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Invited Review

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INVITED REVIEW

Development and application of CRISPR/Cas9 technologies in genomic editing

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Abstract

Genomic editing to correct disease-causing mutations is a promising approach for the treatment of human diseases. As a simple and programmable nuclease-based genomic editing tool, the clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) system has substantially improved the ability to make precise changes in the human genome. Rapid development of CRISPR-based technologies in recent years has expanded its application scope and promoted CRISPR-based therapies in preclinical trails. Here, we review the application of the CRISPR system over the last 2 years; including its development and application in base editing, transcription modulation and epigenetic editing, genomic-scale screening, and cell and embryo therapy. Finally, the prospects and challenges related to application of CRISPR/Cas9 technologies are discussed.

Introduction

The clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein (Cas) system is a bacterial and archaebacterial defense mechanism against phage infection and plasmid transfer in nature. In recent years, this system has garnered increasing attention as an effective and simple genome-engineering tool and has revolutionized the life sciences. It has been recognized for its potentially transformative applications in transcriptional perturbation, epigenetic modulation, base editing, high-throughput genetic screening and generation of animal or cell models of diseases (1).

Based on the effector protein organization, CRISPR/Cas system is classified into two distinct classes that are subdivided into 6 types and 19 subtypes. Class 1 CRISPR/Cas systems utilize multi-protein effector complexes, whereas class 2 CRISPR/Cas systems utilize single-protein effectors (2,3). The diverse CRISPR/Cas systems have a number of features in common, such as the use of short DNA sequences known as ‘spacers’ to direct the targeting of Cas proteins. In addition, there is a requirement for a conserved sequence to aid targeting, called the protospacer adjacent motif (PAM) for DNA-targeted Cas proteins, or the protospacer flanking sequence (PFS) for RNA-targeted Cas proteins (4). The Cas9 endonuclease from *Streptococcus pyogenes*, which belong to the class 2 CRISPR system, has been the most widely used CRISPR system for gene editing. Currently, there are ongoing clinical trials using CRISPR/Cas9-edited human cells, for example, to treat cancer. In the following sections, we review the applications of CRISPR/Cas9 technologies in the last 2 years; including base editing, transcription modulation and epigenetic editing, genomic-scale screening, and cell and embryo therapy (Fig. 1). We will also discuss the limitations as well as regulatory and ethical implications of this transformative set of technologies.

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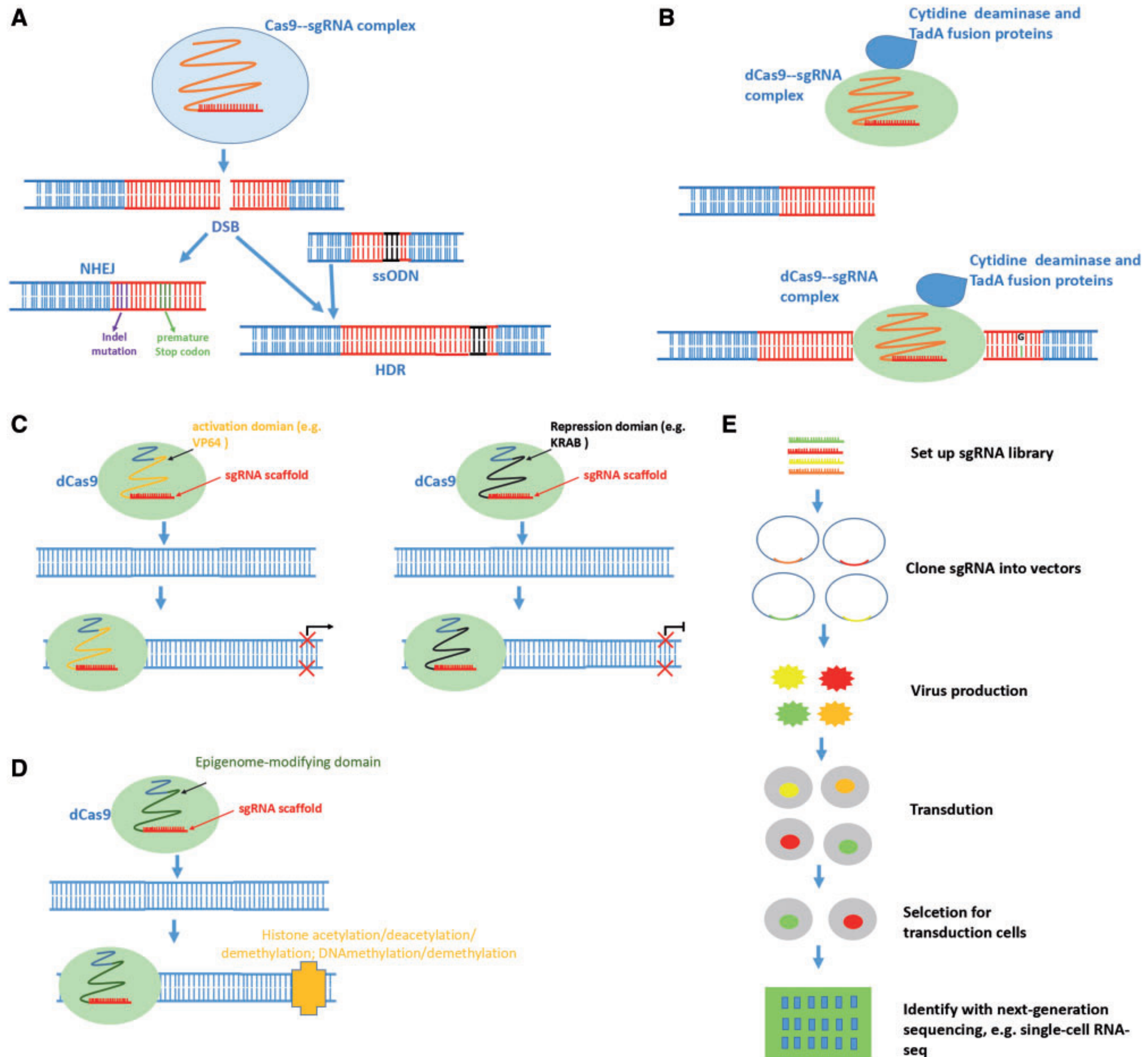

Figure 1. Applications of CRISPR/Cas9 technology. (**A**) Traditional genome editing: CRISPR/Cas9 is used for making DSB at particular sites. These introduced DSBs are mended by either NHEJ or HDR. (**B**) Base editing: A base editor consists of a catalytically modified Cas9, deoxyadenosine deaminase enzymes and TadA fusion proteins complex. Through deoxyadenosine-deaminase action, adenosine is transformed into inosine. (**C**) Transcription modulation: CRISPR transcription modulation system has been developed by modifying dCas9 protein. Combined with activate or repression domain (such as VP64 and KRAB), dCas9 can activate or repress, respectively, DNA transcription without changing DNA sequence. (**D**) Epigenetic modulation: DNA methylation and histone modification; epigenetic effectors merged with dCas9 could change epigenetic states of target loci, such as through DNA methylation or histone modifications. (**E**) Pooled CRISPR screen strategy. In pooled CRISPR screens, gRNAs are synthesized, cloned and constructed as a pool. Pooled screens have dissected genetic networks with the help of NGS like single-cell RNA-seq.

Base Editing

Traditional CRISPR/Cas9 protein-RNA complexes localize to a target DNA sequence through base pairing with a guide RNA, and natively create a dsDNA break (DSB) at the locus specified by the guide RNA (1). In response to DSBs, cellular DNA repair processes result in random insertions or deletions at the site of DNA cleavage through homology-directed repair (HDR) or non-homologous end joining (NHEJ), depending on the presence or absence of a homologous DNA template, respectively. However, HDR-dependent genome editing is limited by low efficiency arising from competition with NHEJ outcomes and from the dependence of HDR on mitosis. Therefore, the primary strategies of modifying eukaryotic genomes using HDR are more unpredictable than the prospection of precise mutation correction (5,6). While researchers have attempted to increase the efficiency of HDR by suppressing NHEJ, the strategies for correction of point mutations using HDR under therapeutically relevant conditions remain inefficient (7,8). In this instance, the third-generation base editor (BE3), a single protein, was designed to allow the direct and stable transformation of target DNA bases into an alternative in a programmable way, without DNA double-strand cleavage or a donor template (9). BE3 contains a tripartite fusion between *Rattus norvegicus* APOBEC1 cytidine deaminase, *S. pyogenes* Cas9n (D10A) and *Bacillus subtilis*

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bacteriophage PBS2 UGI. BE3 results in a higher base editing efficiency than HDR, and a much lower indel frequency than nuclease-mediated approaches. Moreover, multiple studies have shown that BE3 has fewer off-target editing events than Cas9 (10,11).

Subsequently, BE4 was designed based on B3 to increase the efficiency of C: G to T: A base editing by approximately 50%, while halving the frequency of undesired by-products with respect to those generated by BE3. Fusion of this optimized construct to the DSB binding protein Gam from bacteriophage Mu yielded BE4-Gam, a base editor that retains the optimized properties of BE4 while further reducing indel formation (12). DNA glycosylase inhibitor protein (e.g. UGI) was also used to enhance base editing efficiency. In addition to BE3 and BE4, other cytidine deaminase enzymes like activation-induced cytidine deaminase (AID) have also been developed to address the inherent limitations of using DSBs for gene correction (termed 'CRISPR-X'). Target-AID is a BE3-like base editor, and is a synthetic complex of dCas9 and PmCDA1. Target-AID results in the base substitution of C or G into T or A with a shifted deamination window compared with BE3 (13). Genome-wide specificity of base editing approaches continues to be evaluated and improved (10).

Over the last 2 years, base editing tools and related technologies have been successfully used by many researchers in a wide range of applications, including plant genome editing, *in vivo* mammalian genome editing, targeted mutagenesis and knockout studies (13–21). The ability to create or remove a single-nucleotide variation (SNV) in target genes makes BEs valuable tools in genetic, therapeutic and agricultural applications. BEs can also create nonsense mutations by generating premature TGA (opal), TAG (amber) or TAA (ochre) stop codons when targeted to CGA (Arg), CAG (Gln) and CAA (Gln) (15,16,22). This method has the advantage over traditional Cas9 in gene knockout experiments, as Cas9 often induces in-frame indels in a protein-coding gene, which can still produce functional proteins, and frame-shifting indels, leading to translation of out-of-frame polypeptide sequences that can be immunogenic and may have unknown effects in cells (23). Base editing can also be combined with a library of sgRNAs targeted to a gene of interest and used for high-throughput screening of gain-of-function variants in cells (14,18).

Transcription Modulation and Epigenetic Editing

The dCas9 protein is a Cas9 variant which is capable of binding to the target sequence but unable to cleave its target (24). This protein has been adopted as a DNA-binding platform for transcription modulation and epigenetic editing, and engineered by using a variety of effector domains.

In the previous studies, dCas9 use focused on target gene activation (TGA) by fusion with conventional transcriptional activators such as VP64, p65 or a subunit of RNA polymerase (25–28). However, the dCas9-VP64 system was not very effective and was also unable to stimulate robust TGA by using a single-guide RNA (sgRNA) (29–31). Thus, fusion or recruitment of multiple transcriptional activation domains to the dCas9/gRNA complex (32), synergistic activation mediator (SAM) (33,34) or dCas9-Suntag (35,36) were performed to improve the activation capacity and expand the range of applications. These systems could induce very high activation by using several sgRNAs, or even one sgRNA for each target gene, enabling high levels of activation *in vitro* (37–39). However, *in vivo* use remains a challenge (1,40), possibly attributed to the lower efficiency of *in vivo* transferring of dCas9 fusion proteins and the lower level of TGA *in vivo*. In addition, sequences encoding the dCas9/gRNA and co-transcriptional activator complexes exceed the capacity of most common viral vectors like AAV, which is the most promising vector for gene delivery *in vivo* (41). In a recent report, an *in vivo* CRISPR/Cas9 TGA system was created to overcome this problem. In this system, the transcriptional activators were separated from dCas9 to shorten the coding sequence. Other researchers tried to optimize the shortening of gRNAs [14 or 15 base pairs (bp) rather than 20 bp] containing motifs that tether bacteriophage MS2 coat protein domains fused to the MS2: P65: HSF1 (MPH) transcriptional activation complex; this system achieved high efficiency TGA in organs of living animals (41).

dCas9 can also block target gene transcription by fusing a repressive effector domain such as the Krüppel-associated box (KRAB) (27,42). KRAB repression is mediated by repressive histone modifications such as H3K9me3. By utilizing epigenome-modifying repressors, including Lys-specific histone demethylase 1 (LSD1) (43), histone deacetylase (HDAC) (44), DNA methyltransferases DNMT3A and MQ1 (39,45,46), and mSin3 interaction domains (47), the scope of applying CRISPR repression has been extended to epigenetic editing. Similarly, epigenome editing approaches can also be used for targeted transcriptional activation, such as dCas9 fused with a DNA demethylase or a histone acetyltransferase (39,48). Recently, Klann *et al.* (48) developed a CRISPR/Cas9-based epigenomic regulatory element screening (CERES) system, which combines dCas9-p300Core with dCas9-KRAB to obtain both gain and loss of function information by targeting the same regions with a repressor and an activator.

Genomic-Scale Screening

In recent years, tools that use the RNA interference (RNAi) pathway, specifically short-hairpin RNAs (shRNAs), to perturb transcript levels have revolutionized screening approaches (49–51). However, this approach was limited by incompletely abrogate gene expression and high off-target effects, resulting in false positive (52,53). Some studies have shown that CRISPR can be adapted for genome-scale screening by combining Cas9 with pooled-guide RNA libraries and next-generation sequencing (NGS) (54). To date, two applications of CRISPR/Cas9-mediated genomic modifiers have been available for genome-wide screening, either by targeting about 20 000 genes or studying one specific signaling pathway or gene function (55). The general approach for screening of CRISPR is through loss-of-function screens, which is based on indel-prone NHEJ repair or repression of the underlying sequences (CRISPRi). The gain-of-function screen, which uses endogenous HDR or CRISPR activation (CRISPRa) approaches, is also employed for some specific applications (48). In addition, developments in the CRISPR system for RNA targeting or base editing will further broaden the research field of CRISPR screens (56,57).

Given the ever-growing CRISPR toolbox, a variety of high-throughput pooled screening options have become available for genome mutagenesis, transcription and epigenome modification, and base editing. These screens utilize pooled sgRNA libraries generated by cloning chip-synthesized oligonucleotides, which cover the entire human or mouse transcriptome, into lentiviral vectors. Upon transduction of the libraries into cells, sgRNAs inducing a selectable phenotype can be identified by NGS. Traditionally, CRISPR/Cas9 screens have been used to study intracellular phenotypes by combining with positive,

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negative or marker/reporter gene selection (58). Recently, single-cell RNAseq has been developed for the pooled CRISPR screen readout as an alternative strategy ('Perturb-seq'), allowing capture of multi-dimensional transcriptional phenotypes to correlate with their respective genetic manipulation (59–61).

CRISPR/Cas9 library screening enables the identification of critical components in a variety of biological processes. Apart from being widely used to identify essential genes in mammalian cell lines (62), the CRISPR screen has recently been used to analyse synthetic lethal genes (63), HIV host dependency factors (64), genetic vulnerabilities in cancer (65,66), novel targets of diseases (67–69) and resistance phenotypes (70). Moreover, the identification of non-coding sequences and characterized enhancer elements and regulatory sequences belongs to a novel application scope of CRISPR screens. With tiling sgRNAs ('saturating mutagenesis'), researchers could identify non-coding regions involved in resistance to pharmacological inhibitors (71), enhancers required for oncogene-induced senescence (72), or novel enhancer elements (71). CRISPR/Cas9 screens have also been extended to the identification of short-length miRNAs through combining CRISPR/dCas9 with a split-HRP-based reporter system (73). In addition, high-throughput genetic studies combined with CRISPR screens have also been performed *in vivo* by delivering gRNA and nuclease with vectors or using constitutive or inducible Cas9-expressing mouse models that only require gRNA delivery (65,74,75).

Applications in Cell Therapy

The CRISPR/Cas9 system has produced a revolution in the field of cell therapy, mainly involving immune cell therapy and stem cell therapy [42]. Treatment using *ex vivo* gene-editing T cells from patients with cancer or autoimmune diseases have shown promising results (28,76–78); one example being next-generation chimeric antigen receptor (CAR) T cells. The feasibility of using CAR T cells for targeted therapy of malignancies has been established through using electroporation of Cas9 ribonucleoproteins (RNPs) to transfect activated various targets including CXCR4, CCR5, PD-1 and CD7 in human T cells (54,76,79–82). The first clinical trials using CRISPR/Cas9-RGNs to create a PD-1 knockout in T cells have been approved for the treatment of muscle-invasive bladder cancer, castration-resistant prostate cancer, metastatic renal cancer and metastatic non-small cell lung cancer. These phase I clinical trials were commenced in 2016 (83). However, severe side effects due to cytokine release syndrome (CRS), neurotoxicity or on-target off-tumor toxicity are major obstacles for effective treatment of patients. To relieve such limitations related to T cell–based immunotherapies, CRISPR/Cas9 system was used to delete endogenous TCRs and HLA class I to generate the universal allogenic 'off-the-shelf' CAR T cells, or disrupt inhibitory receptors, such as CTLA-4 or PD-1 (76,79,84,85), or target CAR constructs to the endogenous TCR α constant locus in the primary human T cells with gene editing tools (86). Immune cell therapy will achieve greater breakthroughs in clinical application, as the US Food and Drug Administration (FDA) has approved two global CAR-T cell products targeting CD19 for the treatment of acute lymphoblastic leukemia and non-Hodgkin lymphoma in the past year.

Apart from T cells, *ex vivo* editing of induced pluripotent stem cells (iPSCs) and hematopoietic stem cells (HSCs) derived from the patient's somatic cells also likely offer the opportunity for investigating the pathophysiological mechanisms of hereditary diseases. With this technology, researchers have made advances in the treatment of β-hemoglobinopathies, hemophilia B, hearing loss, Alzheimer disease, oculopathy, cardiac diseases and other genetic diseases (Table 1). Notably, the production of organoids from engineered iPSCs has developed rapidly in recent years. Patient-specific organoids offer unprecedented opportunities for studying phenotypes manifested at the cellular level, and provides a framework for both disease modeling and regenerative medicine based on the synthetic reconstitution of tissues with physiologically relevant structural and functional features that could be transplanted into patients (87). With these organoids, the in-depth mechanisms of muscular development and disease (116), epithelial development (117) and some other genetic diseases (118,119) have been revealed. A clinical trial for age-related macular degeneration (AMD) was performed whereby autologous iPSC-derived retinal pigment epithelial (RPE) cells were transplanted into the patient's pathological tissue. Although this treatment could improve a patient's symptoms and avoid immune rejection, the non-ideal therapeutic effects and tumorigenicity of iPSCs are still serious disadvantages (120).

Applications in the Modification of Human Embryo

Gene editing in human zygotes or embryos were previously unthinkable due to the low efficiency of traditional biological technologies. With the rapid development of CRISPR technology, researchers have attempted to edit human embryos using CRISPR and made some progress. Several scientific groups in China have reported genes corrected with the CRISPR/Cas9 technique in human zygotes or 3PN embryos (121,122). Recently, Ma *et al.* (123) described the correction of a pathogenic gene mutation in human embryos using CRISPR technology. They corrected the heterozygous MYBPC3 mutation in human pre-implantation embryos with precise CRISPR/Cas9-based targeting accuracy and high HDR efficiency by activating an endogenous, germline-specific DNA repair response. Other studies were the first to use the base editor system to correct HBB (A>G) mutation within the human embryonic genome (124,125). These breakthroughs highlight the tremendous potential of correcting homozygous and compound heterozygous mutations by base editing in human embryos.

Although the gene targeting efficiencies in the above studies were quite low and were accompanied by significant off-target effects as well as mosaicism, CRISPR/Cas systems have the potential of correcting heritable mutations in human embryos. Ma *et al.* (123) reported that 72.4% of human embryos fertilized with sperm carrying the heterozygous MYBPC3 mutation could carry two copies of the non-mutated gene copy after Cas9-sgRNA proteins injected, as opposed to 47.4% in untreated embryos. Moreover, only one mosaic embryo was detected, with results showing 100% targeting efficiency based on the absence of detected heterozygous blastomeres (123,126). Notably, embryonic genome editing with a base editor showed higher efficiency: targeted deep sequencing on injected embryos revealed that 17 out of 17 (100%) or 6 out of 9 (67%) embryos carried the targeted point mutations at the target site in FANCF or DNMT3B gene, respectively (125). This supports the supposition that base editors can be beneficial for correcting genetic defects in human embryos.

In addition, the above achievements have led to the debate of human embryo genome editing regarding ethical implications, especially clinical applications (127). Although some

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Table 1. *Ex vivo* editing of induced pluripotent stem cells (iPSCs) related to genetic diseases

Associated diseases	Mutant genes	Citation
Sandhoff disease	Exosaminidase subunit beta (*HEXB*)	(88)
Long QT syndrome (LQTS)	Calmodulin 2(*CALM2*)	(89)
Duchenne muscular dystrophy	X-linked dystrophin gene (*DMD*)	(130)
Cleidocranial dysplasia (CCD)	Runt-related transcription factor 2 (*RUNX2*)	(90)
N370S GBA1 Parkinson's disease (PD)	Glucosylceramidase beta1 (*GBA1*)	(91)
Coenzyme Q10 deficiency	Coenzyme Q4(*COQ4*)	(92)
Danon disease	Lysosomal-associated membrane protein 2 (*LAMP-2*)	(93)
Glanzmann thrombasthenia	Integrin subunit alpha 2b (*ITGA2B*)	(94)
Metachromatic leukodystrophy	Arylsulfatase A (*ARSA*)	(95)
Neuronal ceroid lipofuscinoses (Batten disease)	CLN5, intracellular trafficking protein (*CLN5*)	(96)
Niemann-Pick disease, types A and C1	Sphingomyelin phosphodiesterase 1 (*SMPD1*), NPC intracellular cholesterol transporter 1 (*NPC1*)	(97)
Pelizaeus-Merzbacher disease	Proteolipid protein 1 (*PLP1*)	(98)
Pompe disease	Glucosidase alpha, acid (*GAA*)	(99)
Prader-Willi syndrome	15q11.2-q13; Various; Chr.7	(100,101)
Retinitis pigmentosa	MER proto-oncogene, tyrosine kinase (*MERTK*)	(102)
Smith-Lemli-Opitz syndrome	7-Dehydrocholesterol reductase (*DHCR7*)	(103)
Wolman disease (lysosomal acid lipase disease)	Lipase A, lysosomal acid type (*LIPA*)	(104)
Congenital neutropenia (SCN, Kostmann disease)	HCLS1-associated protein X-1 (*HAX1*)	(105)
X-linked RP (XLRP)	Retinitis pigmentosa GTPase regulator (*RPGR*); nuclear receptor subfamily 2 group E member 3(*Nr2e3*)	(106,117)
Leber congenital amaurosis (LCA)	Centrosomal protein 290 [Homo sapiens (*CEP290*)]	(107)
MEN2A	Ret proto-oncogene (*RET*)	(108)
Familial platelet disorder (FPD)	Runt-related transcription factor 2 (*Runx1*)	(109)
Parkinson's disease (PD)	Triplication of the α-synuclein (*SNCA*)	(110)
AD	Presenilin (*PSEN1*)	(111)
Hemophilia B	Coagulation factor IX (*F IX*)	(112)
Hereditary sensual deafness	Myosin VIIA (*MYO7A*); myosin XVA (*MYO15A*)	(113,114)
Retinitis pigmentosa	MEN1	(115)

institutions like the National Academy of Sciences, Engineering, and Medicine in the United States have started the support for modifying human embryos by correcting mutations that cause serious disease when no reasonable alternatives exist (128), further progress is necessary before CRISPR/Cas9 techniques can become a reasonable and safe approach for clinical applications, especially in relation to embryo genome editing.

Limitations and Prospects

Despite being effectively useful in genome modification and functional regulation in biological systems, CRISPR/Cas9 systems have also encountered various inherent challenges. Among them, the off-target effect is the foremost challenge. In the last few years, researchers have attempted to make improvements by optimizing sgRNA, or artificially modifying the protein structure of Cas9, such as the PAM structure (129). In addition, there are investigations into anti-CRISPR proteins which promise to be a useful Cas9 off-switch for avoiding undesirable off-target effects by limiting the amount of time that Cas9 is active in the nucleus (130–132).

The delivery efficiency of Cas9 into cells or tissue is another problem that is preventing successful CRISPR-mediated genome editing. Initial CRISPR-mediated genome editing approaches relied on delivering plasmids or viral vectors encoding Cas9 and sgRNAs. Adeno-associated virus (AAV) vectors, which can facilitate gene transfer and episomal expression in non-dividing cells, are the mostly used vectors in Cas9 delivery. However, its application suffers from limitations associated with the size of encapsulated transgenes, pre-existing immunity against AAV vectors and $CD8^+$ T cell-mediated adaptive response against the AAV capsid (133). Several studies have shown that the delivery format of active as9 protein/gRNA RNP complex through microinjection, liposome-mediated transfection, electroporation or nucleofection (134) has more advantages due to lower off-target effects and rapid gene editing (135,136) when compared with plasmid DNA transfection. However, the *in vivo* delivery formats of mRNA and protein pose certain technical challenges, including inability to globally deliver to the target cells or organs or trigger RNA-sensing innate immune responses in human and murine cells, leading to cytotoxicity (137). Further improvement in the performance of the CRISPR components and means of delivery is necessary to increase the efficiency of *in vivo* genome editing, such as delivering nanoparticles carrying sgRNA and Cas9 protein (138).

In conclusion, there are some difficulties that need to be overcome for effective use of CRISPR-based gene editing, especially in clinical therapy. Nevertheless, genome editing has become a powerful tool for modifying cell lines and organisms to investigate the biology and pathophysiological mechanisms of various genetic diseases. Further studies will continue in order to improve gene targeting accuracy and efficiency, delivery potentials into particular cells, tissues or organs, detection and modulation efficiencies of activity time and activity area of Cas9 *in vivo*, and prediction and treatment efficiencies of undesired mutations caused by gene editing. Another important issue is represented by the ethical concern related to the use of CRISPR technology in humans, and the appropriate ethical and regulatory guidelines that must be developed to judge the reasonable use of these tools. There is anticipation that we can integrally understand the intricacy and diversity of

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CRISPR/Cas in gene editing and thereby improve this technology.

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