Advances in therapeutic CRISPR/Cas9 genome editing

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Targeted nucleases are widely used as tools for genome editing. Two years ago the clustered regularly interspaced short palindromic repeat (CRISPR)-associated Cas9 nuclease was used for the first time, and since then has largely revolutionized the field. The tremendous success of the CRISPR/Cas9 genome editing tool is powered by the ease design principle of the guide RNA that targets Cas9 to the desired DNA locus, and by the high specificity and efficiency of CRISPR/Cas9-generated DNA breaks. Several studies recently used CRISPR/Cas9 to successfully modulate disease-causing alleles in vivo in animal models and ex vivo in somatic and induced pluripotent stem cells, raising hope for therapeutic genome editing in the clinics. In this review, we will summarize and discuss such preclinical CRISPR/Cas9 gene therapy reports. (Translational Research 2016;168:15–21)

Abbreviations: Cas9 = CRISPR-associated protein 9; cccDNA = covalently closed circular DNA; CCR5 = chemokine receptor 5; CFTR = cystic fibrosis transmembrane conductance regulator; CRISPRs = clustered regularly interspaced short palindromic repeats; crRNA = CRISPR RNA; Crygc = crystallin, gamma C gene; DMD = Duchenne muscular dystrophy; DSBs = double-strand breaks; FAH = fumarylacetoacetate hydrolase; HBV = hepatitis B virus; HDR = homology-directed repair; HIV = human immunodeficiency virus; HSPCs = hematopoietic stem and progenitor cells; indels = insertions or deletions; iPSCs = induced pluripotent stem cells; LDL-C = low-density lipoprotein cholesterol; LTRs = long terminal repeats; NHEJ = nonhomologous end joining; PCSK9 = proprotein convertase subtilisin/kexin type 9; RGENs = RNA-guided engineered nucleases; sgRNA = single guide RNA; TALENs = transcription activator-like effector nucleases; tracrRNA = transactivating CRISPR RNA; ZNFs = zinc finger nucleases

INTRODUCTION

Clustered regularly interspaced short palindromic repeats (CRISPRs) are classes of repeated DNA sequences that act in concert with CRISPR-associated (Cas) genes to confer bacterial and archaeal immunity against foreign invading nucleotides such as phages and plasmid DNA.[1] From the 3 types of CRISPR/Cas systems identified so far, the type II system has been studied the most. During this immune response, the invading DNA first gets cut into small pieces and incorporated into the CRISPR locus.[2] The locus is then transcribed as a single noncoding precursor CRISPR RNA (pre-crRNA) that gets further processed into short stretches of mature crRNA. Together with a second noncoding RNA, the trans-activating CRISPR RNA (tracrRNA), the crRNA finally forms a ribonucleoprotein complex with the endonuclease Cas9, which recognizes and cuts the invading DNA.[2]

In 2012, a research team led by Emmanuelle Charpentier and Jennifer Doudna adapted the type II CRISPR system from *Streptococcus pyogenes* for genome editing.[3] By fusing the crRNA to the tracrRNA they generated a single guide RNA (sgRNA), which recruits the Cas9 nuclease to specific genomic locations via standard Watson-Crick base pairing.[3,4] The creation of site-specific double-strand breaks (DSBs)

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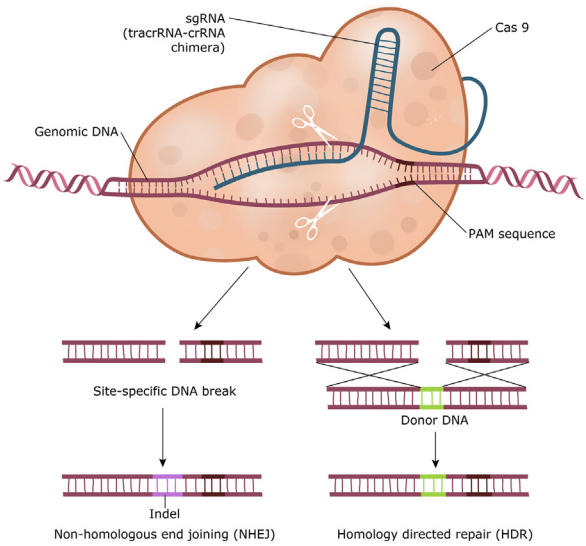

Fig 1. CRISPR/Cas9-mediated genome editing. Cas9 recruitment to the target DNA is mediated by a chimeric single-guide RNA (sgRNA). It contains a protospacer recognizing the target sequence followed by protospacer adjacent motif (PAM). Cas9-induced DSBs are repaired either by NHEJ giving rise to indel mutations or by HDR using a synthetic donor DNA template, which enables the introduction of desired sequence changes. CRISPR, clustered regularly interspaced short palindromic repeat; crRNA, CRISPR RNA; DSBs, double-strand breaks; indel, insertions or deletions; tracrRNA, tracer RNA.

by the CRISPR/Cas9 complex then triggers genome editing through 2 different mechanisms. First, in the absence of a homologous DNA template DSBs can be repaired by nonhomologous end joining (NHEJ), which is an error-prone process that causes small insertions or deletions. Second, in the presence of a synthetic repair template DSBs can be repaired by homology-directed repair (HDR), which enables the introduction of any desired base-pair changes (Fig 1).[5,6]

Before the establishment of RNA-guided engineered nucleases such as the CRISPR/Cas9, programmable DNA-binding nucleases such as zinc finger nucleases (ZNFs) and transcription activator-like effector nucleases (TALENs) were used to edit DNA.[7,8] However, engineering such sequence-specific DNA-binding proteins was time-consuming and challenging, which significantly hampered the widespread usage of these techniques. Because of the ease and speed of designing CRISPR-guided nucleases, the CRISPR/Cas9 system has quickly evolved to be the most widely used DNA editing tool, and has fostered a large number of gene editing studies in a variety of organisms, including mammalians and primates.[9-14] These studies have raised high hopes for CRISPR/Cas9-mediated gene therapy, which aims to repair disease-causing alleles by changing the DNA sequence at the exact location on the chromosome. In this review, we summarize recent studies that demonstrate proof-of-concept for CRISPR/Cas9-mediated gene therapy. The following 2 sections focus on in vivo approaches, which target cells directly in the zygote or adult animals (Fig 2, *A*). Furthermore, ex vivo approaches that aim to modify somatic stem or progenitor cells in culture with subsequent transplantation back into the patient (Fig 2, *B*) have been discussed.

CRISPR/Cas9 zygote editing. Injection of CRISPR/Cas9 components (Cas9 messenger RNA or protein; sgRNA; HDR template) into the zygote or early stage embryo allows modifying the genome in all cells of the organism, including the germline.[12] Thus, this approach results in permanent changes that can be passed on to subsequent generations, offering the possibility to eliminate a genetic disease from an entire family.

Wu et al were one of the first to use this approach to repair a disease-causing mutation in the mouse embryo. They focused on a dominant loss-of-function mutation in the Crygc gene, which causes cataract, a decrease in vision because of clouding of the eye lenses.[15] The authors injected Cas9 mRNA and the sgRNA targeting the dominant Crygc allele into the zygote, leading to the correction of the mutation, with the wild-type allele on the homologous chromosome acting as a template. Another study that used CRISPR/Cas9-mediated genome editing in the mouse embryo corrected a mutation in the gene dystrophin, responsible for inherited disease, X-linked Duchenne muscular dystrophy (DMD).[16] In this case, the authors injected the Cas9 mRNA and specific sgRNA together with a single-stranded DNA oligo into the zygote and achieved HDR from the exogenous DNA template. Although the authors only obtained partially corrected mosaic mice because of the editing occurring after the zygote stage, selective advantage of the corrected skeletal muscle cells still led to a complete rescue of the phenotype. In a very recent study, Liang et al have also demonstrated the possibility of CRISPR/Cas9-mediated zygote editing in human embryos,[14] generating controversy among scientists and in the public.[17,18] The reported study used tripronuclear human zygotes to modify hemoglobin beta, the gene responsible for the blood disorder β-thalassemia. The researchers, however, found that the procedure led to a high number of additional unwanted modifications (off-target effects), arguing against the use of the current technique for clinical applications. Moreover, because existing methods in prenatal diagnostics such as genetic profiling after in vitro fertilization already offer a less risky alternative for selecting against offspring with inborn diseases, we think that germline

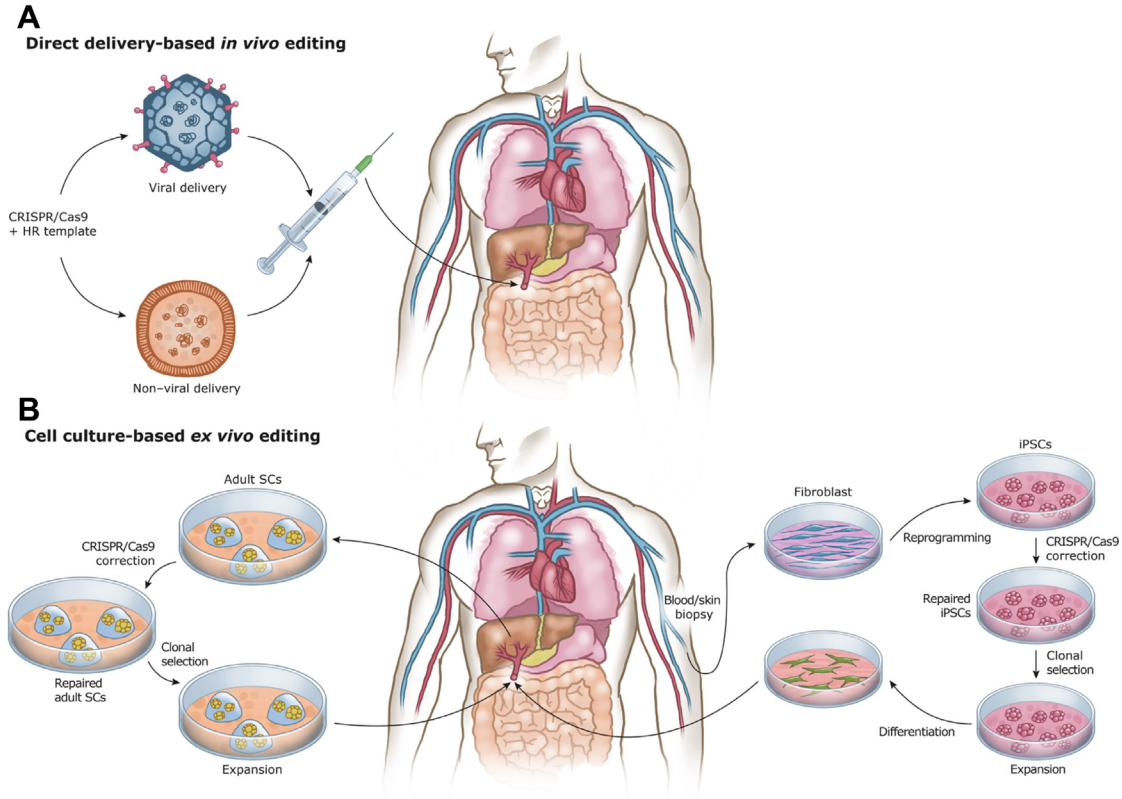

Fig 2. In vivo and ex vivo strategies for CRISPR/Cas9-based gene therapies. (**A**) In in vivo approaches, CRISPR/Cas9 components are directly delivered into the patient using either viral or nonviral vectors for in situ gene editing. (**B**) In ex vivo approaches, genes are edited in patient-derived cells. These can be generated by reprogramming (iPSCs) or direct expansion of somatic stem/progenitor cells, and are transplanted back into the same patient after the correction. CRISPR, clustered regularly interspaced short palindromic repeat; iPSCs, induced pluripotent stem cell.

editing would mainly benefit parents who want to add nonmedically relevant traits to their children. Because of these safety and ethical concerns we therefore do not support the legalization of CRISPR/Cas9-based genome editing studies in human zygotes.

Somatic CRISPR/Cas9-based editing in vivo. One of the first studies that successfully corrected a genetic disease in postnatal animals by in vivo CRISPR/Cas9-mediated genome editing was conducted by Yin et al in a mouse model for type I tyrosinemia.[19] Hereditary type I tyrosinemia is caused by a deficiency of the enzyme fumarylacetoacetate hydrolase (FAH), leading to cytotoxic metabolite accumulation and cell death of hepatocytes. Through hydrodynamic tail vein injection the authors delivered vectors encoding for Cas9 and the specific sgRNA, along with a DNA oligo for HDR directly into the mouse liver. This resulted in the correction of the mutant fumarylacetoacetate hydrolase allele and stabilization of the protein, leading to reduced hepatocellular toxicity and a rescue in weight loss of mice. Of note, tyrosinemia might be particularly suitable for CRISPR/Cas9 gene therapy, as the initially low repair frequency of 0.4% is compensated by the positive selection of corrected hepatocytes.

Another in vivo study focused on the disruption of the gene encoding for the proprotein convertase subtilisin/kexin type 9 (PCSK9) in the mouse liver.[20] PCSK9 is secreted into the plasma by hepatocytes, and limits low-density lipoprotein (LDL) cholesterol uptake and degradation by functioning as an LDL receptor antagonist. Naturally occurring loss-of-function mutations in PCSK9 therefore decrease blood cholesterol levels. To mimic this condition, the authors used adenoviral CRISPR/Cas9 vectors to disrupt PCSK9 in the mouse liver. This led to a decrease in PCSK9 protein levels, an increase in hepatic LDL receptor levels, and subsequently decreased plasma cholesterol levels. Importantly, because this approach is based on the disruption of a gene function by NHEJ, it was possible to reach editing efficiencies of up to 50%, which might already be sufficient for clinical application.

An elegant study by Lin et al further demonstrated that CRISPR/Cas9-mediated genome editing could also be used to cure hepatitis B virus (HBV) infection.[21] In many patients, HBV infections become chronic and trigger liver cirrhosis or hepatocellular carcinoma. Although antiviral therapies for chronic HBV patients have been developed, they usually fail to completely eliminate the virus from the liver. This persistence is because of the high stability of covalently closed circular DNA (cccDNA) intermediates, which serve as templates for viral replication. In their study, the authors modeled chronic HBV infection in mouse livers by hydrodynamic tail vein injections of HBV expression vectors. Importantly, coinjection of the CRISPR/Cas9 system targeting the HBV sequence led to vector cleavage, and ultimately to a reduction in serum hepatitis B surface antigen. Because this HBV mouse model did not produce the actual cccDNA intermediates, the authors eliminated in similar experiments cccDNA of the duck HBV in a human cell line, supporting the possibility of complete HBV eradication by genome editing. Nevertheless, because any residual viral DNA could potentially renew the infection, for clinical applications an extremely efficient CRISPR/Cas9 delivery system would be needed.

In the previously described preclinical studies, proof-of-concept in vivo CRISPR/Cas9-based gene therapy was performed in mice. However, to proceed toward clinical studies several hurdles still need to be overcome. First, methods need to be optimized for efficient and safe delivery of the CRISPR/Cas9 components into the desired tissues. In the past 25 years, many clinical trials for gene therapy with conventional ectopic overexpression vectors have been conducted.[22,23] These studies provide promising results for gene delivery using both viral and nonviral vectors, which could potentially be adapted to deliver CRISPR/Cas9-based vectors. Adeno-associated virus shuttle vectors seem particularly promising because of their high efficiency in transducing a broad range of cell types and their low cytotoxicity and immunogenicity. Importantly, a recent study demonstrated the possibility to pack the Cas9 nuclease together with the sgRNA into a single adeno-associated virus particle by using the significantly smaller Cas9 protein encoded by Staphylococcus aureus.[24] A second challenge for in vivo CRISPR/Cas9-based gene therapy is the low efficiency of HDR compared with NHEJ, which might limit this approach to therapies where gene functions need to be eliminated, or where corrected cells gain a competitive advantage over unrepaired cells. A possible solution to this problem might be generating single-strand breaks by paired Cas9 nickases,[25] as a recent report has shown that it increases the ratio of HDR compared with NHEJ.[26] The third hurdle for applying CRISPR/Cas9 in patients is safety concerns because of off-target effects.[27,28] These unintended DSBs at genomic loci that differ from the target sequence may cause mutations or chromosomal rearrangements because of religation between cuts on different chromosomes. Although the frequency of off-target alterations still remains controversial, recently developed genome-scale methods allow unbiased and highly sensitive detection of unintended DSBs in a bulk cell population.[24,29-32] These studies revealed that the number of off-target mutations strongly varies between individual sgRNAs, from zero to more than 150 sites that are cut at frequencies between 0.03% and 87%. Because even very low off-target rates could be a problem for in vivo gene therapy when tumor driver genes are affected, it will be crucial to enhance CRISPR/Cas9 specificity. Two recent studies showed that this might be possible either by adding 2 guanines at the 5′ end of the sgRNA[33] or by truncating sgRNAs to 17 nucleotides.[34] Moreover, the use of paired Cas9 nickases,[35,36] which generate single-strand breaks rather than DSBs, or the use of catalytically dead Cas9 (dCas9)-FokI fusion protein,[37,38] which can only cut as a dimer, has been shown to greatly reduce off-target mutations.

Somatic CRISPR/Cas9-based editing ex vivo. The ex vivo approach requires protocols for culturing patient-derived stem/progenitor cells, which can be transplanted back into the host after genome editing. In 2007, Takahashi et al[39] developed a method for generating induced pluripotent stem cells (iPSCs) from human fibroblasts. Importantly, iPSCs can be infinitely propagated and differentiated into any cell type of the body, and thereby present a valuable source for ex vivo gene therapies. Moreover, in the last few years, researchers have established protocols to directly expand somatic stem cells in vitro.[40-42] As these protocols do not involve any dedifferentiation steps, they might provide a presumably safer alternative to iPSCs.

One of the first proof-of-concept studies for ex vivo CRISPR/Cas9-based gene therapy was done in iPSCs and tackled the genetic blood disorder β-thalassemia.[43] β-thalassemia is caused by mutations in the hemoglobin beta gene, which reduce the production of hemoglobin and thereby trigger anemia. Currently, the only cure for β-thalassemia is by transplantation of healthy, donor-derived histocompatible hematopoietic stem cells. Corrected patient-derived iPSCs, however, might provide an alternative source for generating transplantable hematopoietic stem cells. Xie et al therefore established iPSCs from fibroblasts of a patient homozygous for β-thalassemia and transfected them with CRISPR/Cas9-based vectors targeted to the disease-causing

allele together with a DNA template for HDR. Homologous recombination events were identified by selecting for a cointegrated antibiotic resistance cassette, which was later excised through transposase-mediated recombination. By using a novel monolayer protocol, corrected iPSCs were then differentiated into fully functional red blood cell precursors, which in the future could potentially be used for transplantations.

In a study directed by Hans Clevers, we recently demonstrated the possibility of CRISPR/Cas9-mediated genome editing in primary somatic stem cells. As a model, we used intestinal organoids, which allow infinite expansion of multipotent intestinal stem cells to correct a prevalent cystic fibrosis-causing allele.[44] Cystic fibrosis is a monogenetic disease that originates from mutations in the cystic fibrosis transmembrane conductance regulator (CFTR), an ion channel that regulates epithelial fluid transport. Loss-of-function alleles lead to an accumulation of mucus in the gastrointestinal and pulmonary tract, causing a number of symptoms such as difficulties in breathing and recurrent infections. In the present study, we first isolated intestinal stem cells from cystic fibrosis patients, expanded them in vitro as 3-dimensional organoid cultures, and then transfected them with CRISPR/Cas9-based vectors targeting the CFTR locus together with a template for HDR. A puromycin resistance cassette within one of the introns allowed selection of clones with integration events. Successful gene correction was further demonstrated by sequencing and in a CFTR-dependent organoid-swelling assay. Importantly, in our study, we could detect only very few off-target effects.

Two independent studies, moreover, used CRISPR/Cas9 to correct alleles causing DMD in patient–derived iPSCs and immortalized cell lines.[45,46] DMD is caused by alterations in the unusually large dystrophin gene, which encodes for a protein that is important for the structural integrity of muscle fibers. Loss of gene function leads to disruptions in muscle fiber structure and ultimately to a weakening of skeletal, respiratory, and cardiac muscles. In the first study, Li et al used CRISPR/Cas9 in combination with a donor template to restore the full-length dystrophin gene by homologous recombination in iPSCs derived from DMD patients that lacked exon 44. Repaired iPSCs were selected and differentiated into skeletal muscle cells, in which the wild-type dystrophin protein was expressed. In the second study, Ousterout et al generated single and multiple exon deletions to restore the reading frame of the dystrophin gene in immortalized myoblasts derived from DMD patients. Importantly, multiplex gene editing allowed to create a large deletion that excises exons 45–55, a mutational "hot spot," harboring more than 60% of disease-causing DMD mutations. This deletion restored dystrophin expression and function in vitro and in vivo after transplantation into mutant mice.

Finally, another ex vivo CRISPR/Cas9-based gene editing study focused on tackling human immunodeficiency virus (HIV) infection. During its life cycle HIV-1 integrates into the host genome of immune cells, where it serves as a template for viral expression. At this stage, HIV infection can become transcriptionally silent, leading to a latent infection. Because the latent virus resides in long-lived cells such as memory T cells, the infection generally persists indefinitely even in the presence of potent antiretroviral drugs. Using genome-editing technologies, researchers are currently testing 2 strategies to tackle latent HIV infection. In the first approach, the viral genome sequence is targeted by nucleases to permanently eliminate the integrated HIV DNA from the genome of infected T cells. Liao et al recently followed this approach using CRISPR/Cas9 and targeted the highly conserved HIV-1 long terminal repeats (LTRs) in infected primary CD4+ T-cells.[47] Stable expression of CRISPR/Cas9-based vectors targeting the LTR region resulted in their disruption and subsequently in reduced virus production and the elimination of the latent reservoir. In addition, the authors could demonstrate that HIV reservoir cell types, which had been generated from human pluripotent stem cells previously transduced with LTR-targeting CRISPR/Cas9 vectors, were resistant to new HIV infection. In the second approach, genome editing is used to convey HIV-1 resistance by modulating chemokine receptor 5 (CCR5), a coreceptor needed for HIV-1 T-cell infection. Mandal et al[48] used CRISPR/Cas9 editing and disrupted this receptor in CD34+ hematopoietic stem and progenitor cells (HSPCs). The authors demonstrated the ablation of CCR5 in HSPCs with an efficiency of roughly 30% using CCR5-targeting CRISPR/Cas9 vectors. Moreover, they reported that these CCR5-ablated HSPC clones retained full multilineage potential after xenotransplantation in mice and contained only few off-target mutations. Importantly, the strategy to mutate CCR5 has already been performed and successfully tested in clinical trials using zinc finger nucleases.[49]

One of the major advantages of ex vivo gene therapy is the possibility to select and analyze corrected cells. Thus, only recombinant clones harboring the correctly edited allele without deleterious off-target mutations will be chosen for transplantation back into the patient. Because of this selection step, the efficiency and accuracy of the CRISPR/Cas9 system is less critical in ex vivo approaches compared with in vivo approaches. On the downside, however, ex vivo editing requires cell expansion in culture, which can lead to additional unwanted genomic alterations. In particular, iPSCs are

prone to accumulate mutations and copy number variations during reprogramming and expansion.[50,51] Although recent studies suggest that growing adult stem cells in 3-dimensional organoid cultures ensure higher genetic stability,[40] extensive in vitro cell expansion might remain a safety concern. Another hurdle for ex vivo gene therapy approaches is the efficient orthotropic transplantation of corrected cells. Currently, cell-based transplantations are well established in the clinics for hematopoietic stem cells, but in other tissues such as liver or muscles approaches are still under development. Nevertheless, despite all the challenges listed, the CRISPR/Cas9 technology holds immense promises for bringing gene therapy into the clinics, and given the tremendous progress that has been made in the last 2 years, we believe this could happen soon.

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