Advance genome editing technologies in the treatment of human diseases: CRISPR therapy (Review)

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Abstract. Genome editing techniques are considered to be one of the most challenging yet efficient tools for assisting therapeutic approaches. Several studies have focused on the development of novel methods to improve the efficiency of gene editing, as well as minimise their off-target effects. Clustered regularly interspaced short palindromic repeats (CRISPR)-associated protein (Cas9) is a tool that has revolutionised genome editing technologies. New applications of CRISPR/Cas9 in a broad range of diseases have demonstrated its efficiency and have been used in *ex vivo* models of somatic and pluripotent stem cells, as well as in *in vivo* animal models, and may eventually be used to correct defective genes. The focus of the present review was the recent applications of CRISPR/Cas9 and its contribution to the treatment of challenging human diseases, such as various types of cancer, neurodegenerative diseases and a broad spectrum of other disorders. CRISPR technology is a novel method for disease treatment, enhancing the effectiveness of drugs and improving the development of personalised medicine.

Contents

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1. Introduction

Since the discovery of the clustered regularly interspaced short palindromic repeats (CRISPR) system in 1987 (1), there have been significant advances in the field of gene therapy. The CRISPR system is an adaptive prokaryotic immune system, which serves as a bacterial defence mechanism against insertion of foreign genomic material and prevents the destructive impacts of mobile genetic elements delivered by phages and plasmids (2). CRISPR/Cas9 has been shown to boost the host immune system using the invading organisms' genetic material in order to protect the host from further invasion. The protective mechanism is completed with the acquisition of spacer sequences by CRISPR-associated spacer (Cas) proteins (3). Cas proteins are guided to the exogenous spacer sequences of foreign nucleic acids by CRISPR-associated RNA (crRNA) (4). The mechanism involves spacer identification and anchoring by Cas proteins, providing protection against further invasion. The existence of CRISPR was discovered in 1987 by Ishino *et al* (1), who cloned a portion of the CRISPR sequence together with the inhibitor of apoptosis gene (1). This discovery resulted in a novel method for gene-based therapeutics for the treatment of challenging disorders (5). Further analysis of prokaryotes, such as *Archaeoglobus fulgidus*, revealed other constituents of the CRISPR system, including non-messenger RNA sequencing, transcription of DNA repeats loci (target DNA sequences that are acquired and preserved in CRISPR loci) to small RNAs (Fig. 1) (6), and the Cas gene family (which is associated with CRISPR loci during immune processes) (7). Furthermore, identification of specific spacer sequences from the viral genome revealed how bacterial systems exhibited phenotypic resistance against the phage (7).

The aim of the present review was to describe the challenges and achievements of CRISPR customisation, taking into consideration various factors that may affect therapeutic outcomes *in vitro* and *in vivo*.

2. DNA repair systems

As a therapeutic method, CRISPR is used for both gene knockout and gene knock-in to correct a specific gene. Generation of double-strand breaks (DSBs) in the target gene results in the initiation of two endogenous DNA repair mechanisms: Non-homologous end joining (NHEJ) and homology-directed repair (HDR).

NHEJ joins a DSB without the use of a homologous DNA template (8,9), resulting in mutations, deletions and insertion of potentially nonsensical genetic material (Fig. 2) (10). Given the functional characteristics of NHEJ, it is considered an error-prone repair pathway, as it may result in the introduction of random indels and frameshifts in the sequence of a gene causing gene knockout, insertion or deletion of an amino acid, incomplete truncation and, ultimately, deactivation of the coded protein (Fig. 2). NHEJ is not limited to a specific cell cycle phase, whereas HDR occurs in the S or G phase and uses the sister chromatid as a template for homologous repair (11). HDR is a more precise DNA repair mechanism, the function of which has become a focus of attention along with site-specific gene editing tools in gene editing technology (9,12-16).

3. Overview of other gene editing tools

Various tools have been developed to perform knock-in and knockout in target genes to improve gene editing, and these have been used as therapeutic tools to treat certain diseases.

Zinc-finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs). ZFNs are sequence-specific nucleases that are frequently found in eukaryotes and were first discovered as repetitive zinc-binding domains in *Xenopus* oocytes (17), which are currently known as zinc finger motifs. Analysis of the ZFN crystal structure revealed the presence of 30 cysteine and histidine residues (each in pair; Cys2-His2) bound to zinc ions that provide stability to their ββα structure (18). ZFNs also contain a non-specific Fok-1 restriction enzyme, which is bound to the DNA-binding domain of eukaryotic transcription factors known as zinc finger proteins (ZFPs) containing Cys2-His2 fingers. Each finger identifies almost three base pairs of DNA sequences of target DNA and assists the binding of the ZFN to a particular sequence (17,19). However, there are difficulties in assembling ZFP fingers to bind to specific extended DNA sequences. Maeder *et al* (20,21) designed and assembled ZPFs that could bind to a specific 200-bp DNA sequence. However, binding may occur at random sites of the genome and, thus, may complicate gene correction. Furthermore, frequent off-target effects in the number of loci is another concern (22,23). Researchers successfully addressed this issue by designing ZFN pairs where one ZFN binds to the forward strand and the other to the reverse strand. Each pair contains distinct heterodimer Fok-1 domains with opposite charges, assisting in the formation of DSBs (Fig. 3) (24-26).

Subsequently, another method for gene editing, termed TALENs, was developed, which is considered more efficient, and has more advanced potential gene therapy applications (27,28). TALENs are fusion proteins composed of TALE and Fok-1 nucleases. The proteins, 33-35 amino acids in length, contain repeat variable di-residues, which are central binding protein regions that may be customized (29,30). Compared with ZFNs, TALENs are more suitable for therapeutic use due to the 1:1 TALE-DNA binding affinity (31) and lower rates of cytotoxicity (32). However, TALENs delivery is dependent on vectors, and developing suitable vectors has proved challenging, as the size of the cDNA that encodes TALENs is larger than the cDNA which encodes ZFNs (Fig. 3) (33).

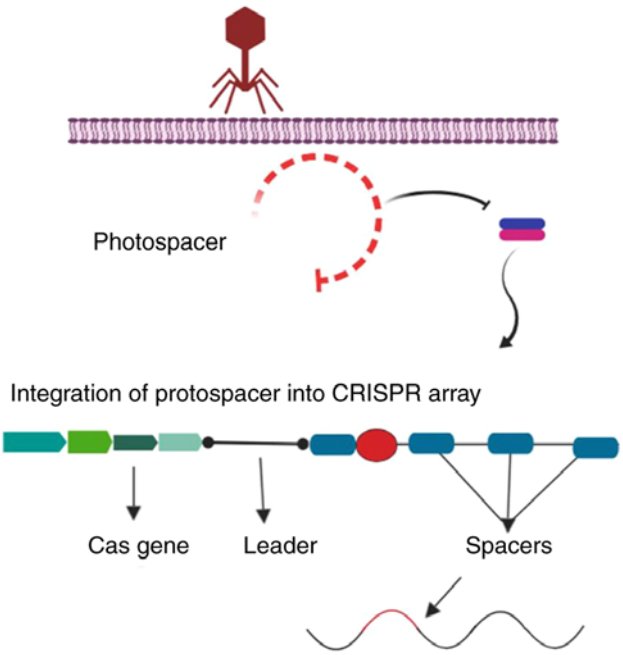

Figure 1. Schematic representation of the CRISPR immune system in the acquisition of foreign genetic material. The CRISPR system consists of a Cas operon containing Cas genes, and a CRISPR array that contains identical repeat sequences and spacers. In the case of viral or plasmid-based invasion, CRISPR acquires the protospacer sequence (red) of the viral DNA, which is achieved via a Cas1-Cas2 complex and integrated into the CRISPR array, which is further transcribed to pre-crRNA. CRISPR, clustered regularly interspaced short palindromic repeats.

CRISPR. To design a customized CRISPR system, a single-guide (sg)RNA must be designed (34). Jinek *et al* (35) and Cong *et al* (5) used the CRISPR/Cas system as a genome editing tool and, since then, it has been extensively studied by researchers to identify other characteristics of the CIRSPR system, which may be used for eukaryotic genome editing and, thus, treatment of various diseases. Liang *et al* (36) used this system to correct a mutation in the haemoglobin subunit β (HBB) gene whilst working on the haemoglobin subunit δ (HBD) gene (which is homologous to the HBB gene) (36).

CRISPR classification. The CRISPR/Cas system is classified into two types, with each type comprising various subtypes (I-V) based on their flanking Cas genes and location of the target on foreign DNA (37), which have improved our understanding of and ability to develop phage-resistant strains and the phylogenetic classification of bacteria. During phage/plasmid invasion, CRISPR/Cas functions in three phases: Adaptation, expression and interference. Each stage is associated with specific characteristics that result in antiplasmid or antiviral immunity (38). Adaptation consists of an integration process by which the invader-derived spacers (known as the spacer sequence) merge with the CRISPR array. In the next step, the CRISPR loci are transcribed into CRISPR-associated RNA (crRNA), which contains the spacer sequence. Subsequently, an endonuclease is produced and uses the spacer sequences as a guide to cleave the invader genome (Fig. 4) (39).

The functional characteristics of the CRISPR/Cas system are defined by the properties of the Cas1 and Cas2 genes. Taxonomic studies initially classified the CRISPR/Cas system

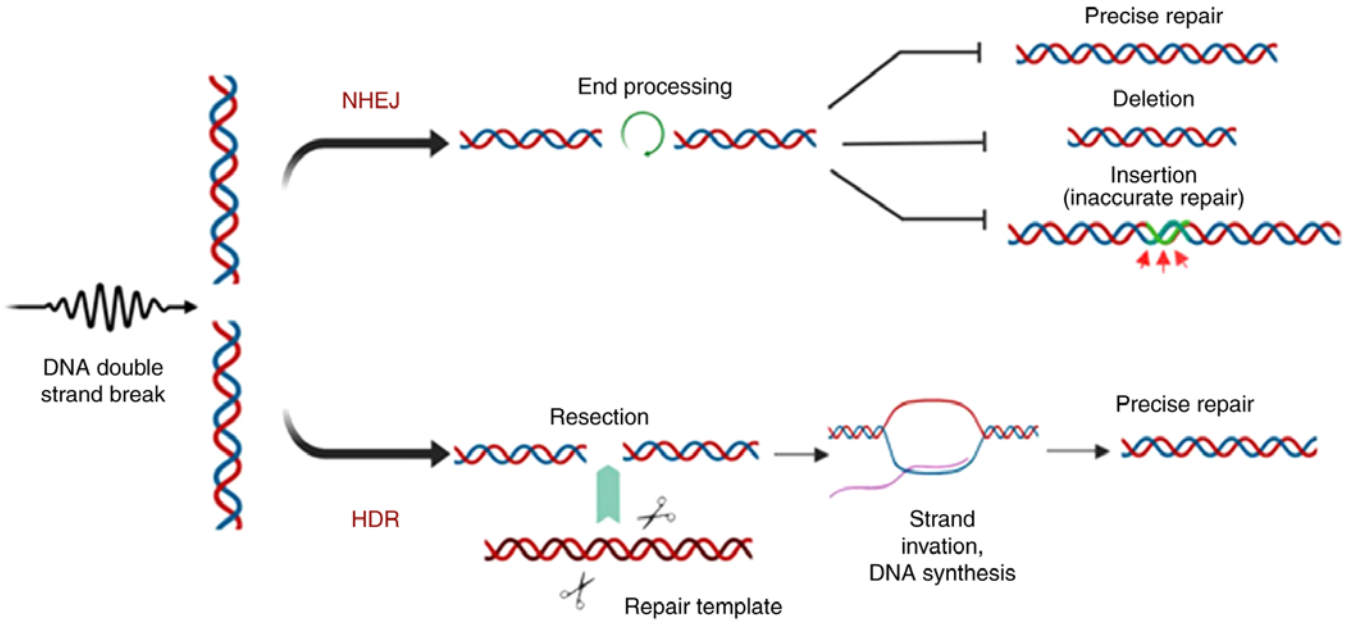

Figure 2. DNA repair mechanisms used for gene editing. Formation of double-stranded breaks to initiate endogenous DNA repair by NHEJ, resulting in accidental insertions/deletions, or by HDR, which uses a template DNA strand for repair. NHEJ, non-homologous end joining; HDR, homology directed repair.

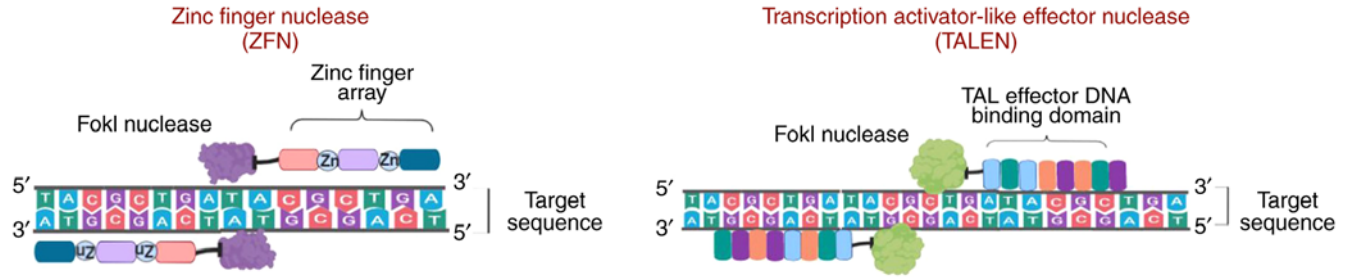

Figure 3. Comparison of ZFNs and TALENs, non-specific nucleases designed to cleave the genome at a specific site. ZFNs, zinc finger nucleases; TALENs, transcription activator-like effector nucleases.

into three types based on the particular marker proteins: Cas3 (type I), Cas9 (type II) and Csm/Cmr (type III) (40). Subsequently, classes IV and V of the CRISPR editing system were added (41). The general classification of the CRISPR system is based on the genes that encode the functional proteins and factors (Cascade, Csm, Cmr complex or Cas9). Class 1 CRISPR systems functions consist of multi-subunit crRNA-effector complexes, and includes type I, III and putative type IV (41). Class 2 only consists of Cas9 as a single protein, which performs all the functions of the CRISPR system, a feature also observed in putative class V (40).

Type I CRISPR is defined by the significance of the Cascade complex followed by the Cas3 nuclease (42). Pre-crRNA, which is the product of a transcribed CRISPR array, is cleaved by Cas6e, resulting in the production of crRNA (43). crRNA, which is associated with Cascade, is responsible for locating the protospacer in the target DNA. Furthermore, another subunit of Cascade, Cas8, identifies and locates protospacer adjacent motif (PAM), which is a short sequence located near the target sequence (44). The PAM sequence is the crucial factor for type I Cascade-Cas immune defence mechanisms; its dysfunction results in an inability of crRNA to recognise the spacers in target DNA by Cascade proteins (45,46), and inhibits R-loop formation between crRNA and target DNA, a process which eventually results in viral evasion from CRISPR screening (47,48). In the presence of a fully functional CRISPR system, recognition leads to activation of the Cas3 nuclease, which creates nicks on the single-stranded DNA of the target (virus or plasmid), resulting in its degradation (Fig. 4) (49).

Unlike type I, type II relies on Cas9 as the sole Cas protein, two types of RNAs, RNase III and tracer RNA (13), and a PAM, which is located downstream of the protospacer sequence in target DNA and recognised by the Cas9 protein (50). A DSB is introduced by HNH nuclease domain cleaving one strand, and RuvC nuclease domain cleaving another (51). However, cleavage requires identification of a PAM sequence by Cas9, which results in dissociation of dsDNA and the formation of an R-loop between the crRNA and DNA. This structural change results in the binding of the tracer RNA to the cleavage target sequence (52,53).

Type III is unique to Cas6, whose endoribonuclease mechanism produces crRNA by cleaving pre-crRNA (5). Unlike previous models of the CRISPR system, this type introduces

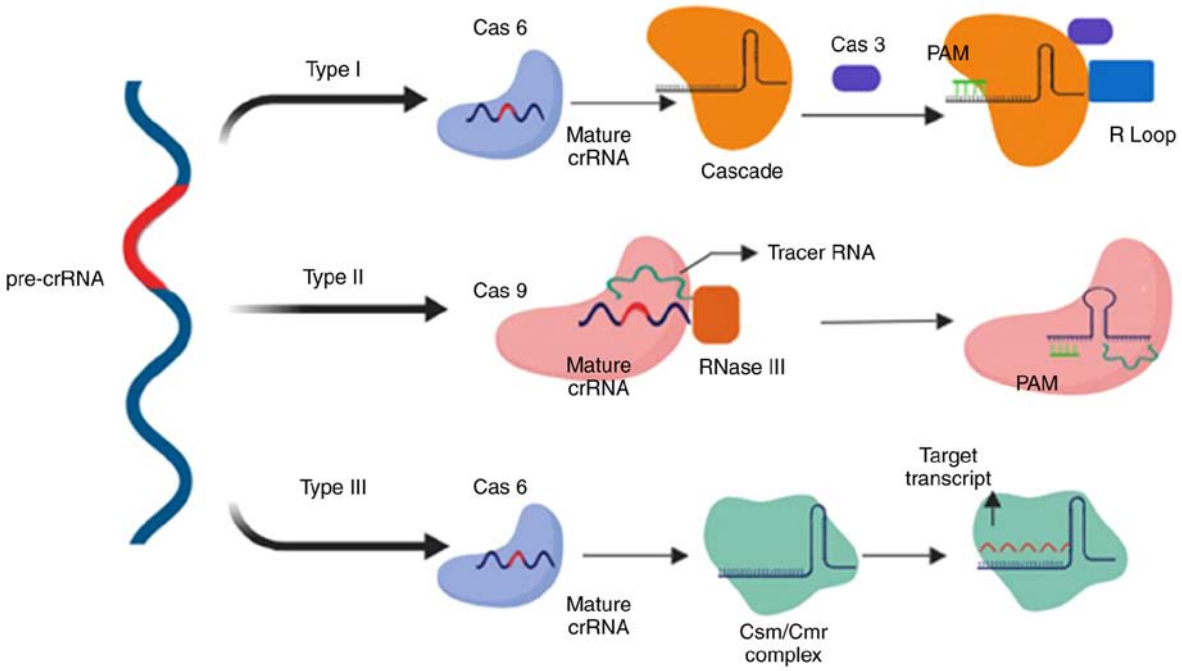

Figure 4. Comparison of type I, II and III CRISPR systems in crRNA maturation and interference. Upon transcription of CRISPR following the acquisition stage, pre-crRNA undergoes a maturation stage, which is processed by Cas6 in type I and III. In type II, the maturation step is performed by Cas9 accompanied by tracer RNA and RNase III. The interference step varies notably between the different types. CRISPR, clustered regularly interspaced short palindromic repeats; crRNA, CRISPR-associated RNA; Cas, CRISPR-associated protein; pre-crRNA, precursor crRNA; PAM, protospacer adjacent motif.

8-nucleotide repeat sequences, known as crRNA tags, as a result of crRNA cleavage by Cas6. The crRNA tag, which is located downstream of the spacer sequence (54-56), undergoes maturation and is modified into six nucleotides (53). The size of the crRNA complex increases and forms Cas10-Csm in type III-A and Cas10-Cmr complex III-B systems (54). In contrast to type I and type II, type III targets both DNA and RNA, producing co-transcriptional crRNA-guided cleavage of the target DNA (57,58). The palm domain of Cas10 cleaves DNA strands (58) and Csm3 (type III-A), and Cmr4 (type III-B) cleave RNA transcripts (59,60). Another notable difference of type III is that the PAM is not necessarily essential for the system to initiate immune mechanisms (Fig. 4).

4. Overview of therapeutic approaches

The identification of the CRISPR system allowed for improvements to gene editing technologies, and may improve current therapeutic techniques in the field of medicine, genetics, embryology, bioinformatics and pathology. Compared with the other genome editing techniques, CRISPR/Cas9 is more cost-effective, easier to use and can be used to perform specific gene knockdown, or base insertions and substitutions with lower rates of mutations with the known mechanism of CRISPR system.

Neurodegenerative diseases. Neurodegenerative diseases are characterised by disruption of neuronal function or loss of neurons that cause progressive central nervous system dysfunction and, thus, are frequently difficult to treat (61). There are numerous pathological neurodegenerative disorders, including Alzheimer's disease (AD), which is a fatal form of progressive dementia, and Parkinson's disease, which is a progressive movement disorder. These neurodegenerative diseases are relatively common (62). Familial AD, which may be caused by point mutations or deletions in the genes encoding amyloid precursor protein (APP), presenilin (PSEN)1 or PSEN2 (62), accounts for <5% of AD cases. Sporadic AD, which develops due to environmental factors, such as ageing or injury following brain ischemia, accounts for >90% of AD cases (63). *In vitro* analyses of induced pluripotent stem cells (iPSCs) that have been treated with CRISPR/Cas9 provide future perspectives in the treatment of neurodegenerative diseases, such as AD (64). Several studies have used the CRISPR/Cas9 system to identify defective and upregulated genes in early- and late-onset AD. Analysis of chromosome 1q31-42 in early-onset familial AD revealed a point mutation in PSEN2 (65), which was found to be correlated with a significant increase in the β-amyloid peptide ratio (Aβ42-43/40) (66), a change in voltage-gated potassium channel expression (67), and an increase in the concentration of calcium in the endoplasmic reticulum of neurons (68), which leads to neurotoxicity, cognitive deterioration and short-term memory loss in patients with AD, primarily due to basal forebrain cholinergic neuron (BFCN) damage. When healthy BFCNs were transplanted into an AD mouse model, a significant improvement in learning ability was observed (69). These findings led to further analyses of BFCNs, which were established from *in vitro* development of iPSCs obtained from the fibroblasts of humans with a PSEN2 mutation (70). The PSEN2N141 iPSC-derived BFCNs, characterised by a lack of electrophysiological properties, were observed to exhibit an improvement in neural activity and amyloid ratio following correction of the PSEN2 gene using CRISPR/Cas9 (71). Other studies performing similar analyses confirmed the effect of the CRISPR/Cas9 genome editing system on cells, the damage of which was associated with AD.

Scientists from the New York Stem Cell Foundation Research Institute expanded their studies on PSEN1 and PSEN2 point mutations in familial AD (72). They demonstrated a correlation between the inflammasome (a component of an innate immune system in a myeloid cell) and PSEN2 mutations using CRISPR/Cas9 (71). A correction of the point mutation on the PSEN2 gene in iPSCs derived from AD patients was performed using CRISPR/Cas9 and template sgRNA, as well as single-stranded oligonucleotides (ssODNs) that could edit the sequence in exon 5 of the PSEN2 gene located on chromosome 1 (1q42.13) (73). *In vitro* gene editing using the CRISPR/Cas9 system reversed the effect of the mutated PSEN2 gene on the increase of amyloid plaques (Aβ42/40) in iPSC-derived BFCNs in the cerebrospinal fluid, which was directly associated with the onset of AD (71). The *in vivo* effect of CRISPR/Cas9 on the mutated APP gene shows the system's ability to reduce neurotoxicity in AD patients. The APPsw allele in the fibroblasts of human patients' with an APPsw mutation was transfected using CRISPR/Cas9 with a gRNA designed to specifically mutate the allele. There was a considerable decrease in heterozygous APP alleles (APPsw/WT) and in the β-amyloid ratio (73). Additionally, the *S. pyogenes* CRISPR system has been shown to target specific desired genes or alleles (e.g., the APPsw allele) without disruption or indel formation in the other alleles, such as APPWT (73). However, *in vivo* analysis in a Tg2576 mouse model (mice with an age-deficient cognitive ability that overexpress mutant APP) using exosome-adeno-associated virus (AAV)1 with CRISPR/Cas9 and gRNA, showed indel formation and DNA frameshifts in the APPsw alleles (73). Although CRISPR/Cas9 may successfully alter the APPsw allele and reduce the β-amyloid ratio in brain cells, further investigations and clinical trials are required to improve the system and to reduce or ideally nullify indel formation.

Cancer therapy and drug discovery. Before the discovery of gene editing tools, several complications in cancer therapy could not be efficiently addressed, as drug resistance had not been effectively identified as a possible cause (74-76). CRISPR/Cas9 has facilitated the identification of genes associated with drug resistance by exposing drug-resistant cells to CRISPR/Cas9 gRNAs, each of which individually knock out a single gene at a time in each cell (77), and the results may be used to introduce alternative drugs with increased efficacy for improved outcomes (77).

For drug design, animal models and human cell lines are the optimal platforms for testing specific drug toxicity and efficacy prior to its use in humans. However, animal models, as well as *in vitro* analysis of human cell lines, may not provide representative and conclusive results regarding the effectiveness of a particular drug. CRISPR/Cas9 has provided a means to modify cells to allow them to more accurately represent human models for different types of cancer. ID8 (mouse ovarian surface epithelium) cells, which were obtained from ID8 mice with ovarian cancer with the TP53 and BRCA2 genes knocked out using CRISPR/Cas9, displayed characteristics of a high-grade serous carcinoma (78). High-grade serous ovarian carcinomas (HGSCs) exhibit reduced activity of TP53 and BRCA genes, loss of ability to form Rad51 foci (associated with DSB repair), and sensitivity to poly(ADP-ribose) polymerase inhibition (79). CRISPR/Cas9 has assisted in the development of more representative models of cancer that are likely to be increasingly used for evaluating drug safety and eliminating drug resistance in human diseases.

In addition to drug design, CRISPR/Cas9 has been proposed as a promising gene editing tool in cancer therapy. The dCas9 (mutated Cas9 without endonuclease activity, with added transcriptional activators on dCas9 or gRNA) is used to target specific genes by either activating or knocking them out, when combined with transcriptional activation or inhibition (80). Epigenome editing is another approach to cancer treatment, in which dCas9 is tethered to histone modifiers involved in DNA methylation to disturb processes associated with cancer progression, as DNA methylation is observed in the majority of cancers (81).

To determine the effects of the CRISPR/Cas9 system on different malignancies, such as leukaemia, K562 human myeloid leukaemia cells, which do not normally harbour a mutation in the isocitrate dehydrogenase (IDH2) gene, underwent a DSB and point mutation on the IDH2 R140Q locus following transfection with a plasmid which contained a sgRNA and CRISPR/Cas9. Following the mutation, gene repair was performed using another transfection with a pBS-SK$^+$ vector with a CRISPR/Cas9 and fluorescent-template DNA followed by sgRNA to check gene correction rate on point-mutated cells (82). The results revealed high levels of H3K9me2, H3K27me2 and H3K4me3 expression, which indicated hypermethylation of chromatin in mutated cells (83). In other studies, CRISPR/Cas9 was used as a tracking device to determine the effect of IL (interleukin)4-induced signal transducer and activator of transcription (Stat)6 activation on the elimination of leukaemia cells by using lentiviral vectors containing Cas9 and sgRNA for Stat6 (84). The results indicated that IL4 and its antileukemic effects were dependent on the ability of acute myeloid leukaemia cells to activate Stat6 (84), highlighting the potential of the CRISPR/Cas9 system as a therapeutic and diagnostic tool in various diseases. Several studies have been performed to assess the therapeutic potential of the CRISPR system for treating different types of cancer. 2CT-CRISPR assay is used to identify the genes causing resistance to immune cells (85). The test consists of two types of target cells and effector cells, such as melanoma cells and CD8$^+$ T cells, respectively. The aim of the assay is to identify factors mediating the growth of melanoma cells following an immune system response, and to design efficient therapeutic methods to augment immunotherapy against cancer cells. These assays were performed *in vivo* on C57BL/6J mice (85). Other studies focused on modifying chimeric antigen receptor (CAR) T cells in order to target cancer cells, and showed that CRISPR/Cas9 was more effective compared with RNA interference, which is only partially effective (86). Thus, scientists improved the efficiency of the technique by using CRISPR/Cas9, TALENs and ZFN, which reduced the off-target effects (87-93). However, the possibility of recurrence following treatment is considered to be a notable disadvantage that should be taken into account when designing the most effective therapy to decrease the possibility of the need for repeated treatments (88). Recent studies have demonstrated that CAR T-cell therapy may a promising treatment for various diseases, including cancer. CRISPR is a system that may be used for improving CAR

T cells. CAR permanent tonic signalling has been shown to reduce antitumor activity (89-93). Human CAR T cells modified with CRISPR/Cas9 gene editing, which contained 4-1BB and CD3z intracellular signalling domains, eliminated tumour cells by targeting the $CD19^+$ B cells that are associated with increased tumorigenesis (89).

Duchenne muscular dystrophy (DMD). DMD is an X-linked recessive muscular disorder caused by certain mutations of the DMD gene, which is located on chromosome 21. The mutation leads to a decrease in the levels of dystrophin, which is the protein responsible for normal muscular integrity (94). Men are more prone to this disease, as they carry one X chromosome (95). Of all the mutations identified in the DMD gene, ~86% are deletions and are present in an exon (96). In an attempt to regenerate muscle tissues to replace damaged tissue, haematopoietic therapy using haematopoietic stem cells obtained using *ex vivo* expansion of myoblasts from satellite cells has been developed. However, this technique was not found to be beneficial for patients with DMD (97), as myoblasts lose their ability to engraft into muscle tissues (98). iPSCS and embryonic stem cells (ESCs) are capable of producing a vast quantity of skeletal myogenic progenitors, exhibit *in vivo* regenerative capacity, as well the ability to synthesize dystrophin (99,100); therefore, ESCs are the optimal cells for performing genome editing to reduce DMD symptoms (99). Different therapies have been developed to replace dystrophin deficiency, such as anti-inflammatory-based techniques, or to restore DMD gene expression, such as cell-based therapies (101). Genome editing techniques, including the ZFN, TALENs and CRISPR systems, are the most efficient of these techniques. *In vitro*, TALENs and CRISPR function by restoring dystrophin synthesis via gene knock-in (insertion of exon 44 in the DMD gene), and has been demonstrated to be effective, with minimal off-target effects. iPSCs that were collected from DMD fibroblasts of a specific patient with DMD with exon 44 missing were transfected with TALENs and CRISPR separately to insert exon 44 by creating indels in adjacent exons (101). *In vitro* removal of exons 45-55, instead of a single exon in the DMD gene in the patient's iPSC-derived cardiomyocytes and skeletal myotubes using sgRNA and CRISPR/Cas9 system, resulted in restoration of dystrophin protein synthesis and, consequently, creatine kinase levels, whose linkage causes muscle instability and disintegration (102). Such a deletion was observed to normalise miR32 (miRNA32) (102), which reduces dystrophin levels in muscular dystrophies, including Becker muscular dystrophy (103). These results indicate the potential benefits of larger deletions, which rectify the dysfunction of other factors affecting the function of dystrophin. *In vivo*, CRISPR/Cas9 has been used to correct mutations of the DMD gene, in mdx mice (mice with a point mutation in the DMD gene and lacking dystrophin expression). sgRNA vector plasmids were used to target exon 23 of the DMD gene and ssODN to activate HDR repair mechanisms and repair the lesions of the DMD gene. However, the results indicated a higher rate of NHEJ-based repair, which resulted in the formation of indels (12).

AIDS. The HIV-1 virus invades host immune cells through the CD4 receptor and interaction with CC chemokine receptor 5 (CCR5) and CXC chemokine receptor 4. Although developmental treatment for HIV-1 with combinational retroviral therapy has improved the quality of life of the patients, it fails to eradicate the HIV-1 virus from the body, resulting in high rates of morbidity and mortality (104). A 32-bp deletion in the CCR5 allele results in the deactivation of the CCR5 gene, which results in a high degree of resistance to HIV-1 infection (104,105). In 2000, a patient with acute myeloid leukaemia and HIV-1 infection underwent bone marrow transplantation (using allogeneic stem cells) from a donor carrying CCR5Δ32/Δ32 cells, which resulted in abrogation of HIV replication, and the HIV-1 virus was not detected in the body (106). Research has focused on the development of homozygous cells using gene editing technologies, such as zinc-finger nuclease (107-109). Human $CD34^+$ hematopoietic stem/progenitor cells (HSPCs) from umbilical cord blood were transfected with ZFNs to knock out the CCR5 locus on chromosome 3 to establish a $CCR5^{-/-}$ clone (108-110). These cells were grafted into non-obese diabetic/severe combined immunodeficient/interleukin $2R\gamma^{null}$ (NOD/SCID/IL2Rγ^{null}; NSG) mice, an ideal rodent model for examining HIV-1 infections (111) and haematopoiesis (112). The results of the experiment revealed HIV-1 replication control (110). Similar studies on $CD34^+$ HSPCs using adenoviral vectors carrying CCR5-ZFN resulted in a more effective knockdown of $CCR5^{-/-}$ and fewer off-target effects compared with plasmid DNA ZFNs (109). These studies were extended to human patients with HIV infection who received CD4 T cells with dysfunctional CCR5 using ZFN60. The results indicated a high number of CD4 T cells. However, the rate of viral replication in cells with non-mutated CCR5 alleles (homozygous) was faster compared with cells with mutated CCR5 allele (heterozygous), which indicates that cells with homogenicity require knockout in both alleles to participate in the disease prognosis (107). Furthermore, using modified cells with ZFN does not result in permanent changes *in vivo*, as modified cells fail to control HIV-1 replication due to the presence of unmodified cells (113). Additionally, the adverse effects of adenoviral vectors must be considered (94). Wild-type (WT) iPSCs have been used to generate homozygous cells that harbour mutations in CCR5, termed CCR5-Δ32 (114). Generation of these cells was performed by transfecting WT iPSCs with a CRISPR/Cas9 plasmid with a specific sgRNA (115). The matured monocytes or macrophages from the modified iPSCs expressed resistance to HIV-1 infection (114-116). Another study on HIV-1-positive patients demonstrated that the presence of Cas9 and gRNA together in T-cells (specifically $CD4^+$ T cells) that have been manipulated genetically by Cas9/gRNA confer resistance to HIV-1 infection (117). The same study experimented with excision of pro-viral HIV-1 DNA from T cells using Cas9/gRNA on the RSBN1 gene, without disrupting the normal function of the gene, which encodes histone demethylase, which is responsible for chromatin structure (117). Astrocyte cell lines were transfected with Cas9 protein with and without plasmids, and double fluorescent protein HIV-1 reporter RGH was utilised to determine the excision sites of pro-viral HIV-1 DNA using a gRNA; the results demonstrated there was a reduction in fluorescent protein in astrocytes with no alterations to their regular function and morphology (118).

Sickle cell disease (SCD). In SCD, a recessive genetic disorder with a prevalence of 250,000 annually worldwide (119), a

modified CRISPR/Cas9 system was used with guide strands that specifically target the HBB and CCR5 genes using the pX330 plasmid in human kidney cells (120). Off-target effects were found to be directly associated with the presence of adjacent acquisition motifs (AAMs) in the PAM sequence, which reduces or nullifies the cleavage of target genes via CRISPR/Cas9 (120). The rate of mutations due to interception of the correction of the genes by Cas9 was directly correlated with the distance between sgRNA and the PAM of the specific protospacer recognised by the particular sgRNA (121). Furthermore, to correct the mutations in patients with SCD, ribonucleoprotein (RNP) consisting of Cas9 protein and sgRNA trG10 (truncated sgRNA G10 that targets the first exon of the HBB gene) along with ssODNs was introduced into human HSPCs collected from blood samples of patients with SCD (121). The results demonstrated that the use of CRISP/CAS9 resulted in efficient gene correction with reduced off-target effects and with optimum activation of HDR, compared with previous studies that used ZFN mRNA electroporation with ssODNs (13). The various diseases that have been treated using the CRISPR/Cas9 system are listed in Table I.

Haemophilia. CRISPR/Cas9 using AAV vectors has been assessed for the development of novel therapeutic methods to treat X-linked genetic diseases (122), such as haemophilia, a challenging disease with a high mortality rate, which is characterised by mutation on the coagulation factor IX (FIX). To restore the function of the F9 gene in patients with haemophilia, an AAV8 vector system carrying codon-optimized SaCas9 cDNA and sgRNA was transfected into hepatocytes in an *in vivo* model of mice with haemophilia B to create DSBs in the exon near the F9 gene (exon 2-8), and insert cDNA into an intron of the gene (123). The results revealed a genotypic and phenotypic correction of haemophilia B mice by targeting hepatocytes without disrupting epithelial cells of the liver morphologically or phenotypically (123). Additional studies extended the therapeutic design using other vectors with capacity for larger constructs. A low dose of adenovirus (Adv) containing Cas9 and specific sgRNA containing a correct donor template (dsDNA) was transfected into hepatocytes of haemophilia B mice, which resulted in F9 gene correction with improved efficacy compared with other vectors (14). However, lack of restoration in coagulation factor was an unfortunate outcome, as the Adv resulted in an adverse immune response due to the presence of vector genome immunogenicity, and an insignificant rate of HDR. Thus, it was hypothesized that recombinant Adv may be more suitable (14). However, integration of cDNA into the host genome can result in genotoxicity by either activating potential oncogenes or damaging functional genes (124). Therefore, using iPSCs compared with vector may be more suitable. *In vitro* and *in vivo* CRISPR studies using human iPSCs from patients with haemophilia B reported interesting results. iPSC cell lines prepared from peripheral blood mononuclear cells from patients with haemophilia B were modified by inserting the complete F9 human cDNA using CRISPR/Cas9; these cells differentiated into hepatocytes, and were subsequently injected into NOD/SCID mice. Analysis of the mice following transplantation revealed secretion of human FIX (125), a promising result that may serve as the basis for future studies.

The results of TALENs have shown promise in haemophilia B genome editing. *In vitro* analysis of canine FIX (cFIX) using both CRISPR and TALENs, showed that TALENs resulted in fewer off-target effects as they act as dimmers, and this may explain the lower numbers of DSBs compared with CRISPR (124). HDR cassettes were designed, which contained 471 bps of the WT coding sequence at the cFIX mutation locus and altered codons at the TALEN/Cas9 binding sites, which were cloned into pscAAV-cFIXWT to reduce off-target effects. Using modified HDR cassettes improved HDR efficiency, thus reducing the off-target effects of gene editing techniques (124).

Autism spectrum disorder (ASD). ASD is a primarily inherited neurodevelopmental condition that is characterised by difficulty in social interactions, with language and communication abnormalities, which may be identified in children during early development. The symptoms have been found to be genetically associated with fragile X, maternal 15q11-13 duplication (83) and 2q37 and 22q13.3 deletion (126). AAVs, specifically AAV9, improves the treatment of Rett syndrome, as AAV9 can effectively penetrate into brain cells (127). To enhance the ability of the vectors for gene delivery, a self-complementary AAV9 vector, along with a codon-optimized version of the major methyl CpG binding protein 2 (Mecp2) gene (a mutation in the Mecp2 gene causing Rett syndrome has been observed in almost 1% of ASD patients) (128). The function of this gene involves transcription regulation by activating and repressing neuron function, and its brain isoform, referred to as MCO, was injected intravenously into Mecp2 knockout mice and was shown to improve behavioural development (22). However, an increased level of Mecp2 in liver cells, as a result of an off-target effect, was observed when a high dose was used for treatment (129), which disrupted liver metabolism and function (130). Different types of AAV vectors, such as AAV8, were used to transfect enhanced green fluorescent protein in astrocytes, which are cells abundantly present in the mouse striatum. The results demonstrated a high rate of protein transport due to the high penetrative ability of AAV8 in brain cells (131), which may be used to design methods to transfer target genes to astrocytes in patients with ASD.

The CRISPR/Cas9 genome editing system has been used in neural and brain cells to deliver specific genes to target cells. RNP induced Cas9 with Simian vacuolating virus 40 nuclear localisation sequence in Ai9 tdTomato mouse neural progenitor cells in the hippocampus, striatum and cortex, and this demonstrated marked gene editing ability *in vitro* and *in vivo*, indicating successful neuron-specific targeting for future ASD treatment (132). CRISPR/Cas9 has been used for Huntington's disease to suppress mutant HTT (mHTT) gene, which is located on chromosome 4 and produces Huntington's disease protein when mutated. *In vivo* HTT gene targeting in HD140Q-KI mice with mHTT using an AVV vector containing the CRISPR/Cas9 system with four gRNAs resulted in a significant reduction of mHTT expression and, thus, a reduction in the expression of Huntington's disease protein in HD140Q-KI mice (133). Therefore, it may be possible to design an efficient gene editing tool to treat ASD or minimise the severity of the symptoms.

One of the most successful studies on Thy1-YFP and Ai9 mice with X-fragile syndrome resulted in a significant reduction

Table I. Strategies using the CRISPR/Cas9 system for the treatment of diseases.

Disease	*In vitro* analysis	*In vivo* analysis	Result	Conclusion	Refs.
AIDS	CRISPR/Cas9 transfected WT iPSCs to generate homozygous $CCR5^{-/-}$	NOD/SCID/IL2Rγ^{null}; NSG mice were engrafted with $CCR5^{-/-42}$ generated from $CD34^{+}$ HSPCs by ZFN HIV^{+} patients were engrafted with $CCR5^{-/-}$ CD4 T cells by ZFN	i) Resistance to HIV-1 infection *In vivo* animal model: Suppression of HIV-1 replication iii) *In vivo* human model: HIV-1 replication could not be controlled	CRISPR appears more promising than ZFN due to long-term effect and ability to mutate both CCR5 alleles	108-112, 114-116
Neurodegenerative diseases	i) Exon 5 of *PSEN2* gene in iPSCs derived from AD patients were corrected using CRISPR/Cas9	ii) Healthy $PSEN2^{N141}$ iPSC-derived BFCNs underwent CRISPR/Cas9 gene correction and were transplanted into AD mice	i) Reversal in elevated amyloid plaques (Aβ42/40) ii) Neurological development	Reduction in AD neuropathological symptoms. Further analysis required for *in vivo* human therapy	70-74
DMD	i) Exon 44 knock-in in *DMD* gene in iPSCs of DMD patients using TALENs and CRISPR ii) 45-55 exon removal of *DMD* gene in iPSC-derived cardiomyocytes	iii) Targeting exon 23 in *DMD* gene in mdx mice	ii) Normal function of miR32 iii) Gene correction, however causing indels due to more NHEJ repair compared to HDR	Larger size deletion by CRISPR corrected errors on factors affecting dystrophin function. However, due to off-target effects, further analysis to modify CRISPR/Cas9 is necessary to reduce off-target effects	101-103
Haemophilia	i) iPSCs of HB patient were transfected with CRISPR/Cas9 and differentiated into hepatocytes ii) Modified HDR cassette containing coding sequence of WT at cFIX mutation locus and modified codon at TALEN/Cas9 binding site	iii) AAV8 using CRISPR-SaCas9 in hepatocytes of HB mice to restore *F9* gene creating DSB and inserting cDNA to intron iv) Injection of *in vitro* generated hepatocytes into NOD/SCID mice	ii) Enhance HDR activation leading to decrease in off0target repair iii) Genotypic correction and phenotypic improvement iv) Secretion of human FIX in mice	Improvements in reduction of off-target effects caused by CRISPR, which is promising for further analysis of CRISPR for treatment in humans	122-125
ASD	i) RNP-induced Cas9 on NPCs of Ai9 tdTomato mouse	ii) AAV9 with MCO was injected into Mecp2 KO mice iii) RNP-induced Cas9 in NPCs of Ai9 tdTomato mouse from hippocampus, striatum and cortex iv) CRISPR-Gold with Cas9 or Cpf1 on Thy1-YFP and Ai9 mice	ii) Behavioural development i and iii) Significant genome editing iv) Reduction in XFS	High dose of Mecp2 in liver cells causing liver metabolism dysfunction However, CRISPR-Gold showed minimal off-target effects and no effects on immune system[98]	129-134

Table I. Continued.

Disease	*In vitro* analysis	*In vivo* analysis	Result	Conclusion	Refs.
SCD	Using pX330 plasmid with CRISPR/Cas9 that contains truncated sgRNA G10 to target first exon in *HBB* gene in human HSPCs from SCD patients	-	Highly efficient gene correction and reduction in mortality	CRISPR showed fewer off-target effects and better HDR function compared with genome editing by ZFN	120-123

CRISPR, clustered regularly interspaced short palindromic repeats; Cas9, CRISPR-associated protein; AIDS, acquired immune deficiency syndrome; HIV, human immunodeficiency virus; DMD, Duchenne muscular dystrophy; ASD, autism spectrum disorder; SCD, sickle cell disease; WT, wild-type; iPSCs, induced pluripotent stem cells; CCR5, CXC chemokine receptor 5; NOD/SCID/IL2R$\gamma^{null,}$ non-obese diabetic/severe combined immunodeficient/interleukin 2Rγ^{null} (NSG); PSEN2, presenilin 2; AD, Alzheimer's disease; ZFN, zinc-finger nucleases; BFCNs, basal forebrain cholinergic neurons; TALENs, transcription activator-like effector nucleases; NHEJ, non-homologous end joining; HDR, homology-directed repair; FIX, coagulation factor IX; cFIX, canine FIX; AAV, adeno-associated virus; DSB, double-strand break; RNP, ribonucleoprotein; Mecp2, methyl CpG binding protein 2 gene; MCO, brain isoform of Mecp2; HBB, haemoglobin subunit β; HSPCs, hematopoietic stem/progenitor cells.

in repetitive symptoms of X-fragile syndrome (XFS) by using CRISPR-Gold (CRISPR designed with gold nanoparticles) to deliver Cas9 or Cpf1 to the striatum via local intracranial injection. CRISPR system side effects included minimal off-target effects and no impact on the immune system, unlike AAVs. Additionally, the metabotropic glutamate receptor subtype 5 gene was selected as an editing target (134,135), since its signalling was found to be overactivated in XFS and other ASD syndromes (136,137). The study reported minimal side effects, indicating successful Cas9 or Cpf1 delivery and gene editing. The same study used CRSPR-Gold effect for gene editing of other cell types, such as glial cells, dysfunction of which is observed in numerous neurological and brain disorders (137,138). Such accomplishments have provided novel insights into CRISPR genome editing technology, and may be used to treat other rare diseases.

5. Factors affecting the use of the CRISPR/Cas9 system

Although the abilities of CRISPR/Cas9 system are clearly established and have been used in various applications, there are concerns regarding off-target mutations, which may limit its future perspectives. Data from several studies indicate that the off-target effects of the CRISPR/Cas9 system are among the most important consequences of this method, regardless of the cell type and target genes (5,14,16,33,36,43). Hybrid R-loop formation between sgRNA and the target DNA may result in double-stranded cleavage of DNA due to RNA-guided nucleases, the recognition of PAM sequences and the presence of adjacent AAMs (139). Additionally, it was demonstrated that such activity results in an increased degree and a high volume of off-target effects by CRISPR/Cas9 during gene treatment, specifically due to dsDNA break and NHEJ function (140). Various techniques and protocols have been designed to optimise the low specificity of CRISPR/Cas9 and to promote HDR-based repair over NHEJ, in order to reduce the mutation rate. Exposure of mini circle-iPSCs to cold shock or low temperatures after treatment with CRISPR/Cas system resulted in increased HDR function and, thus, reduced off-target effects. However, the rate of indel formation was not significantly affected (15). Another study designed to reduce the off-target effects investigated changing the ratio of sgRNA to Cas9 protein, and demonstrated that a higher ratio of sgRNA to Cas9 resulted in reduced incidence of off-target effects (139).

Selection of bacteria for harvesting Cas9 markedly affects the performance of CRISPR/Cas9. For example, several studies investigated the impact of the CRISPR/Cas9 system using three different species of bacteria; *Streptococcus pyogenes* Cas9 (SpCas9), *S. thermophilus* Cas9 (St1Cas9) and SaCas9 (139,141). The analysis of human cells transfected with Cas9 plasmids from bacteria exhibited increased activity, as well as reduced mutation rates, compared with SpCas9 and SaCas9 (124). In addition to the findings mentioned above, the base sequence of the AAM upstream of PAM plays a key role in sgRNA binding with protospacers on the target DNA (142). sgRNAs with a higher ratio of guanine and a lower ratio of adenine are more stable in binding with target DNA compared with sgRNAs with a higher ratio of cytosine (143).

Other challenges include plasmids with low specificity and random integration into the target DNA, which creates tracking obstacles (139).

6. Discussion

Limitations of previous genome editing tools led scientists to develop the CRISPR system, which has reduced the undesired effects whilst increasing efficiency compared with previous methods. CRISPR was designed to reduce off-target effects caused by mutations as a result of DNA breaks, thus resulting in a reduction of unwanted errors. *Cpf*I endonuclease was introduced into the CRISPR system (Class II) to overcome

the challenges mentioned above. *Cfp*I is a single RNA-guided endonuclease that does not require tracer RNA, and studies using Cpf1 of *Francisella novicida* bacterium showed inactivation of RuvC-like domain avoiding dsDNA cleavage (16). In addition to the findings mentioned above, Cpf1 creates 5' overhangs, which can efficiently add a DNA sequence during genome editing via a non-HDR system, in contrast to Cas9, which forms blunt end cuts on target DNA (16).

Furthermore, other studies reported fewer or no off-target effects using *Cpf*1 compared with Cas9 by analysing on-target activity of *Acidaminococcus sp*. BV3L6 *Cpf*1 and *Lachnospiraceae bacterium ND2006 Cpf*1 in human cells, compared with SpCas9 (144). The effect of R-loops and their stability on the occurrence rates of an off-target impact are other factors to be considered in designing the most efficient CRISPR system and potential target sequences in gene editing techniques (145). However, other studies reported that TALENs was more efficient compared with the CRISPR system (29,30,32,33).

CRPSR has helped overcome the challenges of disease therapies by locating target genes that are the causes of drug resistance (88). Techniques, such as 2CT-CRISPR and dCas9, have increased the efficacy of drug therapy. Several factors, such as vector/plasmid and CRISPR selection based on size, have exerted a notable effect on the efficacy and delivery of CRISPR endonucleases to target genes (37,45). Therefore, selecting the most suitable vector with good penetration and a low rate of host immune activation for CRISPR delivery is required to carefully address the treatment of various diseases, such as AIDS, haemophilia, ASD and SCD.

Scientists at the University of Washington used vectors with CRISPR/Cas9 components from either *Streptococcus pyogenes* or *Staphylococcus aureus* to treat DMD (146). The results demonstrated that the expression of dystrophin using dual vectors exhibited increased efficiency compared with a single vector. Furthermore, they achieved a higher yield with SpCas9 compared with SaCas9, as demonstrated by the reduction in the off-target effects in both DMD and SCD (146,147). In addition, certain factors, such as size and PAM sequence recognition, further improve the efficiency of SpCas9 compared with SaCas9 for CRISPR/Cas9 therapy (147). The impact of the CRISPR/Cas9 system on other diseases has been shown to involve off-target effects and, eventually, the formation of indels due to the involvement of NHEJ-based repair, which is associated with an increased risk of mutations (148).

7. Conclusion

The effects of CRISPR/Cas9 on humans requires further investigation, as human iPSCs and mouse iPSCs vary in response based on the specific type of CRISPR system used. Furthermore, the issue of indels as a result of NHEJ-based repair must be reduced in order to reduce the off-target effects caused by indels. Therefore, studies are focusing on designing a CRISPR/Cas system for gene editing with a lower risk of mutations utilizing HDR.

Numerous studies have demonstrated the use of CRISPR, ZFNs and TALENS as powerful gene editing tools. Although ZFNs and TALENS represent important advances in gene editing, their capacity is currently limited for effective use. The discovery of CRISPR, which exhibits higher efficiency and fewer off-target effects, has provided opportunities for scientists to use this technique widely and develop CRISPR-based gene therapy. However, the issue of off-target effects must be addressed and, thus, should be the focus of future studies, with the aim of further developing this technology for use in human gene therapy.

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MA drafted the paper and designed the manuscript structure. NK drafted the manuscript and arranged the data of the studies. Both authors have read and approved the final version of the manuscript for publication.

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Competing interests

All the authors declare that they have no competing interests.

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