

Review

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Site-specific genome editing in treatment of inherited diseases: possibility, progress, and perspectives

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Abstract: Advancements in genome editing enable permanent changes of DNA sequences in a site-specific manner, providing promising approaches for treating human genetic disorders caused by gene mutations. Recently, genome editing has been applied and achieved significant progress in treating inherited genetic disorders that remain incurable by conventional therapy. Here, we present a review of various programmable genome editing systems with their principles, advantages, and limitations. We introduce their recent applications for treating inherited diseases in the clinic, including sickle cell disease (SCD), β -thalassemia, Leber congenital amaurosis (LCA), heterozygous familial hypercholesterolemia (HeFH), etc. We also discuss the paradigm of *ex vivo* and *in vivo* editing and highlight the promise of somatic editing and the challenge of germline editing.

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Finally, we propose future directions in delivery, cutting, and repairing to improve the scope of clinical applications.

Keywords: clinical applications; genome editing; inherited diseases; mutations; principles.

Introduction

From the first description of alkaptonuria as an inherited disease in the early 20th century, humans have recognized hereditary disorders for over 100 years [1]. In the late 1940s, sickle cell disease (SCD) was associated with alteration in a protein of red blood cells [2]. As a milestone, this finding was the first time a “genetic disease” was linked to a mutation of a specific protein and was called a “molecular disease” at that time. In the following years, the discovery of the double helix structure of DNA and the central dogma opened the door to understanding the human genome and genetic diseases. Now we know that mutations in coding and non-coding sequences can cause genetic disorders, some of which are inherited from parents’ germline or occur spontaneously before embryonic development and will also pass to the next generations, such as SCD. These diseases are termed inherited diseases or hereditary diseases [3]. Others are caused by mutations that occur in the somatic cells during a person’s life, such as cancers. According to statistics, there are over 6,000 genetic disorders, with only about one-tenth of them treatable [4, 5]. Individual genetic disease is rare. For example, approximately 1 in 2,000 people are affected by cystic fibrosis. The prevalence is 1 in 500 for familial hypercholesterolemia and hypertrophic cardiomyopathy, 1 in 625 for SCD, and 1 in 5,000 for Duchenne muscular dystrophy (DMD) [4]. However, due to a large number of the diseases, approximately 1 in 21 are affected [3].

The treatment of genetic diseases is challenging. As an example, the treatment options for SCD, the most common monogenic blood diseases affecting millions of people around the world, remain limited, and the lifespan of the patients has not improved during the last decades, even though several drugs reducing the complications were approved and used in the last few years [6]. Allogenic hematopoietic stem cell (HSC) transplantation is the only curative treatment available to less than 15% of SCD patients. Recently, gene therapy through lentiviral vector-mediated addition of an anti-sickling protein, LentiGlobin, has proven to be successful in preclinical and is tested the durability of the safety and efficacy in clinical trials [7]. Also, as a common monogenic disorder, DMD is often on a par with SCD. The patients with DMD first show symptoms before age 5 and usually cannot walk at the age of 12 [8]. Corticosteroids such as prednisolone and deflazacort are the primary treatment currently, aiming to control the symptoms, but their benefits are limited. The estimated annual disease burden/cost of DMD is over 60,000 euros [9]. However, no cure for DMD is available, and the average lifespan is only 20–30 years. In the last few years, the United States Food and Drug Administration (FDA) approved three antisense oligonucleotides (ASOs), Exondys 51, Vyondys 53, and Amondys 45, for treating 14%, 8%, and 8% of DMD patients, respectively [10–12]. Regarding pathogenesis, genetic disorders, especially heritable diseases, are rooted in alterations in the genome. Correction of disease-causing mutations provides a potential therapy achieving permanent cure compared to other treatments.

Genome editing technology provides a powerful tool for treating genetic diseases by targeting pathogenic mutations in a site-specific manner. This technology has been widely used for basic laboratory research and attracts many efforts to prevent and treat various human diseases. These efforts have made enormous progress in pre-clinical studies, and some of the achievements have been successfully translated to clinical studies, such as chimeric antigen receptor T (CAR-T) cell-mediated immunotherapy [13]. This review focuses on genome editing approaches to treat human hereditary disorders. In the first part, we introduce the principles, representative findings in history, and the features of each genome editing tool. In the second part, we discuss the cutting edge of their ongoing therapeutic applications for hereditary disorders in clinics. Lastly, we also highlight this topic's current challenges and future opportunities.

Overview of the genome editing tools

Genome editing modifies DNA sequences at target sites to disrupt or regulate the gene of interest or to repair harmful mutations. The last few decades have witnessed the rapid development and application of this technology, especially the discoveries of zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and clustered regularly interspaced short palindromic repeats-CRISPR-associated protein 9 (CRISPR-Cas9). To precisely change the DNA sequence, homologous recombination (HR) mediated by nucleases (e.g., meganucleases [MegNs], TALENs, ZFNs, and CRISPR-Cas) and CRISPR-Cas-derived agents (e.g., base editors [BEs], prime editors [PEs], and transposases) are currently invented and applied in experimental systems [14, 15]. External nuclease-induced DNA double-strand breaks (DSBs) can be repaired by non-homologous end-joining (NHEJ) and homology-directed repair (HDR), resulting in gene disruption or precise integration and substitution [14]. Efficient HDR induced by nuclease in mammalian cells or organisms is promising for gene therapy [16]. CRISPR-Cas-derived agents independent of DSBs are another promising approach to rewriting the genome and curing genetic diseases [15]. Here, we will briefly summarize these promising approaches' history, basic principles, and application prospects (Figure 1).

MegNs

MegNs, also termed homing endonucleases, are highly specific DNA cleaving endo-deoxyribonucleases. They found in mitochondria and chloroplasts of microbial life, which can be divided into six families based on amino acid sequence and structure motifs: LAGLIDADG, GIY-YIG, HNH, His-Cys box, PD-(D/E) XK, and EDxHD [17, 18]. These enzymes are small in size, and specially recognize and cleave double-strand DNA at specific sites compromising 14–40 bp, which can lead to site-specific recombination events resulting in insertion, deletion, and mutation correction [19]. Unlike restriction enzymes, MegNs generate at individual loci of their genome and facilitate site-specific gene/element conversion from the host to the recipient, thus referred to as homing [19]. One of the most well-studied MegNs families is the LAGLIDADG family, which is generally encoded

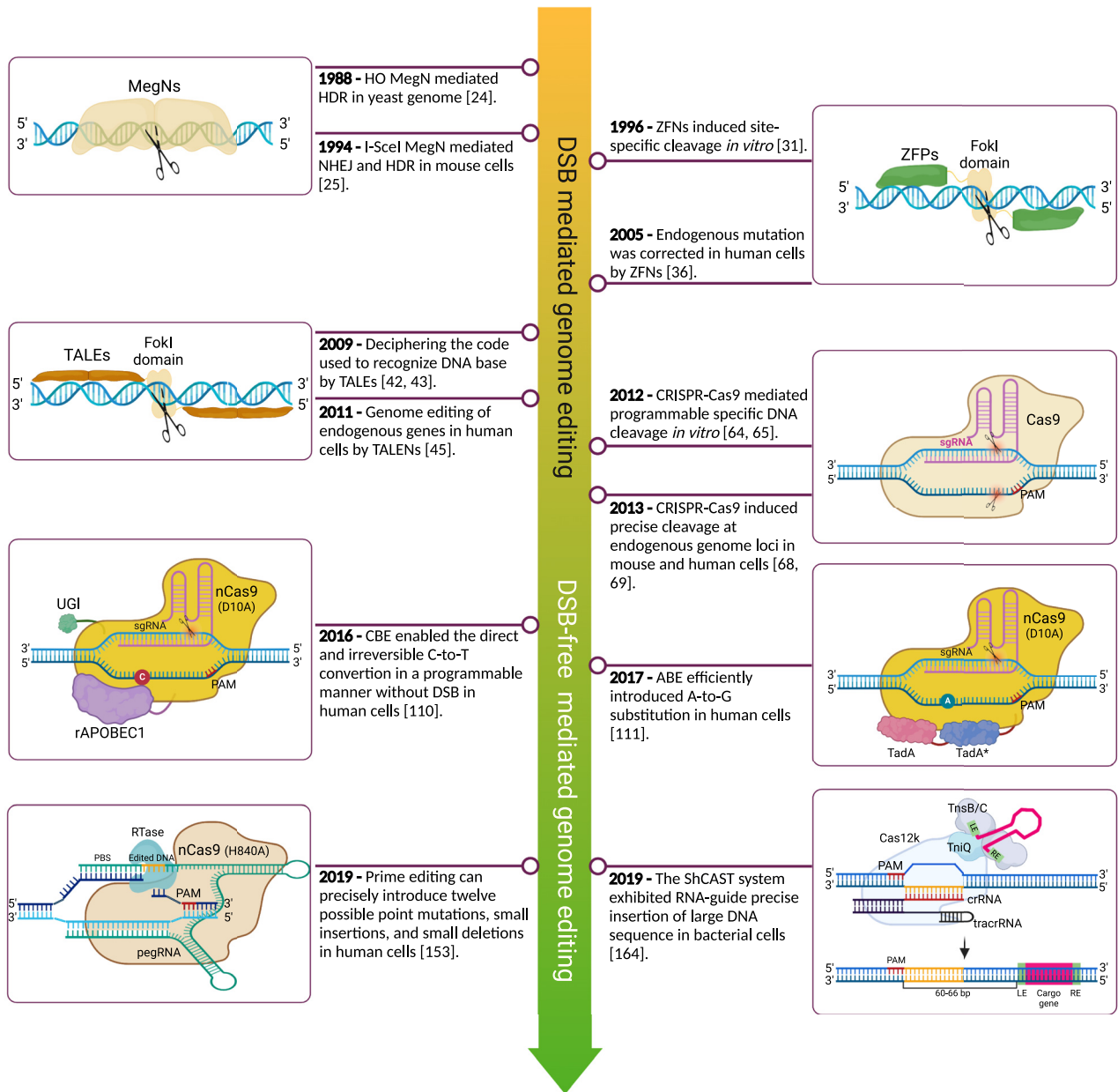


Figure 1: Timeline of the genome editing methods. The significant genome editing events from meganucleases mediated DNA double-strand breaks to transposases induce targeted insertions. After the emergence of CRISPR-Cas9 technology in 2012, it opened a new era of genome editing and derived a series of new agents that can efficiently rewrite the genome DNA. CRISPR-Cas9, clustered regularly interspaced short palindromic repeats-CRISPR-associated protein 9.

within introns or inteins, such as I-CreI, I-SceI, and HO [17]. I-CreI is a homo-dimeric MegNs encoded by an ORF contained within a group I intron in the 23S rRNA gene of *Chlamydomonas reinhardtii* chloroplast, which recognizes and cleaves a 22-bp pseudo-palindromic recognition sequence [20]. I-SceI is a rare-cutting MegNs encoded by a mitochondrial group I intron of *Saccharomyces cerevisiae* with an 18 bp nonpalindromic target [21]. The HO protein makes a DSB at the yeast *MTA* locus, contains two

LAGLIDADG motifs, and has a C-terminal zinc finger domain [22, 23].

MegNs enable inserting intended external DNA into the specific sites by HDR after DSB, which had been proved in model organisms by HO and I-SceI endonucleases [24–26]. In 1988, Rudin and Haber first reported that HO endonuclease stimulates efficient HDR between the homologous flanking sequences after site-specific DSB in yeast [24]. Furthermore, expression of I-SceI in mouse cells carrying

cleavage sites in external DNA or endogenous genome stimulated HR using donor DNA as a template [25, 26], which sheds light on treating genetic diseases through HDR. However, while MegNs show promising applications for genome editing owing to their long DNA recognition sequence, the lack of target site in mammalian cells and the difficulty of engineering DNA-binding and cleave motifs ultimately make them hard for routine applications.

ZFNs

In principle, genome editing originated from the application of ZFNs technology. ZFNs are artificially engineered architectures by fusing the DNA-binding Zinc finger proteins (ZFPs) and the nuclease domain of the IIS restriction enzyme FokI. They are designed to target specific DNA sites [27]. ZFPs enable ZFNs to bind DNA sequences with specificity and contain a tandem array of Cys₂His₂ zinc fingers, one of which consists of approximately 30 amino acids and especially recognizes 3 bp DNA sequences [27, 28]. The FokI nuclease needs to be dimerized to cut DNA sequences, so a pair of ZFPs targeted upstream and downstream of the genomic site of interest need to be designed to induce DSB [29]. One ZFN can especially recognize DNA sequences up to 18 bp, typically 9 bp, depending on the number of fingers and the nature of critical amino acid residues of the DNA binding motif [27, 30].

Generally, DSBs generated by ZFNs can be repaired by NHEJ or HDR pathway, eventually resulting in random indels or exact replacement for genome editing (Figure 2). In 1996, Chandrasegaran's group first constructed hybrid ZFNs to target intended DNA sequences *in vitro*, which opened a new way for genome editing [31]. Subsequently, ZFNs were applied to mutate genes in organisms, such as *Drosophila* [32], *Caenorhabditis elegans* [33], and zebrafish [34, 35]. A breakthrough came in June 2005 when Holmes's group designed ZFNs to repair severe combined immune deficiency mutation of the *IL2Ry* gene in human cells through HDR [36]. The authors proved that ZFNs permanently corrected the DNA sequences at a specific site, encouraging gene therapy in the clinic [36]. However, despite many successful and inspiring achievements at the cellular and organismal levels, some drawbacks limit the wide applications, such as customized protein for every DNA sequence [37], over toxicity to cells [38, 39], and high efficiency of the off-targets [40, 41]. Many efforts have been made to

address these issues to improve the specificity and safety of ZFNs technology [27].

TALENs

Surprisingly, TALENs followed in the footsteps of ZFNs and ignited the genome editing revolution. In 2009, two groups deciphered the code used by transcription-activator-like effectors (TALEs) from plant pathogenic *Xanthomonas* to recognize the DNA sequences [42, 43]. The TALE protein contains three functional domains: a central DNA binding domain of 34 amino acids tandem repeats, two nuclear locational signals (NLSs), and an acidic transcriptional activation domain (AD) [43]. Unlike zinc fingers, which recognize DNA triplets, each repeat domain in the TALE proteins especially recognizes a single base, the specificity of which is determined by the dual residues at positions 12 and 13 of 34 amino acids repeat [42, 43]. These two amino acid pairs are the repeat variable di-residues (RVDs), including NI, NG, HD, and NN. Their TALEs preferentially recognize adenine, thymine, cytosine, and guanine/adenine [43, 44]. Based on ZFNs, TALE truncation variants fused with FokI nuclease (TALENs) enable efficiently cleaving of endogenous human genes at specific sites [45, 46]. Compared to ZFNs, TALENs showed easy operation, improved specificity, and reduced toxicity, improving their applications to fundamental research and gene therapy [47–50]. Notably, many works have further optimized the TALENs for genome editing, such as the assembly of custom TALE arrays [51, 52], the DNA-binding specificity of the TALEs [53], and the delivered contents (plasmids, mRNA, or protein) into the cells [54]. Unfortunately, the discovery and rapid development of CRISPR–Cas technology made the reign of TALENs short-lived (Figures 1 and 2).

CRISPR-Cas

CRISPR-Cas technology originated from the discovery of a series of short, direct repeats (29 nt) interspaced by five intervening nonrepetitive sequences (32 nt) downstream of *Escherichia coli iap* gene in 1987 [55], which were named the clustered regularly interspaced short palindromic repeats (CRISPRs) in 2002 [56]. CRISPRs were later widely detected originating from extra-chromosome and phage-associated sequences in bacteria and archaea [57, 58]. The CRISPR-associated (*Cas*) genes that locate around CRISPR sequences encode proteins with putative nuclease and helicase activity [59]. In 2007,

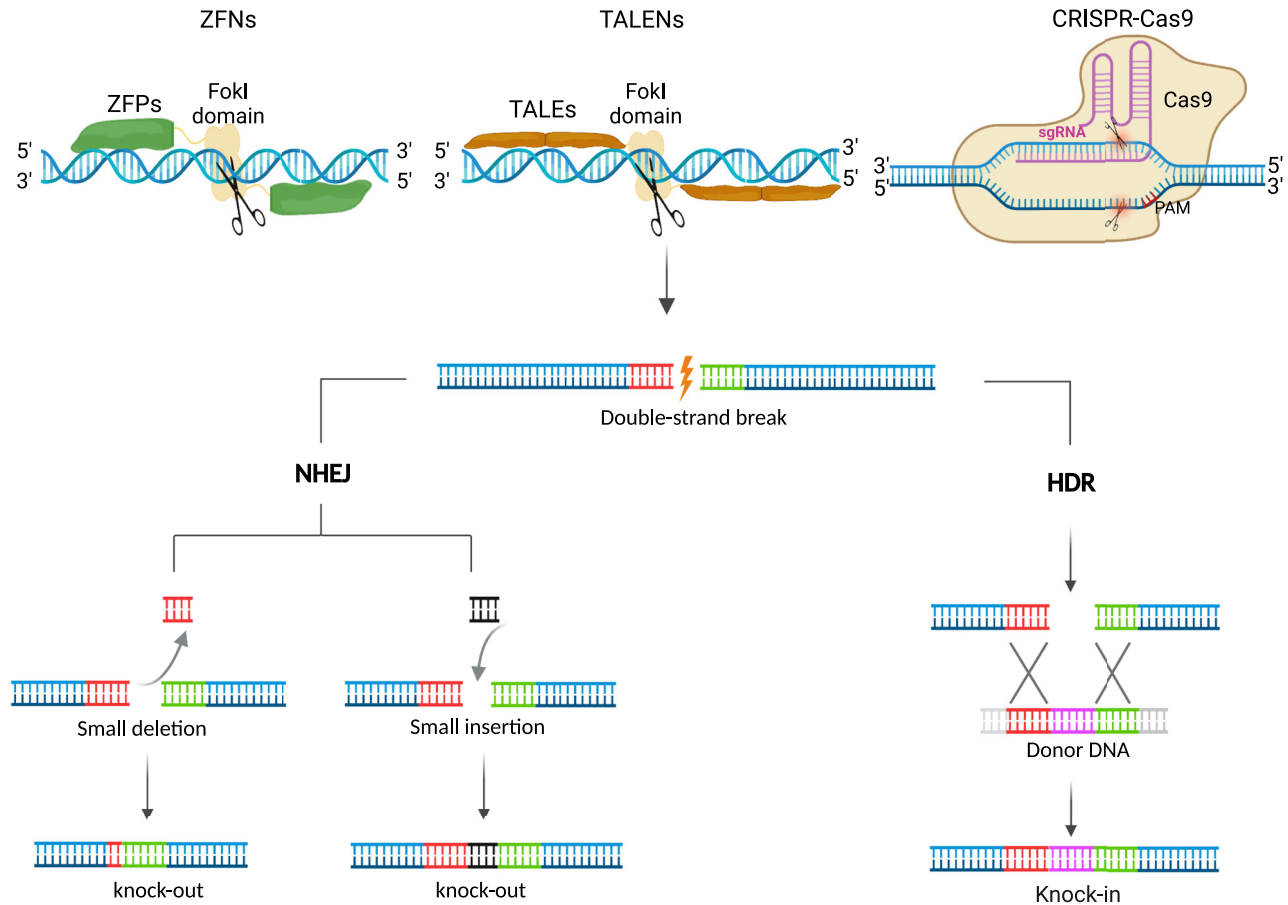


Figure 2: DSBs mediated genome editing. DNA DSBs at the targeted sites induced by nucleases (ZFNs, TALENs, and CRISPR-Cas9), which can be repaired by NHEJ without a donor or HDR in the presence of a donor DNA template. NHEJ can disrupt gene expression by forming indels (deletion and insertion), producing stop codons by frameshift mutations. HDR can precisely restore genetic mutation or insert gene fragments at a specific site. DSBs, double-strand breaks; ZFNs, zinc finger nucleases; TALENs, transcription activator-like effector nucleases; NHEJ, nonhomologous end-joining; HDR, homology-directed repair.

the CRISPR-Cas system was applied to resist phage infection in bacteria using CRISPR and enzymatic machinery of Cas protein—first experimental evidence for CRISPR-Cas as adaptive immunity [60].

The CRISPR-Cas systems can be classified as 1 and 2 based on multiple or single Cas effector modules for CRISPR RNAs (crRNAs) processing and target binding [61]. Furthermore, each class is composed of at least three types and multiple subtypes, including types I (such as Cas3), III, and IV in class 1 and types II (such as Cas9), V (such as Cas12 and Cas14), and VI in class 2 [61]. Besides, the protospacer adjacent motif (PAM), a short 2–5 bp sequences adjacent (e.g., NGG for Cas9; TTTV for Cas12a or Cpf1) to one end of the crRNA-targeted sequence, plays an essential role in recognition of targeted DNA sequences, especially for Type I and II [62, 63].

In 2012, Charpentier's, Doudna's, and Siksny's groups demonstrated that Cas9-crRNA ribonucleoprotein cleaved double-strand DNA guided by crRNA *in vitro*, which paved the way to use the CRISPR-Cas system for genome editing [64, 65]. Cas9 protein was derived from the pathogen *Streptococcus pyogenes* (named SpCas9). It contained two distinct nuclease domains, HNH and RuvC, which cleaved the complementary strand of crRNA and the opposite strand with NGG PAM, respectively [64, 66]. Inactivation of either the HNH (H840A) or the RuvC domain (D10A) in Cas9 creates a variant protein that cleaves only one DNA strand (named nickase Cas9, nCas9), whereas mutating both domains generated dead Cas9 (dCas9) with RNA-guided DNA binding ability [64, 67]. Based on these findings, the CRISPR-Cas9 system was quickly applied to engineer human and

mouse genomes at multiple loci, opening a new age of genome editing [68–70]. Compared to ZFNs and TALENs, the customized RNA-guided CRISPR-Cas9 system is a facile, robust, and low-cost approach for fundamental research and gene therapy (Figure 2) [63].

Although wild-type SpCas9 was widely used in eukaryotic cells and animals, its specific NGG PAM requirement, high off-target, and large protein size (about 1,368 aa) limits its application for gene therapy. To expand the targeting scope of CRISPR-Cas9, the researchers generated various Cas9 variants with altered PAM profiles through structure-guided engineering or protein-directed evolution [63, 71]. Kleinstiver et al. identified SpCas9-VQR (NGA PAM), SpCas9-EQR (NGAG PAM), and SpCas9-VRER (NGCG PAM) through bacterial selection-based directed evolution combined with structural information [72]. Hu et al. used phage-assisted continuous evolution to evolve an xCas9 that can recognize NG, GAA, and GAT PAM sequences [73]. Nishimasu et al. reported a SpCas9-NG harboured relaxed NG PAM guided by structural information [74]. Miller et al. evolved three variants through phage-assisted evolution, including SpCas9-NRRH, SpCas9-NRTH, and SpCas9-NRCH, collectively recognising NRNH PAMs (where R is A or G and H is A, C, or T) [75]. Recently, Walton et al. engineered SpG-Cas9 with high activity to NG PAM and SpRY-Cas9 (where Y is C or T) with NRN and NYN PAMs, which offer capabilities to target almost all genome sites [76]. These PAM flexible Cas9 variants significantly facilitate genome editing. Besides, other CRISPR-Cas enzymes improved the targeting abilities of the genome, such as Cas12a (formerly known as Cpf1) with TTTV PAM [77], Cas12j (formerly Cas Φ) with TTN PAM [78], and Cas12f (formerly Cas14) with TTTR PAM [79]. Collectively, these Cas proteins will expand the future applications of genetic disease treatment.

One major concern associated with using the CRISPR-Cas system for gene therapy was the high efficiency of off-target mutations [80, 81]. Currently, there are many attempts to overcome this concern and improve the specificity of this system, including reusing and engineering Cas proteins and modifying single-guided RNA (sgRNA). First, nCas9 with appropriately paired sgRNAs was applied to introduce two different nicks at the single-strand DNA, resulting in a targeted double-strand DNA break, which showed high fidelity owing to individual nicks in the genome being repaired [82, 83]. Two hybrid nucleases, a programmable DNA binding domain (Zinc fingers or TALEs) fused with SpCas9 variant that disabled DNA and

dCas9 fused with FokI nucleases binding, also increased the specificity of the Cas9 system [84, 85]. Secondly, several studies have evolved showing Cas9 variants reduced off-targets while maintaining editing efficacy, including SpCas9-HF1 [86], eSpCas9 [87], HypaCas9 [88], HeFSpCas9 [89], evoCas9 [90], HiFiCas9 [91], and Sniper-Cas9 [92]. Finally, by computerized analysis, engineering sgRNA structure and optimizing the sgRNA sequence decreased the off-target mutagenesis and improved the editing specificities [93–95].

For gene therapy, adeno-associated viruses (AAVs) are a popular way to package and deliver genes, but the cargo capacity is only about 4.7 kb [96, 97]. An all-in-one AAV delivery system would boost the therapeutic effect. CRISPR-Cas9 system, though, including the promoters, sgRNA, Cas9, and cis-regulatory elements, exceeds the cargo size of a single AAV vector for efficient delivery *in vivo* [98]. The split AAVs with separate SpCas9 and sgRNA, or split-Cas9 architecture, have been developed to overcome this limitation but with reduced fusion protein expression [99, 100]. An all-in-one AAV delivery system would boost the therapeutic effect, and the smaller Cas proteins mediated *in vivo* genome editing raised the potential of efficient gene therapy, including SaCas9 (1,053 aa) [98], CjCas9 (984 aa) [101], Cas12j (700–800 aa) [78], Cas12f (529 aa) [102], etc.

In summary, the multiple CRISPR-Cas systems are a versatile and robust tool for treating genetic diseases. Still, because they require DSBs, they lead to the generation of a minimum of unwanted structural variants [103], chromosomal deletions and rearrangement [104–106], and p53 activation [107, 108].

BEs

In recent years, CRISPR-Cas-derived BEs efficiently converse one base of genome DNA into another in a programmable manner without requiring DSBs or a donor template [15, 109]. DNA base-editors are mainly composed of the nCas or dCas systems for recognizing the specific DNA site and cytosine or adenine deaminase to catalyze nucleotide conversion in a single-strand DNA [109]. Currently, three types of BEs have been developed, including cytidine base editor (CBE, C to T) [110], adenosine base editor (ABE, A to G) [111], and glycosylase base editors (GBE, C to A or C to G) [112, 113]. In addition, recent studies reported a dual adenine and cytosine base editor by fusing both deaminases with nCas9 to simultaneously

achieve C-to-T and A-to-G conversions at the same target site in human cells, in which RNA off-target activity is substantially decreased [114–116]. As approximately half of all pathogenic genetic variants are due to single-nucleotide variants (SNVs) [117], these new BEs offer great potential for treating numerous genetic diseases through permanent DNA transversion.

The first powerful CBE system (BE3), consisting of the nCas9, the rat cytidine deaminase rAPOBEC1, and uracil glycosylase inhibitor (UGI), was developed by Liu's group in 2016 [110], which was used to correct disease mutation and genetic screening by inducing C•G to T•A conversion [118, 119]. There are three steps to convert base C to T for the BE3 system (Figure 3A) [120]: (i) The BE3 complex is targeted to the specific site of genome-guided by the sgRNA; (ii) The rAPOBEC1 changes base C to U within a window of 4–8 bases away from the PAM of the sgRNA, and the nCas9 simultaneously creates a nick in the non-deaminated strand; (iii) Owing to the inhibition of UGI to the uracil N-glycosylase (UNG), which can remove the base U and perform error-free or

error-prone repair and generate different substitutions, the intact deaminated strand is preferred template for endogenous mismatch repair, generating a U•A base pair that is then resolved as a T•A base pair after DNA replication or repair. Subsequently, other cytidine deaminases were also shown to efficiently convert specific base C to T by coupling with nCas9, such as activation-induced cytidine deaminase (AID) [121, 122], human APOBEC3A [123, 124], human APOBEC3G [125, 126], and CDA1 [125] (Figure 3B).

In addition, CBE systems still face some challenges for gene therapy, including low editing efficiency and bystander mutations [118, 119], PAM and editing window limitations [127], DNA and RNA off-target effects [128–131], and packaging constraints [132]. Recently, we have witnessed an improvement in their product purities and efficiencies, expanded the scope of targeting, minimized the undesirable DNA and RNA off-targets and engineered more compact CBE systems (Figure 3B) [133]. To improve the intended editing efficiency (C-to-T mutation) of BE3, additional nuclear-localation sequences [118, 134] and

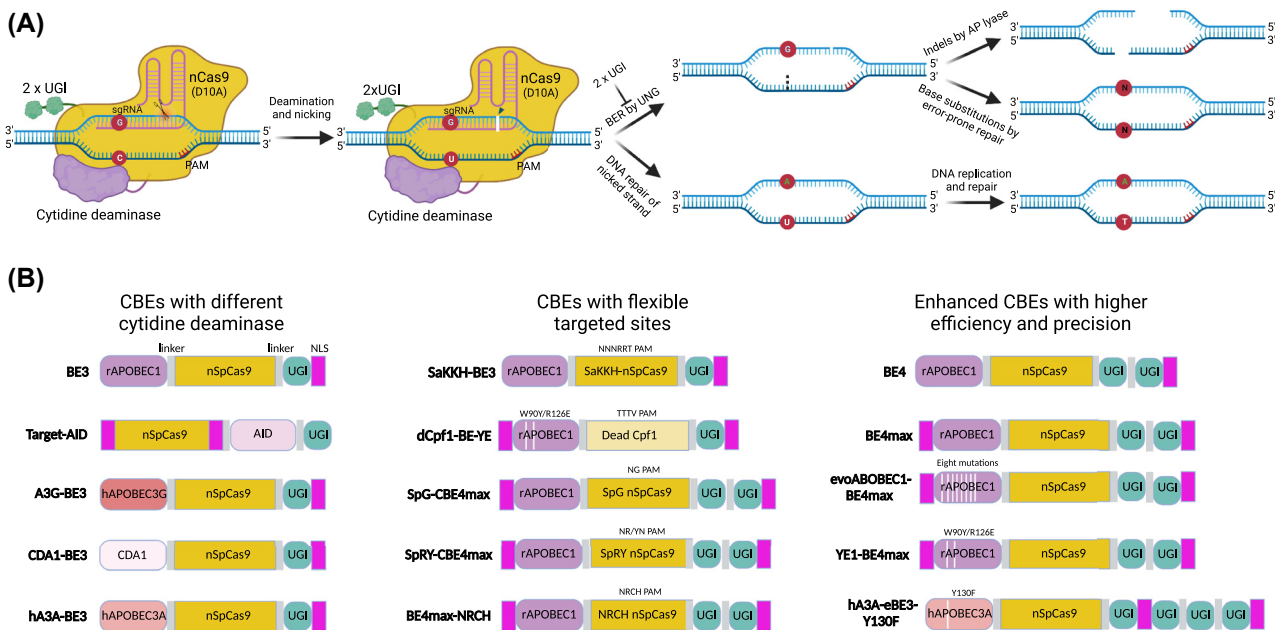


Figure 3: Cytosine base editing. (A) Schematic diagram of a CBE system using nCas9 (D10A) linked with cytosine deaminase and double UGI as an example. The nCas9-sgRNA complex binds to the targeted DNA, forms a single-stranded R-loop, and nicks the non-deaminated strand. The linked cytidine deaminase catalyzes the hydrolytic deamination of cytosine (C) to uridine (U). An additional linked protein, UGI, inhibits the activity of UNG, which excises the U from DNA in cells and initiates BER, with the reversion of the U•G pair to an n•n pair (C•G as the most common outcome). The nick induced by apyrimidinic site lyase (AP lyase) on the deaminated strand combined with the nick on the non-deaminated strand by nCas9 results in the DSB and indels. The intact deaminated strand is preferred for templated repair, and DNA polymerase reads U as thymine (T), generating the U•A base pair to T•A base pair. (B) Schematic diagrams showed different versions of cytosine base editors, including deamination by different enzymes, targeting DNA by various Cas proteins, and evolved CBEs with higher activity and precision. CBE, cytidine base editor; nCas9, nickase Cas9; UGI, uracil glycosylase inhibitor; UNG, uracil N-glycosylase; BER, base-excision repair.

more UGIs [125, 135] both dramatically improved the editing efficiency with fewer indels or by-products. Different CRISPR-associated nucleases fused with evolved cytidine deaminases can increase the target scope and efficiencies of CBEs, such as dCpf1-BE (TTTV PAM) [136], SaBE3 (NNGRRT PAM) [127], SaKKH-BE3 (NNNRRT PAM) [127], SpG-or SpRY-CBE4max [76], evoAPOBEC1-BE4max [137], and CBE-NG [75]. Protein engineering showed that W90Y and R126E mutations in the rAPOBEC1-derived YE1 CBE system showed minimal Cas9-independent DNA and RNA off-targets but retained high on-target activity [138, 139]. Besides, other cytidine deaminases, hAPOBEC3A, hAPOBEC3G, AmAPOBEC1, SsAPOBEC3B, or PpAPOBEC1, derived CBE systems exhibited minimum off-target effects [123, 126, 140]. Due to the limitation of molecular size for AAV delivery, CBE has been split into two AAV vectors for delivery *in vivo*, demonstrating the potential for therapeutic application [132], thus guarantying the single-AAV system as a promising approach for delivery [141]. Thus, CBE is a popular and promising genome editing tool and could potentially reverse about 5,000 pathogenic point mutations associated with human genetic diseases [109].

Drawing inspiration from the CBEs, Liu's group quickly performed seven rounds of evolution for *E. coli* tRNA-specific adenine deaminase TadA and identified a mutant deaminase TadA* that deaminated ssDNA adenosine to inosine (A-to-I editing) in human cells, which was read or replicate as guanine (G) (Figure 4A) [111]. The original ABE version, ABE7.10, consists of wild-type and mutant TadA, nCas9, and sgRNA, which can efficiently convert targeted A•T base pairs to G•C [111]. Recently, several strategies have been adopted to optimize the ABE systems (Figure 4B): (i) ABEmax, an efficient editor obtained by codon optimization of ABE7.10 with bis-bpNLS [142], fused with SpCas9-NG to construct NG-ABEmax with expanded targeting scope [143], the efficiency of which also been improved by NG-ABEmax-KR obtained through human cell-based direct evolution [144]. ABEmax, combined with SpG or SpRY Cas9, targeted almost all PAMs with vigorous activities in the human genome [76]. (ii) Protein evolution of ABE7.10 resulted in ABE8e [145] and ABE8s [146] with only a TadA variant monomer, showing greatly editing efficiency and lower RNA off-target [147]. (iii) V106W or V82G mutation in the TadA* domain [145, 147, 148] or F148A mutation in both TadA and TadA* [131] derived ABEs possess significantly lower RNA off-target effects. (iv) ABEs also catalyzed bystander cytosine deamination at the target site [149],

which was significantly reduced by introducing the D108Q mutation in the TadA* domain [150]. (v) Compact Cas9 ortholog combined with ABE8e was efficiently delivered by a single AAV vector with high efficiency of *in vivo* base editing [151, 152]. These evolved ABEs are functional classes of editing agents because approximately half of the pathogenic single-nucleotide polymorphisms (SNVs) can be corrected by installing A•T→G•C conversions [109].

Currently, the CBEs and ABEs catalyzed DNA base transitions, pyrimidine-to-pyrimidine or purine-to-purine. However, base transversions (pyrimidine-to-purine) are challenging to achieve through base editing [112, 113], which also plays an essential role in repairing pathogenic SNVs [109]. Therefore, the researchers have developed GBE that can efficiently transverse C-to-G mutation in human cells and C-to-A in *E. coli* by replacing the UGI on the BE3 with UNG or directly deleting UNG [112, 113]. Although the optimizations of these BEs have shown explosive growth, there is still a lot of room to maximize their efficiency, specificity, safety, and ability to be delivered *in vivo*.

PEs

PE system is another newly developed genome editing tool that can achieve other functions besides BEs, such as small DNA insertions and deletions, larger deletions, and A-to-C or A-to-T base substitution [153, 154]. The original PE1, a versatile and precise gene-editing method, is composed of a Cas9 nickase (H840A) fused to engineered reverse transcriptase (M-MLV RTase) and a modified sgRNA known as prime editing guide RNA (pegRNA), which can induce all twelve types of base substitutions and small indels without DSBs [153]. In addition, the engineered M-MLV RTase improved the editing efficiency of PE1 (hereafter named PE2). Furthermore, an additional sgRNA was used to introduce nicks on the non-edited strand by nCas9, further boosting the activity of PE2 (designated as PE3 and PE3b) (Figure 5) [153], which quickly applied to genome editing in animals and plants [155–157].

However, a significant determinant of excellent editing efficiency was the selection of the pegRNA, which contains 3' extensions with a primer binding sequence (PBS) that anneals to the target genomic site and an RT template that encodes the intended edit [153]. Recently, computer models from deep learning enabled the quick prediction of the editing efficiency of pegRNA for PE2 in

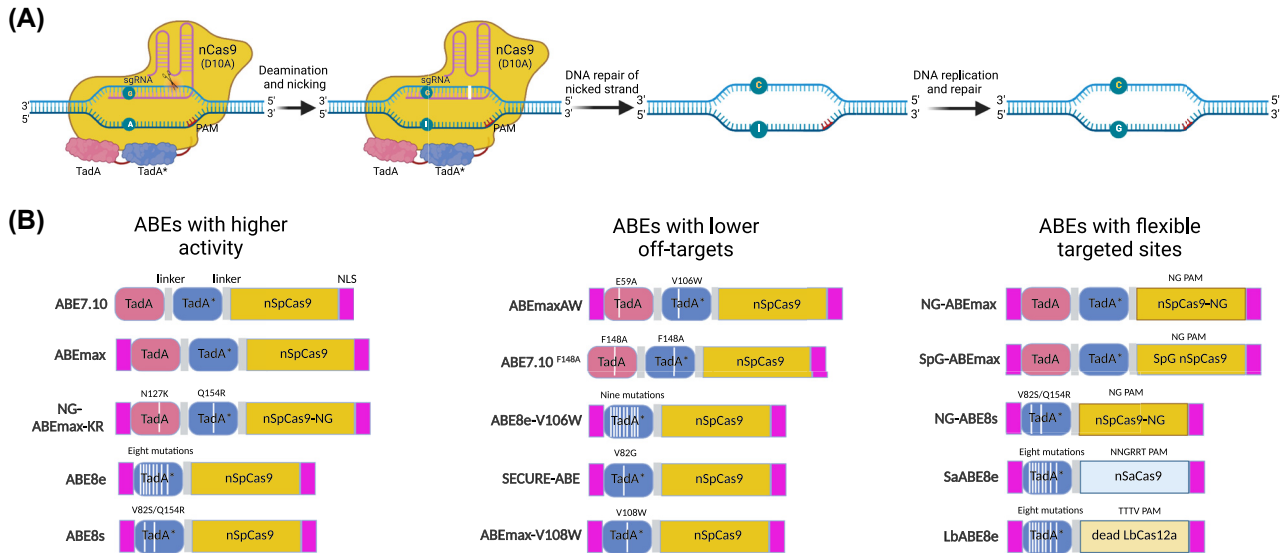


Figure 4: Adenine base editing. (A) Schematic diagram of an ABE system using ABE7.10 as an example. The nCas9-sgRNA complex binds to the non-deaminated strand and forms a nick on it. The fused evolved TadA* catalyzes the deamination of adenosine (A) into inosine (I), which is read as guanosine (G) by DNA polymerase during DNA replication. In the example shown, two deoxyadenosine domains exist, including one wild-type TadA domain and an evolved TadA* domain. Subsequent research found that a single TadA* domain can achieve A•T to G•C base editing. As with uracil, inosine can also be excised by endogenous glycosylases, generating an abasic site that leads to base substitutions or indels, but this process is inefficient. (B) Schematic diagrams showed different versions of ABEs. The evolved ABEs showed higher editing efficiency and lower off-target effects. The Cas variants fused with deoxyadenosine deaminase can target broader genome loci, which are suitable for more genetic mutation repair. ABE, adenosine base editor.

human cells [158]. Furthermore, a web-based pegFinder, for the rapid design and score of pegRNAs from reference was developed [159], which would facilitate the widespread applications of prime editing. It has been shown that engineered pegRNA with stabilized 3' terminus (epgRNA) or same-sense mutations at proper positions significantly improved the editing efficiency [160, 161]. In addition, CRISPRi screens found that DNA mismatch repair (MMR) inhibited prime editing efficiency and promoted undesired indel byproducts, which could be blocked by an engineered MLH1 protein [162]. Thus, the PE4 and PE5 obtained by co-expression of PE2 and PE3 with MLH1 significantly enhanced editing efficiency and reduced indels [162].

While prime editing demonstrates a more versatile new approach to rewriting the genome compared to base editing and nucleases mediated HDR, base editors (CBEs and ABEs) also show higher efficiency and precision when a single target nucleotide is present within the base editing window [15, 153]. Thus, prime editing and base editing offer complementary strengths and weaknesses to make targeted gene mutations through DSB-free genome editing [163]. However, to achieve the therapeutic potential, efficiency, safety, and delivery strategies remain to be stressed for prime editing.

CRISPR-associated transposases (CASTs)

CAST system is emerging as a powerful tool for targeted genomic knock-in and knock-out in bacterial cells. This prevents the need for DSBs in the target DNA and host-cell repair machinery [164–167]. In 2019, two groups demonstrated that Tn7-like transposons from cyanobacteria combined with the CRISPR-Cas system to efficiently perform sgRNA-guided targeted insertions [164, 165]. Strecker et al. reported a CAST system from cyanobacteria *Scytonema hofmanni* (ShCAST) that can insert cargo DNA into a specific site [164]. The ShCAST consists of three main parts: (i) the inactive type V-K (Cas12k) Cas with guide RNA array; (ii) the Tn7-like transposase subunits (TnsB-TnsC-TniQ); (iii) a cargo DNA flanked by the transposon left end (LE) and right end (RE) elements [164]. CRISPR-Cas12k was solely a DNA binding function and efficiently targeted a 24 bp spacer sequence with requisite 5'-NGTN-3' PAM. Among the transposition machinery, TnsB and TnsC specifically recognize the LE and RE sequence and catalyze the cleavage of the cargo DNA. At the same time, TniQ is required for RNA-guided insertions in *E. coli* [164]. ShCAST catalyzes RNA-guided DNA insertion predominantly at

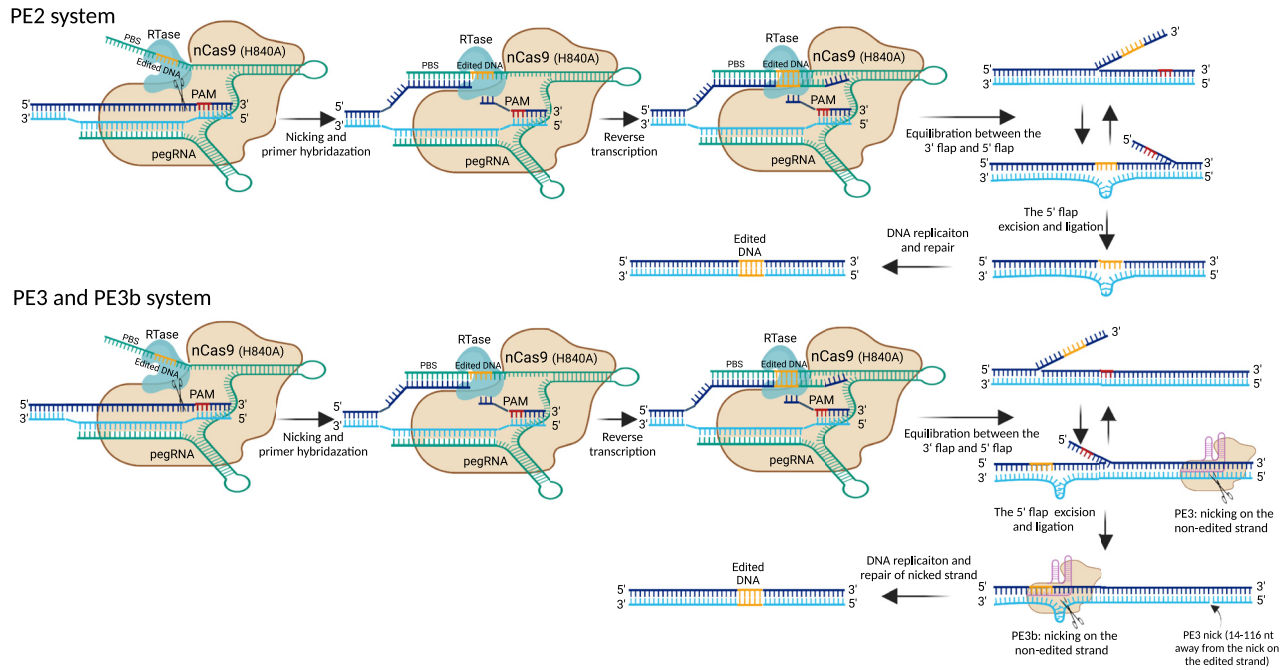


Figure 5: Overview of prime editing by PE2, PE3, and PE3b. The prime editors consist of nCas9 (H840A) fused to RTase via a linker and pegRNA, which consists of gRNA sequence for targeting DNA, PBS for initiating reverse transcription, and RT template for introducing edited DNA. After nicking the PAM-containing strand by the nCas9, the newly released DNA 3' end hybridizes to the PBS sequence of pegRNA to form a primer-template complex. The RTase domain then initiates reverse transcription and adds the edited DNA from the pegRNA into the genomic DNA, resulting in a heteroduplex DNA. After reverse transcription, the PE2 system involves flap equilibrium of edited 3' and unedited 5' flap. Endonucleases excise the unedited 5' flaps, and then ligation and DNA replication/repair lead to stable editing. PE3 and PE3b involve a second nick on the non-edited strand to ensure that the edited strand is used for DNA replication and repair. In the PE3 approach, the second nick is introduced by the nCas9-sgRNA complex in the unedited strand 14–116 nt away from the initial pegRNA nick site. In the PE3b approach, the nCas9-sgRNA complex can only match the edited strand after the edited DNA was incorporated and induces a nick on the non-edited strand, which can prevent the presence of concurrent nicks. PE, prime editor; RTase, reverse transcriptase; pegRNA, prime guide RNA; PBS, primer binding site.

60–66 bp downstream of the PAM sequences in the *E. coli* genome, which displays a T or A preference (about 3 nt) upstream of the insertion site. The insertion only occurred in the forward 5'-LE-cargo-RE-3' orientation, which was highly efficient and can insert up to 10 kb [164].

Meanwhile, Klompe et al. established the VchCAST (Tn6677) system from *Vibrio cholera*. They proved that it had the potential to integrate the donor DNA in two possible orientations at 79 to 83 bp downstream of the PAM sequence (5'-CC-3') [165]. The Tn6677 system contains three plasmids: pQCascade, pTnsABC, and LE-cargo-RE transposon. pQCascade encodes the CRISPR–Cas associated machinery (crRNAs, Cas6, Cas7, and Cas8) and the transposition protein TniQ. TniQ binds to the Cascade complex as a dimer in a head-to-tail configuration and links the Cas machinery and the transpososome (transposase complex with DNA transposon) [168, 169]. Because the Tn6677 system consists of the type I-F CRISPR system, it is more complicated than the ShCAST system. Still, since no crRNA is required, it is more suitable for directing multiple

cargos into the same or multiple genomic targets [170, 171]. Together, the CAST systems stir exciting opportunities in genome editing for specific rearrangements of large DNA sequences or chromosomes. Still, continued efforts need to improve these systems for genome editing in mammalian cells [170].

Progress of genome editing in the treatment of inherited diseases

Therapeutic genome editing aims to target the human genes “hiding” in the cells. In the pathogenesis of inherited diseases, gene mutations often lead to the dysfunction of different systems of the patients. Considering that, it is essential to design and implement a suitable genome editing strategy involving target cell types, the tissues (organs or systems) and their corresponding delivery approaches. In our bodies, somatic cells mainly contribute

to the function of various tissues. In contrast, germ cells are responsible for giving rise to gametes and embryos, a process that could pass the disease-causing mutation to the offspring. Notably, genome-editing therapy in somatic and germ cells is quite different. Therapeutic somatic cell editing only affects the patient himself. However, germ cell editing would potentially introduce genetic changes into the offspring. This will inevitably raise enormous ethical challenges beyond safety issues. In this section, we review the progress of therapeutic somatic cell editing and discuss the possible attempts for therapeutic germ cell editing in research and clinical use.

Ex vivo editing in somatic cells

All therapeutic genome editing in development is based on somatic cell editing (Tables 1 and 2). As mentioned above, editing the somatic cells of the different tissues requires a feasible approach with the considerations of target cells and ways of delivering and editing. Somatic cell editing can be divided into *ex vivo* and *in vivo* (Figure 6).

Ex vivo editing followed by adoptive transfer

Ex vivo somatic editing is suitable for the inherited disorders of the hematopoietic system, such as immune deficiencies, erythrocyte diseases and platelet disorders. The most prevalent attempts aim to cure SCD and β -thalassemia, both caused by a single nucleotide mutation in the gene that encodes the β -globin (*HBB*). In erythrocytes, two β -globin subunits assemble into a tetramer with two α -globin subunits, called adult hemoglobin (HbA), which binds oxygen with high affinity. In SCD erythrocytes, the sickle hemoglobin (HbS), resulting from A-to-T mutant at position 20, polymerizes into fibers under hypoxic or acidic conditions, distorting the erythrocytes to the “sickle” shape with shortened lifespan and reduced function, causing hemolytic anemia and multi-organ damage [172]. Various single nucleotide substitutions, small deletions or insertions within the *HBB* gene cause β -thalassemia, resulting in reduced (β^+) or absent (β^0) β -globin synthesis and anemia [173]. Less than 15%–20% of the SCD and β -thalassemia patients have histocompatible (human leukocyte antigen-matched) healthy donors and are curative by allogenic HSC transplantation [174–176]. HSC gene therapy provides a promising way to treat SCD, which rely on gene correction of autologous HSC followed by transplantation (Figure 6A). Several gene targets involved in regulating the

β -globin gene or γ -globin (forming fetal Hb, abbreviated as HbF), which has anti-sickling effects, have been proposed and proved useful [177, 178]. One of the employed strategies is gene addition by lentivirus in patient CD34⁺ HSCs that are widely tested in clinical trials and reviewed elsewhere [179].

CRISPR-Cas9 in erythrocyte diseases

Currently, mutant *HBB* and *BCL11A* erythroid-specific enhancers (ESE) are the two most popular targeting sites in gene-editing trials. Two clinical trials regard to *HBB* editing by CRISPR-Cas9 for the treatment of severe SCD are in progress at phase 1/2 in the USA [180, 181]. One of them, GPH101 from Graphite Bio, is the first CRISPR-based genome correction therapy that harnesses CRISPR-Cas9 and HDR mechanisms to cut out the mutation and repair with wild-type (WT) DNA sequence [182] (Figure 7A). In this platform, Cas9-sgRNA, is delivered through ribonucleoprotein (RNP) complexes. In contrast, the DNA template is delivered through an AAV6 vector *ex vivo*, and the preclinical data demonstrated this strategy's safety, efficacy, and reproducibility [183]. Furthermore, in 2021, a clinical study of β -globin restored autologous HSCs using CRISPR/Cas9 for β -thalassemia major patients with *CVS-654* mutation started (Shanghai Bioray Laboratories and PLA 923 Hospital) in China [184]. This disease subtype is also known as transfusion-dependent β -thalassemia (TDT), the most severe form of β -thalassemia.

BCL11A ESE attracts more attention and go further in clinical trials. *BCL11A* plays a critical role in HbF-HbA switching via binding to the γ -globin promoter directly, repressing γ -globin expression [185, 186]. Disrupted *BCL11A* expression showed therapeutic potential but negatively affected B-cell differentiation and HSC engraftment [177, 187]. Further studies found that deletion of the *BCL11A* ESE region (especially the site of +58 bp from the transcriptional start site) led to an increase in γ -globin and resulted in survival advantage of the editing cells without affecting their proliferation and differentiation [188–190] (Figure 7B). CTX001 from Vertex Pharmaceuticals, which is autologous CD34⁺ hHSPCs (hematopoietic stem and progenitor cells) modified with CRISPR-Cas9 at *BCL11A* ESE, was used in one SCD patient and one TDT patient and showed a striking curative effect in more than one year follow-up [191]. Of note, no evidence of off-target editing was observed. The multi-site, observational study to evaluate the long-term safety and efficacy of CTX001 in the TDT and severe SCD patients who received CTX001 is ongoing [192]. A phase 3 clinical trial of CTX001

Table 1: Summary of therapeutic genome editing *ex vivo* for inherited diseases in clinical trials.

Editing tools	Diseases	Target cells	Target gene	Delivery	Mechanisms	Drug name	Year	Current status	Sponsor	Trial number ^a
CRISPR-Cas9	Sickle cell disease	HSPCs	<i>BCL11A</i> ESE	NA	NHEJ	OTO923/ HIX763	2020	I/II, Recruiting	Novartis pharmaceuticals	NCT04443907
	Sickle cell disease	HSCs	<i>HBB</i>	Electroporation	HDR?	CRISPR_SCD001	2021	I/II, not yet recruiting	University of California, san francisco	NCT04774536
	Sickle cell disease	HSCs	<i>HBB</i>	RNP electroporation, AAV6 for teampole	HDR	GPH101	2021	I/II, Recruiting	Graphite bio	NCT04819841
	Sickle cell disease	HSPCs	<i>BCL11A</i> ESE	Electroporation of RNP	NHEJ	CTX001	2018	II/III, active, not recruiting	Vertex pharmaceuticals	NCT03745287
	Sickle cell disease (pediatric)	HSPCs	<i>BCL11A</i> ESE	Electroporation of RNP	NHEJ	CTX001	2022	III, Recruiting	Vertex pharmaceuticals	NCT05329649
	Sickle cell disease, β -Thalassemia	HSPCs	<i>BCL11A</i> ESE	Electroporation of RNP	NHEJ	CTX001	2019	n/A	Vertex pharmaceuticals	NCT04208529
	β -Thalassemia	HSPCs	<i>BCL11A</i> ESE	Electroporation of RNP	NHEJ	BRL-101	2020	I/II, Recruiting	Biorary laboratories	NCT04211480
	β -Thalassemia	HSPCs	<i>BCL11A</i> ESE	Electroporation of RNP	NHEJ	CTX001	2018	II/III,Active, not recruiting	Vertex pharmaceuticals	NCT03655678
	β -Thalassemia	HSPCs	<i>BCL11A</i> ESE	NA	NHEJ	ET-01	2021	I, active, not recruiting	EdiGene	NCT04925206
	β -Thalassemia with CVS-654 mutation	HSCs	<i>HBB</i>	Electroporation	NHEJ?	NA	2021	I/II, enrolling by invitation	Biorary laboratories	NCT04205435
CRISPR-Cas12a	Sickle cell disease	HSCs	<i>HBB</i> and <i>HBG2</i> promoters ^b	Electroporation of RNP	NHEJ	EDIT-301	2021	I/II, recruiting	Editas medicine	NCT04853576
ABEs	Sickle cell disease	HSPCs	<i>HBB</i>	Electroporation	T to C	BEAM-101	2022	I/II, not yet recruiting	Beam therapeutics	NCT05456880
ZFNs	Sickle cell disease	HSPCs	<i>BCL11A</i> ESE	mRNA transfection	NHEJ	BIVV003	2019	I/II,Recruiting	Sangamo therapeutics	NCT03653247
	Sickle cell disease	HSPCs	<i>BCL11A</i> ESE	mRNA transfection	NHEJ	BIVV003	2021	n/A, recruiting	Sangamo therapeutics	NCT05145062
	β -Thalassemia	HSCs	<i>BCL11A</i> ESE	Electroporation and AAV	NHEJ	ST-400	2018	I/II, active, not recruiting	Sangamo therapeutics	NCT03432364
	β -Thalassemia	HSCs	<i>BCL11A</i> ESE	Electroporation and AAV	NHEJ	ST-400	2021	n/A, recruiting	Sangamo therapeutics	NCT05145062

^awww.clinicaltrials.gov; ^bThe regions are *BCL11A* binding sites.

Table 2: Summary of therapeutic genome editing *in vivo* for inherited diseases in clinical trials.

Editing tools	Diseases	Target cells	Target gene	Delivery	Mechanisms	Drug name	Year	Current status	Sponsor	Trial number
ZFNs	Hemophilia B	Hepatocytes	Albumin intron 1	rAAVs	HDR	SB-FIX	2016	I, terminated	Sangamo therapeutics	NCT02695160; NCT04628871
	Mucopolysaccharidosis I	Hepatocytes	Albumin intron 1	rAAV2/6	HDR	SB-318	2016	I/II, terminated	Sangamo therapeutics	NCT02702115; NCT04628871
	Mucopolysaccharidosis II	Hepatocytes	Albumin intron 1	rAAV2/6	HDR	SB-913	2017	I/II, terminated	Sangamo therapeutics	NCT03041324; NCT04628871
CRISPR-Cas9	Hereditary transthyretin amyloidosis	Hepatocytes	TTRs	LNP	NHEJ	NTLA-2001	2020	I, recruiting	Intellia therapeutics	NCT04601051
	Hereditary angioedema	Hepatocytes	KLKB1	LNP	NHEJ	NTLA-2002	2021	I/II, recruiting	Intellia therapeutics	NCT05120830
	Leber congenital amaurosis	Photoreceptor cells	CEP290	rAAV5	NHEJ	EDIT-101	2019	I/II, recruiting	Editas medicine	NCT03872479
ABEs	Heterozygous familial hypercholesterolemia	Hepatocytes	PCSK9	LNP	A to G	VERVE-101	2022	I, recruiting	Verve therapeutics	NCT05398029

in pediatric participants with severe SCD and hydroxyurea failure or intolerance was started in 2022 [193]. OTQ923 and HIX763 from Novartis Pharmaceuticals are similar autologous products edited with CRISPR-Cas9, which are also in phase 1/2 for treating adult/children SCD [194]. More recently, a study from Xiangya Hospital, Central South University, reported the preliminary data of an ongoing phase 1/2 trial (NCT04211480) evaluating the safety and efficacy of *BCL11A* ESE editing, autologous, HSPCs (BRL-101 from Shanghai Bioray Laboratories) in two children with TDT. Both patients were clinically well with multilineage engraftment and achieved transfusion independence for more than 18 months without incidence of adverse events [195]. Longer follow-ups of a larger cohort of patients are wanted to confirm whether this treatment can cure pediatric TDT patients. In 2021, EdiGene (GuangZhou) Inc. from China also started a phase 1 study in subjects with TDT, in which HSPCs edited by CRISPR-Cas9 for *BCL11A* ESE will be evaluated (NCT04925206) [196].

CRISPR-Cas12a, ZFNs and BEs in erythrocyte diseases

Except for CRISPR-Cas9, CRISPR-Cas12a and ZFNs were also used for therapeutic *ex vivo*. EDIT-301 from Editas Medicine using Cas12a, targeting a *BCL11A* binding region in *HBB1* and *HBB2* promoters for HbF induction, was tested in phase 1/2 for the efficacy, safety and tolerability in adult subjects with severe SCD in 2021 [197, 198]. Using ZFNs, BIVV003 and ST-400 from Sangamo Therapeutics for treating severe SCD and TDT, respectively, are in phase 1/2. The data in the five TDT patients treated with ST-400 showed that the treatment was generally well tolerated and increased the levels of HbF transiently after reconstitution, which might need resumption of transfusions [199]. In another recent study, four SCD patients were successfully infused with a single dose of BIVV003. They had no SCD-related events or infusion-related reactions with 26 weeks of follow-up, with increased total Hb, HbF, and HbF-containing cells, and clinical improvements [200] (Figure 7B).

Using a custom adenine base editor (ABE8e-NRCH), Liu et al. edited the pathogenic *HBB^S* allele (codon GTG) to a non-pathogenic, naturally occurring variant *HBB^G* (GCG), in HSPCs from SCD patient [201] (Figure 7A). In the 16 weeks of follow-up, edited HSPCs were durable after engraftment in immunodeficient mice with 68% *HBB^G* and reduced propensity for hypoxic sickling. Furthermore, *HBB^S*-to-*HBB^G* base editing alleviates

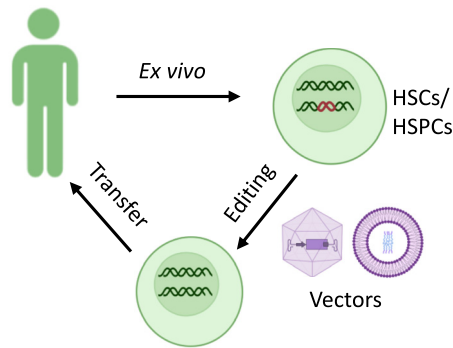
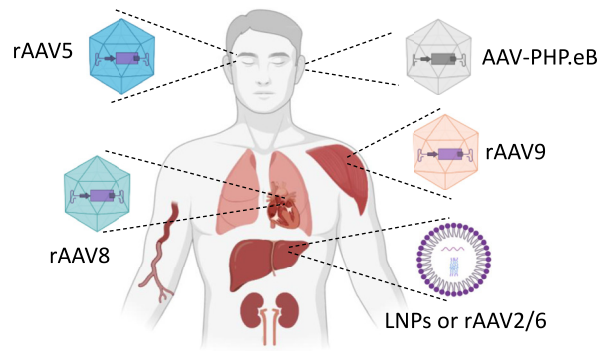
(A) Somatic cell editing *ex vivo***(B) Somatic cell editing *in vivo***

Figure 6: Strategies for therapeutic genome-editing. (A) Schematic diagram for somatic cell editing *ex vivo*. Bone marrow cells of patients are mobilized and HSCs or HSPCs are isolated and cultured *ex vivo*. The genome editing components are packed and delivered into cells, usually by electroporation or AAVs. After quality control, the edited HSCs/HSPCs are infused into the patients for treatments. (B) Schematic diagram for somatic cell editing *in vivo*. The genome editing components are packed into the delivery vehicles with organ tropism, such as AAVs and LNPs, then injected directly into patients. HSCs, hematopoietic stem cells; HSPCs, hematopoietic stem and progenitor cells; AAVs, adeno-associated viruses; LNPs, lipid nanoparticles.

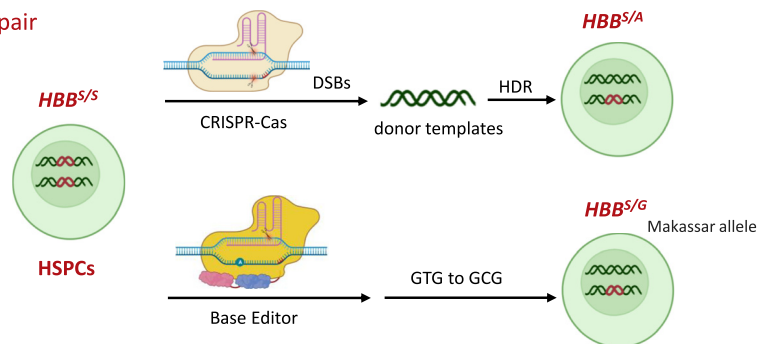
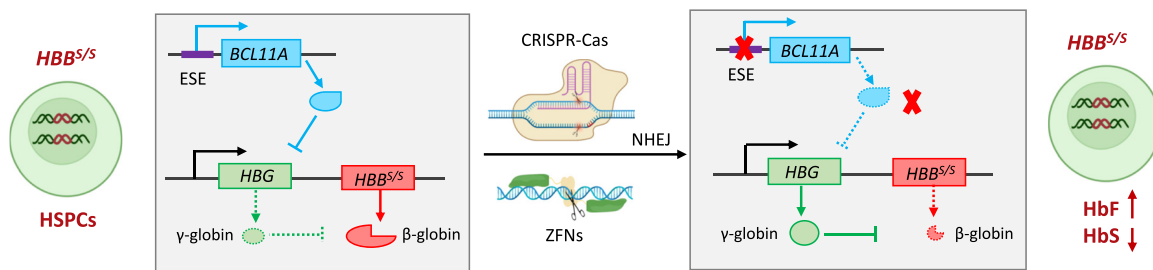
(A) *HBB* Gene Repair**(B) HbF Induction**

Figure 7: Molecular mechanisms of therapeutic genome editing *ex vivo* for SCD. (A) *HBB* gene repair. Upper: The CRISPR-Cas9 recognizes and cuts the locus of *HBB*^{S/S} in HSPCs and DSBs are induced, triggering DNA repairing. At the presence of DNA donor as a template, DSBs are repaired by HDR and the *HBB*^{S/S} is converted to *HBB*^{S/A} or *HBB*^{A/A}. Below: the pathogenic *HBB*^S allele (codon GTG) to a non-pathogenic, naturally occurring variant *HBB*^G (GCG) in HSPCs by ABEs. (B) HbF induction: After birth, *BCL11A* binds to the γ -globin promoter directly, repressing γ -globin expression, a component of HbF that has anti-sickling effects. *HBB*^{S/S} in patients produces sickle β -globin. CRISPR-Cas9 or ZFNs targeting *BCL11A* ESE, will induce DSBs and NHEJ. As a consequence, *BCL11A* expression and the inhibition to γ -globin is disrupted, leading to an increase in γ -globin. SCD, sickle cell disease; HbF, fetal hemoglobin; ESE, erythroid-specific enhancers; HbS, sickle hemoglobin.

pathology in a mouse model of SCD, identifying no off-target mutations with anticipated clinical relevance [201]. These data suggest that this platform provides a promising treatment for SCD. The corresponding manufactured product BEAM-101 was initiated in a clinical trial this year (NCT05456880) [202].

Potential candidates

The above studies give us great hints that many other hereditary diseases of the hematopoietic system also could be cured by transplantation of genome edited autologous HSPCs (Table 1). Fanconi anemia (FA) is an autosomal recessive disorder that leads to bone marrow failure and a predisposition to malignancies. More than 22 gene mutations are involved [203]. CRISPR-Cas9, ZFNs mediated genome editing in FA patient-derived HSCs followed by transplantation in mouse is feasible for correcting disease related phenotypes [204, 205]. Particularly, Paula Ríó et al. showed highly efficient NHEJ-mediated repair of mutated FA genes to generate compensatory mutations that correct the phenotype of FA patient-derived HSCs, where the efficacy of NHEJ is enhanced in FA-deficient cells and secondary mutations in FA genes restored the function of mutated alleles [205]. These studies suggest the feasibility of NHEJ-mediated gene editing in treating FA and other monogenic diseases of the hematopoietic system.

In humans, primary immunodeficiency syndromes (PIDs), which cause dysfunction or nonfunctioning of one or more components of the human immune system, represent a group of monogenic disorders of leukocytes. Over 300 gene mutations cause PIDs, such as *IL2Ry* mutation in X-linked severe combined immunodeficiency (SCID-X1), *ADA* mutation in adenosine deaminase deficiency (ADA-SCID), *WAS* mutation in Wiskott-Aldrich-Syndrome (WAS) etc. [206]. Autologous HSCs transplantation combined with gene addition by lentivirus or retrovirus vectors has attracted much attention in clinical trials for PIDs. The commercial product Strimvelis was approved by the European Medicines Agency (EMA) for the treatment of ADA-SCID in 2016 (reviewed elsewhere [206, 207]), even though long-term efficiency and safety in patients remain challenged. In contrast to erythrocyte diseases, there is a limited NHEJ-based genome editing strategy for PIDs. HDR-based editing is more challenging since the low editing efficiency in primitive HSPCs. For example, only about 5% targeting efficiency of HDR in ZFN-mediated editing of SCID-X1 HSPCs was observed in the previous study [208], which did

not reach the threshold (10%) for functional HSPCs required for immune reconstitution [209]. Optimizing editing reagents and treatment protocols such as delivery method and culture conditions might help its application of genome editing in PIDs.

In vivo genome editing in somatic cells

Delivery systems

In vivo genome editing involves introducing therapeutic components to the cells in the patient's body directly (Figure 6B). There are some advantages over *ex vivo* therapy: (i) it bypasses the complicated steps of cell culture, quality control and cell transfer; (ii) it is transplantation-independent, making it possible to edit cells that are difficult for manipulation *ex vivo* or poor engraftment; (iii) potentially corrects the gene mutations of multiple tissue types when the delivery vehicles are well designed. Sometimes, this might also be a disadvantage of *in vivo* editing. Indeed, the delivery vehicles for *in vivo* are much more challenging than the counterpart used *ex vivo*. The most challenging part is delivering the gene-editing components to the target cells precisely with high efficacy [210]. *Ex vivo* editing strategy often employs non-virus deliver systems such as electro-transfection or microinjection of the RNP (protein-RNA complex), which might be more controllable and suitable for stem cells, mainly because of no genomic integration and no long-term expression and fewer off-target [211]. Instead, viral vectors or nanoparticles are favored for *in vivo* delivery. Viral vehicles, including lentivirus, adenoviruses (AVs), and AAVs, have high efficacy and apply to various tissues. Especially, AAVs are the most popular vehicles used in clinical gene therapy. AAVs can infect the dividing and non-dividing human cells efficiently, making them a promising tool for therapeutic components to deliver and cure diseases of various tissues. Moreover, a variety of serotypes of AAVs show tissue-specific tropism, increasing the specificity of the treatment. The non-integrative property of AAVs into the host genome is another advantage compared to integrating lentiviral vehicles. The integrating feature is a hidden danger both *in vivo* and *ex vivo*. In the phase 1/2 study about transplantation of autologous HSCs transduced *ex vivo* with the LentiGlobin BB305 lentiviral vector in SCD patients, participants developed acute myeloid leukaemia/myelodysplastic syndrome where the transgene was present in the patient's malignant cells [212].

However, AAVs have limited cargo size, with a maximum capacity of 4.7 kb. As a result, when using large nucleases such as SpCas9 (encoding by about 4.2 kb gene), a second AAV for sgRNA and repair template is needed, leading to reduced efficiency as two AAVs need to be introduced into one cell for function [213]. Moreover, sustained expression of genome-editing molecules probably leads to undesired off-target editing or immune reactions in patients [211, 214], which are common problems in applications *in vivo* and *ex vivo* when using viral vectors. Cationic lipid-based nanoparticles and inorganic nanoparticles are emerging delivery systems that could be used in clinical applications of *in vivo* genome editing-based therapy [215–217]. In size, nanoparticles are much bigger than viruses and RNPs, ranging from 50 to 500 nm [211]. They show low cost, low immunogenicity and no genomic integration, but limited tissue targeting spectrum and low systemic delivery efficiency [218].

In summary, several delivery vehicles are available for *in vivo* genome editing, with distinctive drawbacks and advantages. Nonetheless, with efforts in optimization of the types of delivery vehicles, the dosages, the sites of administration and editing strategies, we are delighted to see real progress in the *in vivo* genome editing in treatments of genetic diseases from laboratory research to clinical practices (Table 2).

***In vivo* genome editing of CRISPR for inherited diseases in clinical trials**

NTLA-2001 is the first-ever CRISPR-Cas9-based genome-editing therapy *in vivo*. NTLA-2001 was designed by Intellia Therapeutics to treat hereditary transthyretin amyloidosis (ATTR), including ATTR with polyneuropathy (ATTR-PN) and ATTR-related cardiomyopathy (ATTR-CM) [219]. These diseases are autosomal-dominant disorders and associated with over 100 different missense mutations in the *TTR* gene encoding transthyretin, resulting in misfolds of TTR tetramer, which further develops into TTR protein aggregates, generating amyloid fibrils, protein deposits in multiple tissues [220]. Given that TTRs are exclusively produced by hepatocytes of the liver, NTLA-2001 are designed as a *TTR*-specific guide RNA and human-optimized SpCas9 mRNA packing in lipid nanoparticles (LNP) with liver tropism. After intravenous injection of NTLA-2001, the circulatory LNPs are opsonized by apolipoprotein E (ApoE) and bind to hepatocytes via interaction of ApoE and the low-density lipoprotein receptor (LDLR) on hepatocytes, followed by endocytosis and endosome formation (Figure 8A and B). The mutant

TTRs could be abrogated upon missense or nonsense mutations induced by Cas9 cut and NHEJ-mediated repair. In an ongoing phase 1 clinical study (NCT04601051), NTLA-2001 led to a reduction in the serum TTR protein in a dose-dependent effect, with a 52% decrease in patients who received 0.1 mg per kilogram and an 87% decrease for 0.3 mg per kilogram at 28 dpi, even though mild adverse effects were observed [219].

With a similar delivery system, NTLA-2002 is an *in vivo* CRISPR-Cas9 genome editing drug that is designed to knock out the gene *kallikrein B1* (*KLKB1*) in hepatocytes for the treatment of hereditary angioedema (HAE) (Figure 8C). HAE is a rare, autosomal dominant disorder characterized by severe, recurring inflammatory attacks with swelling in various organs [221]. HAE is caused by a deficiency or dysfunction of the C1 esterase inhibitor, a vital regulator of the complement system and kallikrein-kinin cascades. When the C1 inhibitor loses function, activation of the kallikrein-kinin cascade leads to the uncontrolled generation of plasma kallikrein and excessive production of bradykinin, which causes vascular leakage and subsequent angioedema [222]. Pre-clinical studies proved that knocking out *KLKB1*, encoding a precursor of plasma kallikrein, achieved sustained therapeutically reduction in plasma kallikrein activity in non-human primates (NHPs) [223]. Inspired by the results, Intellia Therapeutics started the Phase1/2 clinical trial of NTLA-2002 in adults with HAE in 2021 [224].

Another CRISPR-Cas9-based genome editing trial in the clinical is EDIT-101, using DSBs and NHEJ to remove the *IVS26* mutation in the *CEP290* gene (NCT03872479) [225]. *IVS26* is a well-known Leber congenital amaurosis (LCA)-associated mutation in the *CEP290*, which is an A-to-G point mutation located within intron 26 (referred to as c.2991+1655A>G), resulting in aberrant splice donor. It accounts for almost one-third of all LCA cases [226, 227]. Jiang et al. subtly developed a pair of sgRNAs that flanked the *IVS26* mutation, induced deletion or inversion of the intervening sequence, and achieved productive editing that met the therapeutic threshold [228] (Figure 9). In humans, EDIT-101 is delivered directly to the photoreceptor cells via the AAV5 vector after subretinal injection. It is worth noting that this is the first *in vivo* administration of CRISPR therapy to a child.

***In vivo* genome editing of BEs for inherited diseases in clinical trials**

The world's first *in vivo* genome editing using BEs in humans, the VERVE-101, was also initiated

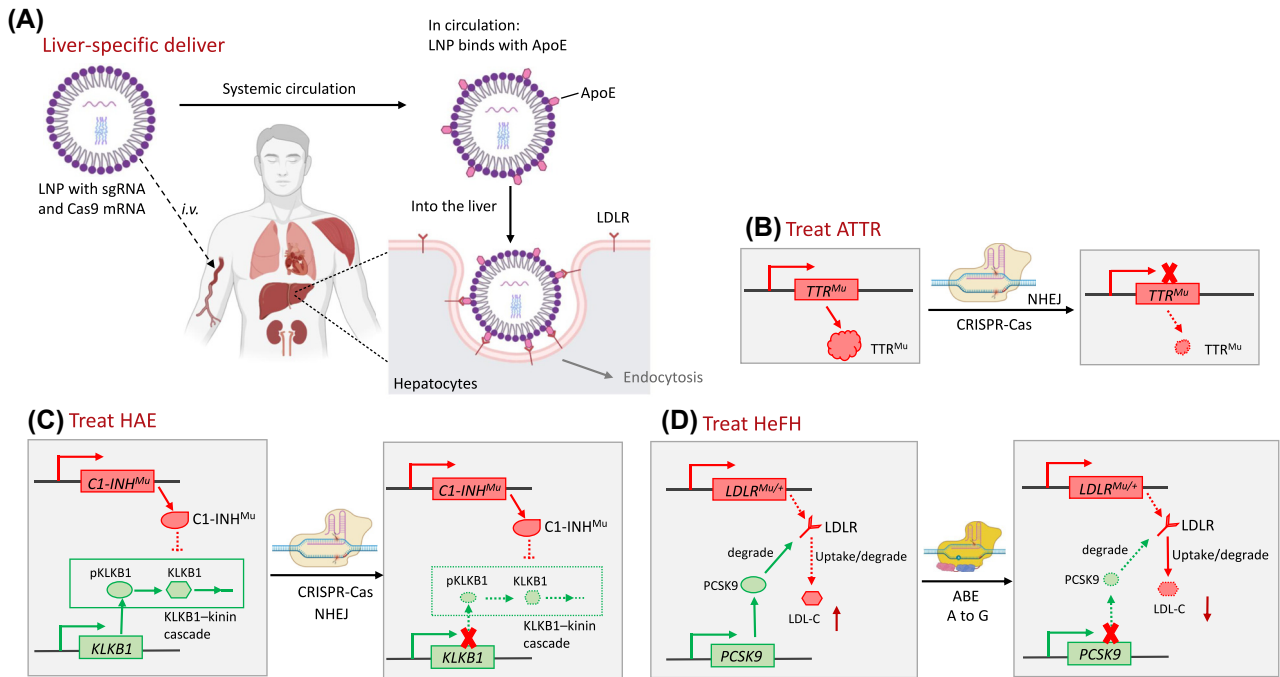


Figure 8: Molecular mechanisms of therapeutic genome editing *in vivo* for hereditary metabolic diseases. (A) Liver-specific deliver of genome editing agents: LNPs with sgRNA and Cas9 are i.v. injected and coated by ApoE in circulation. When circulating into the liver, interaction between ApoE and LDLR take the LNPs into the hepatocytes by endocytosis specifically. (B) In patients with ATTR, missense mutations in the *TTR* gene encoding transthyretin result in the misfolds of TTR tetramer, which further develop into TTR protein aggregates. *TTR*-specific gRNA and Cas9 delivered into hepatocytes via LNPs, abrogate mutant *TTR*s upon missense or nonsense mutations induced by NHEJ-mediated repair. (C) In patients with HAE, mutant C1 esterase inhibitor (encoding by *C1-INH*) lose its function of regulating the complement system and kallikrein–kinin cascades. CRISPR mediated knocking out of *KLKB1* that encoding a precursor of plasma kallikrein, achieved therapeutic outcomes. (D) Loss-of-function mutation in the *LDLR* gene down-regulates LDLR expression, leading to high LDL-C and HeFH. ABEs make a A-to-G conversion at the splice site in the *PCSK9* gene (causing aberrant splicing) for disrupting its expression and degrading of LDLR, subsequently lowers the LDL-C. ApoE, apolipoprotein E; ATTR, hereditary transthyretin amyloidosis; HAE, hereditary angioedema; LDLR, low-density lipoprotein receptor; LDL-C, low-density lipoprotein cholesterol; HeFH, heterozygous familial hypercholesterolemia.

(NCT05398029) [229]. It was designed by Verve Therapeutics and also utilized a LNP-mediated vector to target the liver and ABEs to make an A-to-G conversion at the splice site in the *PCSK9* gene (causing aberrant splicing) for disrupting its

expression and subsequently lowering the LDL cholesterol (LDL-C) levels in patients with heterozygous familial hypercholesterolemia (HeFH) [230] (Figure 8D). In patients with HeFH, a heterozygous loss-of-function mutation in the *LDLR*

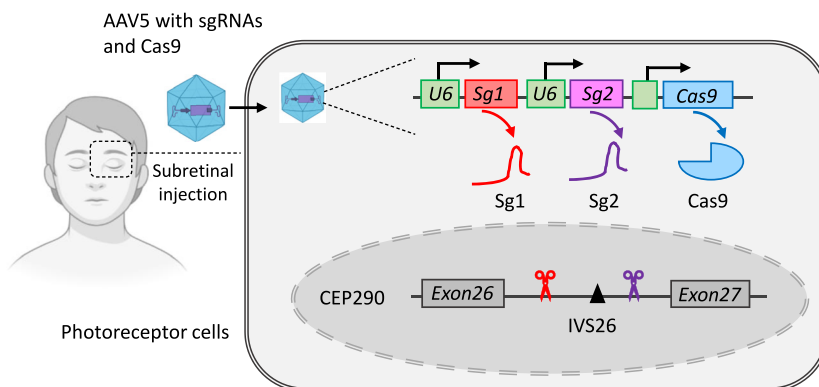


Figure 9: Strategy of therapeutic genome editing *in vivo* for LCA. A pair of sgRNAs and Cas9 are delivered directly to the photoreceptor cells via AAV5 vector after subretinal injection. Cas9 induces deletion of *IVS26* mutation which is LCA-associated mutation in the *CEP290*. LCA, Leber congenital amaurosis.

gene down-regulates *LDLR* expression, leading to high LDL-C in the blood and ultimately resulting in a heart attack or atherosclerotic cardiovascular disease [231]. In a preclinical study, a single intravenous injection achieved near-complete knockdown of *PCSK9* in the liver. It resulted in an approximately 90% reduction in blood levels of *PCSK9* protein and about a 60% reduction in blood levels of LDL-C in NHPs compared to the baseline [232]. Verve Therapeutics plans to develop a second gene editing program that targets the *ANGPTL3* gene, a regulator of cholesterol and triglyceride metabolism, for patients with FH [233]. Notably, in the application of VERVE-101 in patients with HeFH, an issue arose that a low level of LDLR in patients might influence the endocytosis of the LNPs by hepatocytes in the liver as LDLR plays an essential role in LNP uptake.

***In vivo* genome editing of ZFNs for inherited diseases in clinical trials**

Sangamo Therapeutics developed ZFN-mediated *in vivo* genome editing products-SB-318, SB-913, and SB-FIX to treat Mucopolysaccharidosis Type I (MPS I), MPS II, and severe Haemophilia B, respectively [13]. The three drugs were ZFN-mediated genome editing for knocking in therapeutic cDNA to the endogenous albumin locus of hepatocytes precisely, which are delivered by AAV-derived vectors *in vivo* and enable the patient's liver to produce stable circulating therapeutic levels of a corrective protein for the lifetime of the patient [234]. As the first therapy based on ZFN-mediated genome editing, SB-FIX is designed to treat Hemophilia B, caused by the *FIX* (factor IX)-mutation. The editing machinery comprises three liver-tropic rAAVs, two of which carry left and right ZFNs, and one carries the WT *FIX* gene [235]. Under this setup, rAAVs delivered the components into the liver. In addition, ZFNs were responsible for recognizing and cutting albumin intron 1 precisely, followed by HDR with AAV-delivered donor DNA as templates. This clinical trial (NCT02695160) was started in 2016 and terminated in 2021 with only 1 participant enrollment. To date, no study results are available [236].

SB-913 was delivered by rAAV2/6 to insert the copy of the *IDS* gene into the albumin locus for treatment of MPS II (also known as Hunter syndrome), which is an X-linked disorder caused by mutations of the *IDS* gene encoding the iduronate-2-sulfatase enzyme, characterized by accumulation of glycosaminoglycans (GAG), dermatan sulfate, and heparan sulfate in the body, and widespread tissue damage. However, according to the latest results from the 9 subjects in phase 1/2 clinical

trials (NCT03041324), Sangamo Therapeutics has terminated the study due to the lack of observed clinical benefit and serious adverse events including pyrexia, intestinal obstruction, bronchitis, spinal stenosis [237].

SB-318 is a similar drug (also rAAV2/6) to insert the *IDUA* gene (encoding for α -L-iduronidase) for the treatment of *IDUA* mutation-induced MPS I (also known as Hurler syndrome) [238]. The corresponding clinical study (NCT02702115) was also terminated, possibly due to low transgene expression [239].

To solve this problem, the same group designed a proprietary system with CRISPR-Cas9 to insert *IDUA* to the albumin locus through HDR with *IDUA* cDNA or an alternative pathway of NHEJ-mediated whole AAV sequence (with *IDUA* cDNA) integration, which was able to work in both dividing and non-dividing cells [239]. AAV8 instead of AAV2/6 was used and showed efficacy and safety in mice, indicating a potential treatment for a clinical study.

Potential candidates

With efforts in exploring the potential gene targets, genome editing tools and delivery systems, researchers have achieved massive progress in treating monogenic diseases using *in vivo* genome editing therapy (Table 2). These results prompt us to try this therapy further in hereditary diseases of other systems, such as DMD and cystic fibrosis. DMD has attracted lots of investigations regarding genome editing therapy from the beginning. DMD is a progressive and fatal muscle-wasting monogenic disease caused by loss-of-function mutations of the X-linked *DMD* gene encoding the dystrophin protein. This essential scaffolding protein stabilized striated muscles and protected the muscles from contractile-induced damage [8]. Due to the lack of normal function of DMD in the muscle, DMD patients gradually lose the ability to walk at an early age and eventually develop into heart and respiratory failure. *DMD* is one of the largest human genes spanning 2.3 Mb with 79 exons, about 11 kb complete coding sequence, encoding for the 427 kDa protein, and over 7,000 pathogenic mutations are reported. Among them are large deletions spanning one or more exons (about 66%), exon duplications (about 11%), small deletions/insertions (10%–15%), nonsense mutations (10%–15%) [8, 240]. Most mutations lead to a shift in the reading frame that produces a truncated and dysfunctional protein without the essential C-terminal domain. Therefore, reframing or skipping one or two exons would achieve therapeutic benefit in most DMD patients, accounting for 83% as estimated [241].

Numerous *in vivo* experiments in mouse DMD models and other large animal models such as dogs and pigs have shown that NHEJ-mediated exon skipping, deletion or reframing induced by CRISPR or base editing of the splice site could restore functional dystrophin expression and achieve the therapeutic effect (reviewed in elsewhere [218, 240, 242]). However, differences in the sequences and mutations between humans and animals exist. Humanized DMD animal models are needed, providing a powerful platform for developing and verifying sequence-dependent therapies *in vivo* and helping the preclinical studies translated to the clinic. Furthermore, an efficient, safe, and muscle-specific delivery system is lacking and highly desirable. By addressing these challenges, we will see the translation of genome editing therapies for DMD.

Germline genome editing

Germline genome editing includes editing the genome of preimplantation embryos, sperm, eggs and their precursors-germ cells, including spermatogonia, spermatocytes, spermatids from males and oocytes from females (Figure 10). Unlike somatic cell editing, germline editing introduces genetic changes to the next generations, which are heritable. The last raise societal and ethical issues in human application and must be rigorously regulated. No country explicitly permits heritable human genome editing [243]. However, germline genome editing provides potential prevention of the transmission of pathogenic mutations from parent to child and treatments of infertility due to mutations that affect gamete or embryonic development [244].

Heritable genome editing is not a new concept. In many fields, heritable genome editing in embryos (zygotes) is now widely used for producing genetically modified animals, such as mice, rats, pigs, goats, rabbits, dogs, and monkeys, primarily by the CRISPR-Cas9 system as it is easy and efficient [245]. In addition, the editing of spermatogonial stem cells (SSCs) can also be applied to germline-modified animals [246]. In these strategies, mosaic Founder 0 animals require genetic screening and crossbreeding to produce stable and heritable genetically modified animals. In addition, our group and others developed novel haploid embryonic stem cells (haESC) from mice, rats, monkeys, and humans, which could be used for generating germline editing animals [245].

Regarding treatments for hereditary diseases, proof-of-concept studies in mouse models have proved the potential of germline genome editing to cure genetic

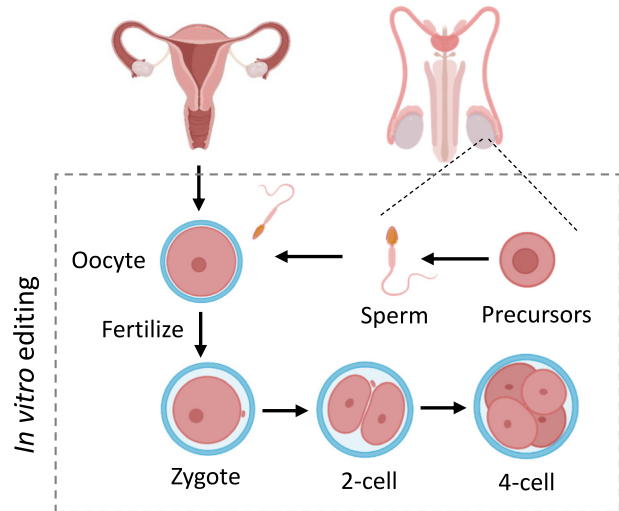


Figure 10: Germline genome editing. Editing the genome of preimplantation embryos, sperm, eggs and their precursors, including spermatogonia, spermatocytes, spermatids from males and oocytes from females.

diseases. Previously, our group co-injected the Cas9 mRNA and sgRNA targeting dominant mutation in the *Crygc* gene that caused cataracts into zygotes and corrected the pathogenic mutation via HDR based on exogenous oligo or endogenous WT allele [247]. With a similar strategy, gene correction of *Crygc* mutation in SSCs was also achieved [248]. Importantly, all the corrected alleles were stably transmitted to the offspring. Olson et al. used CRISPR-Cas9 to correct the DMD mutation in the germline of the DMD mice model and generated mosaic mice with 2%–100% correction of DMD mutations, in which the muscle phenotype was rescued [249]. Recently, Lu et al. used CRISPR-Cas9 to edit the germline of mouse embryos containing a point mutation in *Hbb* that leads to β -thalassemia [250]. Microinjection of Cas9 mRNA and sgRNA into embryos generated 70% of the born mice with corrected genotypes and significantly improved symptoms. In a mouse model of azoospermia, Wang et al. isolated the *Tex11* mutation SSCs and repaired the genetic defect with CRISPR-Cas9-mediated HDR *ex vivo*, restoring spermatogenesis after transplantation into testis [251].

So far, there have been limited studies on the germline editing of human embryos. Using CRISPR-Cas9, Liang et al. conducted editing of *HBB* in tripronuclear (3 PN) zygotes [252]. They found low efficiency of HDR and off-target cleavage in the mosaic embryos. In contrast to these results, Tang et al. demonstrated that CRISPR-Cas9-mediated HDR-based correction of point mutations in *HBB* and *G6PD* of human 2 PN zygotes was

efficient [253]. They did not find evidence of off-targeting, but mosaicism did exist. To eliminate mosaicism, Mitalipov's group co-injected a mixture of Cas9, sgRNA, DNA templates and sperm from the patient heterozygous for the *MYBPC3* mutation into metaphase II (MII) oocytes [254]. This approach avoided mosaicism and achieved over 70% of HDR-mediated repaired embryos. Interestingly, the mutation alleles were repaired using the maternal WT alleles instead of exogenous DNA templates, a process termed gene conversion [244, 254]. Others challenged these results. The main argument was that gene conversion seemed impossible because paternal and maternal pronuclei were separated in early zygotes [255]. Deletion of large fragments by CRISPR-Cas9 might remove the primer-binding site and lead to amplification of only the maternal allele [255, 256]. Mitalipov's group gave additional evidence to support the mechanism of gene conversion, and their further work demonstrated that gene conversion and NHEJ were two major repair mechanisms induced by DSB in preimplantation human embryos [257, 258]. However, Zuccaro et al. showed that the significant repair was mediated by micro homology-mediated end joining (MMEJ) but not NHEJ, resulting in frequent chromosome loss in the context of Cas9-induced DSB [105]. This fundamental question is still under debate, which challenges human germline editing.

In contrast to CRISPR-Cas9, BEs can circumvent the problem induced by DSB in human germline editing. Instead, BEs might lead the trouble with unintended mutations in the target site and base conversion close to the target site [109]. Indeed, CBE and its variants were tested in 3 PN zygotes, 2 PN zygotes, MII and 2-cell embryos in previous studies, involving genes such as *RNF2* [259], *HBB* [260, 261], *FANCF* [260], *FBNI* [262], *OCT4* [263]. Overall, CBEs achieved higher precise editing efficiencies compared to CRISPR-Cas9. Only one study reported ABE editing in 3 PN zygotes targeting multiple gene sites with high efficiency [264]. However, mosaicism and unwanted base conversion proximity to the target sites existed in almost all BE applications in human embryos.

Altogether, human germline genome editing faces many obstacles in theory and technology. The ethical issues also hinder their further applications. Besides safety concerns, informed consent is impossible for the edited generations. The edited generations might also suffer from discrimination. Gene enhancement by germline editing would lead to serious social problems. More seriously, germline editing would introduce unintended mutations, leading to perturbation of the human gene pool [265, 266]. Once the mutations were introduced, dealing with them would be quite complicated. To be

clear, it is a consensus that any germline genome editing for clinical application is forbidden and illegal for a long time in the future. Any research use must be under rigorous regulation according to national law.

Challenges and perspectives

Approximately twenty therapeutic genome editing drugs for inherited disorders are in ongoing clinical trials, as described above. Most studies' preliminary data look promising except for the *in vivo* therapy using ZFNs (Tables 1 and 2). In view of the target cells, all are aiming to edit the somatic cells, including HSCs/HSPs *ex vivo* and hepatocytes or photoreceptor cells *in vivo*. In *ex vivo* studies, therapeutic editing for SCD and TDT are pioneers. The CRISPR-Cas9-based CXT001 is the first to enter phase 3, even though the clinical applications in inherited diseases are generally behind their applications in cancer immunotherapy and treatment of HIV infection. CRISPR-Cas9 is the most used in *ex vivo* genome editing, followed by ZFNs. Emerging tools, such as CRISPR-Cas12a and ABEs, are also used. With the development of tissue-specific delivery methods, genome editing *in vivo* has also come to the clinic. Two CRISPR-Cas9 mediated editing drugs and one ABE mediated drug are reported, all targeting hepatocytes delivered by LNP for treatment of metabolic diseases. Another three ZFN-mediated editing drugs are terminated due to the lack of observed clinical benefit and serious adverse events. Human germline editing is now in the initial stage but full of difficulties for several reasons. First, our understanding of the biology of human development is limited, and the consequence of human germline editing is unforeseeable. Pioneering research has raised the fundamental theory controversy and technological problems. One of the most challenging issues is the ethical problem, such as discrimination, inequality, genetic enhancement and perturbation of the human gene pool. So human germline editing for clinical uses is prohibited and out of reach.

Our ultimate purpose for every drug or treatment is to cure disease with high safety, efficiency, and durability. The era of therapeutic genome editing in humans has already arrived and provided potential durable and curable therapy for inherited disorders, an advantage that traditional therapy is hard to achieve. However, safety and efficiency still limit their further applications. These issues exist in every process of genome editing therapy, including (i) delivery, (ii) recognition and cut, and (iii) repair. Nevertheless, we suppose that, as a rapidly

evolving technology, efforts addressing the following challenges might help their therapeutic outcomes in patients with inherited diseases.

Highly accurate, specific, efficient delivery vehicles are needed

It is more urgent for *in vivo* deliver than *ex vivo* since electroporation is very efficient, simple, and safe for *ex vivo* deliver, which is widely used in the clinic. In contrast, for *in vivo* use, viral delivery systems, especially AAVs, have high expectations because of their efficiency and tissue tropism. As mentioned above, the packaging capacity limits carrying all the editing components into a single AAV and further limits the scope of AAVs. The split-intein dual-AAV strategy has been reported to be useful, but an increasing dose of AAV is required. Another strategy is developing smaller genome editing tools, such as Cas9 ortholog from SaCas9, CjCas9, and Nme2Cas9, SauriCas9. Indeed, the ongoing clinical trial regarding treating LCA used SaCas9 and two sgRNAs packing into a single AAV [228]. AAVs have a prolonged expression of the editing components that might increase the risks of being off-target or trigger an immune response. Spatially or temporally regulating the expression of AAV-delivered editor offers helpful strategies [267]. As a non-viral delivery system, LNPs show advantages for liver delivery with lower immunogenicity, off-target, and toxicity. But LNP-mediated delivery for other organs remains challenging. The physical (changing the charge), chemical (changing the compositions), or biological (conjugating with antibody) modifications of LNPs provide help for targeting non-liver organs. Virus-like particles (VLPs) are emerging delivery vehicles usually assembled by non-infectious retroviral proteins and mRNA or RNP cargos, enabling transiently delivery of the mRNA or RNP into target cells [268]. Liu et al. anticipated that RNP-packaging VLPs would offer the shortest exposure to editors and, therefore, the lowest off-target and would be very popular in future applications [267]. The ability for delivery to different organs and feasibility in pre-clinical studies are also needed.

Highly precise, specific, efficient enzymes are obligatory

Even though various nucleases, nickases, and delivery vehicles are available, none are perfect. ZFNs and

TALENs both tolerate a small number of positional mismatches, but CRISPR-Cas9 tolerates multiple consecutive mismatches, which largely compromise their specificities. Engineering Cas variants and sgRNAs are hopeful of generating editors with higher specificity. Three strategies are reported, including (i) paired Cas9 nickases with two sgRNAs or fused chimaeras with one inactivated Cas9 and one attenuated Cas9; (ii) alanine scanning for screening mutants with precise and efficient genome editing in human cells such as eSpCas9; (iii) engineered sgRNAs such as truncated sgRNAs or hp-sgRNAs [15]. BEs also have bystander editing, transversion mutations, indels at the target nucleotide, and undesired editing at the off-target DNA, impairing their accuracy and specificity. The off-targets are divided into Cas-dependent and Cas-independent [269, 270]. Anyway, unwanted editing at the target and off-target sites should be avoided and waiting for optimization in clinical use.

Repair: expand the scope of applications

In principle, genome editing can correct any mutation for therapy purposes. There are so many types incurable inherited diseases. However, clinical testing therapeutic editing for inherited diseases is no more than ten, accounting for a tiny percentage. In addition to the limitations of delivery vehicles and enzymes we have discussed above, the repairing mechanism is one of the most challenging issues. As we can see, most ongoing clinical trials use the NHEJ-mediated repairing mechanism, indicating that they employ it to induce another “mutation” to cure the pathogenic mutation. Indeed, the precise correction of the pathogenic mutation relies on HDR-mediated repairing, a process that is active in only dividing cells. Moreover, NHEJ is generally more active than HDR [271]. These features limit gene correction in most human cells, such as muscle cells, nerve cells, etc. To expand the therapeutic scope, improvement of donor templates, co-localization of templates with nick, synchronization of the cell cycle, and suppression of NHEJ activity were harnessed to promote the HDR but were not as effective as expected [15, 272]. BEs provided another repairing mechanism without DSBs. Expanding the scope of applications of BEs will be helpful, and developing Cas orthologs or variants recognizing alternative PAMs for targeting a broader region of the genome could achieve this goal [15]. The new finding CAST systems also provided a gene correction technology by a mechanism independent of HDR or base

conversion. They potentially expanded the scope of therapy for inherited diseases after the system was fully developed.

In conclusion, site-specific genome editing systems showed up in clinical trials for several inherited disorders in the last five years. The performances of these systems are exciting, inspiring, and promising, especially the CRISPR-Cas and BEs. Certainly, each of the technologies has its limitations and drawbacks. However, as highly anticipated technologies, they are quick evolving and continuously optimized. With the continuous improvement of delivery vehicles, enzymes, repair pathways in their accuracy, specificity, efficiency, the scope of their applications in treatments of inherited diseases will expand. We anticipate that more clinical trials will be carried out in the next five years, and patients with inherited disorders will benefit from them in the near future.

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