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Progress and challenges towards CRISPR/Cas clinical translation

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ABSTRACT

CRISPR/Cas systems (clustered regularly interspaced short palindromic repeats) have emerged as powerful tools to manipulate the genome for both research and therapeutic purposes. However, the clinical use of this system is hindered by multiple challenges, such as the rate of off-target effects, editing efficiency, the efficacy of HDR, immunogenicity, as well as development of efficient and safe delivery vehicles that can carry these compounds. Tremendous efforts are being conducted to overcome these challenges, including the discovery and engineering of more precise and efficacious Cas nucleases. Moreover, in recent years multiple viral and non-viral delivery approaches have been explored for *in vivo* delivery of CRISPR components. Here, we summarize the available CRISPR/Cas toolbox for genome editing as well as the recently developed *in vivo* delivery vehicles for CRISPR/Cas system. Furthermore, we discuss the remaining challenges for successful clinical translation of this system and highlight the current clinical applications.

Contents

Abbreviations: CRISPR, clustered regularly interspaced short palindromic repeats; crRNA, CRISPR/RNA; tracrRNA, trans-activating RNA; DSB, double-strand break; NHEJ, non-homologous end joining; HDR, homology-directed repair; sgRNA, single guide RNA; DMD, duchenne muscular dystrophy; HBV, hepatitis B virus; HIV, human immunodeficiency virus; CAR-T, chimeric Antigen Receptor T-lymphocytes; SpCas9, *Streptococcus pyogenes* Cas9; StCas9, *Streptococcus thermophiles* Cas9; SaCas9, *Staphylococcus aureus* Cas9; NmCas9, Neisseria meningitidis Cas9; AAV, adeno associated virus; AV, adeno virus; bp, base pairs; nt, nucleotides; Pcsk9, proprotein convertase subtilisin/kexin type 9; OTC, ornithine transcarbamylase; DNMT1, DNA methyltransferase 1; F-LP, folate modified liposome; ARCA, anti-reverse cap analog; e-sgRNA, enhanced sgRNA; TTR, transthyretin; RNP, ribonucleoprotein complexes; LNP, lipid nanoparticles; GN, gold nanoparticles; CLANs, PEG-PLGA-based cationic lipid-assisted polymeric nanoparticles; CML, chronic myeloid leukemia; mGluR5, glutamate receptor 5; Fah, fumarylacetoacetate hydrolase; TILs, Tumor-infiltrating lymphocytes; PD-1, Programmed death-1; Treg, regulatory T lymphocytes; β2m, beta 2 microglobulin; LCA10, Leber congenital amaurosis 10.

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1. Introduction

Since its' discovery almost a decade ago, the CRISPR/Cas systems (clustered regularly interspaced short palindromic repeats (CRISPR) have revolutionized our ability to manipulate genomes easily and rapidly [1,2]. The CRISPR/Cas systems were evolved in bacteria and archaea as acquired immunity against invading DNA phages and plasmids [1]. The CRISPR based immune system can be classified into two distinct classes, class 1 and class 2, depending on the organization of the effector protein complex. Class 1 is composed of three different types I, III, and IV and require multi-protein complexes. Class 2 is divided into types II, V, and VI and is defined by a single-protein effector module. The majority of genome editing applications utilized the class two CRISPR systems, in particular, the CRISPR/Cas9 system. This system is composed of two elements: The Cas9 nuclease that cuts double-stranded DNA and a guide RNA consisting of two RNA molecules the CRISPR/RNA (crRNA) and trans-activating RNA (tracrRNA) that guides the Cas9 protein to a specific location in the genome. To further simplify this system for gene editing purposes, Jinek et al. designed a single guide RNA chimera (sgRNA) connecting the crRNA and tracrRNA by a short loop [1]. Upon target sequence recognition, a double-strand break (DSB) is formed, followed by repairing the target site *via* host cell repair machinery. In eukaryotes DSBs are resolved by two primary mechanisms: Non-Homologous End Joining (NHEJ) [3], a mechanism that usually results in a random insertion or deletion of nucleotides in the break site, and homology-directed repair (HDR) [4], a mechanism that utilizes a template to precisely resolve the DSB. NHEJ is the more prominent DSB repair mechanism in mammalian cells, and by designing sgRNAs to coding sequences, one can theoretically knock-out any gene of interest. HDR mediated repair can be utilized, by the addition of an HDR template, to correct mutated genes, insert genes in specific locations, and to replace a gene with a selection marker gene. Currently, the CRISPR-Cas system has been applied in research for genome-wide screens to study fundamental biological functions, to create rapidly and easily knock-out mice strains, and to identify potential gene targets in diseases [5–7]. The ability to permanently disrupt or correct any desired gene makes the CRISPR/Cas system a game-changer in gene therapy applications. In recent years, several class-2 CRISPR/Cas systems from different bacteria have been discovered (SaCas9, Cas12a, Cas12b, *etc.*) [8–10], this together with the engineering of Cas9 broaden the genome coverage, the possible applications and the efficacy of these systems (*e.g.*, dead-Cas9, High-fidelity Cas9, Cas9 nickase, *etc.*). Multiple therapeutic applications based on the CRISPR-Cas system have been demonstrated in mice for treating genetic diseases (*e.g.*, Duchenne muscular dystrophy (DMD), β-Thalassemia, Fragile-X syndrome, *etc.*) [11–14] and for viral infections (*e.g.*, hepatitis B virus (HBV) and human immunodeficiency virus (HIV)) [15–17]. Moreover, the CRISPR/Cas9 system is used for cell engineering by both disrupting genes and inserting genes in specific locations, for example, in the development of allogenic Chimeric Antigen Receptor T-lymphocytes (CAR-T) [18].

Although these recent advances represent a significant step forward to the future application of CRISPR-Cas systems to the clinic, there are still many challenges to overcome, such as the rate of off-target effects, editing efficiency, the efficacy of HDR, PAM dependency, immunogenicity, as well as translatability of *in vivo* delivery vehicles.

In this review, we discuss the current genome editing toolbox, including newly discovered Cas variants and engineered Cas9 variants. Furthermore, we review the remaining research challenges of the CRISPR/Cas systems and discuss possible solutions. We highlight recent *in vivo* applications of CRISPR-Cas9 for gene therapy in animal models using both viral and non-viral delivery systems and its' translatability to the clinic. Finally, we discuss the remaining hurdles for clinical translation of CRISPR/Cas genome editing and review current and future directions.

2. CRISPR/Cas systems toolbox

CRISPR-Cas systems are currently classified into six types (type I-VI), which can be grouped into two broad classes: class 1 systems, which include types I, III, and IV, and class 2 systems, which includes types II, V, and VI. Class 1 systems form multi-subunit effector complexes, where the crRNA is associated with a wide range of Cas proteins, whereas class 2 systems are characterized by the fact that the crRNA binds to a single Cas protein. The different types were extensively reviewed elsewhere [19,20], therefore in this review, we will focus on class 2 systems that utilize a single nuclease such as Cas9, Cas12, and Cas13 for genome editing and are currently being used for delivery and therapeutics purposes (Fig. 1).

2.1. Cas9

Cas9 is the first and most characterized nuclease, classified as type II CRISPR-Cas system. It can be found in different organisms such as *Streptococcus pyogenes* (SpCas9) [1,21], *Streptococcus thermophiles* (StCas9) [22], *Staphylococcus aureus* (SaCas9) [23], *Neisseria meningitidis* (NmCas9) [24]. Cas9 performs a blunt-ended DSB, which can be repaired either by NHEJ or HDR with a donor template DNA to create a site-specific edit. The different species CRISPR-Cas9 systems share in common the size of the Cas9 (~1000-1600aa) and sgRNA length (~100 nt) but rather differ in the PAM sequence (*e.g.*, 'NGG' (SpCas9), 'NNGRRT' (SaCas9), 'NNNNGATT' (NmCas9) and 'NGGNG' and 'NNAGAAW' PAM for StCas9 [22,25]. Cong et al. were the first to engineer two orthologs of Cas9 (StCas9 and SpCas9) to mediate genome editing in human and mouse cells [25]. After that many other studies showed the use of spCas9 systems for genome editing in different eukaryotic cells such as human cells [21,26–28], mice [29], yeast [30], Arabidopsis [31], rice and wheat [32], *C. elegans* [33], Drosophila [34], Xenopus [35], and non-human primates [36]. Alongside these reports, novel use of the cas9 system was reported by a catalytically inactivated variant of Cas9 called dead cas9 (dCas9). dCas9 lack the endonuclease activity causing gene repression rather than editing by interfering with transcriptional elongation, RNA polymerase binding, or transcription factor binding [37]. One major limitation of therapeutic genome editing in humans is the large size of spCas9 (~1600 a.a), which makes

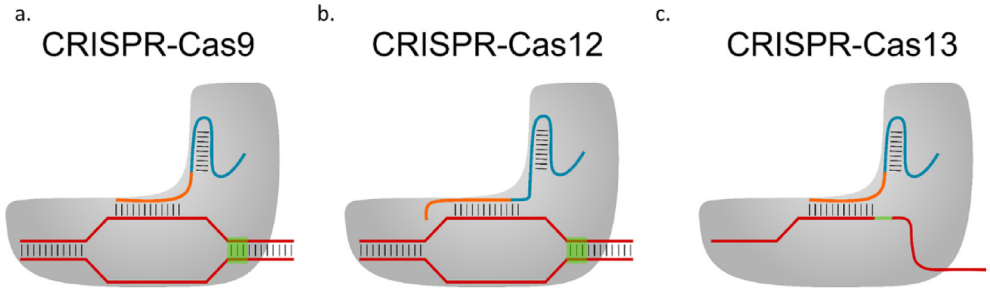

Fig. 1. Schematic comparison of Class 2 Cas proteins. a. Type II Cas9 which induces blunt ended DSB. b. Type V Cas12 which induces a staggered-ended DSB. c. Type VI Cas13 induces a cleavage of ssRNA.

delivering it a great challenge. Therefore, first experiments focused on *ex vivo* manipulation on patients' cells, mostly by electroporation for the delivery of CRISPR/Cas9 [38]. In 2015, Ran et al. reported on smaller Cas9 ortholog from *Staphylococcus aureus* (saCas9, PAM 'NNGRRT'), which was more than 1 kb shorter than SpCas9 and showed high levels of activity in human cells. This allowed the researchers to deliver saCas9, along with a guide RNA, on a single-AAV vector for *in vivo* use [23].

2.2. Cas12

In comparison to the Cas9 family of Cas effector proteins, the Cas12 family is much more diverse. Cas12 family is characterized as type V and comprised of many subtypes (type V-A – V—I). The first Cas12 nuclease that was reported is the Cas12a (also known as Cpf1), which was discovered in the genomes of *Francisella Novicida*, *Prevotella sp.* [39–41]. Its size resembles Cas9 (Size: ~1100–1300 aa) but differs in the crRNA length (only 42-44 nt) and the PAM sequence, which is T-rich. Also, in contrast to Cas9, the Cas12 family causes a 5′ overhang DSB instead of a blunt DSB [42]. There are several advantages of using Cas12a rather than Cas9. First, Cas12a mediated genome editing is more specific than Cas9, which possess a great benefit for therapeutic applications [43,44]. Second, the 5′ overhang DSB created by Cas12a allows for effective insertion of a desirable gene by HDR [45]. Third, the fact that Cas12a utilizes a shorter sgRNA increases the multiplex ability and simplifying the sgRNA design for delivery purposes [46]. Forth, it was recently discovered that Cas12a could digest single-stranded DNA once activated by a target DNA molecule. Unlike Cas12a, the Cas12b family (also known as C2c1) require a tracrRNA similar to SpCas9 [20]. Cas12b showed a significant reduction of off-target effects, thus are more specific than SpCas9 [47,48]. Analysis of metagenomic databases enabled the identification of more Cas12 family effector proteins such as Cas12c [20,49], Cas12d (CasY) and Cas12e (CasX) [50], Cas12f [51], and Cas12e [52].

2.3. Cas13

Cas13 belongs to the type VI family of CRISPR-Cas systems, which targets RNA rather than DNA as previous Cas proteins discussed above [20,49,53–55]. Cas13 size is smaller than other Cas proteins (~900—1300 aa), and its' guide length is 52–66 nt. The complementarity of the crRNA to ssRNA results in a nonspecific cleavage of RNA performed by the RNase domain of the Cas13. This allows resistance to RNA phages ending in programmed cell death due to the cleavage of non-targeted RNA [53,56]. For future therapeutic purposes, it was shown that the Cas13 family could be adapted for use in mammalian cells to mediate targeted RNA knockdown [54,56,57]. These findings make Cas13 a potentially significant therapeutic approach for influencing gene expression, operating at the RNA level, without altering genome sequences.

3. CRISPR/Cas delivery

In addition to the development and the discovery of new Cas nucleases, a major challenge for harnessing this technology for therapeutic applications remain the lack of safe and efficient delivery system. Both viral and non-viral delivery approaches have been explored for CRISPR components delivery in recent years [11,68–70]. However, the large size of CRISPR/Cas nucleases, in addition to sgRNA and HDR template, poses a challenge for encapsulation in a single-vehicle [58,59]. Viral delivery has mainly focused on adenoviruses (AVs) [71,72] and adeno-associated viruses (AAVs) [11,68,69,73]. Non-viral delivery of CRISPR/Cas components in the form of plasmid DNA, Cas9 mRNA and sgRNA, or Cas9/sgRNA RNP has also emerged as a promising delivery strategy, each form of delivery has its' inherent advantages and disadvantages (Fig. 2).

3.1. Packaging challenges

Due to the large size of CRISPR nucleases packaging all the components into a single vector is a major challenge for therapeutic applications [58,59]. This packaging challenge is present in all the formats of delivery (*i.e.*, Plasmid, mRNA and sgRNA, or RNP). In gene-based delivery through adeno associated virus (AAV), the size limit of gene insertion is ~4700 base pairs (bp). However, the SpCas9 gene alone is ~4300 bp. Thus, inserting additional CRISPR components such as sgRNA, DNA template for HDR, or a reporter gene is challenging for a single AAV vector. Several approaches have been investigated to overcome this limitation, for example, splitting SpCas9 and sgRNA into two AAV vectors [60], or to use a smaller Cas9 orthologue (SaCas9) [23].

RNP based CRISPR delivery has multiple advantages over other delivery formats such as rapid editing, low off-target effect, and lack of genome integration risk [61]. However, the RNP complex is large (SpCas9 ~ 160 kDa, sgRNA ~31 kDa) and is moderately negatively charged [58,62], thus packaging these elements through electrostatic interactions might be a major limitation for designing non-viral delivery vehicles. Several approaches have been suggested to overcome this challenge. Rotello et al., have fused the SpCas9 protein to an oligo glutamic acid tag to increase the net negative charge of the protein (Cas9E) [62]. The engineered Cas9E and the sgRNA successfully co-assembled with positively charged arginine gold nanoparticles (Arg-NPs). This delivery vehicle facilitated ~30% gene editing of the PTEN gene in HeLa cells *in vitro*. However, the *in vivo* efficiency of this system is yet to be evaluated.

3.2. Viral delivery of CRISPR/Cas9

In early studies, Cas9 and sgRNA were delivered using AVs, which can easily contain all elements for genome editing. Due to their high packaging capacity, it is possible to express both the Cas nuclease as well as one or multiple sgRNAs from a single vector. Also, large donor HDR template sequences can be included in the same vector. Using AV

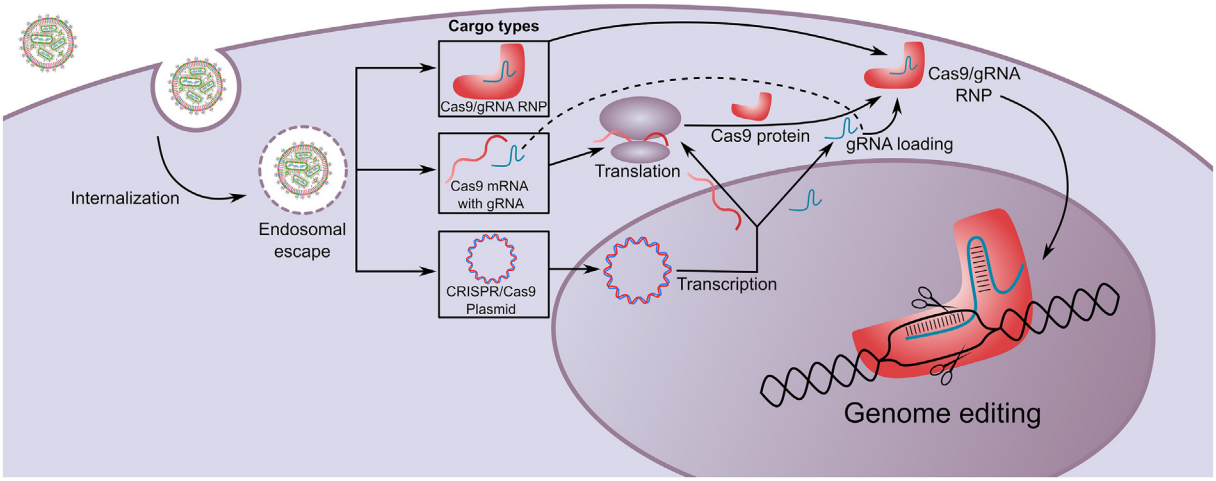

Fig. 2. Genome editing routes by three formats of CRISPR-Cas. Upon cellular entry, the vehicle releases its cargo to the cytoplasm. Three different types of cargo can be encapsulated within the carrier: Cas9/sgRNA RNP, Cas9 mRNA and sgRNA, CRISPR/Cas9 plasmid. 1. Cas9/sgRNA RNP can directly enter the nucleus and cleave the sgRNA target site. 2. Cas9 mRNA with undergo translation processes and the Cas9 will assemble with the gRNA to form RNP. 3. The plasmid will enter the nucleus for transcription, mRNA will exit to the cytoplasm for translation following by RNP formation.

based system by retro-orbital injection resulted in disruption of the Proprotein convertase subtilisin/kexin type 9 (Pcsk9) gene with ~50% of indel in total liver DNA and ~35% reduction of blood cholesterol level in mice [71]. Although AVs can infect both dividing and nondividing cells and is not an integrating virus, AV vectors were shown to elicit severe immune responses and toxicity. Therefore, AVs delivery methods are less used for therapeutic purposes.

The majority of viral systems used in recent years are based on AAVs due to their relatively low immunogenicity and their tissue specificity of different serotypes [73]. Due to the limiting packaging capacity of AAVs, Cas9 and sgRNA are delivered separately using two different AAV vectors. For example, Yang et al. used two AAV8 vectors: one to deliver SaCas9 and the other sgRNA against ornithine transcarbamylase (OTC) and an HDR template of the corrected gene [74]. The two AAVs were injected into the temporal vein of newborn mice. In these mice, a reversion of the OTC mutation was observed in ~10% of hepatocytes, causing an increased survival in mice. In another study, Singh et al. used AAV9 based delivery to target Factor IX in the liver. To increase the tissue specificity of this system, the Cas9 was designed to be under hepatocytes specific promoter. Intravenous injection of both AAV9 vectors resulted in ~50% indels in the liver and a similar reduction in Factor IX activity [75]. This reduction resulted in an emergence of a bleeding phenotype, consistent with hemophilia B. Although viral carriers exhibited relatively high efficiency in gene delivery and expression, concern regarding the potential immunogenicity remains [63]. In addition, limited DNA packaging capacity of viral vectors and the need to split CRISPR components limits the delivery efficiency and, therefore, the editing. Moreover, limitations in the infected cell specificity and the long nuclease exposure time might also result in a high rate of off-target events and adverse reactions.

3.3. Lipid-based non-viral delivery

In recent years there is an ongoing pursuit for developing more efficient and safe delivery vehicles for CRISPR/Cas components *ex vivo* and *in vivo* [76–78]. In comparison with viral delivery, non-viral ones have advantages such as low immunogenicity, biocompatibility, and possibly tissue specificity. Furthermore, the ability to deliver different CRISPR/Cas system formats (*i.e.*, Plasmid DNA, mRNA, and sgRNA and RNP) enable transient expression and therefore favors low frequencies of off-target events and minimizes immunogenicity [58,78]. Moreover, from a pharmaceutical perspective, non-viral delivery systems can be relatively easy to scale up for clinical use.

Traditionally, lipid-based delivery for nucleic acids and proteins was based on cationic lipids (*e.g.*, DOTMA, DOTAP, *etc.*), which enable high cargo encapsulation, high transfection efficiency, and endosomal release. However, due to the constant positive charge, these formulations induce toxicity, adverse reactions, and immunogenic responses [58,78,79]. To overcome these limitations, lipid nanoparticles (LNPs) based on ionizable cationic lipids were developed in recent years [79–82]. These lipids exhibit a cationic charge in the acidic pH equivalent to the pH of late endosomes and neutral charge in physiological pH. This enables high encapsulation efficiency and endosomal escape with minimal toxicity [79,82]. Recently, Patisiran was the first non-viral siRNA-based therapy approved for clinical use for hATTR amyloidosis [83], which opens new avenues for non-viral nucleic acids based therapeutics. However, traditional LNPs formulations were designed for siRNA delivery and are not suitable for large cargoes such as the CRISPR/Cas systems. Therefore, novel ionizable cationic lipids and formulations are developed for longer nucleic acids such as mRNA, plasmid DNA or proteins, which can be used for CRISPR/Cas purposes [84,85].

3.3.1. Plasmid DNA based delivery

Plasmid based delivery of CRISPR/Cas nucleases has several advantages, including low production costs, the ability to control expression using specific/inducible promoters, and inclusion of both Cas9 and sgRNA and even HDR template in a single vector. However, plasmid vectors encoding for Cas9 (4600 bp) and other plasmid elements needed (*e.g.*, sgRNA cassette, bacterial selection, *etc.*) results in large vectors ~10 kb, which pose a challenge for encapsulation in non-viral delivery systems and reduce the delivery efficiency [58,78,79]. Additional disadvantages are the risk of plasmid integration to the host genome, long exposure time to the Cas nuclease, which may result in higher off-target effects, immunogenicity, and the need for nuclear entry, which is crucial for slow or non-dividing cells. He et al. used folate modified liposome (F-LP) to deliver CRISPR/Cas9 plasmid targeting DNA methyltransferase 1 (DNMT1) as a therapeutic approach for ovarian cancer [86]. This study demonstrated that *in vivo* knock out of DNMT1 results in significant tumor inhibition in an ovarian cancer mouse model [86].

3.3.2. mRNA and sgRNA based delivery

An alternative approach that overcomes many of these disadvantages is to use Cas9 mRNA together with sgRNA [76].

Co-administration of Cas nuclease mRNA and sgRNA bypasses the requirement of nuclear entry, eliminates the risk for genome integration, and results in the quick onset of gene editing. Additionally, Cas mRNA delivery provides transient exposure to the Cas nuclease, which was shown to reduce the frequency of off-target editing events and lower risk for Cas nuclease related immunogenicity [63,87]. Miller et al. developed a non-viral vector based on zwitterionic amino lipids for the co-delivery of Cas9 mRNA and sgRNAs [88]. In this proof of concept study, the authors used sgRNA targeting the LoxP site to induce the expression of tdTomato in transgenic LoxP-Stop-LoxP tdTomato mice. Intravenous injection of sgRNA against LoxP resulted in induced expression of tdTomato in the liver, kidneys, and lungs. Although promising, only a minority of the cells were tdTomato positive, and substantially higher editing efficiency is needed for therapeutic purposes. The relatively short stability of Cas9 mRNA and sgRNA is one of the major obstacles of RNA based therapies, which might reduce the editing efficiency [89]. In recent years, extensive research in RNA design results in multiple approaches to modulate and increase RNA stability. These approaches include the use of modified nucleotides [85,90], circular mRNA [91], self-amplifying mRNA [92], *etc.* Regarding mRNA stability, multiple methods have been developed. For example, different cap analogs were used for co-transcriptional capping, such as the anti-reverse cap analog (ARCA) or CleanCap [90,93,94]. In addition, pseudouridine, N6-methyladenosine, 5-methoxyuridine, or inosine can be used to enhance its resistance to RNases and to reduce immunogenicity [84,90,93]. The relatively short length of sgRNAs also enables to chemically modify it (*e.g.*, 2′-*O*-methyl, 2′-*O*-methyl 3'phosphorothioate, 2′-*O*-methyl 3'thiophosphonoacetate, LNA etc). These modifications were shown to increase stability and substantially increase the gene editing efficiency [95]. Yin et al., evaluated the effect of different sgRNA modification patterns on the editing efficiency in cells and in mouse liver [96]. By modifying 70 out of 101 nt of sgRNA with a 2′ hydroxyl (OH) groups and a number of phosphorothioate bonds they developed enhanced sgRNA (e-sgRNA). Systemic co-delivery of native *vs* e-sgRNAs and Cas9 mRNA in LNPs, resulted in up to 70% increase in indel efficiency of GFP and the mouse Pcsk9 gene [96]. In another study, Finn et al., developed LNPs delivery system for Cas9 mRNA and chemically modified sgRNA based on the LP01 ionizanble lipid [97]. As a proof of concept, they targeted the transthyretin gene (TTR) in both mice and rats' livers. Single dose administration resulted in ~70% TTR gene editing in the liver and > 90% reduction in serum transthyretin levels. Furthermore, they have demonstrated that cumulative gene editing is achieved by multiple dosing. However, repeated exposure to the Cas9 protein might induce immunogenic responses. The effect of multiple *vs* single dosing on immunogenicity and treatment efficacy should be extensively evaluated.

3.3.3. Cas9/sgRNA RNP based delivery

Direct delivery Cas/sgRNA ribonucleoprotein complexes (RNPs) is a widely studied strategy in recent years. This strategy has multiple advantages, including rapid editing, high gene editing efficiency, and reduced off-target effects [61,89]. Furthermore, the short exposure time to the Cas nucleases reduces the chances for acute immunogenic responses and toxicity. Although the delivery of the RNP complex is an attractive approach, RNPs are not trivial for encapsulation in traditional formulations. Several considerations should be taken in designing RNP based delivery vehicles, including high loading efficiency, maintaining the Cas nuclease activity, the integrity of the sgRNA, RNP complex integrity, and efficient endosomal release. Zuris et al. utilized the overall negative charge of the Cas9/sgRNA RNP to deliver it to cells using commercially available cationic lipids [98]. Furthermore, to improve encapsulation in cationic lipoplexes, they fused Cas9 to a super negatively charged GFP. Delivery of the RNPs using this approach was highly efficient and induced up to 80% genome editing in cultured human cells. However, the fusion of GFP to Cas9 resulted in inferior overall editing in cultured cells. Lipoplexes encapsulating Cas9/sgRNA against GFP RNPs were injected into the mouse inner ear *in vivo* and edited ~20% of the hair cells. However, the *in vivo* applications of this approach are highly limited and cannot be used systemically. In another study, Wang et al. used the 8-O14B bio-reducible lipid to encapsulate Cas9/sgRNA RNPs in LNPs [99]. Incubation of HEK293-GFP cells with Cas9/sgGFP LNPs resulted in ~70% gene disruption. Furthermore, the authors demonstrated that these LNPs could deliver CRE recombinase to the mouse brain. However, the *in vivo* efficiency of Cas9/sgRNA RNPs was not evaluated. Due to the limitations discussed above, the encapsulation of proteins in general and Cas9/sgRNA RNP in particular in lipid-based vehicles is extremely challenging. Moreover, the pre-existence of an adaptive immune response to Cas9 might pose a significant challenge to the development of *in vivo* therapies. While a single administration of Cas9 RNP based formulations might be well tolerated, immune responses could be generated following treatment. After an immune response is generated, the efficiency of subsequent treatments would be limited, and potential immune-related adverse reactions could be generated. To circumvent these problems, it is essential to develop strategies to minimize Cas9 related immune responses. For example, the development of a more efficient delivery system that could reach therapeutically relevant editing efficiencies within a single administration.

3.4. Inorganic and polymeric delivery

Cationic polymers, cell-penetrating peptides, and inorganic nanoparticles can be used to deliver nucleic acids and proteins similarly to cationic lipids [100,101]. Liu et al. utilized PEG-PLGA-based cationic lipid-assisted polymeric nanoparticles (CLANs) to deliver a plasmid encoding for Cas9 and sgRNA against the BCL-ABL fusion protein in chronic myeloid leukemia (CML) mouse model [102]. Incubation of $CLAN_{pCas9/gBCR\text{-}ABL}$ with K562 CML cell line resulted in ~20% indels at the BCR-ABL locus. Furthermore, the authors evaluated the therapeutic potential of $CLAN_{pCas9/gBCR\text{-}ABL}$ in the K562 xenograft mouse model. Intravenous injection of 1.6 mg per kg of $CLAN_{pCas9/gBCR\text{-}ABL}$ once every other day for seven injections resulted in ~40% increased median survival compared to control groups. In another study, Lee et al. used gold nanoparticles (CRISPR-Gold) to deliver Cas9/sgRNA or Cas12a/sgRNA RNPs to adult mice brain [14]. Intracranial injections of CRISPR–Gold to striatum and hippocampus regions resulted in the editing of neurons, astrocytes, and microglia of up to ~15%, with undetectable levels of toxicity at the doses used. Furthermore, CRISPR–Gold mediated editing of the glutamate receptor 5 (mGluR5) gene reduced local mGluR5 levels in the striatum after an intracranial injection and partially alleviated mice from the exaggerated repetitive behaviors caused by fragile X syndrome. While local gene editing may be beneficial to treat several behavioral deficits, brain wide gene editing may be necessary for the majority of neurological disorders. Moreover, the potential long term accumulation of gold nanoparticles in the brain and consequent toxicity must be further investigated before this approach could proceed to clinical evaluation.

4. CRISPR/Cas system challenges

Despite recent progress in utilizing CRISPR/Cas gene editing for therapeutic applications, there are still many challenges to overcome for the final goal of clinical gene therapy. Here we discuss several leading challenges needed to be addressed for successful clinical translation. For example, the editing efficiency, off-target effects, the efficacy of HDR, PAM dependency, and immunogenicity.

4.1. Editing efficiency

The editing efficiency by CRISPR/Cas systems is affected by multiple parameters, including the sgRNA sequence and modifications, duration of nuclease exposure, delivery efficiency, DSB repair mechanism, *etc.* The repair pathway by which CRISPR/Cas mediated DSB is resolved

plays a major role in determining editing rates. While NHEJ is active throughout the cell cycle [4,77], HDR preferentially operates during the S/G2 phase. Thus, the editing mediated by NHEJ is generally more efficient than HDR. Moreover, the efficiency of HDR is relatively low in mammalian cells and depending on the type of Cas nuclease used (*i.e.*, double Cas9 nickase, Cas12a, *etc.*), homology arm length, and the type of HDR template [9,103–105]. Several reports have demonstrated that Cas9 mediated gene editing in mice results in HDR mediated repair efficiencies of 0.5–20%, while NHEJ-mediated repair occurs at 20–60% [104]. Several approaches have emerged to improve the efficiency of HDR mediated repair. For example, suppression of the NHEJ pathway by using small molecular inhibitors [105–108], silencing of NHEJ machinery [43], cell cycle synchronization as HDR is more efficient in the S and G2M phases of the cell cycle and paired Cas9-nickases [101,103]. One such inhibitor is Scr7, which targets the NHEJ component of DNA ligase IV and has been reported to increase the efficiency of HDR mediated repair by up to 19-fold [43,110]. However, although successful to some extent, these inhibitors might have toxic effects, and its' *in vivo* use is limited. Richardson et al. discovered that the Cas9 asymmetrically releases the 3′ end of the cleaved genomic DNA strand that is not complementary to the sgRNA [101]. They utilized this phenomenon to design the ssDNA HDR template complementary to the non-target strand that is released first by the Cas9. Using the Cas9-nickase together with this approach, they increased the HDR efficiency *in vitro* up to 60%. In another study, Yin et al. used a dual treatment of LNPs encapsulating Cas9 mRNA and an AAV system to deliver sgRNA targeting the fumarylacetoacetate hydrolase (Fah) and DNA HDR template of the wild-type gene in a mouse model of human hereditary tyrosinemia. In this study, a single administration of the treatment resulted in ~6% HDR gene repair efficiency in hepatocytes translated to reduced disease symptoms [68]. Nevertheless, much higher HDR mediated gene repair efficiencies will be needed for the alleviation of most diseases, and further research must be conducted for improving editing efficiencies *in vivo*.

Another challenge is the CRISPR/Cas9 *in vivo* editing efficiency, which is significantly lower compared to *in vitro* editing due to the lack of efficient delivery methods [23]. For example, Yin et al. achieved up to 80% gene editing *in vitro*; however, *in vivo*, efficiency was significantly lower, achieving ~25% gene disruption [70]. Although progress has been made in developing more efficient delivery vehicles for CRISPR components, the editing efficiency reported to date might be sufficient for alleviating certain diseases (*e.g.*, liver tyrosinemia, DMD and to reduce cholesterol levels). However, other diseases such as cancer, require much higher editing efficiencies in order to be alleviated. Furthermore, our tools to reach organs systemically beyond the liver are extremely limited. Therefore, the development of more efficient delivery systems for CRISPR components in general and for systemic delivery, in particular, remains a great need.

4.2. Off–target effects

Multiple studies have demonstrated that the CRISPR-Cas9 system can generate indels at undesired genomic loci due to tolerance of the Cas9 nuclease to mismatches of sgRNA with the off-target site [87,106]. Further pieces of evidence have been reported that the risk of off-target cleavage and reduced specificity is correlated with longer exposure to the Cas nuclease, possibly resulting in unwanted mutations and potential toxicity [107,108]. Significant efforts have been made recently to reduce the off-target events, for example, by using a double Cas9 nickase [105], optimizing sgRNA design [95,104], and by reengineering high-fidelity Cas9 nucleases [43]. Minimizing the off-target events is crucial for Cas9 *in vivo* application as off-target indels could affect cell viability and promote tumorigenesis.

4.3. PAM dependency

Theoretically, Cas nucleases can target any DNA sequence through an engineered sgRNA. The specificity of CRISPR/Cas9 requires a 2–5 nt PAM sequence immediately downstream of the target site, in addition to the crRNA genomic target sequence complementarity, thus, limiting the actual loci to be the target [1]. In recent years several Cas9 orthologs have been discovered, requiring different PAM sequences, such as 'NGG' PAM for SpCas9 [1,21], 'NNNNGATT' PAM for NmCas9 [24] and 'NGGNG' and 'NNAGAAW' PAM for StCas9 [22]. Additional family of Cas nucleases, Cas12a, was lately discovered; this family requires a 'TTTV' PAM [110]. The PAM-dependency of CRISPR mediated DNA cleavage constrains the incidence of targetable sites in genomes. Nevertheless, PAM dependency also increases the specificity of Cas nucleases. Cas nucleases requiring long PAM should have less off-target effects than ones requiring a short PAM. The discovery of new Cas nucleases with different PAM requirements expand our tools to perform a precise genome editing with greater genome coverage.

4.4. Immunogenicity

The bacterial origin of CRISPR nucleases renders them to be recognized by the host immune system and elicit immune responses [63]. The risk for CRISPR related immune responses is greater in gene-based delivery in which the Cas9 gene can permanently integrate into host cells genome. The prolonged or constitutive expression of a bacterial Cas9 protein might be presented on MHC-I molecules, elicit T-lymphocytes mediated immune response as well as anti Cas9 antibodies and elimination of Cas9 expressing cells [63,64]. Furthermore, the most commonly used Cas9 orthologues are derived from *Staphylococcus aureus* (SaCas9) or *Streptococcus pyogenes* (SpCas9), both of which are prevalent human commensals that could be pathogenic. For example, approximately 40% of the adult human population is colonized by *S. aureus*, and 12% of the children under 18 have commensal colonization with *S. pyogenes* [65,66]. Most of the immune responses against these bacteria are against secreted and surface proteins, which are more accessible to the immune system. However, a study conducted by Simhadri et al. detected anti-SaCas9 antibodies in 10% and anti-SpCas9 antibodies in 2.5% of the tested sera (200 patients cohort) [67].

These results indicate that humans are often pre-exposed to Cas proteins and develop immune responses against them. Subsequent exposure to these proteins might result in antibodies dependent clearance of cellular immune responses against Cas expressing cells. Furthermore, these immune responses can result in severe adverse reactions and treatment failure. Present and future clinical trials should monitor and evaluate both humoral and cellular immune responses before and during the treatment course as well as its effect on treatment efficacy.

5. Current clinical applications

Although the clinical development of the CRISPR/Cas9 system is still in infancy, extensive efforts are invested both by academic laboratories and the industry to bring this technology to the clinic. Clinical applications of CRISPR gene editing can be grossly divided into two arms *ex vivo* and *in vivo* delivery (Fig. 3).

5.1. Ex vivo applications

In *ex vivo* therapeutic applications, cells are extracted and isolated from patients, manipulated, and transplanted back to the patient. *Ex vivo* approaches enable higher control on the manipulated cells, superior editing efficiencies than *in vivo* applications, and selection of the desirable clones [111]. Due to the extensive *ex vivo* good manufacturing practices (GMP) and the high accessibility of hematopoietic cells, most *ex vivo* applications are focusing on these cells. Three main applications of *ex vivo* gene editing are the treatment of hereditary diseases

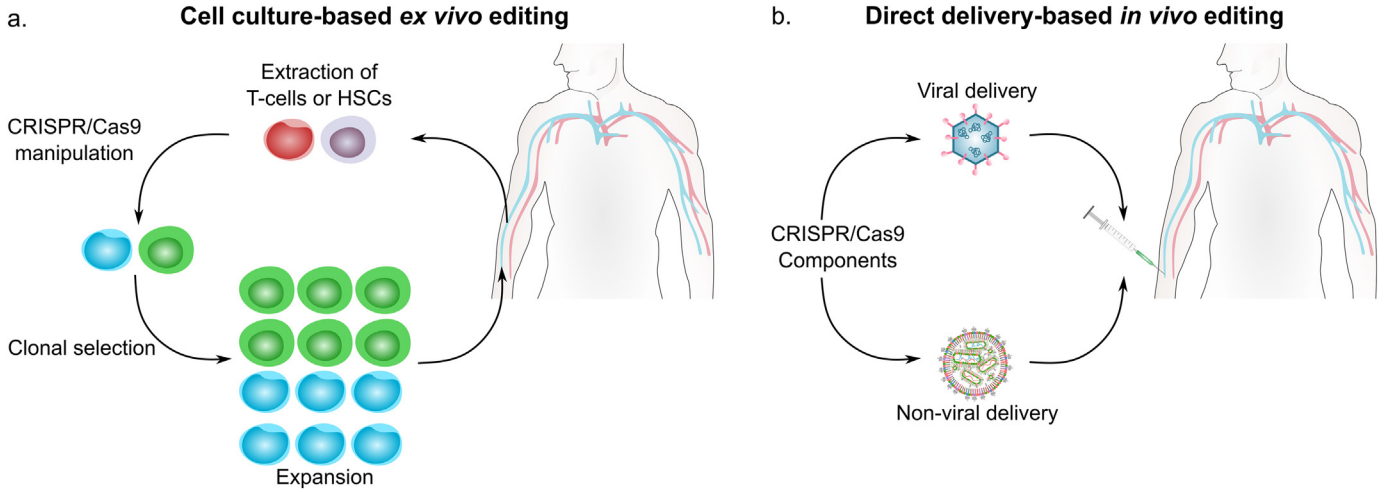

Fig. 3. Current clinical strategies for CRISPR/Cas manipulation. a. Cell culture based *ex vivo* editing. Target cells are extracted from the patient, undergo CRISPR/Cas9 manipulation undergo selection. Only manipulated cells will undergo expansion and will be injected back to the patient. b. direct delivery based *in vivo* editing. CRISPR/Cas9 component are encapsulated in either in viral or non-viral carriers and injected locally or systemically to the patient.

(*e.g.*, β-Thalassemia, sickle cell anemia, *etc.*) [13], viral infection inhibition [16,17] and cancer immunotherapy [18].

β-thalassemia and sickle cell anemia are caused by dysfunction of the hemoglobin protein. Despite a well-understood genetic cause, current treatments result in severe adverse reactions and reduced life expectancy [112]. The only long-lasting curative treatment is bone marrow transplantation from a healthy matched donor. However, this solution is only available for 20% of patients and include high risks for fatal graft *versus* host disease [113,114]. The leading strategy to treat these diseases using genome editing is by inducing the expression of fetal hemoglobin (HbF) in the patient's hematopoietic stem cells (HSCs). HbF, which is usually dormant in adults, can compensate and replace the adult hemoglobin and can alleviate the symptoms of these diseases [115,116]. HSCs are extracted from the patient's blood, expanded *ex vivo*, and treated with Cas9/sgRNA against the transcription factor BCL11a. BCL11a is the negative regulator of HbF. Therefore a knock-out of BCL11a restores HbF expression [115,117,118]. In November 2019, the results of the first phase 1/2 clinical trial conducted by CRISPR Therapeutics and Vertex pharmaceuticals were announced [119,120]. In this initial study, two beta-thalassemia patients were treated, and their total hemoglobin levels elevated to normal levels (11.9 g/dL and 11.3 g/dL) nine months following treatment. Several adverse reactions were observed in both patients, which were considered unrelated to the investigational treatment. Overall, the initial clinical data from this study has the potential to be a curative gene-editing therapy for people with sickle cell disease and beta thalassemia [121]. However, longer patient follow-up on treatment persistence and off-target related safety should be conducted.

The second application of *ex vivo* CRISPR mediated gene editing is virus inhibition either by targeting the viral genome or by targeting the receptors for viral entry. The main focus of this approach is on the treatment of HIV. CCR5 is the main co-receptor of HIV-1, and its disruption had been previously shown in animal models, and patient-derived cells to inhibit HIV infection [15,16]. Mandal et al. delivered CRISPR/Cas9-encoding plasmids into human CD4+ T cells and CD34+ hematopoietic stem progenitor cells (HSPCs) by electroporation, and demonstrated 28% gene editing [121]. CCR5 knock-out HSPCs still retained multi-lineage engraftment potential while minimal off-target effects were observed. In a recent clinical trial, Xu et al. reported on the treatment of a single HIV and related acute lymphoblastic leukemia patient with allogenic CCR5 edited HSPCs [16,122]. Donor CCR5 depleted cells persisted for more than 19 months without apparent gene-editing related adverse reactions. The percentage of CD4+ cells with CCR5 depletion was only approximately 5%, which indicates the need for further research and extensive clinical evaluation to improve this approach.

The third and most clinically advanced application focus on cancer immunotherapy. In this application, Tumor-infiltrating lymphocytes (TILs) or chimeric antigen receptor T-lymphocytes (CAR-T) are edited, expanded *ex vivo*, and injected back to the patient [123,124]. Programmed death-1 (PD-1) receptor is a negative regulator of T-lymphocytes activity and is present on activated T lymphocytes and regulatory T (Treg) lymphocytes [125]. Treatment with Anti-PD-1 antibodies results in positive responses in approximately 20–30% of patients with non-small-cell lung cancer, melanoma, or renal-cell cancer [126,127]. However, due to the essential role of this checkpoint molecule in modulating the immune system, systemic administration of PD-1 blocking antibodies still carries the risk of breaking peripheral immune tolerance and severe adverse reactions [127]. Intrinsic disruption of PD-1 in TILs and CAR-T is an appealing approach to reduce adverse reactions due to systemic checkpoint blockage [128]. Su et al. demonstrated that PD-1 knock-out in patient-derived T cells improved dramatically their anti-tumor activity, such as up-regulation of IFN-γ and increased cytotoxicity towards cancer cells [125]. Second use of CRISPR/Cas9 in the new generation of TILs and CAR-T is to knock-out the endogenous T cell receptor and the beta 2 microglobulin (β2m- a structural protein of MHC class 1 complex). This reduces the risk for graft *versus* host disease and open the opportunity to use allogenic T-Lymphocytes [18]. This approach both increase the safety and shorten the cell preparation process from 3 to 4 weeks to few days. Multiple clinical trials are ongoing using this approach, for example two phase 1/2 trial CTX110 (CD19 CAR-T for CD19+ hematological malignancies) [129] and CTX 120 (BCMA CAR-T for multiple myeloma) [130] by CRISPR Therapeutics (Table 1). In these trials the CRISPR/Cas9 system is used to direct the CAR receptor towards the endogenous T cell receptor locus as well as knocking out the PD-1 and β2m for allogenic transplantation. In these studies, the main objectives include evaluation of adverse event and dose limiting toxicities as well as duration of response follow up for up to 60 months.

5.2. In vivo applications

In *in vivo* applications, the CRSIPR/Cas9 machinery is either locally or systemically delivered to disease-affected cells or organs in the body. To date several viral and non-viral delivery systems have been reported for CRISPR/Cas9 *in vivo* delivery (see section 4). Monogenic genetic

Table 1
Summary of current clinical trial using CRISPR/Cas9 system.

Application	Delivery method	Genes	Disease	Genome editing	Target organ/cells	Phase	Clinical trial Identifier
Ex vivo	Lentivirus and electroporation	HPK1	Acute Lymphocytic Leukemia	NHEJ	Autologous T Cells	1	NCT04037566
Ex vivo	Electroporation	TCR PD-1	Multiple Myeloma Melanoma Synovial Sarcoma Myxoid/Round Cell Liposarcoma	NHEJ	Autologous T Cells	1	NCT03399448
Ex vivo	Electroporation	TCR PD-1	Mesothelin positive solid tumors.	NHEJ	Autologous T Cells	1	NCT03545815
Ex vivo	Not disclosed	PD-1	Advanced Esophageal Cancer	NHEJ	Autologous T cells	1	NCT03081715
Ex vivo	Electroporation	PD-1	Lung cancer	NHEJ	Autologous T cells	1	NCT02793856
Ex vivo	Electroporation	PD-1	Mesothelin positive solid tumors	NHEJ	Autologous T cells	1	NCT03747965
Ex vivo	Electroporation	PD-1	EBV (Epstein-Barr virus) positive advanced stage malignancies	NHEJ	Autologous T cells	1/2	NCT03044743
Ex vivo	Not disclosed	CCR5	HIV-1-infection with hematological malignancies	NHEJ	CD34+ HSPCs	N/A	NCT03164135
Ex vivo	Electroporation	HbF	β-Thalassemia	NHEJ	hHSPCs	1/2	NCT03655678
Ex vivo	Electroporation	HbF	Sickle cell anemia	NHEJ	hHSPCs	1/2	NCT03745287
Ex vivo	Electroporation	TCR, MHCI (β2m)	Multiple myeloma	NHEJ and HDR	Allogenic T cells	1	NCT04244656
Ex vivo	Not disclosed	TCR, MHCI (β2m)	B-cell lymphoma	NHEJ and HDR	Allogenic T cells	1/2	NCT04035434
Ex vivo	Electroporation	TCR, MHCI (β2m)	Refractory CD19+ Leukemia and Lymphoma	NHEJ	Allogenic T cells	1/2	NCT03166878
Ex vivo	Electroporation	TCR	Relapsed or Refractory Leukemia and Lymphoma	NHEJ	Allogenic T cells	1/2	NCT03398967
In vivo	AAV5	CEP	Leber Congenital Amaurosis Type 1	NHEJ	Retina	1/2	NCT03872479
In vivo	Local gel administration	E6/E7	Human Papillomavirus-Related Malignant Neoplasm	NHEJ	Cervical epithelium	1	NCT03057912

disorders are the main targets of *in vivo* therapy (e.g Duchen mascular dystrophy [11], tyrosinemia [70] *etc.*). Because NHEJ occurs more prominently than HDR, most applications have been focused on the NHEJ pathway which leads to gene disruption or exon skipping. For example, in proof-of-concept studies, Long et al. [131], Nelson et al. [12], and Tabebordbar et al. [132], used adeno-associated virus-9 (AAV9) to deliver the CRISPR/Cas9 components to Duchene muscular dystrophy (DMD) mouse model. In this disease, mutations in exon 23 of the dystrophin gene, results in truncated protein and malfunction [12,131]. By cutting out the mutated exon 23, the dystrophin protein expression in skeletal and cardiac muscle is restored causing an improved skeletal muscle function. In another study, Finn et al., developed LNP based system delivering Cas9 mRNA and sgRNA against mouse transthyretin in a single vehicle [97]. This LNP formulation naturally reach the liver which is the target site for transthyretin production. Single administration of these LNPs, resulted with ~97% reduction in serum protein levels that persisted for several months following treatment. Other *in vivo* delivery strategies rely on local injection of CRISPR delivery vehicles to secluded organs (e.g eyes, brain etc). Yu el al., used AAV based delivery into the retinal cavity [134]. Cas9-mediated disruption of the Nrl gene preserved the function of cone photoreceptors in three different mouse models of retinal degeneration. Recently, using a similar approach, the first *in vivo* delivery clinical trial has been initiated by Editas medicine (EDIT-101) for the treatment of Leber congenital amaurosis 10 (LCA10) [135]. In this disease mutation in the CEP290 gene results with degeneration in ocular photoreceptor cells and eventually blindness. The removal of the mutated area will restore of the correct open reading frame and therefore normal protein expression. Expression of the normal protein will hopefully restore the photoreceptor function and vision in these patients.

The relative maturity of *ex vivo* CRISPR/Cas9 applications together with the ability to select manipulated cells, evaluate functionality and easily analyze off-target effects makes these applications the main interest of gene editing companies in the near future. However, the limited number of diseases and applications that could be addressed by *ex vivo* gene editing fuel the research and progress of *in vivo* applications. To date, most CRISPR/Cas9 *in vivo* delivery results in relatively low editing efficiencies [89] which may not be sufficient for treating most diseases. The editing efficiency is mainly depends on the development of new, more robust delivery systems for various tissues. Extensive research is currently conducted in order to develop better, more efficacious delivery systems for multiple applications. Furthermore, multiple new variants and formats of genome editing nucleases are being developed, with improved specificity, lower off-target events, and dual activities. These novel systems can open new treatment opportunities for various diseases with better safety profile.

6. Conclusions

In recent years, CRISPR/Cas technologies have evolved as powerful tools for genome editing and have been widely utilized *in vitro* and *in vivo* both for research and therapeutic purposes. Despite the advances, several challenges still hamper their clinical translation, including low editing efficiency, off-target effects, immunogenicity and the lack of an efficient and safe delivery system. Great efforts have been made with respect to overcoming these challenges. For example, the development of more precise formats of Cas9 nuclease, reducing the duration of Cas9 exposure, modifying the sgRNAs *etc.* Another challenge resides in improving tissue specific genome editing to reduce adverse reactions, as well as undesirable genome editing in adjacent tissues. Multiple viral and non-viral delivery vehicles have been developed for *in vivo* genome editing. Viral vectors can deliver CRISPR/Cas components with relatively high transfection efficiency, however they might induce insertional mutagenesis, high off-target effects due to long exposure time and immune reactions. Moreover, the limited packaging capacity of most viral vectors require to split the CRISPR/Cas9 components which reduce the delivery efficiency. The development of novel non-viral vectors for CRISPR/Cas delivery has brought new opportunities for *in vivo* gene editing. The ability to enable the delivery of the CRISPR/Cas machinery in different formats and tissue specific delivery

represent a promising way of overcoming most of the above limitations and bring *in vivo* based genome editing to the clinic. Nevertheless, the delivery efficiency of current non-viral vectors remains relatively low and as a consequence the correlated *in vivo* genome editing efficiency. Therefore, future efforts should focus on improving and optimizing the performance of non-viral vectors, to meet clinical requirements.

Multiple Clinical trials for both *ex vivo* and *in vivo* applications are at initial stages (Table 1). For example, CTX001 by CRISPR therapeutics is currently being investigated in clinical phase 1/2 trials for patients suffering from β-thalassemia or sickle cell disease. By *ex vivo* manipulation of hematopoietic stem cell they aim to increase the HbF levels and to ameliorate symptoms in patients.

In conclusion, recent progress of *in vivo* gene editing using both viral and non-viral delivery systems have highlighted the promise of CRISPR-Cas9 as a potential therapeutic approach for genetic diseases and viral diseases. However, for clinical translation there are still many hurdles to overcome, such as off-target effects, HDR efficiency, editing efficiency and tissue specificity of the delivery vehicles. In addition, the immunogenicity of the Cas9 nuclease and treatment safety should also be evaluated before clinical translation. However, with the rapid advances in CRISPR technologies and the development of non-viral delivery vehicles, we envision that these hurdles can be overcome in the foreseeable future and pave the way for CRISPR-Cas based genome editing therapeutics for clinical use.

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