Gene Editing on Center Stage

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Smithies et al. (1985) and Jasin and colleagues (1994) provided proof of concept that homologous recombination (HR) could be applied to the treatment of human disease and that its efficiency could be improved by the induction of double-strand breaks (DSBs). A key advance was the discovery of engineered nucleases, such as zinc-finger nucleases (ZFNs) and transcription activator-like (TAL) effector nucleases (TALENs), that can generate site-specific DSBs. The democratization and widespread use of genome editing was enabled by the discovery of the clustered regularly interspaced short palindromic repeats (CRISPR)–Cas9 nuclease system. While genome editing using ZFNs and TALENs has already reached clinical trials, the pace at which genome editing enters human trials is bound to accelerate in the next several years with multiple promising preclinical studies heralding cures for monogenic diseases that are currently difficult to manage or even incurable. Here we review recent advances and current limitations and discuss the path forward using genome editing to understand, treat, and cure genetic diseases.

Setting the Stage: A Brief History of Genome Editing
A little more than 20 years ago, the concept of genome editing (at that point termed gene targeting) came to a new era with the discovery that nucleases could be engineered to create site-specific DNA DSBs that could stimulate homologous recombination (HR; see Glossary) more than 1000-fold [1–3]. Since then, gene editing has transitioned from a niche research technique to a ubiquitous genetic research tool in biomedical science. Genome editing is a process that exploits the natural cellular pathways that repair DNA breaks. DNA breaks can be introduced site specifically in the genome by means of various nuclease platforms. The early platforms employ rational protein engineering of homing endonucleases or DNA-binding proteins, such as zinc fingers or TAL effectors, to confer specificity to a desired DNA sequence. Zinc fingers and TAL effectors are fused to the endonucleolytic DNA cleavage domain of the Fok1 endonuclease to make ZFNs or TALENs. Since the endonucleolytic domain of Fok1 acts as a dimer, a pair of ZFNs or TALENs must be designed to target adjacent sites in the genome to bring the two Fok1 domains together for catalytic activity [1,4–7]. One of the most recent nuclease platforms, based on the bacterial adaptive CRISPR–Cas9 immune system, relies on a 100-nucleotide (nt) single guide RNA (sgRNA) that directs the Cas9 endonuclease [the Cas9–gRNA ribonucleoprotein (RNP) complex] to a specific site in the genome where hybridization of the sgRNA to the target DNA activates Cas9 to cleave the chromosomal target [8–11]. The speed, simplicity, and low cost of sgRNA design and engineering as well as the high activity and specificity of Cas9 has rendered this the preferred nuclease platform and has dramatically expanded the use of genome editing by enabling studies of precise genetic changes and preclinical gene correction therapies. Table 1 gives a brief timeline of some key milestones in the development of therapeutic genome editing.

Highlights
Gene editing is a technique that enables precise changes to be made in the human genome and has with remarkable pace revolutionized genetic research.

The Cas9–sgRNA gene editing platform derived from the bacterial adaptive CRISPR immune system has recently been propelled onto the center stage of gene editing and with its ease of use has become a key methodology in many research laboratories worldwide.

The major hurdles for efficient gene editing in therapeutically relevant primary human cells have largely been overcome as shown in numerous recent preclinical studies and multiple clinical trials are expected to start within the next couple of years.
Table 1. A Lists of Many of the Key Publications in the Development of Therapeutic Gene Editing in Humans*

<table>
<thead>
<tr>
<th>Year</th>
<th>Event</th>
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<tr>
<td>1986</td>
<td>Nuclease-free gene targeting in mouse ES cells</td>
<td>[80]</td>
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<td>1989</td>
<td>TAL effectors are discovered</td>
<td>[81]</td>
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<td>1993</td>
<td>CRISPR discovery begins in bacteria</td>
<td>[82]</td>
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<tr>
<td>1994</td>
<td>Gene targeting can be stimulated by DSBs</td>
<td>[83]</td>
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<td>1996</td>
<td>ZFNs are developed</td>
<td>[84]</td>
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<td>2002</td>
<td>Nuclease-free gene targeting is more efficient using AAV donors</td>
<td>[85]</td>
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<td>2003</td>
<td>Demonstration of an engineered nuclease creating site-specific mutations in a genome</td>
<td>[4]</td>
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<td>2003</td>
<td>ZFN-mediated gene targeting in human cell lines</td>
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<td>2005</td>
<td>ZFN-mediated gene targeting in human primary cells targeting an endogenous gene</td>
<td>[86]</td>
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<td>2007</td>
<td>CRISPR is a bacterial adaptive immune system</td>
<td>[87]</td>
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<td>2009</td>
<td>The DNA-binding code of TAL effectors is deciphered</td>
<td>[88, 89]</td>
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<td>2010</td>
<td>TALENs are developed</td>
<td>[90, 91]</td>
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<td>2011</td>
<td>TALEN-mediated gene editing in human cell lines</td>
<td>[6]</td>
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<td>2012</td>
<td>The Cas9–sgRNA system is developed as a genome editing system</td>
<td>[10]</td>
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<td>2013</td>
<td>Cas9-mediated gene editing in human cell lines</td>
<td>[8, 11]</td>
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<td>2013</td>
<td>Cas9-generated transgenic mice</td>
<td>[92]</td>
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<tr>
<td>2014</td>
<td>Cas9–sgRNA is used as transfected RNPs</td>
<td>[34, 93]</td>
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<td>2014</td>
<td>Cas9-mediated in vivo gene editing in mice treats muscular dystrophy</td>
<td>[48, 49, 94]</td>
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<td>2014</td>
<td>ZFN-mediated gene correction in human HSPCs</td>
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<td>2014</td>
<td>ZFN human trials; engineering HIV resistance in T cells</td>
<td>[95]</td>
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<td>2015</td>
<td>TALEN human trial; TCR knockout for allogeneic CAR T cells</td>
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<td>2015</td>
<td>Cas9–sgRNA gene editing in triploid nuclei</td>
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<tr>
<td>2015</td>
<td>Development of synthetic and chemically modified sgRNAs</td>
<td>[32]</td>
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<td>2016</td>
<td>Cas9-mediated gene correction in human HSPCs</td>
<td>[19, 23]</td>
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<td>2016</td>
<td>Cas9–sgRNA human trials; PD-1 knockout in T cells for cancer therapy</td>
<td><a href="https://clinicaltrials.gov">https://clinicaltrials.gov</a></td>
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<tr>
<td>2017</td>
<td>Cas9-mediated integration of a CAR into T cells</td>
<td>[39]</td>
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*The table is non-exhaustive and we apologize for the omission of many important studies because of space limitations.

**Glossary**

**Adeno-associated virus (AAV):** a single-stranded, nonpathogenic virus that has been used extensively as a gene vector for gene transfer into a variety of cell types. Different naturally occurring and engineered AAV variants (serotypes) exist that differ in the type of cells they infect (termed tropism).

**Clustered regularly interspaced short palindromic repeats (CRISPR)-Cas9:** a bacterial adaptive immune system that integrates small DNA sequences from invading DNA viruses into the bacterial genome and later uses transcribed RNA variants of these DNA segments in conjunction with the Cas9 endonuclease to recognize and degrade infecting viruses with the same sequence.

**DNA endonuclease:** an enzyme that cleaves phosphodiester bonds internally in the endonuclease.

**Hematopoietic stem and progenitor cells (HSPCs):** reside in the bone marrow and comprise stem cells with the capacity to supply a lifelong source of all mature blood cells, and progenitor cells that can supply a short-term source of cells from all or single blood lineages.

**Homologous recombination (HR):** a process where genetic information is exchanged between two homologous DNA strands. In gene editing this process is also sometimes referred to as HDR or synthesis-dependent strand annealing (IDSDA). **INDELs:** insertion or deletion of DNA nucleotides; can be created by error-prone NHEJ.

**Non-homologous end joining (NHEJ):** a DNA repair pathway that is activated on DNA DSBs. The two ends are directly joined by ligation without the use of a repair template. However, the process is error prone and can introduce mutations at the break site.

**Null mutation:** a genetic mutation that leads to a nonfunctional or absent protein.

**Off-target nuclease activity:** engineered nucleases can make DNA breaks at unintended sites in the genome. Although the target site sequence is chosen to be uniquely present in the genome, sites may exist with close homology to the on-

**DNA Break Repair: Behind the Scenes**

The DNA break is the foundation for genome editing and can lead to either gene disruption by error-prone **non-homologous end joining (NHEJ)** that creates **INDELs** or precise gene addition or repair by HR using an exogenously supplied repair template with homology to the targeted site (Figure 1, Key Figure). While disruptive NHEJ is largely uncontrollable in terms of genotypic outcome, HR is a precise process with the ability to alter the genome with nucleotide resolution. However, HR is also theoretically more difficult to achieve because it mainly operates during the S and G2 phases of the cell cycle when a sister chromatid is available for repair [12] and it is in competition with NHEJ since introduced INDDELs or substitutions can prevent further cleavage at the site. Furthermore, HR relies on efficient and nontoxic delivery of DNA repair templates that need to not only enter cells but also efficiently reach the nucleus.
Engineering Genomes
Gene editing provides several means to manipulate the genome. Gene disruption by NHEJ at a target gene can be a definitive method to investigate gene function. In contrast to RNAi, which lowers but never fully abrogates gene expression, NHEJ-based genome editing can completely disrupt gene expression in cells that have INDELs that inactivate all of the alleles of a gene. While NHEJ-based gene editing and RNAi have both largely proved efficient in the study of gene function, there are also examples of RNAi-mediated gene knockdown not fully recapitulating gene disruption phenotypes due to either off-target effects or residual gene expression [13–15]. However, although NHEJ efficiencies can be as high as 95% in primary cells [16], efficiencies can be significantly lower depending on target gene, cell type, and delivery methods. One potentially confounding factor is that the outcome of NHEJ is not controllable, thereby creating a heterogeneous population of cells with different INDELs that may or may not disrupt gene expression or change the function of the protein (Figure 1B). For many applications, it is therefore useful to create clonal cell lines with known genotypes. This also has the advantage of being able to compare different mutations in a gene of interest on an isogenic background.

HR differs from NHEJ in its precision of changing the genome sequence at the target site. For HR, cells need to be supplied with donor DNA that carries the intended DNA changes flanked by DNA segments homologous to the targeted sequence (homology arms) (Figure 1C). In this way, long DNA sequences can be inserted at a specific genomic site or SNPs can be generated. This provides a fully loaded toolbox for efficient and precise manipulation of the genome (Figure 2). Full gene expression cassettes can be integrated into ‘safe harbor’ regions of the genome for safe expression of transgenes [17] or full-length cDNAs or open reading frames of a transgene can be integrated precisely at a start codon of an endogenous gene to put the transgene under transcriptional regulation by the endogenous promoter of the targeted gene [18–20]. For gene therapy, this constitutes a one-size-fits-all strategy for diseases where patients carry different mutations that are scattered throughout the genome. Additionally, a cDNA can be integrated at the start or stop codon for in-frame fusion with the endogenous gene (e.g., to tag the endogenous gene). HR can also serve as an alternative approach to NHEJ by gene-disruptive integration of a reporter expression cassette, which can also enable tracking of cells with gene disruption if the integrated expression cassette contains a gene encoding a fluorescent protein. Multiplexed gene editing systems with different reporter genes have recently been shown to be a forceful method for tracking and enriching for cells with complex genotypes [21,22]. In this approach, integration of reporter gene cassettes enables isolation and tracking of cell populations with biallelic gene disruption of one or more genes (Figure 3) [21,22].

HR can also mediate small genetic changes using a donor template with homology arms flanking the intended genetic change. It is possible to employ small, synthetic single-stranded oligodeoxynucleotides (ssODNs) as a template for the repair of a DSB to create small genetic changes falling under the general category of homology directed repair (HDR). The mechanism [now called single-stranded template repair (SSTR)] by which ssODNs mediate genome editing is not through the canonical HR machinery but instead proceeds through a mechanism that is still being determined and may not reflect a natural way in which cells normally repair DSBs. ssODNs can enable single-nucleotide gene correction for gene therapy purposes or for studying the phenotypic outcome of different gene variants [23]. The ease of synthesizing ssODNs makes the approach somewhat more accessible to researchers, but the range of genetic edits that can be generated is much more limited than when using standard gene-targeting vectors. Finally, the frequency of genome editing may be higher using standard target site and the nucleases may have some off-target activity at these sites.

Protopalsser adjacent motif (PAM): a DNA sequence that is located immediately adjacent to the target site and is essential for Cas9 activity. For Cas9 derived from Streptococcus pyogenes, the PAM sequence is NGG, where N designates any of the four DNA nucleotides.

RNA interference (RNAi): a cellular process that induces post-transcriptional gene silencing by either mRNA destabilization or inhibition of translation.

Serotype: different AAV variants (serotypes) exist that differ in the amino acid composition of the capsid protein. This variation changes the tropism of the AAV (i.e., the ability to infect different types of cells).

Single guide RNA (sgRNA): chimeric single RNA molecules that mimic the fusion of the natural crRNAs and tracrRNAs of the CRISPR system. The sgRNA is approximately 100 nt long and the target specificity is determined by the first 20 nt of the 5’ end.

Single-stranded oligodeoxynucleotides (ssODNs): short, single-stranded DNA strands that can be easily synthesized and purchased from DNA synthesis companies. They are generally up to 200 nt long and can be used as homologous donor DNA with short homology arms during HR for integration of short stretches of DNA.

Transcription activator-like (TAL) effectors: secreted DNA-binding proteins found in Xanthomonas bacteria, which infect a wide variety of plants.

Viral vectors: genetically modified viruses that have been rendered harmless and unable to replicate. Their highly evolved ability to transport genetic material to cells is harnessed by replacing most of the viral genome with recombinant DNA that will be delivered to the cells ( termed transduction).

Zinc fingers: proteins with finger-like structures that recognize a target molecule. Although not a general feature, classic zinc fingers bind DNA and are stabilized by zinc ions.
gene-targeting vectors than using ssODNs [19,23]. Recently, a different approach, termed base editing, has been developed to introduce specific nucleotide changes to the genome. By fusing a cytidine or adenosine deaminase enzyme to a catalytically impaired Cas9 [dead Cas9 (dCas9)], targeted conversion of a C:G to T:A or A:T to G:C base pair can be facilitated [24,25]. However, this engineered base editing enzyme acts on all cytidines or adenosines in a window of approximately 5 nt, which, in conjunction with the requirement for a sgRNA protospacer adjacent motif (PAM), currently limits the number of genomic sites that can be targeted.

Figure 1. (A) Structure and composition of a synthetic chemically modified sgRNA showing the nucleotides of the constant ‘scaffold’ region and nucleotides of the target-specific region (a.k.a. the spacer region), designated N. Chemically modified nucleotides of the sgRNA are depicted as yellow stars. The genomic target site is shown with the NGG protospacer adjacent motif (PAM) in orange and the Cas9 cut site is designated with a yellow triangle. (B) Gene editing by non-homologous end joining (NHEJ) can give rise to a multitude of different genotypes and six different and randomly chosen examples are displayed that introduce either an SNP or insertions, deletions (INDELs), or both. To the right of the sequence, the label shows the genotype, with (-) and (+) designating the number of deleted or inserted nucleotides, respectively. (C) Homologous recombination (HR) gives rise to precise gene editing where a donor template is designed with homology arms corresponding to the regions flanking the Cas9 target site. The intended DNA sequence to be introduced (CHANGE) is placed between the homology arms and is introduced site specifically into the genome following HR.
Cas9–sgRNA: Making It Work in Primary Cells

In the immediate years after the first demonstration of the use of the Cas9–sgRNA system for gene editing purposes, hundreds of papers were published using it but the majority employed the system in easily manipulated cell lines using plasmid delivery. This paralleled the development of the first genome editing platforms (e.g., ZFNs, TALENs). Delivery of ZFN-encoding plasmids to primary cell types like T cells and human hematopoietic stem and progenitor cells (HSPCs) has yielded relatively low frequencies of gene editing as well as plasmid-associated toxicities [26–28]. By contrast, mRNA delivery of ZFNs has been shown to yield higher delivery efficiencies leading to higher gene editing levels as well as less toxicity [26]. Direct cellular delivery of ZFN proteins has also been shown to be well tolerated in primary cells, with the added benefit that the short nuclease exposure reduces off-target activity [29–31]. The same findings translated to the Cas9–sgRNA system, where Cas9 mRNA or Cas9 RNP delivery has been associated with less toxicity and fewer off-target effects in primary cells in contrast to plasmid delivery [32–34]. However, a major deficiency of the system was the high...
instability of the sgRNA leading to low gene editing rates [32]. When using synthetic sgRNAs with both ends stabilized by the introduction of modified nucleotides (e.g., 2’-O-methyl-3’-phosphorothioate) (Figure 1), the Cas9 system performed with unprecedented editing frequencies, with INDELs created at up to 95% of the alleles [16,19,32]. This is now the state-of-the-art method for gene editing in primary cells using the Cas9–sgRNA platform.

HR unavoidably requires delivery of DNA to serve as a repair template. For easy-to-transfect cell lines, plasmid or ssODN donors are the preferred choice due to the ease by which they can be produced. However, in primary cells major obstacles are imposed by poor delivery efficiencies and innate immune responses to incoming DNA. To overcome these hurdles, researchers have turned to viral vectors [e.g., integration-defective lentiviral vectors (IDLVs), adenoviral vectors, adeno-associated virus (AAV) vectors], which have an inherent ability to efficiently ferry DNA to the nucleus while minimizing detection by the immune system [19,26,35–37]. For example, AAV vectors of serotype 6 have been used for efficient donor delivery in primary human T cells and HSPCs, showing HR rates of 30–60% in both cell types [19,36–39]. In the same cell types, IDLV and AAV6 donor delivery were compared and AAV6 was found to be superior [37,40]. However, the major drawback of AAV vectors is their limited packaging capacity. An AAV can hold only about 5 kb in total, which limits the size of the donor DNA and thus potentially the applications that AAV
donor vectors can be used for. Recently it was shown that a large transgene can be split between two AAV donor vectors that are designed to undergo sequential HR, so that the first part of the transgene is integrated first followed by the second part [16]. Since HR is a seamless process, this allows perfect reconstitution of the full transgene expression cassette.

**Drawing the Curtain: The Opening Act for Genome Editing in Humans**

**Ex Vivo Gene Editing in Hematopoietic Stem Cells (HSCs)**

*Ex* vivo gene editing offers the benefit of confining the process of genetic manipulation to a defined subset of cells that is extracted from a patient and transplanted back after gene editing. It also allows, to some extent, quality control of the corrected cell product before administration into the patient, but most importantly it eliminates the need to find immunologically matched donors to replace the diseased tissue. The major challenge is that the target cell must undergo extraction from a patient and *ex vivo* genetic manipulation while maintaining the potential to durably engraft and, in the case of a stem cell, repopulate the target tissue after transplantation.

For the same reasons, HSCs have been a major focus of gene therapy for more than two decades, where retroviral and lentiviral gene transfer to HSCs has provided disease amelioration of and even cures to monogenic disorders of the blood and immune system including X-linked severe combined immunodeficiency (X-SCID), adenosine deaminase (ADA) SCID, adrenoleukodystrophy (ALD), metachromatic leukodystrophy (MLD), Wiskott–Aldrich syndrome (WAS), and sickle cell disease (SCD) [41,42]. However, multiple trials have also shown adverse oncogenic events associated with the uncontrolled semirandom integration pattern of retroviral vectors. These were first observed in 20 X-SCID patients treated in the late 1990s, where five of these patients developed leukemias within a few years after the treatment and an additional sixth patient was recently reported to have developed leukemia 15 years after transplantation (Six et al., unpublished). Hence, there is a clear role for the precision that gene editing technologies provide.

Results from the first gene editing trial were reported in 2014, where researchers used ZFNs to disrupt the CCR5 gene in T cells from HIV-infected patients thereby mimicking the naturally occurring null mutation of the gene, which in a homozygous form renders cells completely resistant to infection by the R5 HIV strain. The *ex vivo* gene editing procedure and subsequent transplantation of the edited T cells were well tolerated in all patients and the edited cells displayed a survival advantage during treatment interruption. However, the gene editing efficiencies were too low to establish a T cell pool with high enough biallelic CCR5 disruption to control the viral load after treatment interruption. The approach is currently being pursued in clinical trials with optimized ZFNs with the aim of editing HSPCs for a life-long functional cure for HIV. Early proof-of-concept studies of genome editing by NHEJ through the creation of INDELS has been applied to the hemoglobinopathies, including SCD and β-thalassemia. In these studies, engineered nucleases were used to genetically recreate hereditary persistence of fetal hemoglobin (HPFH) by inactivating the Bcl11A gene or the Bcl11A erythroid enhancer, or by creating a small deletion in the promoter region of the γ-globin locus [43–45]. As a general strategy, however, the creation of INDELS is not going to be broadly applicable to the correction of disease-causing variants that cause monogenic diseases.

While the community excitedly awaits updates from already-initiated gene editing clinical trials for cancer, HIV, hemophilia, and mucopolysaccharidosis type I (MPSI), a range of published preclinical studies has demonstrated the immense potential of gene editing using HR. For example, among the genetic defects that affect the hematopoietic system, a number of diseases have been approached, including X-linked SCID [26], SCD [19,23], chronic granulomatous disease (CGD) [38,46], and X-linked hyper-IgM syndrome [18]. These studies have
used either a gene correction or a cDNA addition strategy and have targeted either T cells or CD34+ HSPCs. For editing therapies in the latter cell type, the main bottleneck remains reaching editing frequencies in the stem cell population capable of multilineage, long-term hematopoietic repopulation. This is evident from higher gene editing rates in the CD34+ HSPC culture than in mature human blood cells after long-term transplantation of the cells in immunodeficient NSG mice. For some diseases like X-SCID and SCID with an enormous survival advantage of corrected cells, <10% editing frequencies are expected to have a therapeutic effect [47] and these diseases are likely to be the first to be pursued in human clinical trials for hematopoietic disorders.

**In Vivo Gene Editing**

In vivo gene editing is necessary if the affected cells or tissues cannot be harvested from the patient, manipulated ex vivo, or efficiently transplanted back into the patient. Many monogenic diseases affecting a variety of organ systems are potential candidates for in vivo approaches and their efficacy is being demonstrated in several preclinical models. The diseases, and the specific delivery approach, can be broadly grouped by target organ system: (i) musculoskeletal; (ii) central nervous system; and (iii) liver. For in vivo gene editing, the main challenges are the specific and efficient delivery of the gene editing components, and the increased potential for immune-related complications, while limiting genome instability if the nuclease is expressed for prolonged periods. Feasibility depends largely on the tissue of interest and the underlying genetic and molecular mechanisms. Viral and nonviral vectors have been used for delivery into a range of tissues, but editing efficiencies and tissue distribution vary greatly.

Duchenne muscular dystrophy (DMD) is an example of a musculoskeletal disease that could benefit most from an in vivo approach. DMD is caused by mutations in the dystrophin gene leading to progressive skeletal and cardiac muscle degeneration. Genetic repair of the cellular phenotype has been primarily aimed at restoring the reading frame of the dystrophin transcript by creating deletions via NHEJ. Systemic in vivo administration of AAV vectors into DMD mice delivering Cas9 and sgRNAs have been shown to create exon deletions that partially restore the reading frame and result in biochemical and functional improvement in skeletal and cardiac muscle [48,49].

In vivo AAV delivery of genome editing components that depend primarily on NHEJ have also been applied to genetic diseases of the central nervous system such as Huntington’s disease (HD) [50,51], an autosomal dominant form of retinitis pigmentosa [52], and Leber congenital amaurosis 10 [53]. For these diseases the components are directly injected into the retina or the brain. HD is a neurodegenerative disease caused by a dominant CAG-repeat expansion in the first exon of the huntingtin gene (HTT). Genetic strategies attempt to ameliorate the neurotoxic effects of the mutant protein by targeting deletions that result in permanent loss of function of the HTT gene. To anticipate potential deleterious effects of reducing endogenous huntingtin, specific targeting of the expanded allele via specific nucleotide polymorphisms was established [54-56]. The strategy of specifically excising an expanded repeat has been applied to other repeat-expansion diseases [57]. In addition, a self-inactivating Cas9 system has been developed to prevent potential genotoxicity associated with persistent expression of nuclease, which can result from AAV delivery when targeting postmitotic tissues or continued expression until episomal AAV vector is diluted out due to cell division [51].

For diseases where gene correction or gene replacement is key to phenotypic improvement, the goal is to achieve sufficient in vivo HR, which requires efficient delivery of an additional component: the DNA donor template. As discussed above, systems that are based on AAV are
limited by the packaging capacity of AAV and therefore necessitate the use of at least two vectors to deliver all three components. This approach has been used to correct several metabolic diseases of the liver, which have the advantage of requiring a low level of gene correction to restore metabolic homeostasis. Multiple AAVs have been used to deliver all three components for targeted gene correction of the mutant gene in preclinical models of ornithine transcarbamylase deficiency [58] and hemophilia B [59]. In addition, multiple viruses have been used for gene addition into a safe harbor locus using ZFNs in the lysosomal storage disorders MPSI, MPSII, Fabry disease, and Gaucher disease [60,61]. Phase I clinical trials are already underway for MPSI and MPSII.

Several nonviral delivery approaches have also been investigated. Naked plasmid delivered via hydrodynamic tail-vein injection was tested for metabolic conversion of a murine model of tyrosinemia type I into the more benign tyrosinemia type III by knocking out a gene in the same metabolic pathway [62]. Metabolic correction of this disease was improved by systemic delivery of Cas9 mRNA by lipid nanoparticles combined with AAV-mediated delivery of the guide and HDR template for gene correction of a disease-causing mutation [63]. Additionally, systemic injection of gold nanoparticle-conjugated Cas9 RNP and donor DNA has been shown to correct the dystrophin reading frame in a DMD mouse model [64].

Gene Editing for Cancer Therapies
Genome editing of T cells to improve cancer immunotherapy has also entered clinical trials. In the first reports, TALENs were used to knock out the T cell receptor alpha (TRAC) gene and the CD52 gene in allogeneic T cells that had been engineered by a retroviral vector to express a chimeric antigen receptor (CAR) targeting CD19+ leukemic B cells to generate ‘universal’ CAR T cells [65]. CARs are engineered fusion proteins comprising an antigen recognition region (usually a single-chain variable fragment from a monoclonal antibody) and an intracellular domain from the CD3 ζ chain thereby transmitting the same signal as an endogenous T cell receptor (TCR). Intracellular signaling domains from, for example, the CD28 costimulatory protein receptor are usually added to potentiate the signaling. Recently, exciting preclinical second-generation genome-edited CAR T cells have been reported in which a combination of Cas9-gRNA and AAV6 was employed to generate a population of cells in which a CD19-specific CAR is integrated into the gene encoding the constant region of the TCR alpha chain [39]. This both inactivated the endogenous TCR and put the CAR under regulatory control by the endogenous TCR. In a mouse model of acute lymphoblastic leukemia, the resulting CAR T cells displayed much more uniform expression of the CAR and performed with enhanced anticancer potency compared with traditional retroviral delivery of the CAR. Finally, one of the few ongoing clinical gene editing trials uses the Cas9–sgRNA system in T cells extracted from non-small-cell lung cancer patients to knock out the PD-1 protein to override the immunosuppressive interaction with cancer cells that commonly overexpress the PD-1 ligand (PD-L1), allowing evasion from immune recognition. A strong rationale for this strategy has already been provided by the high therapeutic efficacy of PD-1 and PD-L1 inhibitors [66].

Bringing on the Show: Therapeutic Development of Genome Editing
With the explosion of interest in genome editing to treat human disease, there is likely to be a concomitant burst of preclinical proof-of-concept studies, many of which are likely to employ creative new strategies still to be discovered. However, several key steps need to be taken for these proof-of-concept studies to be translated into helping patients. First, more attention must be paid to validating proof-of-concept studies in clinically relevant situations. The base editing systems, for example, have been developed only in easy-to-manipulate and highly abnormal human cancer cell lines. Unfortunately, we have learned that systems that work well in such cell
lines, such as the published high-fidelity Cas9 nucleases [67–69], do not have high activity when applied in a clinically relevant fashion. Thus, it will be important to quantitatively validate approaches in systems that are relevant to clinical translation. Second, for streamlined and efficient translation of preclinical work to the clinic, standards for evaluating safety and toxicity should be established. Regulatory agencies, within the boundaries that constrain them, should work with the field to establish such standards. Finally, once clinical trials are initiated, they should be designed to generate more knowledge about the clinical applications of genome editing. The current standard ‘adverse events’ that are monitored should be supplemented by assays that are directed toward understanding the specific potential toxicities engendered by either ex vivo or in vivo editing protocols. The field of gene therapy has taught us that one can learn valuable lessons from the first trials that then enhance the safety and efficacy of subsequent clinical trials. It is the learning from past trials that has facilitated the renaissance of gene therapy [70–79]. This lesson should not be forgotten as the transformative potential of genome editing is translated to the clinic.

Concluding Remarks and Future Perspectives
While the concept of directly editing the genome to treat diseases is simple, it is only in the past decade that the tools have been developed to make this simple concept experimentally feasible. With these tools, however, we are now poised to generate an entirely new class of therapeutics to treat, or even cure, diseases that previously lacked effective interventions. The Outstanding Questions highlight some vital questions to be answered in the future (see Outstanding Questions). While we search for the answers, we can continue to learn how far our current tools can take us before we have to refine them or pivot to new approaches. There are many potential theoretical problems in moving genome editing into patients, but it is only by testing them in the clinic that we will learn about the actual problems, expected or unexpected, and begin working on solving them. For example, while there is much discussion of the potential off-target activity of the Cas9–gRNA platform, it remains unknown whether those off-target effects have any meaningful clinical relevance, and despite lots of discussion the definitive answer will come only through translation. Nonetheless, there are many reasons to be optimistic that soon an entirely new class of genetic therapeutics based on genome editing will be available to a wide range of patients.

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References

Outstanding Questions
Can gene editing be applied to treat multigenic diseases?
How do we overcome current limitations of delivery efficiency and tissue specificity in in vivo gene editing while balancing nuclease exposure and activity?
What impact will off-target nuclease activity have on gene editing therapies and which genotoxicity assays will be required to assess safety?
How will regulatory authorities assess the risk–benefit ratio for nonfatal diseases and diseases where conventional therapies are already available?
How will future gene editing-based therapies be priced and how will governments and health insurance providers navigate with an expected price of hundreds of thousands of dollars?
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