Received: 7 July 2020 | Revised: 25 August 2020 | Accepted: 7 September 2020

DOI: 10.1002/jcp.30064

REVIEW ARTICLE

Journal of Cellular Physiology WILEY

CRISPR/Cas gene therapy

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Abstract

Clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated enzyme (Cas) is a naturally occurring genome editing tool adopted from the prokaryotic adaptive immune defense system. Currently, CRISPR/Cas9-based genome editing has been becoming one of the most promising tools for treating human genetic diseases, including cardiovascular diseases, neuro-disorders, and cancers. As the quick modification of the CRISPR/Cas9 system, including delivery system, CRISPR/Cas9-based gene therapy has been extensively studied in preclinic and clinic treatments. CRISPR/Cas genome editing is also a robust tool to create animal genetic models for studying and treating human genetic disorders, particularly diseases associated with point mutations. However, significant challenges also remain before CRISPR/Cas technology can be routinely employed in the clinic for treating different genetic diseases, which include toxicity and immune response of treated cells to CRISPR/Cas component, highly throughput delivery method, and potential off-target impact. The off-target effect is one of the major concerns for CRISPR/Cas9 gene therapy, more research should be focused on limiting this impact by designing high specific gRNAs and using high specificity of Cas enzymes. Modifying the CRISPR/Cas9 delivery method not only targets a specific tissue/cell but also potentially limits the off-target impact.

KEYWORDS

animal model, CRISPR/Cas9, gene therapy, genetic disease, genetic disorder, genome editing

1 | INTRODUCTION

Gene therapy is an approach for correcting or replacing an undesirable or unfunctional gene in a cell. Although it can be used in both animals and plants, usually gene therapy refers to human gene therapy. Because humans have so many genetic diseases that are caused by genetic mutation or undesirable expression of certain genes, gene therapy has great potentials to treat and even cure these genetic disorders. Thus, gene therapy has been attracting more and more attention from the scientific and pharmaceutical communities.

The gene therapy study was initiated in the later 1980s and early 1990s. In 1990, Rosenbery and colleagues reported that they used retroviral-mediated gene transduction to introduce the gene coding for resistance to neomycin into human tumor-infiltrating lymphocytes (TIL) and then infused into five patients (Rosenberg et al., 1990). Since then, more study and clinic trials on human gene therapy have been performed. However, due to many limitations, including the difficulty to precisely edit a specific gene, the gene therapy moves very slowly. Recently developed clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated enzyme (Cas) technology and its quick development is evolving the gene therapy field and makes gene therapy truly flexible for the treatment of human genetic diseases.

CRISPR is initially discovered in the genome of prokaryotic organisms, including bacteria and archaea. However, at the first almost one-decade, the scientific community did not recognize its function although the type of nucleotide sequences is widely existed in many bacteria and archaea. At that time, people treated these unique DNA

sequences as a unique feature for an individual bacterium and used them as a biomarker for genotyping (Makarova et al., 2020). As the discovery of CRISPR-associated protein (Cas) and the application of recombination DNA technology, scientists are beginning to realize that CRISPR/Cas system is an adaptive immunity of the prokaryotic defense system to the viruses, and this newly discovered CRISPR/Cas system has been quickly modified to be used as a tool for editing a specific gene in a genome (Makarova et al., 2020).

Since it was recognized in 2012 (Gasiunas et al., 2012; Jinek et al., 2012), CRISPR/Cas9 genome editing technology has been quickly developed and applied in many biological and biomedical fields. Particularly in the past 5 years, CRISPR/Cas9 technology has been significantly modified and improved for different basic and applied research purposes as well as biotechnological application in the fields of agriculture and biomedicine. This quick development includes many milestones, including the development of base editor (Gaudelli et al., 2017; Komor et al., 2016) and prime editing (Anzalone et al., 2019) technology by fusing a Cas endonuclease with other functional enzymes, such as base converter enzymes.

Due to its precision and simplify of genome editing, CRISPR/Cas9 is also employed to perform gene therapy with huge potentials, including editing, regulating, and monitoring an individual gene at the genomic and epigenomic levels (Adli, 2018). In this review, we will first briefly talk about the CRISPR/Cas9 system followed by four major focuses: (1) strategies of CRISPR/Cas9-based gene therapy, (2) CRISPR/Cas9 is a great technology for creating animal models for human genetic disorders, (3) the great potentials and application of CRISPR/Cas9-based gene therapy, and (4) preclinic and clinic application of CRISPR/Cas-based gene therapy. Finally, this review points out the major problems associated with this advanced technology and future directions.

2 | STRATEGIES OF CRISPR/CAS9-BASED GENE THERAPY

Any CRISPR/Cas strategy, which can be used to remove, replace, or correct undesirable genes that cause genetic diseases, can be used in CRISPR/Cas9-based gene therapy. Although it was recognized as a genome editing tool in 2012 (Gasiunas et al., 2012; Jinek et al., 2012), CRISPR/Cas9 technology has been evolutionarily developed in the past less than one decade, many strategies have been developed and applied in a variety of basic and applied research. Among them, gene knockin/out, base editing, and prime editing have shown incredible promising in gene therapy (Figure 1).

2.1 | CRISPR/Cas9 knockout of undesirable genes

There are many undesirable genes in organism bodies, such as oncogenes in the human genome, which include both protein-coding genes and noncoding genes. Noncoding genes are newly discovered class of functional genes, including long noncoding RNA genes and small regulatory RNA genes, such as microRNAs (miRNAs), an important gene regulator and many of them function as oncogene genes and tumor suppressor genes (B. Zhang et al., 2007). In most cases, these genes are not expressed (B. Zhang & Pan, 2009). However, as changing the environment, such as exposing to an adverse environment, for example, cancer-causing agents, these undesirable genes can be induced to express and further cause human diseases, such as cancers. Thus, the removal of these undesirable genes or disease-causing genes is one of the goals of gene therapy.

CRISPR/Cas9 genome editing tool is adopted from the prokaryotic adaptive immune defense system, in which bacteria or archaea use CRISPR/Cas9 system to cut and destroy the invaded DNA from outside of the cells, such as viruses (Makarova et al., 2020). Thus, gene knockout is the first and also well-developed application of the CRISPR/Cas9 system in genome/gene editing. When CRISPR/Cas system is delivered into a cell, the gRNAs will guide Cas enzyme to locate on a specifically targeted DNA sequence with a PAM that is complementary with the gRNA, then Cas nuclease cuts the double strands of DNA and forms a double-strand break (DSB). During the long history of evolution, cells have evolved DNA repair mechanisms for repairing damaged DNAs, including DSBs. The most common repair mechanism is to directly link the two broken DNA molecules together, termed nonhomologous DNA end joining (NHEJ). Because a single CRISPR/Cas9 cutting usually deletes a couple of nucleotides, NHEJ repairment commonly results in the frameshift change for the original DNA sequence although in some cases NHEJ repair also adds a couple of nucleotides in and causing the same consequences. The consequences of NHEJ repair is the silence of the edited genes by frameshift change or inducing nonsense mutations. Thus, the CRISPR/Cas9 system can be used to silence/knockout an undesirable gene for gene therapy.

2.2 | CRISPR/Cas9 knockin/replacement of undesirable genes

Except for the NHEJ repair mechanism, cells also have another repair mechanism, termed homology-directed recombination (HDR) repair (X. He et al., 2016; Platt et al., 2014). Unlike NHEJ repair, HDR repair requires a DNA template for using to correct the DSBs. DNA template can be endogenous or exogenous, such as inserted DNA sequences and homologous sequences in its own genome sequences. If a cell uses its homologous sequences as DNA templates, the HDR will result in DNA duplication and potentially expand genome size although it is contributed a little but more and more will cause increased genome size during the million years of evolution. If the DNA templates come from outside of the genome, it will insert a new DNA sequence into the genome and cause horizontal gene transfer. Thus, scientists use this HDR repair mechanism, as the same delivering the CRISPR/Cas9 system into a cell, a functional DNA template (e.g., gene) is also delivered into the same cell. When the CRISPR/Cas9 cuts a specific DNA sequence, the cells will use the transferred DNA sequences as a template to repair the DSB and finally insert the

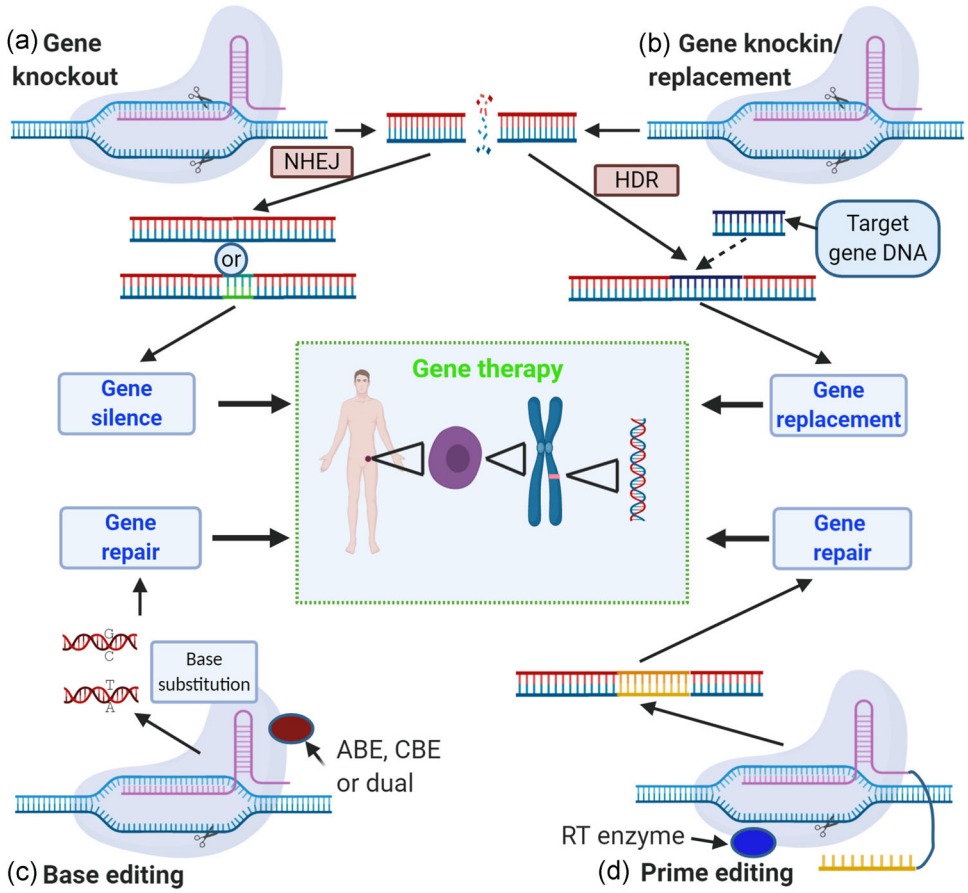

FIGURE 1 Major strategies for CRISPR/Cas9 gene therapy. Both modified Cas9 enzymes and their wildtypes, including dCas9 and nCas9, can be used to gene therapy. All four major strategies, (a) gene knockout, (b) gene knockin/replacement, (c) base editing, and (d) prime editing, can be employed to target an individual gene in our human genome to correct and/or replace the disease-related genes for gene therapy purposes

targeted DNA sequence into the genome. Using this strategy, scientists can insert (knockin) a new gene into a specific location of the genome and also can be used to replace the undesirable gene with a desirable gene.

However, compared with NHEJ, the frequency is very low for HDR repair-based genome editing, particularly this happens most likely only in the divided cells (Devkota, 2018). Thus, CRISPR/Cas9-based on gene knockin/replacement is with a low successful chance.

2.3 | Base editing to alter the point mutation

The principle of CRISPR/Cas9-based genome editing is that there are two functional domains of Cas9, one is HNH and another one is RuvC, each domain cuts one strand of the targeted DNA sequences and forms the DNA DSBs (Jiang et al., 2016; Nishimasu et al., 2014). The Cas proteins that bind to the target site are dependent on gRNA and not related to their own activities. If one or both functional domains are deactivated by changing their amino acids or through structure modification, it does not affect their binding activities. According to this principle, scientists have modified Cas endonuclease to deactivate both HNH and RuvC domains or a single domain to form deactivated Cas (dCas) or Cas nickase (nCas) for various purposes of genome editing, such as screening or monitoring purposes. More importantly and also excitingly, all Cas, dCas, or nCas proteins can be fused with other molecules, including another enzyme without impacting their own functions, including binding function and cleavage functions. Based on this, for the first time, David R. Liu's group at Harvard University developed a CRISPR/Cas base editor (BE) technology to edit a specific base by fused a base-modification enzyme with nCas9 endonuclease (Komor et al., 2016). In their study, they fused a cytidine deaminase enzyme with nCas9 to construct a CBE base editor, termed cytosine base editors (CBEs), which can convert cytidine (C) to uridine (U), and then become thymine (T), through this way they successfully obtained base editing from C to T or G to A (Komor et al., 2016). Later, scientists also developed CRISPR/Cas9 system that converts an A·T base pair to a G·C base pair by fusing adenine deaminase enzyme with nCas9, termed adenine base editors (ABEs) (Gaudelli et al., 2017). Currently, ABEs and CBEs are two classes of CRISPR/Cas DNA base editors, which can convert all four possible nucleotide changes (C to T, A

to G, T to C, and G to A) (Rees & Liu, 2018). Recently, a couple of laboratories developed a dual-deaminase CRISPR/Cas base editor combining adenine and cytosine editor, these dual-deaminase CRISPR/Cas base editors can simultaneously induce adenine and cytosine base editing (Grünewald et al., 2020; Sakata et al., 2020; X. Zhang et al., 2020).

DNA base editors have promising application in treating genetic diseases. This is because the majority of human genetic disorders are associated with a single-nucleotide change, termed as point mutation or single-nucleotide polymorphism (SNP) (Rees & Liu, 2018). Many important human genetic diseases, such as sickle-cell anemia, are associated with a point mutation. Based on a survey of the ClinVar database (Landrum et al., 2016) in 2018, among all recorded point mutations, about 47% are caused by the nucleotide pair change from A·T to G·C, followed by A·T to C·G (15.6%), C·G to T·A (13.5%), and C·G to G·C (10.6%) (Rees & Liu, 2018). At initiated study, Komor et al. (2016) show that CBEs can be employed to correct point mutations in mouse and human cell lines with 35–75% on-target editing efficiency; these point mutations are associated with cancers and Alzheimer's disease (AD). The same group also employed ABE base editing to correct point mutations associated with life-threatening blood-cell disorders, including sickle-cell anemia and hemochromatosis in human cell lines (Gaudelli et al., 2017). Following these two studies, base editing has been employed to correct different genetic disorders using human and animal cell lines as well as animal models. Geurts et al. (2020) successfully employed SpCas9-ABE (PAM recognition sequence: NGG) and xCas9-ABE (PAM recognition sequence: NGN) to genetically and functionally repair the cystic fibrosis (CF) intestinal organoid cells with low or no detected off-target effects based on whole-genome sequencing. X. Li et al. (2020) employed CRISPR/Cas9 ABE base editing to successfully correct the −124C TERT promoter single-nucleotide mutation; the corrected TERT promotor cannot bind to E26 transcription factors, that are required for TERT protein expression, and further reduced TERT gene expression, and finally induced brain tumor cell senescence and proliferative arrest. This suggests that CRISPR/Cas-based base editing can be used to treat cancers. Ryu et al. (2018) also delivered CRISPR/Cas ABE system to muscle cells in a mouse model of Duchenne muscular dystrophy (DMD) and successfully corrected a nonsense mutation on the *dmd* gene; this suggests that CRISPR/Cas genome editing can be used to treat genetic disorders in adult animals and provide successful evidence for human CRISPR/Cas gene therapy.

One of the limitation for CRISPR base editing gene therapy is the potential off-target effect. Off-target effects are a common disadvantage for CRISPR/Cas-based genome editing. Compared with the two commonly used CRISPR/Cas base editors, ABEs show less off-target effects than CBEs. One of the potential reasons is that ABE deaminase binds to its targets more weakly than the CBE deaminase. Weakening the binding forces between CBEs and its targets, by developed a higher-fidelity CBEs, reduced the off-target impact (Rees et al., 2017).

2.4 | Prime editing to correct the gene mutation

Prime editing is a new application of CRISPR/Cas genome editing technology, which was developed in 2019 by the Liu group at Harvard University (Anzalone et al., 2019). Prime editing system introduces two major changes into the traditional CRISPR/Cas9 system. First, a reverse transcriptase is fused with Cas9 nickase. Secondary, a prime editing guide RNA (pegRNA) replaces the traditional gRNA. In pegRNA, except the single gRNA containing both tracrRNA and a spacer, gRNA is also linked to a gene-specific RNA sequence containing a primer binding site (PBS) that is complementary with the target region of the edited DNA sequence (Anzalone et al., 2019). In this novel CRISPR/Cas9 system, the spacer sequence guides the CRISPR/Cas9 system to recognize and locate the DNA region where it binds to; as the traditional CRISPR/Cas system, the tracrRNA sequence is the nCas9 binding site; after nCas9 cuts the opposite strand of DNA and form a single-stranded DNA break, where the broken DNA sequence can serve as a primer to bind the PBS site in the single gRNA and synthesize a new DNA fragment using the CRISPR/Cas9-fused reverse transcriptase. Finally, the newly synthesized DNA sequence will insert and replace the target DNA sequence. Thus, prime editing can replace certain DNA sequence without a donor DNA template (Anzalone et al., 2019). Unlike base editor, prime editing can make any nucleotide change with a single system with less off-target effect. Thus, prime editing has more advantages for repairing a gene than the traditional CRISPR/Cas system or base editor. This new CRISPR/Cas9 system, prime editor, worked well in many human cell lines and mouse cortical neurons with high efficiency of gene editing (Anzalone et al., 2019). The Liu group also employed the newly developed prime editing technology to correct genetic mutations that are responsible for sickle cell disease and Tay-Sachs disease (Anzalone et al., 2019).

3 | CRISPR/CAS9 CAN BE USED TO CREATE ANIMAL MODELS FOR TREATING HUMAN GENETIC DISORDERS

One of the major applications and advantages of CRISPR/Cas genome editing technology is to create animal genetic models for studying and treating human genetic diseases. To obtain inherited traits without mosaic progeny, animal models are usually to be obtained by CRISPR/Cas-edited single-cell embryo/germline, followed quickly move the edited embryo into the female uterus and then breed into a whole animal. Compared with other genome editing method, CRISPR/Cas base editing is widely used for creating animal models for human genetic diseases (Table 1) because of its high efficiency (Rees & Liu, 2018).

Z. Liu, Lu, et al. (2018) generated mouse models harboring clinically relevant genetic mutations at the androgen receptor (*ar*) and homeobox D13 (*hoxd13*) genes by microinjection of ABE mRNA and sgRNA into one-cell mouse embryos. The *ar* and *hoxd13* mouse models can be used to study genetic diseases associated with

TABLE 1 Animal model for studying human genetic disorders created by CRISPR/Cas genome editing technology

Targeted human genetic diseases	Animals	Genes	Created method	References
Human non-small-cell lung cancers (NSCLCs)	Mouse	*EML4–ALK*		Maddalo et al. (2014)
Hepatocellular carcinoma (HCC)	Mouse	P53 and Pten	CRISPR/Cas9	Y. Liu, Qi, et al. (2017)
Androgen insensitivity syndrome (AIS)	Mouse	Androgen receptor (*ar*)	CRISPR/Cas ABE	Z. Liu, Chen, et al. (2018)
Syndactyly	Mouse	Homeobox D13 (*hoxd13*)	CRISPR/Cas ABE	Z. Liu, Chen, et al. (2018)
Hutchinson–Gilford progeria syndrome (HGPS)	Rabbit	*Lmna*	CRISPR/Cas BE3, BE4-Gam or ABE7.10	S. Liu, Wang, et al. (2018)
X-linked dilated cardiomyopathy (XLCM)	Rabbit	*Dmd*	CRISPR/Cas ABE	S. Liu, Wang, et al. (2018)
Ablepharon macrostomia syndrome (AMS)	Pig	*Twist2*	CRISPR/Cas BE3	Z. Li et al. (2018)
Oculocutaneous albinism type 1 (OCA1)	Pig	*Tyr*	CRISPR/Cas BE3	Z. Li et al. (2018)
Pompe disease	Rat	*Acid alpha-glucosidase (Gaa)*	CRISPR/Cas ABE	L. Yang et al. (2018)
Retinitis pigmentosa	African clawed frog (Xenopus laevis)	*Rhodopsin (rho)*	CRISPR/Cas9	Feehan et al. (2017)
Corneal dystrophy	Mouse	Transforming growth factor-beta-induced (TGFBI) gene	CRISPR/Cas9	Kitamoto et al. (2020)

androgen insensitivity syndrome (AIS) and Syndactyly, respectively. AIS is a rare genetic disease that usually affects sexual development before birth and during puberty.

Z. Liu, Chen, et al. (2018) employed CRISPR/Cas base editor to successfully create rabbit animal models for mimicking human Hutchinson–Gilford progeria syndrome (HGPS). HGPS is a rare genetic disease associated with infant prematurely and quickly aging after birth (Pollex & Hegele, 2004). This genetic disorder is majorly caused by a de novo point mutation, p.G608G, in *lmna* gene (Z. Liu, Chen, et al., 2018). By creating a point mutation in rabbit *Lmna* gene, Z. Liu, Chen, et al. (2018) observed the same phenotype as human HGPS, including growth retardation, short stature, bone abnormalities, and loss of subcutaneous fat. This suggests that CRISPR/Cas-generated rabbit model HGPS model, which can be used to precisely mimic the human HGPS genetic disorder.

X-linked dilated cardiomyopathy (XLCM) is an important heart disease with typical phenotype of heart failure because of the weakened cardiac muscle that prevents the heart from pumping blood efficiently. XLCM is caused by genetic mutations in the *Dmd* genes. Recently, Z. Liu, Chen, et al. (2018) generated a rabbit model for mimicking human XLCM genetic disease by inducing point mutations in rabbit *Dmd* gene by CRISPR/Cas base editing. Their rabbit T279A mutant model displayed typical clinical symptoms that are consistent with human XLCM disease but not the same as DMD (Z. Liu, Chen, et al., 2018).

Z. Li et al. (2018) employed CRISPR/Cas9 BE3 base editor to successfully generate two pig models for studying human ablepharon macrostomia syndrome (AMS) and oculocutaneous albinism type 1 (OCA1). AMS and OCA1 are caused by a point mutation in *twist2* and *tyr* genes, respectively. After transferring plasmids with CRISPR/Cas9 BE3 and sgRNA into porcine fetal fibroblasts, they obtained genome editing pigs and these pigs display the same clinical symptoms as AMS and OCA1 in human, respectively.

Pompe disease, also called glycogen storage disease type II (GSDII), is a fatal genetic disorder characterized by progressive loss of heart/skeletal muscle function. A previous study shows that Prompe disease is associated with a point mutation in acid α-glucosidase (Gaa) gene (Kroos et al., 2004). L. Yang et al. (2018) employed CRISPR/Cas ABE base editing to successfully generate a rat model for studying Pompe genetic disease, in which the rats show the similar clinic symptom as our human.

Cancers are now becoming a big global issue. Creating animal models will provide new tools for studying and treating cancers. Maddalo et al. (2014) used CRISPR/Cas9 genome editing to create a mouse model of *Eml4–Alk*-driven lung cancer. The genome-edited mice harbored the fused inverted echinoderm microtubule-associated protein-like 4 (*EML4*) and anaplastic lymphoma kinase (*ALK*) genes and displayed histopathological and molecular features typical of ALK $^{+}$ human non-small-cell lung cancers (NSCLCs).

Except creating animal models for genetic disorders by using CRISPR/Cas genome editing, there are also lots of studies to generate animal and/or human cell lines for studying the pathogenic process. For example, Dabrowska et al. (2020) recently used

CRISPR/Cas9 to generate a series of homozygous HEK293T cell lines for studying human Huntington's disease and employed the new cell lines to test therapeutic reagents. Yao et al. (2020) used CRISPR/Cas9 genome editing technology to successfully generate a Dip2A homozygous knockout 46C ESC cell line by replacing the eighth exon of *Dip2a* gene with PGK-Puro-P2A-mCherry; this CRISPR/Cas9-edited cell line can be used to study DIP2A mutation-associated abnormal brain development and diseases, including dyslexia, autism, and AD. Xu et al. (2020) generated a human embryonic stem cell line by CRISPR/Cas9 knockout of death-associated protein kinase 1 (DAPK1), which can be used to study DAPK1-related human diseases, including tumor metastasis, antiviral responses, AD, and other neurological disorders.

4 | CRISPR/CAS9 HAS GREAT POTENTIALS ON GENE THERAPY

CRISPR/Cas system is a robust and powerful tool not only for altering a gene for its expression and function, but also for screening, monitoring, and regulating those genes. Since it was considered as a genome editing tool in 2012 (Gasiunas et al., 2012; Jinek et al., 2012), CRISPR/Cas system has been widely used in studying gene function in human and/or human-related traits, particularly on human development and genetic disease-related aspects. The past 8 years of CRISPR/Cas-related studies have shown its huge potentials on gene therapy, and CRISPR/Cas9 genome editing can be employed in treating any human diseases associated with genetic mutation or genetic element change (Figure 2). Following are just several examples for the quick development and application of CRISPR/Cas9 genome editing on correcting genetic mutations associated with different human diseases.

4.1 | CRISPR/Cas potentially treats brain and neurological genetic diseases

There are many brain and nerve-related genetic diseases, including AD and Huntington disease (HD). CRISPR/Cas9 genome editing technology provides a powerful tool for discovering, studying, and treating gene targets for these genetic disorders.

AD is a progressive disorder causing brain cells to degenerate and die. Although the exact causes of AD are not fully understood, scientists believe that it is caused by that certain proteins do not work appropriately in the brain and it is caused by specific genetic changes in some cases. Apolipoprotein E4 (apoE4) is one of the most prevalent genetic risk factors of AD; recently, apoE4 is thought to be an important AD therapeutic targets in which converting apoE4 gene to either apoE2 or apoE3 may be an ideal treatment for AD (Safieh et al., 2019). CRISPR/Cas9 genome editing technology, particularly CRISPR/Cas base editing is perfect for making this change because there is a single-nucleotide difference between apoE4 and apoE3 gene (i.e., position 112 is cysteine in apoE3 and it is arginine in apoE4). Komor et al. (2016) employed CRISPR/Cas9 BE3 base editor

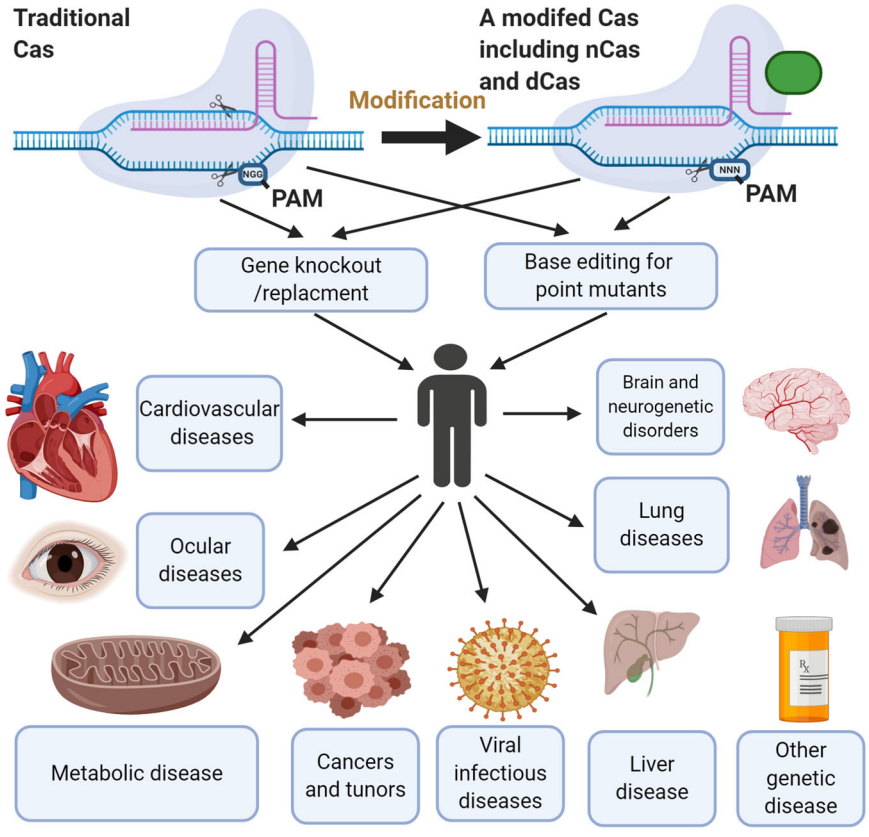

FIGURE 2 Huge potentials of CRISPR/Cas genome editing in treating human genetic diseases. Currently, CRISPR/Cas9 genome editing technology has been widely used to treat a variety of human genetic diseases, which include, but not limited to, cardiovascular diseases, brain and neurogenetic disorders, lung diseases, liver diseases, and cancers, as well as metabolic diseases and viral infectious diseases. Based on the nature of these genetic diseases, it can be achieved by CRISPR/Cas9 gene knockout or replacement; in most cases, it can also be achieved by repairing a single point mutation by base editing or prime editing

to successfully convert apoE4 to apoE3 gene in mouse astrocytes. Wadhwani et al. (2019) converted apoE4 to apoE3 in induced pluripotent stem cells from two unrelated AD patients carrying the E4 allele using CRISPR/Cas9 system. Their study also shows that apoE4 neurons were more susceptible to ionomycin-induced cytotoxicity than the unedited apoE4 neurons, suggesting that apoE4 accelerates tau pathology and neuron death in part by neuron-specific, glia-independent mechanisms (Wadhwani et al., 2019). Study also shows that reducing the ratio of apoE4 and apoE3/apoE2 may also be used to treat AD (Safieh et al., 2019). Offen et al. (2018) knocked out opoE4 using CRISPR/Cas9 system and significantly reduced the ratios of apoE4 and apoE3/apoE2. Recently, Knupp and colleagues (2020) employed CRISPR/Cas9 system to deplete SORL1 gene in hiPSCs and their result showed that SORL1-deficient hiPSC neurons displayed early endosome enlargement, a hallmark cytopathology of AD (Knupp et al., 2020). Aberrant expression of amyloid β peptide (Aβ) is associated with AD etiology; Chiu et al. (2020) recently used CRISPR/Cas9 genome editing to show that calcium and integrin-binding protein 1 (CIB1) are a potential negative regulator of Aβ production.

HD is another major devastating neurological disease, which is caused by an expanded CAG repeat in HTT gene encoding huntingtin protein (Bates et al., 2015). Inhibiting the expression of mutant HHT (mHTT) gene may be a good strategy to treat HD (Rohn et al., 2018) based on a previous RNAi study in which RNAi improved motor and neuropathological abnormalities in an HD mouse model (Harper et al., 2005). Because CRISPR/Cas9 genome editing can be quickly and robustly knockout an individual gene, it has been quickly adopted to test its capability to treat HD using in vitro cell culture and animal models (Rohn et al., 2018). Shin et al. (2016) silenced the mutant HTT in different HD patients' cell lines using CRISPR/Cas9. Monteys et al. (2017) also modified mHTT genes in HD human fibroblast cells and also in an in vivo animal model, BacHD mouse, by using CRISPR/Cas9 genome editing technology. Kolli et al. (2017) employed CRISPR/Cas9 system to target the mutant HTT gene at untranslated region upstream to the open reading frame (uORF) and the at exon1-intron boundary at the mesenchymal stem cells (MSCs) extracted from the bone-marrow of YAC128 mice carrying the transgene for HD. Their study shows that CRISPR/Cas disruption of both the uORF and exon1-intron boundary of mHTT gene inhibited the expression of mHTT gene (Kolli et al., 2017). By using a transgenic HD mouse model with mHTT overexpression (HD140Q-knockin mice), S. Yang et al. (2017) demonstrated that non-allele-specific CRISPR/Cas9-mediated mHTT gene editing could be used to efficiently and permanently eliminate polyglutamine expansion-mediated neuronal toxicity without causing mouse lethality in the adult brain. CRISPR/Cas9-disrupted mHTT gene resulted in about 50% decrease in neuronal inclusions and significantly improved lifespan and certain motor deficits in the R6/2 HD mouse model (Ekman et al., 2019). CRISPR/Cas9 knockout of PD genes PARKIN (PRKN), DJ-1 (PARK7), and ATP13A2 (PARK9) enhanced oxidative stress in independent isogenic human pluripotent stem cell (hPSC) line; knockout of PARKIN also increased the death of dopaminergic neurons (Ahfeldt et al., 2020).

4.2 | CRISPR/Cas potentially treats human immunodeficiency virus/acquired immunodeficiency syndrome (HIV/AIDS)

Although it has been identified for more than 30 years, the HIV remains one of the most serious infectious diseases in the world, particularly in African countries. HIV attacks human immune cells and finally leads to the AIDS. No like other viruses, such as flu, there is still no vaccine available to protect people from HIV infection although significant efforts have been offered by the scientific and industrial communities in the past two decades. No other treatment is available either for getting off HIV from human body although HIV-infected people can take HIV medicine to stop the progression of AIDS. However, the HIV medicine is to be taken daily and is also expensive. Recently developed CRISPR/Cas9 provides new hopes for against HIV and treating human AIDS. As HIV gene is regulated by the long terminal repeat (LTR) promotor when HIV genome is inserted into the host genome, LTR is becoming a target region for HIV treatment (Dampier et al., 2014). In 2013, a study suggests that CRISPR/Cas9 genome editing can be a useful tool for treating HIV infection (Ebina et al., 2013), in which they show that CRISPR/Cas9 system can be used to efficiently cleave and mutate LTR sequences in HIV genome and further affected HIV infection. This is the first report on CRISPR/Cas9 application on treating HIV and these results were further confirmed by subsequent studies (Gao, Fan, et al., 2020; Hu et al., 2014; Zhu et al., 2015). The study performed by Z. Wang, Pan, et al. (2016) also show that certain CRISPR/Cas9-derived mutations in HIV inhibited HIV replication and further caused lethal for HIV. After analyzing 269 HIV-1-infected samples in the Drexel CARES Cohort and the Los Alamos National Laboratory HIV database, Sullivan and colleagues *in silico* found that D-LTR-P4–227913 (package of the top 4 gRNAs) are accounted for all detectable genetic variation within the HIV-1 viral quasispecies (vQS), which can serve the CRISPR/Cas target site for curing AIDS patients (Sullivan et al., 2019). Recently, by designing a single gRNA targeting LTR in HIVs, Gao, Fan, et al. (2020) inhibited all tested HIV infection in T cell cultures by using the CRISPR/Cas12a genome editing system. By designing right gRNAs, Kaminski, Chen, et al. (2016) completely removed the entire genome of HIV genome between 5′ and 3′ LTRs of integrated HIV-1 proviral DNA copies from latently infected human $CD4^+$ T-cells. Their results also show that persistent coexpression of CRISPR/Cas components in HIV-eradicated T-cells protected them from new HIV infection (Kaminski, Chen, et al., 2016).

Except LTR regions, other gene sequences in HIV genome were also targeted to test the HIV activities. Zhu et al. (2015) designed 10 gRNAs targeting 10 HIV sites, including 3 in the LTR, 5 in the *pol* gene, and 2 in the second exon of *tat/rev*. After transferring these gRNAs and humanized Cas9 enzyme into JLat10.6 cells harboring the full-length viral DNA, they found that all gRNAs worked and each target sit in HIV DNA sequence was efficiently edited; the expression of targeted HIV gene was significantly reduced and the HIV production was reduced up to 20 folds (Zhu et al., 2015). Kaminski, Bella, et al. (2016) targeted the HIV DNA sequences within the 5′LTR

and the *Gag* gene for removing critically important segments of the HIV DNA in transgenic mice and rats encompassing the HIV-1 genome by using CRISPR/Cas9; their results show that CRISPR/Cas9 successfully removed the critical segment of HIV DNA sequences that were observed in all examined tissues, including the spleen, liver, heart, lung, and kidney, as well as in circulating lymphocytes. The DNA knockout mice and rats displayed a significant expression level of HIV genes (Kaminski, Bella, et al., 2016); this is the first time to demonstrate that CRISPR/Cas9 technology can be used to eliminate HIV DNA sequences from in vivo mouse and rat models harboring integrated copies of HIV genome. Yin et al. (2017) further demonstrated the feasibility of excising HIV-1 provirus in three different animal models using CRISPR/Cas9 genome editing technology by delivering the CRISPR/Cas9 system using AAV. Their study show that the HIV sequence was successfully removed from the animal models, including the liver, lung, brain, spleen, heart, and colon (Yin et al., 2017).

Studies also show that genome editing HIV-1 co-receptors in host genome can be used to prevent and/or cure HIV infection (Allen et al., 2018). Co-receptors, such as C-C chemokine receptor type-5 (CCR5), is required for HIV-1 to infect susceptible target cells efficiency, these co-receptors are usually transmembrane proteins and mediates HIV-1 entry into target cells (Bleul et al., 1997). W. Wang et al. (2014) knocked out CCR5 gene in HIV-1 susceptible human $CD4^+$ cells by lentiviral vectors expressing Cas9 and CCR5 sgRNAs; CCR5 gene knockout cells were not only resistant to R5-tropic HIV-1, including transmitted/founder (T/F) HIV-1 isolates, but also had selective advantage over wild-type cells during R5-tropic HIV-1 infection. Z. Liu, Chen, et al. (2017) also demonstrated that CRISPR/Cas9 knockout of HIV coreceptors CCR5 and CXCR4 protected T cells from HIV-1 infection. There are other studies showing that silencing CCR5 or CXCR4 gene by CRISPR/Cas9 enhanced host cell resistance to HIV infection (Hou et al., 2015; S. Liu, Wang, et al., 2018; Xiao et al., 2019; Xu et al., 2017).

LEDGF/p75, encoded by the PSIP1 gene, is a cofactor for integrase and is essential for integrating HIV genome into host genome. Lampi et al. (2019) employed CRISPR/Cas9 to site-specifically edit PSIP1 locus to covert the aspartic acid residue in position 366 to asparagine (D366N); the edited cells disrupted the interaction with HIV and inhibited HIV infection but retained LEDGF/p75 cellular function.

miRNAs are an extensive class of small regulatory RNAs, which play versatile roles in almost all biological process, including cancer and various diseases (B. Zhang & Farwell, 2008; B. Zhang et al., 2007). During their functions in host cell responding to and regulating HIV infection (Su et al., 2018), miRNAs are also becoming a target for CRISPR/Cas9 gene editing for treating HIV infection. It was shown that T cells received enhanced resistance to HIV infection by CRISPR/Cas9-disrupting miR-146a in HIV-1 infected MT2 cells, in which miR-146a knockout significantly increased the expression of cytokines and HIV-1 restriction factors and reversed T cell exhaustion markers expression, and further influenced HIV-1 replication (Teng et al., 2019).

All these in vitro and in vivo studies demonstrate the feasibility of CRISPR/Cas9 genome editing on removing HIV genome sequences and reducing the infection of HIV in an individual cell or entire animal body.

4.3 | CRISPR/Cas potentially treats cardiovascular diseases (CVDs)

According to the statistics of the World Health Organization (WHO), CVDs rank number one globally, causing high deaths annually than any other diseases. An estimated 17.9 million people died from CVDs in 2016, representing 31% of all global deaths (https://www.who.int/news-room/fact-sheets/detail/cardiovascular-diseases-(cvds)). Thus, CVDs have attracted more and more attentions form scientific and industrial communities. Because many factors associating with CVDs are related to genetic controls, CRISPR/Cas9 genome editing is also given promising hope for treating CVDs.

It is well-known that high blood lipid level is linked to CVDs; reduced blood lipid levels significantly reduced the risk of CVDs. Proprotein convertase subtilisin/kexin type 9 (PCSK9), initially known as neural apoptosis-regulated convertase 1, plays an important role in the regulation of cholesterol homeostasis (Bergeron et al., 2015). High level of PCSK9 is associated with high hepatic low-density lipoprotein (LDL) cholesterol and premature cardiovascular disease. Reduced the levels of PCSK8 also reduced the risk of cardiovascular diseases (Bergeron et al., 2015). Thus, PCSK9 has become a therapeutic target for CVDs. In 2014, Ding et al. (2014) employed adenovirus to deliver CRISPR/Cas9 system to target *pcsk9* gene in mouse liver; they found that the mutagenesis rate of *pcsk9* was reached to 50% in the liver after 3 days of administrated the CRISPR/Cas9 system which led to a decrease of plasma PCSK9 levels, an increase of hepatic LDL receptor levels, and further a decrease in plasma cholesterol levels by 35%–40%. Following this study, the same research group employed the same CRISPR/Cas technology to target the human *pcsk9* gene in chimeric liver-humanized mice bearing human hepatocytes, and the results further demonstrated that CRISPR/Cas9 induced high on-target mutagenesis (approaching 50%) in the *pcsk9* gene that further reduced the blood levels of human PCSK9 protein with minimal off-target mutagenesis (X. Wang, Raghavan, et al., 2016). Chadwick et al. (2017) also reported that they successfully knocked out *pcsk9* gene in a sequence-specific fashion in the liver in adult mice by using CRISPR/Cas9-based editing. In their genome-edited adult mice, the levels of PCSK9 protein were reduced by more than 50% in plasma and the plasma cholesterol levels were also reduced by 30% (Bergeron et al., 2015). All these suggest that CRISPR/Cas9 can be a therapeutic tool for inhibiting PCSK9 protein levels in the blood and further reduce the risk of cardiovascular diseases.

Recent studies suggest *angptl3* gene is another molecular therapeutic target for CVD gene therapy. *Angptl3* gene is a hepatocyte-specific gene, which encodes angiopoietin-like protein 3. After angiopoietin-like protein 3 is synthesized, it is secreted into the

blood stream and affects the triglyceride levels in the blood (Musunuru & Kathiresan, 2017). Loss-of-function mutations of *angptl3* gene reduced the levels of LDL cholesterol, HDL cholesterol, and triglyceride in human plasma (Musunuru et al., 2010). By employing CRISPR/Cas9 BE3 base editor, Chadwick et al. (2018) silenced *angptl3* gene in 5-week-old male C57BL/6J mice. After 6 days of treatment, all BE3-*Angptl3*-treated mice had significantly lower levels of plasma ANGPTL3 (49%), triglycerides (31%), and cholesterol (19) compared with the wild-type controls (Chadwick et al., 2018). This suggests that CRISPR/Cas9 knockout of *angptl3* gene has the potential to reduce the risk of CVDs.

Other genes are also the potential targets for CRISPR/Cas9 to treat CVDs. By designing suitable gRNAs, Limpitikul et al. (2017) inhibited the expression of calmodulin 2 (*calm2*) without changing the expression of *calm1* and *calm3* by CRISPRi technology in the iPSC, which potentially prevents cardiac arrhythmias due to long QT syndrome (LQTS). Their study also shows that CRISPRi technology can be used to selectively target any *calm* genes for any calmodulinopathy (Limpitikul et al., 2017). Mutations in the β-globin (HBB) gene can be used to treat β-hemoglobinopathies, such as sickle cell disease and β-thalassemia, that affects millions of people in the world. Dever et al. (2016) employed CRISPR/Cas9 system with an HBB DNA template to successfully repair the HBB gene mutation in hematopoietic stem cells with more than 90% targeted integration of HBB gene template. They also used CRISPR/Cas9 to successfully correct the Glu6Val mutation that is responsible for sickle cell disease (Dever et al., 2016).

4.4 | CRISPR/Cas potentially treats cancers

Cancer is a mess of abnormal cells that grow quickly beyond their usual boundaries and further invade the adjoining parts of the body including spreading to other organs. Cancer is the second leading cause of death globally. According to the statistics of the World Health Organization, cancer caused about 9.6 million deaths in 2018 and about 1 in 6 deaths is caused by cancers in the world. Thus, cancer treatment is an urgent need and has a huge market in medicine. Although traditional cancer treatments through chemotherapy, radiation, and surgery have obtained great process, and significantly enhanced the survival rate of cancer patients, it still have not reach the level we hope, and new strategies and/approaches are needed to precisely treat a wide range of cancers.

The majority of cancers are caused by the changes of genetic information, majorly by genetic mutation and/or aberrant expression of certain genes, particularly oncogenes, which include epigenetic change. This provides a tool for gene therapy for treating cancers. The rapid development of CRISPR/Cas9 genome editing technology is becoming a robust tool for altering a specific gene sequence with high efficiency and thus is becoming a promising tool for cancer treatment.

First, CRISPR/Cas9 is becoming a powerful tool for high-throughput screening for target genes for cancer therapy, in which gRNA libraries were used to target many potential genes for loss of function for studying the cell responses, such as accelerated metastasis, influencing immune response and drug resistance. Using CRISPR/Cas9 high-throughput screening technology, Hart et al. (2015) demonstrated that context-dependent fitness genes accurately recapitulate pathway-specific genetic vulnerabilities induced by known oncogene, and new cancer therapeutic targets were identified for cancer treatment. Using CRISPR/Cas9 screening, Shi et al. (2015) identified six known drug targets and 19 additional dependencies in murine acute myeloid leukemia cells. Using genome-wide CRISPR/Cas9 screening, Yau et al. (2017) identified genes affecting the tumor xenograft growth in human colorectal cancer cell lines harboring KRAS mutant or its wild-type cell line; they also found that the chromatin remodeling protein INO80C was a novel tumor suppressor gene in colorectal and pancreatic tumor xenografts with KRAS mutation. Using multi-phenotype CRISPR/Cas9 screening, Gurusamy et al. (2020) identified p38 kinase as a target for adoptive immunotherapies in anticancer T-cells; inhibiting p38 improved the efficacy of mouse antitumor T cells and the inhibition of p38 also enhanced the functionalities of human tumor-reactive and gene-engineered T cells. To show the powerful potentials of CRISPR/Cas9 screening, Behan et al. (2019) employed CRISPR/Cas to genome-wide screen potential therapeutic targets in 324 human cell lines from 30 types of cancers and a promising result was achieved, including the identification of Werner syndrome ATP-dependent helicase (WRN) as a promising new synthetic lethal target in tumors from multiple cancer types with microsatellite instability (Behan et al., 2019).

CRISPR/Cas9 technology also directly studied the treatment of cancers using animal models and human cancer cell lines. KRAS is a well-studied oncogene in various human cancers and always serve as a therapeutic target for cancer treatment. CRISPR/Cas9 knockout of KRAS oncogene inhibited the cancer cell proliferation in HEK293T cancer cell lines; CRISPR/Cas9 inhibition of KRAS gene also suppressed tumor growth in immunodeficient mice (Kim et al., 2018). By using CRISPR/Cas9 technology, Gao, Ouyang, et al. (2020) demonstrated that selective targeting of the KRAS oncogenic mutant allele efficiently inhibited tumor cell development in HEK293T, S549, and H228 cancer cell lines. This phenomenon was also observed in another study by CRISPR/Cas9 inhibition of KEAR oncogenic alleles (Lee et al., 2018).

Epidermal growth factor receptor (EGFR) gene encodes a transmembrane protein that is a member of the ErbB family of receptors. Many studies show that aberrant expression of EGFR gene are associated with lung cancer oncogenesis (Giaccone, 2005; Ohsaki et al., 2000). NSCLC, a major type of lung cancer, has about 15% of it associated with EGFR mutations that play a critical role in lung cancer progression. By using CRISPR/Cas9, selective disruption of EGFR oncogenic mutant allele significantly reduced survival rate of cancer cells and reduced tumor size in a xenograft mouse model of human lung cancer (Koo et al., 2017).

Many other genes were also targeted by CRISPR/Cas9 genome editing technology to study the potentials for cancer gene therapy.

The p53 mutation and aberrant expression of Pten gene are two common events in Hepatitis B virus (HBV) infection-related hepatocellular carcinoma (HCC). To study the role of p53 and Pten in HCC development, Y. Liu, Qi, et al. (2017) disrupted their expression by in vivo hydrodynamic tail vein injection of CRISPR/Cas9 system into adult HBV transgenic mice, their results demonstrated that CRISPR/Cas9-mediated p53 and Pten somatic mutation accelerated hepatocarcinogenesis in adult HBV transgenic mice. Gao and Liu (2017) also show that CRISPR/cas9 knockout of *Pten* gene with Nras knockin induced HCC and hepatic lipid accumulation in mice. Singhal et al. (2020) demonstrated that CRISPR/Cas9 knockout of Ral-interacting protein (RLIP) gene inhibited the proliferation of breast cancer cells both in vitro and in vivo. CRISPR/Cas9 knockout of HBV surface antigen (HBsAg) gene inhibited proliferation and tumorigenicity of HBV-positive HCCs (Song et al., 2018). S. Zhang et al. (2019) successfully knocked *NSD1* out by CRISPR/Cas9 and their result show that genome-edited *NSD1* mutation inhibited cell proliferation, migration, and invasion in human HCC.

4.5 | CRISPR/Cas potentially treats ocular diseases

Ocular diseases are one big class of diseases associated with eye disorders, which affect daily life for millions of people worldwide. The worst ocular diseases can lead to severe vision impairment or complete blindness (Yu & Wu, 2020). Thus, ocular diseases have been attracting more and more attention from scientific and biomedical communities as well as industries. Because many eye disorders are associated with genetic elements, such as retinitis pigmentosa (RP), Leber congenital amaurosis (LCA2), X-linked retinoschisis, and choroideremia, CRISPR/Cas9-based gene therapy also has huge potential for treating genetics-related ocular diseases.

RP is the most common disorder causing blindness and many genes were already identified that can cause RP (Daiger et al., 2007). Among these genes, the Rhodopsin (rho) gene is most prominently associated with the autosomal recessive RP (arRP) (Ayuso et al., 1995), which accounted for 15% of retinal degenerations and 25% of adRP. Thus, *rho* gene has become a promising target for treating RP. The *rho* gene encodes a rod receptor protein that is involved in sense light and initiate the phototransduction cascade in rod photoreceptors by binding to retinal (Sung & Chuang, 2010). There are many genetic mutations in *rho* gene; among these mutations, the p.Pro23Hist (P23H) is the most frequent mutation and P23H mutation alone accounts for ~10% of the adRP cases in the North America (Giannelli et al., 2018). Latella et al. (2016) successfully knocked down the P23H *rho* mutation in HeLa cells engineered to constitutively express the P23H mutant *rho* allele in vitro and also in vivo in P23H RHO transgenic mouse retina. Recently, Giannelli et al. (2018) used adeno-associated viruses (AAVs) for delivering CRISPR/Cas9 system to selectively target the P23H *rho* mutant allele for treating RP. Their results demonstrated that CRISPR/Cas9 can cleave P23H mutation with a high efficiency but not the *rho* wild-type allele and the genetic editing was sufficient to slow photoreceptor degeneration and improve retinal functions (Giannelli et al., 2018). Additionally, Bakondi and colleagues (2016) successfully knocked out S334ter mutation of *rho* gene in rats that model severe autosomal dominant RP; *rho*(S334) also play an important role in retinal degeneration and improved visual function (Bakondi et al., 2016).

Aniridia is a rare eye disorder; aniridia is generally caused by the mutations in the paired box 6 (pax6) gene. The small eye (Sey) mouse is a widely accepted model for aniridia, which carries a spontaneous point mutation causing a premature stop codon in exon 8 of the *Pax6* gene (Mohanna et al., 2020). Recently, Mohanna et al. (2020) used CRISPR/Cas technology to successfully correct the *sey* mutation in both in vitro and in vivo germline in a novel mouse model of aniridia. A recent study by Y. Yang et al. (2020) demonstrated that CRISPR/Cas9 knockout of PDGFRβ attenuated patient vitreous-induced Akt activation and cellular responses intrinsic to proliferative vitreoretinopathy.

5 | PRECLINIC AND CLINIC APPLICATION OF CRISPR/CAS-BASED GENE THERAPY

Its precisely correcting an individual gene makes the huge potentials of CRISPY/Cas9 gene therapy. Since it was employed to edit a gene in human cells, it has been attracting attention from the scientific, biomedicine and industrial fields. In 2016, it was reported that the first clinic trial (Phase 1) for CRISPR/Cas9 gene therapy (NCT02793856), in which Sichuan University, with collaboration with Chengdu MedGenCell Co. Ltd., employed CRISPR/Cas9 technology to knock *PD-1* gene out for engineered T cells for treating metastatic NSCLC. Up to now, more than 30 clinic trials (Table 2) have been registered based on the public available clinic database ClinicalTrials.gov. The majority of CRISPR/Cas9 gene therapy clinic trails are focused on human cancers; however, there are also clinic trails focusing on blood and eye diseases, including sickle cell disease and Rubinstein-Taybi Syndrome. The majority of registered CRSIPR/Cas9 clinic trials came from China; and some are also from the United States, Australia, French, Canada, Germany, and Spain. Most trails in China are not collaborative trails whereas other CRISPR/Cas9 clinic trails are majorly collaborative trails involved in multiple locations and in multiple countries.

The first step of CRISPR/Cas9 gene therapy is to test and make sure this advanced technology is sure with high efficacy. Recently, Stadtmauer et al. (2020) reported the results on their phase 1 clinical trials of using CRISPR/Cas9 genome editing technology to treat human cancers, in which they isolated immune T cell from the patients and disrupted three genes (TRAC, TRBC, and PDCD1) for improving T cell antitumor immunity in vitro; they also induced a cancer-targeting gene, NY-ESO-1, into the T cell; finally, they put back the engineered T cell into the same patients and showed promising results with high efficiency, technical safety, and feasibility in three patients with advanced cancers (Stadtmauer et al., 2020).

TABLE 2 List of registered CRISPR/Cas-based gene therapy clinic trials

Trial registration number	Target disease	Target gene	Clinic phase	Target cell and delivery method	Gender	Age	Established dates	Location of the performed trails
NCT03545815	Solid tumor, adult	PD-1 and TCR	I	Anti-mesothelin CAR-T cells; cells will be infused on Day 0	All	18–70 years (adult, older adult)	6/1/2018–6/30/2020	Chinese PLA General Hospital, Beijing, China
NCT04178382	Severe sepsis		NA	Diagnostic test: PCR-CRISPR / Cas12a detection	All	18 years and older (adult, older adult)	08/01/2019–08/30/2020	The Affiliated Drum Tower Hospital, Medical School of Nanjing University, China
NCT03057912	Human papillomavirus-related malignant neoplasm	HPV16/18	1	CRISPR/Cas9	F	18–50 years (adult)	1/15/2018–1/15/2019	First Affiliated Hospital, Sun Yat-Sen University, China
NCT04074369	Tuberculosis, pulmonary		NA	Diagnostic test: CRISPR-based test	All	18–80 years (adult, older adult)	05/01/2019–10/30/2019	Huashan Hospital, Shanghai, Shanghai, China
NCT04426669	Metastatic gastrointestinal cancers treated with tumor infiltrating lymphocytes	CISH	I/II	T cell, CRISPR/Cas9	All	18–70 years (adult, older adult)	5/15/2020–10/31/2022	Masonic Cancer Center, University of Minnesota, Minneapolis, Minnesota, United States
NCT03164135	HIV-1-infection	CCR5	NA	$CD34^+$ hematopoietic stem/progenitor cells from the donor are treated with CRISPR/Cas9 before transplantation into the patient	All	18–60 years (adult)	5/30/2017–5/20/2021	307 Hospital of PLA (Affiliated Hospital of Academy to Military Medical Sciences), Beijing, Beijing, China
NCT03399448	Multiple myeloma, melanoma, synovial sarcoma, myxoid/round cell liposarcoma	TCR, PD-1, and NY-ESO-1	I	NY-ESO-1 redirected autologous T cells with CRISPR-edited endogenous TCR and PD-1	All	18 years and older (adult, older adult)	9/5/2018–2/29/2020	University of Pennsylvania, Philadelphia, Pennsylvania, United States
NCT04037566	Acute lymphocytic leukemia (ALL) in relapse, acute lymphocytic leukemia (All) refractory, lymphoma, B cell, $CD19^+$	HPK1	I	Autologous T cells engineered to specify CD19 transduced with a lentiviral vector and electroporated	All	18–55 years (adult)	8/1/2019–8/31/2021	Xijing Hospital, Xi'an, Shannxi, China

(Continues)

TABLE 2 (Continued)

Trial registration number	Target disease	Target gene	Clinic phase	Target cell and delivery method	Gender	Age	Established dates	Location of the performed trails
				with CRISPR guide RNA to disrupt the expression of endogenous HPK1 administered by IV injection				
NCT03167450	Sickle cell disease		NA	Examining the knowledge, attitudes, and beliefs of sickle cell disease patients, parents of patients with sickle cell disease, and providers toward the integration of CRISPR in clinical care	All	18 years and older (adult, older adult)	1/16/2020–6/30/2020 (Suspended)	National Human Genome Research Institute (NHGRI), Bethesda, Maryland, United States
NCT03655678	β-Thalassemia, thalassemia, genetic diseases, inborn, hematologic diseases, hemoglobinopathies	BCL11A	I/II	CTX001 (autologous $CD34^+$ hHSPCs modified with CRISPR-Cas9 at the erythroid lineage-specific enhancer of the BCL11A gene). Subjects will receive a single infusion of CTX001 through a central venous catheter	All	2–35 years (child, adult)	9/14/2018–5/31/2022	Stanford University (USA), Columbia University (USA), The Children's Hospital at TriStar Centennial Medical Center/Sarah Cannon Center for Blood Cancers USA), Hospital for Sick Children (Canada), BC Children's Hospital (Canada), University Hospital Regensburg (Germany), University Hospital Tübingen (Germany). Bambino Gesu (Italy), and Imperial College Healthcare (UK)
NCT04244656	Multiple myeloma	BCMA	I	CTX120 B-cell maturation antigen (BCMA)-directed T-cell immunotherapy comprised of allogeneic T cells genetically modified ex vivo using CRISPR-Cas9 gene-editing components	All	18 years and older (adult, older adult)	1/22/2020–1/31/2027	3 locations in USA, 2 locations in Australia, and one location in Spain
NCT04438083	Renal cell carcinoma		I	CTX130 CD70-directed T-cell immunotherapy comprised of allogeneic T cells genetically modified ex vivo using CRISPR-Cas9 gene-editing components	All	18 years and older (adult, older adult)	6/16/2020–4/30/2027	Melbourne, Victoria, Australia

TABLE 2 (Continued)

Trial registration number	Target disease	Target gene	Clinic phase	Target cell and delivery method	Gender	Age	Established dates	Location of the performed trails
NCT04035434	B-cell malignancy, non-Hodgkin lymphoma, B-cell lymphoma		I/II	CTX110 (CD19-directed T-cell immunotherapy comprised of allogeneic T cells genetically modified ex vivo using CRISPR-Cas9 gene-editing components, followed by IV infusion	All	18 years and older (adult, older adult)	7/22/2019–8/31/2026	Multiple (7) locations in USA, Australia, and Germany
NCT03745287	Sickle cell disease, hematological diseases, hemoglobinopathies	BCL11A	I/II	CTX001 (autologous $CD34^+$ hHSPCs modified with CRISPR-Cas9 at the erythroid lineage-specific enhancer of the BCL11A gene). Subjects will receive a single infusion of CTX001 through a central venous catheter	All	12–35 years (child, adult)	11/27/2018–05/31/2022	10 locations in USA, Belgium, Canada, Germany, and Italy
NCT03081715	Esophageal cancer	PD-1		Programmed cell death 1(PD-1) gene will be knocked out by CRISPR Cas9 followed by cell infusion	All	18–80 years (adult, older adult)	3/14/2017–2/28/2018	Hangzhou Cancer Hospital, Hangzhou, Zhejiang, China
NCT03342547	Gastrointestinal infection		NA	CRISPR screen for host factors associated with norovirus infections in stem cell-derived human intestinal enteroid model	All	18 years and older (adult, older adult)	4/18/2018–12/31/2020	Endoscopy Centre, Prince of Wales Hospital, Hong Kong, China
NCT03728322	Thalassemia	HBB	Early phase 1	iHSCs modification and intravenous injection	All	2–60 years (child, adult)	1/1/2019–1/31/2020	No list
NCT04208529	β-Thalassemia, thalassemia, sickle cell disease, hematologic diseases, hemoglobinopathies, genetic diseases, inborn, sickle cell anemia			A long-term follow-up study in subjects who received CTX001	All	18 years and older (adult, older adult)	2/1/2021–9/31/2039	Multiple locations in Germany, Italy, United States
NCT03747965	Solid tumor, adult	PD-1	I	PD-1 CRISPR/Cas9-edited cells will be infused one day after the completion of	All	18–70 years (adult,	11/1/2018–5/1/2020	Chinese PLA General Hospital, Beijing, Beijing, China

(Continues)

TABLE 2 (Continued)

Trial registration number	Target disease	Target gene	Clinic phase	Target cell and delivery method	Gender	Age	Established dates	Location of the performed trails
				conditioning regimen of paclitaxel and cyclophosphamide		older adult)		
NCT03398967	B-cell leukemia, B-cell lymphoma	CD19/20/22	I/II	Universal dual specificity CD19 and CD20 or CD22 CAR-T cells	All	12–70 years (child, adult, older adult)	1/2/2018–5/20/2022	Chinese PLA General Hospital, Beijing, Beijing, China
NCT03166878	B-cell leukemia, B-cell lymphoma	CD-19	I/II	The universal CRISPR-Cas9 gene-editing CAR-T cells targeting CD19(UCART019) will be administered by i.v. injection over 20–30 minutes as a using a "split dose" approach to dosing: 10% on Day 0, 30% on Day 1, and 60% on Day 2	All	12–75 years (child, adult, older adult)	6/1/2017–5/31/2022	Chinese PLA General Hospital, Beijing, Beijing, China
NCT04417764	Advanced hepatocellular carcinoma	PD-1	I	The PD-1 knockout engineered T cells are prepared from autologous origin using CRISPR Cas9 technology. The patients receive 3 or more cycles of PD-1 knockout engineered T cells infusion by percutaneous fine needle liver puncture with a 4-weeks interval. A total of 1 to 3×10^9 PD-1 edited T cells will be infused each cycle	All	18–70 years (adult, older adult)	6/20/2019–6/19/2021	The 3rd Xiangya Hospital of Central South University, Changsha, Hunan, China
NCT03855631	Kabuki syndrome 1	MLL4		Intervention on primary cultured cells. The intervention includes primary cultured cells, reprograming them into mesenchymal stem cells and CRISPR/Case9 gene therapy treatment on patients' cells	All	6 years and older (child, adult, older adult)	2/22/2019–3/31/2020	Arnaud de villeneuve Hospital, Montpellier, Herault, France

TABLE 2 (Continued)

Trial registration number	Target disease	Target gene	Clinic phase	Target cell and delivery method	Gender	Age	Established dates	Location of the performed trails
NCT02863913	Invasive bladder cancer stage IV	PD-1	I	A dose-escalation phase I trial of PD-1 knockout engineered T cells for the treatment of muscle-invasive bladder cancer	All	18–75 years (adult, older adult)	9/1/2016–9/31/2019	Peking University First Hospital, Beijing, Beijing, China
NCT02867345	Hormone refractory prostate cancer	PD-1	I	Peripheral blood lymphocytes will be collected and pogrammed cell death protein 1(PDCD1) gene will be knocked out by CRISPR Cas9 in the laboratory (PD-1 Knockout T cells). The lymphocytes will be selected and expanded ex vivo and infused back into the patients	M	45–85 years (adult, older adult)	11/1/2016–12/31/2020	Peking University First Hospital, Beijing, Beijing, China
NCT02867332	Metastatic renal cell carcinoma	PD-1	I	Peripheral blood lymphocytes will be collected and programmed cell death protein 1(PDCD1) gene will be knocked out by CRISPR Cas9 in the laboratory (PD-1 knockout T cells). The lymphocytes will be selected and expanded ex vivo and infused back into the patients	All	18–75 years (adult, older adult)	11/1/2016–11/30/2020	Peking University First Hospital, Beijing, Beijing, China
NCT02793856	Metastatic non-small-cell lung cancer	PD-1	I	PD-1 CRISPR/Cas9 knockout T cells	All	18–70 years (adult, older adult)	8/26/2016–1/31/2020	West China Hospital, Sichuan University, Chengdu, Sichuan, China
NCT03044743	Stage IV gastric carcinoma, Stage IV nasopharyngeal carcinoma, T-cell lymphoma stage IV, Stage IV adult hodgkin lymphoma, Stage IV diffuse large B-cell lymphoma	PD-1	I/II	PD-1 knockout EBV-CTL cells in treating EBV (Epstein-Barr virus) positive advanced-stage malignancies	All	18–75 years (adult, older adult)	4/7/2017–3/31/2022	Nanjing Drum Tower Hospital, Nanjing, Jiangsu, China

(Continues)

TABLE 2 (Continued)

Trial registration number	Target disease	Target gene	Clinic phase	Target cell and delivery method	Gender	Age	Established dates	Location of the performed trails
NCT03872479	Eye diseases	CEP290	I/II	Evaluate the safety, tolerability, and efficacy of a single escalating dose of AGN-151587 (EDIT-101) administered via subretinal injection in participants with LCA10 caused by a homozygous or compound heterozygous mutation involving c.2991+1655A>G in intron 26 of the CEP290 gene ("LCA10-IVS26")	All	3 years and older (child, adult, older adult)	9/26/2019–3/22/2024	Bascom Palmer Eye Institute, Massachusetts Eye and Ear Infirmary, W.K. Kellogg Eye Center—University of Michigan, Casey Eye Institute—OSHU, USA
NCT04122742	Rubinstein-Taybi syndrome	CREBBP		Correction of CREBBP mutations in SRT patients by CrispR-Cas9	All	10 years and older (child, adult, older adult)	10/8/2019–11/30/2020	Centre Hospitalier Universitaire de Bordeaux, Talence, France

Abbreviation: NA, not applicable.

Among the three patients, two patients are with advanced refractory myeloma and one is with metastatic sarcoma. The edited T cells engrafted at stable levels for at least 9 months in all three patients; during this clinic trail, they did not see off-target effect and clinical toxicities associated with CRISPR/Cas9 gene therapy (Stadtmauer et al., 2020).

6 | CONCLUDING REMARKS AND FUTURE PERSPECTIVES

Currently, CRISPR/Cas9 genome editing technology has been revolutionizing biomedical research and gene therapy. Many experimental researches showed that CRISPR/Cas9 can precisely edit/replace an individual gene and also can be employed to genome widely screen targets for therapeutic purposes. More than dozens of clinic trails have been approved for treating a variety of genetic diseases, including cancers, using the CRISPR/Cas-based genome editing (Table 2 and Figure 2). However, there are still several issues associated with this advanced technology for wide application of CRISPR/Cas gene therapy.

6.1 | Generating more animal models for studying and treating human genetic diseases

CRISPR/Cas genome editing technology, particularly for the base editing and prime editing technology is a robust and powerful tool to precisely generate genetic mutations mimicking human genetic disorders. Many studies show that CRISPR/Cas-based genome editing is opening a new era for creating any genetic disorder-related mutation for generating animal models for human genetic disorder study and treatment. Now, it is becoming a new routine in generating animal models of human genetic diseases by CRISPR/Cas technology. In the future, we need to employ this technology for creating more animal models for studying and treating human genetic diseases.

6.2 | CRISPR/Cas gene therapy targeting limitations and PAM requirement

CRISPR/Cas-based genome editing and gene therapy all require a protospacer adjacent motif (PAM) in the target gene DNA sequence, which is a short DNA sequence with about 2–6 base pairs in length. For example, the most commonly used SpCas9 recognizes NGG PAM. The PAM requirement limits the application of CRISPR/Cas-based genome editing and gene therapy. A study shows that only about 26% of known pathogenic SNPs can be targeted by SpCas9-derived base editors (Rees & Liu, 2018). To solve this problem, scientists have been attempting to expand the PAMs by two different strategies: (1) discovering new Cas9s for genome editing. CRISPR/Cas9 is an adaptive immune defense mechanism in prokaryotes. During the long history of evolution, different bacteria and archaea evolved different Cas proteins that recognize different PAM sequences, for example, SpCas9 recognizes NGG PAM but LbCas12a recognizes TTTV PAM. Since CRISPR/Cas9 system is adopted for genome editing tools, most PAM expansion is contributed by discovering new Cas proteins from new bacteria or archaea resources, including finding Cas13 that can bind to RNA sequences for RNA editing (Makarova et al., 2020). (2) modifying current Cas enzymes. In the last five years, scientists have employed chemically and evolutionarily engineered Cas proteins, particularly the most commonly used SpCas9 protein, for various purposes, including relaxing the PAM requirement to increase the number of targetable DNA locations. Kleinstivar et al. (2015) modified SpCas9 to recognize NGA or NAG PAMs. Nishimasu et al. (2018) further modified SpCas9 protein, which can be used to recognize NG PAM. Recently, two independent laboratories evolutionarily engineered SpCas9 protein to become Cas9-NRNH (Miller et al., 2020) and SpRY (Walton et al., 2020) that further relax the PAM requirement, the modified Cas9-NRNH and SpRY can recognize NRNH or NRN/NYN PAMs. After these modifications, the number of accessible genes is significantly expanded, which will enhance the application of CRISPR/Cas-based gene therapy. However, as the wide application of CRISPR/Cas gene therapy, more reliable Cas enzymes should be discovered or modified with PAM-less requirement. In this way, CRISPR/Cas technology can be used to treat any genetic diseases.

6.3 | Safe and efficient delivery of CRISPR/Cas for gene therapy

Safe and highly efficient delivery of CRISPR/Cas system (components) into the target cells/tissues is a key step for human CRISPR gene therapy. Although there are many approaches developed for delivering Cas proteins and gRNAs into an individual cell, many of them cannot be directly used in human gene therapy due to many factors, including whether or not the method damages the cell, causes toxicity and/or immune response, or off-target impact. Because of the diversity of human diseases, more likely one method is not appropriate to deliver CRISPR/Cas system for treating all of them. Developing the best suitable approach for a specific type of disease may be a better choice for CRISPR/Cas gene therapy. For example, inhalation delivery technology may work better for respiratory diseases (Chow et al., 2020). Recently, He and colleagues (2020) adopted epithelial cell-derived microvesicles (MVs) as a carrier for delivering CRISPR/Cas components into cancer cells and it may become a safe delivery platform of CRISPR/Cas9 for treating cancer patients (C. He et al., 2020).

6.4 | Off-target impact is a big issue for CRISPR/Cas gene therapy

Although scientists have put huge efforts on limiting the off-target impact of CRISPR/Cas9 by designing the high affinity of Cas enzymes

and others, off-target editing is still commonly observed in animal system, which limits the safe and wide application of CRISPR/Cas9-based gene therapy. To limit the off-target impact, there are several strategies that may be focused on in the future. First, choosing a right delivery method may limit the off-target impact and increase the target efficiency. Currently, there are many different delivery systems of CRISPR/Cas9 into cells, including both virus-mediated and no virus-mediated method; different method has different advantage and disadvantage. For example, RNP delivery of CRISPR/Cas9 system into the cells can reduce the off-target impact because that CRISPR/Cas9 system is not inserted into the host genome and the short life time of RNA/protein (Doudna, 2020). Second, continuously identifying and/or modifying current Cas enzymes make them more affinity and higher efficiently bind to the target site and precisely cut the target DNA sequences instead of other sequences. Third, current gRNA is about 20 nt in length, is it possible to design a longer gRNA or higher reliable gRNA to only bind to the target site? Fourth, is it possible to link the Cas enzymes with a special enzyme that has a proofreading function? If so, the proofread function will correct the nontargeted genome editing and the off-target effects can be easily and quickly eliminated (D. Zhang & Zhang, 2020). A recent study used machine learning to train BE-Hive and obtained high efficiency of base editing genotype and lower off-target impact in a target library analysis, which contained 38,538 gnomically integrated targets (Arbab et al., 2020). Another study employed CRISPR Guide RNA Assisted Reduction of Damage (CRISPR GUARD) as a method to successfully protect off-target sites by co-delivery of short-guide RNAs; these short-guide RNAs can be used to be directed against off-target loci by competition with the on-target guide RNA (Coelho et al., 2020). Their results show that CRISPR GUARD can be efficiently used to reduce off-target mutagenesis while retaining on-target editing efficiencies with both Cas9 and base editor (Coelho et al., 2020). As the quick development of CRISPR/Cas9 genome editing technology, the off-target impact will be eliminated in the future.

6.5 | Safety concerns of CRISPR/Cas9 system for gene therapy

Except the off-target impact, the safety of CRISPR/Cas9 genome editing and its components recently also brings more attention from the scientific and industrial communities. First, CRISPR/Cas9 genome editing technology is adopted from a naturally occurring adaptive immune response mechanisms in many bacteria and archaea. Although there are many resources of Cas enzymes (proteins), the most commonly used Cas9 is SpCas9 and SaCas9, which are obtained from *Streptococcus pyogenes* and *Staphylococcus aureus*, respectively. However, both *S. pyogenes* and *S. aureus* are common bacterial species infecting human population at high frequencies (Lowy, 1998; Roberts et al., 2012). This suggests that our humanity may exist the nature response mechanisms against Cas9 proteins and affecting the CRISPR/Cas9 gene-editing efficiency. An early study shows that there is immunogenicity of Cas9 proteins in animal model mice (Chew et al., 2016). A recent study by Charlesworth and colleagues show that there are about 78% and 58% tested people had IgG antibodies in serum against SaCas9 and SpCas9 (Charlesworth et al., 2019). They also found that 78% and 67% of T cells against SaCas9 and SpCas9 in the tested people, respectively. Similar results were also observed in other studies (Ferdosi et al., 2019; Wagner et al., 2019). All these studies suggest that there are pre-existing humoral and cell-mediated adaptive immune responses to Cas9 in humans (Charlesworth et al., 2019) and that pre-existing humoral and cell-mediated adaptive immune responses to Cas9 in humans may affect the efficiency of CRISPR/Cas9-based gene therapy. Thus, this should be considered when we employ CRISPR/Cas9 technology for treating human genetic disorders.

CRISPR/Cas9 genome editing also potentially causes large deletions and rearrangements of DNA. In a CRISPR/Cas9 genome editing experiment in mouse embryos, Adikusuma et al. (2018) observed that CRISPR/Cas9 cleavage can cause large deletions in the target site at an accountable frequency, and some deletion reached to 2.3 kb. In 2019, Cullot et al. (2019) further demonstrated that CRISPR/Cas9 caused unexpected megabase-scale chromosomal truncations from only one Cas9 nuclease-induced DSB in cell lines and primary cells by a p53-dependent mechanism. Some deleted fragments contain 43 genes, including five proto-oncogenes and seven tumor suppressors (Cullot et al., 2019). This is only caused by a single gRNA editing; it may become worse if using two or more gRNAs for gene editing purposes. These large scales of deletions may cause significant consequence to the host cells; thus, it should be avoided during human CRISPR/Cas gene therapy.

p53 is an important tumor suppress gene, which encodes a protein regulating the cell cycle. Recently, the studies showed that Cas9 proteins activated the p53 pathway and caused p-53-inactivating mutations in various human cell lines (Enache et al., 2020), which are further related to human cancers. During CRISPR/Cas9 genome editing, Cas9 cuts DNA and form DSBs; at the same time, the DNA damages caused CRISPR/Cas9 activities p53 pathway and enhance cell DNA repair mechanisms, this will further reduce the genome editing efficiency and increases the potential risk of tumorigenesis (Haapaniemi et al., 2018; Ihry et al., 2018). Thus, the potential negative effects should be closely monitored and reduced during developing CRISPR/Cas9-based gene therapy.

ACKNOWLEDGMENTS

CRISPR/Cas9 genome editing has been developing so quickly. Huge amounts of publications are coming out daily. We greatly appreciate the hard efforts from each lab. Due to space limitations, we are sorry for not citing some papers that have made significant contribution to this field.

AUTHOR CONTRIBUTIONS

Baohong Zhang wrote, revised, and approved this manuscript.

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How to cite this article: Zhang B. CRISPR/Cas Gene Therapy. *J Cell Physiol*. 2020;1–23. https://doi.org/10.1002/jcp.30064