene editing tools has been the most challenging step in vivo. Adeno-associated virus (AAV)-, adenovirus-, and lentivirus-mediated delivery systems have been tested for CRISPR-based gene therapy [53, 54]. Due to lower immunogenicity, non-integrative and high efficiency, AAVs are widely used in CRISPR-based ex vivo and in vivo biological research and therapeutic

development. However, the packaging limit of AAV (~4.7 kb) often hampers its applications. Thus, instead of spCas9 (~4.1 kb), saCas9 (~3.2 kb) is usually employed for AAV-based gene editing, which allows all-in-one CRISPR therapy carrying both saCAS9 and sgRNA sequences on the same vector [55]. Generally, immunogenicity and potential transgene integration are of high concern when viral vectors were chosen for therapeutic development. Therefore, efforts have been exerted in seeking nonviral carriers for CRISPR-mediated gene [53].

Another promising in vivo delivery method involves the encapsulation of CRISPR/Cas into nanocarriers, in the forms of RNA-protein complex (RNP) or coding nucleic acids (DNA plasmids or mRNAs). In particular, delivery by lipid nanoparticles (LNP) achieves efficient targeting of specific tissues and protects the loaded proteins and nucleic acids [56–58]. Advanced LNP technologies for gene editing include self-assembled DNA nanoclews [59], cationic LNP and lipoplexes [60–62], gold nanoparticles [63–65], and zeolitic imidazole frameworks [66]. Most approaches harness electrostatic interactions between guest and host. Despite the promise, delivery of RNPs has been the most challenging due to the strong negative charge of sgRNA, the large size of Cas proteins, and the sensitivity of RNPs to denaturation and degradation during formulation and delivery. To date, the development of stable and organ-specific nanoparticles for delivery of CRISPR toolkits remains elusive.

4 Genome Editing in Dyslipidemia and Atherosclerosis

4.1 Genome Editing: A Driving Force for Dyslipidemia and Atherosclerosis Research

Ever since the applicable invention of CRISPR/Cas9 system in 2012 [67], it has been increasingly used in cardiovascular research (Fig. 1) and fosters delicacy of cellular and animal models for dyslipidemia and atherosclerosis research. Patient-induced pluripotent stem cell (hiPSC) line of carrier of heterozygous p.C310R (c.928 T > C) mutation in *LPL*, encoding lipoprotein lipase, has been reprogramed to model familial hypertriglyceridemia (FHTG). In parallel, researchers generated a mutation-corrected isogenic iPSC line (AHQUi001-A-1) using CRISPR/Cas9 technology [68]. The isogenic pair could differentiate into relevant cell types, such as adipocyte and endothelial cells, and test therapeutic modifications for the patient. Cell banks, such as WiCell, provide as precious resources of isogenic hiPSCs for dyslipidemia

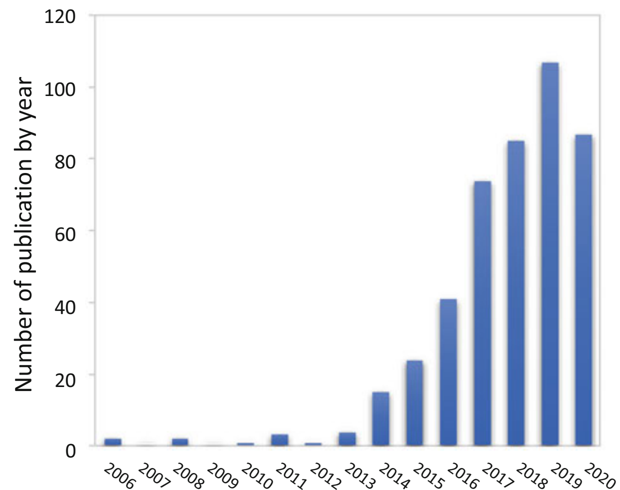

Fig. 1 PubMed search of "gene editing in cardiovascular diseases" for the last 15 years. Numbers of studies by year as indicated, figure adapted from PubMed statistics

and coronary artery disease. Given that the CRISPR/Cas system relies on open chromatin to screen the matched gene code, the efficiency of the gene editing heavily depends on the proliferation and transcription activity of cells. Hyperproliferative cells, such as stem cell and cancer cell, are relatively easy to target with high efficiency. Therefore, gene editing in hiPSC has been valuable in cardiovascular research. It comes with high efficiency for differentiation of many disease relevant cell types that are challenging to access or target, such as hepatocytes, adipocytes, immune cells, endothelium cells, and vascular smooth muscle cells [69].

CRISPR/Cas systems have substantially reduced the time and cost to generate animal models of germline gene knockouts or somatic targeting in vivo. The high efficiency of CRIPSR/Cas allows genetic modification of multiple genes at any time points of an animal's lifespan. The diverse CRISPR tools allow the flexibility in duration of editing, conditional alleles, tissue-specific targeting, and directions of modulation. Yang and Jaenisch et al. have established a protocol to create gene-modified mice by piezo-driven injection of Cas9 mRNA and sgRNA into zygotes. The authors showed that, beginning with target design, the time frame for generation of transgenic mice can be as short as four weeks [70]. Currently, this method and similar others are commonly used for cardiovascular research. For example, Yu and Cowan et al. generated G protein-coupled receptor 146 (GPR146) deficiency mice and showed that the deficiency protected against hypercholesterolemia and atherosclerosis [71]. To establish atherosclerosis mouse models using CRISPR in adult mice, Jarrett et al. performed somatic knockout of *Ldlr* via AAV8 medicated delivery of all-in-one AAV-CRISPR. The approach robustly disrupted *Ldlr* and resulted in severe hypercholesterolemia and atherosclerotic lesions in the mouse aorta [72]. Although the cholesterol increase induced by the somatic *Ldlr* knockdown was not as high as by germline *Ldlr* knockout, it might better model the chronic condition of atherosclerosis which usually develops at higher age [72]. Similar approaches were adopted to generate atherosclerosis animal models in rabbit, pig, and hamster by knocking out *Ldlr*, *Apoe*, or *Lcat* (lecithin-cholesterol acyltransferase) [73–76]. The success of the transgene models, on the other hand, suggested the effectiveness of in vivo CRISPR/Cas system in testing novel gene functions in dyslipidemia and atherosclerosis. Indeed, the novel role of CCC(COMMD-CCDC22-CCDC93) complex in hepatic cholesterol metabolism was explored and confirmed by somatic CRISPR/Cas targeting of *Commd* and *Ccdc22* in mice [77, 78].

However, as for point mutation correction, the editing efficiency of CRISPR/Cas remains low. Omer et al. attempted to correct the loss-of-function mutation E208X in *Ldlr* gene of the mouse liver by AAV-CRISPR/Cas system. The HDR-mediated correction only achieved 6.7% efficiency but resulting in, to some extent, lower serum lipid levels and decreased lesion area [79]. The coming waves of newer types of CRISPR technologies, such as base editor and prime editor, hold potential to improve in this regard.

4.2 Preclinical Investigation of Genome Editing for Dyslipidemia and Atherosclerosis

Gene editing in adult humans, that is, somatic editing, holds the promise to permanently modify one's risk of dyslipidemia and atherosclerosis. In light of the compliance issue with statins, high costs of PCSK9 monoclonal antibodies, and discomfort of lifetime injection of RNA therapies, such once-and-done strategy is attractive. A poll about the acceptance of the gene editing therapy indicated the support from the majority of the participants [80, 81]. Several gene editing strategies against dyslipidemia and atherosclerosis have been intensively investigated in preclinical settings to inactivate pathogenic gene expression, correct disease-causing mutations, mimic atheroprotective effects of natural genetic variations, or insert beneficial transgenes.

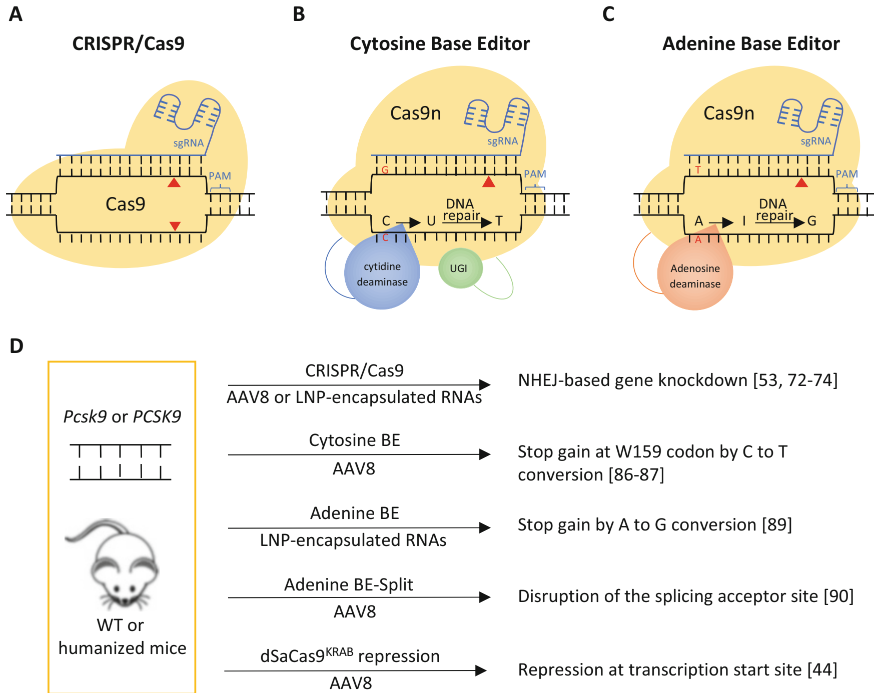

Fig. 2 Therapeutic genome editing of *PCSK9* by CRISPR/Cas and base editing. (**a**, **b**) General overview of DNA editing by CRISPR/Cas9, cytosine base editor (CBE), and adenine base editor (ABE). (**a**) Editing mechanism of CRISPR/Cas9. Cas9 nucleases create double-strand break (DSB) within the guide RNA (gRNA) pairing sequence, usually at 3–4bp ahead of 5′-protospacer adjacent motif [45]. DSB will be fixed through nonhomologous end joining (NHEJ) to create gene knockout or homology directed repair (HDR) to install a genotype or transgene of interest. (**b**) Mechanism of CBE. Cas9 nickase (Cas9n) nicks the top strand, while the cytidine deaminase domain of CBE convers C to U. Uracil glycosylase inhibitor (UGI, an optional component) protects the U intermediate from excision by uracil DNA glycosylase to boost efficiency of base pair editing assisted by nature DNA repair, which ultimately converts a C•G pair to T•A base pair. (**c**) Mechanism of ABE. After DNA nicking, adenosine deaminase domain converts A to inosine intermediate, which will be substituted by G in the subsequent DNA repairing process. ABE replaces A•T to G•C pair. gRNA, guide RNA. (**d**) Overview of strategies investigated to decrease *Pcsk9* or *PCSK9* in vivo. The editing tool is shown above the arrow line and the delivery approach is described below the line. WT, wild type; LNP, lipid nanoparticle; Adenine BE-Split, ABE separated to two domains (split-ABE-Rma573 and split-ABE-Rma674) for virus package, *KRAB* Kruppel-associated box (transcriptional repressor)

The most intensive testing focused on *PCSK9*, given the well-studied biology and rare side effect as a therapeutic target. Gene editing-based therapies allow permanent modification of the culprit and therefore are advantageous as a one shot and one cure for dyslipidemia, especially for FH. Disruption of *Pcsk9* in mice by CRIPSR/Cas9 has been evaluated by AAV- and nanocarrier-based delivery of spCas9 or saCas9 systems [55, 82–87] (Fig. 2d). All led to significant reduction of circulating Pcsk9, plasma total cholesterol (TC), and LDL-C levels. The therapeutic target was further assessed by inactivating the gene using base editing, which result in comparable atheroprotective outcomes [88–91]. A head-to-head comparison of *Pcsk9* gene and base editing in a humanized mouse model showed that the latter introduced no chromosomal translocations, fewer indels, and less new forms of peptides, indicating that it might be a safe strategy for clinical applications [89]. Other gene editing approaches to lower LDL-C level

are pursued, such as CRISPR-/Cas9-based targeting of *Apob* in *Ldlr*$^{-/-}$ mice [55, 72] and replacement of FH mutation of *LDLR* E208X in somatic cells of transgenic mice [55, 79], both of which reduced plasma TC level and atherosclerosis development in mice.

Given that existing lipid-lowering therapies are centered on optimizing cholesterol levels, drugs to reduce levels of non-LDL lipids including TGs and Lp (a) are of an urgent need, particularly for the dyslipidemia patients suffering from obesity, diabetes, or insulin resistance, whose primary risk of atherosclerosis is often related to elevated TGs and other forms of lipids. The attempts beyond LDL-C lowering by gene editing focus on *APOC3* and *ANGPTL3* for reducing TG levels and LPA for decreasing Lp(a).

As naturally occurring loss-of-function (LoF) mutations in *ApoC3* and *ANGPTL3* have found to be atheroprotective [92, 93], CRISPR-/Cas9-mediated inactivation of the two genes was tested to treat hyperlipidemia and atherosclerosis. APOC3, a secretory glycoprotein primarily produced by the liver, inhibits LPL- and hepatic lipase-mediated hydrolysis process of triglycerides in circulation and therefore increases TRL levels. In a human-like animal model (hamster), inactivation of *ApoC3* by CRISPR-Cas9 significantly decreased triglyceride level with no statistical differences in total cholesterol and HDL-C levels, phenocopying APOC3-deficient humans [94]. ApoC3 knockout hamsters also had less atherosclerotic lesions in both thoracic and abdominal arteries, suggesting clinical relevance of APOC3 targeting for the treatment of hypertriglyceridemia and atherosclerosis [95]. In the case of ANGPTL3, an inhibitor of LPL and endothelial lipase, base editing was employed to introduce LoF mutations at Gln-135 site of *Angptl3* in the liver of *Ldlr−/−* mice. This resulted in a median editing rate of 35% in the liver as well as substantially reduced triglycerides (56%) and cholesterol (51%) [96], suggesting a method to treat combined hyperlipidemia and atherosclerosis [96, 97].

LPA, expressed in the liver, encodes for apo (a) that could covalently bound to APOB100, an essential component for both LDL and Lp (a) [98]. Genetic variation of *LPA* was estimated to explain 91% of the variation in Lp(a) levels [22, 99]. Serum Lp(a) level could not be modulated by dietary and lifestyle factors, further necessitating the therapeutic intervention [100]. Lp(a) was also shown as a major carrier of oxidized phospholipids and to induce plaque progression [101–103]. An earlier pioneer study of RNA editing was explored to transform apoB100 mRNA into its truncated form apoB48 by a recombinant adenovirus encoding cytidine deaminase complex (apoBEC-1) to reduce both atherogenic lipoproteins in humanized apoB/apo (a) transgenic mice. This resulted in hepatic editing of human APOB mRNA and reduced plasma levels of human APOB100 and Lp(a). Similar result was observed when the apoB mRNA was edited accordingly in rabbit. These studies demonstrate mRNA editing by apoBEC-1 as a novel approach for lowering plasma concentrations of the atherogenic lipoproteins LDL and Lp(a) [104]. Furthermore, ongoing preclinical studies are investigating the use of base editing to reduce Lp(a) level by inactivating LPA gene.

These proof-of-concept studies demonstrated the feasibility of in vivo gene editing in reducing phenotypes of dyslipidemia and atherosclerosis and triggered industrial interests in developing these further in clinical experimentations. Currently, base editing of *LPA*, *PCSK9*, and *ANGPTL3* are under pharmaceutic development, and ABE-PCSK9 has entered the preclinical toxicology studies. So far, all the tested gene targets address familial dyslipidemia. CRISPR-based therapies could provide personalized treatment for the diseases, which currently cannot be cured. However, whether it could be cost effective to treat nonfamilial forms of dyslipidemia and atherosclerosis should be further investigated. Concerns about its advantage over traditional medications and long-term on- and off-target effects need to be addressed before clinical use. Pilot applications might be firstly available for individuals at high risk for myocardial infarction.

4.3 Further Target Discovery for Dyslipidemia and Atherosclerosis

4.3.1 Gene and Variant Targets Inspired by Human Knockout

Phenotypically healthy humans carrying knockouts of a gene provide evidence that pharmacological knockout of this gene may be safe. For example, LoF variants of *PCSK9* were associated with strikingly low plasma levels of LDL-C, reduced CAD risk [105], and but no apparent adverse health consequences, thus providing reassurance that therapeutic neutralization of *PCSK9* may be safe [106, 107]. Likewise, human knockouts of *ANGPTL3* and *APOC3* led to the development of pre- and clinical drugs for lowering serum levels of cholesterol and triglycerides, and the corresponding alleles related to hypolipidemia are under investigation for treatments using base editing. Increasing discoveries of such "experiments of nature" will be empowered by exome or whole genome sequencing in large-scale biobank cohorts [3]. More gene and allele targets relevant to dyslipidemia and atherosclerosis will emerge [3, 108–111].

4.3.2 Candidate Genes and Variants from Large-Scale Genetic Studies

Genome-wide association studies (GWASs) have discovered over 300 CAD loci and more than 900 loci of blood lipid traits including LDL-C, HDL-C, non-HDL-C, total cholesterol and triglycerides, unveiling novel variants, and genes and pathways underlying dyslipidemia and atherosclerosis with unprecedented speed and mechanistic complexity [20, 112, 113]. GWASs also rediscovered rare variants for dyslipidemia and atherosclerosis, suggesting that beyond these, drug targets are tagged by novel GWAS variants and gene candidates at the many loci associated with lipids and atherosclerosis, especially those loci overlapping for the two traits (Fig. 3). When we explored gene loci shared for CAD and lipids including LDL, TGs, TC, and HDL using the latest statistics of GWAS catalog, EMBL-EBI (2021), we identified 83 loci and classified the mapped genes into related pathophysiological pathways (Fig. 3). Surprisingly, other than the largest portion (~30%) of the genes directly involved lipid metabolism, many genes play roles in known pathways linked to CAD, such as inflammation, angiogenesis, and vascular remodeling. Genes for insulin resistance and glucose metabolism were also identified in our analysis, suggesting that novel genes and pathways for the disease are secondary to dysglycemic regulation. The convenience of CRISPR-based technologies will allow investigation of the novel genetic findings in a high-throughput manner.

Furthermore, by testing causality harnessing genetic information, Mendelian randomization (MR) could identify specific genes as potential therapeutic target and assure efficacy and, importantly, safety before the initiation of drug development [114, 115]. Another genetic approach alerting adverse effect is termed phenome-wide association study (PheWAS), which tests associations of a genetic variant or a gene with hundreds of clinical phenotypes linked to all the organ systems [116, 117]. Using integrative data of individual's genome and electronic health record from large biobank cohort, PheWAS could assess for desirable and adverse clinical outcomes linked to variant and gene of interest. MR and PheWAS provide reassurance for novel gene target selection in pre- and clinical investigations.

4.3.3 Driver Genes and Variants of Systems Genetic Studies

Although compelling efforts have been made to prioritize disease-associated genes utilizing approaches from molecular biology to GWAS, the genetic landscape of atherosclerosis and CAD is not fully elucidated. In the past decade, systems biology based on omic technologies accelerates the understanding of mechanisms underlying complex traits [118, 119]. Systems biology networks, genetic variations, and gene expression with other higher biological layers identify driver variants and genes for complex

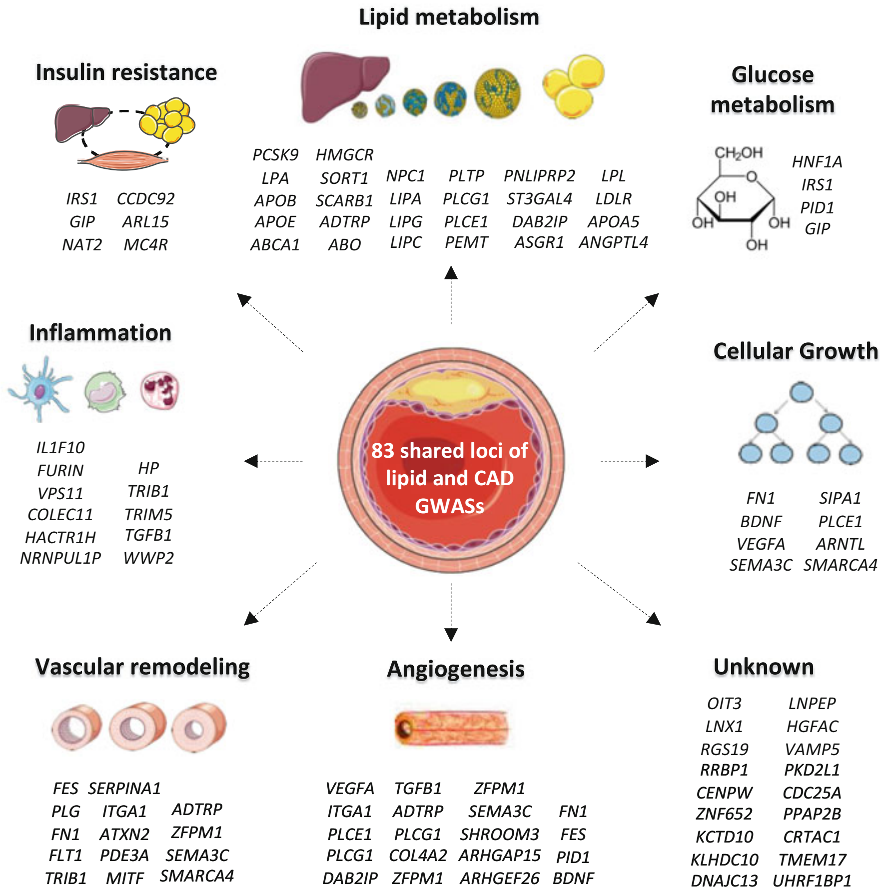

Fig. 3 Genes mapped to 83 shared loci of lipids and CAD GWAS loci and potentially related pathophysiological pathways of atherosclerosis. CAD, coronary artery disease

diseases. Targeting of the key drivers to modulate disease-associated gene or protein networks might enable correction of multiple pathogenic pathways in parallel. Genome editing technologies will play a crucial role in testing related hypothesis and therapeutic potentials [119].

5 Concluding Remarks and Future Perspectives

The possibility of manipulating DNA and RNA has advanced cardiovascular medicine, including understanding gene functions and genetic diseases, as well as the development of novel drug targets. Although the field is still in its infancy, the potentials are exemplified by clinical trials to treat sickle cell disease, to improve effectiveness of chimeric antigen receptor T-cell (CAR-T), or to reverse eye diseases [120]. A clinical trial of base editing targeting *PCSK9* to treat heterozygous familial hypercholesterolemia (HeFH) started in July 2022 (clinicaltrials.gov_NCT05398029). Beyond these examples, many rare genetic disorders, in principle, will be treatable with CRISPR-based therapies.

Despite the exciting progress, many challenges should be tackled before its broader applications. First, tissue-specific delivery of

genome editors has been a long-standing issue. Although AAV systems could allow relatively specific targeting in the liver, brain, muscle, and eye with low immunogenicity [121, 122], they should be further optimized, and many more tissues need to be considered. A new field of research exploiting nanoparticle-based delivery could provide alternative solutions. Second, current genome editing tools strictly rely on specific recognition sequences as well as specific binding sites on the target, such as the protospacer adjacent motif sequences for Cas proteins [45]. The absence of the assisting recognition sequence limits the targeting capability. Therefore, many research teams focus on discovering or engineering editing tools independent of such sequences. Third, substantial variability of editing efficacy depending on genetic loci and cell types was observed, which are partially caused by differences in chromatin accessibility and DNA repairing mechanisms throughout phases of cell cycle. Forth, off-target mutagenesis, although being rare, were detected within sequences of high similarity. While well-designed gRNAs are critical to minimize off-target events, advanced methods have been established to assess unwanted editing in a genome-wide fashion, such as BLISS, GUIDE-Seq, and DISCOVER-seq [123–125]. Finally, a long way has to be gone to fulfill regulatory guidelines and define cost reimbursement for these once-in-a-lifetime therapies. Of note, ongoing therapeutic testing of CRISPR aims to treat patients by modifying their somatic genome. The scientific and social challenges related to human germline editing are discussed elsewhere [126].

Nevertheless, gene editing therapies have to be evaluated carefully case-by-case in extensive pre- and clinical experimentations. Given the recent progress and efforts around the globe to tackle the related issues, genome editing will certainly expand into a new class of therapy to treat many diseases, including dyslipidemia and atherosclerosis.

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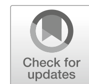

Genome Editing to Abrogate Muscle Atrophy

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Abstract

Muscle atrophy is a multifactor syndrome, which not only decreases the patients' quality of life significantly but also increases the morbidity and mortality of patients with chronic diseases. At present, no effective clinical treatments for muscle atrophy except for exercise are available. The emerging field of genome editing is gaining momentum as it has shown great advantage in the treatment of various diseases, including muscle atrophy. In our current review, we systematically evaluate the etiology and related signaling pathways of muscle atrophy and discuss the application of genome editing in the treatment of muscle atrophy.

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Keywords

Muscle atrophy · Treatment · Genome editing

1 Background

Skeletal muscle is important for body's support, movement, energy consumption, and metabolism [1]. Muscles are the body's largest repository of protein and the source of amino acids [2]. These proteins stay in a dynamic equilibrium during normal physiological conditions, wherein the protein synthesis ratio is equal to that of protein degradation. However, if the protein degradation rate is over that of the synthesis in the skeletal muscle, muscle atrophy happens. Muscle atrophy not only affects the prognosis of patients but also causes a variety of complications [3]. In addition, a massive loss of muscle mass will compromise the efficacy of various different therapeutic interventions [4], which by itself is a poor prognostic indicator. Therefore, maintaining healthy muscles is critical in preventing against metabolic disorders and provide energy to vital organs [5].

The economic burden of muscle atrophy on individuals and the society is enormous. It is urgent to find effective methods of intervention for muscle atrophy. Currently, exercise therapy and nutritional therapy are considered to be good treatment methods for muscle atrophy.

Exercise therapy can not only increase mitochondria and capillary numbers and repair

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the structure of mitochondria in muscle fibers but also increase muscle fiber numbers and improve the exercise ability of patients [6]. In elderly patients with sarcopenia, exercise training can increase the mitochondrial density by 40% [7]. Moreover, it was observed that exercise therapy increased the mitochondrial function while producing more ATP and antioxidant enzymes [8]. But as patients can no longer cope up with their exercise due to the aggravation of their disease, any efforts in this direction will be counterproductive. Therefore, new treatments for muscle atrophy are urgently needed.

Nutrition uptake done through eating is considered to be an effective way to maintain the muscle by providing sufficient energy for muscle activity. This not only increases muscle calories and amino acid intake but also improves muscle protein synthesis [9]. Increasingly, studies have shown that nutritional therapy has a greater impact on the treatment of muscle atrophy in the elderly population [10]. At present, there are several ongoing clinical trials demonstrating the feasibility of nutritional therapy with respect to muscular atrophy, mainly because of its ability to improve the overall quality of life and more importantly to prolong life [11]. However, nutritional treatments are only effective for patients with early muscle atrophy and are still ineffective in patients with severe muscle atrophy.

In recent years, the technology of genome editing has attracted the attention of most scientists in the translational therapy, primarily because this technology allows researchers to better understand the relationship between genetic mutations and their consequent diseases in human and also identify therapeutic methods to successful intervention. Due to the development of genetic engineering in the 1970s, genome editing became more practically promising [4]. Consequently, there has been a rapid progress in developing various methods of genome editing that has shown extraordinary practicality in several fields [4]. Genome editing has been demonstrated to be an effective gene editing tool both in vivo and in vitro, by precisely targeting genes that can successfully alleviate any disease [12, 13]. Some of these techniques thoroughly investigated by researchers are meganucleases, zinc finger nucleases (ZFN), transcriptional activation-like effector nucleases (TALENs), and clustered regularly interspaced short palindromic repeats/associated related nuclease (CRISPR/CAS), all of which has greatly promoted the development of genome editing from proof of concept to successful application in the clinic [14].

2 Muscle Atrophy

Muscle atrophy is a multifactorial syndrome, which occurs in aging and several other debilitating diseases [15]. Muscle atrophy always leads to protein loss, resulting in the reduction of muscle weight, muscle fiber, and muscle strength and eventually leading to muscle dysfunction [16]. Patients with muscle atrophy cannot perform normal activities and have higher incidence rates of other diseases, bringing about a heavy burden to the family and society [17]. Muscle atrophy is the result of various physiological and pathological conditions, such as aging, sedentary lifestyle, physical nerve injury, fasting, chronic heart failure, chronic obstructive pulmonary disease (COPD), kidney failure, and tumor cachexia [18]. In this review, we will focus on the etiology of muscle atrophy induced by physiological and pathological conditions.

2.1 Aging

Due to the persistent metabolic changes that occur during aging, skeletal muscles suffer from a gradual loss of muscle mass and function and eventually lead to muscle atrophy [19]. Epidemiological studies show that starting from the age of 40 years, there is a gradual loss of the skeletal muscle mass at the rate of 1–2% per year [20]. The incidence of senile muscle atrophy was 15% in people over 60 years old and about 30–40% in people around 80 years old [21]. In addition, muscle atrophy caused by aging increases the risk of falls and fractures, which can further endanger the health of older people [7].

The mechanism of muscle atrophy caused by aging is still not fully understood. No effective drugs are available to prevent either the loss of muscle weight or the progressive deterioration of muscle function during aging. Physical activity is the only way to slow down the muscle loss caused by aging [22]. A variety of factors may induce muscle atrophy during aging, classified as (1) internal factors including mitochondrial dysfunction, oxidative stress, hormone imbalance, and inflammation and (2) external factors such as reduced physical activity and malnutrition [23].

Mitochondrial dysfunction is one of the main reasons of muscle atrophy as seen in aging [24]. Mitochondrial dysfunction caused by oxidative damage of mitochondrial DNA is the core mechanism driving the aging processing as proposed in the mitochondrial free radical aging theory [25]. The lack of introns in the mitochondrial genome leads to its low repair capacity, which makes it possible for each mutation to affect the integrity of the gene, thus affecting the function of its protein and subsequently leading to muscle atrophy [24].

Oxidative stress, which occurs in pathological conditions, is usually featured by increased reactive oxygen species (ROS) production. This is considered to be the main cause of excessive protein degradation in muscle atrophy [26, 27]. Skeletal muscle balances the antioxidant mechanism by continuously producing oxidative species such as ROS and reactive nitrogen species (RNS) [27]. Sustained production of ROS could lead to oxidative damage and tissue damage. ROS also inhibits the action of insulin [26]. Additionally, increasing evidences show that oxidative stress can accelerate muscle protein breakdown in multiple ways described as follows. Firstly, it activates both the autophagy pathway and the ubiquitin-proteasome system, which individually promote protein breakdown. Secondly, it activates calpain and caspase-3. Thirdly, ROS produced by the myofibroblast during its oxidative modification accelerates the hydrolysis of protein, thereby enhancing the sensitivity of muscle fibers to proteolysis processing [26].

Hormones have a tremendous effect on the growth, differentiation, and metabolism of the skeletal muscle. They are further required to regulate the normal metabolic activities of the skeletal muscle [28]. In fact, there have been reports suggesting that the imbalance of hormone secretion in aging skeletal muscles can affect the activity of insulin which subsequently leads to muscle loss [29].

Inflammation is another important mechanism contributing toward muscle atrophy caused by aging and an important pathogenic factor bringing about skeletal muscle dysfunction. It has been observed to disrupt muscle homeostasis by activating the FOXO transcription factor family, thus worsening muscle atrophy [30]. Systemic inflammation also causes muscle atrophy. Studies have shown that the inflammatory cytokine interleukin 1β (IL-1β) triggers a cascade of catabolic processes in muscles that accelerate and induce muscle atrophy [31].

2.2 Nerve Injury

Muscle atrophy is a common symptom of peripheral nerve injury. Neuronal disorders lead to muscle atrophy and muscle fibrosis [32]. Muscle atrophy caused by nerve injury is usually devastating and incurable [33]. Denervation-induced muscle atrophy is mainly seen in patients who have undergone trauma, childbirth, and improper exercise caused by nerve injury, slowing initiating sensory and functional impairment of the innervated area accompanied by progressive muscle atrophy [34]. With the rapid advancements in the field of surgical technology, the treatment for neuronal muscular atrophy has made great progress. While nerve repair and autologous nerve transplantation is the standard operation for the treatment of peripheral nerve injury, few patients can still undergo complete recovery of their sensory and motor function after the operation. Therefore, each patient shows different degrees of muscle atrophy with clinical efficacy not being ideal in many cases [35].

Muscle atrophy caused by nerve injury can degrade, disarrange, and shrink the motor endplate [9]. The maintenance of structure and function of the motor endplate depend on normal nerve innervation and electrical activity [36]. As the axon regeneration process takes a

considerable amount of time, there will be scar adhesion and fibrosis after the nerve injury [35]. Limb immobilization gradually degenerates the motor endplate, and it becomes very difficult to reconstruct the nerve-muscle joint [37]. Due to the degeneration of the motor endplate, there is a reduction in the connection between nerves and muscles, and consequently muscle cells lose neuronal nutrition leading to their atrophy [38].

Depletion of skeletal muscle satellite cells is also a key factor involved in muscle atrophy. Skeletal muscle satellite cells (MSCs) have the ability of self-replication and differentiate into mature muscle cells, which determines the normal development, regeneration, and repair function of the skeletal muscle [39]. Therefore, changes in the number of MSC have an impact on the structure, morphology, and function of skeletal muscles [40]. Some studies have found that the number of MSC increases significantly in the first two months of denervation, which may be related to skeletal muscle compensation [41]. Therefore, some of the reasons for muscular atrophy involving MSCs are the death of MSC without regenerative replacement after long-term denervation, and the rate of MSC differentiation is greater than the rate of proliferation because the maintenance of MSC proliferation depends on the muscle activity under innervation [42]. Besides, the muscle tube structures differentiated under denervation do not develop into mature muscle fibers except for nerve reinnervation [43]. This repeated formation of non-innervated muscle tubes also causes MSC depletion, and the reduced number of MSC will eventually lead to skeletal muscle atrophy and regeneration disorders [44].

The process of nerve injury in skeletal muscles can lead to changes in protein metabolism and enzyme activity [45]. After denervation, muscle contraction disorders occur, including reduction of volume, decrease of muscle fiber cross-sectional area and wet muscle weight, increase of myoglobin decomposition, and enhancement of K^+- Na^+-ATPase muscle calcium activity. In addition, due to previously mentioned changes, there is an increase in the fibrillation potential that could accelerate the speed of muscle contraction and promote muscle cell necrosis and even fibrosis [46]. The low gene expressions of key enzymes involved in sugar, fat, and protein metabolism in the skeletal muscle after denervation suggest that the energy metabolism of muscle cells is compromised after denervation [47]. Among them, the low expression of phosphofructokinase Iα, branched amino acid transferase, long-chain fatty acid coenzyme A synthase, and ATP synthase has been observed and reported in skeletal muscle atrophy [46]. These under-expressed key enzymes severely limit the energy supply of skeletal muscles and accelerate muscle atrophy.

Apoptotic protein synthesis and myogenic factor secretion also contribute to muscle atrophy caused by nerve injury [48]. After denervation, an increasing number of innervated muscle nuclei undergo apoptosis, and eventually these muscle cells can no longer maintain their number, shape, and function. Progressively, the number of muscle apoptosis continues to increase, and muscle atrophy occurs [49]. Fas is the main component of the apoptotic pathway observed in the denervation of muscle cells [50]. Often, there is a change in the expression of certain apoptotic genes in the atrophic skeletal muscle, such as Bcl-2 protein, which can inhibit apoptosis and is under-expressed in muscle atrophy [51]. Caspase-3 is a major member of the Fas apoptotic pathway that is quite downstream, and activation of Caspase-3 triggers the apoptotic process [52].

It has been observed that skeletal muscle development and repair defects occur in mice with MyoD and Myf-5 gene knockout [53]. The expression of Myogenin can activate the synthesis of skeletal muscle contractile proteins that are key factors required for skeletal muscle reinnervation after denervation [54]. The level of Myogenin expression determines the ability to undergo nerve reinnervation. Therefore, the role of Myogenin in denervation of the skeletal muscle should be further investigated for its specific role in this process.

The underlying mechanism of muscle atrophy induced by denervation is very complicated. Therefore, investigating key targets which can delay the occurrence of muscle atrophy is important. The need of the hour is to find the upstream

players that trigger muscle atrophy, so as to explore interventional therapeutic options for muscle atrophy induced by denervation.

2.3 Immobilization

Skeletal muscle atrophy occurs when in disuse. The skeletal muscle can quickly adapt to the changes of the external environment, showing corresponding hypertrophy under the action of strong mechanical force and muscle atrophy under the disused state [55]. While the common causes of skeletal muscle atrophy are immobilization and weightlessness, they are important methods in the treatment of the bone and arthropathy [56]. The treatment of many diseases also requires the patient to stay in bed for a long time, and the resulting skeletal muscle disuse-induced atrophy will seriously affect the treatment of the disease and the patient's functional recovery [57]. With respect to skeletal muscle disuse atrophy, many researchers have been discussing the possible ways to prevent and relieve muscle atrophy quickly and restore motor ability.

During the state of disuse, the number of type I slow muscle fiber decreases, while the number of type II fast muscle fiber and mixed fibers increase; however, the number of muscle fiber remained unchanged, while only the number of types I to type II changes [56]. Moreover, there is also nuclear degeneration and nuclear membrane invagination along with lots of lysosomes produced and surrounded by dense matter [55]. The long-term disuse more often increases muscle glycogen consumption and reduces the oxidative capacity of long-chain fatty acids, consistent with decrease in acetyl-CoA dehydrogenase activity in muscles (a key enzyme for fatty acid metabolism) after bed rest [58].

Calpain is also associated with disuse muscle atrophy. Since ubiquitin-proteasome pathway (UPP) cannot degrade intact myofibrils during proteolysis, it first activates calpain to degrade membrane-skeleton proteins that causes myofibrils to fall off the cytoskeleton, thus completing protein degradation [59]. Thus, calpain can be considered as the promoter of skeletal muscle protein degradation in a disused state [60]. When skeletal muscle atrophy occurs, the intracellular calcium levels increase significantly, which in turn promotes the transfer and activation of calpain to the cell membrane, releasing the active catalytic subunit, initiating the degradation of cytoskeletal proteins [61].

Oxidative stress is enhanced under disuse and many pathological conditions and is considered to be the main trigger for the imbalance of protein homeostasis in muscle atrophy [62]. At the beginning of disuse (such as paralysis and fracture), due to the effect of oxidative stress, reactive oxygen species and free radicals produced in the body can greatly oxidize the unsaturated fatty acids in the membrane and form lipid peroxide, thus destroying the normal function of the membrane system, resulting in mitochondrial swelling and enhanced lysosomal membrane permeability [63]. Mitochondrial dysfunction leads to oxidative phosphorylation disorders and insufficient energy production, resulting in reduced protein synthesis [64]. The destruction of the lysosome membrane releases various hydrolases and intensifies protein decomposition. These processes work together to reduce the net content of muscle protein, resulting in muscle atrophy [65].

2.4 Fasting

In the absence of adequate nutrient supply, skeletal muscles undergo degeneration to maintain the normal bodily functions. Thus, muscle wasting has always been observed in fasting [66]. Unlike other types of muscle atrophy, degraded proteins are the source of essential amino acids required for gluconeogenesis during fasting [19]. In addition, the changes in insulin growth factor and glucocorticoid levels caused by fasting also affect the normal activity of the skeletal muscle, which cause disorganization in skeletal muscle metabolism and protein degradation [67].

2.5 Chronic Heart Failure

Heart failure imposes a severe social burden. Muscle atrophy occurred in 30–50% of the patients mainly caused by the elevation of Ang II [68]. Muscle atrophy caused by heart failure seriously affects patient prognosis [69].

The mechanism of impaired skeletal muscle function in heart failure involves several pathophysiological aspects such as energy metabolism, neuroendocrine, and gene expression abnormalities [70]. The primary causes for this condition can be summarized as follows. The energy supply mode has undergone a change, wherein glycolysis is the main energy supply mode of skeletal muscles in heart failure, leading to the reduction of type I fibers [71]. Disturbance of neurohumoral factors, leading to enhancement of sympathetic nerve excitability, and activation of renin-angiotensin system not only cause heart failure but also contribute toward impaired skeletal muscle function, cell apoptosis, and abnormal myosin expression, all of which directly affect skeletal muscle function [72]. Calcium overload and change of calcium pump activity also cause attenuation of skeletal muscle excitability, affecting local tissue sodium-potassium pump activity, and consequently trigger skeletal muscle oxidative phosphorylation dysfunction [73]. These factors interact with each other to form a complex regulatory system, and the gradual accumulation of abnormal components brings about vascular endothelial dysfunction, blood perfusion insufficiency, cell death, and muscle atrophy in the skeletal muscle, ultimately leading to the aggravation of heart failure, and further causes poorer patient prognosis [74].

The renin-angiotensin system (RAS) is an essential regulatory system in the regulation of skeletal muscle function. Angiotensin (Ang II) is the major effector of RAS, which maintains the balance of sodium ions and water in the body by regulating the central nervous system, adrenal, vascular system, and kidney [75]. Additionally, Ang II plays an important role in muscle function regulation in certain diseases, such as Duchenne muscular dystrophy (DMD) [76]. The effects of Ang II on skeletal muscle injury are mainly reflected by the changes in enzyme activity and cellular oxidative activity, ultimately causing muscle weight loss [77]. Furthermore, Ang II could also indirectly trigger muscle protein loss via inflammatory mechanisms. This is because Ang II mediates the catabolism of inflammatory response in damaged muscles by suppressing insulin /IGF-1 signaling pathway [78]. This triggers a series of changes eventually leading to muscle atrophy. Many similar pathological conditions can lead to muscle atrophy, such as chronic kidney disease and chronic diabetes. These pathological conditions causing muscle atrophy can stimulate the RAS system and promote the increase of Ang II circulation in the body.

2.6 Cachexia

Cachexia is characterized by progressive muscle atrophy that cannot be rescued by additional nutritional support. Cachexia occurs in 50% of malignancies and accounts for 20–40% of deaths in patients with malignancies [79]. The mechanism and consequences of skeletal muscle atrophy induced by cachexia are extremely different from those induced by stress and fasting.

Cachexia is a complex metabolic state with complications such as weight loss and muscle atrophy caused by loss of muscle strength [80]. The decrease of protein content is caused by insufficient nutrient transport and abnormal protein apoptosis or autophagy [81]. Protein degradation pathways include ubiquitin-proteasome pathway and autophagy-lysosome pathway.

The pathophysiology of cachexia is characterized by severe deterioration of body functions and organs due to inadequate food intake and metabolic abnormalities, including energy expenditure, excessive catabolism, and inflammation [82]. A large number of literature show that multiple organs are usually involved in the development of cachexia-induced skeletal muscle loss in patients with advanced stages of cancer, including the central nervous system

regulation, white adipose tissue loss, pancreatic dysfunction, and hepatic metabolism imbalance [83].

3 Protein Synthesis and Degradation in Muscle Atrophy

Muscle atrophy is mainly caused by the imbalance between protein synthesis and degradation. The two main protein degradation systems that play a crucial role in muscle atrophy are ubiquitin-proteasome system and autophagy-lysosome system.

3.1 The Ubiquitin-Proteasome System

Ubiquitin-proteasome system (UPS) is activated under several conditions. The classic degradation system requires many enzymes: activating enzymes (E1), conjugating enzymes (E2), and ligating enzymes (E3) [84]. Briefly, ubiquitin links to E1 ubiquitin activator enzyme through a covalent bond and is then transported to E2 ubiquitin binding enzyme. With the help of E3 ubiquitin ligase, the lysine residues are transported to lysosome, and finally the protein labeled with ubiquitin is degraded. As a protein degradation system, the main role of the ubiquitin-proteasome pathway is to degrade intracellular ubiquitinylated proteins [85]. Atrogin-1 and MuRF-1 are considered to be the most important E3 ubiquitin ligases in muscle atrophy, which are observed to be increased in many muscle atrophy models [86].

Some other E3 ligases are also involved in the muscle degradation pathway. TRIM32 has been reported to be another important E3 ligase that degrades thin muscle filaments (such as actin, tropomyosin, and troponins) [87]. TRAF6 is also a member of the E3 ligase family that plays an effective role in mediating the degradation of target proteins in muscle atrophy [87].

ChIP is a ligase of specific ubiquitination of filament C and mediates ubiquitination by causing lysosomal degradation of filament C. Filament protein is an important protein that undergoes folding and unfolding cycles when contact with muscle, and its loss can cause serious muscle injury. Changes in the structure of silk fibroin will cause the chaperone BAG3 composed of HSC70 and HSPB8 to bind to it. Chaperone BAG3 also includes the ubiquitin ligase ChIP [88].

USP14 and USP19 have also been related to muscle atrophy. In addition, many studies have found that E3 ligase is involved in the ubiquitination and decomposition of several muscle proteins, but the exact process and mechanism are still unclear.

3.2 The Autophagy-Lysosome System

The autophagy-lysosome system is another process to carry out protein degradation. This process includes the formation of autophagosome with a bilayer membrane, fusion of the autophagosome and lysosome to form autophagolysosome, breakdown of intima structure after protein hydrolysis in autophagosome, and the contents into material circulation.

Autophagy is also called a nonselective degradation pathway. Early evidence suggests that autophagy not only plays an essential role in maintaining homeostasis in different conditions but also in the transformation of cellular components [89]. Three types of autophagy have been reported in mammals: macro-autophagy, chaperon-mediated autophagy, and micro-autophagy. Currently, most studies on the role of autophagy in muscles are associated with macro-autophagy [90]. Although the micro-autophagy in the skeletal muscle still undetermined, some studies have suggested that when macro-autophagy is blocked, micro-autophagy may be involved in lysosomal glycogen uptake [91].

Current studies have found that the expression of LC3 protein is increased during autophagy while the expression of p62 protein is suppressed. Autophagy is involved in many muscle atrophy

processes [92]. Autophagy participates in the process of muscle fiber atrophy induced by FoxO3 overexpression. Knockdown of the protein LC3 can partially prevent FoxO3-mediated muscle atrophy and superoxide dismutase-induced muscle atrophy [93, 94].

Mitophagy is another type of autophagy that occurs during muscle atrophy. In mammals, Parkin, PINK1, and BNIP3 have all been shown to modulate mitophagy and lead to mitochondrial dysfunction [95]. PINK1 recruits Parkin to the mitochondria, where Parkin recognizes mitochondrial outer membrane proteins via p62 and ubiquitinates outer membrane proteins to enable autophagic vesicles to enter the ubiquitinated mitochondria [96]. It has been reported that NIP3 and BNIP3L can bind directly to LC3 and continuously recruit autophagosomes into mitochondria.

4 Molecular Pathways Underlying Muscle Atrophy

4.1 IGF1-Akt-FoxO Pathway

Insulin-like growth factor 1 (IGF1) is a circulating growth factor secreted by many tissues, including skeletal muscles. IGF1-PI3K-Akt is an important signaling pathway that regulates both protein synthesis and degradation during muscle growth [97]. The IGF1-PI3K-Akt pathway is inhibited by metabolic signals during muscle atrophy. Muscle atrophy leads to a decrease in the phosphorylation levels of GSK3β, FOXO, and mTOR downstream of Akt, thus promoting the degradation of proteins and inhibiting the synthesis of proteins [98]. Also, the inhibition of IGF1-PI3K-Akt-mTOR and IGF1-PI3K-Akt-FOXO pathways can activate the autophagy-lysosome system [89].

Akt pathway promotes muscle growth, and this is further demonstrated in Akt transgenic mice wherein Akt promotes muscle hypertrophy and protects against muscle atrophy induced by denervation [99]. Akt can control protein synthesis through mTOR and FOXO transcription factor. mTOR, a rapamycin complex, is a key kinase downstream of insulin and is associated with cell growth. Akt inhibits Atrogin-1, MuRF-1, and other autophagy-related genes by negatively regulating FOXO transcription factor [100]. Akt phosphorylates FOXO protein, facilitating its transportation from the nucleus to the cytoplasm. The muscle mass of FOXO1 transgenic mice is significantly decreased and fiber atrophy increased, which further prove that FOXO protein can promote muscle atrophy [101].

4.2 NF-κB Pathway

High production of inflammatory cytokines, such as IL-6 and TNF-α, is an important pathological feature of muscle atrophy. TNF-α and other inflammatory factors can activate NF-κB, which leads to the increase of MuRF-1 and promotes muscle atrophy [102]. The NF-κB is a transcriptional regulator and plays an important role in the regulation of immune and inflammatory cytokines. It is also expressed in the skeletal muscle and can regulate the role of inflammatory cytokines in muscle atrophy and dysplasia, especially TNF-α. In inactivating state, NF-κB is inhibited by IκB [103]. In response to TNF-α, IKK complex triggers IκB phosphorylation, which causes ubiquitination and proteasome degradation, and activates NF-κB-regulated gene transcription, leading to muscle atrophy. In transgenic mice, muscle-specific overexpression of IKKβ resulted in severe muscle atrophy by promoting MuRF-1, not Atrogin-1 [102]. Moreover, IL-6 can activate the JAK/STAT3 pathway, thereby inhibiting the IGFR1 pathway, inhibiting muscle growth, and promoting muscle atrophy [104].

4.3 Myostatin Pathway

Myostatin is a protein that inhibits muscle growth. Myostatin is a member of the TGF-β superfamily and has been reported to be involved in muscle growth and atrophy. While on one hand, myostatin promotes muscle atrophy through the myostatin-Actriib-ALK4/ALK5-

Smad2/3 pathway, BMP pathway, and myostatin-Smad-Akt pathway[105], on the other hand, myostatin can inhibit the activation of satellite cells and the proliferation and differentiation of myoblasts through a variety of ways, thus inhibiting muscle growth and promoting muscle atrophy [106].

4.4 β2-Adrenoceptor Pathway

The β2-adrenoceptor is inhibited in the metabolic environment of muscle atrophy. Inhibition of β2-adrenoceptor can further inhibit the PI3K/Akt signaling pathway and promote the activation of the FOXO family, thereby promoting the expression of MuRF-1 and atrogin1 [107]. It can also increase the activity of 26S proteasome and promote the occurrence of muscle atrophy [108]. Like myostatin pathway, the β2-adrenoceptor pathway can regulate muscle atrophy in two different ways.

5 Genome Editing in Muscle Atrophy

5.1 Genome Editing

Correcting aberrant molecules is considered to be an effective therapeutic approach for diseases. Consequently, genome editing emerged as a practical methodology for such an approach. Genome editing, which involves precise manipulation of target genes in the genome of organisms, has shown to have some potential in curing certain diseases. By inserting foreign genetic material, correcting mutations, and replacing the aberrant gene, genome editing can cure the disease by acting at a molecular level [14]. Engineered nucleases are some of the leading tools for genome editing, also known as "molecular scissors," which can produce site-specific double-stranded break (DSB) at a specific location in the genome [109]. With the help of a series of engineered nucleases, it has been possible to perform very effective targeted genome editing and thereby open to newer possibilities of genetic research, so that progress can be made in gene therapy and overall genetic improvement [110].

Genome editing includes the usage of homologous recombination of DNA and nucleases. Homologous recombination is one of the earliest techniques used to edit cell genomes [111]. Homologous recombination is the process of synthesizing DNA fragments using a homologous arm of the genome to be edited. The appropriate mutation rectifying fragments can be injected into the recipient cells, and then these recombine with DNA to replace the part of the genome containing the target [112]. The disadvantage of this method is its low efficiency and high error rate [113]. The key process of nuclease gene editing technology is to create double-stranded breaks (DSB) at the specific genomic locus. Restriction endonucleases can recognize and cleave DNA efficiently at multiple sites; however, the low specificity of classic restriction endonucleases limits the widespread use in genomic editing [114]. In order to overcome this problem, four different types of editing system emerged which are meganuclease, ZFNs, TALEN, and CRISPR/Cas9 system [115]. These four different types of gene editing systems could be applied for the treatment of muscle atrophy (Fig. 1).

5.1.1 Meganuclease

Meganuclease is a deoxyribonuclease characterized by its large recognition site, which can recognize 12–40 base pairs of double-stranded DNA sequences. Meganuclease is the most specific naturally occurring nuclease [116].

5.1.2 Zinc Finger Nuclease (ZFN)

Zinc finger nuclease is an artificially modified nuclease, which is produced by fusing the zinc finger DNA binding domain and DNA cutting domain of the nuclease. By designing zinc finger domains, specific DNA sequences of target genes can be recognized, which enables this nuclease to locate unique targeting sequences within complex genomes. By utilizing endogenous DNA repair mechanisms, zinc finger nuclease can be used to precisely modify the genomes [117].

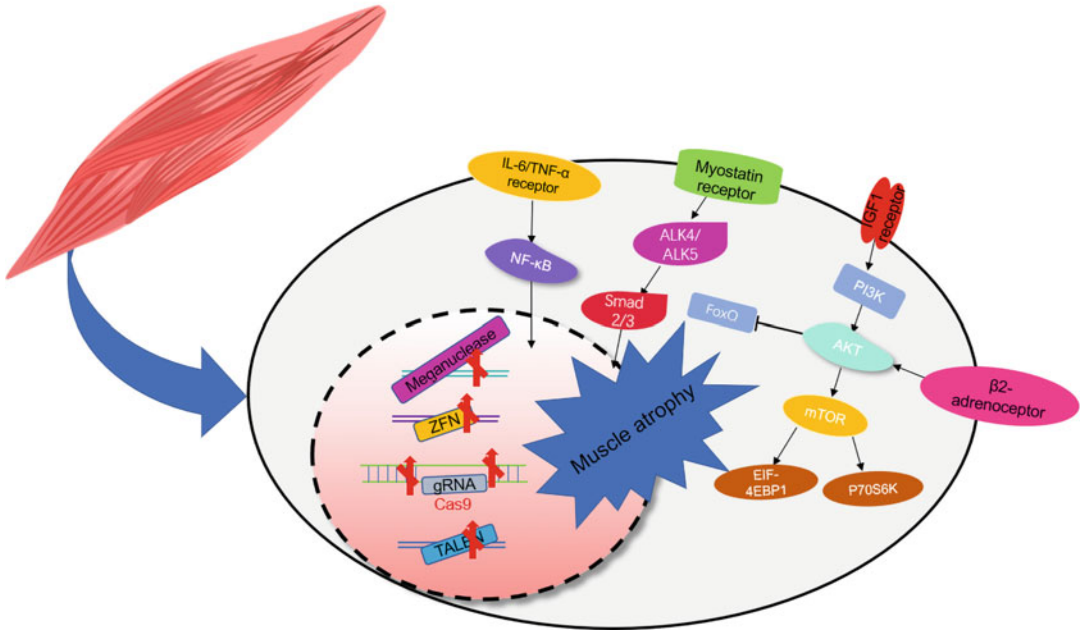

Fig. 1 Genome editing technology for muscle atrophy treatment

5.1.3 Transcriptional Activation-Like Effector Nuclease (TALEN)

TALEN is a restricted enzyme that has been genetically engineered to cut specific DNA sequences. TALEN is obtained by fusing a TAL effector DNA binding domain with a DNA cleavage domain of the nuclease. As TALEN can cut DNA at a specific position when combined with nuclease, it can be designed to bind almost any desired DNA sequence [118].

5.1.4 CRISPR/Cas9 System

CRISPR/Cas9 system is the most widely used gene editing tool. The CRISPR/Cas9 system was originally thought to be an adaptive immune system in bacteria and was later found to edit DNA in mammalian cells [119]. The CRISPR system consists of Cas9 and a directing single-guide RNA molecule (sgRNA), known as transactive RNA. Cas9 binds to sgRNA and recognizes the target DNA sequence via a programmable 20bp sequence located at the 5 'end of sgRNA [120]. There are a number of Cas9 nuclease options available, depending upon the target gene requirement.

CRISPR/Cas9 system has been used for precise genome modification, including targeted gene knock in, simultaneous mutations at two sites, and deletion of small fragments. Because of its high mutation-inducing efficiency, simple fabrication, and low cost, it has been used as a tool for targeted modification of genomes [121]. It has been successfully used to treat a variety of diseases.

CRISPR-Cas9 can edit muscle dystrophy-related genes through nonhomologous connections and homologous recombination repair pathways. Nonhomologous non-ended connection is highly efficient and can be used for splicing within any gene position. Homologous recombination repair is less efficient, but it can achieve accurate gene repair [122]. The CRISPR-Cas9 technology is now widely used in the study of muscle dystrophy-related diseases, which have been successfully studied in the laboratory. One of them has been approved by the FDA (Food and Drug Administration) and is ready for its application in the clinic.

The CRISPR-/Cas9-mediated in vitro editing has shown positive effect on the hematopoietic system [123]. In theory, genome editing could be

useful in the skeletal muscle system in vitro because satellite cells as skeletal muscle adult stem cells can be manipulated. Though the application of this approach can improve local muscle function, the recovery of whole-body muscle function still needs further research [124]. Genome editing in vivo is an effective way to permanently correct the genetic mutations that cause muscle atrophy. However, an efficient delivery system is needed for genome editing to be performed in vivo. In addition to the well-known viral vector delivery systems, physical or chemical delivery methods are also effective methods for delivering, such as delivering the CRISPR/Cas9 genome editing components to target cells using microinjection and electroporation techniques [125].

5.2 Application of Genome Editing in Muscle Atrophy

In recent years, genome editing methods for the treatment of muscle atrophy have gradually come into practice. One of the gene therapy methods, exon hopping, has shown broad applications [126]. In the case of Duchenne muscular dystrophy (DMD), transcoding mutations in DMD can be modified to non-transcoding mutations in DMD by using exons near the artificial RNA antisense oligonucleotide jump deletion gene [127]. The phase II clinical trial of exon 51 jumps, an international multicenter collaboration, has been nearly completed, demonstrating the expression of anti-muscle dystrophy protein in the skeletal muscle of DMD patients and even improved 6-min walking distance without serious adverse effects [128]. Clinical trials of exon 44, 45, and 53 jumps are also underway. Antisense oligonucleotides (RNA drugs) do not alter the DNA of a person's genes in contrast to the way other genome editing methods alter the DNA. This is important especially to avoid the risk of the treatment being passed on to the next generation or spread among the population [129]. And since it belongs to the category of small molecule drugs, immune rejection is minimum. However, as it works on pre-mRNA rather than DNA-level editing, it needs to be administered for life and can target only at specific gene types [128].

CRISPR-Cas9 genome editing technology edits genes through nonhomologous unconnected and homologous recombination repair pathways. While nonhomologous terminal splicing can be used for splicing at any gene location, homologous recombination repair though less efficient can complete accurate gene repair [130]. For example, nonhomologous unconnected exons can be used for exon hopping therapy in DMD, with excision of one or more exons at will, and repair of dynamic mutations in intron or 3′UTR terminal and can be used to treat amyotrophic lateral sclerosis or frontotemporal dementia caused by C9orf72, as well as tension muscle atrophy type I [131]. Homologous recombination repair can theoretically treat any genetically inherited disease at the DNA level. For example, modification of the T base to C in the initiation region of exon 7 in survival motor neuron 2 (SMN2) gene can express relatively sufficient SMN protein to treat spinal muscular atrophy (SMA), and this has been approved by FDA for clinical application [130].

Systemic delivery of AAV has been effectively utilized in the treatment of SMA [132]. In 2017, 15 infants aged 1–8 months received intravenous AAV9 for the treatment of this disease with good clinical results. SMA is an infant motor dysfunction caused by deletion or mutation of the SMN1gene. Of the four subtypes of the disease, type 1 SMA is one of the most common genetic causes of infant death. In March 2019, Zolgensma—a gene therapy based on this study—was approved by the FDA as a result of the infants' survival at 20 months along with significant clinical benefits.

miR-29b is a common regulator that we identified in many types of muscle atrophy [133]. As exercise is an effective treatment for muscle atrophy [134], we further found that exercise training can attenuate muscle atrophy induced by angiotensin II through downregulation of miR-29b [135]. Recently, we reported a CRISPR-/Cas9-mediated miR-29b editing by targets of the biogenesis processing

sites in pre-miR-29b. In mice, this CRISPR-/Cas9-mediated miR-29b editing can effectively prevent Ang II, immobilization, and denervation-induced muscle atrophy. However, large animal studies are required to further pave its application in the clinic [136].

6 Developing Approaches for Muscle Atrophy

Gene therapy is mainly composed of three main components—gene of interest, vector to carry the gene-modifying component, and target cells. There are two types of target cells: somatic cells and germ cells [126]. The genetic modification of germ cells such as sperm, egg, and zygote as target cells is restricted due to ethical concerns, so it is not possible to progress in this regard. Somatic cells are easy to obtain and have a variety of sources. Even certain somatic cells that are not easy to collect, such as nerve cells, retinal cells, liver cells, and muscle cells, can be used in in vivo targeted gene therapy [137]. Therefore, somatic gene therapy was able to develop rapidly in recent years.

The key to the success of gene therapy is how to safely and effectively introduce foreign genes into cells in vitro or tissues in vivo, which is also a major bottleneck faced currently in this field [138]. Therefore, the selection of appropriate delivery tools is essential and an important deciding factor for the safe and effective implementation of gene therapy. Vectors that are currently widely used are divided into two broad categories—viral vectors and nonviral vectors.

Viruses are the smallest and simplest living parasites with no cellular structure. Because of their very high infectability in human cells and their simple molecular mechanism for delivering the genetic material successfully into cells, viral vectors are more preferred to be efficient delivery vectors than the nonviral ones. Due to this, about 70% of gene therapy regimens use viral vectors for gene delivery [139]. However, as most viruses are pathogenic, their genomes must be engineered such that they retain only their functional components required for DNA integration while eliminating the unnecessary pathogenic components from the original viral genome [140]. At present, the most common viral vectors are retroviruses, lentiviruses, adenoviruses, and adeno-associated viruses.

Retrovirus is a type of virus with plus-stranded RNA as its genetic material and synthesizes the complementary DNA strand by reverse transcription in the infected cells. The complementary DNA is randomly integrated into the host cell genome and can be expressed stably for a long time [141]. While the retroviral infection efficiency is high, its toxicity is low, and since the infected cells do not produce any pathological changes, long-term expression of the target gene stable cell line can be established. But at the same time, as the retrovirus can only infect divided cells, the viral integration may accidentally activate oncogenes or insert mutations, posing a certain risk of cancer [142]. Lentiviruses, another type of retroviruses, have larger volumes and can carry larger and more complex genome sequences. While lentiviral vectors also have the ability to integrate transgenes into the host genome for stable long-term expression of products, they pose a similar risk as adenoviral vectors due to the semi-random integration of the genetic material that could potentially lead to the introduction of hazardous mutations [143]. Adenoviruses are double-stranded non-enveloped nonintegrated DNA viruses, suitable to infect almost all cell lines and primary cells, and can also mediate gene delivery in various tissues, such as the liver, lung, brain, blood vessel, and nervous system [144]. However, it takes multiple infections to achieve repair, and repeated treatment can lead to an increased immune response, which not only affects gene expression but also the effectiveness of gene therapy. Adeno-associated virus (ADV) is one of the most widely used viral vectors [145]. ADV causes no pathogenicity to the host and has long-term stable expression in a variety of tissues and cells. Recombinant adeno-associated virus (rAAV) vectors used in gene therapy, after their introduction into the host with recombinant genes, can cause a stable expression of functional proteins in the host cells [146]. This genetic

vector has several advantages such as high biosafety level, wide host range, long expression time, and low immunogenicity making AAV a more sought-after vector for its application in gene therapy.

Many viral vectors have been used for genomic therapeutic delivery, such as lentiviruses, retroviruses, poxviruses, adenoviruses, adeno-associated viruses, and baculoviruses [147]. Among them, adeno-associated virus AAV can successfully deliver genome editing components directly to the muscle tissue. AAV is a non-enveloped DNA virus with two major ORFs that have been successfully used in genome editing delivery systems [148]. Because AAV is not pathogenic either in humans or animals, it is considered a safe delivery system for genome editing [149]. In addition, AAV-mediated CRISPR/Cas9 has been reported to have a favorable therapeutic effect in in vivo genome editing [150]. Therefore, based on these studies, we can conclude that the combination of the delivery system of AAV with CRISPR-/Cas9-mediated genome editing is an effective method for neuromuscular diseases with single-gene mutation treatment.

In addition, adeno-associated virus (AAV)-mediated anti-dystrophy gene therapy focuses on delivery of anti-dystrophy genes into the host through AAV vectors, so that anti-dystrophy proteins can be produced in the host cells due to constitutive expression of the introduced genes. Even just simply increasing the expression of this protein or promoting the transcription of the gene that encodes it may delay the progression of muscle atrophy [151].

The other broad category of gene delivery mechanisms are nonviral vectors. Nonviral vectors can carry DNA across the cell membrane, protecting DNA from degradation by DNA-degrading enzymes before their entry into the cells, lysosomal degradation after their entry into the cells, prevention of their removal from the cell by biodegradation, and non-cytotoxicity [152]. The nanoscale particle size of the nonviral vector contributes toward proper targeting and effectiveness of the vector. At present, cationic polymer carrier, liposome carrier, and nanoparticle carrier are widely used as nonviral vectors.

In conclusion, genome editing is promising in the treatment of muscle atrophy.

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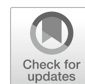
Part IV

Genome Editing in Metabolic Diseases



Genome Editing and Obesity

Davide Masi, Rossella Tozzi, and Mikiko Watanabe

Abstract

Defined as a condition of body fat excess leading to significant morbidity, obesity is a chronic metabolic illness associated with a significant number of diseases. The incidence of obesity does not solely depend on dietary habits, with energy balance being regulated by the complex interactions between genetic, behavioural and environmental factors. Genome-wide association studies (GWASs) have demonstrated that several genes are linked to obesity, and these findings shed light on a growing number of novel potential therapeutics for weight management, including genome editing. In this regard, the cutting-edge technology known as clustered regularly interspaced palindromic repeats (CRISPR)/CRISPR-associated protein (Cas), thanks to its ability to edit DNA or modulate gene expression in eukaryotic cells, undoubtedly enables to understand the genetic mechanisms implicated in obesity and could be a promising tool for its treatment. This chapter summarizes the genetics underlying obesity and currently available obesity treatments, further discussing the research progress of genome editing in the knowledge and treatment of body fat excess.

Keywords

Obesity · Fat · Waist circumference · Nutrition · Genome · GWAS · CRISPR

1 Introduction

The World Health Organization has defined obesity as "the twenty-first century epidemic", since its prevalence has been rising steadily for the past decades all over the world, leading to high morbidity and mortality [1]. Defined as a condition of body fat excess leading to significant morbidity [2], obesity is a chronic metabolic illness associated with a significant number of diseases, including type 2 diabetes mellitus (DM2) [3], cardiovascular diseases [4], obstructive sleep apnoea syndrome (OSAS) [5] and non-alcoholic fatty liver disease (NAFLD) [6]. Many strategies have been proposed for the treatment of weight excess, ranging from dietary patterns and physical activity to pharmacological treatments and bariatric surgery in the most severe cases. The incidence of obesity does not solely depend on dietary habits; in fact, energy balance is regulated

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by the complex interactions between genetic, behavioural and environmental factors [7].

Noteworthy, genome-wide association studies (GWASs) have demonstrated that several genes are linked to obesity, and these findings shed light on a growing number of novel potential therapeutics for weight management, including genome editing. In this regard, the molecular technology known as clustered regularly interspaced palindromic repeats (CRISPR)/CRISPR-associated protein (Cas), thanks to its ability to edit DNA or modulate gene expression in eukaryotic cells, undoubtedly represents a valid technology to understand the genetic mechanisms implicated in obesity and could be a promising tool for its treatment [8].

This chapter summarizes mono and polygenic forms of obesity and currently available obesity treatments, further discussing the research progress of genome editing in the treatment of non-communicable diseases in nutrition, such as obesity.

2 The Genetics Underlying Obesity

Obesity is a multifactorial pathology, and heritable traits and genetics have an indisputable impact on its epidemiology. As a matter of fact, genetic factors have been reported to contribute up to 25% in the determination even of the most common expressions of obesity.

In this regard, only a small number of patients present monogenic forms of obesity (5%), resulting from mutations of specific genes. On the other hand, polygenic forms of obesity, also defined as common obesity, represent a heterogeneous group of disorders that accurately depict the complex interplay between genetic and environmental factors. Noteworthy, several genetic diseases, including Bardet-Biedl's syndrome, Cohen's syndrome, Alstrom's syndrome and Prader-Willi's syndrome, share obesity among their clinical features, though they will not be discussed in this chapter.

2.1 Monogenic Obesity

Monogenic forms of obesity are generally more severe, with a typical juvenile onset, and show a Mendelian-type inheritance, which can be autosomal or recessive [9]. In the late 1990s, molecular studies conducted in agouti *ob/ob* and *db/db* mouse models, presenting a homozygous mutation in the leptin (LEP) gene and its receptor (LEPR), respectively, allowed to achieve new insights into the regulation of energy balance. Upon food intake, the increase in adipocyte-produced leptin circulating levels stimulates proopiomelanocortin (POMC) production in the arcuate nucleus of the hypothalamus. POMC is subsequently processed by proprotein convertase subtilisin/kexin type 1 (PCSK1) into melanocortin peptides such as α- and β-melanocyte-stimulating hormone (α-MSH and β-MSH), which in turn can bind and consequently inactivate melanocortin 4 receptor (MC4R), thus reducing food intake [10].

Monogenic forms of obesity are due to single mutations which generally involve genes coding for appetite regulation molecules, including LEP, *LEP*R, *POMC*, *MC4R*, *PCSK1* and sarcoma homology 2 B adaptor protein 1 (*SH2B1*) (Table 1). The most relevant ones are herein presented:

- LEP/LEPR: Congenital LEP deficiency, resulting from both homozygous frameshift and missense mutation in the *ob* gene, is characterized by severe and early-onset obesity, intense hyperphagia, hyperinsulinemia, advanced bone age and profound abnormalities of T-cell number and function associated with high rates of childhood infection. A similar phenotype can also be observed in children suffering from LEPR deficiency. This condition is caused by a homozygous mutation that truncates the receptor before the transmembrane domain, thus resulting in an aberrant circulating molecule consisting of LEP and LEPR bound together.
- POMC: On the contrary, the *POMC* gene can be affected by a wide range of alterations,

Table 1 Monogenic forms of human obesity

Gene	Type of inheritance	Prevalence	Obesity onset
LEPTIN	Homozygous recessive	Rare	First days of life
LEPR	Homozygous recessive	Moderate	First days of life
POMC	Homozygous or compound heterozygous	Extremely rare	Early childhood
PCSK1	Homozygous or compound heterozygous	Extremely rare	Childhood
SH2B1	homozygote and heterozygote	Extremely rare	
NTRK2	De novo heterozygous mutation	Extremely rare	Early childhood

LEPR leptin receptor, *POMC* proopiomelanocortin, PCSK1 proprotein convertase subtilisin/Kexin type 1, *SH2B1* adaptor protein 1, *NTRK2* neurotrophic receptor tyrosine kinase 2

including homozygous or compound heterozygous missense, cleavage site and complete loss-of-function mutations. The onset is often precocious and not limited to weight excess; patients with POMC deficiency indeed usually present hypoglycaemic crises, hyperbilirubinaemia and cholestasis secondary to isolated corticotrophin (ACTH) deficiency and consequently congenital hypocortisolism in the neonatal period.

- MC4R: Furthermore, the most common monogenic cause of obesity is undoubtedly represented by a heterozygous mutation in the *MC4R* gene, which follows a dominant-type inheritance. Its prevalence has been estimated around 3–6% of the entire population [9, 11], whereas its penetrance is often equal to 100%, depending on environmental- and ethnic-specific factors. Moreover, *MC4R* homozygote patients display a more severe form of obesity than the heterozygotes, who can be in some cases normal or overweight. For this reason, many authors have hypothesized a genetic model characterized by "codominance", with multiple genes modulating the expression and penetrance of the phenotype. Different to what is observed in congenital LEP deficiency, subjects with MC4R mutations show increased lean mass, accelerated linear growth, early hyperinsulinemia and less hyperphagia. The disease also tends to be milder in adults than in paediatric patients. Interestingly, Farooqi et al. found that the severity in the MC4R dysfunction, as observed in in vitro assays, directly correlates with patients' food intake and subsequently with the grade of obese phenotype [9].
- PCSK1: As of today, only heterozygous mutations of PCSK1 have been described. The resulting enzyme deficiency causes an ineffective cleavage of both POMC and glucagon-like peptide 1 (GLP-1), thus leading to severe obesity, high proinsulin levels, hypocortisolaemia and elevated POMC concentrations.
- SH2B1: SH2B1 belongs to a family of scaffold proteins implicated in the downstream signalling of a variety of tyrosine kinase receptors like leptin, insulin and GH and IGF-1 receptors. Its deficiency has been traced to at least seven mutations affecting the C-terminal tails of the four SH2B1 isoforms. Noteworthy, these mutations (both homozygote and heterozygote) have been identified in severe, early-onset obese patients, suggesting a potential pathogenetic role [12].
- NTRK2: Mutations in genes coding for proteins with a predominantly neuronal activity have also been associated with severe childhood obesity and developmental delay. For instance, a deletion or heterozygous missense mutation occurring in neurotrophin receptor TrkB (*NTRK2*) or in its natural ligand, the brain-derived neurotrophic factor (*BDNF*), can be responsible for severe early-onset obesity and impaired food intake regulation, underlining the importance of neuronal plasticity in the hypothalamus for the maintenance of a correct eating behaviour and body weight stability [13].

The great advantage of identifying specific obesity-inducing mutations mainly lies in the fact that it opens up the possibility of highly individualized therapies, capable of reversing the disorder [11]. In this regard, subcutaneous injections of leptin replacement therapy (metreleptin) showed significant beneficial effects in children affected by congenital leptin deficiency, thus resulting in markedly body weight and fat mass reduction [14]. Similarly, encouraging results have also been observed regarding its application in patients suffering from familial lipodystrophies as well [15].

Another promising approach is now represented by setmelanotide, an MC4R agonist. A single-arm open-label multicentre phase 3 trial, conducted on 11 subjects with *LEPR* mutations and 10 with *POMC* mutations, recently evaluated the efficacy of setmelanotide administration, showing a significant reduction in body weight (13–25%), with no side effects other than nausea and hyperpigmentation in the POMC group [10].

Despite these promising novel approaches, it is still difficult to find an effective and completely individualized therapy, as seen in patients carrying heterozygous leptin mutations, who do not show significant reductions in leptin concentrations and therefore do not significantly benefit from the implementation of recombinant leptin. For this reason, it remains mandatory to pursue other treatment strategies.

2.2 Polygenic Obesity

In recent years, a rise in the prevalence of obesity has been reported. Several factors including socioeconomic status, intrauterine environment, sleep deprivation and gastrointestinal microbiome can play a crucial role in the pathogenesis of this disease, along with other well-known determinants such as endocrine disruptors, excessive caloric intake, energy expenditure and sedentary lifestyle. Taken together, these elements suggest that an increasing obesogenic environment may amplify the inherited genetic risk for obesity.

Multiple twin and family studies aimed at establishing the heritability of obesity in order to assess the individual's risk of developing the disease. In this regard, the Framingham Heart Study reported a 40–50% of heritability for increased BMI and similarly the HERITAGE (HEalth, RIsk factors, exercise Training, And GEnetics) Family Study reported a moderate-to-high heritability rate (62–63%) for body fat. As far as waist circumference is concerned, heritability estimates are more heterogeneous, ranging from 37% to 81%, while a smaller rate (6–30%) has been associated with waist-to-hip ratio (WHR) [16]. Such discrepancy could be attributed to the absence of overlapping between the *loci* for WHR and BMI, suggesting that the genetic regulation of fat distribution is independent from that of total adiposity [17].

As previously affirmed, obesity is a complex trait which most commonly does not show a typical Mendelian transmission pattern, since it depends on several susceptibility genes with low or moderate effects, including genes that influence energy homeostasis and thermogenesis, adipogenesis, leptin-insulin transduction and hormonal signalling peptides.

Since the advent of genome-wide association studies (GWASs) in the early 2000s, several single nucleotide polymorphisms (SNPs) have been associated with obesity, allowing for a giant leap towards a better understanding of the genetics underlying fat excess (Fig. 1). In 2007, four reports linked the presence of SNPs in the first intron of the fat mass and obesity-associated gene (FTO) to obesity-related traits. As of today, among 100 different *loci* found to be linked to BMI, FTO remains the genetic region showing the strongest association with obesity development [18].

Moreover, studies conducted on genes regulating the glyco-lipid metabolism and thermogenesis revealed that a specific polymorphism (Pro12Ala) in the peroxisome proliferative activated receptor gamma gene (PPARγ) is associated with lower BMI and higher insulin sensitivity [19].

Similarly, recent meta-analyses involving up to 7000 subjects demonstrated a significant association between BMI and two other polymorphisms such as the Trp64Arg SNP in the β3-adrenergic receptor gene (ADRB3) [20]

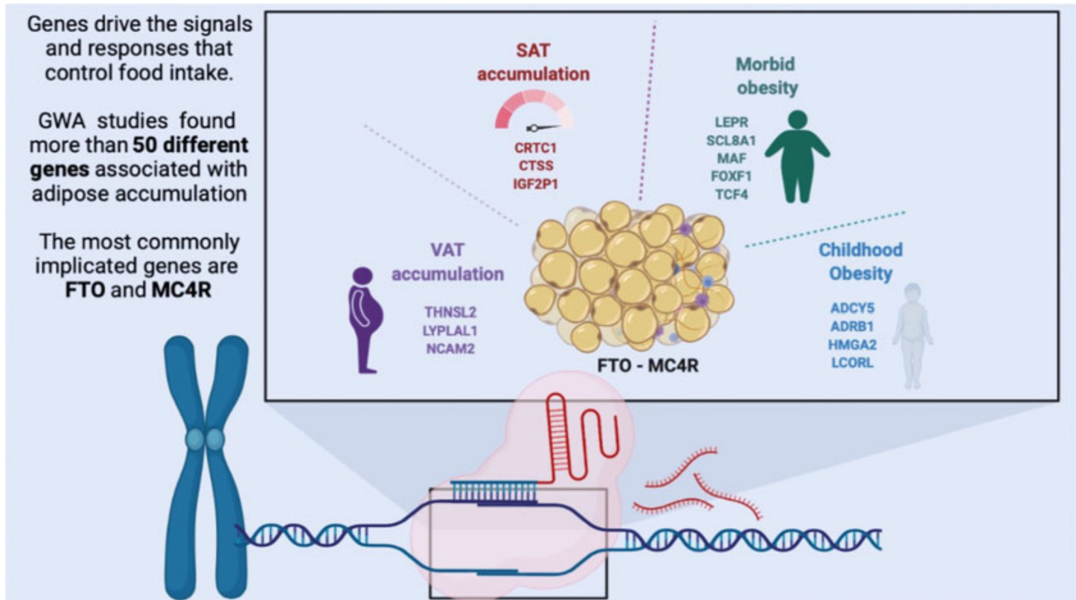

Fig. 1 Major GWAS identified loci associated with obesity-related parameters. *THNSL2* threonine synthase-like 2, *LYPLAL1* lysophospholipase-like 1, *FTO* fat mass and obesity-associated gene, *MC4R* melanocortin 4 receptor, *CRTC1* CREB regulated transcription coactivator 1, *CTSS* cathepsin S, *IGF2BP1* insulin-like growth factor 2 MRNA binding protein 1, *LEPR* leptin receptor, *SCL8A1* sodium-calcium exchanger, *FOXF1* forkhead box F1, *MAF* MAF BZIP transcription factor, *TCF4* transcription factor 4, *NCAM2* neural cell adhesion molecule 2, *ADCY5* adenylate cyclase 5, *ADRB1* adrenoceptor beta 1, *HMGA2* high-mobility group AT-hook 2, *LCORL* ligand-dependent nuclear receptor corepressor like

and the insertion/deletion (I/D) polymorphism in the uncoupling protein 2 gene (UCP-2) [16]. On the contrary, findings regarding the impact on obesity of the 2G866A SNP in the UCP-2 gene still remain inconclusive [21].

As already seen in monogenic obesity, MC4R mutations may also play a relevant role in polygenic forms of obesity, with more than 40 mutations detected [22]. More specifically, the MC4R V103I polymorphism has been found to be negatively associated with obesity, and robust data revealed that, among obese cases, the carrier frequency is about 2%, whereas in non-obese controls the rate is 3.5% [22].

Indeed, polygenic obesity derives from the complex interplay between the above-mentioned genes and the surrounding environment, physical activity, diet and gender, as described in a recent study conducted on a very large European population [23]. Noteworthy, fat distribution-related traits (WHR, WHR-adjusted BMI, WC-adjusted BMI and HIP-adjusted BMI) display a sexually dysmorphic pattern with higher heritability estimates in females compared with males, thus suggesting a stronger genetic contribution to these traits in females [24].

Studies investigating the relationship between dietary patterns and genetic predisposition to an elevated BMI have demonstrated that the genetic association with obesity was stronger among subjects with higher intake of sugar-sweetened beverages, as reported in the Nurses' Health Study (NHS) and in the Health Professionals Follow-Up Study (HPFS) cohorts [25].

Moreover, it should be noted that most GWAS, including those regarding obesity's susceptibility, have largely been conducted in European or East Asian populations, thus causing a gap in the genetic epidemiology of obesity in other populations [26].

3 Currently Available Obesity Treatments

The prevalence of obesity has increased worldwide in the last decades, reaching pandemic proportions. As a consequence, the development of preventive and therapeutic strategies for obesity management has become a major concern of public health and can only be accomplished through a multidisciplinary team [27].

The regulation of body weight is complex, and it is determined by a fine balance between energy intake and expenditure. An initial weight loss of 5–10% of total body weight constitutes one of the main goals in the therapy of obese patients, as it has proven to ameliorate life quality and reduce the risk of future comorbidities and all-cause mortality [28]. Beyond weight and BMI reduction, appropriate treatment aims include improvements in waist circumference and body composition [29]. The decision on which approach is the best one to adopt in order to treat individuals with overweight or obesity depends on many factors that encompass patients' health status, daily energy requirements and available resources.

3.1 Dietary Changes, Exercise and Behaviour Therapy

The first-line therapy for obesity is always represented by lifestyle modifications including both dietary interventions and increased physical activity, though they often fail to produce sustained weight loss [30]. In these circumstances, other weight-loss treatments, briefly discussed further on, may be considered, such as pharmacotherapy and bariatric surgery, depending on BMI risk category. Several obese patients are able to obtain considerable short-term weight loss by changing their eating habits alone, but long-term success is significantly more difficult to achieve [30]. There are many different suggested dietary patterns which vary according to their energy content or macronutrient composition. As far as dietary intervention is concerned, as recommended by the National Institute of Health (NIH), people with overweight and class I obesity should decrease their daily energy intake by approximately 500 kcal/day. Among different existing diets, very low carbohydrate diets have recently been described as novel successful and safe therapies for patients in obesity [31–33], even in patients with non-alcoholic fatty liver disease and chronic kidney disease [34, 35]. The use of food supplements in subjects with obesity has rapidly gained popularity: the benefits, although limited, may be a valid and safe support to lifestyle modifications [36].

Moreover, the addition of regular exercise to a weight-loss programme has considerable effects on obesity management. In this regard, people with obesity should practise at least 150 min of moderate-intensity physical activity per week, both to prevent further weight gain and to increase weight loss [37].

Furthermore, behavioural changes represent a crucial step in the treatment of obesity, as the latter often recognizes a psychological substrate and may be associated with psychopathological patterns such as depression and personality disorders. For these reasons, counselling with mental health experts and support groups can offer significant help in recognizing specific factors, stresses or situations which may have contributed to obesity development [38].

3.2 Prescription Weight-Loss Medication

Along with dietary interventions, in certain situations, pharmacological treatments may be considered in the management of obesity. Weight-loss medications approved by the European Medicines Agency (EMA) include three types of drugs, as follows:

- Orlistat is an intestinal lipase inhibitor which works by decreasing the absorption of fat ingested with the diet of about 30% [39].
- Liraglutide is an incretin-mimetic drug, used at a higher dose than that in diabetes. It works by binding to specific receptors in the

hypothalamic areas involved in food intake, thus increasing the sense of satiety and reducing appetite [39].
- The association of naltrexone and bupropion. The first drug is an opioid receptor antagonist, while the second one is an antidepressant, and they act synergistically at the arcuate nucleus of the hypothalamus, lowering the stimulus of appetite [39].

3.3 Metabolic Surgery

When dietary or pharmacological approaches fail, bariatric surgery may represent a valid option. Less invasive procedures with minor gastrointestinal involvement lead to significantly lower weight loss but have a substantially lower perioperative risk. Bariatric surgery is indicated in patients with severe obesity (BMI> 35 kg/m^2) with comorbidities such as diabetes or high blood pressure or in patients with morbid obesity BMI> 40 kg/m^2 [40].

Numerous lines of evidence indicate that surgery-induced weight loss is strongly associated with a reduction in early mortality and morbidity [41].

Common weight-loss surgeries include the following:

- Restrictive techniques which cause weight loss by decreasing gastric capacity (vertical ring gastroplasty, adjustable gastric banding, sleeve gastrectomy) [42]
- Malabsorptive techniques which shorten the length of the functional intestine and produce weight loss through a reduced absorption capacity of nutrients (biliopancreatic diversion with duodenal switch) [42]
- Mixed or partially malabsorptive restrictive techniques (Roux-en-Y gastric bypass) [42]

4 Latest Strategies for Obesity Treatment

A huge medical need exists for novel weight-loss therapies, with better efficacy and fewer side effects compared to the anti-obesity drugs currently approved. Latest weight-loss approaches, thanks to genome-wide association studies (GWASs), focused on obesity genetics and aimed at identifying additional genetic loci implicated in obesity risk, as novel therapeutical targets.

Among different pathways, in both the central nervous system (CNS) and the periphery, the leptin-melanocortin axis, the opioid system, the GLP-1/GLP-1 system and the FGF21/FGFR1c/b-Klotho axis are the most studied ones since they play an important role in the regulation of feeding behaviour and energy homeostasis [43].

4.1 Genome Editing Tools for Therapeutics in Obesity

CRISPR-based genome editing is a promising therapeutic technology for correcting genetic mutations in model systems ranging from cells in vitro to animals in vivo, and it has recently found possible applications in the treatment of many diseases, including obesity and metabolic syndrome [8, 44].

When energy intake overcomes expenditure, energy excess is stored primarily as lipids in white adipocytes. Mammals have two types of adipose tissue: white adipose tissue (WAT) and brown adipose tissue (BAT). WAT is used for energy storage, while BAT—due to its unique expression of uncoupling protein 1 (*UCP1*)—can transfer energy from food into heat, through the so-called classical non-shivering thermogenesis [45]. BAT is prevalent in newborn babies, representing a defence against hypothermia, but it progressively reduces with age, and in adult humans, BAT may even be absent [46]. In the last decade, growing interest has been directed to BAT, which has been proposed as a novel appealing therapeutic target to treat metabolic disorders [47].

As described by Wang et al., human brown-like (HUMBLE) cells can be created by engineering human white pre-adipocytes using CRISPR-Cas9-SAM-gRNA to activate endogenous uncoupling protein 1 expression. It has been demonstrated that transplantation of

HUMBLE cells in mice highly improves glucose tolerance and insulin sensitivity, through the activation of endogenous murine BAT mediated by arginine/nitric oxide (NO) metabolism. By treating obesity and metabolic disorders in mice, these results highlight the therapeutic potential of CRISPR-engineered HUMBLE cells [48].

Furthermore, it is well known that several genes are associated with obesity, and as a result, another potential approach of genome editing is represented by CRISPR-Cas9 targeting of mutated loci in pluripotent stem cells isolated from obese patients. In this regard, fat mass and obesity- associated (*FTO*) region is the first locus unequivocally associated with adiposity [49]. Over the last years, a clearer understanding of FTO function has been achieved, and a single nucleotide variant (SNV) of FTO allele (rs1421085 T-to-C) has been linked to the development of obesity [50]. Claussnitzer and colleagues have recently showed that this SNV can disrupt a conserved motif for the AT-rich interaction domain 5B (ARID5B) repressor, which can consequently determine an increase in iroquois homeobox 3 (*IRX3)* and iroquois homeobox 5 (*IRX5*) expression during early adipocyte differentiation. This results in the shift from beige to white adipocytes, with a concomitant reduction in mitochondrial thermogenesis and an increase in lipid storage. Interestingly, through the adoption of CRISPR/Cas9 genome editing, the repair of the ARID5B motif in primary adipocytes can revert *IRX3* and *IRX5* function, thus resulting in restored thermogenesis and activated browning expression [50].

Through genome editing strategies, researchers may also have the possibility to create animal models which recapitulate specific human genetic diseases, as well as monogenic forms of obesity. Along this line, CRISPR-Cas9 system has recently been adopted in mice to induce deletion mutations in the leptin (*Lep*) and leptin receptor (*Lepr*) genes—which have been already associated with obesity and diabetes in humans—thus generating obese and diabetic mouse models. In more details, co-microinjection of Cas9 mRNA and sgRNAs which specifically target *Lep* or *Lepr* in C57BL/6J embryos gives rise to mice exhibiting an obese phenotype, even in the first generation. These models are similar to the existing *ob*/*ob* and *db*/*db* lines that show weight gain, hepatic steatosis and hyperglycaemia. When compared to the latters, *Lep* and *Lepr* knockout models are easier to genotype, making them an attractive model for future metabolic disorder research [51].

A wide range of genes have been reported to cause human diseases due to haploinsufficiency that is the loss-of-function mutation in one gene copy which can consequently lead to reduced amounts of encoded protein. In this regard, among the emerging applications of CRISPR-Cas9-based gene editing, there are also novel techniques to treat obesity caused by haploinsufficient genes such as single-minded 1 (SIM1), MC4R, PCSK1, melanocortin 2 receptor accessory protein 2 (MRAP2) in the brain and IRX3 in fat or uncoupling protein 3 (UCP3) in mitochondria [52]. Intriguingly, as reported in a recent study, the CRISPR-mediated activation (CRISPRa)-recombinant adeno-associated virus injection into the hypothalamus leads to reversal of the obesity phenotype in SIM1 and MC4R haploinsufficient mice. These results suggest that CRISPRa can be used as a tool to rescue haploinsufficiency by up-regulating the expression of the endogenous functional allele [53].

Nevertheless, despite the fascinating putative use of CRISPR/Cas9 for treating obesity, strict ethical argument and monitoring regulation are essential when carrying out these therapeutic strategies in human patients in the next future.

5 Conclusions

Genome-wide association studies allowed us to identify additional genetic loci implicated in obesity risk. Thanks to these new discoveries, it has been possible to recognize genetic traits expressed in some populations. This led to the establishment of a genome editing model capable of repairing the associated defect in obesity and metabolic disorders. These futuristic approaches opened up exciting scenarios on how to treat monogenic forms and, even more importantly,

polygenic ones, which are the most common and currently represent a major burden worldwide. However, the analysis of the evidence reveals some aspects to be critically investigated starting from the population-specific interaction and the failure to replicate findings across GWAS. Even more delicate is the ethical question that could arise following the use of this genomic technique in non-communicable diseases in nutrition, unlike applications in fatal and/or oncological diseases.

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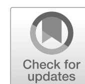

Genome Editing and Fatty Liver

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Abstract

Fatty liver disease is characterized as nonalcoholic fatty liver disease (NAFLD) and alcoholic liver disease (ALD). Fatty liver disease is one of the most common causes of chronic liver disease worldwide among adults and children. It is characterized by excessive fat accumulation in the liver cells. It has a genetically heterogenous background with complex pathogenesis and progressions and is accompanied by significant morbidity, mortality, and healthcare costs. NAFLD's risk factors include metabolic syndrome, abdominal obesity, type 2 diabetes, and atherogenic dyslipidemia. ALD is associated with the excessive consumption of alcohol. Here, we describe the functions of various proteins encoded by gene variants contributing to the pathogenesis of nonalcoholic fatty liver disease and alcoholic fatty liver disease. Advancements in genome engineering technology have generated various in vivo and in vitro fatty liver disease models reflecting the genetic abnormalities contributing toward fatty liver disease. We will discuss currently developed different ALD and NAFLD models using the clustered regularly interspaced short palindromic repeats (CRISPR/Cas9) genome editing tool.

Furthermore, we will also discuss the salient features of CRISPR/Cas9 editing technology and Cas9 variants such as prime and base editors to replicate genetic topographies linked specifically to ALD and NAFLD. The advantages and limitations of currently available genome delivery methods necessary for optimal gene editing will also be discussed in this review. This review will provide the essential guidance for appropriate genome editing tool selection and proper gene delivery approaches for the effective development of ALD and NAFLD models, leading to the development of clinical therapeutics for fatty liver disease.

Keywords

Fatty liver disease · Genome editing · Nonalcoholic fatty liver disease (NAFLD) · Alcoholic liver disease (ALD) · Clustered regularly interspaced short palindromic repeats (CRISPR/Cas9)

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1 Background

Alcoholic liver disease (ALD) and nonalcoholic fatty liver disease (NAFLD) are the leading causes of chronic liver disease and have a worldwide prevalence of over 25% [1]. Both ALD and NAFLD are incredibly complex, multifactorial diseases and have multistage disease presentation and share common histological features such as hepatic triglyceride contents greater than 5%, steatohepatitis, hepatic fibrosis, and cirrhosis [1].

1.1 Pathophysiology of Alcoholic Liver Disease

The relationship between heavy long-term alcohol consumption and progressive damage of the liver cells has been widely accepted; however, only a minority of people who consume substantial levels of alcohol are at the risk of developing progressive liver disease [2]. Several human and animal studies have shown that sustained moderate to high alcohol consumption is associated with early and predictable hepatic steatosis. However, only a small subset progress to more advanced disease such as alcoholic steatohepatitis and hepatic fibrosis [3–5]. Permanent liver damage such as cirrhosis develops ultimately in approximately 10–20% of heavy drinkers [6, 7]. Genetic factors are also believed to contribute a 30–50% risk of developing liver cirrhosis [8]. Among the women and men who consume a similar amount of alcohol, women are more susceptible to developing ALD than men [9]. Twin studies and studies of ethnic differences have also highlighted that the genetic factors determine ALD development risk [10]. The prevalence of ALD is threefold higher among monozygotic twins than controlled pairs of adults independent of alcohol consumption [11, 12]. These findings provide strong evidence that there is a genetic predisposition of alcoholism to organ-specific complications. Heavy drinkers with cirrhosis are more likely to report a paternal death from liver disease than nondrinkers, adding further evidence to the genetic susceptibility [13]. Furthermore, Hispanic Whites (12.6 of 100,000) have the highest mortality from ALD, followed by non-Hispanic African Americans (7.4 of 100,000), non-Hispanic Whites (5.2 of 100,000), and Hispanic African Americans (1.8 of 100,000) [14].

1.2 Pathophysiology of Nonalcoholic Fatty Liver Disease

NAFLD is defined as the excessive accumulation of triglyceride fat in more than 5% of the liver cells without any clear etiology such as viral hepatitis, drug use, and excessive alcohol consumption (>30 g/day for men and >20 g/day for women) [15]. NAFLD includes not only simple fatty liver but also more severe nonalcoholic steatohepatitis called NASH [16]. Histologically, NASH is defined as the presence of macrovascular steatosis, lobular inflammation, and ballooning of the hepatic cells [17]. NAFLD can progress to nonalcoholic steatohepatitis (NASH) due to hepatic injury, inflammation, and fibrosis, which can further progress to liver fibrosis, cirrhosis, liver failure, liver cancer, and many cardiovascular complications [18–23]. NAFLD diagnosis can only be made in the absence of significant alcohol use as the pathology of NAFLD is indistinguishable from the alcoholic fatty liver disease [16]. The ongoing global obesity pandemic has made NAFLD recognized as the most common metabolic disorder worldwide. It is also associated with long-term adverse events and mortality from complications such as liver failure, liver cirrhosis, and cardiovascular complications. Also, NASH is the second most common indication for liver transplantation after chronic hepatitis in the USA [24].

Risk factors associated with NAFLD include obesity, insulin resistance resulting from obesity, and metabolic syndrome. NAFLD can also be related to genetic polymorphisms, and they are also correlated with metabolic disorder and obesity [25]. A recent body of evidence has shown that genetic factors play a critical role in

determining NAFLD prevalence, the severity of the disease, and consequent prognosis. Moreover, studies have shown that NAFLD can also occur in 20% of the nonobese population, in association with recent weight gain, central obesity, dietary, and genetic factors [26].

In Asian countries, the incidence and prevalence of NAFLD are increasing despite lower obesity prevalence compared to the Western world [27]. Therefore, it is crucial to determine the contributing risk factors and the natural course of NAFLD development. According to the available research, the underlying disease etiology is considered to be multifactorial, and genetic factors play a substantial role. Depending on the study design, study methodology, and patient population ethnicity, NAFLD's heritability estimates range from 20 to 70% [28]. Furthermore, the heritability risk for fatty liver disease may be independent of the body mass index (BMI) heritability risk. Some family studies have shown that the prevalence of fatty liver disease could be lower in siblings than in parents of overweight children without NAFLD. It was noted to be more prevalent among siblings and parents of children with NAFLD [29] (Fig. 1).

Serum levels of liver injury markers such as alanine aminotransferase (ALT) and aspartate aminotransferase (AST) are used to diagnose ALD and NAFLD. Liver ultrasound and computerized tomography (CT) scans are used to detect liver steatosis and liver cirrhosis, respectively. Finally, liver biopsy is the standard test to diagnose the severity of the ongoing liver inflammation and the presence of liver fibrosis [30]. There is no approved treatment for fatty liver disease other than weight loss through physical activity and dietary changes

2 Genetic Variants and Fatty Liver Disease

2.1 Candidate Gene Studies

Several epidemiological studies have revealed many key genetic variants associated with fatty liver disease. Since both ALD and NAFLD share the same pathogenesis mechanism, similar gene categories have been selected for study based on the phenotypical characteristics of the encoded proteins [31]. These genes include those involved in alcohol metabolism, lipid metabolism, insulin resistance, and another common group of genes regulating cellular oxidative stress, endoplasmic reticulum stress, and cellular response to injury in hepatic cells. These genes have been selected from a limited pool of relevant genes that are already found associated with fatty liver disease [32].

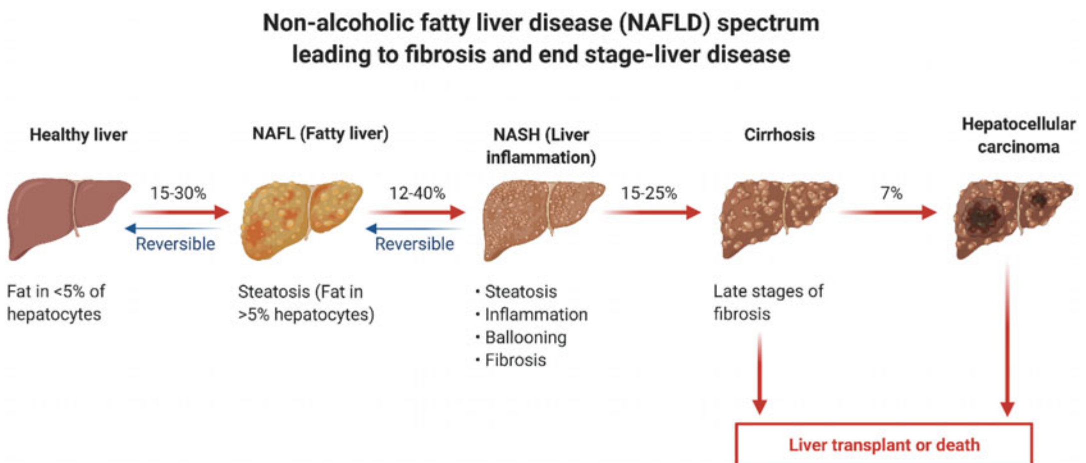

Fig. 1 Schematic illustration of fatty liver disease progression (Created with BioRender.com)

2.2 GWAS Findings

Also, several genome-wide association studies (GWAS) with single-nucleotide polymorphism (SNP) analysis have identified many genetic alleles associated with both ALD and NAFLD [33]. The research and understanding of these genetic variants can better understand the molecular pathology of fatty liver disease occurrence and progression. Some of the NAFLD-associated genetic variants were also subsequently found associated with ALD. The primary gene variants related to ALD and NAFLD have been described in Table 1.

2.3 Transmembrane 6 Superfamily 2 (TM6SF2)

Transmembrane 6 superfamily 2 (TM6SF2) is a genetic variant that encodes a protein containing 351 amino acids. This protein resides in the endoplasmic reticulum (ER) and ER-Golgi apparatus intermediate compartment of human liver cells [25]. Studies in mice have shown that a small hairpin RNA (shRNA)-mediated TM6SF2 impairs very low-density lipoprotein (VLDL) from the hepatocytes [49]. Another study has identified an SNP in the TM6SF2 gene variant (rs58542926, C > T, E167K), which is associated with the development of fatty liver disease predominantly among European individuals. This variant is positively associated with increased levels of hepatocyte triglycerides and liver enzymes such as ALT. These allele carriers are slightly more likely to accumulate fats in the hepatocytes and develop fatty liver disease than those who do not have this allele [34, 50]. Furthermore, some recent studies have identified that the TM6SF2 rs58542926 gene variant is strongly correlated with chronic liver disease and severity of fatty liver diseases such as NASH, liver fibrosis, and hepatocellular carcinoma (HCC) [35, 51]. Another TM6SF2 variant (rs10401969, C > T) was identified in an expression quantitative trait locus (eQTL) analysis, which is also associated with the reduced expression of the TM6SF2 gene variant. Together these data suggest the critical role of TM6SF2 gene variant in triglyceride metabolism in hepatocytes and

Table 1 Genetic variations associated with ALD and NAFLD

Gene	Variant	Function	Phenotype
TM6SF2	rs58542926 C > T (E167K) rs10401969 C > T rs58542926	Hepatic VLDL secretion and triglyceride metabolism ALD associated with cirrhosis	Elevated serum ALT, AST, and triglycerides [34] Low levels of hepatic TM6SF2 mRNA correlated with larger hepatocellular lipid droplets [35] Increased serum lipid profile [36]
GCKR	rs1260326 T>C/T>G (P446L) rs780094 (C > T)	Glucokinase regulator	Associated with an increased hepatic TGs and LDL cholesterol levels, correlated with NASH severity [37] Associated with an increased hepatic TGs and a higher risk of liver fibrosis [38]
PNPLA3	rs738409 C > G rs738409	Triglyceride lipase function ALD associated with cirrhosis	Increased hepatic fat content, associated with a high risk of hepatic steatosis, fibrosis, and HCC [28] Increased serum lipid profile [15, 39, 40]
MBOAT7	rs641738 C > T rs626283	Phospholipid re-acylation ALD associated with cirrhosis	Associated with an increased hepatic TG and NAFLD, higher risk of HCC [41–44] Increased serum lipid profile
HMOX1	rs2071746 A > T	Protects against the hepatic oxidative stress	Increased serum ALT enzyme in pediatric NAFLD patients [45, 46]
ADH	ADH2*B, ADH2*1	Increased hepatic lipid droplets	Associated with ALD, more prevalent in cirrhosis. Also, associated with cirrhosis [47, 48]
ALDH	ALDH2*1 ALDH2*2	Increased hepatic lipid droplets	More frequent in ALD/cirrhosis Less frequent in ALD/cirrhosis [47, 48]

provide evidence of association of NAFLD formation with TM6SF2 gene variant (rs10401969) [52].

A recent candidate gene study has demonstrated an association between the TM6SF2 (E167K), ALD, and cirrhosis. This study provided evidence that this gene variant increases ALD risk and cirrhosis by 1.9-fold [53]. Moreover, a GWAS performed by Buch et al. provided evidence that TM6SF2 (rs58542926) increased the risk of ALD-associated risk of cirrhosis by 1.4-fold ($p = 0.022$). After adjusting for confounders (age, sex, body mass index, and type 2 diabetes), these results pointed out the common pathologic mechanism of ALD and NAFLD [36].

2.4 Glucokinase Regulator (GCKR)

Glucokinase regulator is a gene variant expressed in the hepatocytes and controls glucose metabolism by binding and transporting glucokinase. While some rare mutations in GCKR cannot be detected, it is suspected that p. Arg227Ter mutant allele is associated with the NAFLD in young adults [54, 55]. Recent meta-analyses have demonstrated that single-nucleotide polymorphisms in GCKR (rs1260326 and rs780094) are associated with the NASH and NAFLD picture of the liver [15, 29, 37]. These variants are also associated with increased levels of serum low-density lipoproteins (LDL) and triglycerides. Another epidemiological study further verified that the high TG and glucose levels are correlated with the rs780094 (C > T) variant of GCKR. This variant is also associated with the severity of liver fibrosis [38].

2.5 Patatin-Like Phospholipase Domain-Containing Protein 3 (PNPLA3)

Patatin-like phospholipase domain-containing protein 3 (PNPLA3) gene variant encodes a 481amino acid protein, which is highly expressed in the hepatocytes in humans and adipose tissue in mice. PNPLA3 is also called adiponutrin and is a triglyceride lipase mediating triglyceride hydrolysis in the fat cells [25]. African Americans, Hispanic, and European Americans are found to have SNP in this gene variant (PNPLA3, rs738409, C > G) known by GWAS, and this variant is positively associated with the development of nonalcoholic fatty liver disease [56]. This variant results in the substitution of base guanine for cytosine, changing isoleucine to methionine at codon 148 of gene variant PNPLA3. This subsequent polymorphism results in an increased level of fats in liver cells of these three populations. Several recent cohort studies have shown that the PNPLA3 polymorphism (rs738409, C > G) variant confers a high risk of NASH development, liver fibrosis, and HCC [50, 57, 58].

Furthermore, some theoretical gene studies also have demonstrated the role of PNPLA3 as a modifier of alcoholic liver disease [15]. This variant was also associated with the severity of ALD in a study done on the Mestizo population with an ancestry-adjusted odds ratio of 1.9 [39]. A recent meta-analysis of ten studies has shown that PNPLA3 (I148M) is linked with increased odds of cirrhosis in ALD by 2.1-fold in persons heterozygous for CG sequence and 3.37-fold in persons homozygous for GG allele sequence [40].

2.6 Membrane-Bound O-acetyltransferase Domain-Containing 7 (MBOAT7)

GWAS has identified a single-nucleotide polymorphism in membrane-bound O-acetyltransferase domain-containing 7 (MBOAT7), which encodes an enzyme protein involved in phospholipid re-acylation in the context of phospholipid remodeling in human liver cells [25]. Studies have shown that this genetic variant MBOAT7 (rs641738, C > T) confers an increased risk of hepatic triglycerides and is associated with increased NAFLD severity among Europeans [57, 58]. This variant is also associated with an increased level of liver enzymes such as ALT [41]. Moreover, this SLP also increases hepatocellular carcinoma risk

according to GWAS of an Italian NAFLD cohort [42].

2.7 Heme Oxygenase (HMOX1)

Heme oxygenase (HMOX1) is an enzyme in the liver that has a hepatoprotective effect from the oxidative stress caused by excessive heme in liver cells [25]. Genetic single-nucleotide polymorphisms in this enzyme's promoter region are associated with an increased serum ALT among NAFLD children [43]. Studies in mice have shown that the overexpression of HMOX1 (rs2071746 A < T) has a protective effect on NASH and NAFLD [44, 59].

2.8 Alcohol Dehydrogenase (ADH) and Aldehyde Dehydrogenase (ALDH)

Allelic variants of the genes encoding class I ADH and ALDH can encrypt enzymes with various activity levels. These variant enzymes have a role in modifying the susceptibility of liver damage by alcohol consumption and alcohol dependence leading to ALD [47]. CYP2E1 is another enzyme, and there are several polymorphic loci within this gene. CYP2E1 c2 expresses higher levels of protein and is more common in ALD patients [48].

3 Gene Editing Models for Fatty Liver Disease

Gene editing technology has provided scientists with an unprecedented opportunity to establish various gene models to treat several diseases lacking specific treatment. Some recent advances in gene editing science include the development of CRISPR-/Cas9-associated protein and transcription activator-like effecter nucleases (TALENs) gene editing techniques [60]. The CRISPR/Cas9 technique has revolutionized the gene editing industry and is the most widely used technique. It is easy to use, is not time-consuming, and is cost-effective [61].

3.1 CRISPR/Cas9: A Genome Editing Tool

CRISPR/Cas9 system was originally discovered in the eighteenth century as the molecular basis of the bacterial immune system, providing natural protection to the prokaryotic cells against the foreign viral pathogenic attack. CRISPR/Cas9 was reengineered by Doudna and Charpentier, and they identified it as a two-component more manageable system. It comprises a guide RNA (mRNA) and an enzyme part called Cas9 endonuclease. Furthermore, Church and Zhang practically applied this system for the genetic editing of the cultured human cells. Now, CRISPR/Cas9 has been used in the gene editing of many organisms for the identification and treatment of many diseases.

3.2 Mechanism of CRISPR/Cas9 Genome Editing

Genome editing by using CRISPR/Cas9 is a multistep process. It starts with the recognition and complementary binding to the foreign DNA sequence by gRNA [62]. The Cas9 endonuclease part then recognizes the adjacent protospacer motif (PAM) locus on the target DNA to generate a double-stranded break within the target region, three nucleotides upstream from PAM in a 5-NGG-3 sequence [63–66]. A DNA repair system then kicks off, either homology-directed repair (HDR) or a nonhomologous end joining (NHEJ) to rejoin the already created double-stranded DNA breaks. The HDR-mediated system works to integrate the donor DNA template into the breakpoints, whereas NHEJ-mediated system works to delete a part of DNA to generate deletion mutations. The system is commonly used for short deletions and works highly efficiently. Insertions of single gRNA into the target cells can generate deletions of less than ten base pairs

(bp) in length, whereas injecting multiple mRNAs into the cells can target many different DNA sites to generate large deletions of several kilobases [67]. This technique can also be used to insert or replace the specific DNA sequences in the genome, which requires the introduction of a donor DNA template and CRISPR/Cas9 simultaneously into the target cells.

3.3 CRISPR/Cas9-Mediated Nonalcoholic Fatty Liver Disease (NAFLD) Models

Recently, advancements in the CRISPR/Cas9-mediated gene editing tools have enabled several NAFLD models both in vitro and in vivo. After verifying SNPs' specific links with NAFLD by functional analysis, the genetic variants can be modified to treat NAFLD [46]. Some of the CRISPR/Cas9-mediated NAFLD models have been described in Table 2.

3.3.1 Transmembrane 6 Superfamily 2 (TM6SF2)-Targeted Fatty Liver Disease Models

TM6SF2 rs58542926 (C > T) gene variant has been revealed by GWAS study that it plays a crucial role in cholesterol metabolism and is closely associated with the higher hepatic triglyceride accumulation and resulting high serum ALT levels [34, 49]. Fan et al. explored the pathophysiological role of TM6SF2 by disrupting this gene variant using CRISPR/Cas9 editing technology in mice [68]. They obtained TM6SF2 mutant mice containing a different base pair (C/G) insertion immediately after the gene's start codon. The mice with resultant TM6SF2 mutant gene variant showed a slight increase in the serum triglycerides, but there was no accumulation of hepatic TGs and rise of hepatic enzymes (ALT, AST) when compared with the wild-type mice-phenotype landscapes that did not correspond to features observed in human carriers of TM6SF2 rs58542926 gene variant [49]. These mutant mice showed a different lipid metabolism picture, and their plasma showed lower LDL levels, high-density lipids (HDL), and total cholesterol. These findings were consistent with the genetic studies done on human cells in the past. Another study used CRISPR /Cas9 editing technology to disrupt the TM6SF2 gene in zebra fish. They co-injected fish larvae with Cas9 mRNA and gRNA by targeting specific exons (3 or 4) of TM6SF2. Mutation of both lines showed an increased lipid accumulation in the zebra fish liver [69].

3.3.2 PNPLA3-Targeted Models

As described earlier, the PNPLA3 rs738409 (1148M) gene variant is an SNP associated with both ALD and NAFLD developments and is found in 30–50% of all subjects [28]. Luukkonen et al. determined the biological function of this

Table 2 CRISPR/Cas9-mediated NAFLD models

Target gene	Model organism	Outcome of genotype	Cas9 delivery method	Phenotype
TM6SF2 (rs58542926)	Mouse Zebrafish	Base C/G insertion after the start codon Partial deletion of exon 3 or exon 4	Microinjection Microinjection	A high-fat diet resulted in increased serum TGs [68] Increased lipid accumulation in hepatocytes [69]
GCKR (rs1260326)	Mouse	P446L	Microinjection	N/A (76)
PNPLA3 (rs738409)	Human epidermal carcinoma cell line (A431)	Two-base pair deletion after 146C I148M	Transfection Transfection	Increased hepatic lipids droplets [70] Increased accumulation of neutral lipids [70]
MBOAT7 (rs641738)	Human hepatocellular carcinoma cell line (HepG2)	Deletion of 31-base pair, 91-base pair, or 101-base pair	N/A	Increased hepatic fat accumulation [71]

SNP for the first time. They used CRISPR/Cas9 technique to introduce two different types of PNPLA3 mutations into the human cells [70]. As a result of this procedure, the HDR donor attained two cell lines: a homozygous PNPLA3 knockout (KO) cell line which contains a two-base pair deletion after 146 C and a homozygous PNPLA3-I148M knock-in (KI) cell line with a single (C > G)-nucleotide substitution. Both cell lines showed a frameshift of the PNPLA3 variant and a resultant premature termination of the translation process. Interestingly, the PNPLA3-I148M knock-in (KI) cell line showed increased triglycerides and other neutral lipids. Then they introduced both saturated and polyunsaturated fatty acids into these mutant cells and found that the polyunsaturated fatty acid introduction resulted in more lipid accumulation into both mutant cell lines (PNPLA3-I148M knock-in (KI), PNPLA3 knockout (KO)). They concluded that the PNPLA3 variant might serve as a polyunsaturated fatty acid hydrolase. This gene variant's loss can sequester liver triglycerides in polyunsaturated fatty acid direction, preventing ALD and NAFLD liver picture [65]. Further studies have demonstrated that the PNPLA3-I148M variant increases hepatocellular triglycerides and the risk of NASH, alcoholic, nonalcoholic fatty liver disease, and hepatocellular carcinoma [72].

3.3.3 Miscellaneous Models

Some other gene variants are also associated with the NAFLD development, including the GCKR and MBOAT7 variants. In an experiment, Meroni et al. used BHEJ-mediated CRISPR/Cas9 gene editing technique to generate three human liver cell lines with deleted MBOAT7 gene containing 31-, 101-, and 917-bp deletions [71]. All the mutant cells showed an increased accumulation of fatty acids resulting in NAFLD. The mutant cells with the most extensive base deletion (917 bp) exhibited dramatically saturated and monounsaturated TGs in liver cells resulting in NAFLD. They found that the MBOAT7 variant plays a critical role in NAFLD development.

Similarly, Codner et al. determined the functional role of the GCKR variant. They used HDR-mediated CRISPR/Cas9 gene editing technique to establish GCKR 9446L in mice [73].

4 "Good Fit" Genome Editing Tool Selection for ALD and NAFLD Variant Replication

CRISPR/Cas9-mediated genome editing has opened a new era in the gene editing field; however, certain limitations are still associated with this technology. For instance, there are many limitations in generating precise target-specific point mutations and targeted precise base pair sequence replacement by using this technique. The efficiency of this system is very low (<5%) while performing HDR-mediated nucleotide insertion [74]. Also, nonhomologous end junction (NHEJ)-mediated CRISPR/Cas9 editing can only produce random point mutations and insertions [40]. Researchers have attempted to overcome these issues by generating advanced genome editing tools by using different Cas9 variants. Specific Cas9 variants are useful for reassembling ALD and NAFLD SNPs, and a careful selection of a good fit model from these variants can help establish research models mirroring specific genetic features of fatty liver disease [75].

4.1 Cutting-Edge Cas9 Variants

4.1.1 Base Editor for Fatty Liver Disease Genome Variants

The current gene editing techniques require a template double-stranded DNA breaks to be introduced at the target gene locus to start the gene correction [64]. Moreover, these techniques are inefficient and prone to error in introducing random base insertions and deletions at the locus site of interest [76]. Liu and colleagues used CRISPR/Cas9 variant to develop a base editor for generating specific point mutations. This base editor is unique in the way that it does not even require the donor DNA templates for functioning and can enable the direct irreversible conversion of one DNA base into another in a precise

programmable manner [77]. This base editor uses catalytically impaired Cas9 nuclease enzymes such as deadCas9 or Cas9 nickase. Both enzymes are incapable of inducing double-stranded base pairs (DBSs) [78]. They also linked the CRISPR/Cas9 to cytosine deaminase enzyme to form a cytosine base editor (CBE), which can substitute cytosine (C) for thymine (T) and is extremely efficient in producing point mutations; however, its ability to perform nucleotide substitutions is very limited to conversion of cytosine to thymine only. To address this issue and to broaden the target nucleotide range, they further developed an adenosine base editor (ABE) by linking adenosine deaminase with Cas9 enzymes (deadCas9 and Cas9 nickase) [79]. This new base editor functions by mutating adenosine (A) to guanine (G). Both editors are highly efficient in producing point mutations and are extremely useful for substituting single nucleotides in ALD and NAFLD producing gene variants. For instance, CBE could be used for mirroring TM6SF2 rs10401969 (C > T), TM6SF2 rs58542926 (C > T), GCKR rs780094 (T > C), GCKR rs1260326 (T > C), MBOAT7 rs641738 (T > C) NAFLD genome variants.

4.1.2 Prime Editor for ALD and NAFLD Genome Variants

One of the base editor's limitations is that it can only mutate a specific single nucleotide, and the replacement of a short stretch of DNA is technically challenging. To address this issue, Liu et al. further developed another editor known as a prime editor, which is highly specific in making insertions and point mutations without introducing DNA templates and DSBs within the target cells [80]. The prime editor used a prime editing gRNA (pegRNA) and a CRISPR/Cas9 nickase, further linked to another reverse transcriptase enzyme. The pegRNA is an extended version of the gRNA and contains a prime binding site and a reverse transcriptase template sequence for binding.

Mechanism of Action

Once the pegRNA portion of the prime editor recognizes the target gene sequence, the CRISPR/Cas9 nickase enzyme breaks and introduces a nick in the target genome adjacent to the recognition site called PAM. As a result, reverse transcriptase activates and forms a new DNA fragment by using pegRNA as a template starting at the 3-end sequence. As a result of this process, the newly synthesized DNA strand will incorporate into the target gene's nicked DNA strand. Furthermore, the complementary DNA strand is repaired by using the genome-edited strand as a template strand. Consequently, the prime editor can engender all types of DNA mutations, such as short insertions, short deletions, DNA sequence replacement, and other point mutations [80].

Advantages

The prime editor has several advantages over CRISPR/Cas9 and the base editor. Firstly, it can harvest more precise gene insertions and point mutations than NHEJ-mediated CRISPR/Cas9 genome editing. Secondly, its target range is more flexible than the NDR-mediated genome insertions as it can introduce a point mutation far from the nick site (>30 bp) introduced by nickase [63]. Lastly, the prime editors are capable of generating both transversion (purine base to pyrimidine, A or G to T or C) and transition mutations (purine to purine, C to T or G to A) [66, 67]. These advantages make the prime editor a better choice to generate NAFLD polymorphisms such as PNPLA3 rs738409 (C > G) and HMOX1 (rs2071746 (A > T).

4.2 Gene Delivery Methods for Refining Efficacy of Gene Editing Process

4.2.1 Nonviral Delivery Methods

CRISPR/Cas9-based genome editing is evolving and is one of the most powerful strategies available (Table 3). Currently, various gene delivery methods for CRISPR/Cas9 have been investigated to optimize genome editing efficacy and efficiency in treating different diseases. The components of the CRISPR/Cas9 system can be introduced into the cells in three different

Table 3 Gene delivery methods for genome editing

Type	Method of delivery	Advantages	Limitations	Reference
Nonviral	Liposomes	Simple method, cost-effective, and easy to manipulate	It can be performed in vitro only, endosomal degradation of cargo system, and specific cell tropism	[64, 75, 77, 79]
	Gold nanoparticles	Inert and have low immune reaction and response	N/A	[81]
	Electroporation/ nucleofection	Well-characterized method, delivery to cell population	Can be performed in vitro only; some cells are not suitable	[65, 82]
	Microinjection	Efficient germline cell delivery	Laborious procedure, technically challenging	[67, 83]
Viral	Adeno-associated virus (AVV)	Efficient delivery to both nondividing and dividing cells	Low genome capacity, only 5 kb nucleic acids can be delivered	[84, 85]
	Adenovirus	High genome capacity up to 30 kb nucleic acids, higher efficiency	Induce adaptive immune responses	[86]
	Retrovirus	High efficiency, persistent gene expression	Infect only dividing cells may cause unwanted genome integration	[87]
	Lentivirus	Higher genome capacity of 10 kb causes long-term gene expression	Unwanted gene integration induces a strong reactive immune response	[88]
	Baculoviral	Extremely high genome capacity (>100 kb), minimal immune response	N/A	[89]

platforms. It can be introduced as gRNA and Cas9 mRNA and DNA plasmids which encode gRNA and/or Cas9 and lastly in the form of gRNA and a Cas9 protein [64, 83, 90, 91]. Initial studies have shown the introduction of Cas9 into the cells and mouse embryos in the form of mRNA and DNA plasmids. Afterward, the CRISPR/Cas9 system has been introduced in the form of ribonucleoprotein. However, several studies have reported a little off-target cleavage rate compared to the plasmid DNA form [81, 82, 92].

There are two well-characterized and guaranteed gene delivery methods for CRISPR/Cas9. Electroporation has been the most common in vitro delivery method for CRISPR/Cas9 into the cells, and for in vivo delivery, microinjection has been the standard method for all models in animal cells [65, 93–95]. The efficacy of the electroporation methods is mainly dependent on the type of cell to be engineered. On the other hand, the microinjection method requires a skillful hand and is technically challenging [96, 97]. Specific CRISPR/Cas9 variants such as base editors and prime editors have been introduced into the cells in the form of DNA plasmids [77, 79, 80]. Liposomes are used to deliver these DNA plasmids because they are cost-effective and can easily be managed; however, their use is limited only to in vivo delivery [94]. This delivery method can be affected by the endosomal degradation of the liposomes. An altered way of Cas9 delivery method is the use of gold nanoparticles, which are very effective in introducing the CAS9 into the cells and organs in the form of mRNA or protein [98]. These particles are also inert and produce no or very little immune response if they are better characterized by clinical application.

4.2.2 Viral Delivery Methods

Various viral vectors have been used for gene editing to improve the gene delivery system's efficacy further (Table 3). Adeno-associated viruses (AVVs) is one of these viral vectors; they have been widely used and are known for their efficacy infecting both dividing and nondividing cells [84]. However, this viral vector has a small genome packaging capacity (4.7 kb) relative to the Cas9 size (4.1kb) and is not suitable for

the introduction of the donor DNA template. However, it has been very effective for the smaller CRISPR/Cas9 gene variants such as vjCas9 (2.95 kb), saCas9 (3.116 kb), and Cpf (3.9 kb) [85, 99–101]. Adenoviruses can also be used as viral vectors for gene delivery, and they have a much larger genome capacity to carry than the adeno-associated viruses, but they can induce a strong reactive immune response in host cells [96]. Furthermore, lentiviral or retroviral vectors have a larger gene-carrying capacity than AVVs, but they can induce undesirable genome incorporation into the host cells. They are also linked with causing a strong unwanted immune response in the hosts [98]. Some recent studies have reported the use of baculoviral vectors for gene editing [89, 100]. They possess the essential capacity to carry gRNA expression constructs and nuclease along with the HDR targeting sequences. Baculoviral (>100 kb) vectors have certain advantages over the other viral vectors [102]. Firstly, they have a much larger genome transfer capacity; secondly, they are safer than other viral vectors as they can only replicate within their original host insect cells, leading to fewer chances for host immune response induction [103]. Future studies are needed for baculoviral vectors to validate their clinical application in genome editing.

5 Discussion

Fatty liver disease is the most common chronic liver disease worldwide. It is a multifactorial disease that is influenced by both genetic and environmental factors. Environmental factors are the major determinants of NAFLD and include a sedentary lifestyle, obesity, and inapt dietary patterns. Excessive alcohol consumption is exponentially associated with ALD development. Moreover, certain genetic factors also provide the basis for the onset of the disease and its severity. Several recent cohort studies and GWAS have identified several genetic variants (SNPs) associated with fatty liver disease [39, 49, 56, 59]. However, some functional studies are required to truly verify these SNPs' physiological role in the progression of ALD and NAFLD. Many NAFLD models have been generated using shRNA, siRNA morpholinos, or conventional Cre/loxP recombination technique and have been described in the literature. However, these tools can only inhibit gene expressions but are incapable of carrying out single-nucleotide gene substitutions required for copying NAFLD-associated single-nucleotide proteins (SNPs). The conventional Cre/loxP system can cause single-nucleotide mutations and may lead to NAFLD model mutations; however, it is technically challenging and arduous to perform [49, 104–107].

CRISPR/Cas9 gene editing tool has been swiftly adopted for gene editing and generation of NAFLD models since its development. Recently, remarkable progress has been made to improve the CRISPR/Cas9 gene editing tool efficacy and safety. Safe CRISPR/Cas9 delivery systems can help edit specific diseased tissues in vivo and enhance broad clinical translation [68, 70, 71, 73]. The CRISPR/Cas9 system has a larger Cas9 nuclease, which makes its encapsulation in both viral and nonviral delivery systems a challenge. However, several approaches have been used to overcome these impediments, and these large molecules are delivered as nucleic acids or protein molecules in the hepatic cells. The NHEJ-mediated CRISPR/Cas9 system can produce gene deletions, but its efficacy is very low. Similarly, the efficacy of HDR-mediated insertion is also quite low [64, 65].

Most of the ALD and NAFLD models generated and studied are from animals and do not correlate well with the human data. The current study suggests that certain CRISPR/Cas9 variants such as the prime editor and the base editors can be used to introduce SNPs into NAFLD model generation cells. We recommend using a prime editor for establishing purine-pyrimidine mutations, and ABE and CBE would be suitable for generating purine-to-purine mutations for NAFLD models. Together these tools ensure more sophisticated and precise genome editing.

6 Future Implications

The gene editing field has been revolutionized by the advent of various Cas9 variants and CRISPR/Cas9. However, this gene editing technique is also associated with some off-target events which have been problematic from clinical application standpoints [86, 107]. For instance, base editors can generate some undesirable bystander edits during the gene editing process [71]. Several recent studies on whole-genome sequencing analyses have demonstrated that ABEs are associated with inducing much lower off-target gene effects than CBEs [108]. However, there is not much known about prime editors' off-target effects and more studies need to be done. Also, to optimize the efficacy of the gene editing system for many modern diseases, selecting an appropriate delivery method is imperative.

Furthermore, more studies are needed to elaborate on the precise benefits and shortcomings of the gene delivery systems. Among the available gene delivery options, gold nanoparticles and baculoviral vectors are considered a good fit for in vitro and in vivo gene delivery, respectively. A successful establishment and promotion of genuine ALD and NAFLD models require a systematic strategy for selecting a good fit gene editing tool and a suitable gene delivery method into the target cells. Consequently, these ALD and NAFLD models will identify the underlying disease onset and pathophysiology progression, which can be fixed using proper CRISPR/Cas9 genome editing tools. Lastly, both ALD and NAFLD SNPs can also serve as biomarkers for disease diagnosis and clinical targets for genome therapy.

7 Conclusions

The revolution in genome engineering has helped establish various disease models that can mimic genetic variants. CRISPR/Cas9 genome editing can primarily generate deletion mutations of the target genome, and it has been used to generate both in vivo and in vitro fatty liver disease models. Additionally, Cas9 variants such as prime and base editors can generate specific point mutations and gene sequence substitutions. The successful establishment of the fatty liver disease models for therapeutic development requires a suitable genome editing tool and an efficient and robust gene delivery method.

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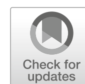

Genomic Editing and Diabetes

Parth Shah

Abstract

The diabetes types and its complications have varying developmental and metabolic pathways. There is an interplay of nongenetic and genetic components in pathogenesis of diabetes and its complications. There are several established genes such as ABCC8, TCF7L2, SLC2A2, and CAPN10 which are known to influence blood insulin and glucose levels. Current management of diabetes types may include lifetime burdensome use of insulin, insulin sensitizers, insulin secretagogues, etc. There has been increasing interest in improving genetic editing tools such as CRISPR/Cas9 and using genetically edited stem cells to alter diabetes disease course or possibly cure it. Current research on microRNAs and long noncoding RNAs may provide insights into the pathways involved in development of diabetes and its complications. Consequently, developing further understanding of genetics and its messenger pathways in diabetes would enhance our ability to develop precise and accurate genetic editing tools which can translate into clinically useful therapeutics.

Keywords

CRISPR/Cas9 · Diabetes · MicroRNA · Long noncoding RNA · Genetic editing

1 Background

In a clinical setting, there are typically two well-accepted diabetes mellitus types: diabetes mellitus type I (DM1) and diabetes mellitus type II (DM2). DM1 is defined by the lack of insulin production by the pancreas whereas DM2 is the result of growing insulin resistance eventually leading to pancreas burnout. As per the World Health Organization (WHO), the incidence of diabetes since 1980 in adults has doubled across each of the regions worldwide [1]. Furthermore, in 2014, an estimated 422 million (8.5%) people worldwide had diabetes with age-standardized prevalence in adults being highest in Polynesia and Micronesia (~25%) and lowest in Northwestern Europe [1, 2]. The global diabetes prevalence in adults rose from 4.7% in 1980 to 8.5% in 2014 [1], and diabetes was directly responsible for ~1.6 million deaths worldwide in 2016 [3]. The incidence and prevalence of DM1 have been rising globally with recent report showing 15 per 100,000 and 9.5%, respectively [4].

Diabetes is associated with several chronic debilitating complications which can be macro- and microvascular, including kidney failure, cardiomyopathy, myocardial infarction, stroke,

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blindness, and peripheral neuropathy. Given the rising prevalence of the diabetes, it is imperative that known risk factors and etiologies and current therapeutic management of diabetes be understood to develop future therapies based on the use of genomic editing.

2 Risk Factors

2.1 Interplay of Nongenetic Risk Factors

The diabetes mellitus type I risk factors and its prevention still remain an enigma. It is known that there is a genetic disposition to DM1, but the trigger(s) of an autoimmune destruction of pancreatic beta cells is unknown. In a male with DM1, the likelihood of child developing diabetes is 1/17, and in women with DM1 with child born at <25 years of age, it is 1/25 and for ≥ 25 years of age, it is 1/100 [5]. Regardless of the gender, if the parent developed diabetes at <11 years of age, the risk of child for DM1 development is doubled [5]. If both parents have DM1, then the risk of child is 1/10 to 1/4 [5]. There is some recent literature to suggest that this destruction may be triggered by infection [6], but more supportive research is needed to prove this etiology.

The diabetes mellitus type II risk factors include weight, centripetal obesity, family history, diet, sedentary lifestyle, age, race and ethnicity, gestational diabetes, and insulin resistance syndrome [7]. It is not entirely clear why there is a higher likelihood that people of Hispanic, Black, Native Americans, Asian, and Pacific Islanders are more likely to develop DM2 [7]. Development of insulin resistance syndrome—also known as polycystic ovarian syndrome in women—increases the risk of DM2. Approximately 50% of women with gestational diabetes mellitus develop DM2 [8]. Over a lifetime, these risk factors are thought to alter proteins involved in cellular glucose transport and signaling pathways leading to insulin resistance, impaired glucose uptake, and ultimately DM2.

2.2 Genetics of Diabetes

There are several genes which are known to regulate insulin production and secretion and glucose metabolism (Table 1). The TCF7L2 gene affects insulin secretion and glucose production. ABCC8 gene is responsible for SUR1 protein which helps regulate blood insulin levels by influencing pancreatic beta cells. Encoded by SLC2A2 gene in humans, GLUT2 is a transport protein which can move the glucose into the pancreas. GCGR is a gene coding for protein which regulates glucagon hormone release which can influence cellular glucose release. Recently, Phase 2 clinical trials have been conducted with an anti-sense oligonucleotide therapy targeting GCGR pre-mRNA which would ultimately lead to degradation of target RNA and inhibit glucagon release. The trials showed some promise in lowering hemoglobin A1C, but there was a trade-off at higher treatment doses indicating elevated liver transaminases and increased hepatic lipid content. Variations in CAPN10 have been associated with development of insulin resistance and ultimately DM2 in Mexican-Americans [9].

2.2.1 Transcription Factor 7-Like 2 (TCF7L2) Gene

The transcription factor (TCF7L2 protein) coded by TCF7L2 gene is known as the strongest influencer in DM2 development out of all genes to date [10]. The polymorphism in rs7903146 of TCF7L2 is most prominently associated with DM2 across multiple ethnic populations [11, 12]. Furthermore, T-allele in TCF7L2 (rs7903146) is associated with significant risk of DM2 when the either parent or both parents are diabetic compared to nondiabetic parents [11]. The T-allele of TCF7L2 (rs7903146) has also been found in women with gestational diabetes mellitus (GDM); consequently, the T-allele GDM women also show failure of early postprandial glucose control and may require insulin therapy [13]. In patients with metabolic syndrome, TCF7L2 variants rs12255372 and rs7093146 have both been associated with development of

Table 1 Genes regulating blood insulin and glucose levels

Gene	Protein	Role
Transcription factor 7 like 2 (TCF7L2)	High-mobility group (HMG) box-containing transcription factor (TCF7L2 protein)	Insulin secretion and glucose production
ATP binding cassette subfamily C member 8 (ABCC8)	Sulfonylurea receptor 1 (SUR1)	Regulate blood insulin level
Solute carrier family 2 member 2 (SLC2A2)	Glucose transporter type 2 (GLUT2)	Regulate glucose uptake by pancreas
Calcium-activated neutral proteinase 10 (CAPN10)	Calcium-sensitive cysteine proteases	Regulate blood glucose levels
Glucagon receptor gene (GCGR gene)	Glucagon receptor (GCGR)	Inhibit glucagon release

impaired fasting glucose, an indicator of DM2 development [14].

Although, the clear mechanism by which TCFL2 leads to DM2 remains unclear, there have been some studies showing its potential impact on some key pathways of insulin resistance and adipogenesis. The Wnt/B-catenin signaling pathway involves TCF7L2 protein as the key factor, and this pathway negatively regulates adipogenesis. In vivo findings have indicated that inactivation of TCF7L2 protein leads to impaired whole-body glucose intolerance and increased adipocyte hypertrophy and inflammation [10, 15]. Data from T-allele of variant rs7903146 in TCF7L2 carrying individuals also points to a possible pathway affecting insulin pulse characteristics of pancreatic beta cells such as decreased orderliness and pulse dispersion independent of hyperglycemia [16]. Further understanding of similar pathways or relationships to DM2 development will permit development of future therapies.

2.2.2 ATP Binding Cassette Subfamily C Member 8 (ABCC8)

The ABCC8 encoding for SUR1 protein is the target for antidiabetic sulfonylurea class of medications which increases pancreatic secretion of insulin into the blood. Up to 5% of diabetes maybe maturity-onset diabetes of young (MODY) which starts in adolescence or in early adulthood. There are 11 different genes which lead to 11 different types of MODY [17]. This is an autosomal dominant condition, and on consistent basis, ABCC8 variant has been found in individuals with MODY. The neonatal diabetes mellitus marked by persistent hyperglycemia in first six months of life occurs in 1:100,000 to 1: 400,000 neonates and can convert to permanent neonatal diabetes mellitus (PNDM) in half of those cases [18, 19]. The gene variation of ABCC8 and KCNJ11 which influence ATP-dependent potassium channels of beta cells has been found in the neonatal diabetes cases [19]. In patients with early-onset diabetes, eight missense variants of ABCC8 (p.R306C, p. E1326K, p.R1379H, p.R298C, p.F1176C, p. R1221W, p.K1358R, and p.I1404V) have been identified [20]. Overall, the MODY types or PNDM may have long-term consequences similar to those of DM2 and need to be medically managed appropriately after patient is diagnosed using appropriate genetic and biomarker tests. Gene variations of ABCC8 and their role in pathogenesis or complicating diabetes can provide future therapeutic targeting for genomic editing.

2.2.3 Solute Carrier Family 2 Member 2 (SLC2A2)

For the DM2 individuals, SCL2A2 C-allele variant appears to play a major role in insulin sensitivity of GLUT2 protein. At the time of diabetes diagnosis, the C variant of allele of SLC2A2 (rs8192675) has been associated with greater diabetes symptoms [21]. The SLC2A2 gene's allele C of rs8192675 which encodes for GLUT2 has been found to be associated with greater metformin-induced glucose reduction as evidenced by hemoglobin A1C (HbA1C), and this effect has been even more pronounced in obese C-homozygote individuals [22].

SCL2A2 mutations can lead to a rare autosomal recessively inherited glycogen storage disease called Fanconi Bickel syndrome. In this condition, glycogen accumulated in the liver and kidneys because of GLUT2 protein defect. The symptoms may include hepatosplenomegaly, rickets, failure to thrive, kidney malfunction, hypoglycemia, bowed legs, and abdominal distension [23]. Overall, the SCL2A2 mutations can create variation in GLUT2 such that it can be the etiology of rare Fanconi Bickel syndrome or affect insulin sensitivity during DM2 and may also impact efficacy of insulin-sensitizing therapy in DM2 patients.

2.2.4 Calcium-Activated Neutral Proteinase 10 (CAPN10)

GLUT4 protein is a protein found in adipocytes and striated muscle cells and is involved in glucose transport as regulated by insulin. CAPN10 is thought to be involved in translocation of GLUT4 protein from Golgi apparatus matrix [24]. The prevalence of DM2 in Mexico in 2015 was ~11.5 million, sixth highest in the world, and CAPN10 is one of the 21 genes which is associated with DM2 in this population [25]. The gestational diabetes mellitus and CAP10 single-nucleotide polymorphisms (SNPs) have been found to be associated. Consequently, in a large population of women, it was found that TT genotype in SNP 63 was most associated with increased risk of GDM [26]. Overall, the CAPN10 variants are associated with DM2, particularly in Mexican ethnicity, and GDM across multi-ethnic women population.

3 Overview of Current Diabetes Management

3.1 Diabetes Mellitus Type I

The treatment for DM1 involves various regimens of injectable insulin which can be short acting, moderate acting, long acting, and ultra-long acting. The insulin regimen may require multiple injections per day which may lead to side effects such as bruising, dizziness, nausea, hypoglycemia, etc. and may impact patient's quality of life. Additionally, the insulin regimen may need to be adjusted based on meal intake and daily activity. The overall management of DM1 requires an inconvenient lifetime commitment to taking insulin.

3.2 Diabetes Mellitus Type II

The development of insulin resistance ultimately leading to pancreas burnout is responsible for the development of DM2. This can be thought of as process over a patient's lifetime. There are many different treatment regimens available for DM2, and these include insulin sensitizers, insulin secretagogues, influencing kidney's glucose excretion, affecting gastro-intestinal absorption of glucose, insulin, etc. There can be different combinations of treatment medications which can be given to optimize glucose control. The insulin injections are reserved for cases of DM2 where other treatment regimens fail to control blood glucose. There are now continuous glucose monitoring and insulin injection devices which patients can wear to optimize the blood glucose levels in more complex DM2 cases. Even though several treatment regimens are available, the key to DM2 therapy lies in its prevention or its complete reversal. In the event of development of DM2 complications, the genetic influence on metabolic pathways leading to the complications needs to be understood to create better therapeutic targets.

4 Genomic Editing

4.1 Genetic Modification of Stem Cells

The genetic makeup of pluripotent stem cells can be modified to generate cellular therapies to address the etiology of a disease with possibility of its cure. As discussed earlier, our current management of diabetes stems from detecting preventative risk factors or finding potential drug targets to control the diabetes after its onset. Through

development of genomic editing, it can be envisioned that someday, one can modify genes to prevent or reverse diabetes. The genetic modification of human pluripotent stems cell-derived beta cells (SC-β) can be used to understand beta-cell development and model their function in diabetes. One of the challenges with β-cell transplantation is recurrent autoimmunity in the host [27]. There is some hope that stem cells derived from other human cell types such as mesenchymal cells, embryonic stem cells, etc. could be used to design a personalized transplantable cell without risk of autoimmunity.

4.2 CRISPR/Cas9 Gene Editing

CRISPR/Cas9 (clustered regularly interspaced short palindromic repeats)-Cas9 (CRISPR-associated protein 9) gene editing technique opens the possibility of understanding developmental cellular biology, regulation of cell function, and its viability which can ultimately lead to pioneering of new diabetes therapies. Consequently, this can provide us the means of treating and possibly curing the disease through genetic correction and transplantation of modified cells. CRISPR/Cas9 uses 20 nucleotide RNA (gRNA) as a guide, and this allows CRISPR/Cas9 to search and excise DNA protospacer exactly three base pairs upstream of protospacer adjacent motif (PAM). Then repairing of the excised DNA is repaired by nonhomologous end joining (NHEJ) resulting in insertion-deletion variant or homology-directed repair (HDR) resulting in desired gene or nucleotide replacement [28].

This tool is user-friendly and cost-effective, but there are few areas which need improvement. The first area of concern is the guide RNA (gRNA) imperfectly matching with excised DNA sites, indicating off-target activity of CRISPR-Cas9 as the accuracy needs to be spot on to be used for clinical purposes [28]. The second area of concern is that there is genome editing frequency restriction imposed by PAM requirements in addition to poor predictability in efficiency of CRISPR-Cas9 excision at varying DNA target sites [28]. The potential clinical use of CRISPR-Cas9 gene tool relies on advancements in understanding of cellular development, function and viability, and in overcoming several challenges posed while using this tool.

4.3 MicroRNAs

There has been an immense recent interest in studying microRNAs (miRNAs) to understand their role in mediating metabolic transitions in DM2 and in complications of DM2. In vivo experiment has shown role of miRNA-26a in β-cell insulin secretion and replication and is thought to regulate peripheral insulin sensitivity through exosomes [29]. This finding is of interest as it shows β-cells being able to influence a broad group of cells. Another study in mice and on pancreatic β-cells of obese and DM2 patients showed overexpression of miRNA-96 which promoted β-cell's proliferative ability and apoptosis inhibition [30]. The impaired glucose tolerance (IGT) is a risk factor for DM2 development, and miRNA-21 was investigated as a potential biomarker for predicting DM2 as it has an impact on generation of reactive oxygen species, manganese superoxide dismutase-2, and 4-hydroxynonenal. In patients who had IGT or DM2, miRNA-21 levels showed a significant positive correlation with glycemic indicators [31]. In women with gestational diabetes mellitus, circulating levels of miRNA-223 and miR-23a were found to be upregulated compared to non-GDM women; hence, these can serve as potential first trimester biomarkers in predicting GDM [32].

The role of miRNA is also being investigated in complications of DM2. A study looked at development of insulin resistance in cardiac cells which causes decreased glucose uptake and increased mitochondrial uptake of fatty acids ultimately leading to dominance of free fatty acid utility for energy production [33]. Furthermore, a lab study of miRNA-320 showed that DM2 cardiomyopathy may be caused by upregulation of miRNA-320 by its induction of CD36 expression leading to cardiac lipotoxicity from increased fatty acid uptake [34]. Insufficient angiogenesis

during the diabetic cardiomyopathy leads to cardiac ischemia, and miRNA-320-3p have been found in exosomes of diabetic cardiomyocytes as a potential regulator of this process [35]. The miRNA-126 and miRNA-210 level assessment in diabetics with and without coronary artery disease (CAD) shows significant correlation in discriminating between diabetes with or without CAD and can serve as potential biomarkers for early detection or progression of CAD in diabetics [36].

Type I diabetes develops due to autoantibodies to islet cells, and T-cells are a major player involved in this destructive process. In obese diabetic mice, induction of miRNA-142-3p has been found to be involved in autoimmunity process, and its inhibition enhances Treg induction/stability leading to reduction in islet cell autoimmunity [37]. Further research into miRNA types involved in the autoimmunity process is warranted. The investigations of miRNA on its influence on pathways leading to diabetes, worsening of diabetes, and causing diabetic complications may allow us to implement genomic editing to influence these pathways.

4.4 Long Noncoding RNA

The long noncoding RNA (lncRNA) and its influence in development of diabetes and its complications warrant consideration when thinking about genomic editing approaches for treatment or cure of diabetes. Two lncRNAs, LY86-AS1 and HCG27_201 expression levels, were studied in diabetic and nondiabetic patients, and it was found that both were expressed at low levels in DM2 patients vs. non-DM2 patients [38]. This would indicate both lncRNA's roles in DM2 and LY86-AS1 serving as a potential diagnostic biomarker for DM2. In DM1, lncRNA which contains single-nucleotide polymorphism Lnc13 rs917997*CC leads to an increase in pancreatic β-cell inflammation [39]. There have also been circulating or placental lncRNA expressions found to be correlated with β-cell dysfunction and insulin resistance which can lead to gestational diabetes mellitus [40].

Increasing evidence suggests that long noncoding RNAs are involved in progression of diabetic cardiovascular complications. The lncRNA Neat1 was investigated in diabetic rat's myocardial tissues, and it was found that during the period of ischemia-reperfusion injury, it was worsened by Neat1's promotion of myocardial apoptosis and autophagy [41]. If findings of this investigation hold true in human studies, it can help us better target diabetic cardiomyopathy. Evidence from animal experiments show that NONRATT021972 lncRNA's atypical expression may contribute to the development of DM2. Furthermore, this NONRATT021972 has numerous functions which may be contributory to pathogenesis of diabetic neuropathy and cardiac autonomic neuropathy, hepatic dysfunction of glucokinase, and myocardial ischemia [42].

5 Conclusion

The diabetes types have varying metabolic pathways which lead to its development and complications. There are some accepted genes and its variants which are found in those who are likely to develop diabetes or have it. Along with the genetic predispositions or changes, there are influential environmental, dietary, and demographic factors which play a role in pathoetiology of diabetes and its complications. Given the developing preclinical evidence, it is necessary to investigate further on the role of genetics and its influence on diabetes from bench to bedside. This understanding will allow us to improve precision and accuracy of genomic editing such that the therapeutics of diabetes and its complications can be developed.

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Conflicts of Interest None to declare.

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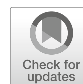

Genome Editing and Protein Energy Malnutrition

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Abstract

Protein-energy malnutrition is a state of disordered catabolism resulting from metabolic derangements or starvation. It is associated with chronic disease, hypoglycemia, hypothermia, serious infections, and even an increased prevalence of morbidity and mortality in countries with poor socioeconomic or environmental factors. Adequate food administration is essential to satisfy the main caloric and nutritional demands of humans. The most significant factors seen in the development of protein-energy malnutrition in areas of high incidence, such as underdeveloped countries, are inadequate food and nutrient supplies. It has been well established that one of the strategies to alleviate undernourishment is the biofortification of staple crops. This is because vegetables and plants are significant sources of

It is worth noting that Sergio Moreno-Nombela and Javier Romero-Parra contributed equally as first authors, while Aiman Tariq Baig and Julio Plaza-Diaz contributed equally as last authors.

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crucial nutrients for human growth and development. To enhance plant nutrition, recent tactics aim to formulated balanced and diverse diets with acceptable levels of vitamins and minerals that benefit human health. New advances in plant biotechnology and animal productivity could control key enzymes in several metabolic pathways, enriching important nutrients such as iron and vitamins and decreasing the content of disadvantageous compounds such as acrylamide-forming amino acids and phytic acids. Numerous biofortified crops such as rice, maize, and wheat have been created to resolve the problem of nutrition deficiencies. Some examples of these methodologies are genome editing engineered nucleases, transcriptional activator-like effector nucleases, zinc finger nucleases, and clustered regularly interspaced short palindromic repeats and associated Cas9 endonuclease which have been created and widely studied for their application, efficiency, and specificity.

Keywords

Protein-energy malnutrition · Gene editing · Malnutrition

1 Background

The World Health Organization describes malnutrition as "the cellular imbalance between the supply of nutrients and energy and the body's demand for them to ensure growth, maintenance, and specific functions" [1]. This disorder is associated with physiological and neurological defects such as growth deficits and tissue damage, reduction in synapses, delayed myelination, and reduced development of dendritic arborization [2]. Although malnutrition can be caused by both deficiency or excess caloric intake [3], this chapter will focus on malnutrition and specifically protein-energy malnutrition (PEM).

PEM is a state of disordered catabolism resulting from metabolic derangements [4] or starvation. It is associated with chronic disease, hypoglycemia, hypothermia, serious infections, and even an increased prevalence of morbidity and mortality in countries with poor socioeconomic or environmental factors [3, 5]. PEM can lead to premature birth, mental disorders, and a higher risk of parasitic disease causing vomiting, among other consequences [2]. The terminology "PEM" envelops a collection of associated disorders that include kwashiorkor, marasmus, and intermediate states of marasmus-kwashiorkor [2].

2 Marasmus

Marasmus, from the Greek word *marasmos* meaning exhaustion, includes insufficient intake of calories and proteins. This results in an overall caloric deficit with depletion of subcutaneous fat stores and muscle wasting. Marasmus is clinically characterized by extreme slimming without any tissue swelling (absence of edema). It is usually contracted by children under five years old as a result of their elevated caloric requirements. These children, apart from being emaciated, emerge weak and lethargic, and their risk of suffering bradycardia, hypotension, and hypothermia is high. As a result of the loss of subcutaneous fat, these kids have the skin that is xerotic and wrinkled but do not have dermatitis. The muscle mass loss is beginning in the groin and axilla. It is followed by the thighs and hips, followed by the abdomen and chest, and finally the facial muscles, whose metabolic rate is lower. Children with marasmus are often apathetic but can become irritable and testy on occasion [3].

3 Kwashiorkor

Another form of PEM, kwashiorkor, which comes from the language Kwa of Ghana means "the one who is displaced," is considered "the sickness of weaning." This is because it is contracted predominantly by children who get

weaned as a result of the birth of a newborn baby and are not capable of raising protein intake through their diet. Kwashiorkor, a term first used as a term in the '30s, appears because of inadequate protein intake even though patients have an adequate caloric intake. The symptoms of kwashiorkor, according to the English Health Service (NHS, https://www.nhs.uk/), are muscle mass loss, an enlarged stomach denoted to as "potbelly," hepatomegaly and hepatic steatosis, regular infections, dermatoses, red and inflamed patches of the skin that darken, and more importantly edema that is distinguishing from marasmus. This edema appears as a result of a mixture of increased cortisol, low serum albumin (hypoalbuminemia), and incorrect inactivation of antidiuretic hormone. Apart from skin changes, children with kwashiorkor suffer changes to their hair texture and color, as it becomes dry, brittle, and depigmented, acquiring a reddish-yellow appearance. Another valid term to define children with this disorder is "sugar baby," because of their inadequate supply of protein but high intake of carbohydrates [3].

A patient with an intermediate state of marasmus-kwashiorkor typically presents as a mixed clinical case with the most common types of both kwashiorkor and marasmus. Those patients are affected with concurrent fat wasting and edema and become atrophied in most cases. The liver grows into a distended tangible fatty liver and the skin and hair turn smooth as well. Findings indicate that kwashiorkor characterizes a maladaptive response to starvation, whereas marasmus indicates an adaptive response to starvation [3].

As we have just seen, these two forms of PEM can result in systemic alterations. On this matter, patients can experience affectations in the endocrine system, symptoms can include reduced levels of thyroid hormone and insulin and increased cortisol and growth hormone levels, and the immune system can also be affected as a result of thymus deteriorate, tonsils and lymph nodes, and impaired phagocytosis. In the gastrointestinal system, a loss of disaccharidases altered intestinal permeability, and pancreatic atrophy, driving to malabsorption, can be seen. The cardiovascular system presents with impaired contractility, bradycardia, hypotension, and consequently a higher risk of arrhythmias. Moreover, these patients possess hematological, neurological, and respiratory alterations, consisting of normochromic anemia and delays in cerebral, motor, and memory functions, as well as a reduction in minute ventilation due to reduced thoracic muscle mass and a damaged ventilatory response to hypoxia as a result of electrolyte imbalances [3].

Other types of PEM have more prevalence among the elderly populations and children in impoverished regions [3], although it is still considered an underestimated problem [6]. Older populations are particularly vulnerable to the development of PEM. It is especially of concern in the older population because it increases rates of morbidity and leads to increased healthcare needs and levels of dependence [7]. One of the main causes of PEM in older population is the insufficient quality of diet as a result of isolation the elderly population is prone to. In line with this, low calorie intake (energy) and high protein intake are nutritional patterns common that are related to PEM in this population. Thus, nutrition is an imperative lifestyle change that is considered as a form of management and treatment in these patients [7]. Treatment of PEM is very critical in this population because it can lead to many other diagnoses such as cardiovascular, liver, renal, and gastrointestinal disorders, among others [8]. PEM also exacerbates other clinical illnesses and is a significant burden on the healthcare system. Interestingly, studies report the frequency of PEM ranges from 20% to 50% among hospitalized patients [5, 9].

According to some studies, there are differences among the sexes when it comes to the prevalence of PEM: females are 45% more prone to be undernourished than men [6]. Looking at different regions of the world, studies indicate that Northern Europe has the smallest malnutrition prevalence (<1%) whereas Southeast Asia holds the highest prevalence (>25%). It is noteworthy that South America and East Asia have a

very small percentage of malnutrition, even with relatively poor socioeconomic conditions, while some advanced areas in Europe have a remarkable prevalence of approximately 17% [6]. This percentage increases to 48.4% if we talk about the risk of suffering malnutrition [10]. Africa and non-Australia Oceania are regions that are understudied; therefore, investigations in these areas are still necessary [6]. People who live in rural areas are more likely to suffer malnutrition and, consequently, complications such as PEM. Rustic areas have fewer access to family support and health assistance and facilities, which explains the increased prevalence [6].

The most traditional treatment for PEM is nutritional interventions; focusing on an increased caloric and protein intake is the main strategy. However, there is no consistent evidence of effective treatments for several of the underlying conditions related to PEM such as pulmonary diseases, heart diseases, and dementia, among others [5]. The nutritional intervention has not been robustly associated with mortality outcomes in these patients; the data does suggest that the PEM associated with chronic illnesses might indicate the severity of their disease [5].

4 Strategic Regulations of Protein-Energy Malnutrition

Adequate food administration is essential to meet the main caloric and nutritional demands of humans [11]. The most significant factors seen in the development of PEM in areas of high incidence, such as underdeveloped countries, are inadequate food and nutrient supplies [12]. On the other hand, developed countries have taken up nutritional strategies to diminish PEM, using nationwide policies to approach dietary modification, supplementation, commercial fortification, and biofortification [13]. Biofortification is a cost-effective and practical procedure that distributes some nutrients to people who may have restricted access to different regimes and foods and other interventions based on micronutrients [14]. The biofortification process increases vitamin and mineral density in a crop through transgenic techniques, plant breeding, or agronomic practices. Biofortified crops will produce quantifiable enhancements in human nutrition and health [15–17]. Efforts have also been made to fight dietary deficits by food-processing approaches and food supplementation; however, many drawbacks have been found [18]. For example, feeding fish with long-chain polyunsaturated fatty acids, omega-3, required for neural development and cardiovascular diseases was attempted [19], to supply the daily requirements; however, the idea was unsustainable [20].

5 Protein Malnutrition and Genetic Modification

As has been well established, malnutrition corresponds to an excess, imbalance, or insufficiency of nutrient intake [21]. It should be noted that a state of nutrient deficiency is not exclusively due to lack of nutrients but also because of an inadequate food supply. Different illnesses such as enteric infections are caused by fungi or bacteria leading to intestinal inflammation with the consequent malabsorption of nutrients [22, 23]. Malnutrition can also be transmitted from mother to child, as affected mothers pass a low birthweight condition to their offspring [22]. To reduce this nutritional disease, one must consider the polymorphisms of genes that modify host genes and the gut microbiome. This affects the effective gathering of nutrients from the diet, the prevalence of enteropathogenic infections, and intestinal inflammation which can result in nutrient malabsorption [22].

As mentioned above, malnutrition is caused by macronutrient or micronutrient deficiencies. Particularly, PEM corresponds to a deficiency of protein ingredients in the diet that exists, constituting a dangerous syndrome that could cause poor brain and body development or even death [24]. Therefore, it is of critical significance to mitigate PEM, especially considering that the mankind is rising, and as a consequence, it is necessary to improve food quality [25]. In this

sense, genome editing has demonstrated to be a significant, preferred, and versatile instrument for crop improvement, together with functional genomics in pulses, cereals, legumes, and fruits and even in animal species in order to create high-quality food in terms of its nutritional use to reduce PEM [25, 26].

PEM is a common condition among children of developing countries [27] causing deficits in their growth, cognitive development, learning ability, and social behavior, among others [28]. Due to the variability of PEM, it is classified into different stages or types: mild, moderate, and acute; the acute stage of PEM being the greatest malnutrition form, which in turn, is subdivided into marasmus and kwashiorkor. Marasmus is predominantly seen in children under one year where they present with deficiency of muscle mass and subcutaneous fat, mainly in the higher limbs and buttocks [24, 29], as well as kwashiorkor [30], where the lack of proteins affects infants manifesting considerable edema in feet and hands [29, 31], as well as fatty liver, hairy discoloration, electrolyte imbalance, irritability, and desquamate rash [32, 33].

Nonetheless, dietary modification, as well as the improvement of agricultural productivity has not yet solved PEM syndrome [24, 34]. Therefore, other strategies and approaches have been evaluated in order to reduce and eradicate PEM. Another approach toward mitigating PEM is the genetic engineering (genome editing) of plants and staple crops as food resources, as well as genome editing in food animals (especially farm animal productivity) which has been the subject of significant controversy.

6 Genetic Engineering: An Opportunity to Control Protein-Energy Malnutrition

Genetic engineering is a terminology that was originally presented in the 1970s to explain the developing area of recombinant DNA technology. Gene editing offers the probability to fix the current biological systems for needed alterations [35]. Recombinant DNA technology began with simple steps such as cloning small DNA sections and producing them in bacteria. It has advanced in an enormous area wherever whole genomes can be moved and cloned from cell to cell operating technique alternatives [35, 36]. Gene editing includes the insertion of leveled DNA at double-stranded breaks created with nucleases. This introduction is monitored through a DNA restoration that happens via both homologous or nonhomologous DNA restoration method. The nonhomologous end joining restoration process involves deletion and insertion in the coding region of the gene [36]. The gene deletion directs to gene silencing/gene knockdown while the gene insertion directs to gene overexpression [36].

7 Strategies to Improve Plants' Nutritional Status Using Genetic Engineering

Plants and vegetables are the principal supply of essential nutrients for normal development and growth. However, 50% of the whole population, mainly Asian and African people, experience deficiency of nutrients as they trust in cereal crops for food [37–39]. The initial verified use of genome editing in plants where the oligonucleotide-directed mutagenesis application, in which oligonucleotides bearing the required nucleotide replacement, would related to triplex DNA and include discrepancy edits by endogenous mechanisms [40]. Mario Capecchi was the first to start gene targeting knowledge and technique, with harnessing double-stranded break (DSB) view for edition of genome [41]. A few years later, the capacity to fix/repair genomes by creating site-specific DSBs was established [42]. After DSBs were created, the cell's restoration system was used to produce the desired genetic product through the imprecise restoration activity of nonhomologous end joining (NHEJ) or the detailed restoration activity of homology-directed repair (HDR) [36, 43–45].

NHEJ can lead to the deletion or insertion of some base pairs and consequently generate functional gene knockouts [46–48]. Chromosomal

deletions, gene inversions, and chromosomal translocations are generated using more than one DSB [49, 50]. On the other hand, HDR creates a correct amendment and allows the sequence to be reshaped in a user-defined approach [51, 52]. HDR is used for genome editing and particular adjustment of the genome with diverse manners of restoration templates, varying from small oligonucleotides to hundred base pairs in distance, all the way up to full genes with homologous ends or even arms flanking the DSB site [53, 54].

To increase the plant's nutrition, recent methodologies aim to offer balanced and diverse diets with acceptable vitamin and mineral levels that improve human health. New improvements in plant biotechnology create it achievable to control the vital enzymes in specific metabolic pathways, enriching crucial nutrients such as iron and vitamins and decreasing the content of disadvantageous compounds such as acrylamide-forming amino acids and phytic acids. Numerous biofortified crops such as maize, rice, and wheat have been created to resolve the problematic of nutrition deficits [55–57].

Methodologies using genome editing engineered nucleases have demonstrated high efficiency. Several genome editing engineered nucleases, reagent-engineered meganucleases, transcriptional activator-like effector nucleases (TALENs), zinc finger nucleases, and clustered regularly interspaced short palindromic repeats and associated Cas9 endonuclease (CRISPR/Cas9) have been created and expansively studied for their efficiency, specificity, and use in numerous systems [58, 59]. The phytopathogen *Xanthomonas oryzae* (Xanthomonas) manufactures transcriptional activator-like effectors (TALEs), which arrive in the cell nucleus and rewrite the transcription machinery to help the pathogen in the plant [60]. These TALEs could be created to bind any DNA sequence [61]. The TALE binding with a nuclease makes an enzyme that can produce DSBs in a site-specific manner in both in vitro and in vivo approaches [62, 63]. The CRISPR/Cas9 is a system constituted with the Cas9 enzyme and a single-guide RNA (sgRNA) molecules [64].

8 Plants, Crops, and Vegetable Nutrition

It has been well established that one of the strategies to combat malnutrition is the biofortification (densification with nutrients) of staple crops, considering that plants and vegetables are essential sources of fundamental nutrients for human growth and development [15]. The HarvestPlus-Consultative Group, through its Biofortification Challenge Program, has focused on the biofortification of some crops such as beans, maize, rice, and pearl millet, among others, mainly targeting Zn, Fe, and vitamin A [65].

An example of biofortification is carried out in certain millet classifications due to its nutritional richness, which contains a high protein amount, iron, zinc, dietary fibers, calcium, potassium, phosphorus, vitamin B, and essential amino acids [66, 67]. However, millet also contains compounds that impair the digestion and absorption of protein, vitamins, and minerals like polyphenols, phytates, and tannins with the ability to reduce cation bioavailability (Fe, Zn, Ca, Mg, and K) by chelation. Thus, millets are susceptible to biofortification by increasing the nutrient collection in milled grains and through decreasing the polyphenol, phytate, and tannin levels to improve the mineral bioavailability [68]. The aforementioned is carried out by taking into account the significance of germplasm description of millets to extend biofortified mixtures and the usage of omic methodologies to increase grain nutrient density [68]. Since PEM is related to protein deficiency, it should be noted that finger millets (one of the existing types of millets) contain a significant proportion of essential amino acids compared to cereals [69]. Indeed, high lysine and tryptophan can be found in this species.

In this sense, different finger millet genotypes were assessed for diversity looking at germ protein abundance with PCR-based methodology, revealing specific marks discerning low- and high-protein genotypes. The aforementioned study has the objective of choosing superior genotypes for use to produce high-quality

proteins in traditional breeding [70]. One of the features of the high amounts of tryptophan and lysine in finger millet is recognized to the transcriptional process in the order of amino acid catabolism genes via Opaque2 (o2), where o2 transformers (*Opm*) decrease lysine ketoglutarate reductase dehydrogenase [71] and upregulate aspartate kinase [72], developing tryptophan and lysine levels in the endosperm. Molecular description of *Opm alleles* is operated with simple sequence repetitions, and single-nucleotide polymorphisms can efficiently recognize quantitative trait loci (QTLs) inducing amino acid levels [73]. Utilizing the functional potential of comparative genomics, elevated tryptophan finger millet genotypes were identified from a global collection using genic simple sequence repeats for *Opm* genes [68]. Remarkably, a 220-bp allele of simple sequence repeats locus OM5 indicator planned from the 27-kDa γ-zein gene of *Opm* was predominant in the great tryptophan-containing genotypes [74]. This indicator can be used in marker-assisted breeding for introgression of *Opm* allele into over-yielding cultivars. Fine mapping of the *Opm* genes related to QTLs can lead to genetic improvement of germ protein quality in small millets and cereals [75]. Hence, crop-specific genes for high protein content can be beneficial in the engineering of other millets, as well as for protein fortification/supplementation in different types of cereals and staple crops.

Another approach to obtain better food nutrition in terms of resources, quantity, and quality is to reduce gene overexpression or gene silencing, respectively [76]. In this manner, genetic engineering biofortification requires knowledge of some key information, such as gene identification and molecular markers. The latter allows improved growth, development, yield, health, and nutritional value among other properties of food crops that include legumes, fruits, cereals, and so on [77]. A possible strategy to regulate PEM is genetic engineering or gene editing. This technique involves the introduction of a targeted DNA using a nuclease which is responsible for the DNA double-strand breaking with a subsequent deletion and insertion in the gene coding region [36] leading to an overexpression.

Many studies reporting improvements in plant's biochemical composition by genetic engineering have been done [78–140] and were extensively reviewed by Praveen Guleria et al. [24]. Some examples of improved nutritional transgenic food are golden rice (rice transgenic rice, abundant in α-carotene), transgenic maize, carrots, soybeans, and potatoes, among others. Table 1 summarizes some examples from these transgenic plants.

There are several methods to obtain transgenic organisms; in the case of golden rice, it was produced by overexpression of the carotenoid biosynthetic pathway genes [92]. Furthermore, a golden rice two was also developed, which indicated 23-fold higher α-carotene content than golden rice due to a substitution in the daffodil *Phytoene synthase* gene with the *maize Phytoene synthase* gene [115]. Transgenic carrot is another case of α-carotene-rich source, where an overexpressing of *α-carotene ketolase Haematococcus pluvialis* cDNA allows an increase of this bio-substance [132]. Similarly, transgenic soybeans represented a good goal in editing genes, as it showed higher amounts of α-carotene than control plants. In fact, the endosperm of transgenic seeds is more orange than usual [133]. It is interesting to note that an increase in the oleic acid and protein levels of transgenic soybean was also seen when the *Phytoene synthase* gene from *Pantoea ananatis* was overexpressed. Considering protein importance, a transgenic rice altered with two novel iron-binding proteins, soybean ferritin and human lactoferrin, has been established [87, 104]. The lactoferrin importance lies in that it binds with two iron atoms, increasing their iron content by 120%. Unfortunately, it has been demonstrated to be inadequate to balance the deficiency of iron in adults [18, 141]. Nevertheless, transgenic rice has shown a greater expression of soybean ferritin gene which showed an enhancement by double threefold its iron content (see Fig. 1) [18, 24, 87].

It should be noted that *Phaseolus vulgaris Ferritin*, *Oryza sativa metallothionein*-like, and *Aspergillus fumigatus phytase* gene expressions were able to improve the iron content by twofold

Table 1 Genetic changes presented in plants for increasing biochemically effective transgenic plants

Reference	Genetically modified plants	Transgene	Introduced adjustment
Falco et al. [79]	Canola	Lysine feedback-insensitive dihydrodipicolinic acid synthase	Increased lysine content
Dehesh et al. [81]	Canola	*Cuphea hookeriana* Acyl-ACP thioesterase Ch FatB2 gene	Greater accumulation of 8:0 and 10:0 fatty acids in seeds
Shewmaker et al. [88]	Canola	Bacterial phytoene synthase gene	50-fold increment in total carotenoid levels
Römer et al. [93]	Tomato	Bacterial carotenoid gene	Increased b-carotene content
Rosati et al. [94]	Tomato	Lycopene beta-cyclase gene	Augmented b-carotene content
Fraser et al. [99]	Tomato	*Phytoene desaturase* gene or *Erwinia uredovora phytoene synthase*	Threefold increase in b-carotene content
Mehta et al. [101]	Tomato	Yeast S-adenosylmethionine decarboxylase gene bonded with a ripening-inducible E8 promoter	Augmented polyamine and lycopene levels. Improved lifetime and juice quality
Goto et al. [87]	Rice	Soybean ferritin gene	Increased iron content
Lucca et al. [97]	Rice	*Phaseolus vulgaris* ferritin gene	Improvement in iron, cysteine residues, and phytase contents
Vasconcelos et al. [106]	Rice	Soybean ferritin gene under endosperm-specific glutelin promoter regulations	Greater zinc and iron content
Kawakatsu et al. [129]	Rice	Lysine-rich binding protein	Augmented lysine content

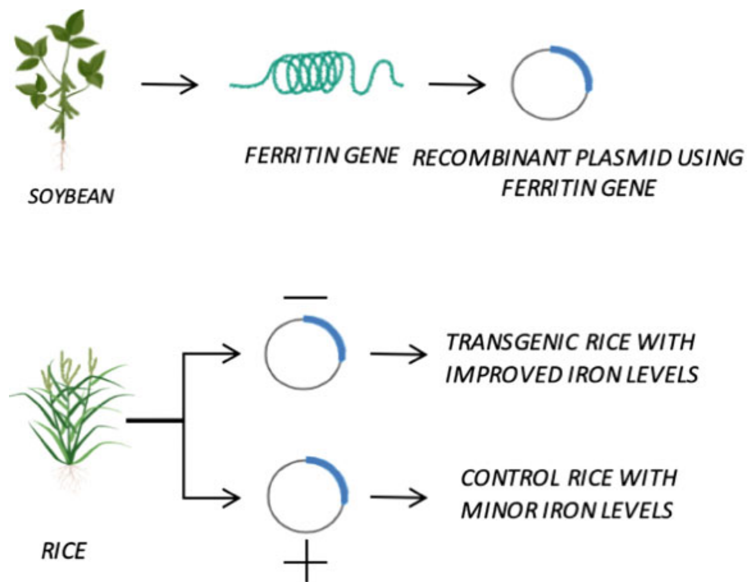

Fig. 1 Improvement of iron-fortified transgenic rice. Ferritin gene was extracted from soybean and hosted to rice plants via genetic engineering strategy [24]

and cysteine sevenfold. Given that cysteine helps in iron absorption, transgenic rice supplemented with cysteine and iron residues can balance iron insufficiency and combat anemia [97]. Concerning the importance of amino acids and proteins, several attempts have been made to

increase these nutrients into the food crop to reduce PEM; several transgenic methods have been developed in order to achieve this task. For example, the expression of the *Corynebacterium* lysine feedback-insensitive dihydrodipicolinic acid synthase (*DHDPS*) gene in canola resulted in a 100-fold rise in lysine expansion. Similar results of this amino acid increase were obtained when co-expression of lysine feedback-insensitive *E. coli* aspartokinase gene along with *DHDPS* gene in soybean was performed [79], and a comparable effect was obtained in the lysine levels of transgenic maize grain [86]. Moreover, potato transgenics where the *Threonine synthase* gene is downregulated by antisense inhibition have been demonstrated to store 239-fold greater methionine than control plants. In the same way, sweet potato has a low percentage of proteins; and then to improve their amino acid, protein, and polyphenolic contents, high expression of the amaranth seed storage albumin gene was made with positive results [142]. Regarding other species, the amaranth plant possesses a fundamental amino acid-rich storage protein named amaratin, which is encoded by the *11S globulin gene*. Thus, overexpression of *11S globulin* cDNA increases the essential amino acid and total protein contents of maize transgenics [111]. Finally, in another effort to solve the PEM problem, a study has reported that overexpressing the rice phosphate transporter gene OsPT2 leads to nitrogen fixation and ammonium assimilation in soybean plants, helping the growth of legumes (whose protein content is high) in nutrient inadequate soil [143].

9 Animal Nutrition

Currently, a higher requirement for animal protein is expected, where it is estimated that milk fabrication will need to grow by 63% and meat production by 76% [144]. In this sense, genetic improvement represents cheaper, faster, healthier, and more efficient animal fabrication, with abridged impact on the environment [25]. From the 1960s to 2005, consumption and production of pigs were increased in kilograms of feed intake. In the case of chickens, the time to obtain 2 kg of mass is condensed from 100 days to 40, the breast meat percentage is augmented from 12 to 20%, eggs produced per year are augmented by 30%, and eggs per ton of feed increased by 80%. Also, in cattle, milk production augmented by 67% [145].

Farm animal genetic engineering differs from that of plants since animals can simply progress from germline cells. Two basic approaches to produce genetically engineered animals are cytoplasmic injection (pronuclear injection or microinjection) and somatic cell nuclear transfer (cloning) [146]. In cloning, primary cells are used in vitro and are transfected by viral transfection or electroporation. Once the chosen DNA modifications are made, the genome-edited somatic cell is joined with an enucleated egg cell to generate a viable, genome-edited embryo. Conversely, microinjection implies a genome editing complex injection into a zygote. However, microinjection usually results in mosaicism (a combination of unedited and edited alleles) [147], and that is why, cloning methods are still extensively utilized in the genome editing process for animals [148, 149], although normally it does entail abortions, birth defects, and early postnatal death [148].

Presently, there are no commercial genetically modified farm animals available worldwide, except genetically modified salmon in the USA and Canada [150]. Nonetheless, market-oriented analyses of genome-edited farm animals have been recently growing [148, 151] indicating that in the near future, genome-edited farm animals as food could be marketed. In any case, genome-edited farm animals exist. In general terms, all of them are produced to increase productivity (e.g., enhanced muscle growth in cows) or increase efficiency of production (e.g., increased antibacterial properties in cow's milk), being the most used molecular mechanisms the SDN-1, SDN-2, and SDN-3 [148, 152]. Nowadays, programs for aquaculture species and major livestock now regularly include genomic selection, which has been an innovatory transformation for food production and selective breeding [25]. The major farm animal genomes have been sequenced [153–155]. Hence, based on the latter, genomic

tools [156, 157] and cheaper and novel sequencing technologies [158, 159] have been a main step forward. This will contribute to the productivity of farmed animals and enhanced modern animal breeding.

Genome editing and transgenic tools offer new opportunities for genetic development. In this manner, genetic modification has positive applications, as well as genome editing to improve farm animal productivity [25]. In the case of genetic modification, the task specifically aims to enhance food production efficiency and upsurge animal health and welfare. One of the most relevant features to improve is the muscle development (body mass) by the liver and pituitary gland, as well as the growth hormone-insulin-like growth factor axis. Through the expression of muscle- and/or liver-derived insulin-like growth factor 1 (IGF-1), muscular hypertrophy can be induced. A study inserted genes encoding two growth-related hormones (growth hormone and IGF-1) into pigs through a microinjection of DNA into zygotes, obtaining positive results for the pigs expressing the transgene encoding growth hormone [160]. Another transgenic pig study (considering only the IGF-1) leads to the obtaining of less fat and leaner tissue by these animals. Unfortunately, the project had to cease due to pigs demonstrating an augmented lethargy, gastric ulcers, lameness, and a minor capacity to reply efficiently to stress [161]. Dairy cattle are susceptible to suffer mastitis owing to *Staphylococcus aureus*; thereby transgenic strategies have been applied such as the case of goats expressing human lysozyme that has been shown to inhibit the mastitis by bacterial growth [162, 163] or transgenic cattle that express the antibiotic lysostaphin in their milk [164]. As was mentioned above, the first genetically engineered salmon for sale, a landmark of genetically modified food animals, has proven to work well for consumption in the USA and Canada. The genetically modified task produced a rapid-growth salmon phenotype relative to wild types due to growth hormone gene integration in Chinook salmon (*Oncorhynchus tshawytscha*) together with a promoter from the cold-water ray-finned fish ocean pout (*Macrozoarces americanus*) [165]. Regarding Atlantic salmon, genome editing targets the dead-end protein encoded by the dnd gene in order to induce sterility in them preventing the escapees from breeding with wild stocks [166].

Genome editing is used to try and make precise and specific changes in the animals' genome increasing their productivity and disease resistance [25]. Up to the present time, the farmed animals in which the myostatin gene has been modified include cattle [167], sheep [167, 168], goat [169], and channel catfish [170]. The pig myostatin gene has been the most commonly targeted [171–177] and extensively studied by researchers, such as Ning Li and colleagues [178], Kang et al. [179], and Wang et al. [172], among others, although development problems and health issues were reported in homozygous knockout pigs. Sonstegard and colleagues knockouted the *KISS1R* gene of pigs, which encodes a receptor for the puberty onset in vertebrates and is intricated in the regulation of gonadotropin-releasing hormone [180]. The latter was done in an attempt to reduce aggressive behavior and to avoid the boar taint taste and odor of non-castrated male pork. Nonetheless, genome-wide association studies further emphasize that the boar taint mechanisms and testicular trait regions have pleiotropic properties, which might influence the genetic intervention applicability for this trait [181, 182].

In polled livestock, the natural hornless cattle is thought to have this particular trait due to one of two alleles [183, 184]. Thus, to reduce physical dehorning in dairy cattle, Carlson and colleagues [185] used TALENs to insert the allele Pc POLLED into the genome of bovine embryo fibroblasts from four lines of cattle. These were cloned with somatic cell transfer, resulting in full-term pregnancies for three of the four lines. Five live calves were created and all of them were regulated to have a likely polled phenotype at birth, where the two surviving calves were confirmed to be polled, strongly suggesting the Pc POLLED allele the causality [25].

Finally, genome editing, combined in some cases with transgenic technology, permits the insertion of novel properties to animal protein

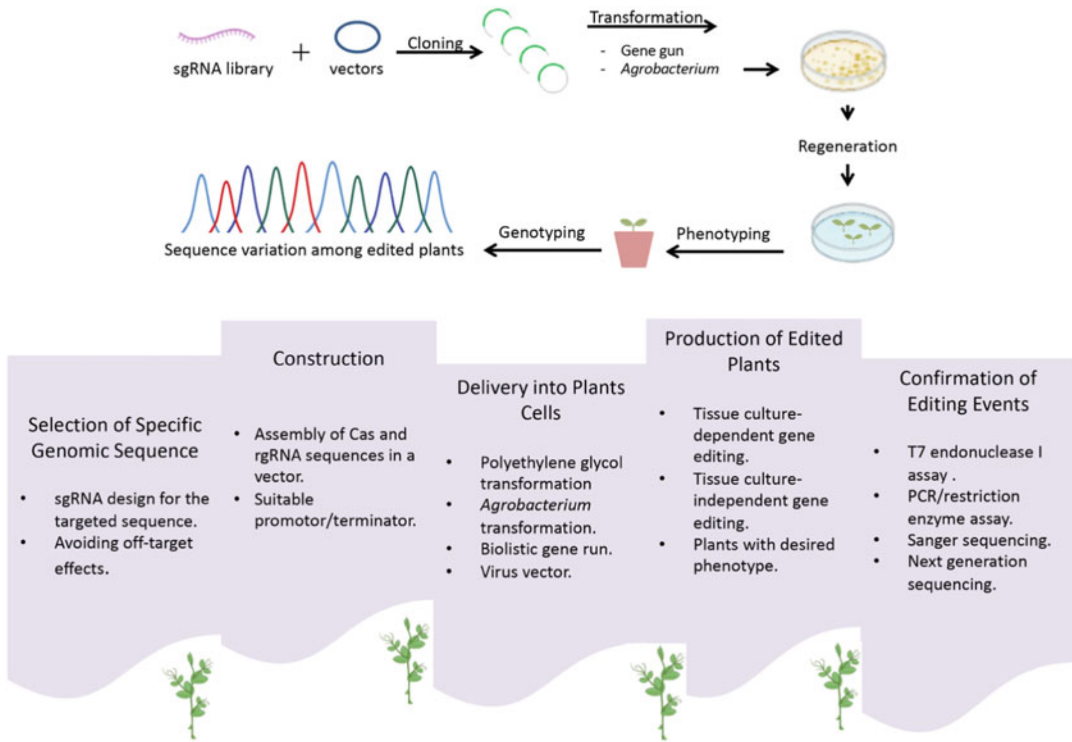

Fig. 2 Gene editing process [64]

that can have possible benefits for the diet in humans. These are the cases of cloned pigs expressing a *fat-1* gene from *Caenorhabditis elegans*. This gene reduces omega-6 to omega-3 fatty acids which is more beneficial [186]; these pigs now encode an omega-3 fatty acid desaturase due to having the *C. elegans fat-1* gene [187, 188]. In fact, Li and colleagues used CRISPR-Cas9 gene editing knowledge for directed integration of the *fat-1* gene from *C. elegans* into the porcine *Rosa* 26 locus [189]. Finally, Fig. 2 summarizes the gene editing process and steps.

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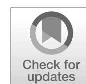
Part V

Therapeutic Implications



Gene Therapy and Cardiovascular Diseases

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Abstract

Cardiovascular diseases (CVDs) are the leading causes of death globally and urgently require new novel therapeutic strategies. Gene therapy is the application of gene modulation technology to treat abnormal gene expression under disease conditions. Viral- and nonviral-based gene delivery systems are the foundation of gene modulation in target cells. Moreover, plasmid- or oligo-based gene modulation tools as well as new advancements in gene editing using CRISPR/Cas technology are currently being tested in a variety of clinical trials. Here, we summarized state-of-the-art gene therapy technologies as well as recent clinical trials and discuss the applications and lessons of gene therapy in CVDs.

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Keywords

Gene therapy · RNA therapy · Cardiovascular diseases

1 Background

Genes, originating from segments of DNA or RNA, are the basic building blocks for the traits that make up organisms [1]. Phenotypic traits derive from a combination of our genetic material and the environment we stem from. This genetic material, including nuclear and mitochondrial DNA, is expressed through the transcription into RNA, which can act as functional molecules themselves or which can be translated into functional proteins [2, 3].

Importantly, gene sequences can exhibit dysfunctional behaviors which are known as mutations, and these mutations have the potential to lead to the development of diseases. These diseases caused by gene mutations are categorized as chromosomal diseases, gene disorders, or mitochondrial dysfunction [4]. In addition, certain infectious diseases such as acquired immune deficiency syndrome (AIDS) as well as some noncommunicable diseases like cancer, are known to be mediated by gene abnormalities. For example, mutations in the DNA repair gene, breast cancer gene 1 (BRCA1), are associated with an increased risk of a variety of cancers such as prostate,

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breast, and ovarian cancers owing to high loads of DNA damage and resulting in genomic instability [5, 6]. It is reported that people with a BRCA1 mutation have an extremely high risk for developing breast cancer (87%) and ovarian cancer (44%) compared to noncarriers of this mutation [7].

Several traditional treatments such as surgery, chemotherapy, and radiation as well as novel approaches such as hormone-based therapies, stem cell therapies, or immunotherapies, are widely used for cancer treatments and for targeting cardiovascular diseases (CVDs) [8–14]. Quite often, traditional therapies are not always successful at correcting the mechanism by which the disease occurs and rather treats the symptoms of the disease instead. Gene therapy, on the other hand, aims to target and potentially correct any genetic mutation causing a disease, providing a new treatment option which focuses on the initial source of any illness [15]. Initially, gene therapy was designed to introduce a new healthy copy where a gene was either mutated or absent in cells via a vector. The restoration of gene function following a therapeutic modification results in the correction of genetic abnormalities stemming from hereditary or environmental processes. With the advancement of gene therapy, however, also comes new techniques by which to manipulate the genome. New mechanisms involving gene editing and inactivation have emerged in recent years such as the CRISPR/Cas system and antisense strategies encompassing RNA-based therapeutics. While the disruption and silencing of genes through direct DNA and RNA editing tools are new and exciting developments in this field, we will primarily focus here on the state-of-the-art vehicle delivery approaches of introducing genes into cells [16].

2 History of Gene Therapy

The concept of gene therapy as a gene modification tool has been around since the 1970s. Despite the beneficial potential in reversing possibly life-threatening mutations, gene therapy also raised deep ethical concerns surrounding genetic modifications [17]. However, the field of gene therapy continued to grow since the 1980s, when the retroviral vector system was developed to efficiently deliver transgenes into mammalian cells and modify preexisting genes [18]. By the 1990s, the first approved gene therapy was applied to two children in the USA who suffered from adenosine deaminase deficiency-severe combined immunodeficiency (ADA-SCID). Two years after the gene therapy treatment, which was performed ex vivo after T cell apheresis using cell culture expansion and reinfusion into the patients after 9–12 days, the integrated vector-mediated ADA gene remained expressed in T cells [19]. This report was the first positive indicator that gene therapy could be an efficient and safe treatment option for patients suffering from immune deficient diseases [19]. Apart from genomic modifications, RNA interference (RNAi), in particular small interfering RNA (siRNA), has also been developed as a gene silencing therapy to block abnormal RNA or protein expression which may lead to disease [20, 21]. In 2003, siRNAs were first shown to mediate Fas cell surface death receptor (FAS) knockdown in vivo, which allowed for a reduced threat from fulminant hepatitis [22]. Notably, the first human trial targeting the vascular endothelial growth factor (VEGF) and kinesin spindle protein (KSP) used lipid nanoparticle (LNP) formulation of siRNAs. This technique was applied to cancer patients in 2013, providing both safe and pharmacokinetically sound evidence that siRNA-mediated gene therapy could be used effectively in humans [23]. In 2008, treatment of Leber's congenital amaurosis (a rare disease typically causing severe visual impairment) by recombinant adeno-associated virus 2 (rAAV2)-

RPE65 became the first effective AAV-mediated gene therapy to show clinical efficacy and disease improvement in patients. Three parallel trials proved that patients who got a single subretinal injection of rAAV2-RPE65, to complement the causative mutation in the RPE65 gene, had long-term improvement in vision and light sensitivity [24–26]. Importantly, the follow-up studies showed persistent visual improvements in patients and did not raise any safety concerns [27–29]. Despite the successes seen with viral vectors in clinical trials, other gene editing techniques were advancing in parallel. With the development of the engineered clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated (Cas) nuclease technology, the ability to manipulate DNA became increasingly simplified, efficient, and cost effective [30, 31]. The CRISPR-Cas technology improved the possibility of gene therapy mediated by engineered cells such as chimeric antigen receptor T cells (CAR-T) [32]. These engineered CAR-Ts were produced to recognize, target, and destroy cancerous cells in a more effective and localized manner [33]. After the first successful clinical trials using engineered CAR-T therapy for lung cancer in 2016, they have since been further utilized in active clinical trials for the treatment of leukemia, lymphoma, and solid tumors [34–38]. The existence of clinical trials using multiple gene therapeutic techniques brings to light the high demand and great advancements of multiple technologies that are being pursued to treat genetic abnormalities.

3 Material and Approach of Gene Therapy

Currently, the field of gene therapy has been broadly studied; however, it is still a therapeutic concept predominantly based in research laboratories with only a limited number of ongoing clinical trials [39]. The efficiency and specificity of gene delivery as well as gene regulation utilized in target cells are the two major obstacles that must be overcome to successfully achieve safe and effective genetic modifications [40]. In this part, we will summarize the viral and nonviral approaches that are currently utilized in gene therapy.

4 CRISPR-Cas-Mediated Gene Editing

Primarily, we see gene therapy as using a vector to replace a mutated or missing copy of a gene. Another form of treatment that is newer to the field of gene therapy involving editing and inactivation is the CRISPR/Cas system. This mechanism avoids the complications and risks associated with some viral vector delivery and the correlating toxicities and safety concerns that have been seen in several clinical trials over the years [41]. The CRISPR/Cas system was the most recent gene editing technique after the foundational mechanisms using transcription activator-like effector nucleases (TALENs) and first zinc finger nucleases (ZFN). This genome editing tool can be delivered to cells through AAV vectors [42], which have a safer history comparative to other viral vectors, as well as through other non-viral delivery strategies. CRISPR/Cas operates via the specific targeting of a segment of DNA in the genome by utilizing a particularly designed single-guide RNA (sgRNA) to identify only the region requiring intervention. Optimizing this specificity allowed by sgRNAs will continue to help reduce off-target effects currently seen by this gene editing technique [43, 44]. The Cas protein, an endonuclease allowing for breaks in DNA at the target site, can also be modified to reduce side effects. These modifications, however, do not make the CRISPR/Cas system superior or inferior to standard gene replacement therapy using viral vectors. Both methods have different advantages and flaws unique to their mode of action and delivery mechanism [41]. The mechanism by which the Cas protein cuts DNA inducing double-stranded breaks (DSBs) can also lead to the unintended activation of apoptosis pathways, such as triggering p53, instead of editing the DNA segment after the break [45]. One recent development with this technique is using the base editing (BE) system.

This allows for a single targeted base pair to be exchanged, for example, a C-G base pair can be exchanged for a T-A by cytidine base editors (CBEs) and the reverse mutation can also be corrected for A-T pairs exchanged for G-C using adenosine base editors (ABEs). This occurs by using a catalytically deactivated Cas9 endonuclease (dCas9) that does not induce DSBs, allowing for single base pair edits [46, 47]. RNA is also edited using CRISPR technology with the endonuclease Cas13 (Cas9 can be modified to target RNA instead of DNA as well; however, Cas13 exclusively targets RNA). Since this system does not require the protospacer adjacent motif (PAM) sequence that is necessary at the DNA editing sites, Cas13 can be more broadly used. There is an additional advantage, in that the Cas13 system does not permanently edit the genome since it is targeting RNA after transcription, resulting in nonpermanent changes (which could trigger immune reactions or lead to incorrect editing with DNA [48]). The method for base pair editing is similar to that of the dCas9 system, using dead Cas13 (dCas13) with the ADAR2 domain to edit adenosine to inosine in what is known as the REPAIR mechanism or with APOBEC1 for exchanging a cytidine to uridine using the RESCUE technique [41, 48–50].

The first CRISPR clinical trial utilized PD-1 edited T cells to treat non-small-cell lung cancer in China. Most patients had minimal side- and off-target effects, and a decrease in disease progression was also seen when edited T cells reached higher levels in patients [51, 52]. Edited PD-1 and CAR-T cells were also used as a combined treatment for the first CRISPR clinical trial to take place in the USA in 2018 to treat myeloma and sarcoma. The study was also deemed a success, in that it provided initial findings in combination with the first Chinese trial that CRISPR editing as a treatment for disease progression seemed to be relatively safe with acceptable side effects. Treatments in both trails did not produce an overwhelming immune response either, which was an early problem that was observed in some of the first clinical trials using gene replacement therapy [53]. Both off-target and on-target mutations were seen in both trials; however, while this safety concern is still valid and needs to be closely monitored in all future DNA editing trials, neither effects were detrimental to the patients and were found to take place primarily in noncoding segments of the genome.

Aside from cancers, CRISPR has also taken its first step in 2019 to treat a genetic disease, sickle cell disease (SCD), by increasing fetal hemoglobin levels in isolated and edited autologous blood stem cells [54]. These stem cells are reintroduced into the body of the patient and can then create a new population of hemoglobin-producing blood cells from the bone marrow. This technique is also quite specific as it involves ex vivo editing of the blood cells directly, which greatly reduces off- and on-target side effects seen with CRISPR editing through a delivery vector into the patient [55]. Overall, CRISPR editing to treat diseases of both genetic and acquired origins is still in its early stages. So far, the clinical trials that have taken place in the last few years have been used to primarily assess feasibility, toxicity, tolerability, and practicality before shifting the focus to successfully cure a disease [56].

5 Nonviral-Mediated Gene Therapy Methods

In the early 2000s, nonviral approaches were not a common tool for gene therapy due to low delivery efficiency and specificity [57]. In the past few years, production and modification of nonviral methods have greatly improved and led to a higher gene transfer efficiency while also allowing for long-term gene expression, not only in vitro but also in vivo. In addition, the low cost, ease of production, and reduced pathogenicity of nonviral applications have important manufactory and safety advantages over viral approaches [58]. Currently, siRNAs or RNA inhibitors, RNA mimics, modified mRNAs (modRNA), and other

oligonucleotide-based molecular products are gaining attention as potential therapeutic materials in the application for gene therapy [59].

6 siRNA and RNA Inhibitors

Since the first RNAi phenomenon was reported in 1990 [60] and the mechanisms behind it were clarified in 1998 [61], siRNAs have become a regular tool to perform gene inhibition in cell culture. SiRNAs are small RNA transcripts with a length of approximately 20–22 nucleotides and can disrupt protein translation by promoting the degradation of RNA transcripts through binding to the targeted mRNA [62]. Similar gene silencing can also be reached by antisense oligonucleotides (ASOs). These synthetic, single-stranded oligonucleotides prevent expression of a target protein by blocking the specific region of target RNA or DNA [63, 64]. For example, locked nucleic acid (LNA) is a kind of modified ASO with a bridged, bicyclic sugar moiety. LNA will bind to the target RNA forming a DNA-RNA hybrid, and RNase H-dependent degradation of the targeted RNA will then be activated [65].

MiR-132 is a breakthrough example of RNA gene therapy and is the first antisense gene therapy to treat CVDs. Since 2011, miR-132 has been reported as a regulator of cardiac fibrosis [66, 67], cardiac hypertrophy, and cardiomyocyte autophagy [68]. A series of preclinical investigations further proved that the inhibitor of miR-132 (antimiR-132) could rescue cardiac hypertrophy and heart failure in mice and more importantly in pigs [68–70]. Notably, CDR132L, the miR-132 inhibitor applied in pigs, is a synthetic LNA-ASO modified with a fully phosphorylated backbone. In addition, large animal investigations reported a safe administration, high cardiac delivery efficiency, and clear reduction of miR-132 expression in the myocardium and plasma [69, 70]. With these promising preclinical results, CDR132L moved forward for the first-in-human study in 2019 involving 28 patients with stable chronic heart failure of ischemic origin (NCT04045405). Safety, pharmacokinetics, and heart failure relevant pharmacodynamic parameters are all intensively evaluated in this phase I clinical trial. After a 1-year follow-up, CDR132L has proved to be safe to administer to patients and can also be well tolerated without an apparent dose-limiting toxicity. Interesting, reductions of NT-proBNP, significant QRS narrowing, and positive trends for relevant cardiac fibrosis biomarkers were reported after CDR132L treatment in heart failure patients with the standard care of treatment [71].

Notably, several siRNA-mediated gene therapies have been approved and applied in the clinics [72]. For example, Alnylam's Onpattro (Patisiran) (NCT01960348) was approved by the Food and Drug Administration (FDA) as a novel RNA interference drug to treat hereditary transthyretin-mediated amyloidosis (hATTR), a rare disease characterized by extracellular amyloid protein deposition leading to multiple organ dysfunction [73]. In addition, several siRNA-mediated RNAi drugs are currently in clinical trials (phase II/III) such as SYL1001Sylentis (NCT 03108664) for dry eye disease or QPI-1007 Quark (NCT 02341560) for non-arteritic anterior ischemic optic neuropathy. Detailed siRNA and RNA inhibitor clinical trials are excellently summarized elsewhere [72, 74].

7 Nucleic Acid Drugs

The first successful genetic transfer in mice occurred in the 1990s with an overexpression of chloramphenicol acetyltransferase, luciferase, and β-galactosidase by an in vitro transcribed (IVT) RNA or DNA plasmid in the skeletal muscle [75]. In the following years, IVT mRNA was introduced for diverse applications, including protein substitution and vaccination approaches for cancer and infectious diseases [76–79]. IVT RNAs are synthesized RNAs that can be transcribed in vitro from DNA templates containing the sequence from either protein coding genes or noncoding RNA transcripts [80]. ModRNAs are IVT RNAs with modified nucleosides or synthetic nucleoside analogues which could reduce the innate immune response of the host cell and

improve tissue specificity. IVT RNAs have come into focus as novel drugs to revise abnormal genetic disorders, allowing for the overall improvement in the field of RNA pharmacology [81].

Although oligo nucleic drugs remain in the initial stages of preclinical or phase I/II clinical trials, some of the pilot investigations have broaden the potential applications of IVT RNA as the future of medicine. For example, in cancer immunotherapy, Melan-A, tyrosinase, gp100, Mage-A1, Mage-A3, and survivin IVT mRNA were utilized in metastatic melanoma patients in a phase I/II trial (NCT00204607) [82]. In addition, several phase I/II clinical trials using IVT mRNAs for the treatment of HIV infections demonstrated the safety of IVT mRNA vaccines and observed the induced responses of immunogens in CD8+ and CD4+ T cells [83–85]. Detailed IVT RNA clinical trials are well reviewed by Sahin et al. [86].

8 Viral-Based Approach for Gene Therapy

The advantage of viral vectors is their high infection efficiency in a broad spectrum of cells, ranging from prokaryotes to many eukaryotic cells. Therefore, recombinant viral vectors have the potential to package and deliver the transgene to the targeted cells. Viral vectors can be divided into genome-integrating vectors as well as non-integrating vectors, classified by whether the transgene can be continuously expressed in dividing cells [87].

Most RNA viruses with single-stranded RNA (ssRNA) or double-stranded RNA (dsRNA) are not able to integrate their genome into the host chromosome, with the exception of retroviruses. One of the best studied retroviruses is the human immunodeficiency virus type 1 (HIV-1) [88]. The first retroviral vectors which were used in human gene therapy trials [19] are derived from the Moloney murine leukemia virus (MLV) [89].

Lentiviruses are a complex subtype of retroviruses which can cause chronic and deadly diseases. Notably, the outstanding feature of lentiviruses is the high efficiency of infection and genomic integration in nondividing and terminally differentiated mammalian cells, including lymphocytes and macrophages. In addition, the ability to transport large genetic payloads as well as their stable long-term transgene expression makes them a very attractive tool for gene delivery [90, 91]. So far, three generations of lentiviral vectors have been developed for transgene modification [92]. First-generation lentiviral vectors originate from a significant portion of the HIV genome, including the gag and pol genes encoding for viral structural proteins and the viral RNA reverse transcriptase, respectively, as well as several additional viral proteins such as the envelope protein (VSV-G) [93]. VSV-G recognizes a ubiquitously expressed receptor such as low-density lipoprotein receptor (LDL-R) [94], which aids in a high transduction efficiency of the lentiviral vector in a wide range of cells [95]. The main improvements that were made to the second and third generations of lentiviral vectors were regarding safety. Second-generation lentiviral vectors were subsequently developed to remove accessory gene factors such as vif, vpr, vpu, and nef. Third-generation vectors split the viral genome into separate plasmids and removed the tat gene to further improve the safety of the vectors [96]. In 2003, the first lentiviral clinical application occurred by delivering a long antisense RNA sequence targeting the HIV-1 envelope gene for anti-HIV therapy [97]. It is important to note that eight years after the study, there was no apparent risk for serious adverse or long-term events occurring in this clinical trial [98].

The Sendai virus (SeV) is a member of the *Respirovirus* genus, a negative sense ssRNA virus from the Paramyxoviridae family. Due to the cytoplasmic gene expression of SeV, the absence of genomic integration is a unique feature of recombinant SeV vectors compared to a retroviral vector [99, 100]. SeV vectors have been used in clinical trials and tested in a live attenuated vaccine [101], in cancer [102], as well as in critical limb ischemia [103] for gene therapy.

A DNA viral vector is an additional virus system that employs double-stranded DNA (dsDNA) or single-stranded DNA (ssDNA) as its genomic materials. Adenoviruses (AdVs) are non-enveloped DNA viruses with a diameter of 70nm, a 36 kb dsDNA, and about 50 viral polypeptides [104]. So far, more than 50 different AdV serotypes have been characterized, and a majority of them can also be observed naturally in humans. In gene therapy, AdV types 2 and 5 were found to be good options for clinical trials due to the fact that they were not already associated with human diseases [105, 106]. In addition, adenoviral vectors have a packaging capacity of up to 8000 base pairs (bps) of foreign DNA, which is sufficient for the delivery of most therapeutic genes. Similar to lentiviruses, recombinant AdVs (rAdV) can also infect dividing and quiescent cells with equal transduction efficiency [107]. Notably, rAdVs can obtain a higher production yield (10^{10}–10^{11} infectious particles/ml) compared to other vector systems such as retroviral vectors [108]. However, rAdVs show no integration into the targeting cell genome, indicating short-term expression particularly in dividing cells. In addition, in vivo applications of adenoviral vectors could lead to cellular immunity and the generation of a humoral response, also reducing the expression or the effect of adenoviral therapy. Furthermore, the generation of neutralizing antibodies of rAdV could strongly reduce their utility, resulting in the difficulties of repetitive treatments [109]. In 1993, the first AdV-mediated gene therapy was performed to transfer cystic fibrosis transmembrane conductance regulator (CFTR) cDNA to treat cystic fibrosis in humans. The benefits of AdV treatment were observed, and no virus-associated adverse effect was detected, indicating that adenoviral vectors were effective at transferring genes to most organs in vivo [110]. However, immunogenicity still limits application of the AdVs in clinical trials [111].

In 1965, a number of "satellite viruses" were observed by electron microscopy (EM) from AdVs prepared in the lab [112, 113]. These small DNA viruses (20–25 nm in diameter) were dubbed AAVs due to their ability to replicate in the presence of AdVs [113]. Two years later, AAVs were first isolated from human tissue [114]. AAVs are one kind of *Dependoparvovirus* within the family Parvoviridae, and they have not only been found in humans but also in nonhuman primates. In addition, they are comprised of an icosahedral protein capsid of ~26 nm in diameter and a single-stranded DNA genome of ~4.7 kb [115]. The wild-type AAV capsid is composed of three types of subunits (VP1, VP2, and VP3). Two T-shaped inverted terminal repeats (ITRs) are located at the ends of the viral genome, and the viral replication and packaging signals are flanked between ITRs. Four rep gene-encoded proteins are the source of viral replication, and capsid subunits are alternatively spliced and translated by cap genes through different start codons [116]. The wild-type AAVs also have the ability to integrate into the human AAVS1 genomic locus [117]. In the early 1980s, the secondary structure of the AAV ITR region only allowed for a very limited number of plasmids cloned with AAV sequences [115]. Until 1984, engineered rAAV2 vectors were generated as a useful tool for gene transfer in mammalian cells and had become the foundation of AAV-mediated gene therapy [118]. rAAVs consist of the same capsid sequence and structure as wild-type AAVs. Importantly, removal of viral coding sequences enlarged the packaging capacity of rAAVs and reduced the genomic integration, immunogenicity, and cytotoxicity of AAVs. However, the gene packaging capacity of rAAVs is still under 5 kbs [119]. The best characterized and most widely applied AAV serotype is the naturally occurring AAV2. Notably, AAV9, a clade F AAV serotype isolated from human liver tissues, demonstrates the ability to bypass the blood-brain barrier [120]. Till now, 13 different human or nonhuman primate AAV serotypes have been classified [121]. However, rAAVs are a major type of AAVs which have been utilized in preclinical investigation and clinical trials. Since the early 1990s, clinical trials mediated by rAAV2 and rAAV1 vectors have been tested in several diseases including CF, hemophilia B, Canavan disease, and α1-

antitrypsin (AAT) deficiency [122–125]. These pilot phase I/II trials demonstrated a good gene expression duration of rAAV therapy as well as proved the safety of injection of rAAV.

9 Gene Therapy in CVDs

In the last three decades, several gene therapies have been tested for cardiovascular disorders including coronary or peripheral artery disease and heart failure [126]. After over 100 clinical trials, there has so far been no successful therapeutic effect reported for gene therapy in CVDs. In angiogenesis, therapeutic attempts are focused on the formation of new blood vessels driven by the production of cytokines which have so far been shown to recover some heart function in animal experiments [127]. For heart failure therapy, the modulation of Ca^{2+} in cardiomyocytes has become the main target of interventional therapy. Similar to therapies in angiogenesis, the beneficial effects of gene therapy in heart failure observed in animal studies did not translate to clinical trials in the last two decades [40]. Here, we will summarize the current clinical trials of gene therapy in CVDs.

To induce the formation of new capillaries or blood vessels, cytokines such as VEGF, FGF (basic fibroblast growth factor), and G-CSF (granulocyte colony-stimulating factor) have been tested in clinical trials as a form of gene therapy for CVDs [128, 129]. This technique has been used in over 20 clinical trials using naked plasmid DNA which carries the VEGF gene, injected into the myocardium of patients with severe coronary artery disease (CAD) in the late 1990s and early 2000s [130–135]. These randomized, double-blinded, placebo-controlled trials failed to show a beneficial effect on either the symptomatic or clinical outcome. One of possible reasons for this is the poor cardiac uptake of the naked DNA plasmid, thus limiting the biological activity in the human heart. Therefore, adenoviral-based cDNA delivery vehicles have also been tested for cardiac gene therapy in clinical trials. For example, AdGVVEGF121.10NH (commercial name: BIOBYPASS, adenoviral vector with a strong CMV enhancer/promoter, and VEGF-A121 cDNA) was used in a series of clinical trials to treat patients with CAD [136]. In preclinical animal studies, myocardium injection of an adeno-vector was able to improve myocardial angiogenesis, increase blood flow, and rescue heart function in the ischemic porcine heart [137–140]. A phase I clinical trial tested in patients with severe CAD also demonstrated that an intramyocardial injection of AdVEGF121 was well tolerated and provided some promising initial findings that showed a trend toward the reduction of myocardial ischemia injury [141, 142]. Interestingly, the Randomized Evaluation of VEGF for Angiogenesis (REVASC) trial reported that AdVEGF121 was associated with significantly improved symptoms and exercise capacity of CAD patients [143]. Unfortunately, other AdVEGF121 trials showed no difference of exercise capacity, time to ischemic threshold, or myocardial perfusion compared to the control patients [144]. Although these completed trials showed no significant beneficial effect in patients, there are still some ongoing clinical studies based on adenoviral vectors, such as three different VEGF-A isoforms in a phase I/II trial (NCT01757223) that had recently begun in 2020 to optimize the therapy.

Another interesting study of gene therapy in CVDs occurred by targeting Ca^{2+} ATPse and SERCA2a, a key factor for Ca^{2+} reuptake by the sarcoplasmic reticulum [145]. Since the early 1970s, the sarcoplasmic Ca^{2+} ATPase was found to be an important molecule for heart function in animal models and was also found to be reduced in different CVDs [146–149]. The idea to restore levels of SERCA2a has been an extremely popular strategy for gene therapeutics in heart failure [150]. In 2007, patients with advanced heart failure were treated with an AAV1 containing the SERCA2a gene to restore protein expression (known as the CUPID trial, calcium upregulation by percutaneous administration of gene therapy in patients with CVDs; NCT00454818) [151, 152]. It was the first phase I clinical trial to use an AAV gene therapy for heart failure and

simultaneously verified the safety and feasibility of the treatment [153]. Unfortunately, the blinded, randomized, placebo-controlled, multicenter study failed to demonstrate positive clinical outcomes. The AAV1- SERCA2a treatment does not improve heart function in patients with heart failure and severely reduced ejection fraction, ischemic and nonischemic etiology (NCT01643330), or left ventricular assist devices (NCT01966887) [154, 155]. Although these current attempts did not show positive therapeutic results, several other approaches are currently ongoing to improve gene therapy in heart failure. For example, a phase I clinical study (NCT04179643) that commenced in 2020 is testing BNP116.sc-CMV.I1c, a chimeric AAV2/AAV8 capsid with a high specificity for cardiac and skeletal muscles with less off-target effects in the liver and lungs [156], in patients with class III heart failure.

10 Future Perspectives of Gene Therapy in CVDs

Critical problems of gene therapy in CVDs involve the insufficient gene transduction into heart tissue or cells [157]. Currently, heart-specific gene delivery technology still limits the application of gene therapy in CVDs. Notably, naked plasmid transfection as well as viral-mediated gene delivery did not cause major safety concerns in most phase I/II trials (summarized above). Transfection of the naked plasmid showed a short-term expression time when compared to the AAV systems which could prolong gene expression [158]. However, the neutralizing antibodies of AAVs reduce the vector transduction efficiency and lead to a big obstacle of AAV application in the clinics [159]. In addition, the high cost of AAV manufacturing for clinical applications is still a challenge for normal patients. Thus, improving cardiac cell specificity, reducing the innate immune response, and reducing production price as well as long-term gene expression and stability are the main goals for the next generation of AAVs used in gene therapy.

11 AAV Engineering for Heart-Specific Therapy

To overcome low specificity in the heart, or more specifically cardiomyocytes, capsid engineering of AAVs tries to improve cell-type tropism. Several AAV serotypes have now been identified since the first AAV was observed, and they have been seen to share similar structures such as genome size and genetic organization. However, the differences are in the amino acid composition of the capsid proteins. Thus, it is possible to obtain chimeric viral particles by AAV engineering through transencapsidation [160]. The capsid reengineering can help to optimize receptor binding and transduction efficiency and more importantly tissue target selectivity of rAAV. Currently, capsid chimera libraries are derived from a variety of AAV serotypes or the random mutation of the capsid region and are a good platform for heart-specific peptide selection [161, 162].

In addition, engineered or random capsid mutagenesis, DNA shuffling, and direct selection are the most commonly used techniques to generate new rAAV variants [163–165]. For example, AAV2i8 and AAV-SASTG, two AAV2 chimeras, achieved a higher cardiac and skeletal muscle transduction efficiency with a lower off-target phenotype seen in the liver [166, 167]. In addition, Pulicherla and colleagues generated engineered liver-detargeted AAV9 vectors which had a similar transduction efficiency to the heart and muscle as wild-type AAV9 but 10- to 25-fold lower infection of the liver [168]. The modification of the AAV capsid could be a solution to improve AAV-mediated gene therapy in CVDs.

12 Successful Viral-Based Gene Therapy in Clinical Trials

While every new gene therapy trial helps advance this technique of repairing the genome, crucial safety concerns have arisen with the development of this treatment option. One of the major benefits of AAVs is their low potential to produce

immunological responses due to the absence of viral protein expression and the extremely limited viral elements present in the vector. The cellular immune response decreases without presentation markers on the surface of cells transduced by the AAV [169]. This is not to say, however, that AAVs cannot produce any immune response. A limitation to using AAVs involves an adaptive humoral response which occurs in an organism when they have been previously infected by an AAV of the same serotype. Neutralizing antibodies (NAbs) have the capacity to neutralize this additional infection from the same AAV serotype in 30–60% of humans [170]. NAbs are capable of limiting this possibly lifesaving gene delivery by blocking AAV transduction into cells of a person who was previously infected. The delivery of alternate serotypes is one possible solution, although some NAbs against one specific serotype have also been seen to neutralize additional serotypes as well [170]. To overcome this obstacle, studies have been performed such as one that simultaneously administered anti-CD20 antibodies in order to reduce the internal titer of NAbs to reduce the neutralization of the added gene therapeutic vectors and to also engineer AAV capsids as was previously discussed above [171].

With many decades of research, gene therapy was eventually successful in clinical trials. In 2017, a study was published where the survival motor neuron 1 (SMN1) gene was delivered to patients born with a mutation or deletion that led to spinal muscular atrophy type 1 (SMA1). SMN proteins are produced primarily through the SMN1 gene, as the SMN2 gene is missing an exon, leading to a reduced protein production from this gene alone. Therefore, having an SMN1 deletion and only copies of SMN2 almost guarantees that a patient will have SMA1 as the SMN2 gene alone produces an insufficient level of protein for neuronal cells. Without the SMN1 gene, motor neurons lose the ability to function, resulting in severe motor disabilities, leading to lifelong ventilation and/or death in 75% of patients before 2 years of age [171–173]. Zolgensma (biologically known as AVXS-101), first approved for use in the USA in 2019, is a gene therapy developed using an AAV9 vector to deliver a healthy copy of the SMN1 gene to motor neurons to hinder disease progression and improve the quality of life of these infants. The study showed improvements in motor function in 11 out of 12 patients in the initial trial with more than half not requiring further ventilation and even two gaining the ability to walk [172]. Interestingly, Zolgensma was not the first FDA-approved drug to treat SMA. In late 2016, an antisense oligonucleotide drug known as Spinraza (nusinersen) was first approved to treat SMA through a multi-dose system approach in patients from the early stages of birth [174]. This treatment option was administered through direct injection into the cerebrospinal fluid four times in the first 64 days of the trial [175]. It was determined at the completion of the study that Spinraza would need to be consistently administered for the duration of the patient's life [173]. It is important to note that the mechanism of action by which this antisense oligonucleotide works is quite different to that of Zolgensma. The aim of Spinraza is to have more full-length SMN proteins expressed in motor neurons by targeting the pre-messenger RNA of the existing copy of SMN2 [175]. Since the therapy only interacts at an RNA level, a continuous treatment plan is required to manage disease progression. Zolgensma, on the other hand, is a direct form of gene replacement therapy that only involves a single administration of a healthy SMN gene via an AAV vector, which can then directly produce full-length SMA proteins without consistent manipulation at the RNA level [173].

Even though the treatment only requires a one-time administration, Zolgensma is currently the most expensive drug on the market, partially due to this single-dose treatment, the cost of developing the drug, and the rarity of the disease itself, highlighting another (in this case, economic) limitation of AAV-based gene therapy [176]. Despite the high costs, this drug is not perfect and can lead to elevated liver enzymes in patients who have taken it, which can cause safety concerns in those with preexisting liver conditions. Other AAV-based therapeutics undergoing clinical trials have also been seen to

cause severe problems, even death, in patients with preexisting liver conditions. The FDA has even halted clinical trials after two patients died while receiving a high dose of AT132 in the Audentes Therapeutics' trial [177]. This AAV8 vector is used to deliver a healthy copy of the myotubularin-1 gene to treat X-linked myotubular myopathy. Since this was the highest dose of AAV gene therapy given to date in a clinical trial and the patients who died as a result also had underlying liver conditions, safety concerns relating to low-dose treatments especially in patients with healthy livers are relatively low. Other trials for AAVs used to treat Duchenne muscular dystrophy have also observed toxicities in patients; however, the knowledge and understanding of gene replacement therapy continues to grow with each trial, especially when complications arise [177]. This was especially true in the case of Jesse Gelsinger who was the first patient to die from an immune reaction to an rAV to treat ornithine transcarbamoylase back in 1999. The severe immune reaction that he experienced that ultimately led to his death was extremely rare as none of the other 4000 patients from other clinical trials experienced the same side effects. The reevaluation that this led to by the FDA to intensely study and determine why and which vectors could be harmful as delivery vehicles has undoubtedly saved lives and ensured safer treatment for all future studies after this tragic loss [178].

13 Novel Therapeutic Target Genes

Current gene therapy candidates in CVDs are mainly focusing on cytokines or calcium-related proteins such as VEGF or SERCA2a. Apart from coding genes which only comprise 1–2% of the human genome [179], noncoding RNA (ncRNA) transcripts (without coding potential) are worth noting as future therapeutic targets. Although the function of most ncRNAs is still unknown, growing evidence has proven that ncRNAs are key modulators in diseases (such as cancer or CVDs) [180]. For exploring a clinical application, numerous independent studies regarding circulating ncRNAs have been reported as biomarkers to predict and monitor the response of CVDs and treatments [181, 182]. Notably, preclinical investigations of ncRNAs are also heading in the direction of potential therapeutic options for CVD patients. For example, a conserved long noncoding RNA (lncRNA) H19 is a powerful ncRNA molecule for the protection of pathological cardiac hypertrophy. Restoration of H19 expression mediated by AAV injection four weeks after induction of chronic left ventricular pressure overload successfully attenuated cardiac hypertrophy in mice. In addition, AAV6-mediated H19 overexpression improves contractility of human engineered heart tissue, highlighting translational potential of H19 [183]. In addition, miRNAs and ncRNAs approximately 20 nt in length have also been evaluated in several preclinical studies for the treatment of CVDs such as miR-181a [184]. Overexpressed miR-181a mediated by AAV9 delivery one week after MI was able to show recovered heart function in mice [185]. Apart from these novel ncRNAs, some traditional protein coding genes were also validated as putative CVD therapeutic targets in preclinical investigations. For example, Tert, a telomerase reverse transcriptase encoding gene, is well known for its role in cellular senescence. Cardiac-specific overexpression of Tert by AAV9 attenuated cardiac dilatation, improved ventricular function, and reduced infarct scarring after an acute MI [186]. Following studies proved that AAV-Tert overexpression protected against cardiac apoptosis and cardiac dysfunction from doxorubicin-induced cardiotoxicity in mice [187]. These encouraging preclinical studies recognized the potential to utilize ncRNAs as well as protein coding genes as novel therapeutic candidates to treat CVDs.

14 Conclusion

After over 30 years from the first gene therapy clinical trial, no successful application has since been reported in CVDs, indicating that this field is still young and needs further development which

is currently pursued with tremendous efforts both in academia and in the pharmaceutical industry. The improvement of gene delivery platforms and preclinical investigation systems, as well as for novel therapeutic candidates, are supporting the development of next-generation gene therapy in rare genetic disorders as well as CVDs.

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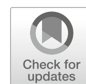

Therapeutics in Metabolic Diseases

Vijayakumar Natesan

Abstract

Metabolic diseases have important effects on the health and healthcare costs of an individual. It adversely affects various body processes. Metabolic diseases are characterized as the accumulation of many conditions that collectively increase a person's risk of atherosclerotic coronary disease, insulin, and diabetes mellitus intolerance, as well as vascular and neurological complications, such as stroke. Rare metabolic disease has also been reported in literatures and clinical research. Understanding the history and causes of the disease, associated symptoms, disease severity, physical and vital evaluations, etc. is recommended to provide or improve some appropriate therapeutic measure. The experience with patients starts with a critical and general presentation to a healthcare provider that may indicate potential conditions such as dyslipidemia, hypertension, and metabolic diseases. The main factors in the treatment and management of metabolic disorders are lifestyle changes. Whenever behavioral changes are not effective or cannot be implemented, pharmacotherapies should be initiated including for most of the rare diseases. Moreover, pharmaceutical molecules are the very commonly used therapies. The prospect of therapy through gene transfer into somatic cells unlocks a new field of treatment and opportunity for people affected by these genetic conditions. Like other medical treatments, many gene therapies can relieve some, though not every indications of a specific disease, which can increase patients' quality of life. Hormone-based therapies are also implemented in the treatment of metabolic diseases. It has been suggested to use herbal extracts with different forms of nano-drug delivery techniques, such as nanobiocomposites, solid lipid nanoparticles, nanoemulsions, green-synthesized gold, zinc oxide, and silver nanoparticles.

Keywords

Metabolic diseases · Rare diseases · Diabetes mellitus · Hypertension · Obesity · Dyslipidemia

1 Background

The cellular-level process of transforming food into energy is called metabolism, and metabolic disorder is any illness or condition that interferes with natural metabolism. Several enzymes are involved in different interdependent metabolic processes to carry out this mechanism. Metabolic disorders impair the cell's ability to conduct substantial biochemical processes involving the

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production or transport of amino acids, carbohydrates, or lipids. The cell's ability to execute substantial biochemical processes including the synthesis or transfer of lipids, amino acids, or carbohydrates is compromised by metabolic disorders. Metabolic diseases are defined as an accumulation of several conditions that collectively increase a human's threat of coronary atherosclerotic disease, intolerance of insulin, and diabetes mellitus, as well as vascular and neurological complications, such as stroke [1–3]. Defining the metabolic syndrome has been long debated, and many definitions are present in the literature [4]. Metabolic syndrome can be declared if three or more of the following symptoms prevail: higher circumference of waist, high triglycerides, increased fasting glucose, elevated blood pressure, and low high-density lipoprotein cholesterol (HDL-C). The criteria for the conditions are presented in Table 1.

There are rare metabolic diseases reported other than the said conditions. They are mostly chronic conditions that could be life-ending. They have no or limited effective therapies. Inherited metabolic diseases are carried over from birth. They could be categorized as single cell(s) or organelle(s) or multiple specific disorders. The former disorder includes symptoms such as allergies and endocrine diseases, and the latter presents with co-morbid conditions with multiple organs and systems. Major rare metabolic diseases are lysosomal, mitochondrial, and protein metabolic disorders. It means that this disease includes metabolic disorders such as complex molecules, energy, and intoxication. In addition to the three major subclasses, carbohydrate metabolic, neurological, hepatic, and vitamin metabolic disorders were also reported [5].

Metabolic diseases have significant impacts on a person's health and healthcare costs. It affects multiple body processes adversely. The increasing incidence of metabolic diseases needs to be understood, as the advancement of the syndrome can be prevented and eventually reversed by the intervention [6–8]. Excess body weight, absence of physical exercise, and hereditary predisposition are the fundamental causes of the metabolic diseases. Over time, the progression of metabolic diseases leads to vascular and autonomic damage [9–11]. Body fat distribution is also important to consider, and the upper body fat plays a dominant role in producing insulin resistance. Abdominal fat can be a greater contributor to insulin intolerance than subcutaneous fat. Nevertheless, obesity and fat accumulation are believed to take part in the progress and advancement of metabolic diseases. High levels of nonesterified fatty acids are expelled from adipose tissue in upper body obesity, allowing lipid to collect in other areas of the body, including the liver and muscle, reinforcing additional insulin resistance.

Microvascular injury is caused by insulin resistance, which may predispose a patient to develop hypertension, vascular resistance, endothelial disease, and inflammation of the vessel surface. The endothelial injury that causes atherosclerotic disease and the production of hypertension will disrupt the body's homeostasis. In addition, many body functions, including elevated vascular resistance and stiffness inducing peripheral vascular dysfunction, systemic cardiac disease consisting of left ventricular hypertrophy, and cardiomyopathy, are significantly impacted by hypertension, which contributes to renal dysfunction [12].

Cumulative consequences of metabolic disease-related endothelial dysfunction and hypertension will also result in ischemic heart disease. Endothelial dysfunction can induce blood thrombogenicity owing to elevated adipokine and plasminogen activator type 1 levels, and hypertension induces vascular resistance from which coronary artery disease can develop. Metabolic disease-associated dyslipidemia can also affect the atherosclerotic mechanism contributing to symptomatic ischemic heart disease [13, 14]. Metabolic diseases could also cause liver damage, by inducing steatosis that can lead to fibrosis, nonalcoholic steatohepatitis (NASH), hepatocellular carcinoma, and cirrhosis. Histologically, lobular inflammation, hepatocyte ballooning, steatosis, pericellular fibrosis, and Mallory bodies are observed in nonalcoholic steatohepatitis. The precise reason of nonalcoholic steatohepatitis

Table 1 Criteria for defining the metabolic diseases [3]

Diseases	Criterion values for declaring the presence of metabolic disease
Circumference of waist	≥40 inches in men; ≥35 inches in women
Triglycerides	>150 mg dl^{-1} or medications for the control of triglycerides
High-density lipoprotein-cholesterol (HDL-C)	<40 mg dl^{-1} in men and <50 mg dl^{-1} in women or medications for the control of HDL-C
Blood pressure	130/85 mmHg or greater or on antihypertensive medications
Fasting glucose	100 mg dl^{-1} or greater or on medications for glucose control

formation is unclear; however, it has been related to metabolic diseases, primarily insulin resistance and proinflammatory status[15].

To provide or develop any effective therapeutic measure, understanding the disease history and causes, related complications, disease severity, physical and vital examinations, etc. are recommended. The experience with patients begins with a critical and general presentation, which may suggest possible disorders like dyslipidemia, hypertension, and metabolic diseases to a healthcare professional. There are genetic conditions that can incline individuals to the progress of metabolic diseases and insulin resistance, but no known genetic community is disposed to metabolic diseases. A patient should then be asked on all other suspected genetic abnormalities. It is therefore important to acquire social and lifestyle records, since there are crucial variables that can greatly influence the progression of metabolic diseases. For metabolic disease diagnosis, a physical examination is necessary since one of the requirements includes waist circumference. In addition, the patient must be tested for physical symptoms of insulin resistance, like acanthosis nigricans, retinopathy, and peripheral neuropathy, if the patient has a disease history. Vascular murmur that may be attributed to atherosclerotic disorder should be diagnosed and treated by a clinician. Xanthomas can be found in dyslipidemia patients. For the identification of metabolic diseases, a detailed physical examination is warranted [16].

The evaluations must be coupled with laboratory research after a detailed historical development of disease and physical examination. Hemoglobin A1C (HbA1c) for resistance to insulin and type 2 diabetes mellitus (T2DM) should be part of the blood tests. To test for an unusually higher levels of triglyceride, lower HDL-C, and elevated low-density lipoprotein levels, a lipid profile must also be tested. A simple metabolic assessment should also be used in the initial assessment to determine renal impairment and evaluate the level of glucose. In order to better examine and help the analysis of metabolic diseases, additional tests such as thyroid analysis, liver panel, uric acid, and C-reactive protein may be suggested. Imaging tests may be requested as required. For example, someone suspected of developing atherosclerotic coronary artery disease should undergo an electrocardiogram to test for symptoms of arrhythmias, infarction, and cardiac ischemia, as well as assess for hypertension with systemic heart diseases. If necessary, cardiac stress evaluations including stress echocardiography and electrocardiogram stress evaluation should be further assessed for patients [17, 18]. The treatments starting from lifestyle modifications, medications, gene therapies, hormone-based treatments, and Phytomedications for metabolic diseases are detailed in this chapter, and Table 2 summarizes the available treatments and disease management.

2 The Lifestyle Changes as Treatment for Metabolic Diseases

Lifestyle changes are key factors in the treatment and controlling of metabolic diseases. Modern treatment incorporates detailed guidelines on food and exercise with behavioral and cognitive techniques. The most successful and healthy interventions to avoid metabolic diseases are lifestyle improvements aimed at improving long-term outcomes, especially in reducing

Table 2 Therapeutics in metabolic diseases: suggested and available treatments with cutoff values

Metabolic disease	Preliminary treatment	Advanced treatment	Management/ control cutoff values
Overweight	Lifestyle changes	Phentermine/topiramate, naltrexone SR or bupropion SR, lorcaserin, orlistat, sibutramine, GLP-1 RA liraglutide, bariatric surgery	Reduction of overall weight to ~10%
High glucose level	Lifestyle changes	Thiazolidinedione, metformin, insulin, dipeptidyl peptidase-4 (DPP-4) inhibitors, sulfonylurea, SGLT-2 inhibitors, GLP-1RAs	HbA1c less than 6.5%
Dyslipidemia	Lifestyle changes	Cholesteryl ester transfer protein (CETP) inhibitors, Colesevelam (resin), statins, niacin, fibrates	LDL less than 160 mg dl^{-1}
Hypertension	Lifestyle changes	Angiotensin-converting enzyme inhibitor (ACEI), thiazide diuretic, beta-blocker (BB), calcium channel blocker, angiotensin receptor blocker (ARB)	Diastolic between 130 and 140 mmHg Systolic between 80 and 90 mmHg

cardiovascular events [19]. Adapting to healthy behaviors is the most important aspect of metabolic disease treatment and control. Various lifestyle modifications will be addressed below as primary treatment for metabolic diseases-related disorders such as hypertriglyceridemia, central obesity, high blood pressure, hyperglycemia HDL-C deficiency, and diabetes impairment. Reducing the body weight in obese people has been found to strengthen all components of metabolic diseases. The improvement in lifestyle should concentrate in two aspects: (1) nutritional advice to combat obesity and correct overweight and (2) energy consumption advice through physical activity to improve exercising capacity in day-to-day life and in their free time while focusing on improving endurance [20].

Lifestyle changes include selecting a particular dietary plan, encouraging moderate to vigorous exercise, and using methodologies for behavioral transformation. Various nutritional strategies effectively yield weight loss, generally a 5–10% decline of initial body weight, which is linked to enhancement in cardio-metabolic problems. Also, weight recovery progresses over time as dietary conformity weakens. Generally, the capacity of a person to implement the changes adopted through a dietetic strategy will need to be viewed as feasible for long-term commitment and retention of weight loss. Thus, what one person finds to be enticing with a dietary prescription, such as high protein, could be very complicated for somebody else, such as someone who chooses low-fat, vegetarian choices. In general, supplementing the nutritional strategy with physical exercise improves losing weight and, more accurately, predicts preservation of weight loss. The use of behavioral intervention strategies, through concrete implementation plans and transparency, helps increase compliance to a diet and exercise schedule. A study on the impact of lifestyle patterns on coagulation of the blood stated that swings in daily habits such as integrating physical activity, losing weight, complete withdrawal of smoking, consumption of fish, reduced alcohol intake, and optimal rest decrease coagulability, encourage fibrinolysis, and decrease platelet aggregation [21].

2.1 Physical Activity and Exercise

Exercise is an important element of lifestyle changes to prevent diabetes mellitus and related metabolic diseases, particularly when introduced to dietary treatment. Studies suggested active walking and comparable exercise or strenuous exercise for half an hour a day can help reduce hypertension [22]. The frequency of T2DM was decreased by 31%, 46%, and 42% with food, exercise, and both diet and exercise combined, respectively [23]. Exercise causes only short-term impacts on blood pressure, aside from a balanced diet. A meta-analysis of multiple

randomized studies found a decrease in blood pressure of 4 mmHg among participants subjected to a physical activity intervention [24].

2.2 Diet

There are three widely adopted nutritional alternatives: diets that are low carbohydrate, very low-fat diets, and diets with reduced fat. In particular, excessive protein and calcium consumption is seriously harmful to renal function, and an eminent possible threat for early atherosclerosis is excessive saturated fat consumption [25].

Due to the positive impact on insulin, serum glucose, and TG levels, reduced glycemic index and higher content of fiber would be ideal. A daily consumption of carbohydrates should be 60% of the total calories and about 50% for people with high-density lipoprotein or low triglycerides. Whole grain products, fruits, low-fat milk products, and vegetables can make up much of the carbohydrates. Burning 200 kcal a day results in an increase in most people's TG/HDL-C ratio [26]. The mean decrease in blood diastolic and systolic pressure was 1.1 and 1.6 mmHg for a kilogram of weight loss, respectively [27].

Weight reduction of obese and hypertensive individuals was attributed to a rise in insulin sensitivity along with a decrease in the blood pressure [28]. A diet containing rich calcium, magnesium, and potassium (low-fat dairy foods, dry fruits, special nuts, meat, vegetables, whole grains, and fish ["DASH diet"]) has greatly decreased blood pressure. Reduced salt intake (~3.8 g day^{-1}) decreased systolic blood pressure in non-hypertensive patients by 7.1 mmHg and in hypertensive patients by 11.5 mmHg when compared to a higher salt intake (~8.6 g day^{-1}). A high salt intake (sodium chloride) has a negative impact on blood pressure, especially in black and elderly people [29, 30]. Also, high-dose intake with omega-3 polyunsaturated fatty acids, 3 g, or high of fish oil a day decrease blood pressure [31]. There is very little exposure to the influence of monounsaturated fatty acids on blood pressure and reducing the risk of heart disease [20].

In medical practice, very low-calorie ketogenic diets (VLCKDs) are progressively used for weight control and the treatment of comorbidities related to obesity. VLCKD is reliant on protein-rich foods produced from soy, green peas, whey, and eggs of high nutritional value. VLCKD is thus distinguished by a low content of lipids, mostly obtained from olive oil, that is, ~20 g a day. VLCKD provides adequate medical oversight [32, 33]. In addition, VLCKD patients should be closely and regularly checked to prevent dehydration and vitamin/electrolyte irregularities, which are possibly related to urinary excretion of ketone bodies and inadequate absorption of micronutrients, by physical assessment (heart rate, blood pressure, anthropometric scales, etc.) and laboratory study [34].

For rare medical conditions, specialized foods are suggested. These special foods contain specific nutrients removed or medications included. For example, maple syrup urine disease might be managed with leucine-, isoleucine-, or valine-free foods. Protein- or amino acid-less foods could be suggested for related metabolic disorders such as urea cycle disorders, lysinuric protein intolerance, non-ketoic hyperglycinemia, etc. Similarly, sucrose, fructose, galactose, or sugar-free foods might help galactosemia and glucose transport disorders Osteoporosis-like disorders need vitamin D and calcium-rich foods [35].

2.3 Alcohol and Smoking Cessation

An elevated risk of hypertriglyceridemia, impaired fasting glucose/diabetes mellitus, hypertension, and visceral obesity is correlated with alcohol in excess as per dietary guidelines. There have been suggested pathways by which alcohol intake could favorably affect the risk of metabolic diseases. In order to enhance insulin sensitivity, moderate consumption of alcohol was found to increase HDL-C, improve insulin sensitivity, and lower triglycerides. The correlations found between alcohol and HDL-C,

serum glucose, serum insulin, and triglycerides are compatible with previous studies [36–43].

Smoking is prothrombotic and atherogenic in all cases. As a result, the threat of acute myocardial infarction, sudden heart arrest, stroke, peripheral artery disease, and aortic aneurysm is raised. Even relatively low dosage levels raise the risk of cardiovascular disease and changes in metabolism [44]. The cessation of smoking could also minimize T2DM-induced chronic injury. The positive effect of smoking cessation in recently diagnosed T2DM patients is reported as decrease in microalbuminuria, delineated as a 30–300 μg/mg ratio of albumin to creatinine, and good control of blood pressure [45]. Smoking cessation may relieve smoking and nicotine-induced ailments and can also contribute to weight increase. Post-cessation weight gain is less detrimental than smoking. Persons with obesity who stop smoking have the greatest demand for treatments to alleviate weight gaining [46, 47]. Obese smokers with anxiety, particularly women, need much more efficient weight management and smoking cessation therapy [48].

2.4 Losing Weight

Current research suggests that a moderate weight loss is correlated with a marked decrease in occurrence of T2DM and other metabolic diseases [49, 50]. There is full acceptance that weight loss is related to major changes in metabolic health abnormalities, including blood glucose level, lipid profile, and blood pressure, and even modest weight loss (7% decrease) in 4 weeks, considering the existence of a large BMI, will boost the metabolic profile [51–53]. With the addition of novel procedures, such as integrating dietary changes with pharmacotherapy, the positive findings were obtained [54] using meal substitution [55] establishing higher physical activity [56] and longer medical treatment and [57] boosting ambitious hopes for a successful cure of obesity and metabolic diseases with changes to lifestyles. Biological pressure to recover weight is among the key causes involved in the long-term loss in weight management [58]. The impact of integrating dietary change with pharmacotherapy aimed at reducing biological pressure to recover weight is also reasonable to determine. The results show that this mixed method appears to increase both the quantities of weight loss and weight loss management [54]. The increase in weight loss is also accompanied by significant changes in many metabolic outcomes and cardiovascular disease risk factors [59]. The central function of cognitive mechanisms in success/failure of weight management and loss indicates that in traditional lifestyle change treatments, cognitive processes should be used in helping patients develop a long-term weight management attitude [60–62].

2.5 Stress Control

Long-term exposure to stress at work will directly impact the autonomic nervous system and neuroendocrine function, leading to metabolic disease development. A case-control analysis found that patients with metabolic diseases in the Whitehall II study had increased production of cortisol and normetanephrine and also decreased variations in heart rate [63]. In the Whitehall II study, decreases in cardiac autonomic function were correlated with metabolic diseases in certain populations and poor job control and social alienation among males [64–66].

Continuous mental stress may decrease resilience and bring imbalance in the metabolism. Improved adrenocortical activity will affect the metabolism of hepatic lipoprotein and insulin sensitivity in the target organ [67, 68]. Cortisol is an insulin antagonist, and the amount of cortisol in metabolic diseases is increased [63]. Low levels of HDL-C and glucose resistance are correlated with high baseline cortisol release [69]. Observational and prospective trials showed that the stress at work was correlated with coronary heart diseases [70, 71]. The biological pathways are also uncertain for the cause [72]. Direct neuroendocrine symptoms and secondary effects mediated by detrimental health practices are possible pathophysiological pathways [63, 64, 73].

There is a dose-response link among work stress exposure and metabolic diseases. After considering other risk parameters, workers with persistent job stress have more than twice the chances of the condition than someone without work stress. The research proves that the biological effects of psychosocial stress pathways lead to heart disease in conjugation with daily life stressors. Likewise, diseases like osteoporosis, depression, and high blood pressure are triggered by increased secretion of cortisol [74]. In addition, higher levels of cortisol lead to the progression of the metabolic disease, including visceral obesity, diabetes and dyslipidemia, and cardiovascular comorbidities [75]. Therefore, it is recommended to have work-life balance to prevent and manage metabolic diseases. People working in high stress jobs should take necessary stress management programs like yoga, stress-busting physical activities, hobbies, and recreational work-offs.

3 The Pharmacotherapy of Metabolic Diseases

The stratification of patients fulfilling the metabolic disease criterion must be focused on their risk profile [76]. The suggested sequence of treatment priorities for high-risk patients must be: (1) atherogenic dyslipidemia, (2) arterial hypertension, (3) glucose resistance, and (4) prothrombotic condition. The treatment target series for lower risk patients must be: (1) unusual non-HDL-C and HDL-C (LDL as the target), (2) pre or moderate arterial hypertension, and (3) fasting hyperglycemia diagnosis for glucose intolerance [76]. Two non-pharmacological methods, that is, tobacco withdrawal [77], diet (comprising phytosterols) and exercise [76], and also pharmacological or surgical therapeutic approaches (bariatric surgery) [78], could be intended to accomplish the objectives and to decrease the long-term peril of explicit T2DM and cardiovascular disease.

Various pharmacological measures are also needed to correct the specific threat factors involved in metabolic diseases appropriately. The usage of many pharmaceutical treatments will pose not only clinical but also moral concerns, which can only be resolved by considering the influence of treatment on specific threat factors [79]. Many other pharmacological approaches addressing risk factors have indicated effects in the overall population in relation to cardiovascular diseases and death. In patients with metabolic diseases, although certain pharmacological treatments may have additional advantages by enhancing greater than one of its risk features, possible adverse consequences of other therapies have also been observed [80–86].

3.1 Pharmacotherapy of Dyslipidemia

The impacts of statins are dose-dependent, and LDL cholesterol can be decreased by up to 60% by high-intensity agents like rosuvastatin [87]. Statins are not only beneficial to decreasing the levels of LDL, but they also decrease cardiovascular death and decrease morbidity [84]. A meta-analysis showed that rosuvastatin, atorvastatin, and simvastatin were all able to mildly decrease the level of triglycerides and very-low-density lipoprotein (VLDL) and raise the HDL-C level [88]. For simvastatin and rosuvastatin, the dose-effect relationship also differs, not for atorvastatin [88].

Ezetimibe effectively restricts the absorption of biliary and dietary sterols, without intruding with liposoluble nutrient absorption. The protein Niemann-Pick C1-Like 1 and the beneficial influence of ezetimibe were also demonstrated in other physiopathological elements of metabolic diseases [89]. In the event of statin resistance, Ezetimibe, bile acid sequestrants, or a mixture have usually been suggested [90, 91]. Resins can also decrease the level of blood glucose in the event of hyperglycemia. In a Cochrane systematic review, a better tolerated resin Colesevelam was shown to support and regulate the glycemic levels when prescribed along with antidiabetic agents [92]. The occurrence of gastrointestinal adverse reactions, the potential impact of rising amounts of triglycerides in

predisposed persons, the high incidence of drug-drug interactions, and the existence of newest agents have restricted its use of bile acid sequestrants.

The proprotein convertase subtilisin/Kexin type 9 (PCSK9) inhibitors are a recent group of agents found to reduce LDL-C. PCSK9 was identified in 2003 with hereditary hypercholesterolemia patients [93], and its ability to cause deterioration of LDL receptors in hepatic cells is a major element in LDL-C level regulation. The linking of PCSK9 to receptors of LDL-C and the lesser levels of LDL cholesterol was blocked by PCSK9 monoclonal antibodies by around 60% [94]. The evolocumab (PCSK9 inhibitor) as an additional to statin therapy has been recently published with proven positive results in cardiovascular disease patients [95]. Nicotinic acid is a typical wide-spectrum hypolipidemic, which reduces LDL cholesterol and triglycerides and raises HDL-C levels most efficiently. It has pleiotropic consequences consistent with adipocyte lipolysis inhibition and reduction of triglyceride and CRP synthesis [96]. The majority of effects have been seen in low to moderate statin dosage trials in conjunction with nicotinic acid.

Cholesteryl ester transfer protein (CETP) inhibitors, fibrates, and statins are the other agents which are shown to improve HDL-C [91]. The impact of HDL-C-directed treatment of cardiovascular disease has not led to a lower number of cardiovascular disease events [91]. Fibrates, a widely used class of lipid-modifying agents, result in a substantial decrease in plasma triglycerides and are usually associated with a moderate decrease in LDL cholesterol and an increase in HDL cholesterol concentrations. Recent investigations indicate that the effects of fibrates are mediated, at least in part, through alterations in transcription of genes encoding for proteins that control lipoprotein metabolism. Fibrates activate specific transcription factors belonging to the nuclear hormone receptor superfamily, termed peroxisome proliferator-activated receptors (PPARs) [97]. They trigger Apo A-I synthesis as well. These pathways collectively contribute to a reduction in triglycerides, a rise in HDL-C, and a change from small LDL to large LDL cholesterol [98]. In combination with statins, fibrates are more effective, especially in patients with overweight and hyperinsulinemia [98, 99]. Generally, fibrates deter cardiovascular illness, and death is less convincing than statins and requires more validation and clarity to assess their beneficial role [90, 100, 101].

3.2 Pharmacotherapy of Obesity

Five medications, phentermine/topiramate, naltrexone SR/bupropion SR, GLP-1 RA liraglutide, orlistat, and lorcaserin, are presently approved for the treatment of obesity. Orlistat, through hindering pancreatic lipase, induces malabsorption of ingested fat, whereas the primary impact of the other medications is to decrease food consumption by control of appetite [102].

For many decades, the cardiovascular effects of pharmacologically mediated weight loss have been controversial [103]. In recent times, the LEADER (Liraglutide Effect and Intervention in Diabetes: Assessment of Cardiovascular Outcome Results) research program has reported positive effects on cardiovascular disease with liraglutide therapy, and liraglutide was permitted for the treatment of obesity at a higher dose (i.e., 3.0 mg) [104, 105]. It remains to be examined if the positive impact shown in LEADER study may be applied to the higher risk patients with no T2DM. However, 13% of risk reduction in cardiovascular disease shown in the LEADER study, coupled with high effectiveness of several aspects of metabolic diseases, is hopeful [106].

Amphetamines has distinct euphoric behavior and also has prospective for violence and addiction [107]. Phentermine via catecholaminergic pathways has stimulant and sympathomimetic activity [108]. In women, an elevated peril of hemorrhagic stroke has been associated with phenylpropanolamine-based appetite suppressants [109]. Dexfenfluramine and fenfluramine act as suppressants of appetite that interact with serotonin release. Due to related heart valve injury and pulmonary hypertension, these substances were also excluded from market [108, 109]. Sibutramine and orlistat are other

medicinal products currently available. By acting on the central nervous system, sibutramine increases satiety, reduces appetite, and decreases the metabolic rate that accompanies weight loss [110]. Compared with placebo, an overall of 43% of sibutramine patients retained 80% of their original body weight loss. Increased sensitivity of insulin, glycemic regulation, and lipid profile of blood in patients with T2DM were followed by weight loss [111]. As shown by a decrease in the levels of leptin and an increase in the concentration of adiponectin, a positive effect on adipocytokines has been observed.

Orlistat, a pancreatic and gastrointestinal lipase inhibitor, prevents about 30% of dietary fat from being consumed [112]. Orlistat decreases the amount of LDL and cholesterol regardless of body weight loss, reduces progression to T2DM, and contributes to better glycemic regulation in patients with T2DM [113].

Rimonabant, the first medicine to aim the endocannabinoid (CB) pathway through hindering the CB1 receptors, has been developed. Siege of central CB1 receptors decreases appetite, while peripheral receptor blockade is assumed to influence liver and adipose tissue, leading to enhanced metabolism of fat, glucose, and lipid and free of changes in body weight [114]. Rimonabant therapy is related with an additional rise of 8–10% in HDL-C and 10–30% decrease in triglycerides and changes in insulin resistance, glycemic regulation in T2DM, besides cytokines and adipokines like CRP [115–118]. The European Medicines Agency authorized rimonabant as an assistant therapy for diet and exercise for obesity treatment in patients with minimum BMI of 30 kg m^{-2} and BMI of >27 kg m^{-2} in overweight patients with related risk factors like T2DM or dyslipidemia.

Weight-loss surgery (bariatric surgery) is the only alternative option nowadays that successfully addresses morbid obesity when other treatments become unsuccessful. Restricted evidence indicates that surgery is highly potent than traditional management for weight reduction in morbid obesity. Surgery led to higher weight reduction compared to traditional management, with life quality and comorbidity changes. However, the relative safety and efficacy of these surgical techniques is not well established.

3.3 Pharmacotherapy of Diabetes Mellitus

Seven forms of antidiabetic drugs are commonly available for T2DM treatment, that is, thiazolidinedione, metformin, insulin, glucagon-like peptide 1 receptor agonists (GLP-1RAs), sulfonylurea, sodium-glucose co--transporter-2 (SGLT-2) inhibitors, and dipeptidyl peptidase-4 (DPP-4) inhibitors, and metformin is the first-line therapy [119]. Impact of metformin versus intense lifestyle modification in metabolic diseases patients showed a 17% decline in metabolic diseases [50].

As the antidiabetic drug, rosiglitazone has recorded an elevated peril of cardiovascular disease, and all potential antidiabetic drugs had to show cardiovascular security in both pre- and post-approval environments [120]. Subsequently, multiple large clinical trials were performed for T2DM patients [81–83, 85, 121–126]. Although DPP-4 inhibitor has only showed neutral effects with respect to cardiovascular disease [121, 125, 126], certain GLP-1RA and SGLT-2 inhibitors were reported to decrease the hazard of substantial detrimental cardiac effects [81–83, 85].

As SGLT2 inhibitor, empagliflozin reduced cardiac effects [85], and similar cardiovascular advantages with both SGLT-2 inhibitor canagliflozin and GLP-1 RAs, liraglutide and semaglutide, were reported [122]. The clinical trials of the SGLT2 inhibitors have jointly shown a 9–26% risk decrease in the composite result of significant undesirable cardiac effects in T2DM patients [81–83, 85, 122]. In comparison, only neutral findings were seen in the Evaluation of Lixisenatide in Acute Coronary Syndrome (ELIXA) analysis examining GLP-1RA lixisenatide [124]. In addition to enhancing glycemic control and showing possible cardiovascular disease advantages with GLP-1RAs and SGLT-2 inhibitors, control of various metabolic disease parameters, like dyslipidemia,

obesity, and blood pressure, has also increased with these medications [81–83, 85, 122].

3.4 Pharmacotherapy of Hypertension

It is advised to commence first-line therapy with one out of five antihypertensive medications: calcium channel blocker, thiazide diuretic, angiotensin-converting enzyme inhibitor (ACEI), or beta-blocker (BB) angiotensin receptor blocker (ARB) according to the hazard level of specific patients [127]. All of these antihypertensive drugs have shown strong advantages of cardiovascular disease as a first line of therapy in a large population [127]. However, possible improvement of metabolic disorders should be discussed with metabolic disease patients, especially in the treatment of BB or thiazides [86, 128]. The Antihypertensive and lipid-lowering therapy to reduce heart attack (ALLHAT) research found an improvement in the incidence of T2DM in thiazide-cured patients in comparison to other antihypertensive treatments, and a raise in total cholesterol in thiazide-treated patients was also reported after 2 years [129].

A related issue was raised with respect to the developing T2DM for BB [86]. BB therapy was correlated with a 28% rise in advent of T2DM in a longitudinal research [128]. While BB may not be regarded as first-line products in metabolic diseases, the third generation, namely, vasodilator BB, nebivolol, and carvedilol, may also have a different place in treatment. Nebivolol has capacity of increasing nitric oxide and antioxidant properties that may justify its unbiased metabolic effects, a positive impact on sensitivity of insulin and the lipid-reducing prospective [130].

ARBs are antagonists of the AT1 receptor, contributing to vasodilation, decreased aldosterone, and vasopressin production, which eventually lower the blood pressure [131]. In addition, peroxisome proliferator-activated receptor (PPAR) gamma activation raised 1.5 more times in human preadipocytes when treated with ARBs irbesartan, losartan, and telmisartan [80]. Earlier, PPAR-gamma agonists were applied to treat both hyperglycemia and hyperlipidemia [80]. The Treat-to-Target Study has repeatedly shown additional metabolic benefits in reducing blood pressure, as administered with ARB irbesartan [131]. The experiments revealed substantial reductions in triglycerides, increases in HDL-C, and decreases in waist circumference and plasma glucose ranges [131]. HDL-C and systemic glucose improvements have been found only in patients with baseline metabolic diseases [131]. In patients with glucose resistance, the advantages of either ACEI or ARB relative to other groups have not been undeniably established [132–134]. Cardiovascular benefits comparable to ACEI or ARB are correlated with dihydropyridine calcium channel blockers and thiazide-like diuretics [132]. Instead, ACEI/ARB is the therapeutic agent of choice in subgroups of T2DM patients, who have proteinuria, diabetic nephropathy, or heart failure [133, 135]. In order to minimize the hazard of cardiovascular events in T2DM patients, bedtime daily dose of antihypertensive medicine tends to be significant [135].

The implementation of a polypill, which offers a fixed drug mixture for AVD protection, has recently been addressed. Statins, ACE antagonists, aspirin, and beta-blockers are applicants for such a pill. It is not acceptable to add beta-blockers to treat patients with metabolic diseases who haven't had AVD, especially in the younger age. Therefore, there appears to be no logical justification for a polypill at the moment, given the heterogeneity and complex pathophysiology of metabolic diseases [20]. The first line of pharmacological therapy is metformin or GLP-1RAs or SGLT-2 inhibitors, statins, GLP-1RA liraglutide, and RAAIs for glucose sensitivity, dyslipidemia, reaching body weight loss, and hypertension, respectively, to minimize the circumference of waist, based on existing evidence on the complications and advantages of the specific agents.

3.5 Therapeutics for Other Rare Metabolic Diseases

The therapeutic methods for metabolic diseases include pharmaceutical molecules, enzyme replacement, antibodies, RNA therapies, and cell and gene therapies. Pharmaceutical drugs or small molecules are common therapeutic approaches for any metabolic syndromes including the rare metabolic diseases. Pharmaceutical molecules are the ones that show the highest number of approvals from regulatory approval [136]. Results from clinical studies showed beneficial effects of lumacaftor and ivacaftor for patients with cystic fibrosis [137]. Lysosomal disorders such as Gaucher disease were successfully decreased by eliglustat like molecules [138]. Antibodies that can act as modulators were also used in the treatments of metabolic diseases. Such examples are monoclonal antibodies like eculizumab for the treatment of hemolytic uremic syndrome and canakinumab for rheumatoid disease [139, 140]. Bispecific antibodies are other line of therapeutics for hemophilic disorders, for example, emicizumab [141]. Protein or enzyme replacement therapies are other strategies in which enzymes are injected as in their original or recombinant protein form. However, very common strategy is to use recombinant enzymes, which are homologues of human types [142]. Though the levels of enzymes required are high, such as 20–40 mg/kg, enzyme replacement therapies are very safe. Oligonucleotide or RNA therapies are recent development such as enzyme replacement therapies. For example, an antisense oligonucleotide was approved by FDA for Huntington disease [143].

4 Gene Therapy

A novel area of treatment and opportunity for people affected by genetic conditions is opened up by the prospect of gene transfer therapy into the somatic cells. Clearly, before effective and complete treatments are made available for several of these illnesses, many technological challenges should be met, and technology must be continuously developed if various conditions are about to be treated. Many gene therapies, like other medical remedies, will relieve some, though not all, effects of a particular condition, which can increase the life quality of patients. Partial enzyme activity replacement in a particular tissue or a group of infected tissues or the cells can delay toxin accumulation in lysosomes, but may not completely inhibit. The development of a long-lasting condition can be inhibited by involvement and can provide very small in the direction of correcting a preexisting degeneration [144].

The lack of certain lysosomal enzymes that destroy specific chemicals, the aggregation of which may induce organ dysfunction, causes many metabolic disorders; some involve mainly visceral organs and other the central nervous system (CNS). Lysosomal defects that induce CNS dysfunction, based on the core deficiency, might need gene transfer into visceral cells, like hepatocytes or hematopoietic cells, or into CNS.

Gene and cell therapies using recombinant viruses were proved efficient in treating hemophilia A and B [145] and spinal muscular disease [146]. However, gene and cell therapies are still at the early stages of research when compared to the other therapies.

5 Natural or Phytomedicines

In metabolic disease treatment, natural-based nanoformulations have demonstrated positive results and should thus be seen as potential candidates instead of or in combination with prescription medications. A new therapeutic solution is nano-sized drug carriers, composed of phytochemicals from traditional drugs blessed with proven pharmacodynamics and pharmacokinetic features. Utilizing herbal extracts with various types of nano-drug delivery technologies have been proposed, such as nanobiocomposites, solid lipid nanoparticles, nanoemulsions, and green-synthesized gold, zinc oxide, and silver nanoparticles. The nano-vehicles give specific characteristics, comprising increased drug

bioavailability and solubility, reduced systemic detrimental side effects, prolonged circulating time, and preferential aggregation in the target organ. Various phytochemicals, including berberine (BBR), curcumin, emodin, oleoresin capsicum, gymnemic acid, naringenin, resveratrol, scutellarin, quercetin (QUE), silybin, baicalin, stevioside, and others, have been used in various therapy methods and metabolic disease alleviation. These phytochemicals were inserted into different systems of nanoformulation, including poly (alkyl cyanoacrylate), polyanhydride and polymeric polyesters, and natural polyesters. Solid lipid nanoparticles (SLNs), liposomes, nanostructured lipid carriers, micelles, green-synthesized nanoparticles (NPs), and other associated nanoformulations are also used in lipid-based drug delivery applications [147]. One of the easiest and environmentally safe approaches for green metal NP production is plant extract-mediated production of NPs [148]. Lycopene, polyphenols, omega-3 fatty acids, and phytoestrogens are some of the dietary supplements that help managing osteoporosis, which is a rare disease as mentioned earlier [35].

6 Other Treatments

Pleiotropic metabolic advantages are seen by the gut hormone GLP1, including increased insulin secretion [149–151] and reduced food intake [152–154]. With regulatory clearance, structurally improved GLP1 paralogues or analogues (like dulaglutide, semaglutide, and exenatide) have advanced to include a new alternative in T2DM treatment and obesity [105, 155], with no evident cardiovascular [82] or psychological [156] detrimental results. Around the same time, antagonists of SGLT2 were effectively progressed for T2DM treatment and appear to decrease body weight as well. SGLT2 inhibitors provide major increase in glycemic function by reducing glucose reabsorption in renal proximal tubules, which is followed by reduced weight loss and blood pressure [85, 157]. The pairing of SGLT2 inhibition and GLP1 agonism could hitherto tend to be an interesting combinatorial method for T2DM management [158, 159]. Many other new groups of peptide-small molecule conjugates and single-molecule multi-agonist peptides have been developed that display outstanding preclinical potency. Other groups have arisen of single-molecule multi-agonist peptides and peptide-small molecule conjugates that display outstanding preclinical potency.

7 Perspective

The occurrence of at least three out of five discrete risk factors is characterized as metabolic diseases: increased triglycerides, dyslipidemia, elevated visceral circumference, increased blood pressure, elevated fasting glucose levels, and reduced HDL-C. The development was confirmed as a significant threat for atherosclerosis and diabetes mellitus emergence. In certain aspects of healthcare, metabolic diseases have emerged as a crucial problem. Its occurrence in children and young adults has also been growing substantially. Owing to a shortage of broad randomized clinical trials, recommendations for management were also discussed. The key approaches for remedy of insulin intolerance and atherogenic dyslipidemia existing in persons with the metabolic diseases are lifestyle approaches aimed at reducing calorie consumption, increasing the levels of physical activity, leading a low stress life, and improving diet composition.

Pharmacological treatments should be initiated whenever lifestyle modifications are not successful or cannot be adopted. The sequence of the treatment priorities must be: (1) repair of lipid defects, (2) arterial hypertension, and (3) glucose intolerance in which a wide variety of the pharmaceutical alternatives are open to clinicians. Drugs with pleiotropic consequences should be attempted first in order to maximize the quantity of individual goals and decrease the cost and side effect burdens of the treatment. There’s really no easy, secure, and reliable pharmacological solution to central obesity yet [4].

Some of the rare diseases have been researched, and drugs are approved by the regulatory authorities in the name of orphan drugs. However, the challenges for developing rare disease drugs are funding, conducting the basic and clinical studies, knowledge base such as training to the clinicians and scientists, and discovery of new drugs. Besides, disease-specific challenges still remain as a critical barrier for new drug development for the rare diseases [160].

Efficient and stable medication formulations with pleiotropic consequences, capable of facilitating concurrent progress for at least three distinct components, the metformin-fibrate-angiotensin receptor blocker, are anticipated to be developed in near future. The metformin-fibrate-angiotensin receptor blocker paradigm used in medical practice would be progressed by advent of novel molecules that enhance sensitivity of insulin, suppress appetite, preserve the mass of beta-cells in pancreas, and facilitate better absorption of free fatty acids. For two discrete research studies, there are promises and related obstacles. First is enhancing accessibility to already available molecules. Secondly, drug finding could limit the increased lipolysis correlated with the visceral obesity and thus reduce flow of free fatty acids to the muscles and liver [4]. Gene therapies are still expected to yield the therapeutics measures, and research activities are undergoing.

To test botanicals for the categorical therapy of metabolic diseases, experiments must be segmented in order to quantify variations in disease severity, age, ethnicity, and genetic diversity in sample populations [161]. In order to allow the standardization of therapeutic formulations, the classification of the active constituents within botanicals, coupled with a perception of their protection, effectiveness, and mechanisms involved, is required. It would be necessary to perform well-designed, controlled clinical studies, once standardized arrangements are produced to show the true efficacy of these formulations for prevention and treatment.

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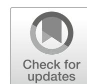

Gene Editing and Human iPSCs in Cardiovascular and Metabolic Diseases

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Abstract

The incidence and the burden of cardiovascular disease (CVD), coronary heart disease (CHD), type 2 diabetes mellitus (T2DM), and the metabolic syndrome are greatly increasing in our societies. Together, they account for 31% of all deaths worldwide. This chapter focuses on the role of two revolutionary discoveries that are changing the future of medicine, induced pluripotent stem cells (iPSCs) and CRISPR/Cas9 technology, in the study, and the cure of cardiovascular and metabolic diseases.

We summarize the state-of-the-art knowledge about the possibility of editing iPSC genome for therapeutic applications without hampering their pluripotency and differentiation, using CRISPR/Cas technology, in the field of cardiovascular and metabolic diseases.

Keywords

Cardiovascular · Gene editing · Metabolism · Epigenetics · Induced pluripotent stem cells (iPSC)

1 Background

The first two decades of the twenty-first century witnessed two revolutionary discoveries that were destined to change the future of medicine in the

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years to come: induced pluripotent stem cells (iPSCs) and CRISPR/Cas9 technology.

In 2006, Yamanaka and Takahashi showed that stem cells with properties similar to embryonic stem cells (ESCs) could be produced from mouse fibroblasts by concomitant introduction of four genes [1]. The two Japanese scientists named these cells induced pluripotent stem cells (iPSCs). In 2007, they successfully extended this approach to human fibroblasts, generating human iPSCs [2]. Few days later, James Thomson's lab also reported the generation of human iPSC using a different combination of transcription factors [3]. Shinya Yamanaka was awarded the Nobel Prize for Physiology or Medicine in 2012. Obviously, iPSC technology was established on the basis of numerous past findings. Three major research field led to the generation of iPSC. The first field was reprogramming by nuclear transfer, shown by Sir John B. Gurdon [4], who eventually was also awarded the Nobel Prize for Physiology or Medicine in 2012. The second field was the one culminating in the birth of Dolly, the first mammal generated by somatic cloning of epithelial cells, by Ian Wilmut and colleagues [5]. Finally, the third field was developed by Takashi Tada's group that in 2001 showed that ESCs also contain reprogramming factors [6].

Clustered regularly interspaced short palindromic repeats (CRISPR) is a family of DNA sequences found in bacteria and archaea [7]. Actually, these sequences derive from DNA chunks of bacteriophages that had previously infected these unicellular organisms. Bacteria use CRISPR to detect and annihilate DNA from similar bacteriophages that might infect them. CRISPR sequences are thus fundamental players in the antiviral defense system of prokaryotes [7, 8].

In turn, the CRISPR/CRISPR-associated system (Cas) is a complex prokaryotic immune system that guarantees resistance to extraneous genetic elements and gives a form of unicellular acquired immunity [7, 9]. From this editing process, a wide variety of applications in the fields of basic biological research, biotechnology, and medical therapies was developed [10]. For this contribution to science, the Nobel Prize in Chemistry in 2020 was awarded to Emmanuelle Charpentier and Jennifer Doudna.

Naturally, the possibility of editing iPSC genome (the previous approaches were time and labor consuming and low editing efficiency) for therapeutic applications without hampering their pluripotency and differentiation, using CRISPR/Cas technology, attracted the attention and the efforts of biomedical researchers worldwide. While the research fields of both iPSC and CRISPR/Cas-mediated genome editing quickly evolve, this chapter sets its high goal in critically summarizing the state-of-the-art knowledge on gene editing and human iPSCs, in particular for their applications in cardiovascular and metabolic diseases, which together account for 31% of all death worldwide (https://www.who.int/health-topics/cardiovascular-diseases/#tab=tab_1).

2 CRISPR/Cas9

In common with other CRISPR/Cas technologies, CRISPR/Cas9 is a technique that takes advantage of the prokaryote evolution that gave rise to an efficient system to fight viruses and plasmids, developing a so called RNA-mediated adaptive immunity [11, 12]. CRISPR sequences are present in ~50% of known bacteria and in ~90% of known archaea [13]. Most applications emerged after the discovery of the type II-A CRISPR/Cas9 system of *Streptococcus pyogenes*. In this bacteria, CRISPR/Cas9 protein possesses a dual-RNA-guided DNA endonuclease, which uses a tracrRNA:crRNA duplex [14] to direct DNA cleavage. The CRISPR locus presents CRISPR repeat-spacer arrays, which are transcribed into CRISPR RNA (crRNA) and trans-activating CRISPR RNA (tracrRNA), and a set of CRISPR-associated (cas) genes, located next to the CRISPR loci, which are an operon that encode Cas proteins with endonuclease activity.

Cas9 is a protein with two helicase domains HNH [15] and RuvC-like [16], essential to avoid the virus invasion [17].

The steps of the immune defense through a CRISPR/Cas9 mechanism are as follows:

acquisition and integration of the foreign DNA, RNA maturation, and interference.

In the first step, CRISPR recognizes the host DNA and integrates it into the spacer loci, thanks to a specific sequence (20 nucleotides) called PAM (protospacer) [18]. In the second step, the RNA polymerase transcribes the precursor crRNAs (pre-crRNA); subsequently, an endonuclease cleaves the RNA precursor in order to generate the active form of CRISPR RNA, and in the last step, a complex of proteins and crRNAs recognizes the foreign RNA or DNA and degrades it [18].

In nature, there exist three types of CRISPR Cas system with reference to the type of DNA/RNA degradation, the third and last stem of the process. The difference between those three is in the multiprotein involvement complex: in the type I, Cas3 helicase recognizes a complex of ribonucleoproteins with the crRNA, through sequence-driven degradation of the foreign DNA. In the type II, Cas9 forms a complex with tracrRNA-sgRNA (single-guide RNA), which is sufficient to generate the crRNA and to degrade the foreign DNA thanks to the RNA guide (gRNA) [19]. In the type III, the protein CRISPR requires Cas5, Cas6, and Cas7 also called repeat-associated mysterious proteins (RAMP) [20] containing RNA recognition motif (RRM) domain that do not recognize the PAM sequences: for this reason, type III is not very specific even though the process is followed by the degradation of the foreign RNA host [21].

Taking advantage of this extraordinary natural mechanism of CRISPR/Cas9, the field of gene editing in eukaryotic cells changed dramatically. In fact, several protocols were developed with the aim of creating therapeutic approaches, in particular exploiting the type II CRISPR/Cas9, in order to achieve site-specific DNA modifications into the DNA, in the sequence(s) of interest.

In this respect, the CRISPR-Cas9 type II from *Streptococcus pyogenes* was modified in order to generate the system tracrRNA:crRNA in one single RNA guide, containing 20 nucleotides that recognize the target DNA sequence at the 5′ end and at the 3′ end the sequence guide that recognizes Cas9. Upon generation of this system, the sgRNA can recruit Cas9 along the DNA target site [22]. In the last step of the process, when sgRNA guides Cas9 to the target sequence, it generates double-stranded break (DSB) that eventually is repaired by the endogenous DNA damage repair (DDR) mechanisms of nonhomologous end joining (NHEJ) pathway or the homology-directed repair (HDR) pathway [23]. In this, thus it is possible to introduce or modify or knock out the specific sequence or gene(s); for this reason, nowadays, CRISPR/Cas9 became one of the most useful techniques for molecular biologists. However, the CRISPR/Cas9 technique still needs to be ameliorated in terms of specificity, due to PAM sequences that are necessary for the genome-targeting scope, low efficiency, and limitations due to the off-target effects [23].

3 CRISPR Cas9 and Cardiometabolic Diseases: Lessons from Animal Models

Cardiovascular diseases (CVD) and metabolic diseases, such as diabetes, are still major health problems with increasing global prevalence (https://www.who.int/health-topics/cardiovascular-diseases/#tab=tab_1; https://www.who.int/health-topics/diabetes#tab=tab_1), and a remaining hurdle is to gain a deeper knowledge of the mechanisms behind the development of both common and less common causes of cardiometabolic mortality and morbidity. The use of animal models has contributed to provide new approaches to improve the diagnostic and the treatment of cardiometabolic diseases [24, 25]. Although hampered by several technical challenges, somatic genome editing may also be useful to study/treat a variety of cardiometabolic disorders in small laboratory animals such as mice. Here, we review few selected studies applying CRISPR/Cas9 technology to model and study CVD and diabetes in animal models, in order to illustrate the potential of this methodology. Patients affected by Duchenne muscular dystrophy (DMD), a severe type of muscular dystrophy, generally present with some degree of cardiomyopathy in adult age. In a mouse model of DMD,

El Refaey et al. [26] demonstrated that systemic administration of *Staphylococcus aureus* Cas9 and gRNA in an AAV vector led to restoration of the defective DMD reading frame and thus dystrophin expression. Ultimately, this led to improvements in cardiac myofiber architecture and papillary muscle contractility, with a decrease in fibrosis. With a similar approach, Amoasii et al. [27] demonstrated an augmented expression of dystrophin—both in the heart and in the skeletal muscle—in a DMD canine model after intravenous treatment with an AAV9 vector that contained Cas9 and gRNA. Another CVD subfield where CRISPR/Cas9 found its applications is nonischemic cardiomyopathy. For instance, phospholamban (PLN) regulates intracellular calcium concentrations through its inhibitory actions on sarcoplasmic reticulum calcium−adenosine triphosphatase (SERCA2). PLN mutations can cause dilated nonischemic cardiomyopathy [28]. Kaneko et al. [29] corrected this defect in the germline using genome editing via CRISPR/Cas9 to silence the PLN gene in a mouse model of severe heart failure. Compared with control mice displaying heart failure, PLN-deficient mice survived longer and had improved cardiovascular performance [29].

Regarding curing diabetes, Chung et al. combined CRISPR/Cas9 interference with a targeted nonviral gene delivery system to treat obesity-induced type 2 diabetes [30]. Fatty acid-binding protein 4 (FABP4) acts as a novel adipokine, and elevated FABP4 concentration is associated with obesity, diabetes, and atherosclerosis [31]. Targeted delivery of the CRISPR interference system against FABP4 to white adipocytes induced an effective silencing of FABP4, resulting in reduction of body weight, inflammation, and in restoration of nutrient homeostasis in obese mice [30]. Srifa et al. have shown that Cas9-AAV6-engineered human mesenchymal stromal cells were able to ameliorate skin wound healing in diabetic rodents [32]. In a complementary and mirroring fashion, CRISPR/Cas9 technology has been used successfully to generate novel mice models of atherosclerosis, autoimmunity, and diabetes, more faithfully recapitulating the respective human conditions [33–36]. The RNA-guided DNA recognition platform included in the CRISPR/Cas9 technology could thus provide a simple and safe approach to regress CVD and metabolic disease in animal models and, in the end, also has a significant potential to improve symptoms and clinical outcome in patients. A major breakthrough occurred in the field of hematological disorders, such as in transfusion-dependent β-thalassemia (TDT) and in sickle cell disease (SCD), two severe monogenic diseases, potentially life-threatening. Autologous transplantation of CD34+ hematopoietic stem and progenitor cells modified with CRISPR/Cas9 to address the respective genetic disorder(s) led to beneficial effects in two patients, in a pilot study privately funded by CRISPR Therapeutics and Vertex Pharmaceuticals [37]. However, in general, additional studies are warranted to evaluate long-term safety and efficacy of CRISPR/Cas9 in animals of bigger size, before larger publicly/privately funded clinical trials could take place in cardiac and/or diabetic patients.

4 Human iPSC and Their Potential for the Modeling of Cardiometabolic Diseases

iPSCs provide an unparalleled tool to study human pathophysiology down to the cellular level. They also have the great potential to be leveraged in the field of precision medicine and, in particular, for personalized drug screening. What are the applications of iPSC for the research and treatment of cardiometabolic diseases? In 2018, the American Heart Association (AHA, on behalf of the American Heart Association Council on Functional Genomics and Translational Biology; Council on Cardiovascular Disease in the Young; and Council on Cardiovascular and Stroke Nursing) issued an official and excellent scientific statement that comprehensively described the use of iPSC for cardiovascular disease modeling and for precision medicine, highlighting the scientific and clinical relevance of the field [38]. Undoubtedly, cardiovascular disease modeling represents the most realistic and productive use of iPSCs to date. iPSCs have

the same genetic landscape of the individual from which they originate; therefore, they are ideal to study illnesses with strong underlying genetic cause. Simultaneously, this genetic cause is “cleansed” by possible environmental/epigenetic influences that accumulate during the lifetime and can be studied separately, simply because iPSCs are reprogrammed into a basal pluripotent state. However, the use of iPSCs as regenerative therapy in cardiometabolic diseases is still in its infancy. The CVD disease types where iPSC has proven useful to uncover the relationship between genotype and phenotype include dilated cardiomyopathy (DCM) (target genes TTN, TNNT2, LMNA, PLN, DES), Duchenne muscular dystrophy (target gene DMD), Barth syndrome (target gene TAZ), hypertrophic cardiomyopathy (HCM) (target gene MYH7), arrhythmogenic right ventricular dysplasia (target gene PKP2), left ventricular noncompaction (target genes TBX20, GATA4), long-QT syndrome type 1 and Jervell and Lange-Nielsen syndrome (target gene KCNQ1), long-QT syndrome type 2 (target gene KCNH2), long-QT syndrome type 3 (target gene SCN5A), Timothy syndrome (target gene CACNA1C), catecholaminergic polymorphic ventricular tachycardia type 1 (target gene RYR2), catecholaminergic polymorphic ventricular tachycardia type 2 (target gene CASQ2), Brugada syndrome (target gene SCN5A), calcific aortic valve (target gene NOTCH1), Williams-Beuren syndrome (target gene ELN), familial pulmonary hypertension (target gene BMPR2), familial hypercholesterolemia (target genes LDLR, PCSK9), familial hypobetalipoproteinemia (target gene PCSK9), Tangier disease (target gene ABCA1), and dyslipidemia (target gene SORT1) (reviewed in [38]. Among these, we will discuss briefly two example studies revolving around the most common applications of iPSC to model CVD: cardiomyopathies and rhythm disorders. DCM is the most common type of cardiomyopathy; cases of familial DCM represent ~30–50% of the total number of cases of DCM in the general population. DCM patients have ventricular dysfunction and heart failure. One of the most commonly mutated gene products in familial DCM patients is TTN (titin), which encodes a component of the sarcomere [39]. Hinson et al. and Schick et al. generated iPSCs from DCM patients harboring either truncating or missense mutations in TTN: when differentiated into cardiomyocytes, these iPSCs displayed sarcomere insufficiency, impaired cell growth, abnormal cell signaling activation, and impairments in the excitation-contraction-coupling system [40, 41]. iPSC-based models are extensively studied also in order to model arrhythmic syndromes, in particular the long-QT syndromes (LQTS), characterized by delayed repolarization of the heart after contraction, which manifests as an increased QT interval on the electrocardiogram and can provoke ventricular arrhythmias and even death [42]. Mutations in 15+ genes have been linked to LQTS, typically affecting the function of ion channels (potassium, calcium, etc.) in cardiomyocytes [43]. There exist three types of congenital LQTS, where arrhythmia is triggered by distinct stimuli [44]. LQTS1, the most common, is triggered by exercise; LQTS2 is triggered by emotional stress and auditory stimuli; LQTS3 happens during sleep. Remarkably, both LQTS1 and LQTS2 have been modeled with iPSCs derived from cardiomyocytes in which mutant genes were inserted with zinc finger nuclease genome editing to target the adeno-associated virus integration site 1 (AAVS1) locus located on chromosome 19 [45]: in this fashion, it is not even necessary to recruit patients with a given mutation in order to study their LQTS.

The metabolic disease types where iPSC has proven useful to uncover the relationship between genotype and phenotype include type 1 and 2 diabetes (T1D and T2D). In diabetic patients, pancreatic insulin-producing cells do not function properly (type 1), or peripheral tissues do not respond properly to the insulin produced by the pancreas (type 2). As seen for CVD, in diabetes as well patient-specific, iPSCs could be promising, as cells derived from the “self” allow autologous transplantation. In a recent study, iPSCs were generated from patients with type 1 or type 2 diabetes and compared them with iPSCs from a nondiabetic control [46]. To induce differentiation of human iPSCs into insulin-producing cells,

the gene expression of the master transcription factor pancreatic and duodenal homeobox 1 (PDX-1), necessary for pancreatic development including β-cell maturation, was induced using an ad hoc adenovirus. Insulin-producing cells deriving from type 1/type 2 diabetes-specific iPSCs began to express pancreatic islet-specific markers such as PDX-1 itself, the transcription factors MafA and Beta2/NeuroD, and insulin, as it was observed in iPSCs generated from a healthy individual [46]. All these iPSC cell lines, from type 1/type 2 diabetes or from healthy controls, displayed similar capacity in glucose-stimulated insulin secretion [46]. This study, and others (reviewed in [47]), paves the way for autologous transplantation employing patient-specific iPSCs in diabetic patients, which will be described in the next chapters. It is thought of foremost importance to fully characterize the gene expression patterns of patient-derived iPSCs before considering clinical applications. Whole-transcriptome RNA sequencing of human iPSC lines from three independent donors, at baseline and at subsequent stages of in vitro islet differentiation, identified key transcriptional regulators of the differentiation process, suggesting that the susceptibility to develop type 2 diabetes could be at least in part mediated via modulation of pancreatic islet development [48]. Accordingly, developmental plasticity and cell identity switch have been recently identified as a valid regenerative strategy for human pancreas aiming at the treatment of diabetes [49].

In some of the studies discussed above, iPSCs were engineered to model cardiometabolic diseases using genome editing technologies other than CRISPR/Cas9. A historical overview of the technological progress of the different tools used for gene editing of human iPSCs, before the recent advent CRISPR/Cas9, will be illustrated in the following section.

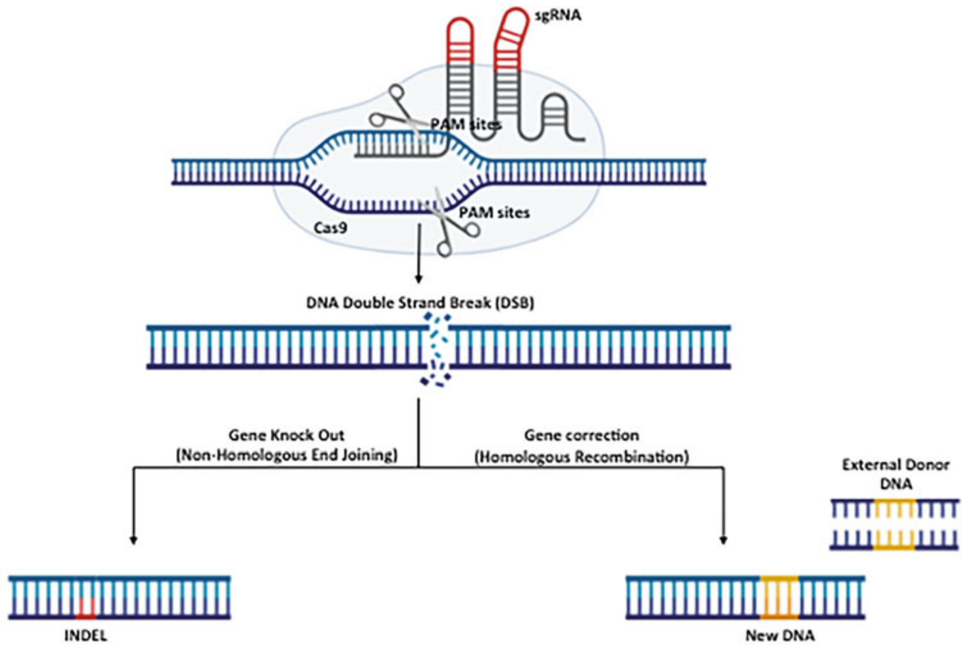
5 Gene Editing in Human iPSC

Before CRISPR/Cas9 development, the first generation of genome editing was based on "targeted gene replacement," a family of techniques aiming to create a localized sequence change triggering null mutations [50]. DNA recombination was achieved triggering DNA double-strand breaks (DDSB) through an endonuclease, eventually repaired by HDR using the designed DNA sequence as template [50]. In 1996, Jasin and colleagues first investigated gene targeting using the megaendonuclease I-SceI [51]. This protein is encoded by *SceI*, a member of the homing endonuclease family [52]. Gene targeting was achieved through the insertion of I-SceI 18-bp recognition sequence inside the target gene. The technique showed a low yield (3–5%) and high risk of unspecific cut discouraging further studies [53]. In 1999, Chandrasegaran and colleagues first introduced zinc-finger nucleases (ZFN) [54]. The most common Cys_2-Hys_2 ZFN is made of 30 amino acids, arranged in a highly conserved ββα configuration. Amino acids on the N-terminal domain, composing the α-helix, are responsible for the recognition of 3-bp in the DNA sequence, while the C-terminal domain is designed to host FokI Type IIS restriction endonuclease cleavage domain [54]. Interestingly, the linker domain of ZFN was designed to induce the dimerization of the protein after recognition of target sequence. As a result, two domains of FokI are placed in a high-density context, triggering its endonuclease activity [55]. Moreover, the introduction of multiple ZFN systems allowed recognition of 18-bp sequences with 25% of yield [52, 56]. ZFN technology found several applications in the hiPSC study. Soldner et al. introduced specific mutations in healthy hiPSC mimicking Parkinson's disease (PD) phenotype [57]. Moreover, ZFN were also employed to study SOD1 mutations in amyotrophic lateral sclerosis (ALS) and to generate Alzheimer's disease (AD) neuron model differentiating hiPSC where specific mutations were introduced [58, 59]. Despite the step forward, ZFN DNA binding domain showed higher affinity for G-rich regions, causing the nonrecognition of several three-base nucleotides and off-target effects. Moreover, high level of expertise in protein engineering was required for their generation [52]. For this reason, in 2007, Botch and colleagues introduced a technology based on

phytopathogenic bacteria of the genus *Xanthomonas* [60]. These bacteria elicit their pathogenicity translocating effector proteins via type III secretion system. Among them, transcription activator-like effectors (TALE) are a family of proteins in charge for host cell reprogramming [60]. These proteins are composed by a nuclear localization, an acid transcriptional activating and a central domain provided of repeat variable di-residues (RVD) responsible for DNA recognition. Boch and colleagues provided the first hint for the development of artificial TALE reporting the RVD code for the recognition of specific DNA base pair. Moreover, they showed that each target base pair must be preceded by a thymine to bind TALE proteins [60]. In order to link their targeting features to the endonuclease activity needed for targeting gene replacement, TALE DNA binding region was coupled to FokI domain. As for ZFN, dimerization of TALE-nuclease (TALEN) following the recognition of vicinal sites triggered FokI activity [61]. Compared to ZFN, TALEN showed several improvements including a decreased cytotoxicity with a less expensive production [62]. For these reasons, TALEN were studied in hiPSC context for generation of several blood disease models [63–65]. Moreover, familiar AD neuron models carrying a prensilin-1 mutation were generated from hiPSC models using TALEN, providing a reliable cell model to further investigate this disease [66]. However, off-target effects and requirement of a thymine at the beginning of the target site restricted TALEN application, pushed for the development of techniques like CRISPR/Cas [67].

6 CRISPR Cas9 and Gene Editing in Human iPSC

In the last decade, CRISPR/Cas9 technology potential was applied to the genome editing of human iPSCs, allowing to make important progress in human disease investigation. Two main approaches have driven the research: the generation of isogenic controls by correcting the mutated gene in diseased iPSCs and the generation of mutated iPSCs lines by knocking out the normal wild type gene in healthy iPSCs (Fig. 1). Both alternatives have opened to the possibility to focus on the pathologies caused by a specific mutation rather than the individual's genetic background, with the great advantages to obtain model of rare diseases in which there are difficulties in obtaining patient biopsies. For example, Horii and colleagues have created a model of immunodeficiency, centromeric region instability and facial anomalies syndrome (ICF), a rare autosomal recessive disorder caused by mutations in DNA methyltransferase 3B (DNMT3B), using CRISPR-Cas9 and gRNA directed to DNMT3B by knocking out (KO) the mutated gene [68]. The KO cells exhibited hypomethylation of satellite two repeats compared to wild-type iPSCs, demonstrating the same effect on methylation patterns observed in ICF patients [68] (1). With a similar approach, Shinkuma's group created a model of dominant dystrophic epidermolysis bullosa (DDEB), an inherited skin disorder characterized by blisters and skin fragility [69, 70]. These researchers created a premature stop codon by NHEJ knocking out the mutated allele of the collagen alpha-1(VII) chain (COL7A1) gene [71], with high specificity for the mutated allele, leaving the normal allele untouched [69]. The genetic modification did not affect the differentiation capacity of the iPSCs into fibroblasts and keratinocytes as well as the COL7 patterns (Fig. 1). The introduction of CRISPR/Cas9 allowed to investigate diseases in which the causative mutation disrupts proper reprogramming of mutated fibroblasts to iPSCs, such as the mutations in Fanconi anemia (FA) genes, in ataxia-telangiectasia mutated (ATM) gene, and ALK2-encoding gene [72–74]. The constitutive activation of BMP type I receptor in patients with activin receptor-like kinase 2 (ALK-2) mutations induces heterotopic ossification [75]. Several studies reported attempts to produce iPSCs from mutated ALK-2 fibroblasts without success or eventually obtaining cells that lost their pluripotency completely [75]. Kim and co-workers corrected the mutation through HDR, using a single-strand oligodeoxynucleotide (ssODN) carrying



Application in iPSC	
Deleted sequence	Reference
DNMT3B	Horii et al., 2013
COL7A1	Shinkuma et al., 2016
CGG repeats in FMR1	Park et al., 2015

Application in iPSC	
Corrected sequence	Reference
ALK-2	Kim et al., 2016

Fig. 1 Schematic overview of CRISPR/Cas9-driven gene knockout (nonhomologous end joining, NHEJ) or gene correction (homologous recombination) and their respective applications in the iPSC field

two-point mutations: the correction of the mutated base and a silent mutation in the PAM sequence, in order to avoid gRNA-mediated cut of the donor DNA. They obtained iPSCs with a corrected ALK-2 gene and a restored mineralization process in the cells [76] (Fig. 1). Other studies used CRISPR-Cas9 to restore normal function in iPSCs generated from stromal cells of patients with rare diseases, like what made by Park and co-workers through the deletion of CGG repeats in fragile X mental retardation 1 (FMR1) gene to cure Fragile X syndrome [77] (Fig. 1). These findings support the tremendous promise to apply CRISPR-Cas9 and human iPSCs to regenerative medicine, through the correction of mutated iPSCs generated from the patients and their reintroduction after differentiation, without the risk of immune rejection. In the following two sections, we will discuss some of the main findings obtained using these new technologies to the fields of cardiovascular and metabolic diseases.

6.1 CRISPR Cas9 and Gene Editing in Human iPSC: Impact on Cardiovascular Research and Therapy

Human pluripotent stem cell-derived cardiomyocytes (hPSC-CM) have emerged as a powerful system to model CVDs as they can recapitulate pathological phenotypes, provide insight into molecular mechanisms, and identify new targets for therapy. Genome editing tools, specially CRISPR/Cas9, allow to evaluate the

causal role of genetic variants in this physiologically relevant cell system. Together, these technologies have vastly advanced our ability to study CVDs creating various models of cardiomyopathy such as Barth syndrome, Fabry disease, and HCM, among others (Table 1). For instance, in 2014, Wang et al. have used patient-derived hiPSCs, CRISPR/Cas9, and tissue engineering in order to replicate the pathophysiology of Barth syndrome cardiomyopathy in tissue constructs. Moreover, using the same model, the authors demonstrated to rescue the phenotype by gene replacement and small molecule treatments [78]. In another study, Mosqueira et al. created 11 isogenic variant models of an HCM-causing mutation in the MYH gene in three independent hiPSC lines, by using CRISPR/Cas9, and subsequently differentiated them into cardiomyocytes for molecular and functional evaluation [79]. These cardiomyocytes reproduced the key cellular features of HCM, characterized by hypertrophy, excessive multi-nucleation, and sarcomeric disarray. Furthermore, they showed an impaired Ca^{2+} functionality with energy depletion, altered abnormalities handling, arrhythmias, and hypo-contractility. The pharmacological rescue of arrhythmias was shown to be achievable. Furthermore, novel long noncoding RNAs (lncRNAs) and possible gene modifiers were identified using these models, proposing new therapeutic approaches for HCM [79].

Genome editing has also contributed to enhance our knowledge about less common cardiomyopathies such as arrhythmogenic right ventricular cardiomyopathy (ARVC) and left ventricular non-compaction cardiomyopathy (LVNC) [81, 82]. CRISPR/Cas9 technology was used to recapitulate the ARVC phenotype characterized by mutations of sodium voltage-gated channel alpha subunit 5 (SCN5A) gene coding for the sodium channels Nav1.5 [81]. In this study, it was observed a reduced sodium current in the mutant model as well as decreased Nav1.5 and N-cadherin clusters at junctional sites, suggesting Nav1.5 as part of a functional complex that involve cell adhesion molecules. These findings provided an alternative explanation to the mechanisms by which SCN5A mutations cause ARVC. In 2016, Kodo et al. generated a LVNC phenotype in induced cardiomyocytes (iCMs) carrying a mutation in the cardiac T-box family transcription factor TBX20 gene. The study identified the LVNC-associated reduced proliferation as a consequence of an impaired TGF-β signaling activation [82].

The use of hiPSCs has brought various advantages also in the DMD therapy research. For example, CRISPR can be used to generate the patient mutations directly in control hiPSC lines, providing unprecedented versatility in facing the great number of DMD mutations. Moreover, iCM models have showed to recapitulate more faithfully the physiology of the human heart [93, 94].

Indeed, iCMs generated from DMD patient hiPSCs were able to reproduce the disease phenotype model quite well, showing significantly greater cell areas, longer resting sarcomere lengths, and a reduced capacity to respond to environmental stimuli compared to control iCMs [95]. One of the earliest studies that used CRISPR treatment on DMD iCMs was carried on by Young's group in 2016 [83]. In this study, multiple hiPSC cell lines were derived from the fibroblasts of patients with deletions in either DMD exons 46–51 or 46–47, or with DMD exon 50 duplication. Young's group, after nucleofecting plasmids containing SpCas9 and gRNAs against DMD introns 44 and 55 into DMD-hiPSCs, differentiated these cells into iCMs, showing a skipping of exons 45–55 and the consequent dystrophin rescue. It was estimated that skipping exons 45–55 could help to treat about 66% of all DMD patients affected by deletion mutations [96]. In 2017, also Kyrychenko's team investigated the effect of multiple exons deletion on DMD hiPSC models, deleting DMD exons 3–9, exons 6–9, or exons 7–11 using CRISPR/SpCas9 [84]. The authors found that deleting exons 3–9 rescued the dystrophin production and also improved Ca2+ kinetics and synchronicity in Ca2+ activity in iCMs. In this case, it was estimated that approximately 7% of DMD patients could benefit by exons 3–9 deletion [96].

Table 1 CRISPR/cas9 and genome editing in hiPSC models for cardiac and metabolic diseases

Disease	Gene mutation	Model phenotype	Genome editing method	Outcome	References
Barth syndrome	TAZ frameshift (c.517delG) and missense (c.328T>C)	Impaired cardiolipin biogenesis and mitochondrial function	cas9-mediated editing	TAZ deficiency in BTHS caused markedly increased ROS production	[78]
Hypertrophic cardiomyopathy (HCM)	c.C9123T-MYH7 (p. R453C-β-myosin heavy chain (MHC)]	Sarcomeric disarray and hypo-contractility, but increased metabolic demands	CRISPR/ Cas9	Generation of HCM human model	[79]
Fabry disease	GLA gene knockout	Enlarged cellular size, increased expression of cardiac hypertrophy genes and Gb3 accumulation	CRISPR/ Cas9	Impaired cytoskeleton dynamics, extracellular vesicle secretion and autophagy process	[80]
Arrhythmogenic right ventricular dysplasia/ cardiomyopathy (ARVD/C)	[p.Arg1898His (c.5693G > A)] *SCN5A* mutation	Prolonged QRS duration	CRISPR/ Cas9	Nav1.5-dependent impairment of sodium current and adhesion protein structure	[81]
Left ventricular non-compaction (LVNC)	Y317* (c. 951C>A) and T262M (c. 785C>T) mutation on the TBX20 gene	Impaired cardiomyocyte differentiation and proliferation	CRISPR/ Cas9, TALEN	Proliferation defect as a consequence of TGF-β signaling abnormal activation	[82]
Duchenne muscular dystrophy (DMD)	Exons 46–51 or 46–47 deletion, or with DMD exon 50 duplication	Muscle membrane fragility and leakage of creatine kinase	CRISPR/ Cas9	Deletion of exons 45–55 restores dystrophin protein function	[83]
	Exons 8–9 deletion	Muscle membrane fragility and leakage of creatine kinase	CRISPR/ Cas9	Deletion of DMD exons 3–9 restores cardiomyocyte functionality	[84]
Neonatal diabetes mellitus	STAT3 K392R missense mutation	Premature endocrine differentiation	CRISPR/ Cas9	STAT3 mutation activate NEUROG3. Its correction reversed disease phenotype	[85]
Pancreatic agenesis	4 bp duplication in exon 2 of GATA6	Generation of truncated GATA6 protein and decreased cell differentiation to definitive endoderm	CRISPR/ Cas9	GATA6 is essential in endoderm and pancreatic development	[86]
Permanent neonatal diabetes mellitus (PNDM)	Homozygous ATG > ATA mutation at codon 1 of the insulin gene	Lack of C-peptide-positive cells, no insulin production	CRISPR/ Cas9	Insulin gene correction led to rescue the insulin production	[87]
Alpha-1 antitrypsin AAT deficiency (AATD)	Point mutation in the A1AT gene (the Z allele; Glu342Lys)	Mutant polymeric A1AT within the endoplasmic reticulum of hepatocytes	ZFN, piggyBac	Gene correction results in normal monomeric A1AT secretion	[88]

(continued)

Table 1 (continued)

Disease	Gene mutation	Model phenotype	Genome editing method	Outcome	References
	α1-antitrypsin (AAT) Z mutation (rs28929474, G>A)	Mutant polymeric A1AT within the endoplasmic reticulum of hepatocytes	CRISPR/Cas9, TALEN	High specificity of CRISPR/Cas9 in gene correction compared to TALEN	[89]
Familial hypercholesterolemia (FH)	g.10891 C>T (c.97C->T, p. Q12X) mutation in LDLR gene	Absence of LDLR expression with elevated levels of LDL-c	CRISPR/Cas9	Insertion of LDLR expression cassette at the AAVS1 genomic site restore LDLR phenotype and function	[90]
Propionic acidemia (PA)	p. Gly407Argfs*14 mutation in PCCB gene	Toxic accumulation of propionyl-CoA and derived metabolites	CRISPR/Cas9	Generation of isogenic control (UAMi-006) from a PA iPSC line	[91]
Primary hyperoxaluria type 1 (PH1)	c.731 T > C mutation (p.I244T) in exon 7 of the AGXT gene	Lack of AGT activity leading insoluble calcium-oxalate crystals formation	CRISPR/Cas9	Integration of an AGXT minigene into the AAVS1 safe harbor locus corrected hepatocytes functionality	[92]

In addition to these valuable findings, genome editing and iPSC had also been used to further our understanding of inherited and drug-induced cardiotoxicity and the essential processes underlying the cardiomyopathy pathophysiology such as cardiomyocyte differentiation and cardiac remodeling [97–100].

6.2 CRISPR Cas9 and Gene Editing in Human iPSC: Impact on Metabolic Disease Research and Therapy

The use of the new genetic editing tools on human iPSCs represents a powerful option also to investigate molecular mechanisms underlying diabetes and liver metabolic disorders, especially for hereditary forms. In the last years, substantial progress in our understanding of hiPSC differentiation toward pancreatic β-cells has been made. Despite these advances, obtaining a pure β-like cell population is still a challenge, because the process of hiPSC differentiation results in a heterogeneous cell population contaminated with other endocrine cell types. Up to the present, hiPSC lines have been generated from several patient populations with diabetes, including those with T1D, T2D, cystic fibrosis-associated diabetes, and other monogenic diabetes such as maturity onset diabetes of the young (MODY), Wolfram syndrome, and mitochondrial diabetes [101].

At the base of most of these diabetes types, there are dominantly inherited mutations in genes involved in β-cell normal functions [102]. A major advantage of generating diabetic iPSC models is represented by the possibility to provide personalized therapeutics and autologous transplantation. In fact, like allogenic donor islet transplantations, current allogenic SC-β replacement therapies, based on edited hESC, would require to administrate immunosuppressor drugs life-long to the patient, unless the cells are incorporated and protected in a macroencapsulation vessel [103, 104]. Despite the use of CRISPR/cas9 editing on diabetes patient-derivated iPSCs is still in its infancy, some

important progresses have been made (Table 1). The main approach is to correct the diabetes causative point mutation in patient-derived iPSC.

The generation of the isogenic cell lines allows to keep the original genetic background of the diabetic patient and to investigate the role of the mutation faithfully. For example, genome editing of h-iPSC was used to assess the mechanism of signal transducer and activator of transcription 3 (STAT3) mutation in a rare case of neonatal diabetes, uncovering its involvement on premature differentiation of multipotent pancreatic progenitors [85]. A similar approach was applied to correct the GATA-binding factor 6 (GATA6) mutation in iPSC from a patient with pancreatic agenesis, revealing the essential function of ß-GATA6 in pancreatic development and cell function [86].

Moreover, Ma et al., after the identification in a patient with permanent neonatal diabetes mellitus (PNDM), a rare form of monogenic diabetes characterized by neonatal hyperinsulinism, of a homozygous ATG > ATA mutation at codon 1 of the insulin gene, have reverted the mutation to wild-type ATG using CRISPR/Cas9 and differentiated both mutant and corrected cells to pancreatic endocrine cells. The followed transplant of endocrine cells derived from the corrected patient stem cells in a diabetic mice models has shown to produce detectable levels of insulin that allowed for the sustenance of normoglycemia [87].

A number of metabolic conditions involve the liver and can cause chronic liver disease, leading to cirrhosis and liver cancer. Today, it is possible to generate human iPSC-derived hepatocyte-like cell (HLC) models, which recapitulate the hepatic functionality in vivo [105–108]. Among various inherited liver metabolic disorders, alpha-1 antitrypsin AAT deficiency (AATD) and familial hypercholesterolemia (FH) have been the most actively studied. AATD is a disease caused by mutations in the AAT gene that lead to develop severe liver diseases, including liver cirrhosis and hepatocellular carcinoma [109]. The mutation promotes spontaneous Z alpha-1 antitrypsin (Z-AAT) polymerization and retention of the Z-AAT polymers in the endoplasmic reticulum (ER) of hepatocytes, with consequent protein overload and liver damage [110]. In 2010, Rashid et al. for the first time generated human iPSC from a patient with AATD and then differentiated these cells into HLCs (AATD-iPS-HLCs) [111]. Gene editing experiments using ZFN or TALEN on these models were conducted to correct the point mutation (Glu342Lys) in the AAT locus that is responsible for Z-AAT production, achieving a different efficacy (4 and 25–33% for ZFN and TALEN, respectively [88, 111, 112]. The corrected AATD-iPS-HLCs showed a proper secretion of normal monomeric AAT in the culture supernatant [112]. Smith et al. later reported the gene correction of AATD-iPS cells using CRISPR/Cas9 system, obtaining a high specificity and efficiency that allowed them to conduct allele-specific gene targeting of point mutations in patient-specific iPSCs [89].

FH is an autosomal dominant hypercholesterolemia due to mutations in the low-density lipoprotein receptor gene (LDLR) or genes related with it. The disease is characterized by an increased levels of serum low-density lipoprotein cholesterol (LDL-c) with consequent xanthoma formation and early cardiovascular disease [113, 114]. The iPSCs generated from FH patient (FH-iPSC) displayed the disease phenotype. FH-iPSCs were genetically corrected using homologous recombination mediated by the CRISPR/Cas9 system integrating a correction cassette at the AAVS1 locus that resulted in restoration of LDLR expression and LDL-c uptake in the differentiated FH-iPSC-HLCs [90]. Recently, other studies were conducted on different inherited metabolic disease such as propionic acidemia, caused by mutations in the propionyl-CoA carboxylase alpha (PCCA) and propionyl-CoA carboxylase beta (PCCB) genes [91]; primary hyperoxaluria type 1 (PH1) caused by a deficiency of the peroxisomal enzyme alanine-glyoxylate aminotransferase (AGT) [92]; and abetalipoproteinemia, caused by MTTP gene mutation [115], confirming the usefulness and versatility of CRISPR/cas9 tool applied to human iPSC and marking the way toward future discoveries.

7 Gene Editing, iPSC, and Clinical Trials

As already mentioned, the combination of iPSC and CRISPR/Cas9 technologies represent a valid tool to model diseases in vitro and in vivo. This section will describe paradigms of the applications of these technologies in preclinical setting, which may lay the foundation for targeted clinical trials, which are currently missing.

Jacków et al. recently proposed a protocol using iPSCs combined with CRISPR/Cas9 techniques as a strategy for the long-lasting treatment of dystrophic recessive epidermolysis bullosa (RDEB), a rare genetic skin fragility disorder due to the COL7A1 gene mutation that encodes for type VII collagen, which in turn is important for stabilizing dermal–epidermal adhesion at the basement membrane zone [116, 117].

Using primary fibroblasts isolated from the foreskin of healthy individuals and also the dermis of two patients with RDEB, the authors obtained iPSCs introducing a vector encoding the four classic Yamanaka's reprogramming factors (Oct4, Sox2, Klf4, and c-Myc) [117], in this way generating RDEB patient-specific iPSCs. Second, they generated CRISPR/Cas9-mediated HDR, using high-fidelity Cas9 (SpyFiCas9) nucleases with no detectable genome-wide off-target effects, to repair the COL7A1 mutation in iPSCs in order to restore C7 expression. With the combination of the two techniques mentioned above, the authors were able to achieve the COL7A1correction in vitro and in vivo, with restoration of skin integrity and type VII collagen after grafting them onto nude mice. These findings are promising to translate this treatment into the clinic [117], However, to this aim, hurdles need to be overcome. On one hand, the iPSCs reprogramming can cause genetic instability [118]; on the other hand, developing xeno-free (without animal products) culture systems of iPSCs is less risky compared to undefined animal-derived components that could introduce variability on the cultures, complicating their therapeutic application in patients.

Inherited retinal dystrophies (IRDs) are a group of neurodegenerative disorders, which was discovered for the first time by Cornelius Franz Donders in 1857 that involves the retina, causing color blindness, tunnel vision, and subsequent progression to complete blindness [119]. In particular, Usher syndrome (USH) belongs to the spectrum of IRD disorders and is characterized by retinal degeneration, retinitis pigmentosa (RP), and hearing loss [119].

USH is caused predominantly by USH2A mutations in exon 13; the first attempts using canonical gene therapy method to treat this disease failed [119]. Applying CRISPR/Cas9 technology to iPSCs obtained from patients with USH in vitro, it was possible to correct the USH2A mutations in exon 13, without any off-target mutagenesis in the corrected iPSCs, which also retained pluripotency and genetic stability [120]. The goal of the latter approach is to develop a strategy for future autologous cell therapy of patents and eventually into clinical trial program.

Another interesting work from Zhanhui Ou et al. reported the combination of iPSC and CRISPR/Cas9 techniques to treat β-thalassemia disease. β-thalassemia is a blood genetic disorder caused by a small deletion in the beta globin HBB gene [121], which causes lack of oxygen in the body, anemia due to shortage of blood cell that in turn causes pale skin, weakness, and fatigue [122]. Generating iPSCs from the somatic cells of β-thalassemia patients, it was possible to obtain hematopoietic stem cells (HSCs), with homologous recombination-based gene correction, which recovers the production of HBB in vitro and with improvement of hemoglobin by CRISPR/Cas9 (HB) production in vivo in immune-deficient mice [123, 124]. The correction was confirmed using markers in BM cell from transplantation and the non-transplantation sides of the mice, and also at protein level, it was confirmed by the presence of HBB protein in the peripheral blood of the mice. This study confirmed that achievement of the genetic correction of β-thalassemia iPSC mutation allows the production of HBB

after hematopoietic differentiation in vivo, highlighting a safe gene therapy strategy of combining iPSCs and CRISPR/Cas9 technology to treat β-thalassemia [121], which can be proposed in the future in a clinical trial study.

Separately, CRISPR/Cas9 is used in preclinical therapy studies for diseases such as Duchenne muscular dystrophy [125], β-thalassemia [126], or Alzheimer's disease [127], and although so far no therapy based on iPSCs has found its way into routine clinical use, as of 2020, a comprehensive and well-conducted meta-analysis using stringent inclusion/exclusion procedure identified 131 studies that could be classified as clinical trials involving iPSCs—77% of them being observational and ~23% being interventional [128]. Only a limited number of trials were focusing on the actual transplantation of iPSCs into patients.

Therefore, despite the potential of the powerful combination of iPSC and CRISPR/Cas9 techniques, this system needs to be ameliorated, and it remained to be verified whether we may ultimately overcome the challenges associated with their clinical use, opening the doors to this fascinating personalized curative medicine to treat several diseases.

8 Limitations and Challenges of iPSC and Editing Technologies

When first described by Yamanaka in 2006, iPSCs represented a giant breakthrough for development of personalized medicine. Fifteen years later, many challenges are still on the table, discouraging iPSC clinical applications [129]. The main bottleneck is represented by iPSCs' tumorigenic potential (Fig. 2). Undifferentiated or immature cells still present in the final iPSC-derived cell product together with still active reprogramming factors and genetic mutations occurred during cell reprogramming may lead to tumor outcome once iPSCs are transplanted [130, 131]. Improved differentiation and purification protocols represent the best strategy to remove potentially harmful iPSCs. Clinically relevant cells can be sorted on the basis of their cell surface antigens, using magnetic or flow

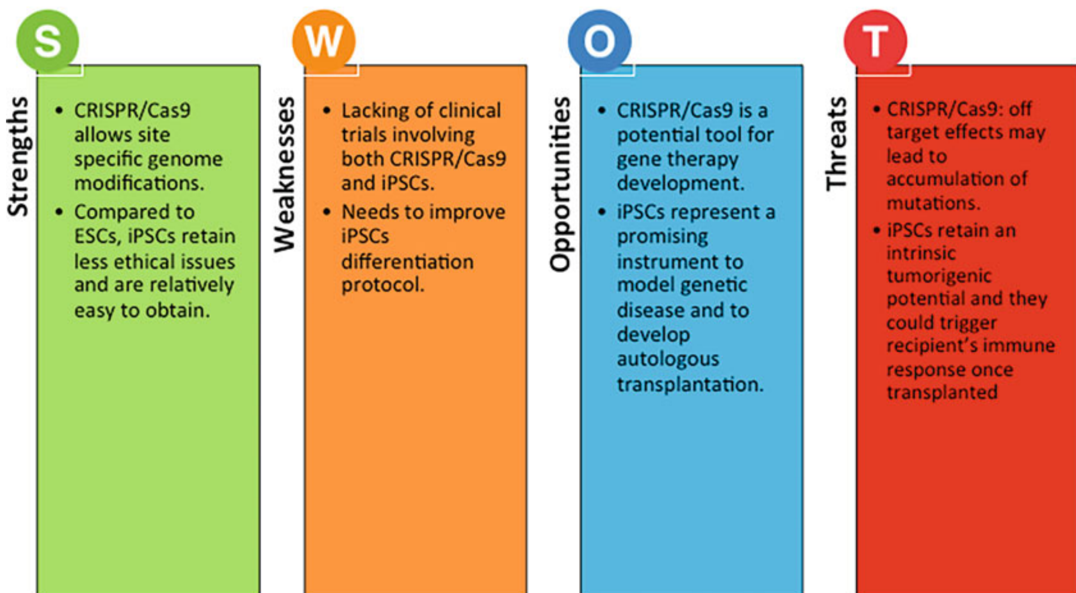


Fig. 2 SWOT (strengths, weaknesses, opportunities, threats) analysis for the utilization of CRISPR/Cas9 within the iPSC technology and clinical applications

cytometry-based methods [129]. However, some cells do not have any specific cell surface marker facilitating their identification. To overcome this problem, Miki and colleagues developed a microRNA-based method (microRNA-switch) for isolation of human iPSC-derived cells. Other strategies are also based on high-throughput screening methods. For this purpose, latest "omics" approaches are critically required, along with analysis of cell survival, integration, behavior, metabolism, and undesired effects [132].

Moreover, fully differentiated cells have a low proliferation rate and an expected lifespan, and therefore, they are eliminated once transplanted. For this reason, iPSCs are preferentially differentiated into progenitor or tissue stem cells, which are able to continuously differentiate [132]. However, optimization of differentiation protocols to obtain human fully is required (Fig. 2). Neural differentiation, for instance, takes usually up to 6 weeks, requiring few more months for functional maturation depending on the neuronal subtype [133]. In 2014, Du and colleagues reported boosting of human fibroblast differentiation into human-induced hepatocytes through the overexpression of the hepatic fate conversion factors, hepatocyte nuclear factor 1 alpha (HNF1A), hepatocyte nuclear factor 4 alpha (HNF4A), and hepatocyte nuclear factor 6 (HNF6), together with the maturation factors, activating transcription factor 5 (ATF5), prospero homeobox protein 1 (PROX1), and CCAAT/enhancer-binding protein alpha (CEBPA) [134]. Similarly, iPSC differentiation can be improved by overexpression of cell lineage-specific factors. For instance, Inamura et al. reported an increased differentiation of iPSCs into hepatocytes after hematopoietically expressed homeobox (HEX) delivery through an adenoviral vector, in combination with overexpression of P450 cytochrome [135]. Interestingly, both HEX and P450 are factors involved in the early stages of hepatic development and liver metabolism, respectively [135]. Alternative strategies focused on transcription factor sequential delivery. Takayama and colleagues first induced the differentiation of iPSC in endoderm cells overexpressing SRY-box 17 (SOX17) or Forkhead Box A2 (FOXA2). SOX17 and FOXA2-derived endoderm cells were in turn efficiently differentiated into hepatocytes overexpressing HEX or HNF1α [136]. As for hepatocytes, several protocols have been established to efficiently differentiate iPSCs into neurons. Pluripotent cells are first differentiated into neural progenitors by embryo body formation or dual SMAD inhibition [137]. Overexpression of three transcription factors, BRN2, ASCL1, and MYT1L, triggers the expression of the neuronal markers β-III-tubulin and MAP2 already in 8 days [138]. Other transcription factors, like ASCL1 and NEUROG2, were tested, showing an improved percentage of differentiated neuronal cells [139, 140]. Further studies focused on protocols optimization to obtain neuronal subtypes. Sun and colleagues recently showed that GABAergic neuron maturation can be improved by overexpressing the four transcription factors: Achaete-scute homolog 1 (ASCL1), distal-less homeobox 2 (DLX2), homeobox protein Nkx-2.1 (NKX2.1), and LIM homeobox 6 (LHX6) [141]. Interestingly, ASCL1 has been also described to contribute to the differentiation of iPSCs into dopaminergic neurons when co-expressed with nuclear receptor-related 1 protein (NURR1) and LIM homeobox transcription factor 1 alpha (LMX1A) [142]. Similarly, motor neuron differentiation is ameliorated by overexpression of neurogenin-2, ISL LIM homeobox 1 (ISL1) and LIM homeobox 3 (LHX3) [143].

The transient overexpression of the cardiac progenitor marker ISL1 has been reported to improve both mouse and human ESC differentiation in myocardial cells. ISL1 is in turn activating the production of cardiomyocyte markers such as actin alpha cardiac muscle 1 (ACTC1), myosin light chain 2V (MLC2V), and myosin heavy chain 7 (MYH7) [144, 145]. A further improvement has been made with mouse ESCs overexpressing the three cardiogenic transcription factors, GATA4, myocyte enhancer factor 2C (MEF2C), and TBX5, and achieving a cardiomyocyte derivation efficiency of 60% [146]. Despite the encouraging data coming

from ESC differentiation, it looks like transcription factor-dependent differentiation is not yet enough good to produce high functional cardiomyocytes. For this reason, combination of new transcription factors, also in combination with small molecules improving cell differentiation, needs to be tested.

As shortly described, a significant issue encountered in ESC clinical application is represented by the allogenic immune rejection triggered after transplantation. To overcome this problem, imposition of an immunosuppressive regimen is required [147]. However, the treatment turns to be toxic for the patients, improving the chance of tumor outcome [148]. iPSC-derived cells, as consequence of their autologous origin, were though to provide a useful strategy to avoid immune rejection and immunosuppression [147]. However, results appear to be controversial. As showed by Kruse and colleagues, iPSCs are encoding for the natural killer (NK) membrane receptor NKG2D, triggering recipient's NK-mediated immune response [149]. Using a C57/BL6 mice model, Zhao and colleagues proved that differentiated cells derived from isogenic iPSCs (B6 iPSCs) were able to trigger the autologous immune system response once transplanted [150]. The effect was a consequence of mis-regulated immunogenic proteins like the tumor antigen Hormad1, triggering T-cell-mediated immune response [150]. Moreover, using a humanized mouse model (Hu-mice), reconstituted with a fully functional human immune system, Zhao and co-workers investigated the differences among autologous human iPSC-derived smooth muscle cells (SMCs), appearing to be highly immunogenic, and autologous human iPSC-derived retinal pigment epithelial (RPE) cells, which are immune tolerated even in non-ocular sites [151]. Gene expression analysis showed an upregulation of the immunogenic proteins Zg16 and Hormad1 in iPSC-derived SMCs when compared with iPSC-derived RPE [151]. Abnormal expression of immunogenic proteins can be explained by an epigenetic signature inherited by iPSCs from parental cells, which can be cell line specific as showed in iPSC-derived SMCs and RPE, resulting in a different expression of antigens like Zg16 and Hormad1. In addition, several mutations and chromosomal translocation detected in iPSCs may lead to new immunogenic determinants driving recipient's immune response [152]. The lack of immunogenicity of iPSC-derived RPE has been also showed in humans, with the first patient treated using autologous iPSC-derived RPE cells against macular degeneration in 2014 [153].

Of note, new strategies aiming to improve iPSC-derived cell immune tolerance excluding the imposition of an immunosuppression regime have been investigated. Pearl and colleagues showed that iPSC-derived cell engraftment in mice was improved after short-term treatment with calcineurin inhibitor Tacrolimus, aiming to inhibition of CD34+ T-cell-mediated immune response [147]. However, immune tolerance in humans must be evaluated, since, compared to mice, up to 50% of T cells show a memory phenotype. In this context, Hu-mice may work as reliable model to study iPSC engraftment in a human immunocompetent context [152].

From the CRISPR/Cas9 point of view, the most important concern is represented by target specificity, mostly determined by the 20nt sequence of the gRNA (Fig. 2) [132]. Off-target effect could lead to lethal genetic mutations improving iPSC tumorigenicity or host immune response. In this context, gRNA design can be optimized through algorithmically based in silico predictive models aiming to detect and quantify off-target effects and generally referred as bias and unbiased methods [154]. Bias method goal is the gRNA sequence optimization through algorithms developed on databases where authors identified CRISPR/Cas9 off-target effects under specific conditions. Bias algorithms have been further categorized as conventional algorithm and scoring-based algorithm [154]. In conventional algorithms, gRNA sequence is aligned to the target genome, and off-target effects are visualized as sequence homology. In scoring-based algorithms, most appropriate gRNA sequence is retrieved through a given scores,

and it is ranked based on identified off-target sequences [154, 155]. In addition, a percentage of GC contents between 40% and 60% and a length up to 17 bp improve the stability of the gRNA:DNA duplex. Duplex stability can be also improved through 2′-O-methyl-3′-phosphonoacetate incorporation into the gRNA ribose-phosphate backbone or including a hairpin structure at the 5′-end of the gRNA sequence [154].

Together with bias methods, unbiased strategies aim to detect unintended cleavages in vitro and in vivo, mapping the Cas9 cleavage sites within a genome with a high-throughput sequencing methods [154]. Of note, a critical point to improve CRISPR/Cas9 efficiency is represented by replacement of the broadly used *Streptococcus pyogenes* Cas9 with both engineered and orthologous alternative isoforms showing higher selectivity.

Wienert et al. recently introduced a new method named discovery of in situ Cas off-targets and verification by sequencing (discover-Seq) [156]. Discovery-seq prevents the application of oligodeoxynucleotides avoiding any cytotoxic effect, turning to be an efficient tool to map Cas9-related off-target effects also in iPSCs. Off-target analysis is based on a Chip-Seq mapping the MRE11 sites, a DNA-repair factor, which has been showed to be tightly associated with Cas9 cleavage sites [156]. However, further studies are needed to develop an ultra-precise CRISPR/Cas9 and to finally see its applications either in iPSC technology or in genetic disease treatment.

9 Perspective

Although there are still many issues to solve, we wish to emphasize that considerable and exponential progress has been made during the last ~15 years since iPSCs were first generated. With further refinement of iPSC technology using CRISPR/Cas9, it may not be long before doctors will use this resource to better understand or even to treat cardiometabolic and other diseases.

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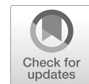
Part VI

Future Prospects



Prospective Advances in Genome Editing Investigation

Gaetano Isola

Abstract

Genomic editing technology has been developed since 2010 through the use of some techniques, such as the clustered regularly interspaced short palindromic repeat (CRISPR) DNA sequences/CRISPR-associated (Cas) type-9 method, or through genetic manipulation tools derived from host response systems from some microbes (e.g., bacteria) against plasmids and viruses. The introduction of the CRISPR/Cas9 method as a genome-editing instrument represented an important step in the advancement of the genome-editing method thanks to the ease and effectiveness of use as well as the great adaptability to different biomedical areas. This paragraph will discuss all conceived technologies and new perspectives that can be applied in treating some associated genetic disorders, such as cardiovascular diseases, metabolic diseases, inflammatory diseases, and tumors by means of reversible and modulating control of gene expression epigenetics using genetic editing techniques.

Keywords

CRISPR/Cas9 · Genome editing · Cardiovascular diseases · Metabolic diseases · Oral diseases · Inflammatory diseases · Cancer · Epigenetic

1 Advances in Genome Editing Investigation

The advent of new generations of sequencing technologies and DNA microarrays has provided both clinicians and researchers with fundamental tools, which are useful for genetic polymorphism research [1]. At present, millions of types of structural variant single-nucleotide polymorphisms (SNPs) have been identified in various systemic diseases, with increasingly innovative methods of investigation [2].

In this research area, the ability to accurately and quickly genotype patients affected by certain pathologies has allowed researchers to identify the different genetic loci involved in the etiology of various complex diseases through genome-wide association methods [3, 4].

Specifically, genomic editing technology has been developed since 2010 through the clustered regularly interspaced short palindromic repeat (CRISPR) DNA sequences/CRISPR-associated (Cas) type-9 method, or through genetic manipulation tools derived from host response systems from some microbes (e.g., bacteria) against

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plasmids and viruses. However, in 1950, Muller [5] and Auerbach [1] demonstrated that the rate of mutagenesis at gene level could be improved or reduced by radiation or biochemical treatment. Subsequently, specific studies alternated between inserting transposons inserted into the body with the main function of altering some genomic sequences sites, including genomes 1 and 2.

The genome-editing technique involves the use of methods designed to generate double-stranded DNA breaks in specific chosen in genomic sequence positions. The breaking of the double strand allows activating a specific cellular DNA repair mechanism and, consequently, improves the efficiency of genomic alternation at different orders. In doing so, the induced genomic alteration can no longer occur randomly and rarely but with a well-defined frequency of about 1 in 10 sequences. This method, therefore, has begun to make it possible to assume a possible human genome changing or replacing for therapeutic targets [6].

The double-strand break obtained by genomic editing can determine different types of gene modifications, mainly related to the two usual methods, which it happens in cell restorations through the different filament breaks (Fig. 1) [7, 8].

The double-strand break technique is centered through extremely programmable nucleases that, through double-stranded breaks, cause variations in specific genome portion of interest, which are subsequently restored by well-defined cell-repair methods.

Such methods are nonhomologous end junction (NHEJ) and homology-directed repair (HDR) (Fig. 1). The repair allows the development of gene insertions, deletions, or replacements in a specific well-defined portion in order to correct or replace gene dysfunction [9, 10].

In the NHEJ technique, the free ends of the DNA molecule generated through specific double-strand breakdown are then assembled [10] through a predefined repair process, which can be used in all types of cell jambs. However, NHEJ has been shown to be a repair process with several imperfections that sometimes evolve into the insertion or semi-random deletion of some "Indel" type base pairs in the DNA. Once inserted in the encrypting portion of a specific gene, the indel determined a frameshift mutations or in-frame gene deletions. Once inserted in the specific gene sequence, the indel will represent frameshift mutations or in-frame deletions. This process will mix some portions of the amino acid sequence or the early truncation of some genomic protein products, which can finally lead to the addition or removal of some target protein amino acid portions [11].

However, suppose the modification of the genome is used in order to produce two double-stranded chromosome disruptions; in that case, the DNA segment that is inserted between the interruption created can be misplaced if the free ends at the margins are conjuncted. This mechanism determines the portion, the entire gene, or even more gene removal of a specific chromosomal region.

Therefore, unlike NHEJ, which can cause unpredictable consequences (even there are large deletions that also extend to other genes besides the affected one), the HDR mechanism is high specific repair workflow that determines to carry out gene mutation corrections, which are characteristics of some diseases. However, as the HDR technique is limited to exclusive parts of the cell cycle, the HDR method also has some disadvantages. One is represented by a less efficient editing, which occurs when increasing cells are involved [12, 13]. Secondly, HDR is a technique that does not develop in nonproliferating cells. This makes it significantly limiting in cardiovascular diseases, that is, when it should be used on postnatal cardiomyocytes and other typical types of cells affected by cardiovascular disease. Finally, mediated HDR editing requires a highly customized repair model that is difficult to orchestrate.

Therefore, the HDR method limits make the interruption or deletion of genes obtained by the NHEJ technique more practicable in the correction of mutations or gene insertion when it is not possible to practice HDR, with important repercussions in the therapeutic application of genome editing.

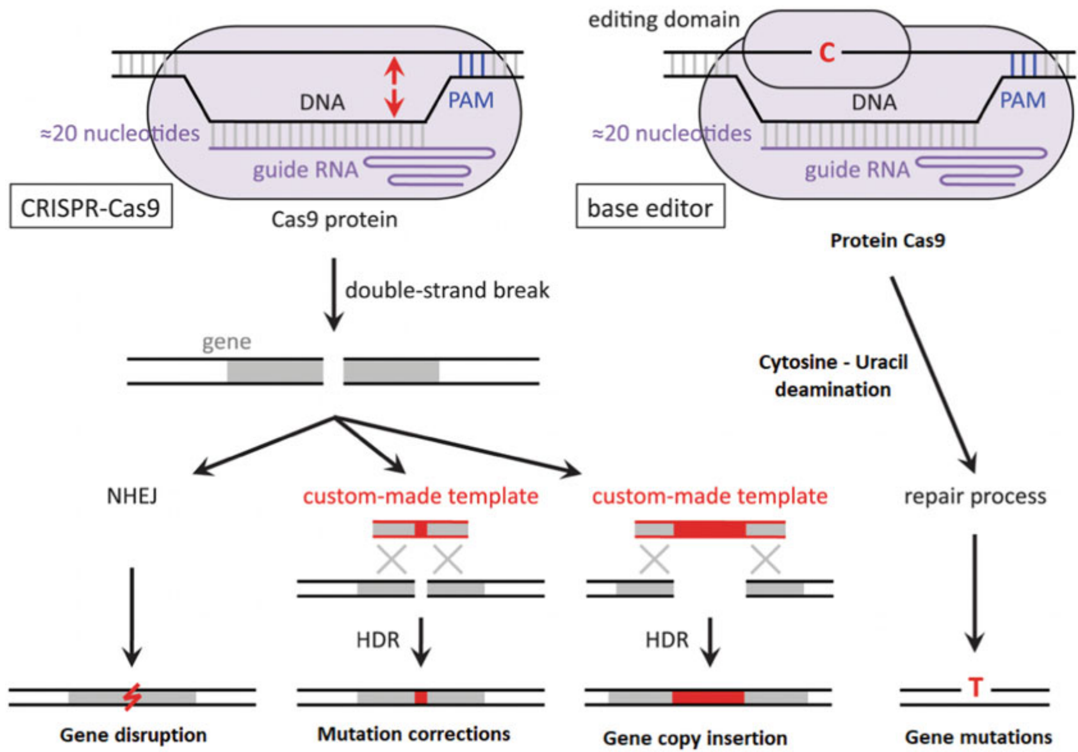

Fig. 1 Genome editing against cardiovascular disease. Left side, the CRISPR-Cas9 is a specific technique to a certain portion of the protospacer-adjacent DNA genome (PAM) and from the protospacer sequence in the guide RNA. The fracture of the genomic portion is subsequently repaired through some portions called nonhomologous junction (NHEJ) or direct homology repair (HDR). Right side, the basic editor does not determine a specific double-stranded fragmentation, but a conversion of one or more cytosine bases into a uracil base takes place, which is subsequently replaced with a thymine base. This process finally determines a complementary change on the opposite side of the chain of guanine-adenine sites [7]

2 CRISPR/Cas9 Editing Properties and Limits

The introduction of the CRISPR/Cas9 method as a genome-editing technique [14, 15] represented an important step in the advancement of the genome-editing method thanks to the ease and effectiveness of use as well as the great adaptability to different biomedical areas. CRISPR/Cas9 systems are implemented through adaptive immune mechanisms capable of correcting intrinsic errors in the DNA that are the basis of various diseases. Genome editing mainly includes techniques such as modifiable nucleases, which are zinc finger nucleases [16], effector nucleases similar to the transcription activator [17] meganuclease [18], and, finally, the CRISPR/Cas9 system that is implemented through short palindromic repeats at regular intervals associated with the CRISPR locus [19].

The CRISP/Cas9 method considers that, following the viral infection, the contact of a bacterial cell to external DNA sequences in its cytoplasm determines a specific actions in its host immune system that starts to aggregate foreign DNA sequences into its bacterial genome. These sequences are then expressed and translated as ribonucleic acid (RNA) portions, which bind the Cas9 proteins. Then, the host immune system recognizes the foreign sequences through RNA molecules and, through Cas9 or similar

proteins, neutralizes these DNA sequences through cleavage [20].

The CRISPR/Cas9 system is composed by both RNA portion and a protein. Specifically, the Cas9 protein scans and unwinds double-stranded DNA, recognizes and binds specific DNA and RNA sequences, and produces a double-stranded DNA breakdown. In the CRISPR-Cas9, a long component comprises hundreds of nucleotides and a combination of two RNA portions used in CRISPR-Cas9 systems of a bacterial nature. The modification induced by the CRISPR/Cas9 sequence relays a different RNA binding complex in some DNA portions that host complementary sequences to the protospacer sequence motif (PAM) [21].

Compared to other techniques, CRISPR/Cas9 allows it to be revised to a wide range of use, just in order to determine a protein RNA-DNA protein complex (dCas9 protein), which, combined with an RNA director, can be customized in a specific DNA binding domain that can be linked to other portions (Fig. 2) [22], which sometimes can allow specific double-stranded breakdown in some target genome sites that could lead to unexpected results [23, 24].

Furthermore, it is sometimes hard to determine cell production that contains alleles with HDR-mediated modification while the heterozygous alleles or some other cells that had alleles with more specific HDR-mediated homozygous changes. This possibility is highly limiting in situations where there is a need of beneficial intervention in order to correct a single mutation [25].

However, the CRISPR/Cas9 system represents a highly specific and reference tool for the treatment of various pathologies with genetic-based dysfunctions and for the study of these forms of diseases on both human and animal models. More specifically, the therapy carried out through genomic editing can determine the restoration of the correct gene function or compensate for some gene mutations. If the gene mutation cannot be repaired because of a specific genomic environment, a pseudogene would be triggered to exchange the mutated genetic factor [26].

Given the rapid progression of therapeutic tools through genome editing techniques, different preliminary systems were developed, which could give good possibility to overcome the current limits in various forms of human pathologies.

The following paragraphs will address the newly conceived technologies that can be applied in treating some associated genetic disorders, such as cardiovascular diseases, metabolic diseases, inflammatory diseases, and tumors by means of reversible and modulating control of gene expression epigenetics using genetic editing techniques.

3 Advances in Genome Editing Investigation in Cardiovascular Disease Applications

In recent years, research on cardiovascular diseases (CVD) linked to genetic disorders is going to renew in a strong revolution through the use of CRISPR/Cas9 or other editing genome techniques. The CRISPR/Cas9 is the most common genome editing platform that comprises an RNA-driven nuclease (Cas9), which determines double-stranded DNA breakdown in CRISPR/Cas9 that determines small indel gene mutations or insertions of some DNA fragments in some cardiovascular forms of diseases [27, 28].

Specifically, the fusion of Cas9 with other protein fractions allows better directing of enzymatic mechanisms in some genome portions, including transcriptional or repressor proteins, chromatin modifying, and reverse transcriptase in cardiovascular disease models [29, 30].

Actually, several pieces of evidence have shown that genomic variants represent the etiology of different forms of cardiomyopathies. These genomic variants pose a problematic therapeutic challenge for both physicians and patients. In fact, the pathogenicity's determination should include a careful analysis in the specific genomic context of that pathology and evaluate the genomic interactions with reference to the environmental context, also predicting the presence of patient-derived pluripotent stem cells (iPSCs). The iPSCs can be distinguished into a multitude

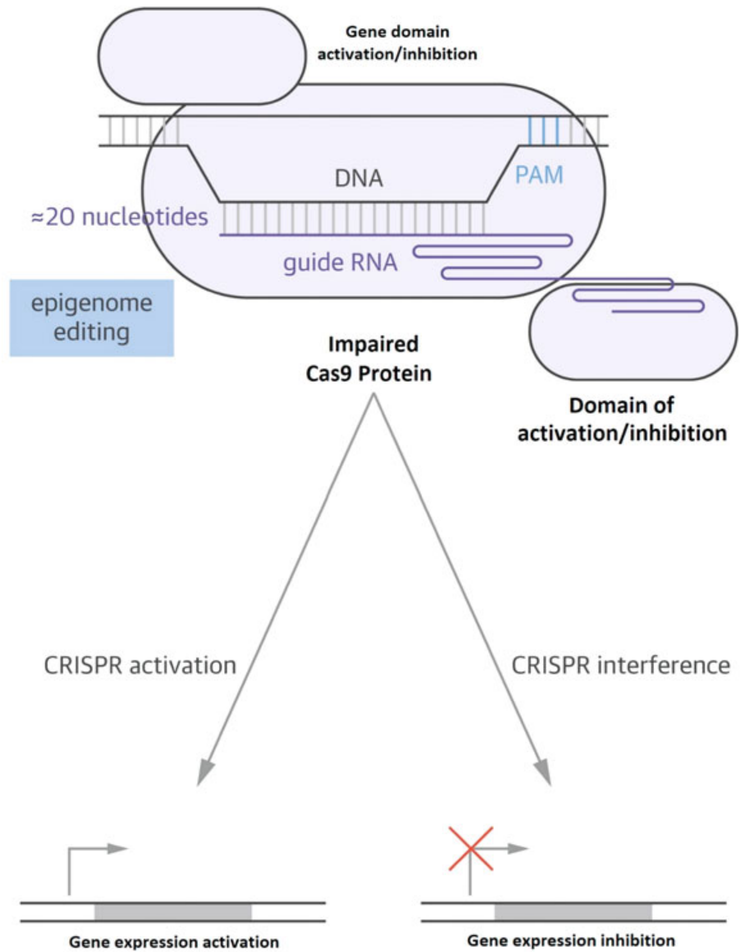

Fig. 2 Cas9 (dCas9) technique that can determine gene transcriptional inhibition or activation without altering the DNA sequence [22]

of type of cells involved in CVD, as macrophages or cardiomyocytes. In this regard, Ma et al. [31] found iPSCs in healthy subjects using a genetic variant of the gene MYL3 encoding the specific light chain of myosin 3, which is an important sarcomeric protein underlying a cardiovascular disorder.

In fact, it has been shown that MYL3 gene mutations are related to some forms of hypertrophic cardiomyopathy in a highly damaging and disabling condition [32]. In this pathology, CRISPR/Cas9 editing system was applied to produce different isoforms of iPSC with some specific and corrected homozygous alleles in wild form that were distinguished in cardiomyocytes (iPSC-CM) [31].

CRISPR/Cas9 editing using iPSC is a system applied in the analysis of gene interactions. A group of authors [33] have shown that tropomyosin1 (TPM1) and vinculin (VCL) gene interaction had an influence on the grades of dilated cardiomyopathy (DCM) cases. In these forms, the pathogenicity and mutations associated with TPM1 and VCL that significantly influenced the altered contractility cardiomyocytes and their organization into sarcomeres were determined by techniques using cells generated from human iPSCs of the same patient and through the CRISPR/Cas9 genome (hESC) [34]. Similarly, other studies have shown that, through iPSC analysis methods, there were some forms of cardiomyopathy associated with Duchenne muscular dystrophy [35].

Moreover, important enhancements have been performed with gene editing for the study of the cardiovascular system, specifically in the autosomal dominant disorders in which this technique represents an exclusive chance for the destruction of the allele-specific gene.

Pathologies directly related to cardiac disorders have also been demonstrated to be the correct target for genome editing. Some authors have shown that in animal models affected by transgenic cardiomyopathy, Cas9 was shown to specifically determine the ablation of the expression of some genes at myocardial level [36]. Specifically, it has been highlighted that the CRISPR/Cas9 deletion inhibits the Mhy6 gene (gene coding for some cardiac alpha-myosin heavy chains), causing, after 10 days post-birth, a dilation of the atria and ventricles, thinning of the walls of the ventricle, cardiac dilatation, and heart failure [36]. However, other studies have revealed that administering some adeno-associated virus (AAV) forms of gRNA that targeted Sav1 and Tbx20 determined slight growth in the hypertrophy of the heart and some associated biomarkers [37]. Moreover, the use of AAV9 to deliver gRNAs targeted to the protein that encodes JPH2 (Junctophilin 2-protein binding T tubule membranes to the sarcoplasmic reticulum) occasioned in ventricular dilation and hypertrophy and failure and finally in death in some neonatal mice [38].

Genome editing has also been shown to be useful in cases of familial Wolff-Parkinson-White (WPW) syndrome, a condition that determines a paroxysmal supraventricular tachycardia. Usually, this pathology can cause severe progressive heart failure or sudden cardiac death from ventricular tachyarrhythmia if patients are not treated promptly by surgical ablation [39]. However, in this regard, in a mice WPW model that contain PRKAG2 gene mutation [39], it was shown that the AAV9-Cas9 editing technique and gRNA leveling the 1589A>G sequence resulted in a clear improvement of the pathology, also having a key part in the H530R PRKAG2-WPW syndrome in the prevention of cell death [39]. Surprisingly, the use of genome-editing techniques has shown to improve and restore the expression of proteins associated with cardiomyocytes in most of the models analyzed, together with an improvement in the physiological muscle tissue histology [40].

The abovementioned evidence has underlined the CRISPR/Cas9 to be very useful for obtaining modifications in the long-term run for otherwise genetic diseases that are difficult to be approached. On the basis of the aforementioned studies and despite the evidence presented above, they are still preliminary, and it can be defined that the genome-editing technique is useful for analyzing and early avoiding unwanted mutations in cardiovascular diseases, with highly promising results in the coming years.

4 Advances in Genome Editing Investigation in Metabolic Disease Application

Diabetes mellitus and metabolic syndromes are highly prevalent chronic diseases worldwide and cause important systemic impairments if not well treated. There are specific genetic mutations and complex gene-environment interactions based on the etiopathogenesis of metabolic diseases. In this regard, in recent years, there is increasingly more evidence regarding the human stem cells associated to specific genetic modeling and genome-editing techniques.

Specifically, there are some techniques associated with specific genome editing such as conventional homologous recombination (HR), zinc finger nuclease (ZFN), effector nucleases similar to the activator of transcription (TALEN), or the CRISPR/Cas system [41] that have been demonstrated, in several preclinical models, as highly useful techniques associated with hPSCs as donor cells in order to create mutations in hPSCs.

The first studies in this regard compared the usefulness of using ZFN, enzymes containing DNA binding domains fused to a DNA cleavage domain (usually derived from a bacterial enzyme IIS FokI located in the C-terminal portion) [42]. These DNA binding domains are capable of recognizing specific base pairs that enable

homing to target DNA surveyed by DNA cleavage [43, 44]. However, for DNA cleavage to occur, there is a need of ZFN pairs that bind the opposite strands of DNA in a specific region while stimulating an endogenous DNA repair mechanism [16] that, associated to homologous donors, could lead to a target allele substitution.

Following the ZFN uses, effecter nucleases similar to transcription activators (TALEN) has been discovered from bacterial proteins of xanthomonas that contains amino acid domains of tandem repeats capable of having specific DNA targets. The TALEN represent amino acid variables 12 and 13, which determine the strong bond with DNA. If well applied, TALEN can be customized for targeted use or to DNA through DNA cleavage domain fusion with which TALE-like nucleases (TALEN) are generated [45].

Compared to the ZFN method, TALENs are simpler to design and prototype, with greater cell specificity associated with fewer side effects [46, 47]. However, the CRISPR/Cas9 method has been shown to be better compared to ZFN and TALEN since it is based on the RNA that positions itself in DNA, unlike TALEN and ZFN that depend on the use of customized DNA target proteins. Furthermore, the CRISPR/Cas9 technique has been shown to be technically more suitable and easier to use in precisely cutting the target DNA [48].

Advancement in techniques has led to the development of the CRISPR method, an organized model with repetitions ranging from 24 to 48 base pairs. These sequences would be followed by reverse-oriented DNA sequences. After the phage attack, the bacterium responds by first transcribing the spacer and then the palindromic DNA into a long RNA-like molecule. In turn, this molecule is then split by RNase III, involving Cas9 and transactivating CRISPR RNA (tracrRNA), which carries short space derived RNA (CRISPR RNA; crRNA) into the cell [49]. The CRISPR/Cas systems currently in use come from bacteria such as *Streptococcus thermophilus*, *Streptococcus pyogenes*, *Neisseria meningitides*, and *Treponema denticola*.

There are several risk variants with specific alleles associated with type II diabetes or with states of impaired fasting hyperglycemia (HOMA-B and HOMA-IR) (Table 1) [44, 50].

However, even if the overall impact of gene variant was demonstrated in less than 10% of type II diabetes cases, understanding the genetic variant mechanisms could have significant consequences for treatments of diabetes or metabolic diseases [51]. In this regard, hPSCs' impact associated with genome modification methods, such as the transcription factor 7 gene similar to 2 (TCF7L2), could lead to highly innovative results. In this regard, TCF7L2 has been considered for the survival and function of pancreatic beta cells [52], especially in the more aggressive variants of type II diabetes [53]. It has been demonstrated that, through its epigenetic variants, TCF7L2 can influence the therapeutic response to sulfonylureas [54] and precisely modulate the function of pancreatic islets [55].

However, the currently evident mechanisms appear to possess great potential for preventive therapy of type II diabetes through hPSC and genome editing. Therefore, in the next few years, certain developments will allow extrapolating results deriving from these studies that can be applied to routine clinical practice, especially on gene variants associated with type II diabetes and different forms of familial metabolic disease on a strictly genetic basis.

5 Advances in Genome Editing Investigation in Inflammatory Disease, Oral Diseases, Malformations and Cancer

In recent years, a growing body of evidence has shown an important role of genomics and metagenomics in the genesis of various inflammatory and neoplastic diseases, associated with a number of studies that are increasingly trying to find mediators for early disease diagnosis. Since saliva is an accessible and easy to collect medium, several studies have highlighted how a saliva sample, which can be regularly obtained during a routine medical or dental visit, could be useful in obtaining early biomarkers of meta-genomic

Table 1 Some genetic causes of metabolic syndromes

Disease	Genes
Forms of diabetes	
Genes associated with type 1 diabetes	Variants or single-nucleotide polymorphisms
HLA DR-DQ, HLA-B, HLA-A, INS	
PTPN22	R620W, 1858C/T
IL2RA/CD25	rs706778, rs3118470, rs41295061, rs35285258
CTLA4	A17T
IFIH1/MDA5	rs1990760
CLEC16A/KIAA0350	rs2903692, rs725613, rs17673553
ERBB3	rs2271189, rs11171747, rs2292399
PTPN2	rs2542151, rs1893217
UBASH3A/STS2	T946A
IL18	rs1946519, rs1946518, rs187238
RANTES	rs4251719, rs2306630, rs2107538
Genes involved with type 2 diabetes	Variants or single-nucleotide polymorphisms
TCF7L2 (strongest)	rs7903146, rs7901695, rs12255372, rs10885409, rs12573128
PPARG	Pro12Ala (P12A)/rs1801282
KCNJ11	Glu23Lys (E23K)/rs5219, C42R
HHEX/IDE	rs1111875, rs7923837, rs5015480, rs7923837
SLC30A8	Arg325Trp/rs13266634, rs3802177, rs2466293
CDKAL1	rs7756992, rs7754840, rs10946398, rs9465871, rs4712523, rs4712524, rs6931514
IGF2BP2	rs4402960, rs1470579, rs6769511
CDKN2A/B	rs10811661, rs1412829
ABCC8	Ala1369Ser (A1369S)/rs757110, Y356C
MTNR1B/ADCY5	rs10830963, rs1387153, rs2877716, rs1374645, rs2166706, rs10930963
KCNQ1	rs2237892, rs2237895, rs2237897, rs231362, rs2283228, rs163182, rs2299620
GCKR	P446L, rs780094, rs1260326
GCK	rs1799884, -30G/A polymorphism in promoter, rs4607517
HNF1A	G319S, rs7957197
ADAMTS9	rs4607103
G6PC2	rs560887, rs552976
JAZF1	rs864745
CDC123/CAMK1D	rs12779790, rs10906115
IRS1	rs2943641
WFS1	rs4689388, rs1801214
DUSP9	rs5945326
Genes involved with metabolic syndrome and obesity	Variants or single-nucleotide polymorphisms
GNPDA2	rs10938397
LYPLAL1/SLC30A10	rs4846567, rs2605100, rs11118316
HMGCR	rs7703051, rs12654264, rs3846663, rs3846662
PTP-1B/PTPN1	rs718049, rs2282146, rs1885177, 1484insG, P303P, P387L
CD36	rs1049673, rs3211931, rs3211938, rs1194197, rs1761667
SFRS10-ETV5-DGKG	rs7647305
MSRA/TNKS	rs545854
NPC1	rs1805081
MAP2K5/SKOR1	rs2241423

(continued)

Table 1 (continued)

Disease	Genes
Forms of diabetes	
NRXN3	rs10146997, rs11624704
PCSK1	rs6235, rs1799904
GRB14	rs13389219
VEGFA	rs6905288, rs9472138
INPPL1/SHIP2	rs2276047, rs9886, rs2276048
AHSG	rs2077119, rs4917
GHRL	rs696217, rs26802
NISCH/STAB1	rs6784615
LEPR	rs1137101
ADAMTS9	rs13060013
NUDT3	rs206936
GPR120	R270H
KLF9	rs11142387
LRP1B	rs2890652

diseases. Moreover, it has also been shown that even the human microbiome can significantly affect systemic health [56], by influencing environmental and innate factors and conditioning the genomic importance of an individual through a meta-genomic approach. For these reasons, large-scale health and dental education should be increasingly stimulated, in order to be able to prepare in the future large-scale genome editing screening, accompanied by other noninvasive salivary-type genetic analyzes.

Genome-editing approaches through CRISPR/Cas-associated nuclease systems are currently one of the most recent approaches in the field of genomics [23, 57] for the treatment of different pathologies with a personalized or precision approach. In this regard, the treatment of various forms of inflammatory diseases and cancer through a personalized approach using targeted pharmacotherapies with genetic testing is currently the last evidence on the genomic applications. The therapeutic occasion for several inflammatory or cancer diseases (especially oral cancer) is enormous and includes the improvement of treatment strategies, tissue engineering, and interventions related to metagenomic lesions of microbiome origin [58].

For the craniofacial district, genome procedures have been recently developed; however, the evidence is limited due to the few studies present in the literature.

Caries and periodontitis are the extremely common infectious diseases of the oral facial district and the human body, mainly due to dysbiosis of the oral microbiome associated with bacterial plaque. In this regard, CRISPR loci have been associated with most human oral microbiota [59]. A group of authors [60] have reported that the CRISPR system of an oral bacteria responsible for caries, the *Streptococcus mutans (S. mutans)*, may play an important part in bacterial/antibiotic resistance prevention through gene resistance. This important discovery carries out the scientific community's interest to exploit *S. mutans* antibiotic resistance by targeting its CRISPR system. In this regard, studies evaluating CRISPR loci in the oral biofilm of periodontitis and healthy patients found a strict correlation between altered CRISPR components and oral bacteria [61]. It can be hypothesized that the CRISPR system may be closely linked to the delicate balance of the oral microbiome and may be one of the main targets, in the future, for modulation of the oral microbiome against periodontal disease and other inflammatory-based diseases. Alternatively, it could be hypothesized to use CRISPR in patients with infectious-based pathology in order to improve the host's inflammatory response and modulate it accordingly.

Furthermore, epigenetic anomalies have become a presumed source of various cancer forms and craniofacial anomalies [62, 63]. In this regard, oral and head and neck cancer are one of the most common malignancies, with an incidence nearly of 70,000 cases in the United States in 2020 [64]. With an epigenetic origin, head and neck cancer could greatly benefit from CRIPSR/Cas9 techniques. Specifically, Cas9 epigenetic alterations could be applied in order to analyze transcriptional regulations in some forms of disease and early cancer and could be also extended in some other forms of oral and craniofacial pathologies.

Preliminary studies in this area corroborate the involvement of LDB1 and fibronectin in regulating tumor cells' invasiveness [65, 66] by identifying new therapeutic targets such as MUL1-HSPA5 and p75NTR [67–69]. It can be concluded that even for various forms of cancers, CRISPR has been shown to provide greater therapeutic possibilities.

6 Future Directions

In recent years, several more innovative and versatile genome-editing systems have been analyzed and are still under development in the field of genome editing, with the possibility of introducing certain variations in the subject stem cells to define specific therapeutic effects. Cell phenotyping of genome-modified stem cell-derived will represent a highly innovative frontier in gene-editing therapies.

It was already reassured evidence of the therapeutic role of genome editing to treat various systemic diseases, such as hypercholesterolemia, hypertriglyceridemia, ventricular tachycardia, metabolic diseases, and Duchenne muscular dystrophy and various malformations and pathologies of the craniofacial district, as well as in some forms of cancers. However, among the different in vitro and animal models, one of the most reliable seems to be represented by the CRISPR/Cas system, which can be adapted according to the specific editing enzyme, the design, the type of target cell.

At present, the genome-editing technique has shown to be very promising in various in vitro or animal models, but, in the coming years, it will be a tool of wide use in the routine clinical setting for the treatment of various pathologies with a precise and customized approach.

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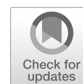

Enabling Precision Medicine with CRISPR-Cas Genome Editing Technology: A Translational Perspective

Nazma F. Ilahibaks, Maike J. Hulsbos, Zhiyong Lei, Pieter Vader, and Joost P. G. Sluijter

Abstract

Genome editing technologies, particularly CRISPR-Cas (clustered regularly interspaced short palindromic repeats (CRISPR) associated nucleases), are redefining the boundaries of therapeutic gene therapy. CRISPR-Cas is a robust, straightforward, and programmable genome editing tool capable of mediating site-specific DNA modifications. The rapid advancements from discovery to clinical adaptation have expanded the therapeutic landscape to treat genetically defined diseases. Together with the technical developments in human DNA and RNA sequencing, CRISPR-directed gene therapy enables a new era to realize precision medicine where pathogenic mutations underlying monogenic disorders can potentially be corrected. Also, protective or therapeutic genomic alterations can be introduced as preventative or curative therapy. Despite its high therapeutic potential, CRISPR-Cas´ clinical translation is still in its infancy and is highly dependent on its efficiency, specificity in gene corrections, and cell-specific delivery. Therefore, this chapter focuses on the challenges and opportunities the CRISPR-Cas toolbox offers together with delivery vehicles to realize its use for therapeutic gene editing. Furthermore, we discuss the obstacles the CRISPR-Cas system faces for successful clinical translation and summarize its current clinical progress.

Keywords

CRISPR · Genome editing · Gene therapy · Precision medicine · Translational medicine

1 Introduction

Many therapies are directed toward symptomatic treatment and are aimed to delay disease progression, such as corticosteroids for Duchenne muscular dystrophy (DMD), or digoxin for heart failure [1, 2]. Gene therapy aims to treat or prevent human disease by correcting, removing, or

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replacing pathogenic DNA or RNA [3]. The clinical implementation of gene therapy offers new therapeutic possibilities for patients suffering from hereditary diseases with limited therapy options or who do not benefit from current therapies to delay disease progression. Gene therapy originates from viral plasmids encoded with exogenous DNA to be randomly integrated into the host genome, evoking transient or longtime expression. Unfortunately, the use of viral vectors for gene therapy is paired with the risk of adverse immunogenicity and insertional mutagenesis [4]. Therefore, programmable genome editing tools are being investigated and clinically translated, including meganucleases [5], zinc-finger nucleases (ZFNs) [6], transcription activator-like effector nucleases (TALENs) [7], and CRISPR-Cas9 [8]. Meganucleases, ZFNs, and TALENs operate via protein-DNA interactions to recognize the target DNA sequence and require protein engineering for each new target, limiting more general tool developments. Moreover, difficulties associated with targeting specificity, requiring complex cloning and protein engineering strategies, have hampered their broad adaptation and application [9]. In contrast, CRISPR-Cas9-mediated genome editing is a highly versatile tool as it operates via RNA-mediated DNA target recognition. Hence, CRISPR-Cas9 can be easily programmed to target new sites or multiple sites simultaneously, that is, multiplexing, via the design of complementary RNA(s), enabling the development of more generic tools [10, 11].

CRISPR-Cas9 is part of the microbial adaptive immune system and the first nuclease of the CRISPR-Cas system to successfully mediate genome editing in eukaryotic cells [8, 10]. The CRISPR RNA (ctRNA) enables the recognition of the target gene via a ~20 nt spacer sequence, and the transactivating CRISPR RNA (tracrRNA) facilitates ctRNA maturation and Cas9 recruitment [11]. Hybridization of ctRNA and tracrRNA forms a synthetic single-guide RNA (sgRNA), enabling site-specific DNA recognition followed by a protospacer adjacent motif (PAM) via Watson-Crick base pairing. Upon complex formation of sgRNA and Cas9, a ribonucleotide protein (RNP) complex is formed whereby sgRNA recognition of target sequence and PAM engages Cas9 nucleolytic activity causing a double-strand break (DSB). The Cas9-mediated DSB is repaired by either nonhomologous end-joining (NHEJ) or homology-directed repair (HR). In NHEJ, the DSB leads to direct rejoining of the lesion, ultimately disrupting the gene via small deletion or insertion mutations (indels), potentially causing exon skipping or a frameshift [12]. Concerning HR, a DSB in the presence of a DNA donor template can lead to insertion and correction of the gene [11–16]. Both repair pathways can be applied for therapeutic purposes by correcting disease-causing mutations or by introducing a therapeutic mutation to rescue the phenotype [17].

Over the years, the CRISPR-Cas toolbox has expanded with the discovery and engineering of CRISPR-associated nucleases and repair pathways. Their application has advanced into (pre)clinical models for inborn genetic diseases, such as DMD [18–22], hereditary tyrosinemia [23], as well as acquired diseases including cancer [24], infectious diseases [25], atherosclerosis [26–28], and other cardiovascular disorders [29, 30]. Despite rapid advancements in the use of CRISPR-Cas technology as gene therapy, its trajectory is paved with safety and efficacy considerations for successful clinical translation. The endeavor to translate safe and effective CRISPR-Cas as gene therapy requires a multitude of choices, including which gene editing nuclease to employ, what type of genomic edit is required, what type of delivery vehicle is appropriate, and which available preclinical models adequately recapitulate the disease. This multitude of considerations regarding safety and efficacy per step makes the CRISPR-Cas translation pathway an intricate process. In this review, we discuss the CRISPR-Cas translation trajectory by giving an overview of available Cas nucleases capable to introduce a genomic modification or correction. We assess the choices in the CRISPR-Cas payload to enable efficient genome editing together with a compatible delivery vehicle. Next, we highlight the challenges associated with the CRISPR-Cas system for its clinical translation, including off-target mutagenesis, editing

efficiency, immunogenicity, and preclinical models. Finally, we give an overview of CRISPR-Cas adoption in the clinic and assess the obstacles to overcome in the realization of precision medicine with CRISPR-Cas gene therapy.

2 CRISPR-Cas Toolbox for Genome Manipulation

Advancements in CRISPR-Cas engineering and discoveries of natural variants have expanded the genome editing manipulation toolbox [31]. The native programmability makes CRISPR-Cas systems a powerful tool for therapeutic gene editing. CRISPR-Cas systems are classified into Class I and II based on their effector nucleases required in the interference process underlying the prokaryotic adaptive defense system [32]. These two classes are subclassified into types based on the catalytic Cas nuclease and their respective mechanism of action. Class I systems contain multi-subunit effector nuclease complexes, including type I, III, and IV CRISPR systems. In contrast, Class II CRISPR systems require a single effector nuclease and include type II, V, and VI [33]. Class I CRISPR systems' application is limited by the current lack of knowledge and the need for multi-subunit nucleases to mediate genome editing [34]. Conversely, class II systems are widely investigated as a single nuclease enables straightforward genome editing. We therefore reviewed the class II Cas nucleases with respective DNA repair mechanisms. Type II Cas9 and type V Cas12 variants cleave DNA, while type VI Cas13 endonuclease activity is directed toward RNA.

2.1 Cas9 Nuclease

CRISPR-Cas possibilities for genome editing emerged from applying the type II Cas9 from the *Streptococcus pyogenes* to mediate targeted genome cleavage in mammalian cells [8, 10] (see Fig. 1a). Since then, different rationally engineered Cas9 nucleases or natural orthologs have been investigated for DSB genome editing in mammalian cells, which are summarized in Table 1. The Cas9 nuclease consists of two catalytic domains, RuvC and a higher eukaryote and prokaryote nucleotide-binding (HNH) domain. Cas9 mediates DNA cleavage upon sgRNA target recognition of the DNA strand, following a small PAM (NGG) sequence. The post-cleavage activity catalyzes a 5'-blunt end at the target site leading to a DSB [34, 62]. The DSB triggers endogenous DNA repair systems to rejoin the lesions via NHEJ, causing gene disruption or HR-mediated gene insertion in the presence of an exogenous present DNA template. These repair mechanisms serve as therapeutic editing strategies, such as disrupting the *PCSK9* gene as cholesterol-lowering therapy [49, 63] or correcting *Fah* mutation for hereditary tyrosinemia [64, 65]. Furthermore, CRISPR-Cas9 can be harnessed for multiplex editing to remove numerous pathogenic mutations in parallel, particularly helpful for treating DMD [20, 22, 66] or Leber congenital amaurosis type 10 (LCA10) [67]. By introducing a mutation in the RuvC and HNH domains, CRISPR-Cas9 has been repurposed as a DNA binding domain to mediate transcriptional interference for gene repression via CRISPRi or activation via CRISPRa, see Fig. 2a, b [68, 69]. Alternatively, base editors are made by catalytically inactivating Cas nuclease via a D10A mutation creating nickase (nCas) or together with H840A forming "dead" Cas (dCas) fused to a deaminase. Base editors enable RNA-mediated recognition and base editing of single-strand DNA (ssDNA), where they can install a point mutation without a DSB, see Fig. 2c. This feature makes base editors a valuable expansion of the CRISPR toolbox since point mutations account for more than half of pathogenic human genetic variants [70]. Base editors are classified in cytosine base editors (CBE) or adenosine base editors (ABE) mediating C→T or G→A conversion or A→G, and T→C transition mutation, respectively [71–73]. Unfortunately, base editors cannot correct eight transversion mutations, that is, C→A, C→G, G→C, G→T, A→C, A→T, T→A, thereby leaving certain indel mutations

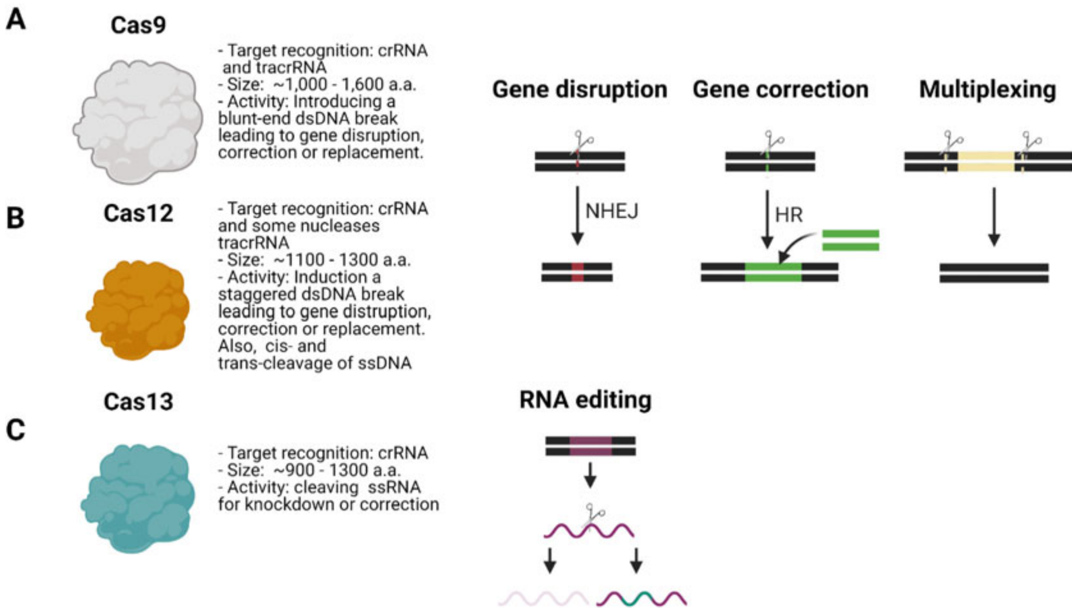

Fig. 1 Overview of Class II CRISPR-associated nucleases and respective modes of genome editing The CRISPR-associated nucleases (**a**) Cas9 and (**b**) Cas12 rely on RNA-guided recognition of a specific genomic target site to introduce a DSB. The DSB triggers DNA repair, leading to gene disruption via NHEJ, gene correction by HR in the presence of donor DNA or gene replacement in the presence of multiple sgRNA's. CRISPR-associated nuclease (**c**) Cas13 relies on crRNA for ssRNA target recognition and enables RNA deletion or correction

unchanged. For this reason, reverse transcriptase was fused to nCas to create prime editors, which courts all possible base-pair conversion together with a prime editing guide RNA (pegRNA). The pegRNA is responsible for DNA target recognition, hybridization with the ssDNA to engage reverse transcription to incorporate information of pegRNA the target site, see Fig. 2d [70, 74].

2.2 Cas12 Nucleases

Cas12 nucleases represent type V CRISPR systems and consist of a single RuvC-like nuclease domain that catalyzes dsDNA and ssDNA cleavage, see Fig. 1b. In contrast to Cas9 nucleases, most Cas12 nucleases only require crRNA to mediate a DSB upstream from the "TTN" PAM sequence, resulting in a 5′ staggered end cut [75]. The most well-known variant is Cas12a, formally known as Cpf1, which can target dsDNA and ssDNA complementary to a ~42–44 nt crRNA [76]. Cas12a-mediated germline genome editing has successfully corrected DMD mutations in *mdx* mice [77]. Also, Cas12a mediates genome editing with a higher specificity than Cas9, which is advantageous for enabling safe gene therapy [39]. The shorter crRNA streamlines gRNA design for delivery and improves multiplex opportunities [78]. Alternatively, Cas12b, also known as C2c1, requires tracrRNA like Cas9 but shows a higher on-target specificity [79, 80]. Interestingly, the Cas12b and Cas12i variants predominantly nick dsDNA [81, 82]. All Cas12 orthologs applied for mammalian genome editing mediating a DSB have been summarized in Table 2.

2.3 Cas13 Nucleases

Type VI is represented by Cas13 nucleases and consists of two HEPN domains. These HEPN

Table 1 Type II Cas9 natural and engineered nucleases investigated for mammalian genome editing by introducing a DSB

Nuclease	Name	Species	Substrate	WT, variants, mutants	sgRNA component (s)	PAM sequence (N = A/T/C/G; V = A/C/G; D = A/G/T; Y = T/C; R = A/G)	Protein size (amino acids)	Reference
Cas9	SpCas9	*Streptococcus pyogenes*	dsDNA	WT	crRNA, tracrRNA	NGG	1368	[8, 10, 35]
			dsDNA	VQR	crRNA, tracrRNA	NGAN	1368	[36]
			dsDNA	EQR	crRNA, tracrRNA	NGAG	1368	[36]
			dsDNA	VRER	crRNA, tracrRNA	NGC, NGCG	1368	[36]
			dsDNA	Sniper-Cas9	crRNA, tracrRNA	NGG	1368	[37]
			dsDNA	eSpCas9	crRNA, tracrRNA	NGG	1368	[38]
			dsDNA	SpCas9-HF1	crRNA, tracrRNA	NGG	1368	[39]
			dsDNA	SpCas9-NG	crRNA, tracrRNA	NG	1368	[40]
			dsDNA	xCas9	crRNA, tracrRNA	NG, GAA and GAT	1368	[41]
			dsDNA	HeFSpCas9	crRNA, tracrRNA	NGG	1368	[42]
			dsDNA	HiFi Cas9	crRNA, tracrRNA	NGG	1368	[43]
			dsDNA	HypaCas9	crRNA, tracrRNA	NGG	1368	[44]
			dsDNA	evoCas9	crRNA, tracrRNA	NGG	1368	[45]
			dsDNA	SpG	crRNA, tracrRNA	NGN	1368	[46]
			dsDNA	SpRY	crRNA, tracrRNA	NRN, NGN	1368	[46]
	SauriCas9	*Staphylococcus auricularis*	dsDNA	WT	crRNA, tracrRNA	NNGG	1061	[47]
			dsDNA	eSa-SauriCas9	crRNA, tracrRNA	NNGG	1061	[47]

(continued)

Table 1 (continued)

Nuclease	Name	Species	Substrate	WT, variants, mutants	sgRNA component (s)	PAM sequence (N = A/T/C/G; V = A/C/G; D = A/G/T; Y = T/C; R = A/G)	Protein size (amino acids)	Reference
			dsDNA	SauriCas9-KKH	crRNA, tracrRNA	NNRG	1061	[47]
	SmacCas9	*Streptococcus macacae*	dsDNA	WT	crRNA, tracrRNA	NAA	1338	[48]
			dsDNA	iSpy Cas9	crRNA, tracrRNA	NAA	1338	[48]
	SaCas9	*Staphylococcus aureus*	dsDNA	WT	crRNA, tracrRNA	NNGRRT, NNGRR	1053	[49]
			dsDNA	eSaCas9	crRNA, tracrRNA	NNGRRT, NNGRR	1053	[38]
			dsDNA	KHH	crRNA, tracrRNA	NNNRRT	1053	[50]
	ScCas9	*Streptococcus canis*	dsDNA	WT	crRNA, tracrRNA	NNG	1375	[51]
			dsDNA	Cas9-Sc++	crRNA, tracrRNA	NNG	1375	[52]
	CjCas9	*Campylobacter jejuni*	dsDNA	WT	crRNA, tracrRNA	NNNNRYAC	984	[53]
	FnCas9	*Francisella novicida*	dsDNA	WT	crRNA, tracrRNA	NGG	1629	[54, 55]
			dsDNA	RHA	crRNA, tracrRNA	YG	1629	[55]
	Nm1Cas9	*Neisseria meningitidis*	dsDNA	WT	crRNA, tracrRNA	NNNNGATT	1082	[56]
	Nm2Cas9	*Neisseria meningitidis*	dsDNA	WT	crRNA, tracrRNA	NNNNCC	1082	[57]
	St1Cas9	*Streptococcus thermophilus*	dsDNA	WT	crRNA, tracrRNA	NGGNG, NNAGAAW	1121	[36, 58]
	St3Cas9	*Streptococcus thermophilus*	dsDNA	WT	crRNA, tracrRNA	NNAGAAW, NGGNG	1409	[58]
	BlatCas9	*Brevibacillus laterosporus*	dsDNA	WT	crRNA, tracrRNA	NNNNCND, NNNNCNAA	1092	[59, 60]
	GeoCas9	*Geobacillus stearothermophilus*	dsDNA	WT	crRNA, tracrRNA	CRAA	1087	[61]

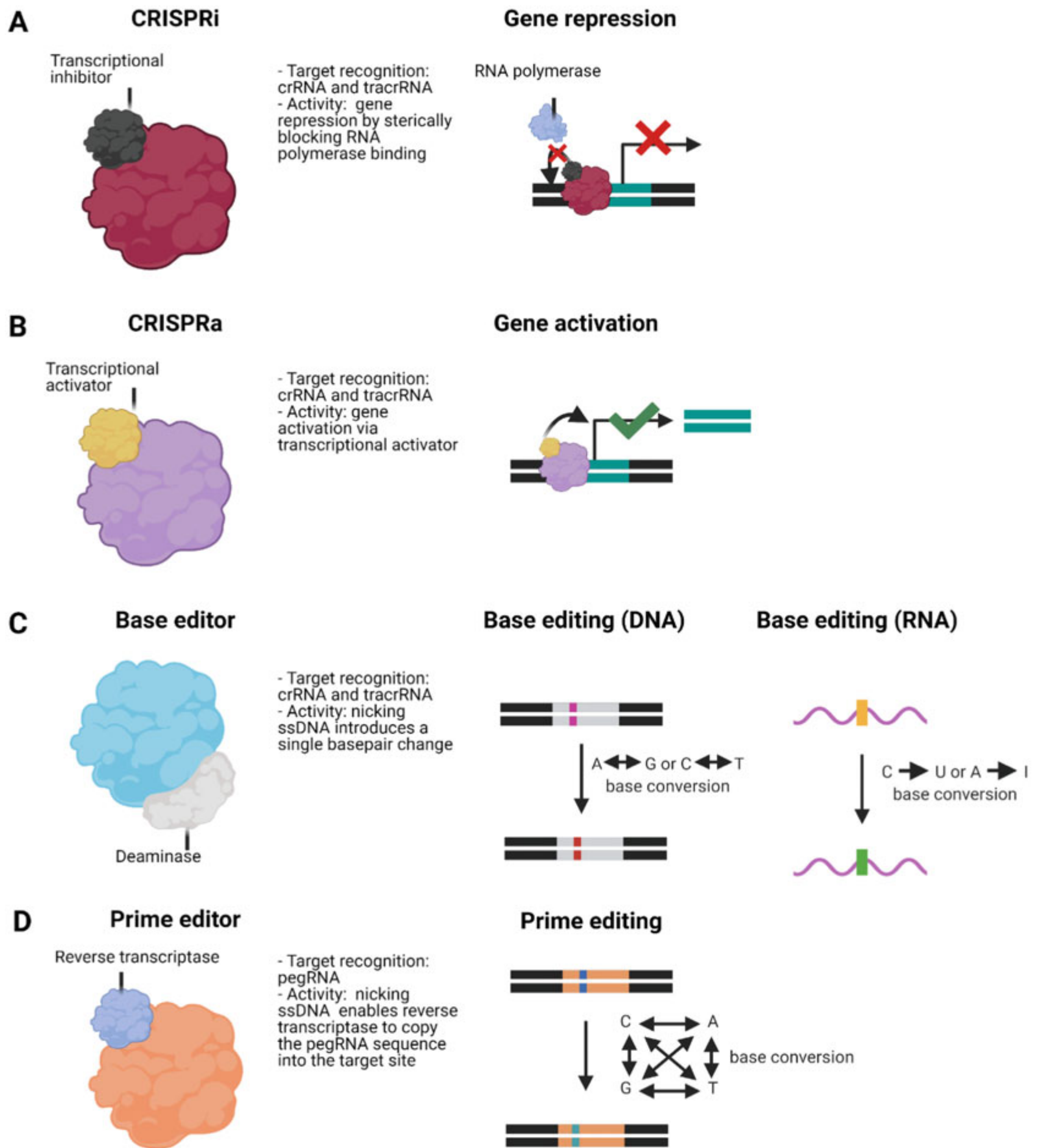

Fig. 2 Overview of rationally engineered CRISPR-Cas nucleases for genome editing. New modification of CRISPR-Cas nucleases have expanded the opportunities or genomic manipulation. Transcriptional control of gene expression can be modulated via (**a**) CRISPRi leading to gene repression or (**b**) CRISPRa leading to gene activation. (**c**) Base editors are able to nick DNA or RNA fostering a A·G or C·T base conversion in DNA and A·I or C·U conversion in ssRNA (**d**) Prime editors can nick DNA, leading to all possible base-pair conversion depending on the pegRNA

domains display RNase activity leading to exclusive RNA cleavage, see Fig. 1c. Cas13 requires a ~60–66 nt crRNA carrying a ~28–30 nt spacer sequence complementary site to the RNA target region [93]. The emerging variants in this type for RNA interference are Cas13a (C2c2) and Cas13b

Table 2 Type V Cas12 nucleases investigated for mammalian genome editing by introducing a DSB

Nuclease	Name	Species	Substrate	WT, variants, mutants	sgRNA component(s)	PAM sequence (N = A/T/C/G; V = A/C/G; D = A/G/T; Y = T/C; R = A/G)	Protein size (amino acids)	Reference
Cas12a	LbCas12a (Cpf1)	*Lachnospiraceae bacterium*	ssDNA, dsDNA	WT	crRNA	TTN, TTTN, TTTV, CTTV, TCTV, TTCV	1228	[75, 83, 84]
	FnCas12a (FnCpf1)	*Francisella novicida*	ssDNA, dsDNA	WT	crRNA	TTN, TTTN, TTTV	1300	[75, 85]
	AsCas12a (AsCpf1)	*Acidaminococcus* sp. *BV3L6*	ssDNA, dsDNA	WT	crRNA	TTN, TTTN, TTTV, NTTV, TCTV, TTCV	1353	[75, 83, 84, 86]
			ssDNA, dsDNA	AsCas12-RVR	crRNA	TATV	1353	[87]
			ssDNA, dsDNA	AsCas12-RR	crRNA	TYCV	1353	[87]
	ArCas12a	*Agathobacter rectalis strain 2789STDY5834884*	ssDNA, dsDNA	WT	crRNA	TTN	–	[88]
	BsCas12a	*Butyrivibrio* sp. *NC3005*	ssDNA, dsDNA	WT	crRNA	TTN, TTTV	1236	[88, 89]
	Mb2Csa12a (Mb2Cpf1)	*Moraxella bovoculi AAX08_00205*	ssDNA, dsDNA	WT	crRNA	TTTV	–	[89]
	Mb3Cas12a (Mb3Cpf1)	*Moraxella bovoculi AAX11_00205*	ssDNA, dsDNA	WT	crRNA	TTTV, TTN	–	[89]
	PrCas12a	*Prevotella ruminicola strain BPI-34*	ssDNA, dsDNA	WT	crRNA	TTN, TTTN	1252	[88]
	HkCas12a	*Helcococcus kunzii ATCC 51366*	ssDNA, dsDNA	WT	crRNA	TTN, TTTN	1310	[88]
	PxCas12a	*Pseudobutyrivibrio xylanivorans strain DSM 10317*	ssDNA, dsDNA	WT	crRNA	TTN, TTTN	1207	[88]
Cas12b	BhCas12b (C2c1)	*Bacillus hisashii*	ssDNA, dsDNA	BhCas12b v4 mutant	crRNA tracrRNA	TTTN, GTTN, ATTN	1108	[82]
	AacCas12b (AacC2c1)	*Alicyclobacillus acidoterrestris*	ssDNA, dsDNA	WT	crRNA tracrRNA	TTC	1129	[90]
	AaCas12b	*Alicyclobacillus acidiphilus*	ssDNA, dsDNA	WT	crRNA tracrRNA	TTN		[80]

	AkCas12b	*Alicyclobacillus kakegawensis NBRC 103104*	ssDNA, dsDNA	WT	crRNA tracrRNA	TTTN		[80]
Cas12d	Cas12d (CasY)		ssDNA, dsDNA		Short-complementarity untranslated RNA (scoutRNA), crRNA	TR		[91]
Cas12e	DpbCas12e (DpbCasX)	*Deltaproteobacteria*	ssDNA, dsDNA	WT	crRNA tracrRNA	TTCN	980	[92]

(C2c6), which can be used for knockdown or correction of disease-relevant transcripts in mammalian cells [94, 95]. By solely mediating RNA knockdown, these nucleases could be a safer approach to gene therapy as they only inactivate RNA pathogenic transcript without altering the DNA. The Cas13 variants investigated for mammalian RNA interference have been summarized in Table 3.

Collectively, these CRISPR-Cas variants have expanded the possibilities of CRISPR-Cas-mediated therapeutic genome editing. Yet, the specificity and delivery per variant remain to be determined for its future clinical application.

3 CRISPR-Cas Payloads and Delivery

Even though the CRISPR-Cas toolbox offers diverse therapeutic options to modulate gene expression, the simultaneous intracellular delivery of the different CRISPR-Cas components remains a significant barrier for its application in human patients [101]. The success of intracellular delivery of multiple CRISPR-Cas components is determined by the CRISPR-Cas payload and delivery vehicle's efficacy and safety. CRISPR-Cas components can be delivered through a plasmid, messenger RNA (mRNA)-sgRNA, or as a ribonucleotide protein (RNP) complex directly. Various physical, viral, and nonviral drug delivery systems have been investigated to facilitate CRISPR-Cas intracellular delivery, see Fig. 3 [15]. Each CRISPR-Cas payload and delivery strategy has its own inherent (dis)advantages, which will be discussed in the following sections.

3.1 CRISPR-Cas Payloads

To achieve genome editing with CRISPR-Cas, the intracellular introduction of the Cas-sgRNA-encoded plasmid, mRNA encoding Cas with complementary sgRNA, or CRISPR-Cas RNP is essential. Plasmid-based Cas-sgRNA is considered cost-effective because it can be manufactured in a standard laboratory setting. Compared to mRNA and RNP delivery, plasmid-based delivery results in a longer expression time in cells and later onset of genome editing due to transcription and translation steps [102]. However, this prolonged expression increases the risk of off-target activity. An advantage of plasmid delivery is that multiple components, including Cas (~4600 bp), sgRNA (~100 bp), and exogenous DNA for HR, can be integrated into one plasmid. This simultaneous integration potentially increases genome editing efficiency since all components can be packed and delivered simultaneously to the target cell. However, this approach results in a large plasmid (~10 kb), reducing encapsulation efficiency in delivery vehicles [103–106]. Furthermore, plasmid delivery requires nuclear entry and translation and is accompanied by the risk of host genome integration, immune response, and off-target effects resulting from prolonged expression [15].

Direct Cas mRNA-sgRNA delivery enables rapid genome editing compared to plasmid-based delivery because there is no need for transcription [101]. Furthermore, the transient expression associated with Cas mRNA reduces the chance of off-target activity and leads to the faster onset of genome editing. Also, mRNA-based delivery enables efficient transgene expression together with low cytotoxicity in (primary) cell lines [107]. Even though the transient exposure reduced the toxicity and off-target effects, it may compromise gene editing efficiency. Despite RNA-based payload having many advantages over plasmid-based delivery, it has a poor stability character and is prone to RNase degradation [101, 108]. Hence, RNA modifications are being investigated to enhance their stability after intracellular delivery [109].

CRISPR-Cas RNP delivery does not require cell-driven expression, thereby enabling faster and more efficient on-site genome editing than plasmid and mRNA delivery. Furthermore, CRISPR-Cas RNP delivery has the most transient exposure of all payloads in cells, resulting in minimal off-target activity [110]. Also, RNP delivery is associated with low toxicity and immunogenicity [111]. CRISPR-Cas RNP delivery disadvantages are its high cost and intrinsic

Table 3 Type VI Cas13 nucleases investigated for mammalian RNA interference

Nuclease	Name	Species	Substrate	WT, variants, mutants	sgRNA component (s)	PAM sequence (N = A/T/C/G; V = A/C/G; D = A/G/T; Y = T/C; R = A/G)	Protein size (amino acids)	Reference
Cas13a	LshCas13a (C2c2)	*Leptotrichia shahii*	ssRNA	WT	crRNA	N.A.	1389	[96, 97]
	LwaCas13	*Leptotrichia wadei*	ssRNA	WT	crRNA	N.A.	1182	[96, 98–100]
	LbaCas13	*Lachnospiraceae bacterium NK4A179*	ssRNA	WT	crRNA	N.A.	1437	[99, 100]
Cas13b	PspCas13b	*Prevotella* sp. *P5-125*	ssRNA	WT	crRNA	N.A.	–	[95, 99, 100]
	PsmCas13b	*Prevotella* sp. *MA2016*	ssRNA	WT	crRNA	N.A.	–	[99, 100]
	CcaCas13b	*Capnocytophaga canimorsus*	ssRNA	WT	crRNA	N.A.	–	[99, 100]

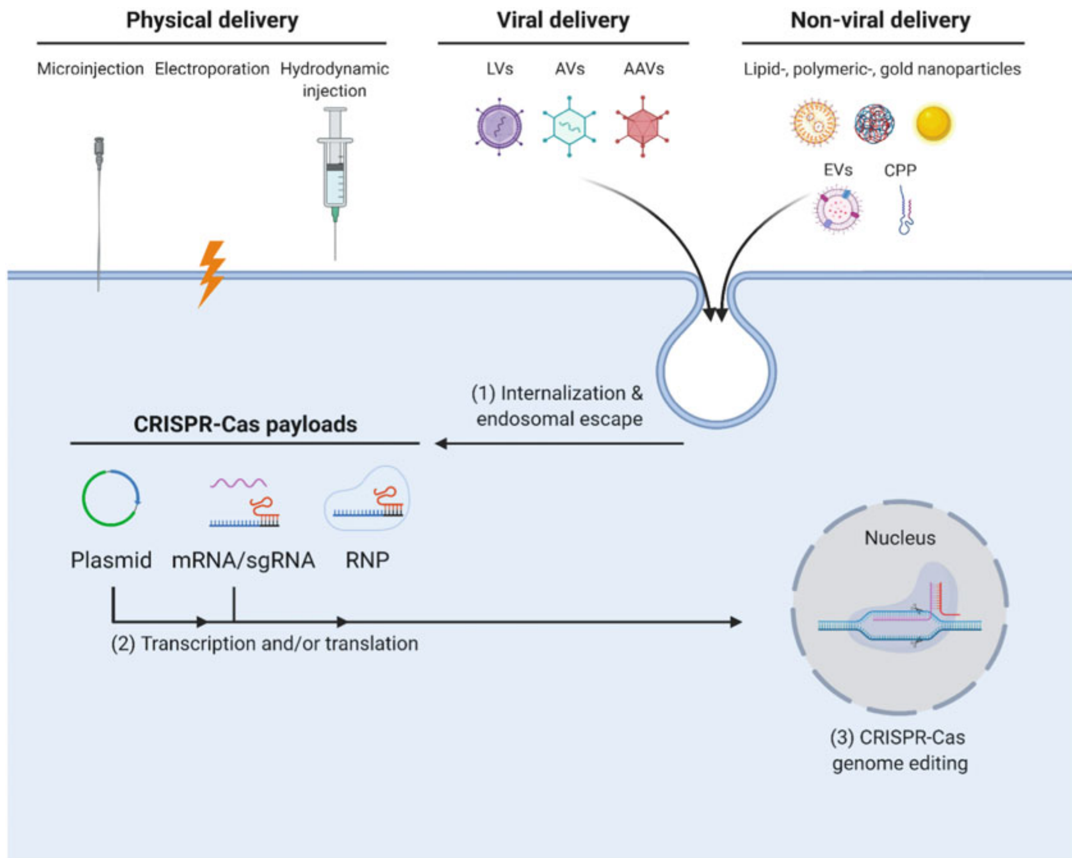

Fig. 3 Overview of CRISPR-Cas delivery methods and payloads. Physical delivery methods directly introduce CRISPR-Cas payloads intracellularly. Conversely, viral and non-viral delivery methods need to be taken up by the cell and escape the endosome for releasing their CRISPR-Cas payload intracellularly. Depending on the payload, transcription and/or translation is required to enable genome editing in the nucleus

physicochemical properties of the RNP, including the Cas protein's relatively large size, instability, and low cell permeability.

In short, each CRISPR-Cas payload has their own inherent benefits and drawbacks to mediate genome editing through persistent or transient expression. Even so, the payload must be successfully introduced intracellularly via delivery vehicles for a functional CRISPR-Cas RNP complex to mediate site-specific genome editing. To that end, the delivery vehicle's ability to promote efficient uptake and endosomal escape in target cells is crucial for effective CRISPR-Cas genome editing [112].

3.2 CRISPR-Cas Delivery Vehicles

CRISPR-Cas delivery methods can be classified into three categories: viral, nonviral, and physical delivery methods. For viral delivery, adeno-associated viruses (AAVs), lentiviruses (LVs), and adenoviruses (AVs) have been investigated [113]. Among these, AAVs are the most investigated vehicle for CRISPR-Cas in vivo delivery. AAVs have low immunogenicity and high transduction efficiency and are nonintegrating into the genome, and their tropism enables tissue-targeted delivery. AAV's limitation is its limited packing size (±4.5 kb) making

it challenging to deliver all CRISPR-Cas components simultaneously. Therefore, a dual AAV system with one carrying Cas9 and the other sgRNA together with exogenous DNA for HR are used [114]. Alternatively, smaller Cas9 orthologs like SaCas9 (±3.15 kb) can be loaded into AAV vectors. However, this requires the design of new sgRNA as SaCas9 operates via different PAM sequences [49]. Despite AAVs' promising features, their production is expensive, and they carry the risk for genome integrations [115], carcinogenesis, and eliciting an immune response [15].

Alternatively, nonviral delivery methods may address the constraints associated with viral delivery [116, 117]. CRISPR-Cas nonviral delivery methods include physical methods and nanoparticle delivery for CRISPR-Cas plasmid, mRNA/sgRNA, or RNP delivery. Physical methods include electroporation [118], microinjection [119], and hydrodynamic injection [120]. Electroporation entails electric pulses for transient opening up the cell membrane allowing cargo into the cells. Despite being highly efficient, the electrical current induces cell death and remains more suitable for ex vivo therapy, as is the case for hematologic diseases [121]. Microinjection employs a microscope to guide a needle for the manual injection of CRISPR-Cas through the cell membrane. The laborious nature, technical difficulties, and need for microscopy-guided injection per cell make it an inefficient delivery method for human application. Alternatively, hydrodynamic injection rapidly introduces nucleic acid solutions in vivo in relatively large volumes, that is, volumes of 8–10% of the bodyweight [122, 123]. Unfortunately, hydrodynamic injection promotes high blood pressure, brief cardiac dysfunction, liver expansion in vivo, and even hepatoxicity in patients [123, 124]. Despite the efficiency of physical methods, their application remains to be invasive and laborious or have safety concerns to be successfully adopted for direct CRISPR-Cas delivery in patients.

In contrast, nanoparticle delivery may overcome the limitations of viral and physical methods, particularly concerning their safety for clinical translation and packaging size [125, 126]. Synthetic nanoparticle delivery systems Cas9-sgRNA plasmid delivery include inorganic [127], lipid-based [125, 126, 128, 129], and polymer-based delivery systems [125, 130–133]. For nucleotide delivery, both polymer- and lipid-based vectors have been widely investigated in vivo and in clinical trials [125, 126]. Lipid and polymer nanoparticles are compatible with all CRISPR-Cas payloads. However, their organ-specific delivery efficiency is still poor. Moreover, upon delivery to the target cells, the lipid or polymeric nanoparticle needs to escape from the endosome to release its cargo intracellularly. Henceforth, there is ongoing research to determine the optimal polymers [134] or lipids [135], inclusion of targeting moieties to promote these nanoparticles uptake and enhance endosomal escape. Alternatively, the use of extracellular vesicles (EVs), that is, cell-derived nanovesicles carrying biomolecules used for endogenous cell-to-cell communication, has been proposed as a drug delivery platform because of their intrinsic nature to carry biomolecules to target cells. Hence, EVs pseudotyped with the vesicular stromatic virus glycoprotein have been demonstrated to be highly efficient for CRISPR-Cas RNP delivery [136, 137]. Yet, EV clinical translation still awaits overcoming challenges related to manufacturing, scalability, standardization of production, purification, and quality controls. Finally, other CRISPR-Cas RNP delivery methods such as gold nanoparticles [14, 138], induced transduction by osmocytosis and propanebetaine (iTOP) [139], and transmembrane internalization assisted by membrane filtration (TRIAMF) [140] are potential alternatives and are claimed to be highly efficient. Alternatively, for RNP delivery, spherical DNA structures called nanoclews [134] and cell-penetrating peptides (CPP) [141] have controllable architecture, desirable size, and safety profiles. However, gold nanoparticles, iTOP, and TRIAMF are not suitable for in vivo application due to potential toxicity, requiring high salt concentration of Cas solubility or reliance on membrane filtration, respectively [101, 140]. Moreover, DNA

Table 4 Overview of clinical trials using the CRISPR-Cas9 system

Disease	Phase	Target gene	Delivery method	Intervention/treatment	CRISPR-Cas9 strategy	ClinicalTrials.gov identifier
Metastatic lung cancer	I	PD-1	Electroporation	Autologous PD-1 KO T-cells	Ex vivo	NCT02793856
Esophageal cancer	N.A.	PD-1	Not disclosed	Autologous PD-1 KO T-cells	Ex vivo	NCT03081715
Advanced hepatocellular carcinoma	I	PD-1	Not disclosed	Transcatheter arterial chemoembolization combined with autologous PD-1 KO T-cells	Ex vivo	NCT04417764
Mesothelin positive solid tumors	I	PD-1, TCR	Electroporation	PD-1 and TCR KO CAR-T cells	Ex vivo	NCT03545815
Mesothelin positive solid tumors	I	PD-1	Electroporation	Autologous PD-1 KO T-cells	Ex vivo	NCT03747965
Relapsed or refractory renal carcinoma	I	TCR, β2m, CD70	Electroporation	CTX130: Allogeneic CD70-specific CAR-T cells	Ex vivo	NCT04438083
Relapsed or refractory T- or B-cell malignancies	I	TCR, β2m, CD70	Electroporation	CTX130: Allogeneic CD70-specific CAR-T cells	Ex vivo	NCT04502446
Relapsed or refractory B-cell malignancies	I	TCR, β2m, CD19	Not disclosed	CTX110: Allogenic CD19-specific CAR-T cells	Ex vivo	NCT04035434
Acute lymphocytic leukemia and lymphoma	I	HPK1	Electroporation	XYF19 CAR-T cell: CD19-specific CAR-T cells carrying CRISPR-Cas9 encoded lentiviral vector for endogenous HPK1 KO	Ex vivo	NCT04037566
Refractory or relapsed leukemia and lymphoma	I, II	TCR, CD19, CD20 or CD22	Not disclosed	Allogenic CD19, CD20 or CD22-specific CAR-T Cell	Ex vivo	NCT03398967
Metastatic gastrointestinal cancers	I, II	CISH	Not disclosed	Autologous neoantigen-specific TILs	Ex vivo	NCT04426669
Relapsed or refractory multiple myeloma	I	TRAC, MCH 1, BCMA		CTX120: Allogeneic BCMA-specific CAR-T cell		NCT04244656
β-Thalassemia	I, II	BCL11A KO to restore fetal hemoglobin expression	Electroporation	Autologous CRISPR-Cas9 Modified CD34+ human hematopoietic/ progenitor stem cells	Ex vivo	NCT03655678
β-Thalassemia	I	HBB	Not disclosed	Autologous HBB corrected inducible hematopoietic stem cells	Ex vivo	NCT03728322
Sickle cell disease	I,II	BCL11A KO to restore fetal hemoglobin expression	Electroporation	Autologous CRISPR-Cas9 modified CD34+ human hematopoietic stem/progenitor cells	Ex vivo	NCT03745287

(continued)

Table 4 (continued)

Disease	Phase	Target gene	Delivery method	Intervention/treatment	CRISPR-Cas9 strategy	ClinicalTrials.gov identifier
Sickle cell disease	I,II	BCL11A KO to restore fetal hemoglobin expression	Not disclosed	Autologous CRISPR-Cas9 modified CD34+ human hematopoietic stem/progenitor cells		NCT04774536
Epstein-Barr virus associated malignancies	I,II	PD-1	Electroporation	Autologous PD-1 KO T-cells	Ex vivo	NCT03044743
Human immunodeficiency virus	N.A.	CCR5	Not disclosed	Autologous CCR5 Modified CD34+ hematopoietic stem/progenitor cells	Ex vivo	NCT03164135
Kabuki syndrome 1	N.A.	KMT2D	Not disclosed	Autologous KMT2D KO mesenchymal stem cells	Ex vivo	NCT03855631
Leber congenital amaurosis 10	1	CEP290	Subretinal injection in the eye	EDIT-101: AAV5 mediated delivery of CRISPR-Cas9 to correct disease-causing mutations in CEP290 gene	In vivo	NCT03872479
Human papillomavirus-related malignancy	1	E6, E7	Cervical epithelium	TALEN or CRISPR-Cas9 mediated KO of E6 and E7 oncogene	In vivo	NCT03057912

BCMA B-cell maturation antigen, *CISH* cytokine-induced SH2 protein, *KO* knockout, *TRAC* TCR alpha constant, *MHC 1* major histocompatibility complex 1

nanoclews and CCP are burdened with poor stability and the need for chemical RNP anchoring.

To conclude, each CRISPR-Cas payload in combination with delivery method has its own advantage and disadvantage, which need to be carefully considered, as they both affect the safety and efficacy for CRISPR-Cas' clinical development.

4 CRISPR-Cas: Challenges for Clinical Implementation

Despite the significant developments in the therapeutic application of CRISPR-Cas system, its clinical translation into gene therapy still faces safety and efficacy concerns. Here, we review the obstacles associated with the successful translation of CRISPR-Cas as gene therapy, including off-target mutagenesis, genome editing efficiency, immunogenicity, and preclinical models.

4.1 Off-Target Mutagenesis

CRISPR-Cas efficacy is determined by the sgRNA specificity to mediate on-target DNA cleavage without binding to comparable genomic sequences leading to off-target mutagenesis. As sgRNA has a high mismatch tolerance, it enables Cas nucleases to cleave alternative sites comparable to the target sequence [142]. Consequently, researchers have engineered Cas9 to enhance on-target specificity [39, 44] and improve gRNA design to reduce off-target activity [143, 144]. Furthermore, genome-wide sequencing tools have been develop to detect CRISPR-Cas' on- and off-target activity, such as BLISS, HTGTS, GUIDE- and DIG-seq [145–148]. It should be noted that these off-target prediction tools have been investigated referencing the standard human genome. Henceforth, its predictive nature cannot take interindividual genetic variations into account. In essence, sgRNA should display a

high on-target activity and consider natural human genetic variations to minimize CRISPR-Cas' off-target mutagenesis for precise genome editing [106, 149, 150].

4.2 Genome Editing Efficiency

The broad application of CRISPR-Cas as gene therapy is dependent on its efficiency to mediate genetic modifications and ability to target the whole genome. The genome editing efficiency is dependent on the DNA repair pathway. Precise CRISPR-Cas9 HR has a lower editing efficiency than NHEJ as it is only active in the S/G2 phase of the cell cycle. Fortunately, HR editing efficiency can be improved via different Cas nucleases [151], using ssDNA donor template and through suppressing NHEJ [152]. Nonetheless, high HR efficiency is required for therapeutic genome editing to correct disease-causing mutations, warranting more research into this area.

As Cas9 is the most common investigated CRISPR-Cas system for gene therapy, it is limited to act at endogenous target sites bearing a "NGG" PAM sequence thereby constraining the targetable genomic loci. To overcome this restriction, efforts have focused on rationally engineering Cas nucleases and discovery of natural variants in order to expand the PAM flexibility of the CRISPR-Cas system [41]. In effect, CRISPR-Cas application has increased to mediate precision genome editing at more targetable genomic sites.

4.3 Immunogenicity

Immunogenicity and preexisting antibodies against the CRISPR-Cas system are two critical risk factors for CRISPR-Cas' clinical translation. As CRISPR-Cas nucleases have a bacterial origin, they are recognized by the host immune system evoking an immune response [153]. Furthermore, delivery vehicles could elicit an immune response, which reduces the safety and efficacy of the CRISPR-Cas therapy.

Also, preexisting antibodies toward Cas9 orthologs in humans challenge CRISPR-Cas therapeutic translation [154–157]. As result, the systematic administration of Cas RNP complexes are potentially being neutralized, and the anti-Cas T cells may recognize Cas-associated peptides and destroy target cells harboring CRISPR-Cas [158]. Furthermore, in vivo studies showed that cytotoxic T cells neutralized and annihilated AAV-mediated CRISPR-Cas genome editing [159, 160]. Therefore, CRISPR-Cas interactions with the immune systems require more research and may be overcome by transient transgene expression, immunosuppression, or engineering of Cas proteins [158, 161]. Additionally, the humoral and cellular response should be closely monitored in current and future clinical trials to assess the treatment's safety and efficacy.

4.4 Preclinical Studies

Adequate preclinical models are required to successfully determine the efficacy, toxicity, and safety profile of CRISPR-Cas-based therapies. Selecting animal models, which accurately recapitulate the pathology of interest, is fundamental to obtain clinically relevant insights. Therefore, many mouse models harboring genetic mutations underlying human hereditary disorders have been tested to assess CRISPR-Cas-mediated genome editing efficiency. However, many genetically engineered rodent models do not entirely capture the progression of the pathophysiology [158]. For example, *mdx* mice used for studying DMD show mild disease progression and relatively smaller lifespan reduction than human clinical symptoms. Conversely, the DMD canine and pig model are characterized with severe disease progression, cardiomyopathy, and premature death, which resembles the phenotype of DMD more accurately [18, 162, 163]. Consequently, studying CRISPR-Cas-based therapy in larger preclinical models will enable more relevant extrapolation of the results to humans.

Furthermore, testing CRISPR-Cas efficiency in preclinical studies needs to consider genetic discrepancies. Each preclinical study with

different animal models requires surrogate RNA compatible with its genome sequence. However, it remains questionable how representative surrogate sgRNA is for assessing therapeutic efficacy and dosage for human applications [158]. The complete extent to which preclinical studies have investigated CRISPR-Cas therapeutic potential cannot be covered in this review; therefore, we refer to [164].

5 CRISPR-Cas in the Clinic

Although CRISPR-Cas clinical development is still in its infancy, ongoing clinical trials are already investigating the therapeutic potential of CRISPR-associated nuclease Cas9. In this section, we summarize the progress and challenges of the CRISPR-Cas9 system per clinical application, which can be broadly categorized in ex vivo and in vivo *strategies*.

5.1 Ex Vivo Strategies

With ex vivo strategies, somatic or inducible pluripotent stem cells (iPSCs) from the patients are being isolated. Subsequently, these cells are edited with CRISPR-Cas9 and expanded, and the successfully engineered cells are transplanted back in patients for therapeutic purposes [165]. The advantage of ex vivo administration is the ability to select correctly CRISPR-Cas-modified cells for transplantation back into the patient. However, the extensive cell culture expansion required for ex vivo administration may lead to undesirable genomic alterations. Especially iPSCs are prone to errors in reprogramming and expansion regarding copy number variations and accumulative mutations [166–168]. There remains a safety concern using these cells, even though researcher has increased their genetic stability through 3D culture organoid cultures [169]. Furthermore, ex vivo approaches require more steps compared to in vivo approaches.

Currently, ex vivo CRISPR-Cas therapies are mainly investigated for the treatment of β-thalassemia [121], sickle cell disease [170], cancer immunotherapy [171], and conferring resistance for viral infections [172, 173], see Table 4. At present, CRISPR-Cas9 ex vivo therapy for treating β-thalassemia and sickle cell disease involves knocking down the BCL11a transcription factor leading to restoration in fetal hemoglobin expression in autologous hematopoietic stem cells [174]. Furthermore, conferring resistance to human immunodeficiency virus (HIV) infection is being investigated by knocking out the co-receptor CCR5 in CD34+ hematopoietic stem/progenitor cells [173]. Finally, CRISPR-Cas9 ex vivo therapy is mainly applied in immuno-oncology by modifying chimeric antigen receptor T-lymphocytes (CAR-T), using tumor-infiltrating lymphocytes (TILs) or knocking out the programmed death-1 (PD-1) in autologous T cells. Also, new studies focus on the knocking-out T cell receptor (TCR) and β2-macroglobulin (β2m) in CAR-T to improve safety and allow the use of allogenic T cells [171]. These clinical studies will provide pieces of evidence on the safety and efficacy to facilitate site-specific genome editing and catalyze CRISPR-Cas9 clinical translation.

5.2 In vivo Strategies

In vivo administration entails a systematic or local injection of CRISPR-Cas elements to exert genome editing at the desired target site. Many monogenic diseases require in situ genome editing, which is realized via CRISPR-Cas in vivo delivery to the desired cell type in need of genetic repair. A major challenge of in vivo administration remains tissue-specific delivery of all CRISPR-Cas components [175]. Consequently, current clinical trials employing CRISPR-Cas in vivo administration have targeted accessible organs, including the eye and cervix.

The first clinical trial using in vivo delivery of CRISPR-Cas9 is directed to treat LCA10, a hereditary monogenic retinal dystrophy disorder. The delivery is realized by AAV5 carrying plasmid-encoded with Cas9 and two sgRNAs

and is administrated via sub-retinal injection to correct the pathological mutations in the CEP290 gene [67]. To treat cervical cancer, a polymer-based suppository carrying plasmid-encoded CRISPR-Cas9 is administrated via the vagina to treat human papillomavirus-related malignancy by removing E6 and E7 oncogene [176, 177].

6 Conclusion

The rapid advancements in CRISPR-Cas engineering, together with an increasing number of preclinical investigations and clinical trials, enable new therapeutic opportunities for various pathologies in the future. Currently, CRISPR Therapeutics received FDA market-approval for their ex vivo CRISPR/Cas9 edited CAR-T therapy (CTX110™) to treat relapsed or refractory CD19+ B-cell malignancies. For in vivo applications, CRISPR-Cas clinical adaptation as gene therapy may well lead to market approval in the next five to ten years [31]. Even though this is a promising prognosis, CRISPR-Cas as gene therapy still has a long road ahead with many challenges to overcome. These challenges include the realization of precision genome editing, overcoming immunogenicity, and eliminating off-target mutagenesis. Furthermore, adequate preclinical models and clinical trials are needed to establish the safety and efficacy profile of CRISPR-Cas-based gene therapy. Here, a difference in genomic DNA effectivity and therapeutic benefit might be key to distinguish.

Moreover, tissue-specific delivery and high gene-editing efficiency are other challenges to overcome for CRISPR-Cas in vivo applications [175]. Hence, extensive research is focused on developing robust and effective delivery systems for CRISPR-Cas delivery into various tissues. Also, advancements in CRISPR-Cas nucleases to increase their fidelity, reduce off-target effects, and increase sgRNA specificity can promote gene-editing productivity [39, 178]. Altogether, advancements in delivery systems and CRISPR-Cas nucleases enable the creation of novel CRISPR-Cas systems for therapeutic intervention with better safety and efficacy profiles.

Furthermore, the CRISPR-Cas translational pathway faces numerous issues regarding societal and ethical concerns. From a social perspective, genome editing is costly and raises the concern to only be accessible to wealthy individuals in the future. Consequently, this increases the existing disparities in access to health care [179]. Ethical concerns were raised when He Jiankui triggered international controversy for creating the first genome edited human babies carrying modified CCR5 gene to promote genetic resistance to HIV infection. Consequently, a global moratorium was called in place with legislation banning all clinical uses related to human germline editing [180]. Moreover, germline genome editing raised ethical concerns on the implications of transferring unwanted mutations to next generations, including but not limited to potential side effects after birth, concerns about breeding (eugenics), and informed consent. Hence, strict regulation and international consensus should be established to regulate germline gene therapy and prevent "designer babies" and nonessential therapies.

In short, CRISPR-Cas gene therapy has a profound opportunity to improve healthcare outcomes. Yet, expediting its clinical development requires the efforts of scientists, clinicians, the pharmaceutical industry, bioethicists, healthcare economists, and regulatory officials to safeguard the realization of safe, effective, and affordable CRISPR-Cas-based gene therapies [31, 158].

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