EngMedicine 1 (2024) 100035

Contents lists available at ScienceDirect

EngMedicine

journal homepage: www.sciencedirect.com/journal/engmedicine

Review

Engineering CRISPR for emerging therapeutic applications

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ARTICLE INFO

Keywords:
CRISPR
Base editing
Prime editing
CRISPR-directed integrase
Epigenetic editing
RNA editing

ABSTRACT

Clustered regularly interspaced short palindromic repeats (CRISPRs) were originally identified in bacterial and archaeal genomes as an adaptive immune response against invading viruses and phages. The discovery of the ability of CRISPR–Cas9 to cut targeted DNA sequences at predetermined sites has introduced a new era of precision and flexibility in genome editing. Building on this classic CRISPR–Cas system, the discovery of other diverse CRISPR systems and their further engineering have produced various powerful tools for gene editing and modulation at the genomic, chromatin, and RNA levels. This review summarizes the fundamental knowledge regarding CRISPR–Cas systems and explores the engineering strategies of these versatile systems for novel therapeutic applications. We discuss the evolution from classic CRISPR editing (CRISPR 1.0) to advanced methodologies such as base editing, prime editing, CRISPR-directed integrases, epigenetic editing, and RNA editing. This review focuses on the related principles, therapeutic applications, challenges, and future outlooks.

Introduction

Clustered regularly interspaced short palindromic repeats (CRISPRs) were initially identified as mysterious repetitive sequences in bacterial genomes in the 1980s. However, their true function remained elusive until the early 21st century [1–3]. The pioneering work of Mojica et al. in the early 2000s elucidated the role of CRISPR in adaptive immunity in bacteria and Archaea and laid the foundation for this field [4–6]. In 2012, this system was first harnessed for genome editing, demonstrating that CRISPR–Cas9 can be programmed to target and cut any specific DNA sequence at a predetermined site [7]. This discovery was transformative, earning Doudna and Charpentier the Nobel Prize in Chemistry in 2020 [8]. Their work, along with that of Feng Zhang and George Church, who independently demonstrated CRISPR–Cas9's genome editing capability in human and mouse cells, revolutionized genetic engineering by introducing a precise, easy-to-use, and versatile tool for genome manipulation [9,10].

The evolution of CRISPR technology has been rapid and multifaceted. The first generation of CRISPR tools, based on double-strand breaks (DSBs) and endogenous DNA repair, was soon followed by more sophisticated techniques such as base editing and prime editing. Base editing, developed by David Liu's group, enables the direct conversion of single DNA bases, circumventing DSBs and related genome toxicities [11,12]. Prime editing, another innovation by Liu et al., further refines genome editing, allowing for precise small insertions, deletions, and all possible point mutations [13]. The integration of CRISPR-directed integrases introduced new possibilities for inserting long DNA sequences [14]. CRISPR-based epigenetic editing, which makes reversible modifications at a specific gene locus, can activate or suppress gene expression without DNA sequence changes or breaks, expanding the scope of CRISPR technology beyond DNA editing to gene regulation without altering the genetic code [15,16]. CRISPR-based RNA editing is an emerging technique that can target and edit RNA transcripts to modulate protein expression or induce mutations without altering DNA sequences [17,18].

Since its discovery, CRISPR has been considered a powerful tool for disease treatment. On December 8, 2023, the U.S. Food and Drug Administration (FDA) approved the first CRISPR-based treatment, CASGEVY™ (also known as exagamglogene autotemcel or exa-cel) for sickle cell disease (SCD). This therapy employs autologous CD34+ hematopoietic stem cells that have been edited at the BCL11A gene site using CRISPR/Cas9. This eliminates the repression of fetal hemoglobin (HbF) production, which is a form of hemoglobin that predominates before birth and is postnatally replaced by adult hemoglobin [19,20].

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https://doi.org/10.1016/j.engmed.2024.100035
Received 17 February 2024; Received in revised form 17 April 2024; Accepted 28 April 2024

CASGEVY™ can increase HbF and total hemoglobin levels, restore the normal morphology of red blood cells, and alleviate the symptoms of sickle cell anemia following a single administration [21]. The landmark approval of CRISPR therapy marks a significant advancement and will catalyze the development of CRISPR-based treatments. Furthermore, engineering CRISPR for precise and versatile genome editing or gene modulation without DSBs is expexted to unlock tremendous new therapeutic opportunities, ranging from the treatment of rare genetic diseases to the prevention of severe chronic diseases [14,16,22–24]. The diversification of CRISPR applications underscores its versatility and highlights its potential as a cornerstone technology for gene therapy (Fig. 1).

CRISPR–Cas systems

CRISPR–Cas systems were originally adaptive immunity systems in Archaea and bacteria [4–6], and have been adopted for genome editing and engineering. The discovery of various CRISPR–Cas systems and mechanistic studies of these systems through comparative genomics, structural biology, and advanced biochemical approaches have greatly elucidated CRISPR functionality [2]. This understanding, forged with the creative design and engineering of the CRISPR machinery, has led to the development of various CRISPR-based genome-editing tools [7–9,11,25].

Diversity in CRISPR systems

The role of the CRISPR–Cas system as an adaptive immune mechanism in prokaryotes was experimentally validated in 2007 in *Streptococcus thermophilus* [26]. In this study, phage sequences in bacterial CRISPR spacers conferred resistance to the corresponding phage; however, this resistance was lost when the matching protospacer was removed. Further studies have demonstrated that CRISPR–Cas can restrict plasmid transformation using sequences that match CRISPR spacers [27]. In 2011, the functionality of the *S. thermophilus* CRISPR–Cas system was replicated in *Escherichia coli*, providing heterologous protection against plasmid transformation and phage infections [28]. This study emphasized the crucial role of Cas9 in CRISPR-mediated interference. Since 2013, the purified Cas9-CRISPR-guide RNA (gRNA) complex has demonstrated its capability to cleave target DNA in vitro, paving the way for its application in the genome editing of various cells, including human nerve cells and mouse kidney cells [7,9,10].

To date, highly diverse CRISPR systems have been identified. They can be categorized into two classes (1 and 2) and six types (I–VI) [29]. Class 1 systems, found in both bacteria and Archaea, constitute 90 % of the identified CRISPR systems and involve a complex of multiple Cas proteins, including types I, III, and IV. By contrast, class 2 systems, which represent the remaining 10 % and are exclusive to bacteria, utilize a single multidomain effector protein and encompass types II, V, and VI. The simpler architecture of class 2 effector complexes has garnered significant interest in the development of genome editing technologies [7,30,31], such as Cas9 (type II) [7], Cas12 (type V) [32], and Cas13 (type VI) [18].

CRISPR–Cas9 systems

Cas9 is the most extensively used multidomain effector protein. *Streptococcus pyogenes* Cas9 (SpCas9), the prevalent form of Cas9, is composed of 1368 amino acids and features two primary lobes: the recognition (REC) and nuclease (NUC) lobes [33]. The NUC lobe has two critical domains: the RuvC and HNH nuclease domains. The RuvC domain targets the non-complementary DNA strands (non-target DNA strands), mirroring the protospacer sequence, whereas the HNH domain cleaves the complementary DNA strands (target DNA strands) aligned with the CRISPR RNA (crRNA) sequence (Fig. 2). Together, these domains act at precise locations on the target DNA to generate blunt ends [33].

The functionality of the type II CRISPR–Cas system, which includes Cas9, is contingent on two RNA components as follows: crRNA and transactivating crRNA (tracrRNA) [10]. Each crRNA contains a 20-nucleotide guide sequence derived from a CRISPR spacer at its 5′ end, which pairs with corresponding sequences in the target DNA, and a 19–22 nucleotide repeat-derived sequence at its 3′ end that hybridizes with tracrRNA. crRNAs encoded within the CRISPR array of the host genome are initially transcribed into long pre-crRNAs comprising multiple crRNA units. This pre-crRNA is processed with tracrRNAs by RNase III into multiple distinct crRNA–tracrRNA complexes. Each crRNA–tracrRNA complex is associated with the Cas9 protein, guiding it to the target DNA sequences [34]. With further RNA engineering, the use of a single guide RNA (sgRNA), which fuses crRNA and tracrRNA, has been instrumental in simplifying genome editing processes. sgRNA enhances the precision and efficiency of Cas9 by facilitating the recognition of specific target sequences [7].

The Cas9–crRNA complex requires a protospacer-adjacent motif (PAM) to engage its target DNA sequence. Located on non-targeted DNA strands, PAM is recognized by Cas9's PAM-interacting domain [35]. This interaction is crucial because it initiates DNA unwinding and subsequent base pairing between the crRNA (or sgRNA) and target DNA [36]. The specificity and efficiency of Cas9-mediated DNA targeting and cleavage are significantly influenced by the PAM sequence. Cas9 variants derived from various species recognize specific PAM sequences that are typically rich in guanine (G) bases. The necessity of a PAM sequence enhances targeting specificity while concurrently limiting the selection of potential genomic sites for editing [33].

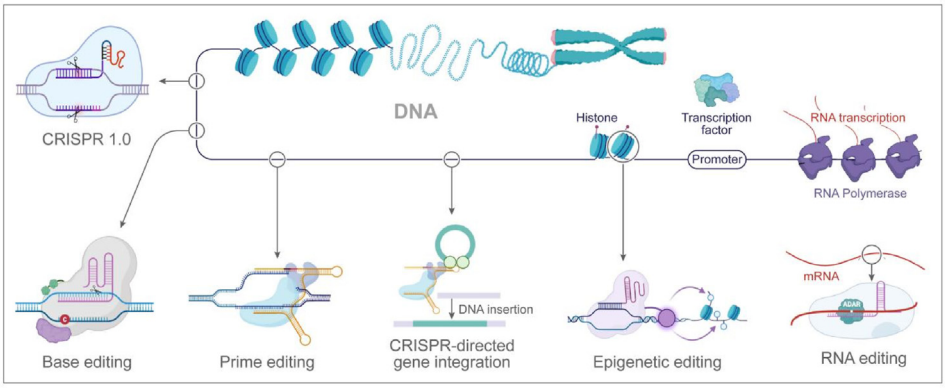

Fig. 1. Engineering clustered regularly interspaced short palindromic repeats (CRISPR) for emerging therapeutic applications. Engineered CRISPR technologies unlock various gene manipulation applications, including double-strand break-based classic CRISPR–Cas systems for gene knockout/knock-in (defined as CRISPR 1.0), base editing, prime editing, CRISPR-directed integrases, epigenetic editing, and RNA editing. Created with BioRender.com.

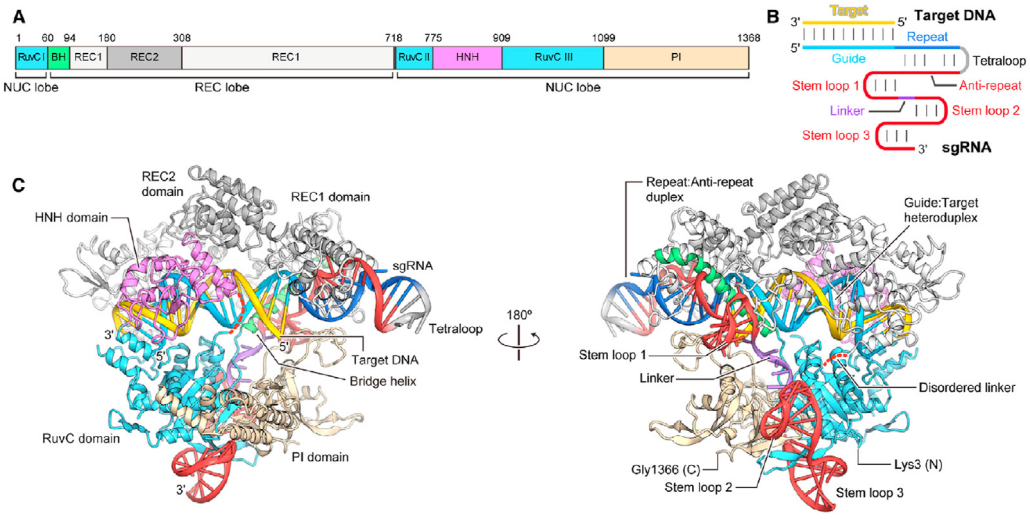

Fig. 2. Architecture of the *Streptococcus pyogenes* Cas9 (SpCas9)–single guide RNA (sgRNA)–DNA complex. A) Illustration of the domain structure of SpCas9. B) Binding of the sgRNA to its target DNA sequence. C) Ribbon structure of SpCas9–sgRNA–DNA complex. Adopted from Ref. [33].

CRISPR–Cas12 systems

Cas12 systems and type V CRISPR–Cas systems have broadened the scope of CRISPR-based gene-editing tools. Despite their high diversity [37], the Cas12 family exhibits unique features that distinguish them from the Cas9 systems. Lacking the HNH domain found in Cas9, Cas12 nucleases possess only a RuvC nuclease domain and recognize thymine-rich (T-rich) PAM sequences located 5′ upstream of the target region on the non-target DNA strand [30,37]. This differs from the guanine-rich (G-rich) PAM sequences recognized by Cas9 on the 3′ side of the target DNA. Furthermore, Cas12 enzymes create staggered DSBs distal to the PAM sequence compared with the blunt-end cuts produced by Cas9 [32]. Herein, we used Cas12a (formerly known as Cpf1) as an illustrative example of a type V system [32].

Cas12a has a molecular architecture comprising approximately 1200–1500 amino acids. Similar to Cas9, Cas12a has REC and NUC lobes. However, Cas12a recognizes the PAM sequence "TTN," although variations such as *Acidaminococcus* sp. Cas12a can recognize "TTTN" [32,38,39]. Cas12a enzymes do not require tracrRNA for the maturation of crRNA and can effectively target and cleave DNA using crRNA alone [38]. Upon binding to the target DNA, the Cas12a–crRNA complex induces nicks in the DNA strands, resulting in sticky end-like DSBs with four or five nucleotide overhangs. In addition to cis-cleavage of the target DNA, Cas12a is known for its trans-cleavage activity on non-target DNA, a process that occurs independently of PAM recognition and specific target sequences, and has been leveraged to design tools for nucleic acid detection [40].

CRISPR–Cas13 systems

Cas13 belongs to the type VI CRISPR–Cas system. Cas13 proteins specialize in cleaving single-stranded RNA (ssRNA) instead of DNA because they lack a DNA catalytic domain. Structural analyses of Cas13a revealed two conserved higher eukaryote and prokaryote nucleotide-binding (HEPN) domains, each harboring an RNA cleavage site. CRISPR–Cas13 system consists of a Cas13 effector protein and a CRISPR RNA (crRNA), which acts as the sole guide RNA and recognizes target RNA sequences [41]. Unlike the "PAM" requirements for Cas9 and Cas12, a protospacer-flanking site is needed for the RNA-targeted processing for CRISPR–Cas13 systems. Another feature of the Cas13 system is collateral activity, which refers to the nonspecific degradation of RNAs by nonspecific RNase activity after Cas13 recognizes its target RNA. This phenomenon induces infection-triggered cell death to protect against viral spread among bacteria. The collateral activity of Cas13 depends on HEPN1 domain activation, which is triggered by the binding of the target RNA to the REC1 and REC2 domains of Cas13, forming a crRNA-guided RNA recognition module [30,41].

The collateral cleavage activity of Cas13 is variant-dependent and can be modulated. For example, selecting a Cas13 variant with low collateral activity, such as PspCas13b, can minimize the off-target effects of Cas13 by increasing the specificity and fidelity of target RNA binding and cleavage [42], which can be used for RNA editing [43]. Cox et al. have revealed that fusing the catalytically inactive PspCas13b to the ADAR2 deaminase domain ($ADAR2_{DD}$) generated an A-to-I RNA editor for programmable RNA editing and demonstrated high specificity for target sequences [18].

Engineering CRISPR for therapeutic applications

Since the first application of CRISPR in genomic editing in 2013, it has emerged as a powerful tool for therapeutic development [7,9,10]. The approval of the first CRISPR-based therapeutic, CASGEVY™, will further catalyze the development of CRISPR-based treatments [44].

CRISPR 1.0

The generation of site-specific DSBs is a fundamental mechanism leveraged by CRISPR–Cas editing technologies to manipulate the genome. The advent of RNA-guided genome editing systems using CRISPR–Cas proteins has significantly advanced DSB-based genome editing [44]. DSBs induced by DNA nucleases are predominantly repaired via two mechanisms: error-prone end-joining pathways and error-free homology-directed repair (HDR) [45,46]. End-joining, including non-homologous end-joining (NHEJ) and microhomology-mediated end-joining (MMEJ), directly ligates broken DNA ends, potentially resulting in insertion or deletion (indel) mutations [47]. In contrast, HDR employs homologous sequences from sister chromatids or exogenous DNA templates to facilitate the integration of desired sequences into precise genomic locations [45].

DSBs resulting from Cas9-induced DSBs are predominantly repaired by NHEJ-mediated single-base insertions and deletions, as well as MMEJ-driven deletions [46]. The mutagenic nature of end-joining has been utilized to disrupt genes to treat diseases caused by pathogenic mutations, showing promise in both clinical and pre-clinical settings. This approach has been demonstrated in trials aimed at inactivating BCL11A, a repressor of HbF and γ-globin, in hematopoietic stem and progenitor cells to treat SCD and β-thalassemia [21] (Fig. 3). In vivo disruption of

transthyretin using end-joining approaches has shown potential for the treatment of transthyretin amyloidosis [21]. Furthermore, dual-DSBs strategy targeting the CEP290 gene's aberrant splicing site has also revealed meaningful vision improvement in clinical trials for Leber congenital amaurosis [48,49]. Similarly, dual DSBs have successfully removed mutant exons and thus restored the open reading frame of the dystrophin gene in Duchenne muscular dystrophy (DMD) cells and animal models [50]. This approach has been extended to eliminate pathogenic trinucleotide repeats in models of Huntington's disease [51] and fragile X syndrome [52]. Moreover, the predictability of MMEJ-mediated DSB repair outcomes has been harnessed to correct microduplications that cause limb–girdle muscular dystrophies and Hermansky–Pudlak syndrome [53,54]. Machine learning-based models for predicting the results of end-joining repair could enhance the precision of such genome-editing interventions [46,55].

HDR is particularly useful for precise genome editing owing to its ability to introduce functional gene knock-ins. The therapeutic potential of HDR has been underscored by the successful enhancement of the antitumor activity in T cells by knock in chimeric antigen receptor gene via HDR [56]. However, compared with the more common error-prone end-joining repair processes, the infrequent occurrence and low efficiency of HDR pose a substantial challenge for the direct application of HDR in CRISPR-based in vivo gene therapy [45,46]. This highlights the need for further research and development to enhance the efficiency and reliability of HDR techniques for therapeutic purposes.

Base editing

Base editing allows the direct conversion of one base pair to another without requiring DSBs or donor templates [11,12]. Briefly, base editors consist of a deaminase enzyme tethered to Cas9 nickase (or other Cas nickases) through a flexible linker that can convert either cytosines to uracils (cytosine base editor, CBE) or adenines to inosines (adenine base editor, ABE). Cas9 nickase is guided by a gRNA to the target site, where it binds and unwinds the DNA, exposing the ssDNA of the non-complementary strand at the PAM-distal region [57]. The deaminase then accesses and modifies the target bases in the ssDNA window. The resulting U:G or I:C mismatches are then resolved by cellular DNA repair machinery, leading to permanent C:G to T:A and A:T to G:C transitions. A wide range of single-nucleotide substitutions can be generated using different gRNAs and base editor variants, covering many pathogenic point mutations that cause genetic diseases in humans [58].

Protein engineering and the directed revolution have advanced base-editing technology. The first DNA base editor (CBE1) is based on a catalytically deactivated Cas protein (dCas) fused with rat APOBEC1 as the deaminase enzyme, which can edit cytosines within a 5-nucleotide window (positions 4 to 8) of the PAM site [11]. CBEs have been enhanced through the integration of a uracil glycosylase inhibitor (UGI) peptide and the utilization of Cas nickase. The UGI inhibits the cellular DNA repair machinery from removing the uridine intermediate, thus prolonging its presence at the target site and enhancing the efficiency and

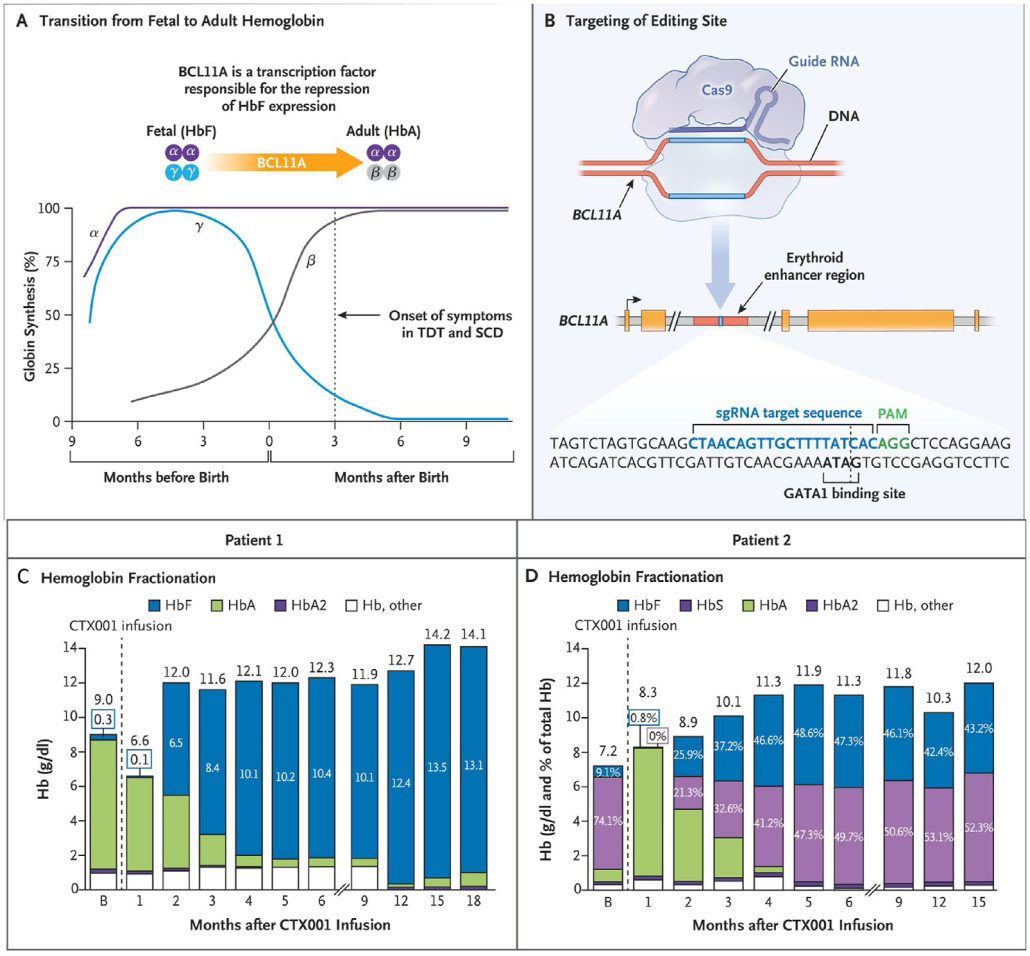

Fig. 3. Therapeutic effects of CASGEVY™ (previously exagamglogene autotemcel [exa-cel], CTX001). A, B) Schematic mechanism. C) Representative therapeutic effect in a patient with transfusion-dependent β-thalassemia. D) Representative therapeutic effect in a patient with sickle cell disease. Adopted from Ref. [21].

fidelity of the edit [25]. The Cas nickase introduces a break into the unedited DNA strand, prompting repair machinery to use the deaminated strand as a template. Deaminating one strand and resynthesizing its complement solidifies the alteration of the target base pair. Further optimizations of the base editors include the refinement of linker components, the addition of a second UGI domain, and optimization of the nuclear localization sequence (NLS) and expression [59]. Simultaneously, ABE development involves the fusion of adenosine deaminase (ADAR) with Cas nickase, as demonstrated by Liu et al., in 2017 [12]. Briefly, adenosine (A) was deaminated using ABE to form inosine (I). DNA polymerases read inosine (I) as G during the DNA repair process, resulting in the replacement of the original A:T at the target site with G:C (Fig. 4). ABEs distinguish themselves by superior precision in editing, resulting in no conversion of A:T to non-G:C and minimal occurrence of indels, and have advanced rapidly towards therapeutic applications [57].

Several studies have demonstrated the therapeutic potential of base editing for correcting disease-causing mutations both in vitro and in vivo. For example, base editing has been used to correct mutations associated with β-thalassemia [60], SCD [61], tyrosinemia [62], cystic fibrosis [63], DMD [64], and congenital immunodeficiencies [65]. Moreover, base editing has been used to introduce beneficial mutations or to disrupt genes for therapeutic purposes, such as enhancing blood coagulation factor IX levels in hemophilia B [66], reducing cholesterol levels by disrupting PCSK9 in vivo [67], and conferring HIV resistance by mutating CCR5 in human CD4+ T cells [68]. Overall, base editors represent a novel strategy for genome editing in disease treatment.

Prime editing

Prime editors have emerged as a new generation of gene editors that are versatile in inducing precise small insertions, deletions, and all possible point mutations (C:G to A:T, C:G to G:C, C:G to T:A, T:A to A:T, T:A to G:C, and T:A to C:G) in the absence of DSBs. Prime editing is based on the fusion of Cas9 nickase with a reverse transcriptase (RT) and utilizes a specially designed pegRNA. The pegRNA consists of a spacer sequence that hybridizes to the target strand, a Cas9-binding scaffold sequence, an RT template encoding the desired modification, and a primer binding site (PBS). The pegRNA guides Cas9 nickase to the target site and provides the RT with a template to encode a desirable sequence into the genomic DNA.

The mechanism of action for prime editing is more complex and multi-step compared to the aforementioned Cas editing strategies and can be divided into several discrete steps. First, the Cas9 nickase introduces a single-strand break in the non-target strand, freeing a single-stranded DNA segment at the 3′ end (3′-ssDNA). Next, the 3′-ssDNA pairs with a PBS of pegRNA guide, enabling the RT to elongate the exposed 3′-ssDNA based on the pegRNA template containing the desired edits. This extension produces two overlapping DNA segments: an unmodified 5′ flap and a modified 3′ flap. The unmodified 5′ flap is selectively removed by endogenous structure-specific endonucleases, allowing the modified 3′ flap, which carries the desired edits, to anneal with the target DNA strand. This process forms a heteroduplex—a DNA double helix with one edited and one unedited strand. Finally, the cell's inherent DNA mismatch repair (MMR) machinery recognizes this heteroduplex and uses the information on the edited strand to correct the unedited strand, thus rendering the edit permanent.

Prime editing can achieve versatile gene corrections and low off-target activity in non-dividing or poorly dividing cells, which represents a significant advancement in genome-editing technology. Since the first report on prime editing, its performance and versatility have been enhanced. The original prime editor (PE1) consisted of wild-type Moloney murine leukemia virus RT (MMLV RT) attached to a Cas9 nickase, which only achieved a modest base substitution efficiency of 0.7–5.5 % [13]. Anzalone et al. improved PE1 by engineering MMLV RT with specific mutations to increase its thermostability and binding efficiency, resulting in PE2 (Fig. 5). PE3 includes the mutated M-MLV RT of PE2 and a second sgRNA that nicks the non-edited strand and further increases the editing efficiency by favoring the repair of non-edited strands based on the edited strands [13]. Prime editing efficacy is highly limited by the MMR process for installing desirable edits. The manipulation of the MMR cellular repair mechanism has generated major improvements in prime editors, resulting in PE4 and PE5 [69] (Fig. 5). They inhibited key MMR factors, such as MLH1 and MSH2, using an engineered MMR-inhibiting protein (MLH1dn), which significantly improved prime editing outcomes in various cell types. PE4, which transiently expresses MLH1dn with PE2 (PE2+MLH1dn), increases the editing rate by 7.7-fold. Meanwhile, PE5, which transiently expresses MLH1dn with PE3 (PE3+MLH1dn), increases the editing rate two-fold compared with PE3.

Prime editing has shown great potential for therapeutic applications. For example, prime editing has been used to correct mutations associated

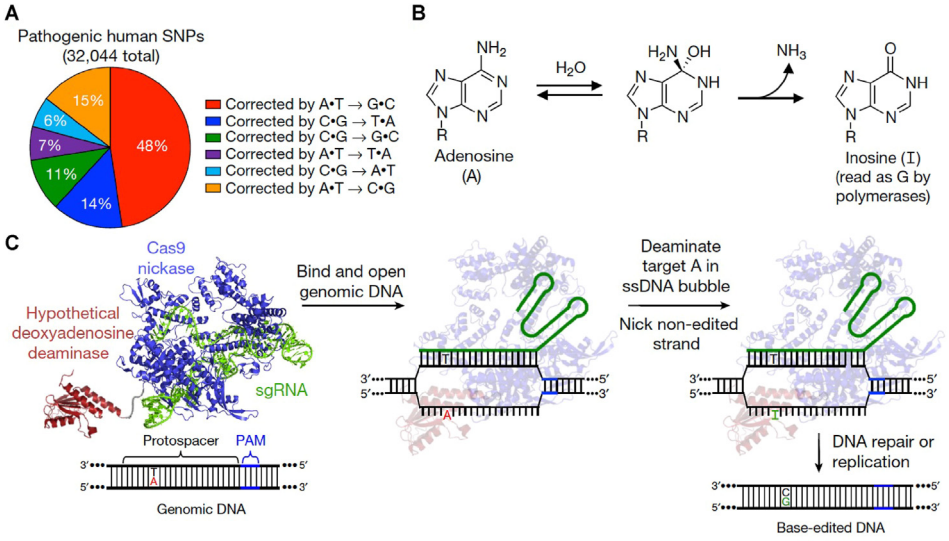

Fig. 4. Adenosine base editors (ABE) convert A:T to G:C at the target site. A) A:T to G:C conversations have promising therapeutic applications for pathogenic human SNPs. B, C) Mechanism of ABE-mediated A:T to G:C base editing strategy. Adopted from Ref. [12].

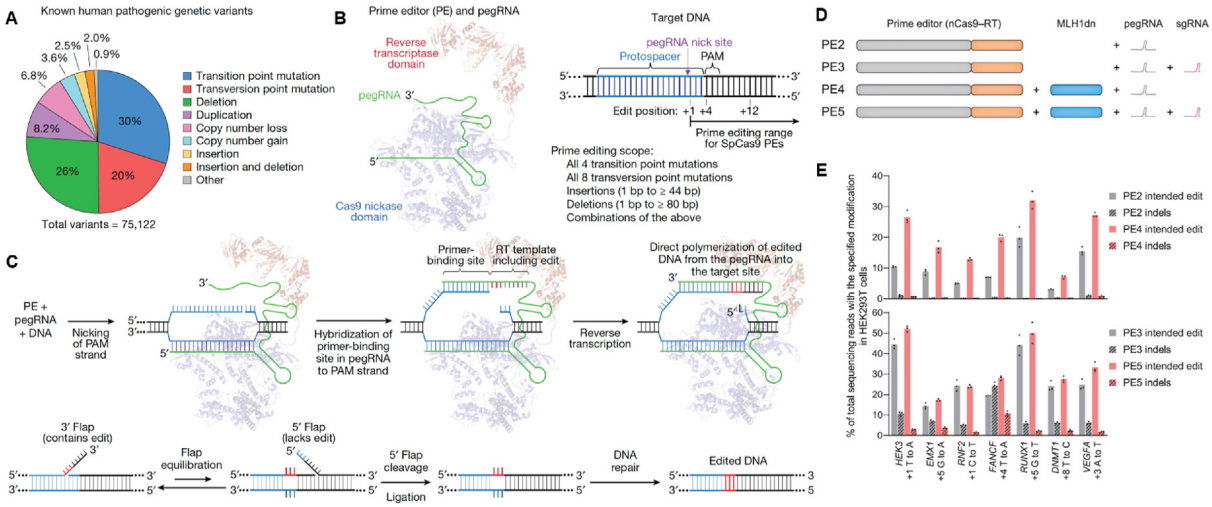

Fig. 5. Prime editing overview. A) Prime editing has promising therapeutic applications for human pathogenic genetic disease by inducing point mutations, small insertions, and small deletions. B, C) Schematic mechanism of prime editing (PE2). D, E) Engineering and iteration of prime editing and comparison of editing efficacy of PE2, PE3, PE4, and PE5 in mammalian cells. Adopted from Ref. [13,68].

with SCD [23], phenylketonuria [70], α1-antitrypsin deficiency, hereditary tyrosinemia [71], and Leber congenital amaurosis [71]. Moreover, prime editing has been used to introduce beneficial mutations or disrupt genes for therapeutic purposes [72–75]. However, prime editing also faces challenges and limitations that must be addressed before clinical translation. A major challenge is the delivery of prime editors, which are composed of large proteins and RNA components, to target tissues and cells, particularly for in vivo applications. Another challenge is the optimization of prime editor components such as Cas9 variants, RT variants, and pegRNA designs to improve editing efficiency, specificity, and fidelity.

CRISPR-directed gene integration

Despite progress in CRISPR, inserting long DNA sequences into the genome remains challenging, especially for in vivo therapeutic gene editing and the editing of non-dividing cells. Engineered CRISPR machinery, in theory, can direct transposases or integrases to specific gene sites and mediate precise and efficient integration without DSBs and HDR. Therefore, CRISPR-directed gene integration could potentially overcome a few of the limitations of classic CRISPR–Cas9-mediated HDR, including low HDR efficiency, high indel formation, and off-target effects.

CRISPR-associated transposases are naturally present in bacterial cells. These systems, that combine CRISPR RNA array elements, Cas genes lacking nuclease activity, and transposase genes, have been discovered through computational analysis. They employ Cas domains that lack nuclease activity to target specific genomic loci and facilitate DNA integration using transposase modules. For instance, the CRISPR-associated transposase (CAST), a Cas12k–Tn7 CRISPR-associated transposase system, enables genomic insertion in *E. coli* [76]. Catalytically inactive Cas12K binds to the DNA and generates an R-loop. Tn7 facilitates the insertion of the cargo gene downstream of PAM. CAST from the cyanobacterium *Scytonema hofmanni* has been utilized to insert large DNA sequences into specific *E. coli* genome locations. Efforts have been made to extend this technology to genome editing in mammalian cells, albeit with lower efficiency, indicating a need for further improvements.

The development and advancement of prime editing have opened a new avenue for CRISPR-directed gene integration. Yarnall et al. developed a technology called Programmable Addition via Site-specific Targeting Elements (PASTE), which builds on the prime editor and adds recombinases serine integrases, catalyzing the integration of large foreign DNA cargo (Fig. 6). This system, which incorporates Cas9 nickase, RT, pegRNA, DNA cargo, and large serine integrases, enables programmable cargo integration of up to 36 kb, achieving efficiencies of 50–60 % in cell lines and 4–5% in primary human hepatocytes and T cells. PASTE was further refined to enable orthogonal integration, allowing the simultaneous insertion of three distinct genes at three separate genetic loci. Additionally, it has been engineered for sequence replacement, facilitating simultaneous deletion and insertion of sequences using paired guide RNAs. Comparative genome-wide sequencing has demonstrated the superior specificity and insertion purity of PASTE over traditional methods, such as homology-independent targeted integration and HDR. When evaluated against other prime-editing-based insertion techniques, PASTE showed an 8.3- to 42.1-fold increase in integration efficiency at the three specific endogenous sites. In terms of therapeutic applications, PASTE is compatible with a wide range of viral and non-viral DNA templates. The authors also extended the application of PASTE to in vivo studies, demonstrating programmable gene insertion into the mouse liver. PASTE offers easy retargeting of different genes, requires only a single plasmid dose for delivery, and is effective in both non-dividing and primary cells. This development paves the way for the site-specific multiplexed insertion of large genes without DNA repair pathways, which has significant implications for biomedical research and the development of therapies.

Epigenetic editing

Beyond the genomic DNA sequence, chromatin accessibility and DNA and histone modifications are important for controlling gene activation or repression. DNA can be modified at specific sites, such as the 5-carbon methylation of cytosine residues (5-mC) [77]. Meanwhile, histones can be modified at lysine residues, such as K4, K9, or K27 of H3, with methyl, acetyl, and other chemical groups [78]. These chromatin marks dynamically regulate gene activation or repression, essential for cellular function and operation [77,78]. The development of CRISPR technology has provided new opportunities for epigenetic editing or engineering by directing epigenetic modifiers to adjusting gene expression [15]. This is achieved by fusing dCas9 or dCas12a with epigenetic modifiers such as histone acetyltransferases (HATs), histone deacetylases (HDACs), DNA methyltransferases (DNMTs), and transcriptional activators or repressors and directing them to specific genomic loci via gRNAs. Epigenetic editing has several advantages over conventional CRISPR–Cas9 genome editing, including no genome sequence changes or breaks, reversible modifications, and fewer ethical concerns [79,80].

Various types of epigenetic editing have been reported for different chromatin markers (Table 1). For instance, CRISPR-DNMT editors use dCas fused with DNMTs such as DNMT3A or DNMT3L to increase DNA

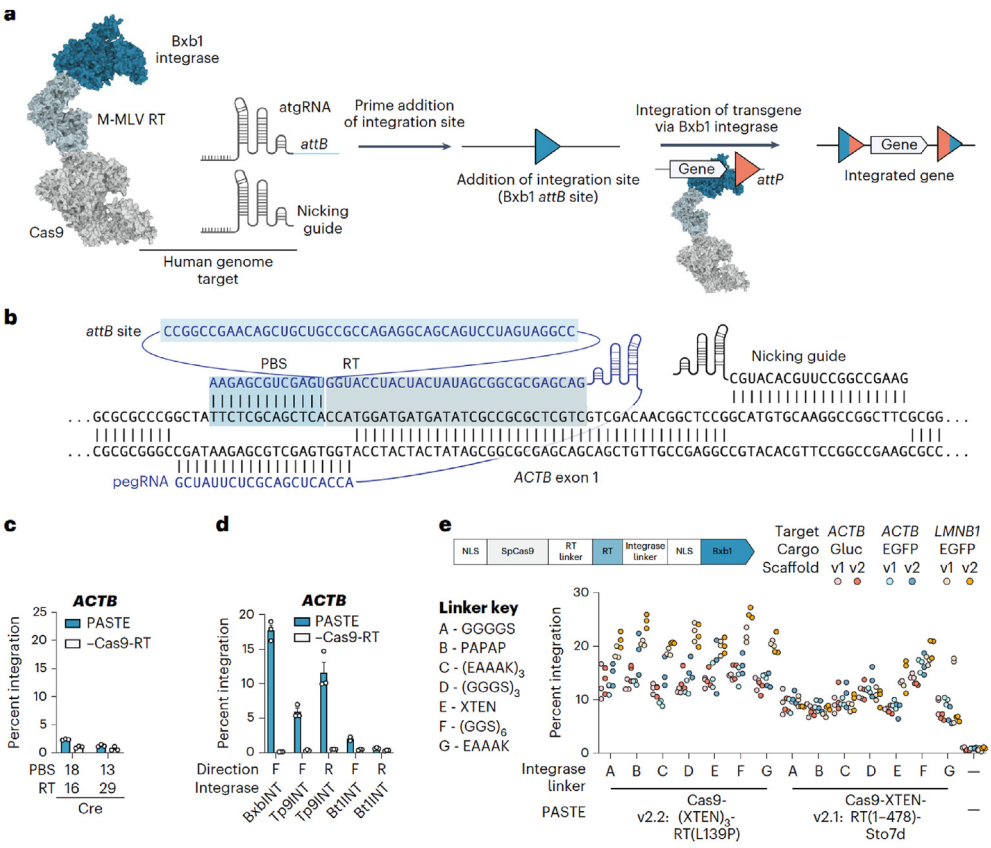

Fig. 6. Overview of programmable addition via site-specific targeting elements (PASTE) editing technology. A, B) Schematic mechanism of PASTE editing. C–E) Targeted integration and optimization of PASTE editing. Adopted from Ref. [14].

methylation and gene silencing at the target loci [81–83]. CRISPR-TET editors use dCas fused with TET enzymes such as TET1 to decrease DNA methylation and gene activation at the target loci [81,84,85]. CRISPR-HAT editors use dCas fused with HATs such as p300 or CBP to increase histone acetylation and upregulate gene expression at the target loci [79,86]. Conversely, CRISPR-HDAC editors use dCas fused with HDACs such as HDAC3 to decrease histone acetylation and gene expression at the target loci [16,87]. To regulate gene activity, CRISPR-DNMT editors target methylation at H3K4 for upregulation and at H3K9 and H3K27 for downregulation, using enzymes such as PRDM9, LSD1, and EZH2 [88,89]. CRISPR activators integrate dCas with transcriptional activators, such as VP64 or VPR, enhancing transcription factor recruitment and gene expression [90,91], whereas CRISPR repressors use dCas fused with repressors, such as KRAB, to decrease gene expression by recruiting co-repressors and histone modifiers [15,92].

Epigenetic editing holds great promise for various therapeutic areas such as cancer, inflammation, metabolic disorders, and neurological diseases [16, 100,101]. For example, in the Fragile X syndrome, FMR1 is silenced by DNA methylation. Targeted demethylation at the FMR1 locus using epigenetic editing tools has shown promise for reactivating the expression of this gene [16,81]. Tumorigenesis is associated with extensive epigenetic alterations. For instance, tumor suppressor genes such as p16INK4a are silenced in cancer through DNA methylation. CRISPR epigenetic editors can modify methylation status, potentially reactivating these genes and inhibiting tumor growth [100,102]. In cardiovascular diseases, the epigenetic editing of genes involved in lipid metabolism and inflammation may prevent cardiovascular events. At the 2023 American Heart Association conference, Chroma Medicine reported that targeted epigenetic editing of PCSK9 could achieve >80 % PCSK9 silencing in nonhuman primates and last for >10 months, which represents a significant milestone for the clinical application of durable epigenetic silencing [103]. These examples highlight the broad applicability and promise of epigenetic editing for treating various diseases. However, challenges such as the specificity of epigenetic modifications must be addressed before these approaches can be widely applied in clinical settings

Table 1
Epigenetic and transcription modulation.

Editor type	Location	Effectors	Results
Histone methylation	H3K4me1/2/3	PRDM9 [93], DOT1L [93], UBE2A [93], SMYD3 [94]	Transcriptional upregulation
	H3K9me2/3, H3K27me2/3	KRAB [88,95], G9A [96], SUV39H1 [96], EZH2 [96], FOG1 [96]	Transcriptional repression
Histone acetylation	H3K27ac	p300 [79,97], CBP60 [86]	Transcriptional upregulation
DNA methylation	DNA 5-mC	DNMT3A [81,82], DNMT3A + DNMT3L [83]	Transcriptional repression
DNA demethylation	DNA 5-mC	TET1 [81,85]	Transcriptional upregulation
CRISPR activator	Transcription factors	VP64 [98], VPR [90], SAM [86]	Enhanced recruitment of transcription factors and RNA polymerase II and increased gene expression
CRISPR silencer	Transcription factors	KRAB [96], SID4X [99]	Recruitment of co-repressors and decreased gene expression

since off-target epigenetic changes in distant genomic loci are often observed due to the spatial effect of chromatin. As research advances, strategies to overcome these hurdles will potentially lead to the development of novel epigenetic therapies.

RNA editing

CRISPR-based RNA editing is a novel approach for regulating gene expression without altering the underlying DNA sequence. It involves fusing dCas proteins to RNA-editing enzymes to target and modify specific RNA transcripts, resulting in reversible and transient RNA modulation. By fusing dCas proteins with RNA-editing enzymes, such as ADARs or cytidine deaminases (APOBECs), targeted RNA transcripts can be edited by converting specific nucleotides into different ones. For example, dCas13 fused with ADAR2 can induce A-to-I editing of RNA [17,18] while dCas9 fused with APOBEC1 can induce C-to-U editing of RNA [104]. RNA editing can also introduce premature stop codons, affect mRNA stability and splicing patterns, alter the secondary structures of RNA molecules, and modulate protein functions.

The therapeutic potential of CRISPR-based RNA editing spans various diseases by targeting RNA for knockdown, repair, or modification. For instance, researchers employing a mini-dCas13X-based RNA ABE have successfully targeted dystrophin gene mutations in a humanized mouse model of DMD, leading to the restoration of both dystrophin protein expression and function in multiple muscle tissues [105]. The same system has been used to correct transcript mutations in myosin VI and rescue hearing loss in mice [106]. Thus, RNA editing may be a feasible and promising tool for disease treatment. Compared with RNA, these dCas13-based systems can achieve higher targeting specificity and more versatile RNA manipulation [107]. However, several hurdles still limit the progress towards further clinical translation. First, the therapeutic effect of the CRISPR RNA editor is transient and needs to be re-dosed frequently. Second, the RNA editing machinery is more complex than siRNAs or oligonucleotides, which present more delivery challenges. Third, the immunogenicity, efficiency, specificity, and accuracy of RNA editors warrant a more rigorous and comprehensive investigation. Therefore, appropriate indications and applications should be assessed to balance the clinical benefits and costs of CRISPR-based RNA-editing technologies [105,106]. Eukaryotic programmable RNA-guided endonucleases such as Fanzor proteins are attractive alternatives to CRISPR-based RNA editors and are equipped with a unique set of structural and functional attributes adapted to eukaryotic cellular environments, which can significantly expand the RNA editing toolbox [108].

Perspectives and conclusions

Delivery

The delivery of CRISPR-based gene editors to target cells or tissues is a crucial aspect of the CRISPR technology, significantly affecting its efficiency, specificity, and therapeutic applicability. CRISPR-based gene editors can be delivered via either viral or non-viral systems. Viral systems, including adeno-associated virus (AAV) vectors, lentiviral vectors, and their derivatives, are pivotal in gene therapy because of their efficient gene delivery capabilities. However, the application of viral CRISPR-based therapeutics faces several challenges. AAVs have payload capacity limitations that restrict the size of the CRISPR components that they can deliver [109]. While lentiviruses carry risks associated with genome insertions that can lead to insertional mutagenesis [110]. The immunogenicity of viral vectors can also trigger an immune response, thereby undermining their efficacy and safety [109–111]. Furthermore, large-scale manufacturing of viral vectors poses significant challenges in reproducibility and cost [112]. In contrast, non-viral vectors have become increasingly attractive for delivering CRISPR components, either in ribonucleoprotein (RNP) complexes or mRNA + gRNA formats. The choice of non-viral carriers depends on the cargo format because their physicochemical properties influence the encapsulation mechanism. Various nanoparticles, nanocapsules, virus-like particles, exosomes, and engineered extracellular vesicles have shown promising results in RNP delivery, both in vitro and in vivo [113,114]. Nonetheless, the clinical translation of these RNP-based non-viral approaches is limited by their modest editing efficiency.

The most advanced non-viral delivery system for in vivo gene editing is lipid nanoparticle (LNP)-based delivery of mRNA and gRNA. Intellia Therapeutics' lead gene editing candidates, NTLA-2001 in phase 3 clinical trials (Knockout of Transthyretin in Transthyretin Amyloidosis) [115] and NTLA-2002 in phase 2 clinical trials (Knockout of Kallikrein B1 in Hereditary Angioedema) [116], have demonstrated significant clinical efficacy with a single-dose injection of LNP-mRNA + gRNA. Despite the success of LNP-mRNA + gRNA in liver editing via an ApoE-mediated mechanism, its application for extrahepatic delivery faces significant hurdles [114,117]. To overcome the barriers associated with extrahepatic delivery, several key factors must be optimized. First, the biophysical properties (size, surface charge, and hydrophobicity), pharmacokinetics, biodistribution, and extravascular transportation in the desired organ should be fine-tuned to ensure sufficient bioavailability in the target tissues [114,118]. The protein corona that forms around nanoparticles is instrumental in this process because it can mediate cell recognition [118], change nanoparticle size and surface properties [119], and induce clearance by the reticuloendothelial system [119]. Second, cell targeting and internalization direct gene editors to the right cell population, resulting in desirable phenotypic changes and therapeutic effects. Surface modification of nanocarriers with specific targeting ligands, including antibodies [120], antibody derivatives [121], peptides [122], aptamers [123], and small molecules [114,124], is a promising strategy. Lastly, endosomal escape and nuclear trafficking are required for efficient gene editing. The endosomal escape rate of nanocarriers is generally <10 %, which represents a notable space for further improvement [125]. NLSs have been commonly applied to protein sequences to enhance nuclear import [59]. However, efficient non-viral methods for the delivery of DNA templates into the nucleus for HDR or integration remain lacking.

Other critical considerations for the in vivo delivery of gene editors include the route of administration, off-target effects, duration of editing effects, and manufacturing feasibility. In addition to systemic injection, local injection directly into target organs, such as the eye (for retinal diseases) [122], brain (for neurodegenerative disorders) [126] or lung (for pulmonary diseases) [127], offers higher specificity, although this requires performing invasive procedures. Delivery device innovation, along with delivery systems, may accelerate the advances in local delivery of gene editors. Off-target effects are a significant concern for the systemic delivery of gene editors, especially when considering the risks associated with irreversible genome changes [25,110], editing of germline cells [128], imperfect precision, and genome toxicity [46,110], and appropriate selective turn-on of editing in target cells or turn-off of editing in non-target cells [129]. For the duration of gene editing, transient editing, such as LNP-mRNA + gRNA, is typically preferred over persistent editing for a long period, such as AAV or *Lentivirus*-delivered editors, because transient editing can potentially reduce unforeseen off-targeting editing risk over persistent editing [115,116]. Transient conditional editing that activates gene editors temporally using external stimulators, such as light-activated systems, is intriguing for certain applications [130]. Lastly, scaling up and ensuring manufacturing reproducibility are major hurdles in the clinical translation of viral and non-viral vectors for in vivo gene editing [112,113,117]. Innovations in manufacturing technology and adopting manufacturing into a design system early on will hopefully increase the chance of success for clinical translation.

Safety and ethical considerations

The safety and ethical considerations of CRISPR–Cas systems and related genome-editing technologies are crucial factors that influence their application in research and therapeutic contexts. Classic CRISPR–Cas9 systems induce DSBs at the targeted genomic locations, however,

the specificity of these systems are not absolute and can lead to off-target cleavage, which can result in unintended mutations, deletions, or insertions in the genome [46]. Base editors, designed to convert a single base pair into another without inducing DSBs, exhibit reduced off-target activity compared with the classic CRISPR–Cas9. However, base editors can still induce off-target base conversions outside the intended genomic sites, and the deaminase component can act on DNA and RNA in a gRNA-independent manner [131], potentially leading to unintended modifications. Prime editing, a more recent development, offers a "search-and-replace" genome editing model, allowing for precise edits without DSBs. Although it promises higher accuracy and fewer off-target effects than CRISPR–Cas9, the complexity of the prime editing system requires further investigation to fully understand the off-target profile of this technology [132]. CRISPR-directed integrases offer a mechanism for inserting large DNA sequences without DSBs and although they can theoretically reduce off-target cleavage effects, the specificity and integration efficiency of these systems are poorly quantified [14]. Epigenetic editing, which modulates gene expression without altering the DNA sequence, may pose fewer long-term risks, however, off-target epigenetic effects exist. Off-target dCas binding and the long-range effects of chromatin modifications on distal gene loci could impact gene expression patterns in unforeseen ways [16]. RNA editing alters the RNA sequence post-transcriptionally, offering a transient and potentially reversible approach to gene therapy. Although this reduces the risk of permanent genomic alterations, the specificity of RNA targeting and the potential off-target effects on the transcriptome require detailed investigation [18].

The potential to create heritable genetic modifications using CRISPR editors raises ethical questions, especially regarding germline editing [128]. The possibility of unintended consequences, such as genetic defects or the introduction of new diseases, necessitates stringent regulatory frameworks and ethical guidelines governing research and clinical practice. The reversible nature of epigenetic modifications addresses ethical concerns related to permanent genetic changes [16]. However, the broad implications of altered gene expression patterns and the potential for heritable epigenetic changes warrant a thorough ethical evaluation [133]. The temporary nature of RNA editing may lessen the ethical concerns associated with permanent genome editing. However, off-target RNA modifications with potential effects on protein and cellular functions require careful examination [18,104,134].

Conclusion

CRISPR technology has revolutionized genome editing and gene therapy, opening new avenues for treating various diseases and disorders. The rapid advancement and diversification of CRISPR tools have enabled the precise and versatile manipulation of DNA and RNA sequences, as well as the epigenetic and transcriptional regulation of genes. The first FDA approval of a CRISPR-based therapy for SCD indicates this technology is promising. However, many challenges and limitations need to be overcome before the widespread clinical application of CRISPR-associated gene-editing technologies, such as improved specificity, efficiency, delivery, and safety of CRISPR systems. Furthermore, the ethical and social implications of CRISPR must be carefully considered and addressed by the scientific community and society at large. CRISPR and CRISPR-based emerging technologies are powerful transformative toolboxes that have reshaped the landscape of biomedical research and innovation.

Funding sources

Z. Liu was supported by the New Cornerstone Science Foundation through the New Cornerstone Investigator Program and the XPLORER PRIZE, the National Research Program of China (2021YFF0701800 and 2020YFA0211100), the National Natural Science Foundation of China (T2321005 and 52032008), the Collaborative Innovation Center of Suzhou Nano Science and Technology, and the 111 Program of the Ministry of Education of China.

CRediT authorship contribution statement

Xiaoqi Sun: Writing – review & editing, Writing – original draft, Conceptualization. **Zhuang Liu:** Writing – review & editing, Supervision, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Xiaoqi Sun reports a relationship with Editas Medicine that includes: former employment and stock holder. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

The author is an Associate Editor for this journal(EngMedicine) and was not involved in the editorial review or the decision to publish this article.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.engmed.2024.100035.

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