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Review

Ex vivo cell-based CRISPR/Cas9 genome editing for therapeutic applications

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ABSTRACT

The recently developed CRISPR/Cas9 technology has revolutionized the genome engineering field. Since 2016, increasing number of studies regarding CRISPR therapeutics have entered clinical trials, most of which are focusing on the *ex vivo* genome editing. In this review, we highlight the *ex vivo* cell-based CRISPR/Cas9 genome editing for therapeutic applications. In these studies, CRISPR/Cas9 tools were used to edit cells *in vitro* and the successfully edited cells were considered as therapeutics, which can be introduced into patients to treat diseases. Considering a large number of previous reviews have been focused on the CRISPR/Cas9 delivery methods and materials, this review provides a different perspective, by mainly introducing the targeted conditions and design strategies for *ex vivo* CRISPR/Cas9 therapeutics. Brief descriptions of the history, functionality, and applications of CRISPR/Cas9 systems will be introduced first, followed by the design strategies and most significant results from previous research that used *ex vivo* CRISPR/Cas9 genome editing for the treatment of conditions or diseases. The last part of this review includes general information about the status of CRISPR/Cas9 therapeutics in clinical trials. We also discuss some of the challenges as well as the opportunities in this research area.

1. A brief history of CRISPR

The term CRISPR, or the clustered regularly interspaced palindromic repeats, was first used by Jansen et al., in 2002 to describe a novel family of repetitive DNA sequences presented in the genomes of prokaryotes [1]. These unusual repeated sequences were first detected in 1987 by Nakata et al. in *Escherichia coli* [2], and then recognized to be widespread in archaea and bacteria by Mojica et al., in 2000 [3]. After Jansen et al. identified the CRISPR-associated (Cas) genes in 2002 [1], Mojica et al. [4], Vergnaud et al. [5], and Bolotin et al. [6] revealed the exrtachromosomal and phage-associated origins of the spacers that separate the individual direct repeats, in 2005. Speculations about CRISPR arrays as immune memory and defense mechanism against virus envisions were then proposed [6,7], while the first experimental evidence for CRISPR/Cas system based adaptive immunity was reported by Horvath et al., in 2007 [8]. Following the series discoveries on the basic function and mechanism of CRISPR systems [9–12], in 2010 Moineau et al. demonstrated the Cas9 enzyme (formerly named Cas5, COG3513, Csn1 or Csx12), which is guided by spacer sequences, cleaves target DNA [13].

In 2012, Doudna, Charpentier et al. [14], and Siksnys et al. [15] proved that *Streptococcus pyogenes* and *Streptococcus thermophiles* Cas9 can be guided by CRSIPR RNAs (crRNAs) to cleave target DNA *in vitro*, through forming a double-strand break (DSB). In 2013, Zhang et al. [16], Church et al. [17], and Doudna et al. [18] independently demonstrated the applications of engineered CRISPR/Cas9 systems for genome editing in mammalian cells. This represented a giant leap forward in the genome engineering field and triggered a tremendous number of studies regarding the use of the CRISPR/Cas9 platform for eukaryotic gene editing in a wide range of species, including flies [19,20], zebrafish [21–24], frogs [25], mice [26–28], rats [26,29], pigs [30], and monkeys [31,32].

The first clinical trial of CRISPR in human was initiated in 2016 at the West China Hospital [33], in which CRISPR/Cas9 edited immune cells were used to treat patients with lung cancer (ClinicalTrials.gov Identifier: NCT02793856; Closed study). In 2018 – the same year in which He, a scientist in China, announced the birth of human CRISPR-edited babies [34,35], provoking international discussions surrounding the ethical implications of genome editing technology [36,37] – clinical trials of CRISPR-based genome editing therapeutics started in the US for the treatment of cancer (ClinicalTrials.gov Identifier: NCT03399448), β-thalassemia (ClinicalTrials.gov Identifier: NCT03655678) and sickle cell disease (ClinicalTrials.gov Identifier: NCT03745287), along with many others (more details can be found in section 4). It should be noted

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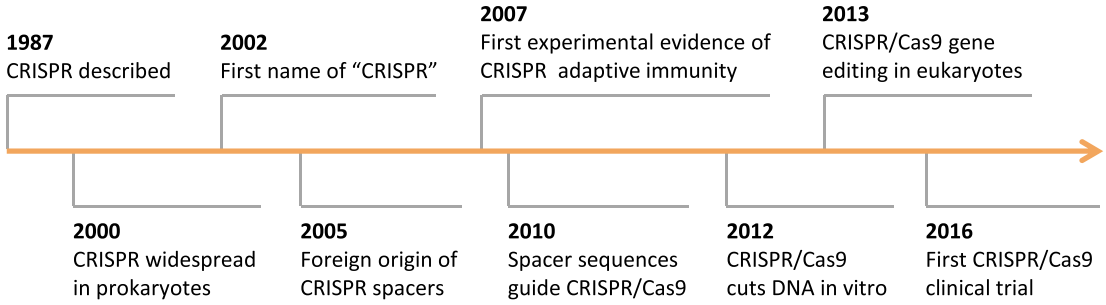

Fig. 1. A brief timeline of CRISPR/Cas9 development.

that He's research institution has denied prior knowledge or approval of his research, which was met with wide international criticism. By contrast, all of the FDA-sanctioned clinical trials have been carried out under the typical medical and ethical guidelines of the FDA. A brief timeline of CRISPR development has been summarized in Fig. 1. Now that CRISPR is in the international spotlight and has demonstrated some preliminary success, professionals from academia, industry, and regulatory agencies are expected to work together to realize the tremendous therapeutic potential of the revolutionary CRISPR technology in the most scientifically, medically, and ethically rigorous fashion.

2. CRISPR/Cas9: biology, functionality, and applications

The CRISPR-mediated adaptive immunity in prokaryotes involves three steps: (1) the identification of DNA from a pathogenic species and subsequent incorporation of the pathogenic sequence into the prokaryote's own DNA in the form of a spacer sequence, (2) the transcription and maturation of crRNA based on that spacer sequence, and (3), the crRNA-directed cleavage of target pathogenic nucleic acids, effected via Cas enzymes [38,39]. Different CRISPR/Cas systems (type I, II, III and the less common IV) use distinct nucleic acid recognition and degradation mechanisms [38]. The effector complexes of type I, III and IV contain multiple subunits, while the type II effector complex consists of single multidomain protein, Cas9 [40]. The wild-type Cas9 protein has two putative nuclease domains, HNH and RuvC-like (Fig. 2A) [38–40]. The Cas9 targeted activity can be guided by two pieces of RNAs that form a duplex, i.e., the crRNA and *trans*-activating-crRNA (tracrRNA), to introduce a DSB in target site on DNA chain, adjacent to the protospacer adjacent motif (PAM) sequence [38]. In prokaryotes, generally the CRISPR array (containing the pathogenic sequence) is transcribed into pre-crRNA, which is then cleaved into crRNA by the tracrRNA with homology to the short palindromic repeat. The tracrRNA helps recruit RNase III and Cas9 and produce mature crRNA. The tracrRNA, crRNA, and Cas9 then complex together and seek out DNA sequence that complementary to the crRNA. Once the crRNA has bound to the target DNA via strand base-pairing, the HNH domain of Cas9 cleaves the DNA strand complementary to crRNA and RuvC-like domain cleaves the opposite strand to generate blunt ends [39]. Doudna and Charpentier et al. demonstrated the feasibility of fusing crRNA and tracrRNA into a single guide RNA (sgRNA) to complex with Cas9 and guide the target DNA cleavage [14]. The therapeutic potential of CRISPR lies in the fact that the targeted cleavage activity of the Cas9 protein can be guided via a synthetic sgRNA or crRNA/tracrRNA molecule, allowing the researcher to target a wide variety of genomic sequences.

DNA breaks in the genome can be repaired by either non-homologous end joining (NHEJ) or homology-directed repair (HDR) as shown in Fig. 2B [39]. Taking advantage of these pathways, specific gene disruption, deletion, correction, and insertion can be achieved [38]. Wild type Cas9 nuclease can also be engineered into variants with additional engineering applications such as Cas9 nickase (a mutant which can only cleave a single strand of the DNA duplex, generated via a mutation in either the HNH or RuvC-like domain which results in the specific inactivity of that single domain) or catalytically inactive Cas9 (dead Cas9 or dCas9, generated by inactivating both the HNH and RuvC domains) [41]. Cas9, either wild-type or the engineered mutants, can also be fused with other functional ligands or protein domains, introducing the capability of designing novel CRISPR systems which allow for specific gene labeling, silencing, activation, epigenetic modification, enhanced specificity, or even (DNA and RNA) single base editing [41,42]. The CRISPR/Cas9 enabled genetic and epigenetic engineering has led to its broad application ranging from basic biology (e.g. creating cellular and animal models) to biotechnology (e.g. improved fuel and food productions) and medicine (e.g. facilitated pathogen detection, drug development, and gene therapy) [39,43]. More in-depth reviews focusing on one or several aspects of the history, biology, development, and applications of CRISPR/Cas9 genome editing systems have been published [44–49].

One of the necessary conditions for successful eukaryotic genome editing using CRISPR/Cas9 system is the presence of guide RNA (sgRNA or crRNA/tracrRNA duplex) complexed Cas9 protein in the nucleus [48]. To achieve this, the Cas9 could be introduced in either protein, mRNA, or DNA (plasmid or viral genome) formats; the guide RNA can be either *in vitro* transcribed (IVT) RNA, chemically synthesized RNA, or encoded in viral genomes to be expressed directly by the target cell [48]. The challenge is that neither the protein nor the nucleic acid components can bypass the physicochemical barriers of the cells and tissues unaided, which makes a delivery system necessary. Physical methods (e.g. microinjection and electroporation) [50–52], viral

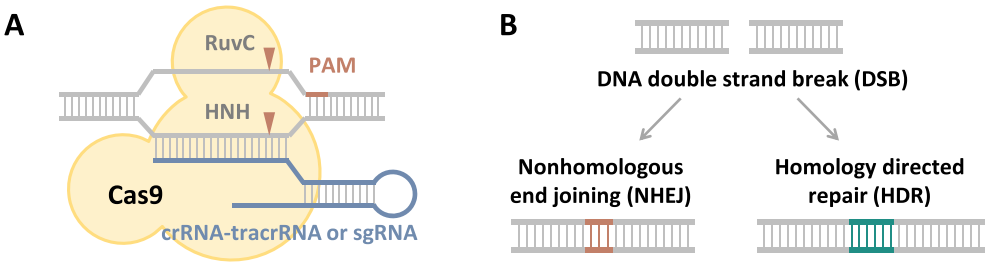

Fig. 2. Schematic illustration of the structure and function of CRISPR/Cas9. (A) RNA-guided Cas9 nuclease for double-strand DNA cleavage. (B) The DSBs in genome could be repaired through NEJH or HDR pathways.

vectors (e.g. adenovirus and lentivirus) [53–55], and non-viral carriers (e.g. lipids, polymers and inorganic nanoparticles) [56–61] have been developed for intracellular delivery of CRISPR/Cas9 systems. The advantages and disadvantages of different delivery systems (materials of carriers and formats of cargos) have been reviewed previously [41,48,62–64].

Here, we review the rapid advance of CRISPR/Cas9 gene editing systems for therapeutic applications. As this review is not intended to be a comprehensive summary of potential CRISPR therapeutics, we focus on the *ex vivo* delivery systems aiming for the development of CRISPR medicine. Considering many reviews have been published that are organized according to the delivery materials used in CRISPR/Cas9 systems [48,63,65–68], this review aims to provide a different perspective for biomaterials scientists working in this field by focusing on the types of diseases/disorders in which *ex vivo* CRISPR/Cas9 technology can be useful. We will review the current status of CRISPR therapeutics in clinical trials. At the end, we discuss some of the challenges as well as opportunities that biomaterials scientists working in this field are facing.

3. Therapeutic targets for *ex vivo* cell-based CRISPR/Cas9 genome editing

Ex vivo genome editing is a therapeutic approach in which the genome of particular cells are edited *in vitro,* and then those modified cells are transplanted back into the patient to exert a therapeutic effect (specifically in which the therapeutic effect is a result of the genome editing). This approach is in direct contrast to *in vivo* genome editing approaches, in which the CRISPR/Cas9 or other genome editing components are directly introduced into the patient via local or systemic delivery and exert their therapeutic effect on-site [64,69]. Compared with the *in vivo* strategy, the *ex vivo* editing strategy requires more steps (e.g. cell collection, isolation, expansion, editing, selection, and transplantation) and may be better suited for targeting a specific organ rather than the whole organism [70]. However, it largely avoids the tremendous *in vivo* delivery challenges which have been described extensively in other review papers [41,48]. Furthermore, the *ex vivo* approach may have particular safety benefits, especially regarding off-target gene editing. *In vivo* approaches must worry about unintended off-target editing events, either in the form of unintended delivery to an off-target cell type, or in the form of unintended editing of an off-target locus in the genome. *Ex vivo* approach avoids this problem by only editing exactly the intended cell type, and allowing an opportunity to screen for successful editing. In this section, we highlight the *ex vivo* applications of CRISPR/Cas9 for therapeutic genome editing. The targeted conditions, genome editing strategies, and related references have been summarized in Table 1.

3.1. Human immunodeficiency virus infection

The human immunodeficiency virus type 1 (HIV-1) requires CD4 and C–C chemokine receptor type 5 (CCR5) or C-X-C chemokine receptor type 4 (CXCR4) to infect host cells [71,72]. Homozygosity with a 32-bp deletion in *CCR5* allele (CCR5Δ32) results in resistance to HIV-1 infection [73]. The medical achievement from the “Berlin patient” (Timothy Brown) indicated the treatment of HIV infection through stem cell therapy [74].

In 2014, Ye and Kan et al. used a combination of CRISPR/Cas9 and piggyBac technologies to generate induced pluripotent stem cells (iPSCs) homozygous carrying a CCR5Δ32 mutation that is identical to the naturally occurring and HIV-1 protective mutation (Fig. 3A) [75]. The DSB in genome DNA at exon 4 of *CCR5* gene was generated from CRISPR/Cas9, which was delivered into cells as plasmids through electroporation. A piggyBac transposon vector was inserted through HDR and the CCR5Δ32 mutation was formed after transposase excise. The authors showed that CRISPR/Cas9 yielded 100% homologous recombination and 33.3% biallelic targeting efficiency. Furthermore, no off-target mutation was found after checking 13 top-scoring potential sites in the genome. Finally, the iPSCs with the CCR5Δ32 mutation were differentiated into monocytes and macrophages *in vitro*, which were then demonstrated to be resistant to HIV-1 infection (CCR5-tropic HIV–1SF170; Fig. 3B). This study provided both a possible way for cell therapy of HIV-1 infection and inspiration for gene correction or mutation in other genes.

Using electroporation and plasmids of CRISPR/Cas9 components, Torbett and Slukvin et al. also demonstrated that the *CCR5* gene disruption in human iPSCs induced selective resistance against CCR5-tropic HIV, and that a dual guide RNA system can significantly increase the frequency of biallelic editing without compromising specificity [76]. This study also demonstrated that macrophages from CCR5-edited iPSCs were resistant to CCR5-tropic HIV-1 (R8 Bal and SF162), not the CXCR4-tropic virus (LAI and NL4-3).

Using adenovirus vectors, Hu et al. delivered CRISPR/Cas9, targeting the open reading frame (ORF) on exon 4 of *CCR5* gene, into primary $CD4^+$ T cells and disrupted CCR5 expression through the NHEJ pathway [77]. The edited cells were proved to be resistant to HIV-1 (Bal and a transmitted/founder strain CH042). Another HIV-1 coreceptor, CXCR4, was knocked out in primary human $CD4^+$ T cells, by Doudna and Marson et al. by delivering Cas9:sgRNA ribonucleoprotein (RNP) through electroporation [78]. Both the *CXCR4* gene disruption (through NHEJ or HDR pathways) and protein expression were characterized, while the susceptibility of edited $CD4^+$ T cells against CXCR4-tropic HIV-1 needed further examination.

It should be noted that certain indel (insertion and deletion) mutations in CCR5 gene induced by the NHEJ pathway can have the same HIV-1 protective effect on the cellular level. However, one consequence of the NHEJ pathway is that it lacks precise control over the specific mutation it generated, and thus in addition to the desired indel, may

Table 1
Summary of the targeted conditions, genome editing strategies, and key references of the *ex vivo* cell-based CRISPR/Cas9 genome editing for therapeutic applications discussed in this review.

Targeted conditions	Genome editing strategies	References
Human immunodeficiency virus type-1 infection	CCR5 or CXCR4 knockout	[75–78]
Cancer	PD-1 knockout	[78,81]
	CAR knock-in	[83,84]
Duchene muscular dystrophy	DMD exon-skipping	[91,92]
Chronic granulomatous disease	CYBB point mutation correction	[101,102]
Monogenic diabetes	INS point mutation correction	[108]
Sickle cell disease	HBB point mutation correction	[111]
	BCL11A knockout	[112]
β-thalassemia	BCL11A knockout	[112]
Hereditary tyrosinemia type 1	FAH point mutation correction	[117]
Cystic fibrosis	F508del correction	[121]

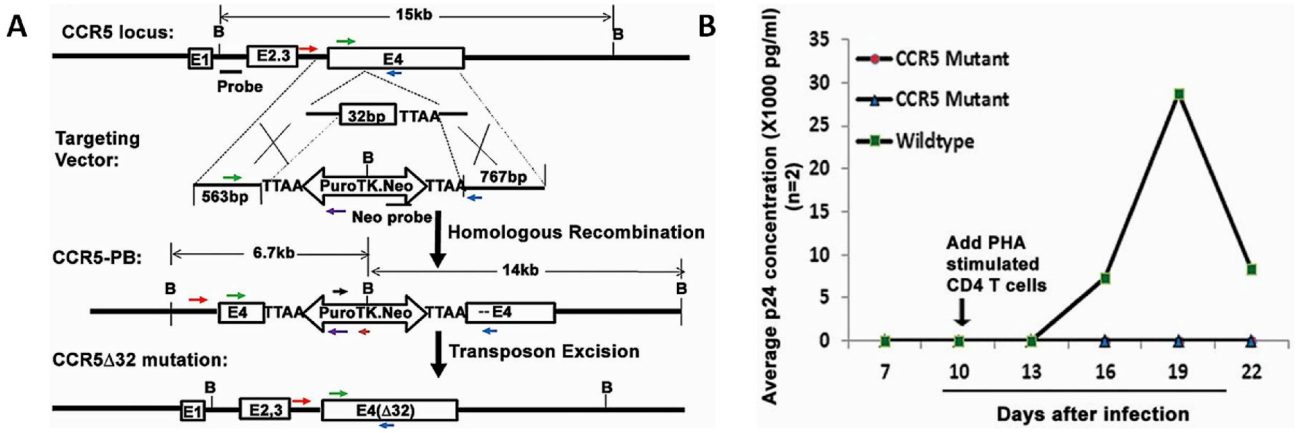

Fig. 3. CRISPR/Cas9 enabled CCR5Δ32 deletion mutation and HIV protection. (A) Combination of CRISPR/Cas9 (or TALEN) and piggyBac technologies to generate CCR5Δ32 mutation in exon 4 of *CCR5* gene. B, BamHI; E, exon. The PuroTK.Neo cassette was inserted to enable cell selection and southern blot analysis. The red, green and blue arrows indicate different primers for PCR. (B) HIV-1 challenge of monocytes/macrophages derived from CCR5Δ32 mutation and wild-type iPSCs. PHA (phytohemagglutinin) stimulated $CD4^+$ T cells were added to amplify HIV-1 for detection. Reprinted with permission from Ref. [75]. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

generate other mutations whose health implications are yet to be understood. The CCR5Δ32 deletion mutation, induced by the more complicated and more precise HDR or HDR/piggyBac strategies, recreates a very specific allele which has been found naturally occurring with minimal adverse outcomes, and thus should have fewer safety concerns. Until the long-term evaluation of NHEJ-generated CCR5 indels can be thoroughly done, the CCR5Δ32 deletion mutation strategy would likely be the more favorable therapeutic approach. Even though the efficiency of HDR is usually much lower than the NHEJ pathway, an *ex vivo* approach may allow for the opportunity for screening, selection, and enrichment of the cells with specifically the correct mutation prior to transplantation. This opportunity would not be available for an *in vivo* treatment strategy. More research is necessary to further develop this idea.

3.2. Cancer immunotherapy

In the checkpoint blockade immunotherapy, the programmed death-1 receptor (PD-1) has been identified as a cell surface receptor on chronically activated or exhausted T cells that can inhibit T cell-mediated tumor cell clearance [79, 80]. Monoclonal antibody against PD-1 has been approved for advanced malignancy, while the systemic administration of PD-1/PD-L1 (ligand of PD-1) blocking antibodies elevates the risk of breaking peripheral tolerance. The knockout of PD-1 expression in T cells through genome editing holds great promise in cancer immunotherapy. In this context, using electroporation of CRISPR/Cas9 RNP, Doudna and Marson et al. reduced primary human T cell surface PD-1 expression by targeting the exon 1 of PD-1 gene [78]. Huang and Liu et al. also showed the successful genetic disruption of PD-1 in human primary T cells obtained from cancer patients, using an electroporation protocol and plasmids that encode Cas9 and sgRNA [81]. After studying the genotype and phenotype of the reprogrammed T cells, the authors showed that, comparing to the control T cells, the edited cells had enhanced cytotoxicity against PD-L1 expressing tumor cells.

Although the *in vivo* anti-cancer activity was not evaluated in these studies, there is no doubt that the CRISPR holds great promise in checkpoint blockade cancer immunotherapy. Actually, the very first human CRISPR clinical trial used CRISPR/Cas9-mediated PD-1 knocked out T cells for the treatment of patients with non-small cell lung cancer (ClinicalTrials Identifier: NCT02793856) [33]. There are several other open or closed clinical trials involving CRISPR/Cas9-mediated PD-1 knocked out T cells for cancer therapy and some of them are highlighted in section 4.

Besides the checkpoint blockade immunotherapy, another direction for cancer immunotherapy is the CAR (chimeric antigen receptor) T therapy. CARs are synthetic cell surface receptors that direct the reprogrammed T cells to find and kill cancer cells expressing certain ligands. Two CAR T cell therapies, Kymriah and Yescarta, have been approved by the US Food and Drug Administration (FDA) [82]; these therapies are CD-19 directed autologous T cells transduced by lentiviral vector (Kymriah) and γ-retroviral vector (Yescarta).

In 2017, Sadelain et al. reported a two-in-one strategy for simultaneous T-cell receptor (TCR) knockout and CAR knock-in (Fig. 4A) [83]. The CAR knock-in enables T cells to target and kill specific antigen-expressing tumor cells and the endogenous TCR knock-out generates universal CAR T cells that are incapable of mediating the graft versus host disease (GvHD) in allogeneic transplantation. Using a combination of electroporation of Cas9 mRNA and gRNA, and adeno-associated virus (AAV) vector carrying CAR sequence, the exon 1 of T-cell receptor α constant (*TRAC*) gene was targeted by CRISPR/Cas9 and CD-19 specific CAR (1928z) was integrated through HDR. Unlike retrovirally encoded CAR (RV-CAR) which showed variegated expression, CRISPR/Cas9 induced homogenous and consistent expression of CAR in multiple donors. Even though CRISPR engineered CAR T cells did not show notable differences against RV-CAR regarding cytotoxicity and proliferation response *in vitro*, it induced greater response and prolonged median survival at different doses in acute lymphoblastic leukemia NALM-6 mouse model (Fig. 4B). The authors demonstrated that the new CAR system averts tonic CAR signaling and establishes effective internalization and re-expression of CAR, delaying effector T-cell differentiation and exhaustion. A similar TCR replacement system has been realized in primary T cells by Sewell et al. using a lentiviral vector encoding CRISPR/Cas9 [84]. The simultaneous endogenous αβ TCR knockout and cancer-reactive γδ TCR expression resulted in efficient redirection of $CD4^+$ and $CD8^+$ T cells against established blood cancer cell lines and primary blasts.

These studies showed the advantages of CRISPR/Cas9 genome editing technology originated from its high controllability. Comparing with the virus-mediated gene integration, the CRISPR/Cas9 HDR pathway makes gene knock-in highly precise. In addition, one step of CRISPR/Cas9 HDR integration can result in two positive effects – CAR knock-in and TCR knockout.

Moreover, in order to make the universal CAR T cells for potential allogeneic adoptive transfer, Wang et al. used electroporation method to transfect Cas9 protein and IVT sgRNA *in vitro* [85]. CAR T cells with two (*TRAC* and *B2M* (beta-2 microglobulin)) or three (*TRAC*, *B2M* and *PD-1*) genes disrupted were generated and tested for anti-cancer

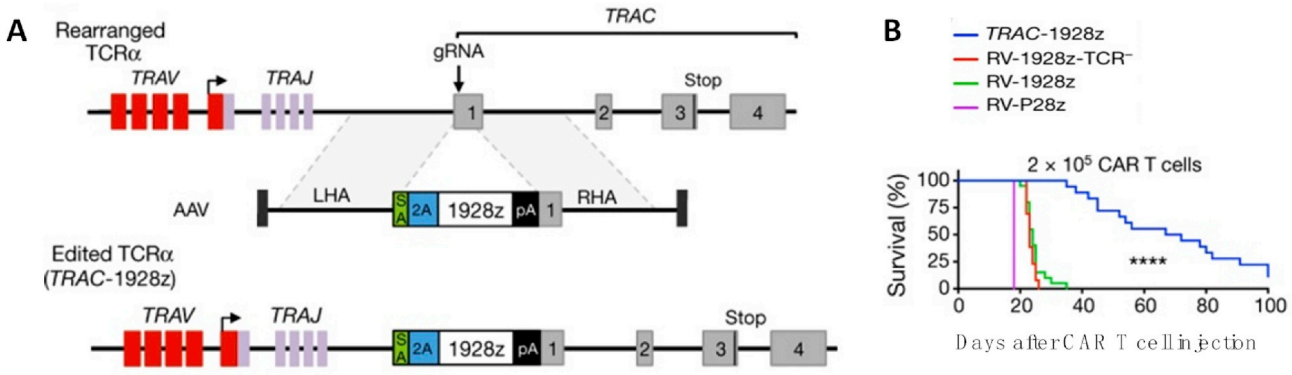

Fig. 4. CRISPR/Cas9 enabled CAR T therapy. (A) CAR insertion into the *TRAC* locus mediated by CRISPR/Cas9. LHA and RHA, left and right homology arm. (B) Kaplan-Meier analysis of survival of NALM-6 mice after CAR T cell injection. TRAC-1928z, TRAC disrupted and CD19-specific 1928z integrated CRISPR/Cas9 CAR T cell; RV-1928z-TCR-, CRISPR/Cas9 generated TCR- T cells were transfected by RV-1928z retroviral vectors; RV-1928z/P28z, T cells transduced with RV-1928z or RV-P28z (PSMA (prostate-specific membrane antigen)-specific CAR) retroviral vectors. Reprinted with permission from Springer Nature and Copyright Clearance Center: Springer Nature, Nature, Ref [83]. Copyright 2017.

functions *in vitro* and *in vivo*. In another study, using electroporation to deliver Cas9 mRNA and sgRNA, June and Zhao et al. generated universal CAR T cells with *TCR*, *B2M* and *PD-1* double or triple disruption [86].

There is no doubt that the CAR T cell therapy is potent and flexible, supported by the FDA approval of Kymriah and Yescarta, as well as hundreds of CAR T cell related clinical trials. In the future, we expect that continued research towards the discovery of new cancer cell-surface markers will facilitate the development of CAR T cell therapies with more specific and/or diverse targets. Furthermore, through gene and/or cell engineering, the side effects of CAR T cell therapy (e.g. cytokine release syndrome, anemia, neutropenia, etc.) would be reduced or eliminated. Together, this will allow for the development of even more powerful and effective CRISPR-based CAR T therapies.

3.3. Duchenne muscular dystrophy

In the most common fatal genetic disease of childhood, Duchenne muscular dystrophy (DMD), mutations disrupt reading frame and prevent the translation of dystrophin, which is a vital protein connecting the cytoskeleton of muscle fiber to the extracellular matrix [87,88]. Around 63% of such mutations occur between exons 45 to 55 of *DMD* gene [89]. Interestingly, it has been shown that a large-scale genomic deletion of exons 45–55 (which would include the deletion of the disease-bearing mutation) results in a truncated but partially functional form of the protein and improved therapeutic outcomes [89,90]. Thus, this exon-skipping approach has been proposed as a potential therapeutic strategy for DMD.

In 2016, Spencer and Pyle et al. used CRISPR/Cas9 (electroporation of plasmid DNA encoding Cas9 and gRNA) to generate in-frame deletion of *DMD* gene exons 45–55 through NHEJ pathway, in patient-derived iPSCs (Fig. 5A) [91]. A pair of gRNAs targeting intron 44 and intron 55 were chosen, and deletions of up to 725 kb in the *DMD* gene were observed. The dystrophin protein function was restored in cardiomyocytes and skeletal muscle cells derived from reframed iPSCs from multiple patients. Correct localization of dystrophin and β-dystroglycan was observed from the skeletal muscle cells derived from CRISPR-edited iPSCs in a mouse model after cell transplantation (Fig. 5B).

By targeting *DMD* gene exons 45–55, Gersbach et al. restored the dystrophin reading frame through introducing shifts within exons or deleting one or multiple exons. DMD patient immortalized myoblasts were transfected with plasmid DNA encoding Cas9 and sgRNA by electroporation [92]. The restored human dystrophin expression was observed *in vivo* following cell transplantation into immunodifficient mice.

In addition to these advances, the *in vivo* gene editing for DMD treatment has also been reported. In 2016, Wagers et al. [93], Gersbach et al. [94], and Olson et al. [95] used AAV vectors to deliver CRISPR/Cas9 to the *mdx* mouse model of DMD, locally and systemically. The same strategy, exon-skipping, was used in these studies and partial functional dystrophin recovery as well as improvement of muscle biochemistry were achieved. In general, if the *in vivo* CRISPR/Cas9 delivery system can produce clinically relevant editing efficiency, and the short-term and long-term side-effects are carefully evaluated [96], it may be more advantageous than *ex vivo* genome editing, as the procedures are simpler and repeated administration is easy to do. However, the *ex vivo* testing of CRISPR/Cas9 systems for DMD treatment can still provide useful information to guide and optimize gRNA design, and to enable quick formulation screening.

3.4. Chronic granulomatous disease

Chronic granulomatous disease (CGD) is a rare genetic disease, characterized by persistent and life-threatening infections resulted from the inability of phagocytotic cells to generate reactive oxygen species (ROS) [97,98]. This disease can be treated with antibiotics, however the patient will be on the antibiotic treatment for life. Heterologous bone marrow transplantation provides a curative therapy, but is usually complicated by the GvHD [99]. Like in many other genetic disorders, genome editing technologies like CRISPR can make autologous stem cell transplantation possible. Although many genes are involved in the ROS-producing nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and mutations on any of them could induce CGD, more than 60% of CGD are resulted from the loss-of-function of the cytochrome *b*-245 heavy chain (CYBB) protein (or GP91PHOX), which is located on the X chromosome [100].

In 2015, Moore et al. used CRISPR/Cas9 to correct a point mutation (T > G) in the intron 1 of *CYBB* gene in CGD patient-derived iPSCs (Fig. 6A) [101]. Plasmid DNAs carrying Cas9, gRNA, and donor sequence were introduced into iPSCs through electroporation. Correction of the *CYBB* gene through HDR resulted in the restoration of ROS production in the differentiated monocytes and macrophages (Fig. 6B). Another contribution of this study is that the authors demonstrated the T > G mutation resulted in exon skipping within the mRNA of CYBB.

Ravin and Malech et al. corrected a C > T single point mutation in the exon 6 of *CYBB* gene, the most frequent mutation (6%) in the NIH cohort of CGD patients, in patient-derived hematopoietic stem cells [102]. In this case, IVT Cas9 mRNA, sgRNA and single-strand oligonucleotide (ssODN) as donor DNA template, were transfected into stem cells using electroporation. Function of NADPH oxidase and superoxide radical production of myeloid cells derived from the corrected progenitor cells were restored. Long-term persistence of corrected and

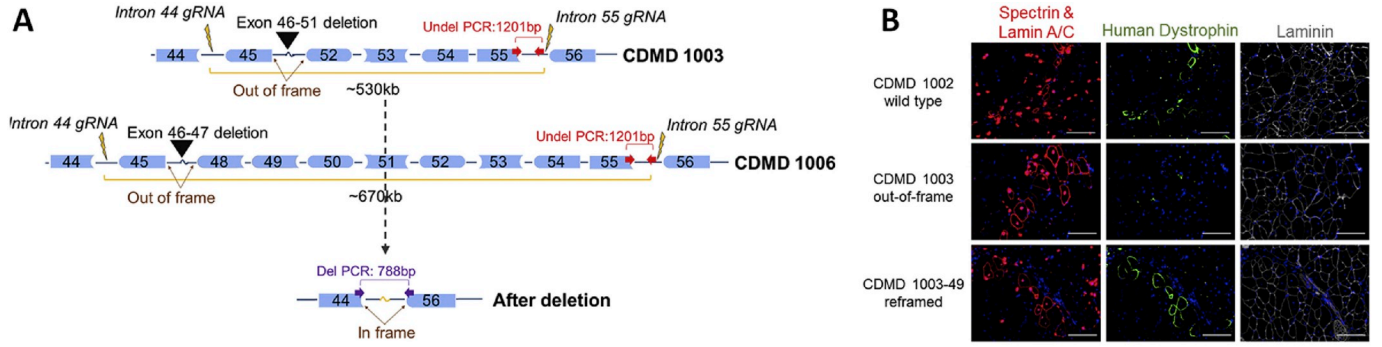

Fig. 5. CRISPR/Cas9-mediated in-frame deletion of *DMD* gene. (A) CRISPR/Cas9 mediated exons 45–55 deletion through NHEJ and following reading frame restoration. CDMD, Center for Duchenne Muscular Dystrophy. (B) Human dystrophin restoration in wild-type, out-of-frame, and reframed MyoD OE cells engrafted into mice model. DAPI was used to stain nuclei; Spectin and lamin A/C were used to identify human cells; Laminin was used to show all fibers. Scale bar = 100 μm. Reprinted from Ref. [91], Copyright 2016, with permission from Elsevier.

transplanted cells was achieved in mouse model. The safety of this CRISPR/Cas9 gene editing system was evaluated by whole-exome sequencing, and no indel mutations were found outside *CYBB* gene.

Therefore, both studies demonstrated that single nucleotide base mutation in CGD can be corrected *ex vivo* by CRISPR/Cas9 through the HDR pathway. To demonstrate their therapeutic effects, these strategies for CGD therapy needs to be further validated in small and large animal models. Additionally, besides HDR, the point mutation – or more specifically, single base change – in CGD or other types of disorders, could also be potentially corrected by DNA base editing, which was first developed by Liu et al., in 2016 [103]. There are two classes of DNA base editors that can mediate four transition mutations, i.e., C to T, A to G, G to A, and T to C [103,104]. While the T to C base correction as in the case of Malech's study can be potentially corrected by the adenine base editor (ABE), the G to T conversion in the case of Moore's study is not feasible yet. This also represents an opportunity, as such a base editor enzyme can be used not only in the treatment certain CGD cases, but also many other pathogenic point mutations [42]. Another possibility is to use the most recently developed prime editing technology [105], which will be discussed in section 5.

3.5. Monogenetic forms of diabetes

The most common diabetes, i.e. type 1 and 2, are polygenic, that is they may be caused by multiple genes that affect the production and function of insulin [106]. The monogenetic forms of diabetes, which account for 1–4% of all diabetes patients in the US, present with a phenotype identical to type 1 diabetes, however is not mediated by immune rejection [106,107]. Two main forms of monogenetic diabetes are neonatal diabetes mellitus (NDM, which can be permanent (PNDM) or transient (TNDM)) and maturity onset diabetes of the young (MODY), which usually occur at different ages. Because of the monogenic feature and absence of autoimmunity complications of NDM and MODY [106], *in vitro* gene correction of stem cells followed by transplantation could be useful to restore the glucose homeostasis.

In 2018, Egli et al. identified a G > A point mutation on exon 2 of the *INS* gene of a PNDM patient, and corrected this mutation in iPSCs (Fig. 7A) [108]. DNA vectors encoding Cas9 and gRNA, as well as ssODN template carrying a neutral single nucleotide polymorphism (SNP) were transfected into iPSCs through electroporation. The CRISPR HDR-repaired cells were able to differentiate into pancreatic endocrine cells, and restored insulin production and secretion were observed. Mice transplanted with these edited cells could maintain normal

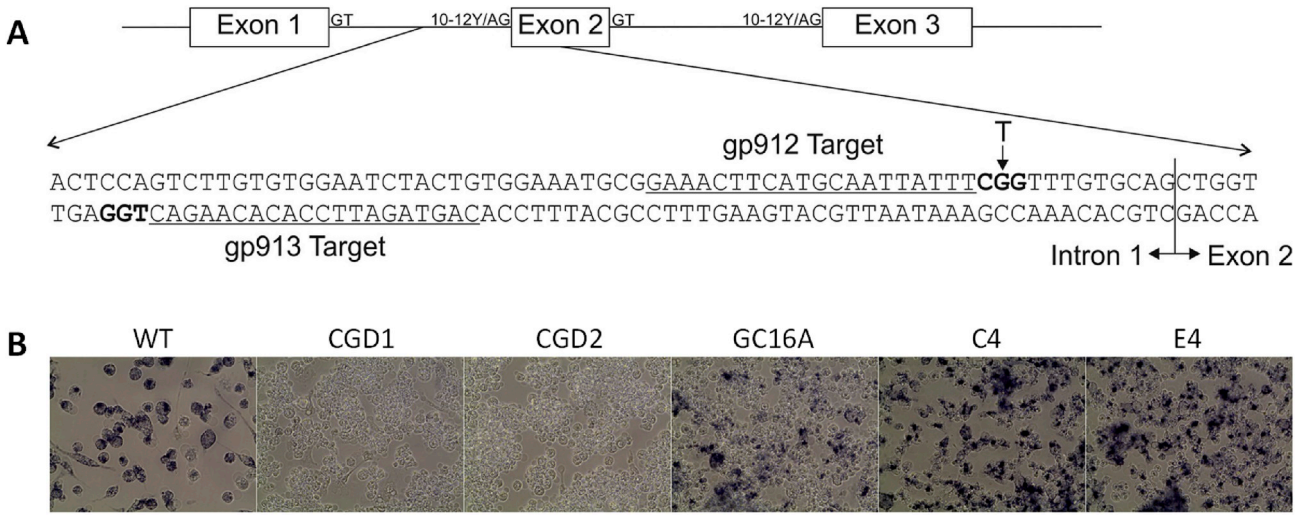

Fig. 6. CRISPR/Cas9-mediated point mutation correction in *CYBB* gene through the HDR pathway. (A) CGD2 patient's T > G mutation and target sites of CRISPR/Cas9 in the *CYBB* gene. The consensus splice donor (GT), splice acceptor (AG), and polypyrimidine tract (10-12Y) have been shown. (B) ROS-production (indicated by the dark precipitations; nitroblue tetrazolium (NBT) assay) was rescued from CRISPR/Cas9 mediated *CYBB* gene correction in CGD2 iPSC-derived myeloid cells. Images show macrophages from WT NHDF1 iPSCs (WT), P47Phox mutant iPSCs (CGD1), *CYBB* mutant iPSCs (CGD2), mixed pool CGD2.GC16A of CRISPR/Cas9 edited iPSCs (GC16A), and two corrected single-cell clones from CGD2.GC16A. Reprinted from Ref. [101], under the terms of the Creative Commons Attribution License (CC BY).

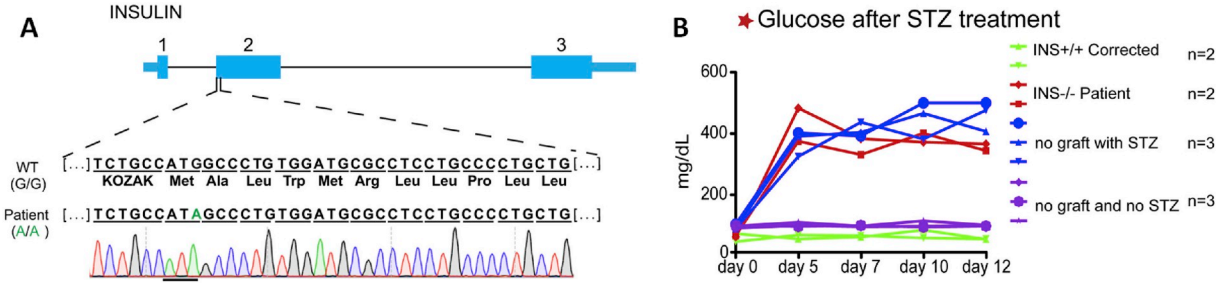

Fig. 7. CRISPR/Cas9-mediated point mutation correction in the *INS* gene. (A) The wild-type sequence and patient with G > A homozygous mutation (highlighted in green) in exon 2 of the *INS* gene. (B) Blood glucose level of mice with or without transplantation, after STZ treatment. STZ, streptozotocin, which was used to ablate mouse endogenous β cells. Reprinted from Ref. [108], Copyright 2018, with permission from Elsevier. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

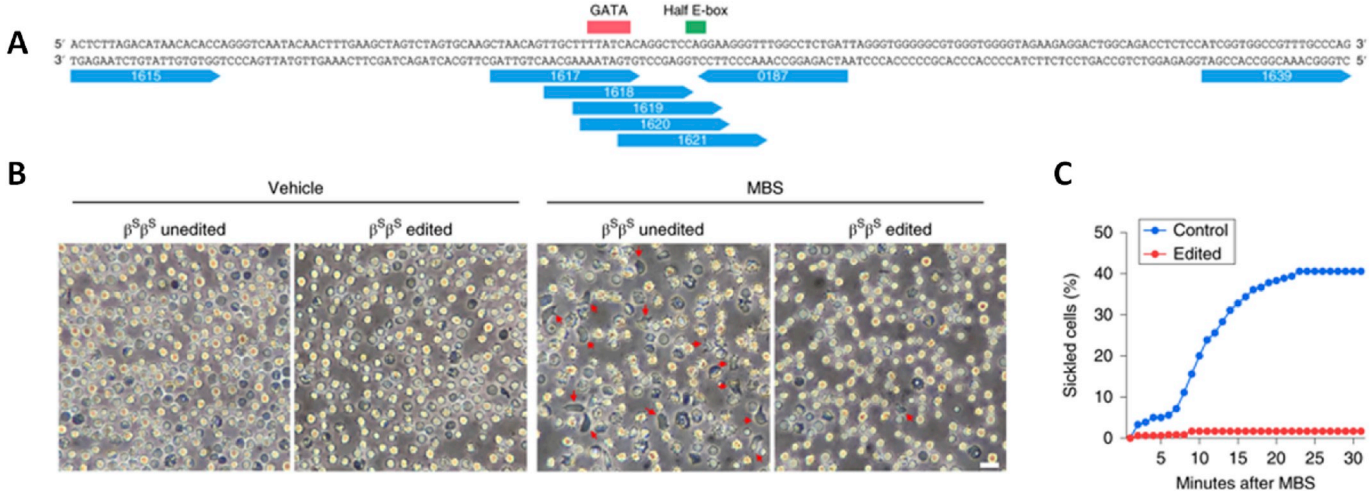

Fig. 8. Targeted *BCL11A* enhancer disruption mediated by CRISPR/Cas9. (A) Eight CRISPR/Cas9 target sites (highlighted in blue) in BCL11A enhancer DNase I hypersensitive site. (B) Images of enucleated erythroid cells *in vitro* differentiated from bone marrow of mice transplanted with unedited or *BCL11A* enhancer edited CD34$^+$ HSPCs, with and without MBS treatment. Red arrows indicate sickled cells. MBS, sodium metabisulfite. Scale bar = 10 μm. (C) Quantification of *in vitro* cell sickling. Reprinted with permission from Springer Nature and Copyright Clearance Center: Springer Nature, Nature Medicine, Ref [112]. Copyright 2019. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

glucose homeostasis, even after the ablation of endogenous β cells by streptozotocin (STZ; Fig. 7B).

This study demonstrated the possible application of CRISPR/Cas9 in autologous stem cell transplantation for the treatment of monogenic non-autoimmune insulin-dependent diabetes. The limitations are, as also pointed out by the authors, the occurrence of off-target events and the formation of teratomas in engrafted mice. The formation of teratomas and other neoplasms is an inherent and long-standing challenge in iPSCs therapies, while the off-target effects of CRISPR/Cas9 can potentially be attenuated or avoided by optimizing transfection conditions and engineering CRISPR components, which will be discussed in section 5.

The single point mutation studied by Egli et al. can also potentially be corrected by the base-editor, which does not require template DNA sequence as in the HDR strategy. Generally speaking, the therapeutic potential of newly developed base editors have not been fully explored yet, which represents an opportunity as the base editor system is simpler and probably more efficient.

3.6. β-Hemoglobinopathies

Sickle cell disease (SCD) is one of the most common monogenic disorders and can result in serious mortality and morbidity [109]. SCD is caused by a point mutation, A > T, in the β-globin (*HBB*) gene [110]. The production of the mutated hemoglobin distorts red blood cells into the sickle shape which give SCD its name. The premature cell breakdown and blockage of small blood vessels result in anemia, pain and organ damages. Currently, there are two FDA approved medications, i.e. Endari and Siklos, to reduce SCD severity. Allogeneic stem cell transplantation is a curative option but complicated by the GvHD. Mutated gene correction and functional hemoglobin restoration in patient derived hematopoietic stem and progenitor cells (HSPCs) would make the autologous transplantation possible.

There are at least two strategies using genome editing technology to make engineered SCD stem cells suitable for autologous transplantation. The first one, which is also the most straightforward strategy, is to correct the point mutation in *HBB* gene. In 2016, Kohn et al. demonstrated the application of CRISPR/Cas9 to correct SCD mutation in patient bone marrow derived HSPCs [111]. Using a combination of electroporation of IVT Cas9 mRNA and lentiviral vector carrying gRNA and donor DNA sequences, the SCD point mutation in CD34 $^+$ HSPCs were corrected through HDR. The CRISPR/Cas9 treatment did not affect cell differentiation throughout culture process, and at the end of erythroid differentiation, production of wild-type human hemoglobin A (HbA) was achieved.

The second strategy involves the reduction or disruption of BCL11A production, which can restart the fetal hemoglobin (HbF) production. The rationale behind this is that the switch of fetal to adult hemoglobin expression is mediated by BCL11A protein and the presence of HbF in SCD can provide protection from red blood cell sickling [113,114].

Bauer et al. targeted the +58 erythroid enhancer of BCL11A in CD34 $^+$ HSPCs using CRISPR/Cas9 (Fig. 8A) [112]. Cas9 RNP with

chemically modified synthetic sgRNAs were delivered via electroporation and targeted gene disruption was achieved through NHEJ pathway. The reduction of BCL11A expression correlated well with the production of fetal γ-globin, and the edited SCD patient HSCs were prevented from sickling (Fig. 8B and C). Another class of β-globin disorder, β-thalassemia, could also be treated by the induction of HbF re-expression, through the BCL11A reduction as demonstrated in the study of Bauer et al. [112].

CRISPR/Cas9 platform offers multiple therapeutic choices (by targeting different genes or different loci in the same gene) for each genetic disorder. However, as discussed above in the context of genome editing for HIV and cancer immunotherapy, different editing strategies may require separate analyses of the safety and efficacy. As one example specifically in the case of β-hemoglobinopathies, a recent study revealed that the genome editing of *HBG1/2* promoter leads to robust HbF expression *in vivo*, while editing BCL11A enhancer resulted in erythroid defects [115]. Nevertheless, by targeting *HBB* or BCL11A enhancer, clinical trials are on the way in regards to the SCD and β-thalassemia gene/cell therapy enabled by CRISPR/Cas9 technology (more information can be found in section 4).

3.7. Hereditary tyrosinemia type 1

Hereditary tyrosinemia type 1(HT1) is a rare autosomal recessive genetic disorder caused by mutations in the fumarylacetoacetate hydrolase (*Fah*) gene [116]. The lack of FAH enzyme leads to tyrosine and its metabolites accumulating in and damaging the liver, kidney and central nervous system. Current HT1treatment depends on the FDA approved nitisinone (NTBC or INN) along with a diet restricted in tyrosine and phenylalanine. Liver organ/cell transplantation offers a curative therapy for HT1, but like many other cell therapies, allogeneic hepatocyte transplantation is restricted by donor resources and the GvHD complications, and precise gene correction is required for autologous transplantation.

Lillegard and Hickey et al. corrected the *Fah* mutation in mouse hepatocytes through HDR using CIRSPR/Cas9 (Fig. 9A) [117]. Cas9, gRNA and donor template were transfected by a dual AAV system. No off-target effect was observed after checking seven possible sites. The authors transplanted the corrected hepatocytes into HT1 mouse model through splenic injection and demonstrated that the edited hepatocytes can proliferate extensively *in vivo*. The mouse model can be phenotypically rescued after transplantation and the gene correction is durable (Fig. 9B). This study demonstrated the potential of CRIPR/Cas9 gene editing in the treatment of HT1 and maybe other forms of inherited metabolic liver diseases.

Although the *ex vivo* gene therapy of HT1 mediated by retroviral vectors has been reported previously, the site-specific genome engineering enabled by CRISPR/Cas9 as demonstrated in this study would be more preferred in clinical applications. It would be more advantageous if the mutated *Fah* gene could be repaired *in situ* for HT1 therapy. In this context, Anderson et al. corrected the *Fah* gene in a HT1 mouse model through hydrodynamic injection of ssODN and plasmid expressing Cas9 and sgRNA [118]. Apparently, the direct *in vivo* genome editing, which does not require hepatocytes isolation, *in vitro* culture, and transplantation, is more convenient, but the *ex vivo* gene therapy followed by cell transplantation can still be a potential alternative curative strategy for HT1.

3.8. Cystic fibrosis

Cystic fibrosis (CF) is a genetic disease caused by the mutations in the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene and the malfunction of the translated CFTR protein [119]. Failure of CFTR channel protein to maintain fluid and electrolyte homeostasis in epithelia results in thick and sticky mucus in various organs, which leads to complications like infections, inflammation, and malnutrition, etc [120]. Progress has been made in the management of CF symptoms, with drugs such as Kalydeco, Orkambi, Symdeco, and Cayston recently being approved by FDA. However, just like other types of genetic disorders that have been discussed, the complete cure of CF relies on the correction of mutated genes.

The most common mutation of CFTR is deletion of phenylalanine in *CFTR* gene exon 11, called F508del. In 2013, Clevers et al. used CRISPR/Cas9 to correct the F508del mutation through HDR, in cultured intestinal stem cells of CF patients (Fig. 10A) [121]. Plasmids encoding Cas9, sgRNAs targeting exon or intron 11, and HDR template were delivered by Lipofectamine 2000. Correction of F508del was confirmed and the restoration of CFTR function was demonstrated in the organoid system (Fig. 10B). The authors also checked sixty potential off-target sites for two different gRNAs, and found one heterozygous insertion mutation.

Considering the multi-organ involvement nature of CF, the *in vitro* genome editing followed by cell transplantation may not be a primary option for clinical applications. However, this study showed the strong potential of using CRISPR/Cas9 in CF gene therapy and the CRISPR design strategy can be directly transferred to *in vivo* formulations. One of the opportunities is to develop safe and efficient CRISPR/Cas9 delivery systems for *in vivo CFTR* gene mutation correction through local or systemic administration.

4. CRISPR therapy in clinical trials

Currently, there are 28 studies registered in the ClinicalTrial.gov website relevant to CRISPR (by searching the keyword “CRISPR”) [122]. After screening out the programs that involve only CRISPR for gene function study, biomarker and gene target identification, model development, etc., 14 studies regarding CRISPR therapies are in the status of “open study” i.e. those in the recruiting and not yet recruiting

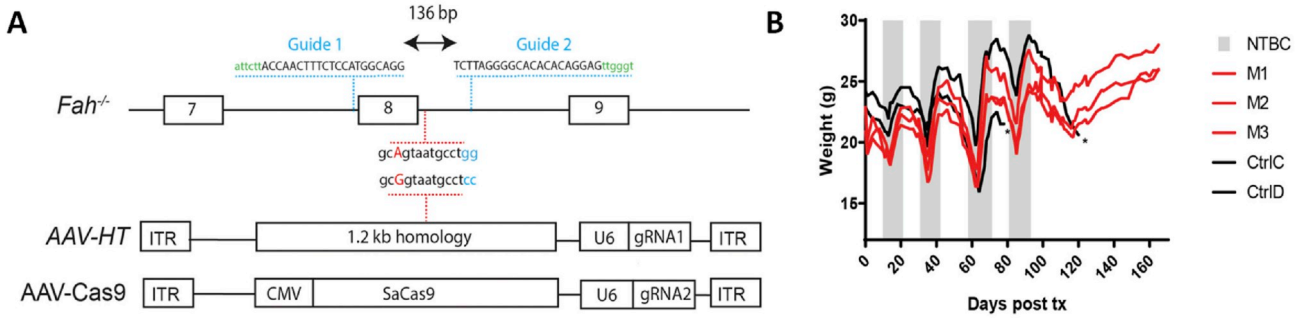

Fig. 9. Point mutation correction in *Fah* gene enabled by CRISPR/Cas9. (A) Homozygous point mutation at *Fah* locus, CRISPR/Cas9 target sites, and the AAV vectors design. (B) Weight charts of HT1 male mice transplanted with CRISPR/Cas9 edited hepatocytes and control mice. Female group can be phenotypically rescued from liver failure with one fewer NTBC cycle. NTBC, 2-(2-nitro-4-trifluoromethylbenzoyl)-1,3-cyclohexadione. Reprinted from Ref. [117], under the terms of the Creative Commons Attribution-NonCommercial-NoDerives License (CC BY-NC-ND).

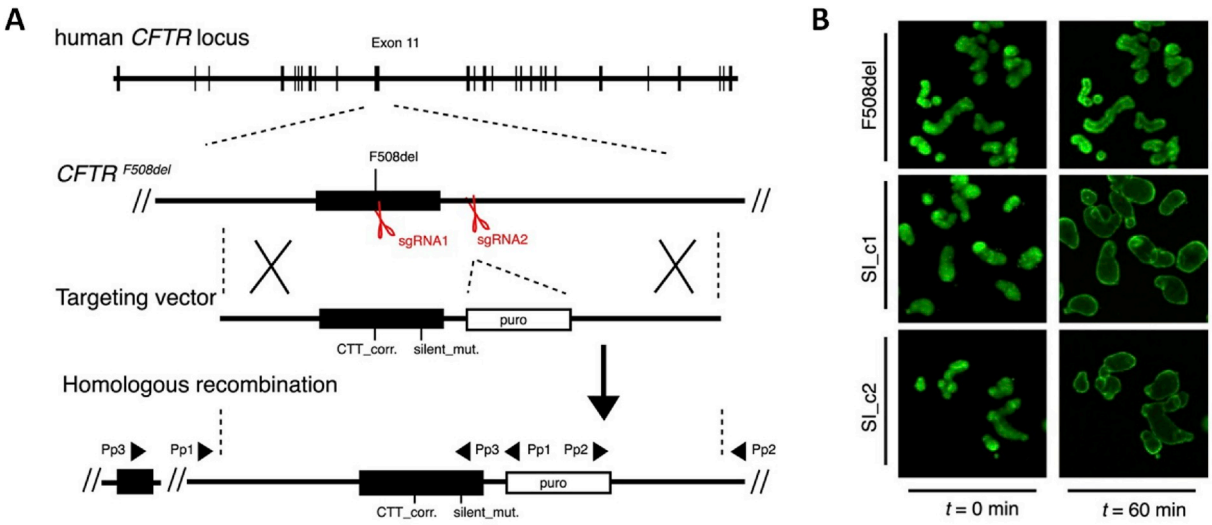

Fig. 10. CRISPR/Cas9 mediated HDR for the correction of *CFTR* mutation. (A) A silent mutation was introduced downstream of the CTT F508del correction and allows allele-specific PCR testing. Pp1, Pp2 and Pp3 indicate different primers for PCR. (B) Images of calcein-labeled and forskolin-stimulated small intestine organoids. Forskolin activates CFTR by increasing intracellular AMP, resulting in fluid secretion and organoids swelling. F508del, uncorrected organoids from patient; SI_c1/2, clones derived from small intestine organoids targeted by sgRNA1 and sgRNA2, respectively. Reprinted from Ref. [121], Copyright 2013, with permission from Elsevier.

Table 2
The general information about CRISPR-based therapeutics in clinical trials in the status of open study.

NCT Identifier	Targeted conditions	Description of main biological
NCT03164135	HIV-1 infection	Allogeneic CD34$^+$ HSPCs edited by CRISPR/Cas9 at the CCR5 gene
NCT03399448	Multiple myeloma Melanoma Synovial sarcoma Myxoid/Round cell liposarcoma	Autologous NY-ESO-1-directed T cells edited by CRISPR/Cas9 to eliminate endogenous TCR and PD-1
NCT03545815	Solid tumor, adult	CRISPR/Cas9 edited PD-1 and TCR knockout mesothelin-directed CAR T cells
NCT04037566	Leukemia Lymphoma	Autologous CD19-directed T cells edited by CRISPR/Cas9 to eliminate endogenous HPK1
NCT03655678	Thalassemia	Autologous CD34$^+$ HSPCs edited by CRISPR/Cas9 at the erythroid lineage-specific enhancer of the BCL11A gene
NCT04035434	B-cell malignancy Non-Hodgkin lymphoma B-cell lymphoma	Allogeneic CD19-directed T cells edited by CRISPR/Cas9 to insert CAR and eliminate endogenous TCR and MHC1
NCT03745287	Sickle cell disease	Autologous CD34$^+$ HSPCs edited by CRISPR/Cas9 at the erythroid lineage-specific enhancer of the BCL11A gene
NCT03728322	Thalassemia	CRISPR/Cas9 edited patient-specific iHSCs at the HBB gene
NCT03747965	Solid tumor, adult	CRISPR/Cas9 edited PD-1 knockout mesothelin-directed CAR T cells
NCT03166878	B cell leukemia B cell lymphoma	Allogeneic CD19-directed CAR T cells edited by CRISPR/Cas9 to eliminate endogenous TCR and B2M
NCT03398967	B cell leukemia B cell lymphoma	CRISPR/Cas9 edited universal dual-specific CD19 and CD20 or CD22-directed CAR T cells
NCT03690011	T-cell acute lymphoblastic leukemia T-cell acute lymphoblastic Lymphoma T-non-Hodgkin lymphoma	Autologous CD7-directed CAR T cells edited by CRISPR/Cas9 to eliminate endogenous CD7
NCT03044743	EBV positive advanced stage malignancies	Autologous T cells edited by CRISPR/Cas9 to eliminate PD-1
NCT03872479	Leber congenital amaurosis 10	CRISPR/Cas9 in AAV vectors for subretinal injection

Note: MHC1, major histocompatibility complex class 1; EBV, Epstein-Barr virus.

stages. The general information about these studies, including NCT Identifier, targeted conditions and main biologicals used, are summarized in Table 2.

It is obvious that, except for one case (NCT03872479; sponsored by Allergen and Editas Medicine; using viral vectors for local CRISPR/Cas9 delivery *in vivo)*, *ex vivo* CRISPR/Cas9 edited cells were used in all the other studies. Nine of the *ex vivo* studies concern cancer immunotherapy (sponsored by University of Pennsylvania, Chinese PLA General Hospital, CRISPR Therapeutics, Baylor College of Medicine, etc.), three for β-hemoglobinopathies (sickle cell disease and β-thalassemia; sponsored by Vertex Pharmaceutics, CRISPR Therapeutics, Allife Medical Science and Technology), and one for HIV-1 infection (sponsored by Affiliated Hospital to Academy of Military Medical Sciences). The general CRISPR/Cas9 gene editing strategies employed for biological development in these cases, i.e., CAR insertion and/or TCR disruption in cancer immunotherapy, HBB correction or BCL11A reduction for β-hemoglobinopathies therapy, and CCR5 modification for HIV-1 infection treatment, are all discussed in previous sections.

5. Challenges and opportunities

The first major challenge to the *ex vivo* gene editing approach is the delivery method. When examining the *ex vivo* CRISPR field as a whole, electroporation is generally the most common method to deliver CRISPR components for *ex vivo* genome editing. In other studies, viral vectors or synthetic materials (Lipofectamine) were used. This represents one potential opportunity for improvement. Although the electroporation can be applied to all cell types and at all stages of cell

cycles, its efficiency is reliant on the electrical properties of the cells and it has been reported to occasionally cause cell death and cargo damage [123]. Generally speaking, synthetic carrier systems would be less invasive than physical methods of transfection, and benefit from a lack of both immunogenicity and propensity of genome integration when compared with viral delivery [41,124]. Lipid-based transfection methods are simple to perform, due to the availability of commercial delivery kits such as Lipofectamine family of reagents. But the toxicity issues and cell morphological changes associated with Lipofectamine can be a concern [58,60,125]. From a delivery perspective, there is an opportunity here to develop novel synthetic delivery systems (lipids, polymers, and inorganic nanoparticles) [48,57,126,127] that can transfect CRISPR encoding DNA, RNA or RNP for *ex vivo* applications, in a highly efficient and safe way, especially into particularly hard-to-transfect cells.

Second, as has been shown in many cases, there are usually more than one genome editing strategy that may be deployed for the treatment of any given disease. For example, a given disease may be treated by base editing, HDR, NHEJ, exon skipping, etc. However, certain strategies may potentially be more favorable than others for a particular application, for example, naturally occurring Δ32 mutation in *CCR5* gene versus random indel mutations [75], and insertion of CAR under certain promoters versus at other random locations [83], etc. The CCR5 Δ32 deletion mutation can be achieved through a combination of HDR and piggyBac technology, which involves multiple steps [75]. On the other hand, the NHEJ-mediated random indel mutations in *CCR5* gene could also potentially induce CCR5 receptor knockout, which is a much easier editing strategy [77]. Even though the NHEJ-mediated CCR5 edited immune cells were proven to be HIV resistant, the long-term biological effects of the edited cells must be carefully examined for translational purpose. This is illustrative of the fact that there are many decisions to be made when undertaking to translate the basic research of CRISPR-mediated gene editing to the clinic. From a perspective of CRISPR/Cas9 system design, even though each condition or disease is different, it should be kept in mind that specific gene knock-in or knockout with higher controllability and predictable biological effects would be more preferred.

Third, the off-target effect is a huge concern in any genome engineering tool, including the CRISPR/Cas9 systems [128]. The frequency of off-target editing differs depending on the chemical nature and transfection method of Cas9, gRNA, as well as the targeted conditions, cells, and gene locus. In this context, RNP delivery is considered to have the most direct and transient effect which can potentially contribute to the minimization of off-target effects, yet has been rarely used in the *ex vivo* studies discussed above. This may be due to the relative difficulty of RNP delivery compared with nucleic acids. Cas9 and gRNA engineering have also been showed to be able to improve the editing specificity and reduce off-target effects [129–132]. However, these strategies have largely not been employed in the *ex vivo* CRISPR/Cas9 genome editing systems. Although the cleavage specificity of CRISPR/Cas9 systems was considered to be determined by the PAM and 20-nt guide sequences, off-target cleavage of DNA sequences could still occur with even three and more mismatches in the PAM-distal part of the guide RNA [133,134]. Molecular engineering of Cas9 protein and guide RNA can be useful to achieve CRISPR/Cas9 genome editing systems with high specificity and efficiency [135,136]. Opportunities also exist here for biomaterials scientists to develop safe and efficient synthetic carriers to deliver engineered/optimized CRISPR/Cas9 RNP complexes *ex vivo*. Even though the off-target effect was not observed in many of the *ex vivo* studies we have discussed in previous sections, it should be more carefully evaluated for translational studies. Whole genome sequencing to ensure gene integrity after genome editing will be preferred in order to facilitate the translation of *ex vivo* CRISPR therapies [137,138].

Fourth, even though most of the currently open clinical trials involve CRISPR/Cas9 engineered cells for cancer immunotherapy, and both the checkpoint blockade and CAR T strategies have achieved great success, there is still room for improvement in order to develop new and potentially better therapeutic strategies. CRISPR/Cas9 has been demonstrated to be able to or has the potential to develop better candidates like site-specific CAR insertion [83], universal CAR T cells [86], bi-specific or multi-targeting CAR T cells [139], reduced or controllable side-effects [140], etc. On the other hand, previous proof-of-concept studies have shown the applications of CRISPR/Cas9 in managing many conditions and diseases [75,91,101,108,112,117,121], other than cancer immunotherapy and β-hemoglobinopathies. From a perspective of translational study, vast opportunities exist by optimizing or re-designing previous CRISPR/Cas9 editing systems to achieve higher specificity and efficacy and pushing them into clinical trials, for the treatment of diabetes, immune disorders, metabolic diseases, and many others.

Fifth, as the potential of using base editors to treat diseases and conditions resulted from point mutations has been briefly discussed in previous sections [42,141], opportunities also exist in utilizing other newly developed genome editing technologies such as the prime editing, or PE, as therapeutics [105]. The PE system uses a reverse transcriptase fused Cas9 nickase and a multifunctional guide RNA, which enables gene insertion, deletion, and base conversion [142]. Comparing with the traditional Cas9-mediated HDR pathway, the PE offers a template-free, DSB-free, and more efficient directed repair. Comparing to the currently available base editors, the PE allows all twelve possible base conversions (C to T, T to C, etc), and PE is more precise without bystander editing theoretically. However, in some cases, the base editors can induce higher efficiency. Overall, great opportunities exist in the CRISPR/Cas9 field, for both molecular biologists and biomaterials scientists, to develop and utilize more precise and versatile genome editing tools. There is no doubt that more and more gene editing tools will be available and best choice will be reliant on the features of the tools as well as the genetic origin of each disease or condition.

Finally, almost all the *ex vivo* CRISPR/Cas9 genome editing cases that have been discussed involve single gene disruption and/or correction/insertion. The efficient editing of new disease-relevant single-gene targets, multiplex gene editing, as well as mitochondrial gene editing will fully realize the therapeutic potential of CRISPR/Cas9 technology, in the treatment of monogenic [143], multifactorial inheritance disorders (e.g. Alzheimer's disease and heart disease etc.) [144,145], and inherited mitochondria diseases [146,147].

To summarize, in one way, CRISPR has enabled us to achieve what was impossible; in another, it offers an innovative way to re-do what have been done previously [148,149]. Vast opportunities exist in this area, but it requires the close collaboration between chemists, biologists, clinicians, as well as policy-makers to protect and advance human health using the CRISPR technology.

Acknowledgements

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