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THE PRESENT AND FUTURE

STATE-OF-THE-ART REVIEW

Genome Editing

The Recent History and Perspective in Cardiovascular Diseases

Kiran Musunuru, MD, PhD, MPH

ABSTRACT

The genome-editing field has advanced to a remarkable degree in the last 5 years, culminating in the successful correction of a cardiomyopathy gene mutation in viable human embryos. In this review, the author discusses the basic principles of genome editing, recent advances in clustered regularly interspaced short palindromic repeats and clustered regularly interspaced short palindromic repeats–associated 9 technology, the impact on cardiovascular basic science research, possible therapeutic applications in patients with cardiovascular diseases, and finally the implications of potential clinical uses of human germline genome editing. (J Am Coll Cardiol 2017;70:2808–21)
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An adult man has been diagnosed with familial hypertrophic cardiomyopathy, with severe enough disease to warrant the use of an implantable cardioverter-defibrillator and antiarrhythmic medications. Upon genetic testing, he is found to be heterozygous for a 4-bp deletion in the *MYBPC3* (myosin-binding protein C, cardiac type) gene. In light of this finding, each of his children would have a 50% chance of inheriting the *MYBPC3* mutation and being at risk for severe hypertrophic cardiomyopathy. To avoid this possibility, the man's sperm are used for in vitro fertilization of oocytes. At the same time that the sperm are injected into the oocytes, clustered regularly interspaced short palindromic repeats (CRISPR)-CRISPR-associated 9 (Cas9) and a synthetic deoxyribonucleic acid (DNA) molecule containing the correct *MYBPC3* sequence are also injected with the intent of cleanly correcting the mutation carried by the sperm. This procedure results in many embryos in which the correction has successfully occurred, with no effects on the rest of the genome. The embryos are ready for transfer into the womb of a mother, who would carry to term a child who would be free of the father's disease.

Although this vignette might have the feel of science fiction, in fact this very sequence of events has already occurred in real life (1), published in a report in August 2017 and widely announced in the press. In this case, there was never any intent for the embryos to be carried to term, but the embryos were viable and in principle could have given rise to live people. This highlights the dramatic progress that has taken place in the field of genome editing. How did we get to this point?

PRIMER ON GENOME EDITING

It is important to recognize that genome editing did not suddenly emerge out of a vacuum but built on decades of work by numerous investigators seeking to improve the ability to target specific alterations into specific genes within the genomes of cells, whether in model organisms such as mice or in human cells. Traditional gene targeting, such as that used in mouse embryonic stem cells to make "knockout" mice, is

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From the Cardiovascular Institute, Department of Medicine, Perelman School of Medicine at the University of Pennsylvania, Philadelphia, Pennsylvania; and the Department of Genetics, Perelman School of Medicine at the University of Pennsylvania, Philadelphia, Pennsylvania. This work was partially supported by National Institutes of Health grants R01-HL118744, R01-GM104464, R01-DK099571, and R01-HL126875, and the American Heart Association. Dr. Musunuru has reported that he has no relationships relevant to the contents of this paper to disclose.

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technically challenging and relies on the process of homologous recombination (2). A custom-made piece of DNA is introduced into cells and serves as the template for homologous recombination. For the custom-made template to work, it must have sequences that match the sequences around the target site in the genome—regions of homology, also termed homology arms. A desired alteration, whether a single nucleotide change or a longer DNA sequence to be inserted, is placed between the homology arms in the template. Homologous recombination causes "crossing over" to occur between the matching homology arms in the genome and in the introduced template and stably copy the alteration into the genome. Spontaneous homologous recombination with a custom-made template occurs at a very low frequency—on the order of a 1-in-a-million event. While there are methods to enrich for cells in which the desired event has occurred and discard the other cells (which has made it possible to generate genetically modified cells and animals in the laboratory setting), the frequency of recombination is far too low to be useful if the goal is to produce a therapeutic effect in cells in a live human being.

Genome editing takes advantage of tools that produce double-strand DNA breaks at desired locations in the genome. The double-strand break activates the cellular DNA repair machinery and, in doing so, can improve the efficiency of altering the genome by orders of magnitude. Instead of being 1-in-a-million events, alteration of the genome can routinely occur with higher than 1-in-10 frequency. This dramatic improvement in efficiency has for the first time made it feasible to undertake a "rewriting" of the human genome for therapeutic purposes.

Genome editing that is instigated by double-strand breaks can achieve several types of changes, tied to the 2 major ways in which the cell repairs double-strand breaks (**Figure 1**) (3). In nonhomologous end joining (NHEJ), the DNA molecule's free ends created by the double-strand break are rejoined (4). NHEJ is the default repair pathway in the sense that it operates in all types of cells at all times. NHEJ is a less-than-perfect repair process that occasionally results in the semirandom insertion or deletion of DNA base pairs, termed "indels." Indels introduced into the coding sequence of a gene will either represent frameshift mutations or in-frame insertions or deletions; the former will usually result in scrambling of part of the amino acid sequence and premature truncation of the protein product of the gene, and the latter will add or remove amino acids from the protein. Either can interfere with or even "knock out" the function of the protein. If genome editing is used to produce 2 double-strand breaks on the same chromosome, the portion of the DNA molecule between the breaks might be irrevocably lost if the far free ends are rejoined. The consequence could be deletion of part of a gene, an entire gene, or a chromosomal region with multiple genes.

The second way in which a cell can repair a double-strand break is homology-directed repair (HDR). Unlike NHEJ, HDR is normally limited to proliferating cells that have doubled their chromosomes and thus have duplicate chromatids on each chromosome (i.e., are in S phase or G2 phase). Akin to homologous recombination, HDR requires a repair template with homology to the DNA sequences flanking the double-strand break. Ordinarily the repair template is a duplicate chromatid (which has the identical sequence) or a matching chromosome (e.g., the paternally inherited chromosome that is paired with the maternally inherited chromosome—perhaps not identical in sequence, but similar enough to provide homology) (5). A synthetic, custom-made DNA template with a desired alteration flanked by homology arms, when introduced into a cell, can instead be used by HDR and result in stable copying of the alteration into the genome (6).

Whereas NHEJ can have unpredictable consequences—indels of varied sizes occur, sometimes as large as kilobases, which can affect genes beyond just the target gene—HDR is a much more precise repair process that makes it suitable for correcting disease mutations. However, HDR has 3 principal disadvantages compared with NHEJ. First, HDR-mediated editing generally occurs with less efficiency than does NHEJ-mediated editing in proliferating cells, due to HDR being limited to only part of the cell cycle. Although exceptions have been observed in vitro in transformed cells (in which DNA repair mechanisms are often dysregulated) (7), and certain chemicals have been shown to inhibit NHEJ or enhance HDR in cultured cells in vitro (8–10), in vivo studies appear to confirm this rule (11,12). Second, HDR does not normally occur at all in nonproliferating cells, a substantial limitation with respect to postnatal cardiomyocytes and other cell types relevant to cardiovascular disease. Third, HDR-mediated editing requires a custom-made repair template, and delivery of the template into cells is nontrivial. Together, these characteristics make NHEJ-mediated disruption or deletion of genes more feasible than HDR-mediated correction of mutations or insertion of

ABBREVIATIONS AND ACRONYMS

AAV = adeno-associated virus

Cas9 = clustered regularly interspaced short palindromic repeats–associated 9

CHD = coronary heart disease

CRISPR = clustered regularly interspaced short palindromic repeats

CRISPRa = clustered regularly interspaced short palindromic repeats activation

CRISPRi = clustered regularly interspaced short palindromic repeats interference

DNA = deoxyribonucleic acid

HDR = homology-directed repair

hPSC = human pluripotent stem cell

iPSC = induced pluripotent stem cell

NHEJ = nonhomologous end joining

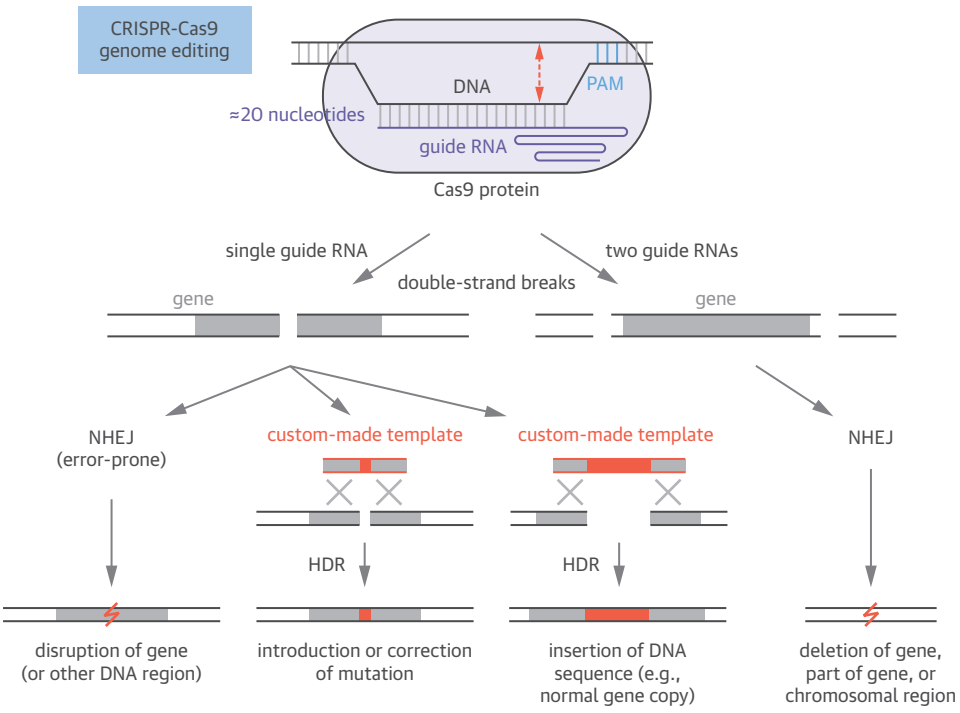
PAM = protospacer-adjacent motif

RNA = ribonucleic acid

TALEN = transcription activator-like effector nuclease

ZFN = zinc-finger nuclease

FIGURE 1 Genome Editing Based on CRISPR-Cas9



Clustered regularly interspaced short palindromic repeats (CRISPR)-CRISPR-associated 9 (Cas9) recognizes a genomic site defined by complementary base pairing of ≈20 nucleotides in the guide ribonucleic acid (RNA) (protospacer) and the presence of an appropriate protospacer-adjacent motif (PAM) in the deoxyribonucleic acid (DNA) (shown in **blue**). The **orange arrows** indicate the sites of Cas9-mediated DNA cleavage to generate a double-strand break. Different outcomes occur depending on how many guide RNAs are used, whether a custom-made DNA repair template is provided, and whether the double-strand break is repaired by nonhomologous end joining (NHEJ) or homology-directed repair (HDR). Reprinted with permission from Chadwick AC, Musunuru K. CRISPR-Cas9 genome editing for treatment of atherogenic dyslipidemia. Arterioscler Thromb Vasc Biol 2017 Aug 24 [E-pub ahead of print].

genes. This has important implications for the viability of therapeutic applications of genome editing.

GENOME EDITING AND NEW TYPES OF EDITING WITH CRISPR-CAS9

STANDARD GENOME EDITING. A common characteristic of genome-editing tools is that each can produce double-strand breaks at user-specified sites in the genome, after which the cell repairs the breaks by either NHEJ or HDR in a tool-independent manner. Several protein-based tools are available for genome editing: zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and meganucleases (13). ZFNs and TALENs are modular proteins that comprise arrays of DNA-binding motifs (either zinc fingers, which typically bind 3 DNA base pairs each, or TAL repeats, which bind 1 DNA base pair each), which provide specificity for particular binding sites in the genome, attached to enzymatic domains. ZFNs and TALENs are each employed as a pair of proteins, binding 2 nearby DNA sequences and combining their enzymatic domains to create a double-strand break. In contrast, meganucleases are adapted from naturally occurring, large, DNA-cleaving enzymes that specifically bind to long stretches of DNA sequence (as long as 40 base pairs). Whereas work on ZFNs and meganucleases has taken place over the last few decades, TALENs were first introduced in 2010. ZFNs, TALENs, and meganucleases share the disadvantage that, as proteins, they must be reengineered for each new genomic site to be targeted, a process that can take days to months. Nonetheless, each has been used productively for a variety of research applications and, in the case of ZFNs, clinical trials for the treatment of HIV infection (14).

The introduction of CRISPR-Cas9 systems as genome-editing tools in early 2013 (15-19) represented a watershed in the field of genome editing—and, in retrospect, in biomedical research—for

3 reasons: 1) their ease of use; 2) their efficacy compared with other tools; and 3) their adaptability to applications beyond genome editing. CRISPR-Cas9 systems are based on an adaptive immune mechanism discovered in bacterial species and used by bacteria to protect against foreign DNA molecules. Upon exposure of a bacterium to foreign DNA sequences within its cytoplasm (e.g., viral infection), its immune system can incorporate pieces of the foreign DNA sequences into the bacterial genome, whereupon they are expressed as ribonucleic acid (RNA) molecules that bind to Cas9 proteins or other similar proteins. If the bacterium is re-exposed to a foreign DNA sequence, the immune system can identify the sequence via matching to the corresponding RNA molecule and use Cas9 or other proteins to neutralize the DNA sequence by cleaving it.

Each adapted CRISPR-Cas9 system comprises a protein and an RNA molecule (**Figure 1**). The Cas9 protein serves a variety of functions—it can act to scan and unwind double-strand DNA, it can recognize and bind particular DNA sequences, it can recognize and bind RNA sequences, and it can produce a double-strand break in DNA. In the streamlined CRISPR-Cas9 system now most commonly used for genome editing, the RNA component is a "guide RNA" that is about 100 nucleotides in length (it represents a fusion of 2 RNA molecules used in natural bacterial CRISPR-Cas9 systems). Cas9 binds to this guide RNA, which itself can hybridize to 1 strand of double-strand DNA via its first ~20 nucleotides, a sequence termed the protospacer. Cas9 also binds to several adjacent nucleotides in the DNA, termed the protospacer-adjacent motif (PAM). Thus, a tripartite complex of protein, RNA, and DNA is formed. Once formed, the complex produces a double-strand break in the DNA.

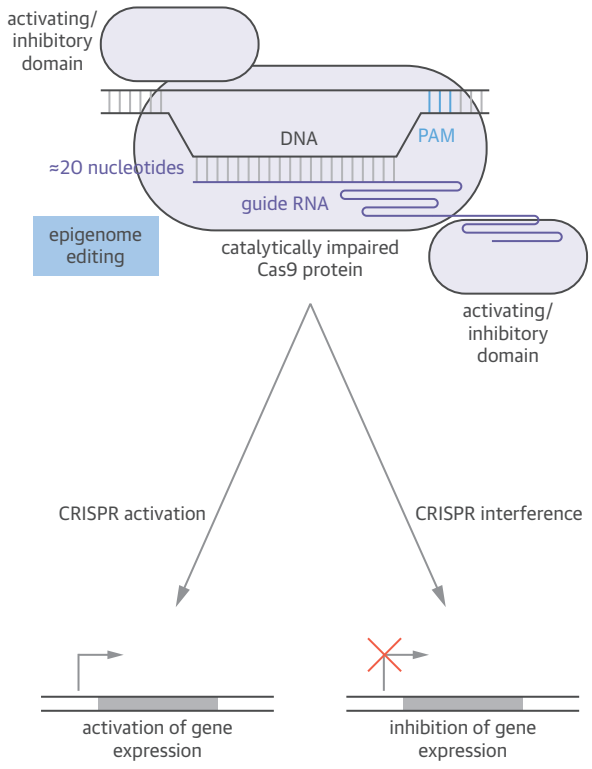
The specificity of CRISPR-Cas9 is encoded in the protospacer sequence of the guide RNA. Changing this sequence redirects the protein-RNA complex to bind a different site in the DNA that harbors a sequence that is complementary to the protospacer sequence and is adjacent to a PAM. Because of this feature, it is far easier to change the target genomic site of CRISPR-Cas9—a simple change of the first ~20 nucleotides of the guide RNA, which can be done in 1 day—than it is for ZFNs, TALENs, and meganucleases. Of note, different bacterial species have different CRISPR-Cas9 systems. The most commonly used version is from the species *Streptococcus pyogenes*, although a version from the species *Staphylococcus aureus* is being increasingly used because its Cas9 protein is smaller (and therefore easier to deliver into cells in vivo) than *S. pyogenes* Cas9 (20,21). These 2 CRISPR-Cas9 systems, as well as CRISPR-Cas9 systems adapted from other bacterial species, are also distinguished by the differences between the sequences of their respective guide RNAs as well their PAMs (*S. pyogenes* Cas9 binds to the PAM sequence NGG, where N is any nucleotide, whereas *S. aureus* Cas9 prefers to bind NNGRR, where R is either guanine or adenine).

The ease of generating guide RNAs makes it feasible to create a large library of guide RNAs all at once, for example, a library that covers all of the genes in the genome. This has permitted genome-wide screens in cells (22,23). Another advantage of CRISPR-Cas9 is its multiplexing capacity. If one wishes to target 2 genes at once, one can mix Cas9 with 2 different guide RNAs matching the 2 gene sequences, and CRISPR-Cas9 complexes will form and create double-strand breaks in the 2 genes simultaneously. With the use of several guide RNAs, one can potentially target several genes at the same time. To delete a specific region of DNA with NHEJ, one can use 2 guide RNAs that target sites flanking the DNA region (**Figure 1**).

In general, CRISPR-Cas9 displays higher efficiency than other genome-editing tools when used "out of the box" (i.e., no particular attempt at optimization for activity at a specific genomic site, which typically has been performed for ZFNs by screening thousands of possible proteins). In a study performed shortly after CRISPR-Cas9 was introduced for use in genome editing, CRISPR-Cas9 outperformed TALENs at numerous genomic sites in human cells in a head-to-head comparison (24). Other studies established that CRISPR-Cas9 was more efficient than had ever been observed with ZFNs and TALENs when injected into single-cell embryos of mice (12,25) and then other animals, up to and including nonhuman primate embryos (26) and then, finally, human embryos (1,27). CRISPR-Cas9 also proved to be highly efficient in somatic (i.e., nongermline) tissues in live animals (28).

EPIGENOME EDITING. A third major advantage of CRISPR-Cas9 over other tools is that it has been adapted to a variety of applications beyond standard genome editing. It is straightforward to alter the Cas9 protein so that it can continue to form a protein-RNA-DNA complex but is catalytically dead (i.e., cannot generate a double-strand break in the DNA). This so-called dCas9 protein, in combination with a guide RNA, can serve as a customizable, high-affinity, sequence-specific DNA-binding domain to which other domains can be attached (**Figure 2**). Alternatively, an extension of the guide RNA can be used as a tether for other domains. Addition of a transcriptional activator domain yields a complex that can increase

FIGURE 2 Epigenome Editing



Use of catalytically impaired Cas9 (dCas9), with tethering of a regulatory domain to either Cas9 or the guide RNA (or both), can result in transcriptional activation or inhibition of a target gene without alteration of the DNA sequence. Abbreviations as in Figure 1.

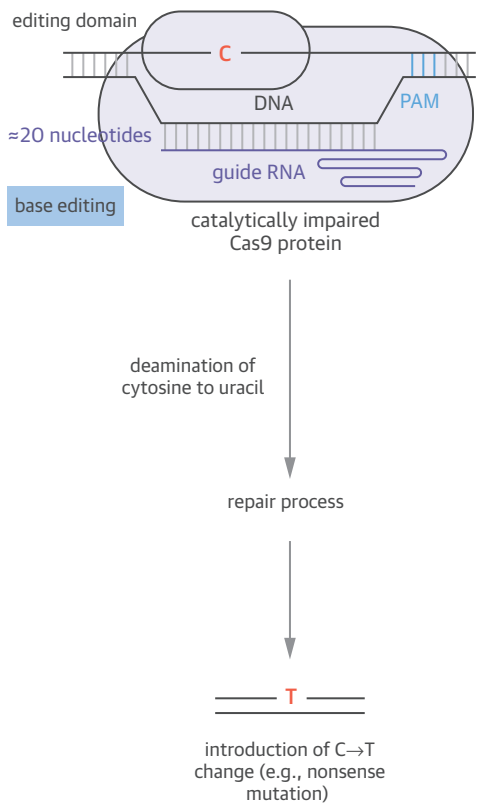
the expression of a target gene when positioned at the promoter of the gene or other regulatory regions, a phenomenon known as CRISPR activation (CRISPRa) (29-32). Addition of a transcriptional repressor domain can have the opposite effect—CRISPR interference (CRISPRi), by analogy to RNA interference, the standard method of knocking down gene expression (33). CRISPR activation and inhibition are considered to be forms of "epigenome editing," as no alterations are made to the genomic DNA sequence. In some cases, transient expression of modified CRISPR-dCas9 platforms can produce stable, long-term epigenetic changes (34,35). As with standard CRISPR-Cas9 genome editing, CRISPRa and CRISPRi can be multiplexed through the simultaneous use of multiple guide RNAs, allowing for increased or decreased expression of sets of genes in tandem. CRISPRa and CRISPRi can also be combined with large-scale guide RNA libraries to perform genome-wide screens in cells (36,37).

BASE EDITING. Recently, CRISPR-Cas9 has been adapted so that it cannot directly generate double-strand breaks in DNA but can nevertheless alter specific nucleotides in the DNA sequence, a phenomenon known as "base editing." Addition of a cytosine deaminase domain adapted from an RNA-editing or DNA-editing enzyme to the dCas9 protein confers the ability to convert cytosine bases near the dCas9 target site (as determined by the guide RNA) into uracil bases (**Figure 3**) (38-41). Ordinarily, uracil bases in DNA would be removed by base excision repair, but addition of yet another specialized domain to dCas9 can serve to inhibit this process. dCas9 can be altered so that it can introduce a nick (single-strand DNA break) on the opposite DNA strand, which triggers the mechanism of nick repair in which several bases are removed from the opposite strand and then replaced with bases complementary to the uracil-containing strand, resulting in the introduction of adenine opposite to uracil (instead of the original guanine). After the protein-RNA-DNA complex disassembles, base excision repair removes the uracil and introduces thymine opposite to adenine (instead of the original cytosine).

The end result is a C-to-T edit at a user-specified site in the genome. If the edit occurs on the sense strand of a protein-coding sequence, there will be a C-to-T change in a codon. Alternatively, if the edit occurs on the antisense strand, there will be a G-to-A change in a codon. In principle, base editing potentially allows for specific alterations—such as the correction of disease-causing mutations—to be made without the limitations of HDR-mediated editing (restriction to proliferating cells, need for custom-made repair template) or the unpredictability of NHEJ-mediated editing (semirandom indels of varied sizes). Base editing can occur with high efficiency in vitro (38-41), in embryos (42), and in somatic tissues in live animals (43), making it a valuable addition to the editing toolbox.

LIMITATIONS OF CRISPR-Cas9. The remarkable efficiency of CRISPR-Cas9 in editing the genome lies at the heart of its most significant limitations. Perhaps the most concerning limitation is the capacity for CRISPR-Cas9 (or any other genome-editing tool) to introduce double-strand breaks and, therefore, mutations at off-target sites throughout the genome. Studies to ascertain the seriousness of the issue have had mixed results (29,44-48). Empirical evidence confirms that off-target effects are most likely to occur at sites with sequence similarity to the desired target site, although there are not yet reliable means to predict at which sites and with what

FIGURE 3 Base Editing



Tethering of an editing domain (cytosine deaminase) to catalytically impaired Cas9 (either dCas9 or a nickase form of Cas9) can result in site-specific alteration of cytosine-guanine base pairs to thymine-adenine base pairs without the need for double-strand breaks. C = cytosine; T = thymine. Abbreviations as in Figure 1. Reprinted with permission from Chadwick AC, Musunuru K. CRISPR-Cas9 genome editing for treatment of atherogenic dyslipidemia. Arterioscler Thromb Vasc Biol 2017 Aug 24 [E-pub ahead of print].

frequency they will occur. A variety of methods to assess for off-target effects across the genome in an unbiased way are available for use in cultured cells in vitro (20,49–53) but have not yet been extended to use in vivo. Even if an off-target site is known, current deep-sequencing technologies cannot reliably detect mutations that occur at <0.01% frequency. Although off-target mutations can potentially confound the interpretation of findings in genome-edited cellular and animal models, the most serious concern lies with therapeutic applications. If administered to billions of cell in the human body, even extremely rare mutations could have deleterious consequences, such as perturbation of an oncogene or tumor suppressor gene that results in the eventual growth of cancer. At the same time, it should be recognized that perturbation of the vast majority of locations in the 6.2 billion bases of the human genome in a limited number of cells would have no health consequences for a patient. Unsurprisingly, substantial effort has been invested in reducing the risk of off-target mutagenesis with CRISPR-Cas9, either through modification of the guide RNA or modification of the Cas9 protein itself (e.g., changing key amino acids to create "high-fidelity" versions of Cas9) (54–56).

Another limitation is an excess of efficiency at the desired target site. Attempts to cleanly introduce specific alterations at a genomic site with HDR, which is active only in proliferating cells in S or G2 phase, can be compromised by NHEJ, which is active in all cells in all phases of the cell cycle. It can be difficult to generate cells in which one allele contains a desired HDR-mediated edit and the other allele remains intact (heterozygous) or cells in which both alleles contain a desired HDR-mediated edit (homozygous), because the alleles are more likely to instead be disrupted by NHEJ. This phenomenon is particularly troublesome if a therapeutic intervention requires correction of a mutation. In an adult mouse model of ornithine transcarbamylase deficiency, attempted HDR-mediated correction of the disease mutation actually worsened the disease; although only a few percent of alleles were corrected, a large proportion of alleles received indels that disrupted residual gene function (11).

In contrast, there can be an inability to target a desired genomic site. One limitation of the original *S. pyogenes* CRISPR-Cas9 is the necessity for a PAM DNA sequence, NGG, which occurs on average 1 in every 8 base pairs but in some cases may not occur near the desired target site—a particular issue if one wishes to correct a specific mutation. A second limitation is that there is considerable variability in the efficiency of on-target mutagenesis from site to site, even for sites that are a few base pairs apart. The reasons for this variability are unclear, and it is hard to predict a priori if a site will be amenable to genome editing. One way to overcome these limitations is to use CRISPR-Cas9 systems from other bacterial species that have different PAM sequences, the idea being to expand the range of candidate target sites near a mutation. Another way to overcome these limitations has been to engineer the *S. pyogenes* and *S. aureus* Cas9 proteins so that they can recognize other PAM sequences (21,57).

Another substantial limitation is the size of CRISPR-Cas9, complicating delivery into cells in vivo. The *S. pyogenes* CRISPR-Cas9 system is too large to fit

into a single adeno-associated viral (AAV) vector, which mandates the use of either multiple AAV vectors or an alternative viral vector with a larger cargo size, such as adenovirus or lentivirus, each of which is less preferred for use in human therapeutics due to safety concerns. One potential solution is the use of lipid nanoparticles to deliver RNAs encoding components of the system (58), although this approach has not yet been demonstrated to result in efficient in vivo genome editing. Another potential solution is to use smaller CRISPR-Cas9 systems such as that from *S. aureus*, which can be accommodated in single AAV vectors (20).

Some of the aforementioned limitations can potentially be overcome with base editing, which provides a means to introduce or correct certain types of mutations at a much higher efficiency than HDR-mediated genome editing, along with a greatly reduced rate of indels due to the fact that base editing does not require the generation of double-strand breaks. Furthermore, early analyses suggest that base editing has a more favorable off-target effect profile than standard CRISPR-Cas9 genome editing (59). However, base editors are currently limited to C-to-T edits, and they are larger than Cas9 and thus even more challenging to deliver into cells in vivo.

CARDIOVASCULAR RESEARCH APPLICATIONS

It is difficult to overstate the impact of genome editing on biomedical research, as attested by the thousands of reports in which genome-editing tools have been used during the past few years. Space limitations prevent a comprehensive discussion of every published application of genome editing in cardiovascular research; rather, this section highlights 2 broad themes that have emerged—disease modeling with animals and disease modeling with human pluripotent stem cells (hPSCs) (**Central Illustration**).

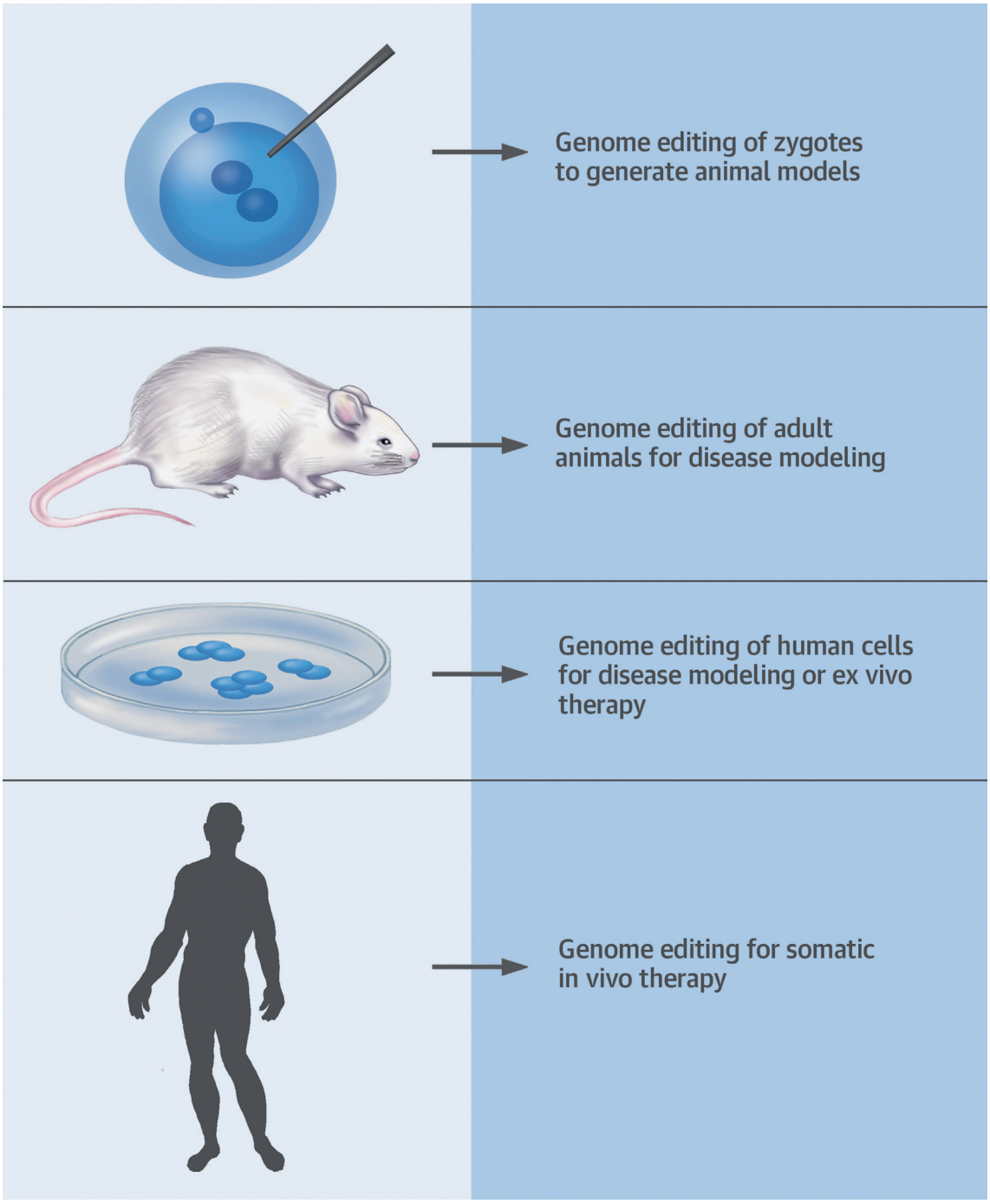
ANIMAL MODELS. Genome-editing tools have been employed for the creation of animal models to study a variety of cardiovascular diseases. Zebrafish have proven to be quite amenable to modification by genome editing and have been used to model vascular development and cardiac development and regeneration (60). The ease of use and adaptability of CRISPR-Cas9 enables makes it feasible to create numerous strains of zebrafish with distinct mutations in the same gene, something not possible with the classical genetic techniques of RNA interference and morpholinos. A notable example demonstrating the utility of CRISPR-Cas9 was its use to generate a series of mutants spanning the Titin protein, linked to cardiomyopathy (61). In finding that mutations in the C-terminal part of the protein were associated with more severe cardiomyopathy than those in the N-terminal portion, the study uncovered a novel internal promoter midway through the gene that produces a distinct, shorter gene transcript that partly reverses the effects of N-terminal Titin mutants.

Genome-editing technology has had an even greater impact with respect to the generation of knockout and knock-in mouse models of disease (62). CRISPR-Cas9 has proven to be so efficient when injected into single-cell mouse embryos (zygotes) (12,25) that it has already begun to supplant the standard method of generating genetically modified mice (mouse embryonic stem cells). For knockout mice, CRISPR-Cas9-induced NHEJ efficiently introduces knockout frameshift mutations into the target gene in the embryos. Knock-in mice are readily generated via HDR with CRISPR-Cas9 coinjected with a custom-made repair template bearing the knock-in mutation or insertion (e.g., reporter gene). Besides interrogating gene function, these approaches are useful for interrogating regulatory elements in the noncoding genome (63). A cardinal advantage of these approaches is that they can yield full knockout or knock-in mice in a single generation—a matter of weeks, rather than the months to years entailed by the standard method.

The same approach of embryo genome editing that has been so fruitful in generating mouse models has also made it possible to generate genetically modified animals of a variety of species in which disease modeling had previously been difficult or unachievable. This has the potential to be a boon in cardiovascular research, in which mouse models often poorly phenocopy human diseases. Genetically modified rats, rabbits, and pigs have been reported for a variety of cardiovascular conditions, ranging from hypertension to hyperlipidemia to aortopathies (60,64–66), with investigation of these conditions underway.

An alternative to germline genome editing (i.e., embryo editing) to perform disease modeling in animal models is the use of somatic in vivo genome editing, in which the editing occurs in an organ in a live animal. Although CRISPR-Cas9 delivered by viral vectors has proven to be effective in easily accessible organs such as the liver (20,28), its effectiveness has been more limited in other organs such as the heart, where delivery is more challenging (67). As described previously, one obstacle to delivery is the large size of Cas9. A workaround is the use of transgenic mice in which Cas9 is endogenously expressed from the

CENTRAL ILLUSTRATION **Applications of Genome Editing**



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Genome editing may be used to generate animal models, either through germline modification or somatic modification; to modify human cells, including human pluripotent stem cells and primary human cells; and for therapeutic purposes in human patients.

mouse genome—constitutively, conditionally, or in a tissue-specific manner (67–69). The much smaller guide RNA is easier to deliver by a viral vector, nanoparticles, or other means. In one example, a cardiac-specific Cas9 transgenic mouse was used to perform somatic in vivo genome editing in the heart; although the efficiency was not high, sufficient disruption of the *Myh6* gene was achieved for the mice to develop hypertrophic cardiomyopathy (69). In another example, a conditional Cas9 transgenic mouse model was used to individually, partially deplete 9 different genes in the heart and thereby define roles for junctophilin-2 and ryanodine receptor-2 in T-tubule stabilization and maturation, respectively (70).

hPSC MODELS. In principle, hPSCs have substantial advantages over standard transformed or immortalized cultured cell lines, as they have normal human karyotypes and can be differentiated into various cell types relevant to cardiovascular diseases, including cardiomyocytes, vascular endothelial cells, smooth muscle cells, hepatocytes, and macrophages. A large number of studies have generated induced pluripotent stem cell (iPSC) lines from patients with cardiovascular diseases. Most of these studies have used iPSC lines from healthy people as control subjects. Although these types of studies can be informative, they can suffer from substantial confounding due to poor matching of the patient-specific and control cell lines—differences in genetic background, sex, ethnicity, epigenetics, pluripotency, capacity to differentiate into the desired cell type, and other characteristics.

Genome editing provides the most rigorous means to eliminate these confounders, as it can be used to correct a pathogenic mutation in a patient-specific iPSC line or, conversely, to introduce a pathogenic mutation into an iPSC line from a healthy person (or knock out a gene, change a single nucleotide variant, etc.). The latter approach is particularly useful when the mutation is very rare in the population and no patient is available to generate iPSCs. With either approach, investigators are able to compare well-matched, isogenic cell lines that differ only with respect to the mutation. ZFNs, TALENs, and CRISPR-Cas9 have all been used to generate isogenic hPSC lines, although CRISPR-Cas9 has proven to be the most efficient of the tools in hPSCs (24).

In one example, iPSC-cardiomyocytes from 2 patients with mutations in *TAZ* (tafazzin) and with Barth syndrome, a mitochondrial disorder affecting muscle function and causing dilated cardiomyopathy, exhibited abnormal sarcomere assembly, impaired contractility, and excess levels of reactive oxygen species (71). Genome editing was used to introduce *TAZ* mutations into iPSCs from a healthy individual, and the edited iPSC-cardiomyocytes displayed the same abnormalities vis-à-vis the matched, nonedited iPSC-cardiomyocytes, strengthening the link between *TAZ* mutations, the cellular phenotypes, and the clinical features of Barth syndrome. Genome-edited hPSC lines have likewise been informative in studying other cardiomyopathies (72–76), lipid metabolism (77–82), vascular disorders (83), valvular disease (84), and arrhythmia disorders (85,86).

In an example of epigenome editing, CRISPRi was used to perform disease modeling in iPSC-cardiomyocytes (87). A variety of cardiovascular disease genes were knocked down in differentiated cells; knockdown of the *HERG* potassium channel resulted in prolongation of action potential duration.

THERAPEUTIC APPLICATIONS

Clinical trials to modify T cells and other hematopoietic cells are already underway (14) or have been announced. These involve ex vivo genome editing of a patient's cells, followed by transplantation of the cells back into the patient's body. Ex vivo therapeutic applications have a few advantages over in vivo therapeutic applications: the delivery of genome-editing tools into cells is much simpler, there is less risk of genome editing of nontarget cell types, and there is the potential to sort out or select just the appropriately edited cells for transplantation and thereby improve the efficiency and safety of the therapy. Although there is the theoretical possibility of isolating or generating stem cells from a patient, editing the cells, converting the cells into the relevant cardiovascular cell type, and transplanting the cells back into the body with engraftment into the target organ (e.g., heart), it is not yet clear whether this will prove to be a viable and practical approach. For the foreseeable future, genome-editing therapies to prevent or treat cardiovascular diseases will be limited to in vivo applications, most likely targeting hepatocytes or cardiomyocytes within the body.

One attractive strategy for the prevention of coronary heart disease (CHD) is the disruption of genes involved in atherogenic dyslipidemia. Such genes include *PCSK9* (proprotein convertase subtilisin/kexin type 9), *ANGPTL3* (angiopoietin-like 3), and *APOC3* (apolipoprotein C-III). With each of these genes, naturally occurring loss-of-function mutations are associated with both reduced blood lipid levels and reduced risk of CHD (in the case of *PCSK9*, up to 88% reduced risk of CHD) (88–92); furthermore,

individuals with 2 loss-of-function mutations and no residual gene activity are healthy with no apparent adverse consequences (93-95). These observations have made these genes attractive therapeutic targets, with treatments already available to patients or in clinical trials (90,96-99). A shared feature of all of these treatments—whether based on antibodies, RNA interference, or antisense oligonucleotides—is that they have limited half-lives (days to months), necessitating repeated administrations over the lifetime for a patient to experience the full clinical benefit. An arguable advantage of genome-editing therapies targeting 1 or more of these genes is that a single administration would offer prolonged and possibly lifelong protection against CHD, due to the permanence of the alterations in the genome.

In proof-of-principle studies in mice, *S. pyogenes* CRISPR-Cas9 delivered by an adenoviral vector was used for disruption of the mouse or human *PCSK9* gene in the liver, where the protein product is expressed and secreted into the bloodstream (28,100). In another study, *S. aureus* CRISPR-Cas9 delivered by an AAV vector was used for the same purpose (20). NHEJ disrupted the majority of mouse *Pcsk9* alleles in the liver, resulting in a reduction of blood PCSK9 protein levels by >90% and a reduction of blood cholesterol levels by ~40%; off-target mutagenesis was not detected at a variety of potential off-target sites with sequence similarity to the *Pcsk9* target site (20,28). In a more recent study, a base editor designed to convert the codon for Trp-159 in *Pcsk9* into a stop codon (i.e., specific insertion of nonsense mutations, rather than semirandom indels) successfully converted a large proportion of alleles in the livers of adult mice, with no evidence of off-target mutagenesis (43). The success of the base editing study presages the possibility of efficiently and precisely correcting pathogenic gene mutations in vivo.

Disruption of endogenous genes could in principle be used to address a variety of cardiovascular diseases. In one study, disruption of hepatic *Apob* (apolipoprotein B) resulted in the normalization of cholesterol levels and atherosclerosis in a mouse model of familial hypercholesterolemia (101). However, genome editing of *Apob* resulted in marked hepatic steatosis in 2 mouse studies (20,101), suggesting that it might not be a safe strategy in human patients. Disruption of the *TTR* (transthyretin) gene in the livers of patients with familial amyloid cardiomyopathy could halt and potentially even reverse the disease (102), as has been demonstrated for familial amyloid polyneuropathy with an RNA interference therapy targeting the same gene. In patients with dominant mutations linked to myocardial diseases such as hypertrophic cardiomyopathy or catecholaminergic polymorphic ventricular tachycardia, specific disruption of the mutant allele with sparing of the wild-type allele in cardiomyocytes (which could be performed by using a genome-editing tool that is matched to the mutant sequence but not the wild-type sequence) could prevent disease (103,104). For certain diseases, targeted deletion of a portion of a mutant gene could be ameliorative. This has been demonstrated for Duchenne muscular dystrophy in several studies in a mouse model, where simultaneous use of 2 guide RNAs with Cas9 removed mutant exons in some proportion of *Dmd* alleles in skeletal muscle and in the heart, resulting in some restoration of protein activity and improved muscle function (105-107). However, the efficiency was low in skeletal muscle and especially in cardiomyocytes compared with the liver. This raises the question of variability in the efficiency of CRISPR-Cas9 genome editing among different organs in vivo, either due to differences in accessibility to CRISPR-Cas9 delivery or due to differences in intrinsic activity of CRISPR-Cas9 within various cell types.

Alternative strategies for the prevention or treatment of cardiovascular diseases—especially those with genetic causes—would entail HDR-mediated gene correction or insertion of extra copies of a wild-type gene in target organs. The challenge is that HDR is generally less efficient than NHEJ and is inactive in nonproliferating cells such as postnatal cardiomyocytes. As described earlier, an attempt to treat adult mice with ornithine transcarbamylase deficiency with HDR-mediated correction of the disease mutation actually worsened the disease due to the inefficiency of HDR compared with NHEJ-mediated disruption of the partially active mutant gene (11). Novel HDR-independent methods such as base editing will be needed to achieve these strategies.

Questions of safety remain to be addressed before therapeutic genome editing with CRISPR-Cas9 can be implemented for the benefit of patients with cardiovascular diseases. The biggest concern is off-target mutagenesis. Although in vivo CRISPR-Cas9 genome editing studies in mouse models generally have found little evidence of off-target mutagenesis, the cost of next-generation sequencing means that only a limited number of genomic sites can be assessed in any given study, and the intrinsic limitations of next-generation sequencing means that very rare mutations cannot be detected. Novel in vivo techniques to perform unbiased scans for rare off-target events throughout the genome will be needed. A second concern is unintended on-target mutagenesis. In one

study in which AAV-delivered CRISPR-Cas9 was used to disrupt a gene via NHEJ, a large proportion of the disrupted alleles harbored insertions of viral sequences (101). Also possible are very large indels that disrupt not just the target gene, but also neighboring genes. These consequences can be mitigated with refinements of CRISPR-Cas9 technology such as base editing, which does not require double-strand breaks. A third concern is that viral delivery of CRISPR-Cas9 will elicit immune responses to the Cas9 protein due to prolonged expression, a phenomenon that has been observed in mice (108). This could be addressed by devising ways to limit Cas9 expression, such as including an extra guide RNA in the vector that will result in self-cleavage of the Cas9 gene (109). An alternative is to use a nonviral means to deliver CRISPR-Cas9, such as encapsulating short-lived Cas9 messenger RNA molecules in lipid nanoparticles (58).

Until these safety issues can be definitively resolved, therapeutic genome editing should be restricted to those patients for whom the potential benefits greatly outweigh the potential risks, for example, patients with acutely life-threatening genetic disorders, or elderly patients with very high risk for future coronary events and maximal indications for lipid-lowering therapy but who are unlikely to live long enough to develop cancer from off-target mutagenesis.

HUMAN GERMLINE GENOME EDITING

The ability to efficiently and safely (i.e., without off-target mutagenesis) modify the human germline was heralded by the August 2017 report of the correction of a pathogenic *MYBPC3* mutation in human embryos (1). Several features of this report were particularly noteworthy. A potential risk with CRISPR-Cas9 treatment of an embryo is that different cells in the embryo will be modified in different ways, due to CRISPR-Cas9 continuing to be active after the zygote has begun dividing into multiple cells. This could lead to chimerism in the resulting offspring, a phenomenon that is commonly observed in CRISPR-Cas9-generated mouse models. In the August 2017 report, the investigators largely eliminated chimerism by formulating CRISPR-Cas9 as a short-lived synthetic protein-RNA complex and coinjecting it into the oocyte at the same time as the sperm. Another finding in the report was that correction of the *MYBPC3* mutation by HDR occurred at high efficiency but apparently did not use the custom-made repair template coinjected with CRISPR-Cas9. Instead, the normal copy of the *MYBPC3* gene from the oocyte nucleus was used as the template to repair the mutant copy from the sperm nucleus. Although this is encouraging in that it might not be necessary to use a custom-made repair template if attempting to repair a heterozygous mutation in an embryo, it also raises the possibility that repairing a homozygous mutation in an embryo—where there is no naturally occurring repair template within the zygote genome—might not be straightforward. No off-target mutations were detected in the corrected embryos, suggesting that it might be possible to edit the human germline safely. Finally, the corrected embryos were viable and, in principle, could have been carried to term and resulted in living people.

Thus, the barriers to human germline genome editing no longer seem like they will be technological—further refinements of genome-editing tools in the coming years will undoubtedly improve the efficacy and safety of embryo editing even further—but rather will be social, ethical, and legal in nature. The specter of "designer babies" initially elicited calls for a blanket moratorium on genome editing of human embryos (110). Since then, a number of organizations have been considering the issues around human germline genome editing in a more measured way. The U.S. National Academy of Sciences and National Academy of Medicine hosted an International Summit on Human Gene Editing, and its final published report supported basic science research using genome editing of human embryos to study reproductive biology, as long as it is performed within an appropriate regulatory framework (111). The American Society of Human Genetics statement on human germline genome editing went a step further in saying that there is "no reason to prohibit in vitro germline genome editing on human embryos and gametes, with appropriate oversight and consent from donors, to facilitate research on the possible future clinical applications of gene editing" and there should be "no prohibition on making public funds available to support this research" (112).

Going beyond basic science applications to the clinical use of human germline genome editing is a knottier question. One potential category of clinical use would be the treatment or prevention of a severe genetic disorder that would result in early loss of life or poor quality of life. The August 2017 report of the correction of a pathogenic *MYBPC3* mutation in human embryos would fit in this category (1). In most scenarios in this category, standard in vitro fertilization paired with pre-implantation genetic diagnosis—a well-established procedure—would result in some mutation-free embryos and arguably make it unnecessary to perform germline genome editing. This would be true for a heterozygous, dominant *MYBPC3*

mutation; around 50% of embryos from in vitro fertilization would not have the mutation. However, there are scenarios in which no mutation-free embryos would be available: 2 parents with a recessive disorder such as cystic fibrosis or sickle cell disease, or a parent with 2 dominant mutations such as those that cause Huntington's disease. Although these scenarios might be rare, they would mandate the use of genome editing in order for the parents to have healthy descendants.

A second potential category of clinical use would be the reduction of risk of common, serious adult diseases. Targeting of *PCSK9* to reduce the risk of CHD or editing of the *APOE* ε4 allele to reduce the risk of Alzheimer's disease would fit into this category; unlike with the aforementioned Mendelian disorders, these diseases have an intermediate probability of occurring in unmodified offspring. A third potential category of clinical use would be the selection of advantageous or otherwise desirable traits ("enhancement") that would not affect the lifespan of the offspring. It does not take much of a stretch of the imagination to envision the last category being forcibly applied within a population for the purpose of eugenics, which clearly would not be an acceptable use of the technology.

A survey of several hundred attendees at an American Heart Association conference assessed opinions about the clinical use of human germline genome editing (113). There was substantial support for clinical use in scenarios in which there is no other means to have a healthy biological child (61%); somewhat less support of clinical use to reduce the risk of a child having a serious medical condition such as premature CHD (45%); and almost no support of use to increase the odds of a child having a desired trait such as athletic ability (2%). These findings accord well with data from other similar surveys (114,115). Although these findings suggest that achieving consensus on appropriate uses of human germline genome editing will be difficult, what already seems to have broad support is the notion that engagement of the public is imperative in navigating the thicket of social, ethical, and legal issues. In the American Heart Association conference survey, only 19% would support clinical use if the public were not consulted, although it remains to be seen exactly how public engagement can most effectively take place.

CONCLUSIONS

Genome editing and related approaches are already transforming cardiovascular research, and they have the potential to have a similar impact on the practice of cardiovascular medicine as the future unfolds. Considering how far the field has advanced in just the 5 years since the introduction of CRISPR-Cas9 systems in early 2013, we can undoubtedly expect remarkable progress in the next 5 years.

ADDRESS FOR CORRESPONDENCE: Dr. Kiran Musunuru, Perelman School of Medicine, University of Pennsylvania, 3400 Civic Center Boulevard, Building 421, 11-104 Smilow Center for Translational Research, Philadelphia, Pennsylvania 19104. E-mail: kiranmusunuru@gmail.com.

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