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Recent advances in prime editing technologies and their promises for therapeutic applications

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Prime editing (PE) is a groundbreaking genome editing technology offering unparalleled precision in targeted genome modifications and has great potential for therapeutic applications. This review delves into the core principles of PE and emphasizes its advancements, applications, and prospects. We begin with a brief introduction to PE principles, followed by a detailed examination of recent improvements in efficiency, precision, and the scale of feasible edits. These improvements have been made to the PE systems through guide RNA engineering, protein engineering, DNA repair pathway screening, chromosomal or epigenomic modification, and in silico design and optimization tools. Furthermore, we highlight *in vivo* studies showcasing the therapeutic potential of PE to model and treat genetic diseases. Moreover, we discuss PE's versatile applications in saturation genome editing and its applicability to nonhuman organisms. In conclusion, we address the challenges and opportunities linked with PE, emphasizing its profound impact on biological research and therapeutics.

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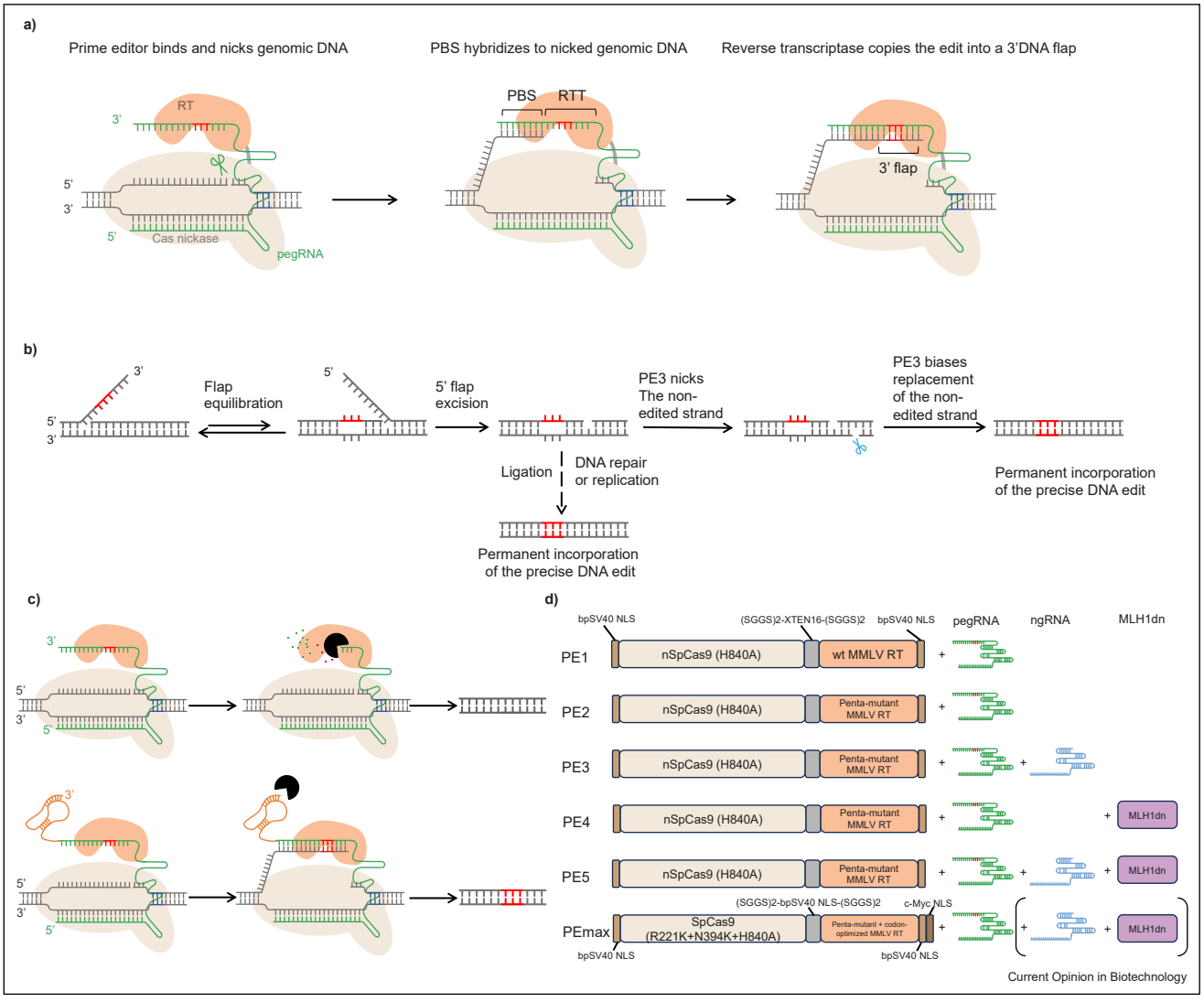
Introduction

The emergence of programmable gene editing tools has transformed life sciences by empowering researchers to execute precise and targeted genomic alterations in living cells. The advent of the Clustered Regularly Interspaced Short Palindromic Repeats-Cascade (CRISPR–Cas) technology has greatly accelerated genome editing research and applications. However, the efficiency of homology-directed repair (HDR) following Cas nuclease-induced double-stranded DNA breaks (DSB) for gene editing is generally low and often leads to uncontrollable insertions and deletions (indels) and chromosomal loss, inversions, or translocation via non-homologous end joining, raising safety concerns [1,2].

Prime editing (PE), a newly developed genome editing technique, offers a solution to these limitations, enabling accurate DNA modifications without DSBs [3]. PE systems consist of two main components: a fusion protein of reverse transcriptase (RT) and *Streptococcus pyogenes* Cas9 nickase (nSpCas9, H840A), namely the prime editor, and a prime editing guide RNA (pegRNA) (Figure 1a) [3]. The pegRNA comprises three essential regions extending from the single-guide RNA (sgRNA) scaffold: a 5′-protospacer region, a 3′- RT template (RTT), and a primer-binding site (PBS) that follows the RTT (Figure 1a). Programmed by the protospacer sequence, the pegRNA navigates the prime editor to the targeted genomic locus, hybridizes with the target strand, and unwinds the DNA double helix to expose the nontarget strand as single-stranded DNA. Consequently, the nSpCas9 (H840A) creates a nick in the nontarget strand to generate a 3′-flap for the PBS to hybridize, allowing the RT to leverage the information encoded in the RTT region of the pegRNA to introduce the desired edit onto the nontarget strand (Figure 1a). This process results in an edited 3′-flap, which can anneal to the target strand and displace the unedited 5′-flap (Figure 1b). The 5′-flap can then be excised, and the annealed 3′-flap can be ligated onto the phosphate backbone (Figure 1b). An additional nicking guide RNA (ngRNA) can be introduced to nick the target strand at a proximal locus to bias cellular replacement of the nonedited strand. The final step involves the cellular DNA repair or replication mechanism to copy the edit to the target strand, thereby making the desired prime edit

Figure 1



Schematic of PE systems. **(a)** Prime editors consist of three major components: SpCas9 nickase (H840A), MMLV-RT, and pegRNA [2,3]. PE1 uses wild-type MMLV-RT, while PE2 and beyond use engineered MMLV-RT. The currently proposed mechanisms for PE-mediated editing follow 1) pegRNA–Cas9 complex-mediated target binding and nicking of the nontarget strand; 2) base-pairing of PBS and the nontarget strand; 3) RTT- and RT-mediated synthesis of desired edit; 4) replacement of unedited nontarget strand flap by the newly synthesized flap containing the desired edit; and 5) DNA repair by cellular mechanisms for permanent incorporation of the DNA edit. Green scissors and unannotated nicks denote nicking by PE with pegRNA. Blue scissors denote nicking by PE with ngRNA. **(b)** A more detailed proposed mechanism of how the edited 3′-flap replaces the original 5′-flap, followed by 5′-flap excision, 3′-flap ligation, target-strand nicking mediated by an additional ngRNA in PE3, and permanent incorporation of the precise DNA edit [2,3]. **(c)** Comparison between regular and epegRNA, and an illustration of the proposed mechanism of how the 3′-pseudoknot in epegRNA protects pegRNA degradation at the 3′- end from exonucleases (denoted in black). **(d)** Schematic representation of different generations of prime editors. When PE2, PE3, PE4, and PE5 pair with the PEmax architecture, they are referred to as PE2max, PE3max, PE4max, and PE5max, respectively. bpSV40 NLS: bipartite SV40 nuclear localization signal; c-myc NLS: c-myc nuclear localization signal.

permanent (Figure 1b, d). PE enables a broad variety of gene editing outcomes, including base transitions and transversions, insertions, and deletions.

Nonetheless, the overall editing efficiency of PE remains low [4], and current PE systems struggle with long-fragment edits, including insertions greater than a few hundred base pairs or deletions exceeding one kilobase pair. Efficient editing with PE also often requires meticulous pegRNA design and comprehensive screening. Furthermore, the precise mechanism of PE-mediated DNA editing is not entirely understood yet. Recent research efforts have concentrated on addressing these obstacles, yielding significant progress in understanding and enhancing PE's performance and potential, thus setting the stage for more efficient and diverse applications.

This review delves into the most significant advancements in PE within the recent two years that enhanced the efficiency, precision, and scale of feasible edits. Our discussion spans various improving strategies, including pegRNA engineering, protein engineering, alternations in DNA repair pathways, chromosomal and epigenomic modification, and using in silico design and optimization tools. Additionally, we highlight the applications of PE in therapeutically treating human genetic diseases, engineering cellular pathways, detecting genetic variants, and editing nonhuman organisms. Last, we address the challenges and prospects associated with PE that could further broaden its profound implications for biological research and therapeutics.

Prime editing guide RNA structural engineering

PE efficiency is generally tied to the pegRNA design, particularly its 3′-extension that encodes both the PBS and the RTT. Vulnerability of this 3′-extension to cellular ribonucleases can diminish PE's potency due to potential degradation [5]. Nelson and colleagues addressed this issue by incorporating structured RNA motifs from viral pseudoknots, evopreQ$_1$ or mpknot, to the 3′-end of pegRNAs, thereby enhancing pegRNA stability and preventing the 3′-extension degradation (Figure 1c) [5]. The resulting engineered pegRNA (epegRNA) improved PE efficiency 3–4-fold in human cell lines and primary human fibroblasts without increasing off-target editing activity [5]. Similarly, multiple other research groups introduced other motifs such as the viral exoribonuclease-resistant RNA motif, human telomerase RNA, G-quadruplex, and stem-loop aptamer to the 3′-extended portion of pegRNAs, resulting in similar fold improvement compared with epegRNA in mammalian cells [6–8]. Furthermore, Yuan and co-workers designed a multiplex pegRNA array with 3′-interval sequences, which likely enhances PE via a similar mechanism [9]. In their drive-and-process array for multiplex PE, multiple pairs of ngRNAs and pegRNAs are co-expressed and processed from a compact engineered tRNA-driven array [9]. In addition, Li et al. stabilized the pegRNA secondary structure by changing each non-C/G pair to a G/C pair within the second stem loop of the pegRNA scaffold, leading to a 2.77-fold increase in the efficiency of insertions and deletions by PE [10].

In contrast to the conventional pegRNA design, where the spacer and template sequences are linked in a single pegRNA, Liu and colleagues designed a split pegRNA system. In their design, a conventional sgRNA directs the nSpCas9. At the same time, an independent linear or circular PE template RNA, containing the PBS and RTT, recruits the RT to the target site via MS2 coat protein–MS2 aptamer binding [11]. The split pegRNA strategy shows comparable efficiency to the conventional pegRNA and offers increased flexibility and stability [11]. The compelling data from these studies support adopting strategies to stabilize pegRNA structures, such as epegRNA, for PE applications.

Paired prime editing guide RNAs for enhanced efficiency and scalability

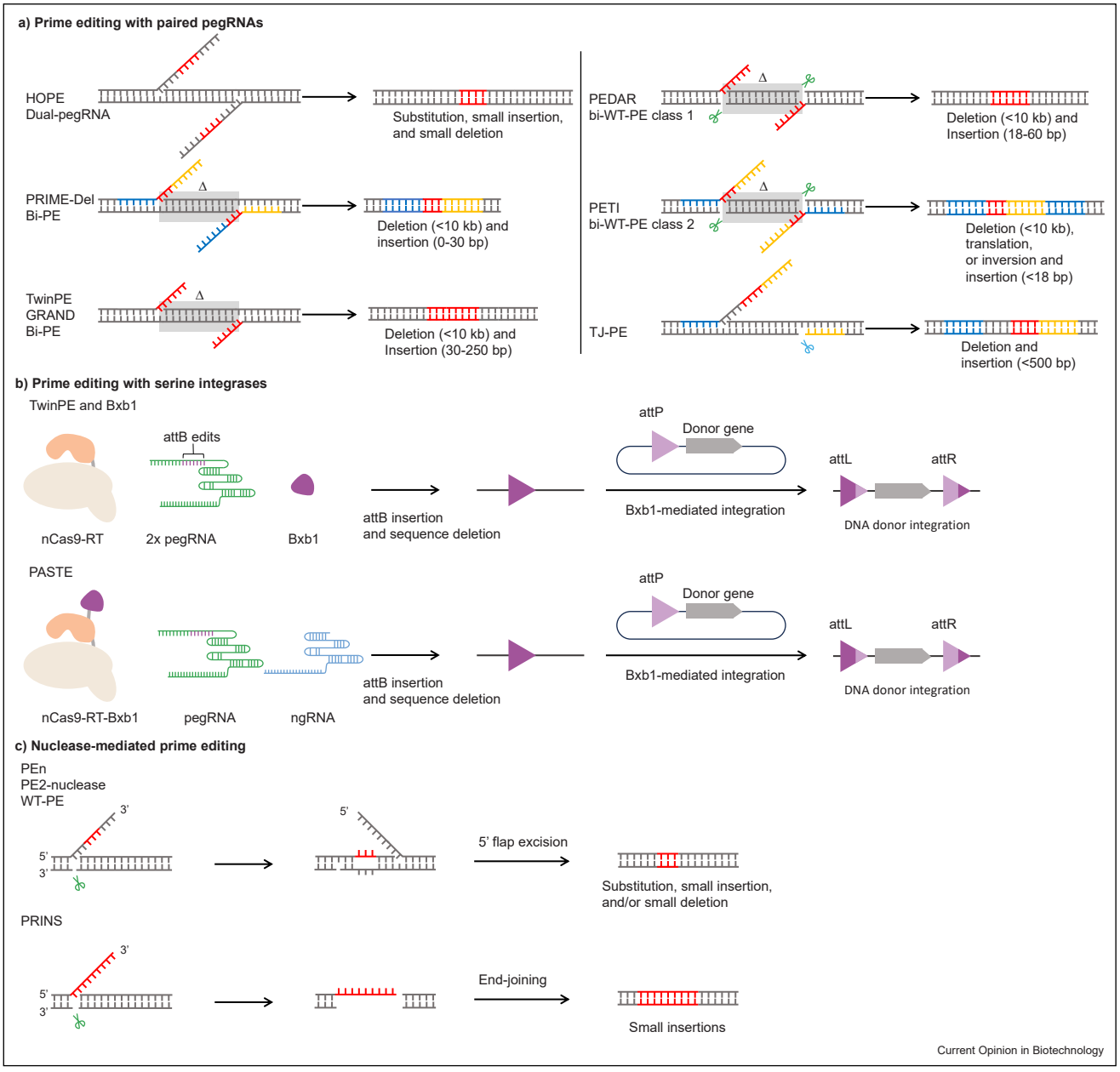
In the quest to improve overall PE editing efficiency and its capability to manage long insertions and deletions, recent innovations have seen the rise of dual-pegRNA systems. These strategies include homologous 3′-extension-mediated prime editor (HOPE) [12], dual-pegRNA [13], PRIME-Del [14], twinPE [15], genome editing by RTTs partially aligned to each other but nonhomologous to target sequence within duo pegRNA (GRAND) [16], and bi-directional prime editing (Bi-PE) [17] (Figure 2a). Distinct from single pegRNA designs, these methods, though varying their 3′-extension design and DNA cleaving strategy, exploit pairs of pegRNAs to target both DNA strands at the targeted loci.

The dual-pegRNA and HOPE systems use a pair of pegRNAs targeting two proximal genomic loci (< 50 bp from one another) to generate 3′-flaps containing the intended edit and some homology with downstream genomic sequence [12,13]. The resulting 3′-flaps can anneal to each other to form a duplex. Subsequent 5′-excision of the unedited genomic region and ligation of the nicks then permanently incorporate the edits into both DNA strands (Figure 2a) [12,13]. Dual-pegRNA resulted in a 17.4-fold increase in PE efficiency in plant cells, and HOPE showed greatly improved product purity in human cells compared with the PE3 system [12,13].

Utilizing pegRNA pairs targeting genomic loci at greater distances from each other allows longer sequence changes with PE. Using distant pegRNA pairs, PRIME-Del can mediate large deletions (up to 10 kb) at endogenous genomic sites with 1–30% efficiency (Figure 2a) [14]. The pair of pegRNAs can also encode a short insertion that can be concurrently installed at the deletion junction. Similarly, the twinPE system shows efficient deletion of a dystrophin (*DMD)* exon (around 600 bp) with 30% efficiency in human cells [14]. In addition to efficient long-fragment DNA deletions, twinPE demonstrates a 20-fold improvement compared with PE3 for a 108-bp fragment insertion (from 0.8% to 16%) with a concomitant 90-bp sequence deletion [15]. Notably, the synthesized 3′-flaps in the twinPE system can be designed without homology to the genome, and they only require 20 nucleotides of overlap on their 3′-end (Figure 2a) [15]. Independently, comparable strategies have been mirrored in GRAND and Bi-PE (Figure 2a) [16,17].

Despite the prowess of these paired pegRNA systems, direct insertion of DNA cargo beyond a few hundred

Figure 2



Advanced PE strategies. **(a)** PE with paired pegRNAs. TJ-PE uses one pegRNA with two PBS, one paired with the target strand and the other with the nontarget strand [2,12–17,21,31,32]. Δ denotes DNA deletion. Blue scissors denote nicking by PE with ngRNA. Green scissors and unannotated nicks denote nicking by PE with pegRNA. Red DNA bases represent the edited DNA with heterologous sequences. Blue and orange DNA bases represent homologous sequences on the 5′- and 3′-sides of the edited DNA, respectively. **(b)** PE for recombinase recognition site integration followed by large-fragment DNA insertion mediated by the integrases [2,15,18]. **(c)** Nuclease-based prime editors utilize Cas9 nuclease to create DSBs for enhanced PE efficiency, however, at the cost of frequent formation of unintended by-products [2,27–29,32]. In subpanels **(a)** and **(c)**, DNA in red denotes the intended edit, and DNA in blue and orange denotes base-pairing.

base pairs is not readily achievable [2]. This gap was bridged by synergizing twinPE with site-specific serine integrases [15]. TwinPE can efficiently insert 38-bp Bxb1 *attB* or 50-bp *attP* sequences at specified target DNA sites in human cells (up to 90%) [15]. Once these sequences are integrated, the Bxb1 large serine integrase can recognize them and achieve site-specific integration of a plasmid donor of more than 5 kb (Figure 2b) [15]. Moreover, by installing *attB* and *attP* sites, this technique allows targeted sequence inversions of 40 kb

between the iduronate 2-sulfatase (*IDS)* gene and its pseudogene *IDS2* associated with Hunter syndrome in human cells [15]. In another independent study, the programmable addition via site-specific targeting elements (PASTE) system can insert sequences for integrase-mediated insertion of donor DNAs as large as ~36 kb in human cell lines (Figure 2b) [18]. PE and integration steps in PASTE are performed by a single-protein fusion of nSpCas9 (H840A), RT, and Bxb1 recombinase. In contrast, these steps can be performed sequentially or as a single transfection using the twinPE system (Figure 2b). PrimeRoot editors enabled targeted DNA insertions up to 11.1 kilobases in plants with a similar method [19]. Although the current efficiency for integrase/recombinase-mediated large DNA insertion following PE-mediated *attB*|*attP* site integration remains low, this strategy opens a significant path for programmable long-fragment DNA manipulation. With more integrases and recombinases being computationally identified and experimentally characterized [20], we anticipate the emergence of increasingly efficient and reliable PE systems for precise large-fragment DNA manipulation.

Template-jumping prime editing (TJ-PE) was also developed, taking inspiration from the insertion mechanism of non-long terminal repeat (non-LTR) retrotransposons (Figure 2a) [21]. By leveraging a singular pegRNA that harbors the insertion sequence and two PBSs, one PBS matching the sequence of the pegRNA target site and the other matching the ngRNA target site, 200-bp and 500-bp DNA fragments were inserted into human cells with 50.5% and 11.4% efficiency, respectively [21]. Further, an exon was rewritten by TJ-PE in the liver of tyrosinemia-I mice to reverse the disease phenotype *in vivo* [21].

Investigation of DNA repair mechanisms for advanced prime editing systems

After prime editors synthesize an edited 3′-DNA flap at a target locus, endogenous cellular pathways are essential to permanently incorporate the edit into the host genome. Although the exact post-synthesis mechanism remains elusive, recent CRISPR interference screens revealed that DNA mismatch repair (MMR) can obstruct PE and promote undesired indel by-products [22]. In eukaryotes, MMR works by selectively replacing DNA strands with nicks to fix DNA heteroduplexes containing base mismatches or small insertion–deletion loops (IDLs). This repair process involves either the MSH2–MSH6 or the MSH2–MSH3 complex binding to the DNA heteroduplex and recognizing base mismatches and IDLs shorter than 13 nucleotides. MSH2 then recruits the PMS2–MLH1 complex, which incises the nick-containing strand near the heteroduplex. Subsequently, exonuclease 1 removes the heteroduplex at these cut sites. Polymerase δ then resynthesizes the excised DNA strand, and ligase 1 seals this newly synthesized strand [22]. MMR can revert the nicked heteroduplex formed when the prime-edited 3′-DNA flap anneals to the genome, adversely affecting the editing efficiency. Correspondingly, knockdown or knockout of MSH2, MSH6, MLH1, and PMS2 genes improves PE2 editing efficiencies by up to 5.8-fold and increases the purity of the editing outcome [2,22]. Similar findings have been independently reported by other researchers, including a study that verified the localization of MLH1 and MSH2 at prime editor-targeting sites [23–25].

Chen et al. developed PE4 and PE5 systems, which incorporate the transient expression of an engineered MMR-inhibiting dominant-negative MLH1 protein (MLH1dn) with PE2 and PE3, respectively (Figure 1d). This approach increases the efficiency of substitution, small insertion, and small deletion prime edits by an average of 7.7-fold and 2.0-fold compared with PE2 and PE3 systems, respectively, and improves the edit-to-indel ratios by 3.4-fold in MMR-proficient cell types [22]. In tandem, they introduced the PEmax architecture, enhancing PE efficiencies via nuclear localization signal engineering, codon optimization of the Moloney murine leukemia virus RT (MMLV-RT), and the introduction of mutations that increase SpCas9 nuclease activity (Figure 1d) [22]. PEmax, currently the state-of-the-art choice for PE applications, enhances PE efficacy by an average of 2.8-fold in Hela cells [22]. Transient perturbation of MMR via MLH1dn helps improve PE editing outcomes *in vitro* and *ex vivo* in MMR-proficient cells. However, adding an MLH1dn domain increases the difficulty of *in vivo* delivery due to increased molecular size and packing complexity using common delivery vectors.

An alternative tactic to bypass MMR is introducing silent mutations close to the intended edit, thus increasing PE efficiency without perturbing MMR activities globally or introducing an MMR-inhibiting protein [22]. Similar observations have also been reported where introducing same-sense mutations in the RTT can enhance PE efficiency [10,26]. However, further research is needed to understand how these silent mutations facilitate MMR evasion and to create reliable prediction and design tools.

Nuclease-based prime editing

Conventional nickase-based PE systems utilize nSpCas9 (H840A) and need to navigate a complex maze of DNA repair processes and intermediates to incorporate the edit. This intricate process of 3′-flap annealing, 5′-flap displacement, 5′-flap excision, and heteroduplex resolution leads to high risks for the edit to be unsuccessful [2]. Creating a DSB instead of a DNA nick

may circumvent these steps and engage other DNA repair pathways, thus rescuing editing efficiency in genomic loci where nickase-based PEs display inefficiency. Nuclease-based PE systems, including PEn, primed insertion strategy (PRINS), PE2-nuclease, and prime editing with wild-type Cas9 (WT-PE), were developed by replacing the nSpCas9 (H840A) in PE with wild-type SpCas9 nuclease (Figure 2c) [27–29]. When integrating a 53BP1-inhibitory ubiquitin variant with PEn, upgraded PEn showed increased efficiency in introducing RT-dependent edits [30]. These systems did show increased PE efficiency. However, the undesired by-products, such as indels, outnumbered the desired edits.

The nuclease-based PE systems were also combined with paired pegRNA strategies to develop systems, including PE-Cas9-based deletion and repair (PEDAR) [31], bi-directional prime editing with wild-type Cas9 (bi-WT-PE) [17], and prime editor nuclease-mediated translocation and inversion (PETI) [32], that enable more precise and predictable long-fragment deletions, inversions, and translocations than Cas9-based homology directed repair (Cas9-HDR) (Figure 2a). For instance, PEDAR successfully removed a 1.38-kb pathogenic insertion within the fumarylacetoacetate hydrolase (*Fah)* gene in the tyrosinemia-I mouse model $Fah^{\Delta Exon5}$, precisely repairing the deletion junction to restore FAH expression in the liver [31].

Nuclease-based PEs have shown increased editing efficiencies at genomic loci where nickase-based PEs display inefficiency. They also outperformed wild-type Cas9 systems by preventing unwanted large on-target deletions [31]. However, these improvements often accompanied increased indel frequencies, imprecise prime edits, and increased off-target edits compared with nickase-based PEs.

Optimized prime editing systems for *in vivo* delivery

Overcoming the challenge of efficient *in vivo* delivery, particularly the limited packing capacity (~4.7 kb) of widely used adeno-associated virus (AAV) vectors, is vital for broadening the application of PE systems (> 6.3 kb) [33]. Recent research efforts have concentrated on four major strategies: untethered RT, truncated RT, smaller RTs from diverse species, and leveraging split-intein systems.

Studies by Grünewald, Liu, and their respective colleagues [11,34] showed that an untethered RT and nCas9 achieved on-target editing efficiencies and off-target editing frequencies similar to an intact PE in human cells. The untethered RT design paves the way for a dual-AAV vector delivery, where nSpCas9 (H840A) is packaged in one AAV, and RT, pegRNA, and ngRNA are packaged in the other AAV for *in vivo* PE delivery. In addition, multiple independent studies have truncated the RNase-H domain of MMLV-RT (RT-ΔRNase H) to minimize the overall size of PE systems while maintaining comparable or higher activities than PE systems with full-length MMLV-RT [33–38].

Recently, Doman and colleagues surveyed RTs from diverse phylogenetic origins, evaluating 59 enzymes spanning 14 classes [39]. Using phage-assisted continuous and noncontinuous evolution, they evolved the *Escherichia coli* Ec48 retron RT to create PE6a and evolved *Schizosaccharomyces pombe* Tf1 retrotransposon RT to create PE6b [39]. By combining the mutations in the evolved Tf1 RT (PE6b) with additional rationally designed mutations used in the rdTf1 (rationally designed Tf1), PE6c was further developed [39]. They also combined the most promising mutations that emerged while evolving the PE2 with MMLV-RT ΔRNase H and created PE6d. In addition, the authors constructed and assayed SpCas9 mutants bearing single or combinatorial mutations that developed in the directed evolution process and created PE6e–g by combining the best-performing SpCas9 variants with MMLV-RT ΔRNase H [39]. These PE6 variants are smaller than previous PE1–PE5 systems but have comparable PE efficiency and offer unique benefits in different scenarios. For instance, PE6a is the smallest among these variants and can be used when the delivery size is the limiting factor [39]; PE6c and PE6d can accommodate highly structured RTTs, PE6b and PE6c may rescue PE efficiency at sites where MMLV-RT-derived prime editors show inefficiency, and PE6e–g can further enhance PE efficiencies at certain sites [39].

For effectively deploying PE via dual AAVs for *in vivo* applications, researchers have been compelled to split the coding sequence into fragments, ensuring each is below the 4.7-kb threshold [33]. These fragments are attached to either the N-terminal or C-terminal of an intein sequence and, upon delivery and expression, reassemble through an intein-mediated splicing mechanism [33]. Current research focuses on determining optimal sites within the nSpCas9 domain to facilitate intein sequences from widely used split-intein systems, such as those from *Nostoc punctiforme* and *Rhodothermus marinus* [11,33,35,36,40–42]. These advances facilitated the effective correction or installation of mutations *in vivo* in genes that are therapeutic targets for human genetic diseases [11,33,35–37,40–42]. Many of these *in vivo* edits showed promising results in rescuing enzyme expression and functions, thus alleviating disease symptoms [11,33,35–37,40–42]. For instance, by systematically identifying bottlenecks that limit AAV-mediated PE *in vivo* and introducing optimized v1em and v3em PE-AAV with enhanced prime editor protein expression, pegRNA stability, and modulation of DNA repair [33], the resulting PE editing efficiency reached up to 42% in the mouse brain cortex, 46% in the liver, and 11% in the heart [33].

Chromatin dynamics on prime editing efficiencies

The role of chromatin structure in the efficiency of PE has gained interest in recent research [43–45]. Kim et al. have used statistical models to elucidate the influence of critical epigenomic and sequence features at target sites [43]. In a recent study by Li and co-workers, they identified a positive correlation between the transcriptional activity of a gene and its editing efficiency, whereas an inverse relationship was observed with the distance from the transcription start sites [44]. Notably, enhancing the expression of a gene using the CRISPR activation system significantly enhanced the desired editing outcome [44]. Meanwhile, Park and colleagues proposed different strategies to modulate chromatin structure and thus increase PE efficiency, incorporating proximal dead sgRNAs and chromatin-modulating peptides [45]. They successfully generated insulin like growth factor 2 (*Igf2)* mutant mice with germline transmission, inducing a dwarf phenotype [45]. These insights and strategies underscore the importance of understanding and utilizing chromatin structure to maximize PE outcomes.

In silico design tools and models for prime editing

PE efficiency varies widely across pegRNAs with different PBS and RTT lengths [3]. Designing optimized PE systems is becoming more complicated as researchers strive for improved performance. Tools such as PrimeDesign and PrimeVar [46], PE-Designer and PE-Analyzer [47], and pegFinder [48] aid in the design and analysis of PE experiments. Moreover, PE efficiency and precision can be influenced by various factors, including the composition and structure of DNA sequence at the target locus, epigenomic context, and the protospacer, PBS, and RTT sequence in the pegRNA. Models such as prime editing guide prediction [49], DeepPE [4], Easy-Prime [50], and Modeling insertion efficiency for Prime Insertion Experiments (MinsePIE) [25] have been developed to enable more precise in silico prediction of PE performance. By leveraging high-throughput PE data and deep-learning models to predict editing outcomes based on user-defined parameters, these models reduce the need for extensive *in vitro* or *in vivo* testing. Additionally, Chen and co-workers have provided a detailed review of PE, and Doman and colleagues have written a detailed protocol for designing and conducting PE experiments [2,51].

Therapeutic potential and cellular pathway modification

Since the first *in vivo* PE editing was demonstrated in mice, more PE studies have emerged in disease-relevant cells and mouse models [52]. For instance, PEs have been applied in patient-derived induced pluripotent stem cells and hematopoietic stem and progenitor cells (HSPCs) [53,54]. Further, Geurts et al. used PE to create organoids harboring the tumor protein p53 (c .747G > T; p.R249S) mutation, commonly seen in hepatocellular carcinoma, and functionally repaired the cystic fibrosis transmembrane conductance regulator F508del mutation, the most prevalent mutation associated with cystic fibrosis, in patient-derived intestinal organoids [55]. Schene and co-workers employed PE to introduce a mutation in the gene encoding beta-catenin, mimicking liver cancer proliferation and development. They also tested PE's capacity to rectify a prevalent disease-causing biallelic deletion (c.629_631delCCT, p.S210del) on the *DGAT1* gene in intestinal organoids from patients with diacylglycerol-acyltransferase-1 deficiency and liver organoids from a patient with Wilson disease [56].

Jang and co-workers applied PE2 and PE3 *in vivo* by hydrodynamically injecting a fumarylacetoacetate hydrolase ($Fah^{mut/mut}$) mouse model and repaired the genetic liver disease hereditary tyrosinemia type I [57]. Remarkably, they showcased *in vivo* PE of the retinoid isomerohydrolase *RPE65* mutation (c.130C > T; p.R44X) to ameliorate visual function in the *Rd12* mouse model of RPE-related Leber congenital amaurosis (LCA), rescuing the genotype and phenotype of LCA [57]. Meanwhile, Böck and colleagues used dual-AAV-delivered PE to introduce adrenoceptor beta 1 A187V mutation in the cortex, a naturally occurring variant of the beta1-adrenergic receptor previously linked to increased activity and natural short sleep, observing an increase in the activity and exploratory behavior of treated animals [58]. Furthermore, Qin et al. demonstrated the potential of PE in reversing retinal degeneration in a phosphodiesterase 6B-associated retinitis pigmentosa mouse model, enabling the mice to complete a visually guided water-maze task [59].

Highlighting PE's potential in hematology, researchers corrected the sickle-cell disease (SCD) allele in patient-derived HSPCs. The outcomes were promising, as an average of 42% of human erythroblasts and reticulocytes, isolated from mice 17 weeks post transplantation of prime-edited HSPCs from patient donors, express HBB^{A} [60]. These edited cells showed similar characteristics to healthy cells, reduced sickle hemoglobin, and increased resistance to hypoxia-induced sickling [60]. Another study also independently demonstrated the *in vivo* repair of the SCD mutations in hematopoietic stem cells in an SCD mouse model (CD46/Townes mice) using PE [61]. PE has also been applied to create mouse models with phenotypic inheritance for human diseases, including cataracts [62]. Further showcasing *in vivo* applications, Ely and co-workers developed a Cre-inducible prime editor, $Rosa26^{PE2}$, in the mouse germline, allowing for rapid, precise engineering of a wide range of mutations in cell lines and organoids derived from primary tissues [63]. These applications underline the immense potential of PE for

treating and modeling genetic disease across various tissues and cell types and for molecular pathway reprogramming.

Prime editing for genetic variant characterization

The recent integration of PE into large-scale genomic research has added a new dimension to the functional characterization of genetic variants in their natural environment [64]. Erwood and colleagues utilized the PE system to perform saturation gene editing (SGE), scoring, and classifying PE-created variants. Their work centered around the NPC intracellular cholesterol transporter-1 gene, where mutations cause Niemann–Pick disease type C [64]. Similarly, PRIME, a pooled PE screening approach, was also developed to simultaneously characterize many coding and noncoding gene variants. PRIME successfully pinpointed crucial nucleotides in an *MYC* proto-oncogene enhancer and examined many noncoding variants linked to breast cancer and thousands of other variants from ClinVar [65]. Chardon et al. also developed prime-SGE and assayed single-nucleotide changes at scale in oncogenes for their ability to confer drug resistance [66]. Similarly, PE-mediated SGE was developed for in planta screening [67]. Large-scale, precise genetic variant creation and classifications via PE will improve clinical diagnoses, treatment, disease prevention, and genetic counseling, enabling personalized patient care.

Applications in diverse organisms

Beyond its applications in human cells and mouse models, PE has been expanded to various other organisms, including plants [68]. Enhanced PE efficiency was achieved by using two pegRNAs in rice and the developed PlantPegDesigner, a design tool for pegRNA use in plants [13]. Recently, Zong et al. developed engineered plant PPE (ePPE) via RNase-H domain removal, incorporation of a viral nucleocapsid protein, and rational mutagenesis [38]. Moreover, Jin and co-workers recently wrote a detailed protocol for PE in monocot plants using PlantPegDesigner and ePPEs [69].

Branching out of the broader spectrum of life, PE has also been successfully applied in a variety of other organisms, including the moss *Physcomitrium patens* [70], the cornerstone model organism, *Drosophila melanogaster* [71], sheep [72], dogs [73], and the model vertebrate, zebrafish [74]. These applications highlight the versatility of PE across different species.

Conclusions, challenges, and prospects

PE has demonstrated tremendous potential in advancing biological research and gene therapies. However, several challenges and prospects must be addressed and explored to thoroughly achieve its capabilities. One primary challenge resides in discerning the mechanisms influencing PE efficiency. The roles of cellular processes influencing PE, including DNA repair mechanisms such as MMR and mechanisms involved in DNA replication, need to be extensively investigated. Insights could be further enriched by obtaining crystal structures capturing the live action of prime editors, identifying cellular enzymes localizing at PE-targeted sites, and drawing parallels with similar mechanisms such as the non-LTR retrotransposon-mediated DNA integration [75]. These studies would facilitate the development of advanced PE technologies that work efficiently in diverse tissues, cell types, and cells at different stages of the cell cycle.

PE editing efficiency, scale, and specificity also present areas for enhancement. It is essential to increase desired edit rates further, accommodate large-fragment insertions, deletions, and replacements, and reduce indel rates, particularly in the PE3 and strategies introducing nicks on both strands of DNA. Doman and colleagues have demonstrated successful directed evolution of RTs other than MMLV-RT to achieve efficient PE [39]. Continuing to discover and engineer more diverse PE systems with different effectors, including RTs from various species and DNA polymerases, could further advance the technology. While PE demonstrated no detectable RNA-independent off-target effects in human cells [76], low PE off-target effects due to pegRNA design and nSpCas9 H840A-induced DSBs have been observed [77–79]. A recent report also showed that prime editors can induce detrimental transcriptional responses that reduce editing efficiency and hematopoietic repopulation in xenotransplants, as well as generate DNA double-strand breaks and genotoxic byproducts [80]. Further research is needed to minimize these off-target effects, including developing more precise in silico prediction tools and systematic enhancement. Expanding the PE toolbox is another vital step, specifically for multiplex genome editing and perturbation systems. Last, delivering prime editors into targeted cell types and tissues remains a significant challenge for *in vivo* PE, and improvements await.

With PE holding a promising position in genetic editing and ongoing research striving to enhance the system in various ways, the future looks bright for the application of PE in biological research and therapeutics.

Data Availability

No data were used for the research described in the article.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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