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Invited Mini Review

Applications of CRISPR technologies to the development of gene and cell therapy

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Advancements in gene and cell therapy have resulted in novel therapeutics for diseases previously considered incurable or challenging to treat. Among the various contributing technologies, genome editing stands out as one of the most crucial for the progress in gene and cell therapy. The discovery of CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) and the subsequent evolution of genetic engineering technology have markedly expanded the field of target-specific gene editing. Originally studied in the immune systems of bacteria and archaea, the CRISPR system has demonstrated wide applicability to effective genome editing of various biological systems including human cells. The development of CRISPR-based base editing has enabled directional cytosine-to-thymine and adenine-to-guanine substitutions of select DNA bases at the target locus. Subsequent advances in prime editing further elevated the flexibility of the edit multiple consecutive bases to desired sequences. The recent CRISPR technologies also have been actively utilized for the development of *in vivo* and *ex vivo* gene and cell therapies. We anticipate that the medical applications of CRISPR will rapidly progress to provide unprecedented possibilities to develop novel therapeutics towards various diseases. [BMB Reports 2024; 57(1): 2-11]

INTRODUCTION

The Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) are originally discovered as bacterial immune systems and were subsequently investigated as versatile tools for genome editing (1-3). Generally, CRISPR system consists of a combination of single or multiple protein components with nucleic acid-cutting functions and a guide RNA that determines the target nucleic acids. In the cases of DNA targeting CRISPRs, double-strand DNA breaks (DSB) occur at the target loci with a complementary sequence to the guide RNAs (Fig. 1A). In eukaryotic cells, when CRISPR-mediated DSB triggers the intracellular DNA repair mechanisms, such as non-homologous end joining (NHEJ), homology-directed repair (HDR), and microhomology-mediated end joining (MMEJ), that results in induction of gene editing (4-6). DSB repair leads to the generation of Insertion and Deletion (INDEL), resulting in frameshift and premature stop codon, inducing knockout. This is being considered as a clinically useful approach for gene therapy.

While CRISPR gene editing methods have shown high efficiencies, the CRISPR genome editing via DSB could lead to unexpected genetic modifications (7). Large scale chromosomal rearrangement and DSB induced p53 DNA damage responses are some examples of such a problems that can affect cell viabilities and characteristics (8, 9). In addition, the off-target phenomena could potentially raise safety concerns in the development of genetic disease treatments using CRISPR. Such unintended mutation by off-target gene editing were shown as more probable at the DNA sequences in the genomic DNA that were partially complementary to the guide RNA. To address the issues of unintended outcomes of CRISPR genome editing, various CRISPR technologies with improved accuracy are being developed (10, 11).

In addition to off-target effects, another notable issue is the inherent variabilities in the resulting modified sequences found in genome editing by DNA break and repair (12-15). In the process, uneven insertions, modifications, and deletions may occur in the DNA sequences during the process of intracellular DNA repair mechanisms (16). The inherent variabilities pose a significant technical problem in medical application of CRISPR gene editing that utilizes DSBs to correct pathogenic single nucleotide variants (SNV) or precisely replace specific bases (17). To address the issue of gene editing heterogeneity caused by DSBs, researchers sought to investigate various CRISPR gene editing applications that enables more precise gene editing (18-20). One of these approaches is the base

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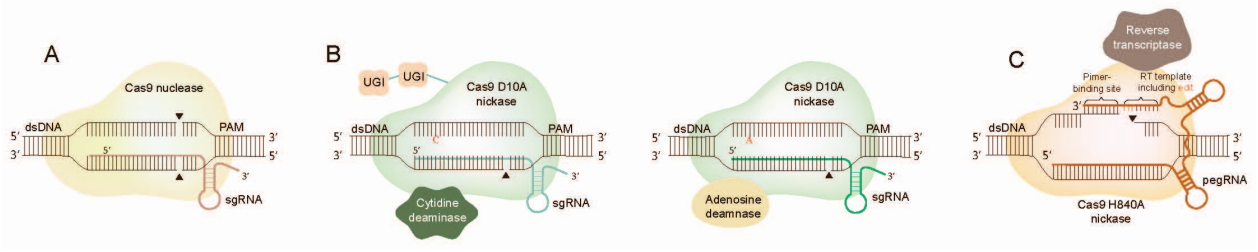

Fig. 1. Schematics of various CRISPR gene editing technologies. (A) CRISPR gene editing technology that utilizes double-strand DNA breaks in the target loci. (B) Base editing CRISPR technology that uses base modifying enzymes to replace single bases at the target sequences. (C) Prime editing that utilizes reverse transcriptase to simultaneously edit multiple bases in the target DNA sequence.

editing method, which has been demonstrated to replace a single base more accurately at a target gene location by combining a base modification enzyme with a CRISPR protein (Fig. 1B) (18, 19). The first base editing methods typically involved the substitution of cytosine with thymine or adenine with guanine within a window of a few consecutive DNA sequences bases. While several studies expanded the capabilities of base editing, simultaneous substitutions of consecutive sequences were still difficult. Further research showed that another CRISPR technology called prime editing could overcome the sequence limitations of base editing by integrating the CRISPR proteins with reverse transcriptase (Fig. 1C) (20).

Furthermore, recent advances in CRISPR technologies also enabled targeted regulation at epigenetic and RNA levels. Nonetheless, in this review, we will focus on DNA editing CRISPR technologies to provide a brief overview of the mechanisms of CRISPR system specifically can edit target DNA and describe how base editing and prime editing methods can achieve higher accuracies in genome editing. Next, we described some of the medical applications of CRISPR technologies for developing gene and cell therapy.

CRISPR GENOME EDITING VIA DOUBLE STRAND DNA BREAK AND REPAIR

The molecular mechanism of CRISPR-mediated DNA cleavage

Gene editing technologies have developed through various generations. The first generation included zinc-finger nucleases (ZFN), while the second generation comprised transcription activator-like effector nucleases (TALEN). The discovery and advancement of third generation CRISPR brought rapid progress in the gene editing technology (21-24). The CRISPR system is a form of adaptive immunity that allows bacteria and archaea to respond to exogenous viral invasions (5). In bacteria, the CRISPR system serves as a memory that contains a portion of the sequences of foreign DNA that previously invaded those bacteria. Therefore, the CRISPR system targets and cleaves foreign DNA with a sequence matching the guide RNA produced using this sequence information. Hence, prokaryotes utilized CRISPR to defend themselves by cleaving and degrading foreign nucleic acids, such as DNA or plasmids carried by invading bacteriophages.

The CRISPR system provides a significant advantage over ZFNs and TALENs, especially in the creation of comprehensive sets of vectors for targeting a multitude of sites (25), including entire genome-wide libraries. Another noteworthy benefit of CRISPR system is its ability to multiplex, enabling the concurrent utilization of multiple guide RNAs to target various sites within a single cell (25, 26). This streamlines the simultaneous manipulation of multiple genes or the precise engineering of deletions in a specific genomic region.

For the CRISPR-Cas protein to specifically cleave the target DNA, not only must the guide RNA have a complementary sequence but also a specific protospacer adjacent motif (PAM) sequence must be present in the DNA. This restriction reduces the risk of CRISPR cleaving genetic DNA of its own. As CRISPR research advanced, it became evident that bacteria possess various CRISPR systems with diverse biochemical properties. Some DNA targeting CRISPR could bind to DNA without cleavage capabilities and other CRISPRs could target RNAs (27, 28). The diverse CRISPRs could be classified into two classes (class I and class II) and more specific types based on their characteristics (3). Among the large varieties of CRISPR systems in bacteria and archaea, some of them have been widely utilized for genome editing in eukaryotic cells (2).

In particular, the CRISPR-Cas9 (SpCas9) protein discovered in Streptococcus pyogenes, showed robust DNA cleavage activity that could be utilized for effective gene editing in eukaryotic cells (4). It functions as a ribonucleoprotein complex, consisting of a single SpCas9 protein and guide RNA. The protein structure encompasses two distinct lobes—the recognition (REC) lobe and the nuclease (NUC) lobe (29). The two lobes of the Cas9 protein bind to the single-stranded guide RNA and establishes a stable DNA:RNA hybrid duplex with the DNA strand that complements it through Watson-Crick base pairing (30-33). This duplex formation is pivotal in inducing specific DSBs. Within the SpCas9 ribonucleoprotein structure, the DNA:RNA duplex created by the guide RNA and the target DNA single strand bears a negative charge, recognized by positively charged amino acids situated between the two

lobes of CRISPR-Cas9. For DNA target recognition by SpCas9 target DNA, a specific region known as the "seed sequence," which is located within 10 bases from PAM sequence (generally rich in guanine and NGG in the case of SpCas9), plays a critical role. This seed sequence is particularly sensitive to base pair mismatches. Consequently, each of the 20-base DNA sequence that form base-pair with the guide RNA in the SpCas9 system have varying levels of importance in accurately recognizing and cleaving the target DNA sequence within the genome (34). Base pair mismatches occurring within the seed sequence of the target DNA strand and the guide RNA have been observed to have negative impact on the cleavage efficiency depending on their location. Additionally, apart from the sequence that complements the guide RNA, the PAM sequence is another crucial component within the target DNA for recognition and cleavage (35-38). The PAM sequence does not form base pairs with the guide RNA but it directly interacts with the amino acids of the CRISPR-Cas protein, for example the amino acids Arg1333 and Arg1335 of SpCas9, forming a phosphate lock loop that leads to the heteroduplex formation of the guide RNA and target DNA (30). The structural elucidation of the SpCas9 system unveiled the molecular mechanism underlying specific DNA cleavage of the target gene within the genome, achieved through design of the guide RNA with complementary base sequences.

CRISPR Genome editing in eukaryotic cells during the DNA repair process

CRISPR Genome editing in eukaryotic cells by DNA break and repair are conducted in two steps after introduction of CRISPR systems, such as the SpCas9, into eukaryotic cells. The initial step of the CRISPR gene editing process involves the generation of DSBs at the desired location within the eukaryotic genome. As described above, the Cas protein utilized the guide RNA to induce DSB at the target site of the cellular genomic DNA. Next, the cell activates the cellular DNA repair pathways, and the repair process of the cleaved DNA induces genome editing at the site of DNA breaks. The results of CRISPR genome editing include insertions or deletions of variable sequences at the target site or insert externally delivered DNA sequences. In eukaryotic cells, the repair of DSB involves three processes: NHEJ, homologous recombination (HR), and MMEJ (39-41). These DNA repair processes function in a mutually exclusive or complementary manner, depending on the *in vivo* and cellular context (39, 42, 43).

The NHEJ operates throughout all cell cycles and involves four steps: 1) recognition of DNA breaks, 2) joining of the cut DNA and the formation of a synapse, 3) end processing, and 4) ligation. Various proteins, including Ku70/80, MRE11, Artemis: DNA-PKcs complex, and XLF:XRCC4:DNA ligase IV complex, collaborate in this repair process. NHEJ is inherently error-prone because there is no template DNA with a homologous sequence during the process of cutting and joining DNA ends (40, 44). Consequently, variable changes may occur in the DNA base sequence in NHEJ mediated repair. In cases of insertion or deletions in the coding region, there is a risk that the gene may be knocked out due to a frame shift or the introduction of premature stop codon.

The HR process is primarily active during the S and G2 phases of the cell cycle. This process is initiated by the coordinated action of various proteins, including Rad51, Rad52, Rad54, BRCA2, and RPA. Homologous recombination proceeds through several key steps, including 1) DNA end recognition and resection, 2) homologous pairing and DNA strand exchange, 3) DNA heteroduplex extension and branch migration, and 4) resolution of Holliday junctions (45, 46). In the homologous recombination process, the homologous locus on the sister chromatid serves as a template for repairing the cleaved DNA, allowing for error-free DNA repair without introducing mutations.

Additionally, alongside the canonical NHEJ (c-NHEJ), MMEJ is an alternative NHEJ process, which is known to function during G1 and early S phases of the cell division cycle. Unlike the c-NHEJ process, MMEJ repairs DNA breaks by utilizing short homologous sequences near the DSB site (47, 48). This process initiates with protein components, such as PARP1, MRE11, CtIP, and XRCC1: DNA ligase 1 or 3, binding to the site of the DNA break. Each end of the cleaved DNA is joined using a 10-base microhomologous sequence. This error-prone splicing process typically leads to the deletion of DNA sequences.

CRISPR technology exploits these repair processes, facilitating the insertion of exogenous DNA sequences into the genome or the elimination of target genes through DSB and subsequent repair. However, the concurrent and competitive operation of different DNA repair pathways following a DSB can pose challenges in predicting the sequence mutations at the repaired genomic location (43, 45).

Therapeutic application of gene editing by DNA-cleavage and repair

Utilizing CRISPR to induce precise DSB at specific genomic locations in eukaryotic cells, has significantly broadened the capacity of genome editing (25, 26, 49). The methods have been harnessed to develop gene therapy methods targeting pathogenic DNA variations identified by clinical genomic studies, offering the possibility of disease alleviation and treatment. Several studies demonstrated medical treatment technologies utilizing CRISPR gene editing, involving the precise cutting of DNA double strands. In this review, we introduce some of the key findings from pertinent gene and cell therapy studies that restored or eliminated pathogenic genetic mutations by CRISPR technology (Table 1).

Ex vivo genome editing

Some studies demonstrated *ex vivo* application of CRISPR technology to develop cell therapy using patient-derived cells. Scharenberg *et al*. showed the development of CRISPR-mediated cell therapy for Gaucher disease caused by lack of

Table 1. Applications of therapeutic CRISPR genome editing for human diseases

Disease	Gene	CRISPR type	Reference
Gaucher	CCR5	SpCas9	(50)
Wolfram syndrome	WFS1	SpCas9	(51)
Myeloproliferative neoplasm	V617F	SpCas9	(52)
Huntington's disease	HTT	SpCas9, SaCas9, LbCpf1	(53, 54)
Cancer	HPV18 E6	eiCRISPR	(55)
Sickle cell disease	BCL11A	Cas9 (CTX001)	(56)
Transthyretin amyloidosis	TTR	SpCas9	(58)
Corneal dystrophy	TGFBI	SpCas9, AsCas12a, SaCas9	(59)
Retinitis pigmentosa	Rho-P23H	SpCas9	(60)

glucocerebrosidase (GCase) (50). CRISPR genome engineering of hematopoietic stem cells (HSC) to knock-in GCase into the CCR5 safe-harbor locus resulted in functional restoration of GCase expression in monocyte- macrophage lineage cells. Maxwell *et al*. applied CRISPR genome editing to correct pathogenic mutations of WFS1 gene in induced pluripotent stem cells (iPSC) from patients with Wolfram syndrome, a disorder that causes diabetic symptoms (51). The transplantation of the corrected iPSC into diabetic mouse model resulted in improved insulin secretion for 6 months, suggesting the potential for autologous beta cell transplantation therapy. Smith *et al*. demonstrated the application of CRISPR technology to human induced pluripotent stem cells to target the V617F mutation in the JAK2 gene associated with myeloproliferative neoplasm, as well as the Z mutation in alpha-1 antitrypsin (AAT) (52). The CRISPR genome engineering of patient-derived iPSCs were conducted by positioning the mutations in the seed region of the guide RNA. Shin *et al*., and Monteys *et al*. demonstrated the application of CRIPSR to fibroblasts from patients with Huntington's disease (HD) by targeting abnormal expansion of CAG repeat expansion sequence in the Huntingtin (HTT) gene (53, 54). Shin *et al*. utilized their knowledge on the single nucleotide polymorphism (SNP) of the patient to conduct genome engineering of only the pathogenic allele. Monteys *et al*. demonstrated that exon 1 could be specifically excised by targeting the PAM base sequence unique to pathogenic alleles in humanized HD model mice (BaCHD) and patient fibroblasts. Enzyme-inducible CRISPR (eiCRISPR) was developed to selectively activate select group of cells (55). In eiCRISPR, the guide RNA was initially blocked by a deoxyribozyme (DNAzyme), and upon high expression levels of NAD(P)H: quinone oxidoreductase (NQO1) in cancer cells the DNAzyme was selectively released and unlocked the guide RNA. This study showed that CRISPR system can be activated in select cells to decrease the risk of unintended genome engineering. Erythroid-specific CRISPR-Cas9 editing resulted in the development of effective cell therapy for transfusion-dependent β-thalassemia and sickle cell diseases (56). In the genome engineered cell therapy, the enhancer region of BCL11A was edited by CRISPR-Cas9 that resulted in the increased expression of γ-globin. Recently, FDA approved the cell-based gene therapy, Casgevy, along with Lyfgenia, a similar approach, for their efficacies in treating sickle cell disease (57).

In vivo genome editing

The potential for developing CRISPR gene therapy was also demonstrated for targeting genetic diseases through *in vivo* delivery. In a clinical trial study by Gilmore *et al*, intravenous infusion of lipid nano particles (LNP) containing SpCas9 mRNA and sgRNA demonstrated notable therapeutic effect to transthyretin amyloidosis, a fatal disease caused by accumulating misfolded transthyretin (TTR) proteins (58). A single dose delivery decreased the serum level or TTR proteins greater than 95%, suggestive of significant potential as *in vivo* gene therapy. Another study by Christie *et al*. utilized CRISPR to correct mutations in the TGFBI gene that cause corneal dystrophy in mouse model: R124C, R124H, R124L, R555Q, and R555W (59). Various CRISPR systems with different PAM sequences, including SpCas9, AsCas12a, and SaCas9, were employed to effectively position the pathogenic mutations in the different PAM regions. Li *et al*. demonstrated the delivery of DNA plasmids encoding Cas9 proteins by subretinal injection to model mice with retinitis pigmentosa caused by the Rho-P23H mutation (60). CRISPR gene therapy resulted in specific removal of the pathogenic mutant alleles that caused the eye diseases. Consequently, the expression of the mutant gene was reduced by over 50% in the pathogenic mouse model, leading to a significant delay in the degeneration of photoreceptor cells.

CRISPR GENOME EDITING WITHOUT DOUBLE-STRAND DNA BREAK

Development of base editing and prime editing

Although DBS mediated CRISPR gene editing technology has improved in precision, the CRISPR methods inherently led to some introduction of variable nucleotide sequence mutations, insertions, or deletions into the target DNA sequences (12-15). Consequently, the CRISPR gene editing could face difficulties

when applied to tasks requiring precise alteration of a specific base, such as correcting pathogenic SNVs (17). Some studies sought to utilize the homologous recombination DNA repair pathway reduce the sequence heterogeneity at the gene edited loci (6, 61-64). However, it has been challenging to effectively eliminate the indels that generally result in variable changes in the DNA sequences during the DSB repair process (65).

To address the issue of gene editing caused by DSB, efforts have been made to explore technologies that can perform gene editing without cutting DNA double strands (18-20). One such approach, base editor technology, has demonstrated the ability to replace a single base at a target site by utilizing a base modification enzyme in conjunction with engineered CRISPR system that induces DNA nick instead of DSB (18, 19). Base editing techniques can typically substitute single bases within specified sequence regions containing 10 or fewer bases in the target DNA. They offer the capability to direct changes in DNA bases, switching from cytosine to thymine or adenine to guanine. Furthermore, depending on the objectives of gene editing, the sequence region for base substitution can be intentionally extended or reduced beyond the 10-base limitation.

Development of base editing technology provided an alternative approach that circumvents the use of DSBs (18, 66). The first cytidine base editors (CBE) employed either engineered SpCas9 nickase (nCas9) or catalytically dead SpCas9 (dCas9) fused to cytidine deaminase enzyme. A single amino acid mutation (D10A) in nCas9 inactivates the RuvC nuclease domain, and therefore nCas9 can only cleave that DNA target strand that hybridize to the guide RNA via HNH nuclease domain. In dCas9, both RuvC and HNH nuclease domain are inactivated by two mutations (D10A and H840A). Cytidine base editors induced conversion of cytidine into uridine in the genomic DNA within the target sequence range, resulting in the incorporation of adenine, in place of guanine. One of the initially developed base editing technology, known as BE1, utilized a fusion protein comprising SpCas9 and rat APOBEC1 (cytidine deaminase) connected by 16-residue linker (18). BE1 can convert cytidine to thymine within 5 bases of the guide RNA target site. During the base editing process, cytidine bases are transformed into uridine bases by cytidine deaminase, forming a G:U wobble base pair with guanine present on the opposing DNA strand. Subsequently, during DNA replication, an adenine base substitution takes place on the strand opposite to the uridine base. However, an analysis of how G:U pairs are resolved in cells revealed that the enzyme uracil DNA glycosylase (UDG) can remove the uridine base, ultimately restoring the G:U pair to its original G:C state. This process could have negative impact on the base editing efficiency, and researchers sought to improve BE1 by inhibiting the function of the UDG enzyme. Accordingly, a 3-fold more efficient BE2 was developed by fusing uracil DNA glycosylase inhibitor (UGI) with BE1. Both BE1 and BE2 base editing operated with minimal reliance on non-homologous end joining and homologous recombination mechanisms, resulting in an extremely low rate of base sequence insertion or deletion, below 0.1%.

Subsequent studies have sought to increase the efficiency of base editing and develop BE3. The maximum achievable base editing efficiency, aimed at forming a G:U wobble base pair, is limited to 50% because both DNA strands can serve as templates for replication. To enhance the base editing efficiency, intentional single-stranded DNA breaks were induced in the DNA strand containing the guanine base within the G:U pairs. Upon single-strand DNA break, the intracellular mismatch repair (MMR) mechanism recognizes the unedited guanine as damaged DNA and replaces it with adenine. BE3 demonstrated 2-6 fold higher conversion rate compared to BE2. However, the increased efficiencies of BE3 were also accompanied by the drawback of a relatively high likelihood of introducing base sequence insertions or deletions. Subsequent studies further improved the efficiency and precision of base editing by utilization of other cytidine deaminase, fusion of bacteriophage-derived Gam proteins that bind to DNA cleavage sites, and optimization of codon usage (67-69).

Base editing technology for base substitution in other directions beyond cytosine to thymine and guanine to adenine conversions has also been investigated (70). An adenine base editing (ABE) method enabled replacing adenine with guanine or converting thymine into cytosine by combining a CRISPR-Cas9 with E. coli transfer RNA adenosine deaminase (ecTadA) (19). In adenine base editing, adenine is transformed into guanine through the process of conversion to inosine by the ecTadA enzyme. The efficiencies of adenine base editing technologies have been steadily improved. ABE 7.10 technology increased an adenine-to-guanine conversion efficiency of 68%, surpassing the efficiency of homologous recombination (69). Furthermore, a more efficient ABEmax was developed by optimization of codon usage and nuclear localization signal sequences (69).

While advancements in base editing techniques have enabled precise editing of single bases, some problems including bystander editing, incapability of transverse mutation and the accurate editing of consecutive base sequences remained challenging (ref). To overcome this limitation, a different method called prime editing was developed (20, 71-73). Prime editing, akin to base editing, performs gene editing without inducing DSBs, but its molecular mechanism differs. A key distinction lies in the use of reverse transcriptase (RT) in the prime editing method, replacing the base conversion enzyme. Additionally, the prime editing guide RNA (pegRNA) employed in this method serves dual functions: it acts as an RNA template for DNA reverse transcription while also containing a segment that recognizes the target sequence as a guide RNA.

The initially developed SpCas9-based prime editing is conducted in three steps: 1) DNA single-strand cleavage, 2) DNA polymerization by reverse transcriptase using pegRNA as a template, and 3) DNA repair. In the initial step, a modified nickase SpCas9 protein (H840A) cleaves a single strand within

the DNA target. In the second step, the 3′ end of the pegRNA, featuring approximately 13 base sequences complementary to the target DNA, forms a DNA:RNA heteroduplex at the position of the DNA single-strand break. The Moloney murine leukemia virus (M-MLV) reverse transcriptase within the prime base editing protein then polymerizes DNA, utilizing the DNA:RNA heteroduplex as the primer and the pegRNA as the template. In this process, the mutant sequence, designed in the internal template region of pegRNA, is generated as a DNA sequence. The third and final step involves the introduction of the mutant base sequence formed through the reverse transcription process into the genome via intracellular DNA repair mechanisms. The first prime editing technique, called PE1, exhibited limited efficiency, and an improved PE2 was developed by optimizing the amino acids of the reverse transcriptase. Higher efficiencies were achieved in PE3 method by introducing additional DNA nicks at the uncorrected DNA strand near the genome editing site (44). The intentional nick activates endogenous DNA repair system that results in preferential incorporation of the prime edited strand into the genomic DNA sequence. In PE4 and PE5, the prime editing efficiency was increased by inhibition of DNA MMR via transient expression of MMR inhibitor proteins (74). A subsequent study showed that prime editing could be further enhanced by utilizing proximal dead sgRNA (dsgRNA) and chromatin-modulating peptides (75). DsgRNA is a 14- or 15- nt short variation of sgRNA that enables the binding of Cas9 to the target without catalytic DNA cleavage. The application of dsgRNA led to increased genome editing efficiency, likely via chromatin modification in the vicinity of the target site.

While PE and BE techniques are effective for editing relatively small gene regions, but their efficiency for inserting thousands of bases is limited. Although HR methods exist for inserting larger sequences, they face challenges with low insertion efficiency and the occurrence of indels. To tackle these challenges, ongoing studies are exploring innovative approaches, one of which is the Programmable Addition via Site-Specific Targeting Elements (PASTE) method (76). PASTE utilizes a CRISPR–Cas9 nickase fused with both a reverse transcriptase and serine integrase. This fusion enables targeted genomic recruitment and integration of desired payloads, providing a promising solution for efficiently incorporating large sequences into the genome.

Therapeutic applications of base editing and prime editing technologies

Base editing technologies have been utilized *ex vivo* and *in vivo* for developing gene therapy for neurological diseases. An *in vivo* base editing study conducted intracochlear delivery of BE3 ribonucleoprotein in mouse model to increase the regeneration of sensory hair cells through precise base editing of β-catenin (77). Introducing the S33F mutation into the β-catenin effectively blocked protein phosphorylation, resulting in the upregulation of Wnt signaling. In contrast, DSB mediated gene editing of the beta-catenin gene did not induce effectively Wnt activation. Another study employed BE3 cytosine base editor to correct pathogenic single-base mutations in the APOE4, a gene linked to late-onset Alzheimer's disease (18). BE3 delivery successfully corrected single nucleotide mutations that cause C158R and Y163C substitutions in APOE4 in mouse astrocytes with efficiencies of up to 74.9%. Arbab *et al*. demonstrated development of ABE based gene therapies for spinal muscular atrophy (SMA), a disease caused by the absence of survival motor neuron (SMN) protein (78). Effective A-to-G conversion in SMN2 gene was conducted in cells and SMA mouse model that expressed truncated non-functional SMNΔ7 protein. Notably, one-time intracerebroventricular injection of adeno-associated virus (AAV) serotype 9 that encoded adenine base editor induced an average of 87% conversion of SMN2 gene in the SMA mouse model. The results suggested that SMA could be potentially cured by a single dose of gene therapy based on adenine base editor. Ryu *et al*. demonstrated the delivery of AAV vectors encoding ABE7.10 to Duchenne muscular dystrophy (DMD) mouse models that had a nonsense mutation in the dystrophin gene (79). Microinjection of AAV to DMD mouse embryos resulted in effective correction of the disease-causing premature stop codon with editing efficiencies up to 95%. Also, notably, intramuscular administration of the AAV into the tibialis anterior muscle in DMD mice restored dystrophin expression by 17% and improved muscle function.

Base editors were also applied to develop gene therapies for blood disorders. Gehrke *et al*. demonstrated the application of high precision BE3 with engineered human APOBEC3A (eA3A-BE3) to correct point mutations in human HBB promoter that results in β-thalassemia (80). Gaudelli *et al*., showed the applications of ABE to induce specific base changes in the promoter region of fetal hemoglobin genes, HBG1 and HBG2, that resulted in continuous expression of the genes and consequential resistance to sickle-cell anemia and some of beta-globin-related diseases (19). ABE has also been used to correct pathogenic single base mutations associated with hereditary haemochromatosis, a serious disease that leads to excessive iron absorption (81). Hereditary hemochromatosis is most commonly caused by a G-to-A mutation at position 845 within the HFE gene, resulting in the C282Y mutation. Single administration of split AAV ABE7.10 system to hereditary haemochromatosis mouse model corrected more than 10% of the HFE point mutation and alleviated aberrant iron metabolism in the liver.

Base editors have also been utilized for investigation of therapeutics towards various diseases. An application of BE3 to correct the Y163C oncogenic single-base mutation in the TP53 gene in human breast cancer cells, achieved an editing rate of 7.6% (18). Notably, the rates of unintended insertions and deletions in BE3 base editing were significantly lower than gene editing by DSB-mediated CRISPR gene editing. McAuley *et al*. showed that severe combined immunodeficiency caused by

mutations in CD3δ could be corrected by ABE (82). The the correction of CD3δ mutations in the patient hematopoietic stem and progenitor cells (HSPC) restored the ability for T-cell differentiation in artificial thymic organoid system. Furthermore, transplantation of the edited human HSPCs to immunodeficient mice resulted in 88% reversion of CD3δ defects, suggesting the potential of ABE based autologous cell therapy for CD3δ SCID patients. Reichart *et al*. applied ABE8e to correct a pathogenic mutation in cardiac myosin heavy chain that causes hypertrophic cardiomyopathy (HCM) that is currently an incurable disease (83). The authors targeted a dominant missense pathogenic variant R403Q in the myosin heavy chain by delivering split ABE8e system in dual-AAV9 vectors. Notably, a single dose of the AAV9 vectors edited more than 70% of the mutations in the ventricular cardiomyocytes. However, higher doses could induce unintended by-stander mutations that may raise safety concerns, suggesting that the therapeutic window may be narrow.

Prime editing technology enabled genome editing of multiple consecutive DNA bases with relatively less sequence constraints compared to base editors. Consequently, some mutations can be targeted by both base editor and prime editor, while some therapeutic applications can be implemented exclusively by prime editing. Hong *et al*. demonstrated the application of both ABE and PE to edit the loss-of-function mutations in COL7A1 gene that causes recessive dystrophic epidermolysis bullosa (RDEB) (84). The pathogenic COL7A1 mutations within RDEB patient-derived fibroblasts were corrected by ABE or PE. Both gene editing methods showed functional restoration of the COL7A1 gene of the RDEB fibroblasts in intradermal injection into mouse and skin grafts. Jang *et al*. demonstrated *in vivo* hydrodynamic injection of PE2 and PE3 into mice model to target a point mutation in FAH gene that causes genetic liver disease hereditary tyrosinemia (85). AAV delivery of PE2 to mouse eye corrected the pathogenic mutation in the RPE65 gene that causes Leber congenital amaurosis. The prime editors showed precise correction of the disease-causing mutations without detectable off-target effect. Qin *et al*. utilized an enhanced prime editor, called PE^{SpRY}, to edit mutations that results in retinitis pigmentosa (RP), a serious vision related disorder with progressive and irreversible loss of retinal photoreceptors (86). The PE^{SpRY} system utilizes SpRY, a modified SpCas9 with significantly reduced PAM constrains, that enables more flexible selection of the target sequences (87). The delivery of PE^{SpRY} via dual split AAV to correct the pathogenic mutations in the phosphodiesterase 6β (PDE6β) gene associated RP mouse model resulted in significant improvement in the symptoms. The results suggested that PE^{SpRY} based gene therapy may be useful in alleviating or preventing vision loss by RP. Jang *et al*., utilized prime editing to target 12 types of oncogenic KRAS mutations with a universal pegRNA (88). The prime editing achieved up to 47% restoration of various altered amino acids back to glycine.

DISCUSSION

Advancements in CRISPR gene editing technology have opened up possibilities for the development of gene treatments that were once considered challenging or unattainable. Diverse CRISPR based methods are being developed for medical applications. Currently, CRISPR gene editing technologies can be broadly categorized based on whether they induce changes in the DNA sequence. Technologies modifying DNA sequences encompass those that cleave DNA double strands, such as gene editing technology, as well as base editing technology, which replaces a single target base, and prime editing technology, which modifies contiguous base sequences. Conversely, technologies regulating the expression of target genes without altering the DNA base sequence include CRISPRi, CRISPRa, and Cas13 technology, which cleaves RNA. While not covered by the review, CRISPR methods without DNA alterations also hold potential for therapeutic applications.

Improvements in efficiency and precision of CRISPR gene editing are critical to meet the high safety standards in medical applications. It is also important to accurately deliver CRISPR genome editing to specific target genes in selected subgroup of cells, tissues, or organs. As gene editing technology progresses, the potential to choose and apply the most suitable method for various research goals is expanding. Nonetheless, challenges persist in applying CRISPR technologies to gene and cell therapies. Issues concerning the accuracy and efficiency of CRISPR technology must be addressed. In the case of developing treatments involving *in vivo* CRISPR delivery, prior research to anticipate potential immune rejection within the body is crucial. Ongoing research endeavors are dedicated to mitigating these issues and expanding the application of CRISPR gene editing technology. We anticipate that, while some technical challenges currently remain, continuous improvement and evolution of CRISPR technologies will lead to wider applications in translational research in the future.

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CONFLICTS OF INTEREST

The authors have no conflicting interests.

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