Genome Editing: A New Approach to Human Therapeutics

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Abstract

The ability to manipulate the genome with precise spatial and nucleotide resolution (genome editing) has been a powerful research tool. In the past decade, the tools and expertise for using genome editing in human somatic cells and pluripotent cells have increased to such an extent that the approach is now being developed widely as a strategy to treat human disease. The fundamental process depends on creating a site-specific DNA double-strand break (DSB) in the genome and then allowing the cell's endogenous DSB repair machinery to fix the break such that precise nucleotide changes are made to the DNA sequence. With the development and discovery of several different nuclease platforms and increasing knowledge of the parameters affecting different genome editing outcomes, genome editing frequencies now reach therapeutic relevance for a wide variety of diseases. Moreover, there is a series of complementary approaches to assessing the safety and toxicity of any genome editing process, irrespective of the underlying nuclease used. Finally, the development of genome editing has raised the issue of whether it should be used to engineer the human germline. Although such an approach could clearly prevent the birth of people with devastating and destructive genetic diseases, questions remain about whether human society is morally responsible enough to use this tool.

INTRODUCTION

There have been four major approaches to curing disease through the history of medicine, but we are now at a stage at which a fifth approach—the use of precise genetic modification of cells, or genome editing—is poised to complement these other four. Perhaps the most important strategy to improving the health of humans was the recognition of the importance of clean water and sanitation in preventing the development of disease. Unfortunately, these seemingly basic public health measures still remain elusive in certain parts of the world. Almost as important as clean water and sanitation to improving the health of humankind was the development of vaccines, biologics that specifically activate the immune system to prevent disease. The elimination of smallpox from the world and the drive toward eliminating polio stand as hallmarks for the impact vaccines have had on improving global health. Once effective sterile techniques and the rudiments of anesthesia were developed in the nineteenth century, surgery became another effective method to cure disease, whereby diseased tissue was either physically repaired or surgically removed. The strategy most associated with Western medicine is the development of drugs to cure certain diseases. The transformational properties of antibiotics, for example, in this quest cannot be overstated. With increasing sophistication, drugs have moved from being small molecules to more complex molecules such as antibodies. But as we enter the modern era of genetics and genomics, these four stalwart approaches to curing disease may have reached their limits. Instead, a fifth strategy, the ability to precisely manipulate the genome of a patient's cells for therapeutic purposes (genome editing), is now ready to provide a therapeutic complement to the diagnostic power of genetics. In this review, I focus on the principles, development, and early therapeutic applications of genome editing. However, it is also becoming a transformative research tool, an application that has been well reviewed elsewhere (1).

DEVELOPMENT OF GENOME EDITING

Genome editing, previously called gene targeting, is a method to modify the nucleotide sequence of the genome with base pair specificity and thus represents true genetic precision therapy. Although recombinant adeno-associated virus (rAAV) can be used to stimulate genome editing through mechanisms that still require further study (2, 3), this review focuses on genome editing mediated by engineered nucleases. In nuclease-mediated genome editing, researchers design an engineered nuclease to create a specific DNA double-strand break (DSB) in the genome. Using the DSB, we gain entry into the relatively stable DNA molecule and hijack the cell's own DSB repair machinery to create specific genomic modifications, including single-nucleotide substitutions, deletions both small and large, and the insertion of novel DNA sequences into a precise genomic location. The development of therapeutic genome editing arose out of three different independent lines of thought. The first was the importance and power of gene targeting and the ability to precisely modify cell genomes. The key role that yeast, both *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, played in biomedical research highlights this achievement because it is relatively facile to modify genes in those organisms in a specific fashion (4, 5). Moreover, the importance of gene targeting as a research tool was formally recognized when the Nobel Prize in Physiology or Medicine was awarded in 2007 to Drs. Oliver Smithies, Mario Capecchi, and Martin Evans for their pioneering work in the genome editing of mouse embryonic stem cells, from which they derived mice carrying a defined mutation (6–10). Even before Dr. Smithies published his work on using homologous recombination (HR) to create modified mouse embryonic stem cells, he used HR to precisely modify the *HBB* gene in human somatic cells (11). The efficiency of the modification process without a DSB was too low (on the order of 10^{-6} to 10^{-7}) to be of therapeutic use, but the attempt anticipated the potential therapeutic utility.

The second was the discovery that a DSB, when introduced into a specific location in the genome of mammalian cells, can stimulate HR and gene correction of a defective gene (12–14). This stimulation can range from 100- to 50,000-fold, depending on the cell type and experimental conditions, such that up to 5% of cells in a population could have a genetically defective gene corrected (15–20). These seminal experiments were done by integrating a defective reporter gene containing the 18–base pair recognition site for the I-SceI homing endonuclease. The defective reporter gene could be corrected by the simultaneous introduction of the I-SceI endonuclease, which would create a DSB in the gene, along with a donor DNA plasmid that contained the genetic information to fix the defective reporter. When the cell repaired the defective reporter by HR using the provided donor plasmid DNA as a template, the gene would be corrected and the frequency of that process easily measured. Although they demonstrated that human somatic cells could be modified by HR at high frequencies, the limitation of these studies was that the I-SceI endonuclease could not be redesigned to recognize natural target sites in the genome. Thus, the system was a powerful method to study the process but could not be applied to modify natural sites in the human genome.

The third line of investigation was the development of a method to engineer a nuclease to recognize a new target site. Chandrasegaran and his colleagues (21, 22) first showed that by fusing a zinc finger DNA binding domain to the nuclease domain from the FokI type IIS restriction endonuclease, they could create a new enzyme that could cut DNA at a site determined by the zinc finger DNA binding domain. In collaboration with Carroll and his colleagues (23–27), they then showed that such chimeric nucleases, now known as zinc finger nucleases (ZFNs), could stimulate HR in extrachromosomal DNA in *Xenopus* oocytes and create mutations in *Drosophila melanogaster*, but only when a pair of ZFNs were made to allow the FokI nuclease domain to dimerize. Whereas I-SceI and ZFNs both created DSBs that could stimulate gene correction in reporter genes in human somatic cells (18, 28), ZFNs represented a significant breakthrough because, unlike homing endonucleases (of which I-SceI is a family member), ZFNs could be reengineered much more easily to recognize novel target sites, including natural sites within the human genome (29). Thus, designer nucleases could be made to stimulate genome editing in human somatic cells at high frequencies. In the first modification of the *IL2RG* gene, in which mutations are causative of X-linked severe combined immunodeficiency (SCID-X1) (the most common form of severe combined immunodeficiency), the frequencies of genome editing by HR were even higher than those achieved using I-SceI, the gold standard at the time (30).

NUCLEASE-MEDIATED GENOME EDITING ACTS AS A PRECISION THERAPEUTIC BY COOPTING THE ENDOGENOUS DNA DOUBLE-STRAND BREAK REPAIR MACHINERY

Nuclease-mediated genome editing can create a variety of precise genomic modifications after a specific DSB is introduced by hijacking the cell's endogenous DNA DSB repair machinery. Cells use multiple mechanisms to repair a DSB, but the two major pathways are nonhomologous end-joining (NHEJ) and HR (31–35).

In NHEJ, the break is first processed to create ends that can be ligated together. Then, using a series of steps involving the Ku70, Ku80, DNA-PKcs, DNA ligase 4, and XLF proteins, the free ends are stitched together. Generally, the ends created by an engineered nuclease break are repaired with high fidelity by NHEJ (36). But if the site is iteratively broken and repaired, NHEJ will eventually create small insertions or deletions at the site of the break. This iterative process will continue until the insertion or deletion at the site of the break destroys the ability of the engineered nuclease to either rebind or recut at the site. In genome editing, therefore, NHEJ

can create small insertions or deletions at the site of the engineered nuclease target site, thereby inactivating genetic elements such as coding regions of genes, specific regulatory elements, or other genetic elements. Overexpressing enzymes that process the ends of the breaks—TREX2, for example—such that the DSB cannot be repaired in a nonmutagenic fashion, can increase the frequency of insertions or deletions (37). When two DSBs are created in the genome, either the canonical or noncanonical NHEJ pathway can join the two DSBs, creating a translocation if the two DSBs are on separate chromosomes or a chromosomal deletion if they are on the same chromosome (38, 39).

In HR, the ends of the break are processed to create free 3′ single-stranded ends, and these free 3′ tails then invade into an undamaged DNA duplex to create a D-loop. After strand invasion, high-fidelity polymerases use the undamaged DNA as a template to synthesize new DNA, followed by resolution of the heteroduplex. In mitotic cells, the normal undamaged DNA template is the sister chromatid, and the process is thought to occur through the synthesis-dependent strand annealing mechanism, a noncrossover HR mechanism, rather than through a crossover HR mechanism (the primary mechanism in meiotic HR). In genome editing, the HR machinery, for reasons that are not understood, will use an extrachromosomal piece of DNA as its template (a donor). For high frequencies of nuclease-mediated genome editing by HR, the donor should have homology arms of greater than 400 base pairs (40). Interestingly, the frequency of HR-mediated genome editing does not seem to depend on the size of the change induced, with high frequencies of editing being possible when just a single nucleotide change is created or when an entire cassette of genes (>10 kilobases) is integrated (41, 42). This is probably related to the fact that HR uses high-fidelity, highly processive DNA polymerases to synthesize new DNA from the donor molecule. The frequency of HR-mediated genome editing can be increased by arresting cells in the G2/M phase of the cell cycle, by inhibiting sister chromatid cohesion (thus inhibiting a competing template for the HR machinery), and by other small molecules (30, 43–47). Although HR is considered a high-fidelity form of repair, in genome editing, we coopt this pathway to create defined nucleotide changes in the genome. In sum, HR-mediated genome editing proceeds by a so-called copy and paste mechanism and allows us to change the genomes of cells with high frequency and single-nucleotide precision.

The mechanism by which a cell chooses to repair a DSB by either NHEJ or HR remains a subject of active study. Both repair mechanisms are essential in human cells, but defects in HR cause cell death within one to two cell divisions, whereas defects in NHEJ require more cell divisions and may be the result of telomere dysfunction rather than DSB repair dysfunction (48, 49). Although researchers commonly assert that genome editing by NHEJ is more common than by HR, under optimal conditions, the frequency of HR-mediated genome editing can exceed that of NHEJ-mediated genome editing (40).

A DIVERSE GENOME EDITING TOOLBOX

Both NHEJ- and HR-mediated genome editing require a highly active nuclease to create the site-specific DSB. These nucleases now comprise four standard platforms (**Table 1**): meganucleases, ZFNs, transcription activator–like (TAL) effector nucleases (TALENs), and CRISPR/Cas9. Two hybrid platforms for engineered nucleases are also used, namely megaTALs and CRISPR/Cas9-Fn fusions.

Meganucleases, also known as homing endonucleases, are a large class of natural genetic parasites that catalyze their own propagation using DSB-mediated HR (50, 51). They are characterized by a long, specific DNA recognition site intertwined with catalytic nuclease activity. Investigators have used multiple approaches, including structure-based design, phage display, and yeast surface

Table 1 Summary of different nuclease platforms

Nuclease type	Year of first use in human cells	Target site length (base pairs)	Ease of reengineering	On-target activity[a]	Specificity[a]	Clinical development
Homing endonuclease or meganuclease	1994	>18	Very difficult	++	ND	Target validation
megaTAL	2013	>25	Difficult	++++	ND	Preclinical
ZFN	2003	18–36	Difficult	++++	++	Phase I/II
TALEN	2011	24–38	Easy	++++	++++	Phase I
CRISPR/Cas9	2013	22 (*Streptococcus pyogenes* Cas9)	Easy	++++	+++++	Preclinical

Abbreviations: ND, not determined; TAL, transcription activator–like; TALEN, TAL effector nuclease; ZFN, zinc finger nuclease.
[a]The plus signs denote relative activity and specificity for a first-generation nuclease of that class. With further engineering, a given nuclease usually can be improved to have better on-target activity and specificity.

display, to reengineer meganucleases to recognize novel target sites (52–56). The success of these engineered meganucleases has usually been moderate, but the coexpression of TREX2, an end-processing 3′ exonuclease, can transform a poorly active nuclease to one with high on-target activity (37). The expertise needed to reengineer a meganuclease to a therapeutically relevant target site has precluded this platform from being widely adopted, although academic groups and biotechnology companies continue to develop the system.

ZFNs are artificial proteins with two domains (22, 29). The enzymatic nuclease activity is derived from the FokI type IIS restriction endonuclease. They derive their specificity from the zinc finger DNA binding domain. These domains consist of arrays of individual zinc fingers, which are approximately 30 amino acids organized into β-β-α structure in which specific amino acids in the α helix mediate binding to a 3–4-base-pair sequence in the major groove of the DNA molecule. A full zinc finger DNA binding domain for a ZFN consists of an array of 3–6 individual zinc finger modules that create a target binding site of 9–18 base pairs. Because the FokI nuclease domain needs to dimerize to be catalytically active, a pair of ZFNs must be engineered for each target site, creating an overall binding specificity of 18–36 base pairs. The utility of ZFNs is that the zinc finger DNA binding domain can be engineered to recognize a wide variety of different sequences. This engineering can be done through various methods, including modular assembly (28, 57–59), phage-based selection (60, 61), bacterial-based selection (62–66), or a hybrid approach of bacterial selection–modular assembly–empiric changes, as first developed by Isalan et al. (67) and then adopted by Sangamo Biosciences as part of a proprietary platform (30, 68). Researchers have made active ZFNs using a variety of platforms for research purposes, but only those ZFNs made by Sangamo Biosciences have shown sufficient activity and specificity for development as a therapeutic, although a nonproprietary method of making similar quality ZFNs is also being developed in academia.

TALENs share a similar overall structure to ZFNs in that they consist of an engineered DNA binding domain fused to the FokI nuclease domain (69–73). In TALENs, however, the DNA binding domain is derived from TAL effector proteins in which 12–20 individual TAL repeats are arranged in an array whereby each repeat binds to a single base pair as determined by a repeat variable diresidue (RVD) code (74, 75). Each TAL repeat consists of a nearly identical, approximately 34-amino-acid sequence in which amino acids 12 and 13 of the repeat (the RVD)

mediate binding to a single DNA base through specific interactions. In the simple, natural RVD code, the following RVDs (listed first) correspond with a specific base (listed second): NN:G, HD:C, NI:A, and NG:T. However, a complete exploration of all possible RVDs revealed recently that alternative RVDs may provide improved specificity in certain circumstances as compared to the natural RVDs (76). TALENs are engineered by stringing together a series of TAL repeats that contain the appropriate RVDs to mediate binding to the intended target site. They can be generated by a variety of methods, including by gene synthesis, a Golden Gate cloning system (70), commercial purchase, or a high-throughput assembly mechanism that can be adapted to robotic protocols (77). Approximately one-third of engineered TALEN pairs show high activity at their intended target site.

CRISPR/Cas9 nucleases are derived from the bacterial adaptive immune system and consist of a protein component (Cas9) and an RNA component (guide RNA) (78–81). In mammalian genome editing, the two-part bacterial RNA component (crRNA and tracrRNA) is fused into a single-guide RNA (sgRNA) (78, 82, 83). Cas9 is multifunctional protein with helicase, nuclease, and sgRNA binding activity. The Cas9 nuclease activity is activated when it unwinds double-stranded DNA and the sgRNA hybridizes to its target site. Thus, in contrast to the other nuclease platforms that mediate specificity through protein-DNA interactions, the CRISPR/Cas9 system generates specificity through RNA-DNA hybridization. The current most commonly used Cas9 system for genome editing is derived from *Streptococcus pyogenes* (spCas9). A 3-base-pair NGG protospacer adjacent motif (PAM) sequence immediately adjacent to a 20-base-pair target sequence defines the target site for spCas9, giving an overall specificity of 22 base pairs. Scientists have employed Cas9 proteins from other species with different PAM sequences and directed protein evolution to evolve spCas9 to use other PAM sequences, further increasing the already tremendous flexibility of the CRISPR/Cas9 system (84–86).

In addition to these four core platforms, there are variant platforms in which (*a*) the nuclease is engineered to create a DNA nick rather than a DNA break (46, 87–90), (*b*) a TAL repeat is fused to an engineered meganuclease to increase binding affinity (mega-TAL) (91), and (*c*) the Cas9 nuclease is inactivated to create dead-Cas9 that is then fused to the FokI nuclease domain (Cas9-Fn fusions) (92, 93).

Introducing Nucleases into Target Cells

The most effective method to deliver the nucleases into primary cells or primary somatic stem cells ex vivo is as RNA or ribonucleoprotein (RNP) molecules (94–96). With this method, the duration of nuclease expression is reduced as compared to delivery through a DNA-based expression vector, thus limiting the potential for off-target effects. Moreover, by delivering the nuclease as RNA, one mitigates the activation of the deleterious interferon response—which causes both cell death and a decrease in editing frequency—that occurs when primary cells are transfected with naked DNA molecules.

The genome-editing activity of nucleases can be enhanced by exposing cells to which the nuclease has been delivered to a transient cold shock—a 16–24-h period in which cells are cultured at 30°C instead of 37°C (97). The full mechanism by which cold shock enhances editing is not clear, but it seems to be at least in part the result of increased expression of the nuclease (97).

Donor DNA Molecule

For HR-mediated genome editing, in addition to having a highly active site-specific nuclease, an engineered donor DNA fragment must also be provided to the cell as a template for the repair

of the DSB by the HR machinery. In contrast to HR-mediated editing of mouse embryonic stem cells, which requires isogenic arms of homology with one arm at least 10 kilobases in length for maximal efficiency, the arms of homology for nuclease-mediated genome editing can be as short as 400 base pairs (the frequency does decrease significantly if the homology arm length is 200 base pairs) (40). Although the need for isogenic DNA has not been formally studied, high frequencies of HR-mediated genome editing can be achieved after the induction of a DSB using arms of homology that are not isogenic. The arms of homology should be centered around the site of the break to achieve maximal efficiencies because if the DSB site is at a distance from the site of the desired modification with intervening homology sequences, the HR process will abort before the desired change is introduced, thus decreasing the efficiency of creating the intended genomic change. The defined genetic modification should be placed between the homology arms and can be as small as a single nucleotide or at least as large as 10 kilobases without a seeming drop in editing efficiency (42, 98). The efficiency of genome editing by HR is directly proportional to the amount of donor delivered into the cell (18, 40, 46).

Although the donor vector has most commonly been delivered as plasmid DNA or small polymerase chain reaction (PCR) fragments (99) via transfection or electroporation, for primary cells the donor molecule seems to be more effective if delivered via a mechanism that avoids activating an innate interferon response. This can be done by delivering the donor vector as an integration defective lentivirus or rAAV vector (95, 100–104). The donor vector can also be delivered in vivo to tissues via a hydrodynamic injection and probably through nanoparticles as well (105).

Researchers have also used single-stranded oligonucleotides as donor molecules to template precise nucleotide changes in the genome (106). The mechanism of oligonucleotide template genome editing is not understood but is clearly different than standard HR, as it does not require Rad51, an essential protein for HR. Because the mechanism of oligonucleotide modification is not known, genome editing by oligonucleotides falls under the generic class of homology-directed repair. The efficiency of genome editing using an oligonucleotide as a template can be quite high, particularly in cell lines, and investigators have used it to make precise modifications in pluripotent cells (107). One of the important considerations for any donor molecule is the frequency at which it integrates in an uncontrolled and mutagenic fashion into the genome (108).

The ultimate therapeutic use of genome editing will require combining the tools of genome editing with appropriate delivery systems and understanding the relevant cell type being modified. Thus, as genome editing becomes more streamlined and routine, its translation will depend on disease-specific expertise rather than specific expertise in editing itself.

DNA IS THE TARGET

One of the most important challenges in therapeutic development is target validation. In the case of genome editing, the universal shared target is the DNA molecule. That is, in contrast to targeting proteins as therapeutics, in which every target is different, genome editing targets the relatively homogeneous polymeric DNA molecule. Thus, within a single class of nuclease, two nucleases that target different DNA sequences will still be nearly identical to each other at the primary, secondary, tertiary, and quaternary structure levels. Although the genome is epigenetically modified, strikingly, all nuclease platforms can edit genes with high efficiency in a variety of epigenetic states, including those in which the gene is actively suppressed. Thus, in contrast to targeting proteins, no DNA target in the genome is undruggable using the powerful array of genome editing tools now available.

The challenge, therefore, is not to determine the molecular target (which is always DNA) but instead to determine what genome sequence change would be therapeutically curative. A powerful source of information for target validation is human genetics. The vast array of human genetic polymorphisms provides a rich natural data set for which genotypes are safe and which might provide clinical benefit. If a certain genetic sequence is a therapeutic endpoint, for example, then human genetics can provide target validation, and editing provides the mechanism to achieve that endpoint. That is, the existence of even a single healthy individual with a certain DNA sequence who is cured of or resistant to a certain disease provides powerful evidence that recreating that genotype in another individual would be both safe and therapeutically beneficial. This evidence is unparalleled in drug development. A simple example is that patients who have the mutation causing sickle cell disease in only one *HBB* allele do not have sickle cell disease, and thus converting the homozygous disease genotype (SS) to the heterozygous carrier genotype (SA) by genome editing would convert someone from having sickle cell disease to not having the disease. A second example is that people who contain biallelic mutations in the *CCR5* gene are near completely resistant to HIV infection but are otherwise almost completely healthy. Thus, this human genotype-phenotype relationship (which is more than simple correlation because CCR5 protein is an essential coreceptor for initial HIV infections) validates the hypothesis that using genome editing to generate biallelic mutations in *CCR5* might be a method to functionally cure someone of HIV. Multiple other examples illustrate how human genotypes and genetics provide powerful validation for curative genome editing strategies.

THERAPEUTIC GENOME EDITING USING MUTAGENIC NONHOMOLOGOUS END-JOINING

Three primary approaches use mutagenic NHEJ genome editing of cells as a potential therapeutic: (*a*) knocking out functional genetic elements by creating spatially precise insertions or deletions, (*b*) creating insertions or deletions that compensate for underlying frameshift mutations, and (*c*) creating defined genetic deletions. These are illustrated in **Figures 1** and **2**.

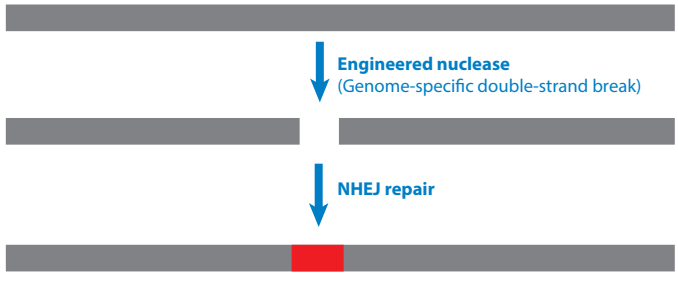

Figure 1

Therapeutic genome editing using NHEJ with a single DSB. An engineered nuclease is designed to create a specific DNA DSB in the genome. After delivery into the cell, it creates the DSB, and the cell activates the NHEJ repair pathway. Although most repair events of a nuclease-induced break are precise, after repeated cutting and repair, the NHEJ repair machinery will create insertions or deletions at the site of break (*red bar*). Deletions (1–10 base pairs) are more common than insertions (a single to hundreds of base pairs), and both can be variable in size. In this way, genetic elements, including protein-coding genes, miRNAs, and regulatory elements, can be disrupted. In addition, insertions and deletions can lead to frameshifts that can restore the reading frame of other frameshift mutations. Abbreviations: DSB, double-strand break; miRNA, microRNA; NHEJ, nonhomologous end-joining.

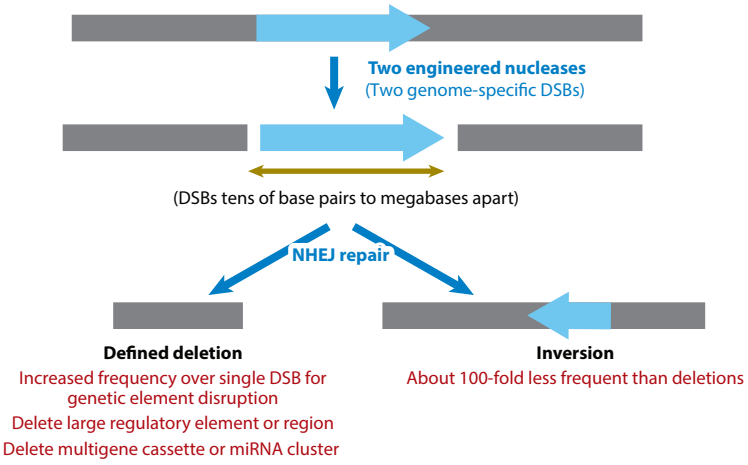

Figure 2

Therapeutic genome editing using NHEJ of two simultaneous DSBs. Two engineered nucleases are designed to create breaks at two different sites on the same chromosome arm. The binding sites should be nonoverlapping to allow simultaneous creation of DSBs but can be megabases apart. The concurrent DSBs can be repaired by NHEJ to create a deletion (the intervening genetic element is removed) or an inversion (the intervening genetic element is reversed in orientation). Deletions are much more common than inversions. Deletions are efficient mechanisms to inactivate genetic elements (as in **Figure 1**) but are also a method to remove large DNA regions. Abbreviations: DSB, double-strand break; miRNA, microRNA; NHEJ, nonhomologous end-joining.

Gene Knockout

Highly active engineered nucleases can generate insertions or deletions at their target site in >90% of alleles under optimal conditions (1, 94). When insertions or deletions are created in the coding region of a gene, most, if not all, will create a gene knockout. In contrast to small hairpin RNA– or small interfering RNA–mediated gene knockdown, biallelic genome editing causes complete knockout of the gene rather than simply a decrease, and the effect is heritable and permanent. The most advanced application of this approach is the generation of HIV resistance by knocking out the *CCR5* gene (68, 109, 110). Human genetics validates knocking out *CCR5* as a target because there are healthy people with biallelic mutations in the *CCR5* gene who are consequently resistant to HIV infection because CCR5 protein is an essential coreceptor of HIV entry into cells, particularly during a primary infection. Moreover, a single HIV patient (the Berlin patient) whose hematopoietic system was replaced by a *CCR5* mutated hematopoietic system following allogeneic hematopoietic stem cell transplantation (allo-HSCT) is the only case in which a person seems to have been cured of HIV (111). The general strategy is to use engineered nucleases to create biallelic mutations in the *CCR5* gene in autologous cells and thereby engineer resistance to HIV. Researchers have engineered highly active ZFNs, TALENs, CRISPR/Cas9, and megaTALs to target *CCR5* and create high frequencies of insertions or deletions in the gene (68, 72, 91, 94, 109, 112–114). The strategy has advanced to clinical trials for ZFNs in which HIV-infected patient T cells are treated ex vivo with ZFNs to knock out CCR5 and then reinfused back into the patient (110). The early results of this first-in-human use of genome editing demonstrated that the modified T cells persisted and may have had a selective advantage over unmodified cells and that there were no adverse events, the primary endpoint of this Phase I trial. One striking aspect of this

clinical trial is that it took less than five years to go from the initial proof-of-concept laboratory experiment to a Phase I clinical trial. In the future, as the development and regulatory pipeline for genome editing becomes more established, the time from concept to clinical trial may become even shorter. Researchers have also used nucleases to create T cells such that they would not cause an allogeneic graft-versus-host disease reaction by knocking out the T cell receptor (115) and by simultaneously knocking out the T cell receptor and engineering the cell to kill leukemia cells (116, 117).

In addition to ex vivo genome editing of hematopoietic stem and progenitor cells or T cells, investigators have performed exciting proof-of-concept studies in vivo in mice. One example is the use of CRISPR/Cas9 to create biallelic mutations in the *PCSK9* gene (118). Human genetics demonstrates that people with single allele mutations have lower low-density lipoprotein (LDL) cholesterol levels and that rare individuals with biallelic *PCSK9* mutations have even lower LDL cholesterol levels and yet are completely healthy (119–121). Ding et al. (118) used the CRISPR/Cas9 system to create biallelic modifications in the mouse liver *PCSK9* gene, resulting in lower cholesterol levels in mice.

Scientists have also developed the strategy of mutating genes through engineered nucleases as a way to treat viral infections by targeting viral genomes directly (122–125). The concept is that by mutating essential viral genes, an effective antiviral therapeutic can be developed. Multiple significant challenges hinder the success of this approach, including the risk that the virus genome will evolve to escape the nuclease and the problem of delivering the nuclease safely and effectively in vivo to the cell types that are either infected with the virus or susceptible to viral infection.

An important challenge in the in vivo use of nucleases for genome editing (whether it be for gene knockout, targeted gene insertion, or the treatment of viral infections) is avoiding an immune response to the fundamentally foreign nuclease protein. In the few in vivo mouse studies performed thus far, researchers have not evaluated carefully the potential immune response to the nuclease. Moreover, the gene therapy field has learned that the immune response in mice and even larger mammals such as dogs often underestimates the pathologic immune response to in vivo gene therapy protocols in humans. The exact solution to this problem remains to be determined but probably lies in part in making sure the nuclease is not expressed for a prolonged period of time.

Insertions and Deletions to Restore Reading Frame

Frameshift mutations are one cause of different genetic diseases and an important cause of mutations in the *dystrophin* gene that cause Duchenne muscular dystrophy (DMD). DMD is an example of how large-scale insertion or deletion events cause a devastating human disorder. It is an X-linked recessive disease defined by frameshift errors in the *dystrophin* gene, a 2.6-million-base-pair DNA sequence arranged in 79 exons. About 60% of the mutations are characterized by large insertions or deletions that abrogate the function of dystrophin protein, destabilizing the dystrophin-associated protein complex. By age 12, male patients lose their ability to walk, and they die in their early 20s owing to a failure of the diaphragm and cardiac muscle to promote respiration. Although there are multiple approaches to treating this disease, no treatments yet modify the disease course, much less cure DMD.

Gersbach and his colleagues (126) engineered nucleases to create insertions or deletions prior to a DMD frameshift mutation. One-third of these insertions or deletions would compensate for the pathologic mutation, leading to restoration of the correct reading frame in the downstream coding region and functionally restoring dystrophin expression.

Creating Defined Deletions

If one creates two DSBs on the same chromosome using engineered nucleases, tone can create a defined deletion between the two breaks (39). These breaks can be as close as a few base pairs to each other or as far as a megabase apart. Although the deletion frequency generally decreases as the deletion size increases, the size of the deletion is not the only determinant of frequency, as larger deletions can sometimes be induced with higher frequency than smaller deletions.

Investigators have used the ability to create defined deletions for two different purposes. The first is to increase the frequency of knocking out a gene. When a single nuclease is used, the inactivation of the gene depends on the iterative cutting and repair of the site until a small insertion or deletion occurs. When two DSBs are made simultaneously, however, the frequency of gene inactivation is increased because a defined deletion between the two breaks is created (113). The second is to use two DSBs to remove a large segment of the genome. This has been used to delete (*a*) a pathologic exon or set of exons from a gene (127, 128); (*b*) repeat expansions such as the dominant triplet repeats that cause Huntington's disease, myotonic dystrophy, or Friedreich's ataxia (129); and (*c*) a specific regulatory region that then alters the expression of the therapeutic protein either directly or indirectly (130).

A rare but measurable result of creating two DSBs is that the intervening DNA segment can be released and then reinserted in the opposite orientation, thereby causing an inversion (131). This not only would inactivate a gene but has also been proposed as a therapeutic strategy to treat human genetic diseases that are caused by chromosomal inversion, particularly the inversion within the *Factor VIII* gene that is the most common cause of hemophilia A (132). The frequency of inversions, however, is approximately 100-fold lower than that of deletions, and this low frequency is a barrier to easy translation.

THERAPEUTIC GENOME EDITING USING HOMOLOGOUS RECOMBINATION

Although several different therapeutic applications use editing by NHEJ, the broadest applications of therapeutic editing will harness genome editing by HR. Other creative and innovative therapeutic applications to HR-mediated genome editing are likely to be developed in the future, but here I focus on four major types (**Figure 3**): gene correction, functional gene correction, safe harbor gene addition, and targeted transgene addition.

Gene Correction

Thousands of genetic diseases are caused by point mutations in single genes. In fact, recent whole-genome sequencing efforts suggest that such diseases may be more common than previously recognized (133, 134). A potentially elegant approach to curing these genetic disorders is to use HR-mediated genome editing to correct disease-causing mutations directly. In this strategy, researchers design engineered nucleases to make a DSB close to the site of the mutation (<30 base pairs); the nuclease, along with the donor fragment that contains the wild-type gene sequence, is then delivered into the therapeutic cell of interest either ex vivo or in vivo. After HR occurs using the donor fragment, the underlying genetic mutation is corrected (18, 30, 135).

For genetic diseases of blood cells, the therapeutic efficacy of a gene correction strategy is validated by the curative properties of allo-HSCT. The patient's entire hematopoietic system is replaced with a functional hematopoietic system from someone who does not have the genetic disease, with the result that the patient is cured. This approach has been appropriately called

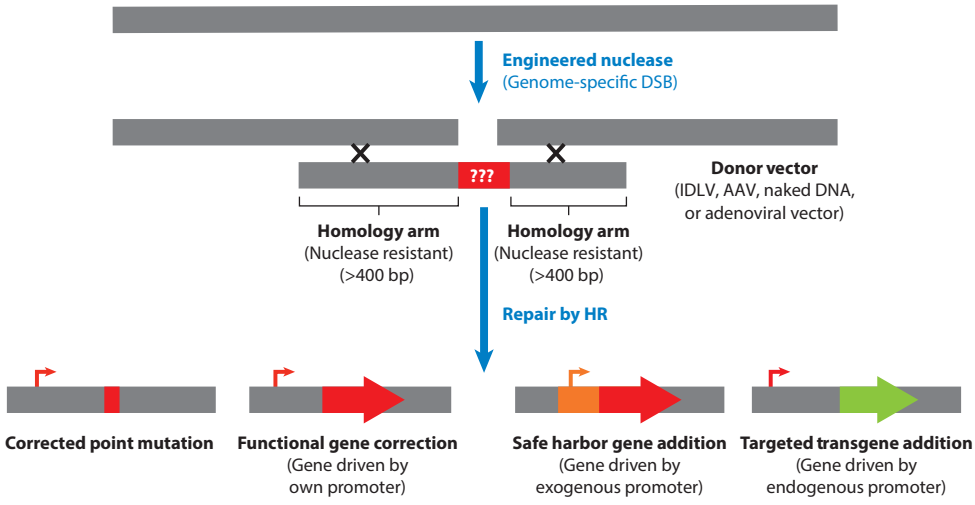

Figure 3

Therapeutic genome editing using HR. An engineered nuclease is designed to create a specific DNA DSB in the genome. The nuclease is delivered into the cell along with a donor vector that contains arms of homology that flank a new DNA sequence (*red box with question marks*). After homologous recombination, the cell will integrate the new DNA sequence precisely at the site of the break, which can lead to the correction of a point mutation, functional gene correction, safe harbor gene addition, or targeted transgene addition. Abbreviations: AAV, adeno-associated virus; DSB, double-strand break; HR, homologous recombination; IDLV, integration defective lentivirus.

allogeneic gene therapy because allogeneic cells correct the underlying genetic mutation (136). An alternative approach to using genetically correct cells from someone else would be to cure the disease by genetically correcting the patient's own cells using genome editing. Given the toxicity of allo-HSCT and the difficulty in finding appropriate immunologically matched donors, the use of genome editing of autologous cells would be substantially better. There are now multiple examples of scientists employing nuclease-mediated genome editing to correct disease-causing mutations using various different nuclease platforms (see 105, 137–144).

Functional Gene Correction

Unlike sickle cell disease and Hutchinson-Gilford progeria, in which all patients have the same mutation, most genetic diseases are caused by mutations scattered throughout the gene. An alternative to designing unique nucleases and donor vectors to uniquely correct each mutation is to design a single set of reagents that would functionally correct all the mutations in a single gene. In this strategy, a wild-type cDNA is targeted by HR to integrate at the endogenous initiation start codon (98). In this way, the cDNA is regulated by all the gene's natural regulatory mechanisms such that it is expressed in the correct cell at the appropriate developmental stage and at an endogenous level. By targeting the cDNA to the endogenous start codon, all downstream disease-causing mutations would be functionally corrected, and only mutations that deleted the target site or promoter mutations would not be corrected. Subtle variations on this process, including modifying the codon usage of the cDNA and including an intron to increase expression, might be needed to replicate the endogenous gene expression level exactly. In most cases, however, the

cDNA alone will likely be sufficient to functionally rescue the phenotype of the defective gene. Highly active editing reagents could conceivably be developed to correct every gene in the human genome once the general strategy is streamlined and validated. This approach has been described for the *HBB* gene, mutations in which cause β-thalassemia and sickle cell disease (98), and the *IL2RG* gene, mutations in which cause SCID-X1 (95).

Safe Harbor Gene Addition

When precise regulation is not required or when supraphysiologic levels of a therapeutic transgene are desired, HR can be used to integrate a transgene expression cassette (containing both a promoter and a gene cassette) into a specific location in the genome (42, 145–147). Two important factors should be considered when choosing such a genomic site: (*a*) position effects, such that the integration event will not disrupt or interfere with the function of neighboring genes, a site also known as a safe harbor; and (*b*) the newly integrated gene will not be subjected to silencing—the regulatory elements around that genomic site will promote the sustained expression of the transgene. By targeting a transgene cassette to a specific location, one avoids the problem of insertional mutagenesis that is an inevitable part of integrating transgenes into genomes using retroviral or lentiviral vectors. Proposed physical criteria for defining a safe harbor include such factors as it not being in the coding region of a gene or lying a certain distance away from known oncogenes (148). Although these are reasonable screening criteria, as researchers become increasingly knowledgeable about the genome, it is apparent a myriad of functional genetic elements (e.g., microRNA clusters, long noncoding RNAs, enhancer regions, elements yet to be discovered) throughout the genome cannot be fully captured by a list of physical criteria based on our current knowledge of protein-coding genes. Instead, the ultimate proof that a specific genomic location is safe will be determined by functional criteria: Does the integration of the expression cassette disrupt the behavior of the cell in an unintended fashion?

Although people have proposed a site within an intron of the *PPP1R12C* gene as a safe harbor (the *AAVS1* site) (146, 149), integration into this site can disrupt the expression of the protein phosphatase encoded by this gene, and careful functional characterization of long-term cellular behavior has not been carried out to validate that integrations into this site would not perturb cellular function. Nonetheless, researchers have used this site to integrate a variety of transgene expression cassettes as a potential strategy to express therapeutic proteins to treat α-thalassemia (150) and chronic granulomatous disease (151, 152).

Investigators have also used the *CCR5* gene as a safe harbor site for transgene insertion (147). Because there are healthy people with one or two alleles of the *CCR5* gene disrupted, the use of this location as a safe harbor has foundations in human genetics. This gene has been used as a safe harbor to express a variety of different transgenes, including genes that confer resistance to HIV infection (41) and as a locus to overexpress secreted therapeutic proteins such as erythropoietin (153).

The concept of using a safe harbor to express a therapeutic gene has also been shown in mice. For example, researchers engineered fibroblasts to secrete high levels of platelet-derived growth factor-β (PDGF-β). Using genome editing, a PDGF-β expression cassette was inserted into the ROSA26 locus, and the engineered cells enhanced wound healing (154).

The development of TALENs and the CRISPR/Cas9 system and the ease with which scientists can engineer active and specific nucleases to a wide variety of genomic sites presents a still unrecognized opportunity to more fully explore different safe harbors, each of which might have specific characteristics making it advantageous for specific clinical applications. That is, we can

almost certainly find better safe harbors than *CCR5* or *AAVS1* with more study. In addition to the specificity of genome editing compared to lentiviral integration, safe harbor gene addition can be distinguished from lentiviral integration because the prior can accommodate larger payloads. In the integration of anti-HIV genes into the *CCR5* gene, for example, the gene cassette was >10 kb in length and contained four different genes driven by two different promoters (41). This capacity for larger payloads with multigene cassettes facilitates the use of genome editing for synthetic biology. The larger payload also permits the simultaneous introduction, along with the therapeutic gene or gene cassette, of (*a*) selection markers to enrich for modified cells (98, 155, 156) and/or (*b*) safety switches such that modified cells could be eliminated if they started to cause pathology or after they had served their intended therapeutic purpose, such as in wound healing (157–160).

Transgene Expression Using Endogenous Regulatory Elements

In the safe harbor gene addition strategy, the transgene is expressed using an exogenous promoter. A more sophisticated approach is to knock a transgene into a specific site such that it is expressed using endogenous regulatory elements. In this approach, the therapeutic transgene expression is governed specifically by the natural regulatory elements of the locus the transgene is knocked into; it is thus expressed at the same level and developmental stage and in the same cell type as the endogenous gene. For example, knocking in *Factor IX* gene behind a strong liver-specific promoter in mice rescued a clotting defect in the mice by achieving high levels of circulating expression of the desired transgene without having to achieve high frequencies of genome editing (3, 161, 162). Researchers are developing a similar approach to knock lysosomal storage disease enzymes in vivo into the albumin locus to rescue deficiencies in these enzymes. Although the albumin locus was chosen because it is such a strong promoter, one can also target transgenes to loci such that they will be expressed only in a lineage-specific manner, at different levels, or in response to specific developmental or environmental signals (98).

The ability to modify the genome precisely by HR also opens up the possibility of engineering human cells for therapeutic purposes using the principles of synthetic biology. In this case, entire cellular pathways are reorganized to confer on the cell-specific functions that can be tuned precisely by either exogenous or endogenous stimuli.

THERAPEUTIC EDITING OF PLURIPOTENT CELLS

An alternative to using somatic cells is genome editing of autologous induced pluripotent cells (iPS cells) as a source of genetically corrected cells for transplantation (163). The potential advantage of using this strategy is that single edited iPS clones can be identified and sequenced fully to identify clones that have no off-target effects. Furthermore, because iPS cells have tremendous replicative capacity and can be induced to differentiate into essentially any cell in the body, they would be a source for many corrected cells to be used for curative intent that are not otherwise available from the patient. Although editing of human iPS cells can be done without engineered nucleases, the efficiency of the process and thus the ability to generate many corrected clones to characterize increases dramatically by using engineered nucleases or rAAV (164). The challenge of the general approach is to generate large, clinical-scale numbers of the highly purified, transplantable, and differentiated cell type desired. Currently, for example, cells derived from iPS cells have an embryonic phenotype rather an adult phenotype and thus may not be of the quality necessary to rescue postnatal phenotypes. However, a mouse model of sickle cell disease has shown important proof of concept of this strategy (165).

LEVELS OF EDITING EFFICIENCIES NEEDED TO ACHIEVE THERAPEUTIC BENEFIT

The potential therapeutic effectiveness of genome editing depends on both the potency of the edited cells and the underlying disease pathology. For certain genetic diseases, SCID-X1 being an excellent example, human experiments of natural spontaneous reversion mutations have demonstrated that only a small number of stem or precursor cells, perhaps as few as one, need to be corrected (166). This small number can be explained by the selective advantage that corrected cells have over uncorrected cells. For other genetic diseases, such as sickle cell disease and β-thalassemia, the selective advantage is not as great but is still present, thus suggesting that correction efficiencies of stem cells in the 2–10% range should be sufficient to cure the disease. Moreover, genome editing can be used to increase the potency of modified cells. For example, the correction of certain metabolic or clotting diseases requires a specific level of protein in the circulation. Direct correction of the endogenous gene would require the generation of a high frequency of corrected cells to achieve a therapeutically appropriate level. However, by engineering cells by genome editing to secrete supratherapeutic amounts of protein, each cell becomes more potent, and thus fewer cells need to be modified to have a clinical benefit. In the end, therefore, no one frequency of editing is needed. Instead, there is a close interaction between the underlying disease pathology, the editing event engineered, and the modified cell type. In certain circumstances, this means that a seemingly low frequency would be effective, and for several indications, these frequencies are already being achieved.

ASSESSING SPECIFICITY, TOXICOLOGY, AND SAFETY OF GENOME EDITING

The precision of genome editing not only gives the potential to generate a more homogeneous population of genetically modified cells with better and more consistent potency over other methods of genome engineering, but the same precision should also result in a greater degree of safety. However, DSBs are a known method of creating genetic instability and cancer (167, 168), and thus nuclease-mediated editing, as with all therapeutic modalities, must be evaluated formally for safety. The challenge is that no single assay or suite of assays has been demonstrated to accurately predict the safety of using genetically modified cells for therapeutic purposes (169, 170).

With the discovery that engineered nucleases could stimulate HR in human somatic cells came the recognition that these nucleases had toxicity from cutting DNA at off-target sites (18). That is, although the engineered nuclease was designed to recognize a single genomic target, it could also create DSBs at unintended off-target sites. Here I discuss some of the approaches to evaluating specificity and minimizing off-target cutting.

There are five current approaches to assessing the specificity of a given engineered nuclease (**Table 2**). The first involves using bioinformatics prediction algorithms to identify potential off-target sites, followed by next-generation sequencing to assess the frequency of insertions or deletions at those sites (171, 172). This approach can identify a fraction of bona fide off-target sites and is a useful first step in assessing specificity. As the algorithms improve as more data on the nature of off-target sites are gathered, this approach will become even better. The second involves using in vitro assays to evaluate which target sites the engineered nucleases might bind to and then using this to identify potential off-target sites in cells (68, 173–176). This approach is also able to identify a subset of off-target sites but is limited because the conditions used to identify sites in vitro are not the same as those in cells. The third approach is to use unbiased DSB capture-based assays in cells. Since the first work with I-SceI, researchers have discovered that random fragments of DNA could be integrated in a nonhomologous fashion into the sites of

Table 2 Nuclease specificity assessment

Assay	Basic description
Off-target site evaluation	
Bioinformatics	Use bioinformatics to identify potential off-target sites and then experimentally measure the frequency of insertions or deletions at those predicted sites.
In vitro to in vivo	Use in vitro assays to experimentally measure binding to potential off-target sites, use that data to make prediction about potential genomic off-target sites, and then experimentally measure the frequency of insertions or deletions at those predicted sites.
Double-strand break capture assay	Use a plasmid DNA molecule, AAV vector, IDLV vector, or oligonucleotide to capture the off-target breaks that an engineered nuclease might create in a genome-wide unbiased fashion.
Gross chromosomal rearrangement assay	Use LAM-PCR and next-generation sequencing to measure the frequency at which gross chromosomal rearrangements occur after the expression of a nuclease.
γ-H2AX or 53BP1 foci formation	Use microscopy or flow cytometry to quantify the double-strand breaks a nuclease creates in a cell without having to determine where those breaks are occurring.
Functional toxicity assays	
Cell cycle analysis	Use cell cycle assays to determine if nuclease expression perturbs cell cycle progression.
Cell proliferation or survival	Use quantitative measures of cell survival or proliferation to determine if nuclease expression affects these processes.
Clonogenicity assay	Use barcode marking to determine if nuclease expression alters the clonal makeup of a large population of cells.
Lineage reconstitution	Analyze whether modified cells, after transplantation into an immunodeficient mouse, are able to reconstitute appropriate cell types without cell transformation.

Abbreviations: AAV, adeno-associated virus; IDLV, integration defective lentivirus; LAM-PCR, linear amplification–mediated polymerase chain reaction.

breaks, and this phenomenon has been observed with all nuclease platforms (40, 177, 178). The capture of random DNA fragments into DSBs is an aberrant and mutagenic form of repair and demonstrates the lengths a cell will go to to repair a DSB. Thus, several different groups have applied this aspect of biology and bioinformatics as a method to identify off-target sites by capturing DSBs with nonintegrating viral vectors or oligonucleotides (179–181). The use of oligonucleotides seems to be a particularly good method because large molar amounts of oligonucleotides can be delivered into cells, increasing the probability that an off-target break will be captured by the oligonucleotide before it is repaired by some other mechanism. The need to deliver large molar amounts of oligonucleotides into cells may limit this approach's application in primary stem cells, as stem cells may not tolerate such a challenge. The fourth approach identifies the frequencies and types of genome rearrangements an engineered nuclease might make in an unbiased fashion (182, 183). Because genome rearrangements would be the most concerning type of engineered nuclease mutation, this method has tremendous importance in assessments of the safety and specificity of engineered nuclease–mediated genome editing. Finally, the fifth method involves staining for DSBs in a cell without overtly identifying where these breaks are occurring (62, 184). Using this method, an unbiased assessment of the number of DSBs an engineered nuclease is creating in a cell is measured. This enables a quantitative comparison among different nucleases or different formulations of the same nuclease. This method can be made more sensitive by using cell types that are defective in DSB repair (185).

A hallmark of genome editing is its hit-and-run nature, in that the editing process is transient but ends up leaving a permanent genetic mark. Thus, sustained expression of the nuclease is not necessary. Studies have shown that editing by HR is essentially complete within 24 h after

delivering the nuclease (185). Moreover, sustained expression is not desirable because the longer a nuclease is expressed, the greater chance it has to create a DSB at an off-target site. Thus, researchers have shown that minimizing the half-life of the nuclease by regulating its expression through small molecules or by delivering the nuclease as RNA or protein rather than as a DNA expression vector all minimize the off-target activity of a given nuclease (94, 185). In addition to minimizing the duration of expression, using a modified FokI nuclease domain that has been engineered to not homodimerize can also minimize off-target effects for ZFNs and TALENs (62, 184, 186, 187). Although the use of the obligate heterodimer form of the FokI nuclease is an effective method for reducing activity at off-target sites, it also comes at a cost of reducing activity at the desired target site. Thus, an assessment must be made for each situation as to whether the benefit of an improved on- to off-target ratio exceeds the potential loss of on-target editing activity.

When evaluating the specificity of nuclease-mediated genome editing, researchers must put the frequency of off-target modification into the context of the normal genetic variability among humans and the ongoing genetic instability of all cells. Recent whole-genome sequencing efforts have demonstrated that humans are born with tremendous genetic variability, with each individual containing millions of single nucleotide variants and approximately 500,000 insertions and deletions, many of which lie in exons and create frameshift mutations (188). In addition, investigators predict that stem cells spontaneously acquire about three mutations per cell division (189, 190), which is in part the result of every cell having to repair 20–40 spontaneous DSBs and hundreds to thousands of other forms of DNA damage per division. Although this ongoing and continuous mutation frequency is an important contributor to 50% of humans eventually developing cancer, it highlights the tremendous ongoing natural genetic mutation frequency. Researchers have yet to determine if engineered nucleases, which almost always create small insertions or deletions in noncoding regions of the genome, contribute significantly to this mutational burden or if they are merely a rounding error in what cells normally encounter.

Whereas measurements of specificity are critical in the development of a therapeutic genome-editing process, the ultimate assessment of toxicology should be no different than that of any other therapeutic intervention: Does it have a deleterious effect? That is, the safety assessment of a kinase inhibitor, for example, is not dependent on identifying the degree to which it inhibits all other kinases, although that is important and interesting information, but instead on how the drug affects cellular behavior and organismal phenotypes. Thus, functional toxicity is a critical aspect of assessing the safety of a genome-editing process (169). When assessing the toxicology of a therapeutic editing process, scientists must distinguish between toxicity that transiently cripples cell behavior but has no long-lasting consequences, toxicity that permanently cripples a cell such that it will be less potent in its activity compared to other cells, and toxicity that creates a cell that seems normal initially but is predisposed to transform into a malignant cell over time. Four major assays measure the functional effects of genome editing (**Table 2**); they assess the effect on (*a*) cell cycle dynamics (191); (*b*) short-term cell survival proliferation (62, 185); (*c*) clonal dynamics in large, heterogeneous populations (192); and (*d*) long-term cell behavior such as multilineage reconstitution for hematopoietic stem and progenitor cells or T cell persistence without leukemia development after transplantation into immunodeficient mice (169, 193).

THERAPEUTIC GENOME EDITING THAT WOULD RESULT IN GERMLINE MODIFICATION

One of the most powerful research uses of nuclease-mediated genome editing is its ability to create genetically modified organisms by zygote injection (1). Researchers have applied this technology

in a variety of organisms using ZFNs, but with the ease of engineering using the CRISPR/Cas9, multiple companies and academic core facilities have adopted it to create new animal models with precise genetic alterations. This broad use, combined with experiments demonstrating that engineered nucleases can alter the genetic content of human tripronuclear zygotes and human mitochondria in zygotes, has engendered discussion of whether genome editing should be used in a setting in which it would change the genetic content of future generations (194–199). Although curing disease using genome editing of somatic cells is ethically uncontroversial, multiple genetic diseases may be impossible to completely cure using somatic cell genome editing. Genome editing of germ cells or zygotes provides a theoretical way to correct disease-causing mutations, thus preventing a newborn from ever being born with the disease.

The inefficiency and unpredictability of direct zygote modification, along with its inability to assess off-target effects fully, mean that this method is unlikely to be reproducibly effective. Other approaches—such as editing spermatogonial stem cells, the precursor stem cells to sperm—however, might be efficient, be predictable, and allow one to assess fully for off-target effects (200). The question is whether this should be done. One's ultimate view on this question will depend on one's ethical framework. Society will come to the best decision when people listen to a variety of diverse viewpoints, including those from scientists, doctors, patient advocacy groups, lawyers, ethicists, religious leaders, politicians, and so on.

The issue of therapeutic genome editing that might result in germline modification with the goal of preventing someone from being born with a genetic disease also needs to be put in the context of methods that people already use for the same purpose. These methods include the prohibition of marriage between two people who would have a 25% chance of passing along an autosomal recessive genetic disease to their child; the use of preimplantation genetic diagnosis to implant only embryos that will not have a genetic disease, eliminating embryos that would have the disease; and the selective termination of pregnancies once the fetus is determined to have a genetic disease. Each of these approaches results in the prevention of genetic disease but also in the selection of one genetic background over another in future generations. The question is whether proactively editing genomes to prevent the birth of children with genetic diseases is any more ethically troublesome than reactively selecting for embryos that will not have the genetic disease and eliminating those that will. In addition, our progressive ability to bring people into adulthood who in the past would have died in childhood from their genetic disease and who can have their own children (an unequivocally good thing!) does result in a subtle skewing of the genetic makeup of future generations by keeping the allele frequency for the mutation higher in the population. Thus, the humane and patient-centric medical care that we can rightly now provide is subtly altering the genetic makeup of future generations already. Thus, if genome editing could be shown to correct only the disease-causing mutation with no other genetic changes, is it ethically troublesome to try to eliminate that mutation from the population? This is a particularly important point for those mutations for which there is no benefit and which cause tremendous burdens to individuals, families, and societies and have been passed down through generations in certain unlucky families.

Countervailing these points is the belief that proactively correcting disease-causing mutations crosses an ethical threshold that is different than the passive selection of genetically corrected embryos. Moreover, the malignant history of eugenics in our past, and even the prevailing subtle suggestions in our culture today that some genetic makeups are better than others, suggest that we are not morally ready to take on the task of using therapeutic editing of the germline responsibly and that, by opening up this possibility, we risk creating a dystopian future. This concern should not be readily dismissed.

As a pediatrician, I am aware that the power of prevention over treatment is inculcated into our practice. Thus, the ability to use this approach to prevent devastating genetic diseases is tremendously appealing. And whereas I feel from a practical perspective that therapeutic germline genome editing is not in the near future, I also see the subtle and not so subtle ways that people are discriminated against; creating one more mode for discrimination is troubling. Although we as scientists and doctors have a role to play in the discussion as experts in the field, our expertise is not definitive, and the ultimate decision is not solely ours to make. Instead, it is one that society should make after hearing from a diverse variety of viewpoints and perspectives.

SUMMARY

For decades, researchers have recognized the ability to precisely manipulate the human genome with nucleotide precision (genome editing) as a strategy to treat or even cure human disease. Initially, the frequency of genome editing of human cells was too low to be of potential therapeutic use. But with the explosion of genome editing tools, including a diverse array of nuclease platforms and different methods to deliver a donor vector into cells, the frequency of genome editing has now increased to therapeutic levels in multiple preclinical cell types. Moreover, the first Phase I clinical trials using nuclease-based genome editing have shown no serious adverse events. Thus, we are potentially at the dawn of a new era in human therapeutics in which we can rewrite the genome precisely as a method to prevent, cure, or ameliorate human disease. The coming years will be exciting as the creativity and ambition of scientists and biomedical researchers are married to a toolkit that allows them to carry out efficiently what once was only a figment of people's imagination.

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I am a consultant for CRISPR Tx and have equity interest in the company that is managed in accordance with the conflicts of interest policies of Stanford University. The company had no input into any of the content in this review.

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