One Prime for All Editing

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https://doi.org/10.1016/j.cell.2019.11.030

Many targeted base transversions, insertions, and deletions remain challenging due to the lack of precise and efficient genome editing technologies. Recently, Anzalone et al. reported a versatile approach to achieve all types of genome edits, shedding new light on correcting most genetic variants associated with diseases.

Genome editing by CRISPR/Cas systems has been widely used in biomedical research and holds clinical potential, for instance, for use in correcting disease-associated genetic variants (Hsu et al., 2014; Knott and Doudna, 2018). Directed by a single-guide RNA (sgRNA), Cas nucleases such as Cas9 or Cas12a can bind to and cut targeted genomic DNA in living cells for engineering. Two DNA repair pathways, non-homologous end joining (NHEJ) and homology-directed repair (HDR), are responsible for repairing DNA double-strand breaks (DSBs) induced by CRISPR/Cas (Ceccaldi et al., 2016). NHEJ, the major DSB repair pathway, leads to random insertions or deletions (indels) and is widely used for gene knockout. DSBs can also be resolved through HDR to induce precise sequence replacement, given an exogenous DNA template; however, HDR is inefficient in most cell types and is inevitably accompanied by the formation of unwanted indels. In the past few years, a variety of CRISPR/Cas-based technologies have been further developed to fulfill different requirements for biomedical research. By conjugating a Cas moiety with a nucleotide deaminase moiety, base editors (BEs) carry out C-to-T (or G-to-A) and A-to-G (or T-to-C) base substitutions with no need of DSB repair (Rees and Liu, 2018). However, these BEs can yield only base transitions, not base transversions. To fill these technological gaps, the field has aspired to a multi-functional genome engineering technology that can install all types of genetic changes. Recently, that dream came true. By conjugating a Cas9 nickase with a reverse transcriptase (RTase), Anzalone et al. revolutionarily developed a "search-and-replace" genome editing technology, referred to as prime editor (PE), to rewrite genomes with almost any intended changes, including all types of base substitutions, small indels, and their combinations (Anzalone et al., 2019). PEs employ a prime editing guide RNA (pegRNA) to encode the desired genetic information, which is eventually incorporated into the genome after RTase-mediated reverse transcription (RT) (Figure 1A).

In order to enable targeted RT in a programmable manner, a pegRNA consists of three parts: a typical sgRNA containing a spacer region for Cas9 targeting, a primer binding site (PBS) for RT initiation, and a RT template with edits for targeted DNA changes. Protein engineering and elaborate guide RNA designs led to the advent of three PE systems, from PE1 to PE2 and then to PE3 and PE3b, with stepwise improvement in editing efficiency and/or product purity (Figure 1B). PE1 was made by fusing Moloney murine leukemia virus (M-MLV) RTase with Cas9 H840A nickase, which nicks the spacer-cognate DNA strand to enable its hybridization with the PBS of pegRNA and provides a 3′ end for RTase-directed primer extension. PE1 installed base transversions and small indels *in vitro* but showed limited efficiency in cells (Anzalone et al., 2019). To boost editing efficiency in cells, Anzalone et al. screened different mutants of M-MLV RTase, resulting in the construction of PE2. Compared to PE1, PE2 induced significantly higher editing efficiencies at tested genomic loci. By co-transfecting an additional sgRNA (nicking sgRNA) to nick the unedited DNA strand in the PE3 system, Anzalone et al. took advantage of the endogenous mismatch repair pathway to retain the information of the edited strand, further enhancing the editing efficiency. However, as two single-strand breaks were induced nearby on opposite DNA strands, the PE3 system induced NHEJ and thus exhibited higher indel frequencies. To suppress unwanted indels, Anzalone et al. redesigned the nicking sgRNA in PE3b to fit the edited DNA sequence, which ensures that the second nicking occurs after the resolution of edited strand flap (Figure 1B). Thus, PE3b temporally separates the two nicking steps, substantially reducing the generation of unwanted indels.

By applying PE3 and PE3b, Anzalone et al. efficiently induced transversions and small insertions or deletions to install or correct genetic mutations associated with human disorders in HEK293T cells. It was also shown that PE3 and PE3b were applicable in other human cell lines and mouse primary neurons, albeit with lower editing efficiencies than those in HEK239T cells (Anzalone et al., 2019). Although the *in vivo* application of PEs in

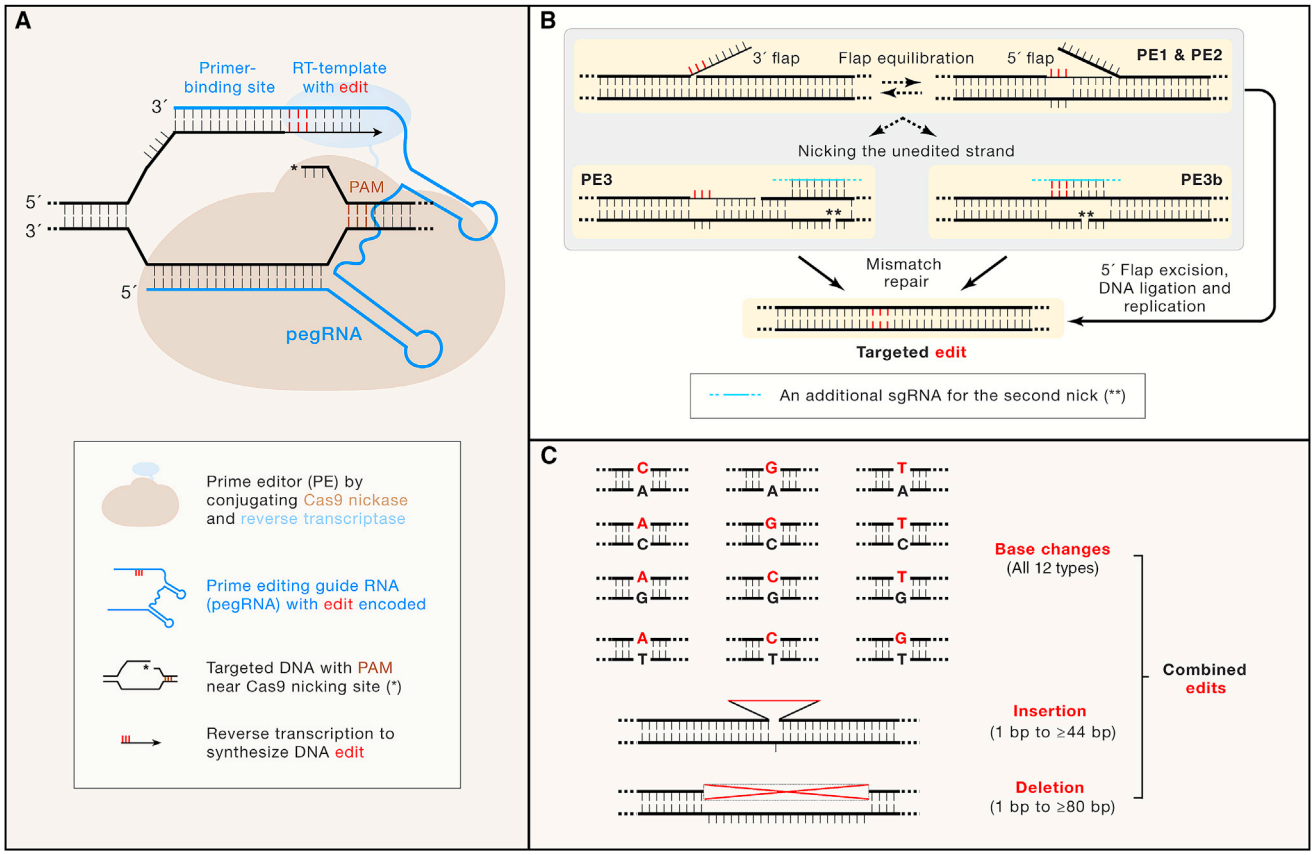

Figure 1. Development and Application of the PE System

(A) Schematic of the PE system. By using the genetic information encoded in a pegRNA, the PE system conjugates a Cas9 nickase with a RTase to achieve targeted edits via RTase-mediated primer extension from the 3′ end of the nick generated by Cas9 nickase.

(B) Development of different PEs with stepwise improvement of editing efficiency and/or product purity. After the RTase-mediated primer extension, PE1 and PE2 generate an edit-containing 3′ flap, which can be transformed to a 5′ flap through flap equilibration (top panel). The excision of 5′ flap and subsequent DNA ligation and replication will eventually fix the edits in target DNA (bottom panel) despite relatively low efficiency. To enhance editing efficiency, the PE3 system is developed by adding into the PE2 system an additional nicking sgRNA to generate a second nick in the unedited DNA strand (middle panels), thus triggering mismatch repair to install the edits more efficiently (bottom panel). To suppress unwanted indels triggered by simultaneous nicking on both DNA strands in PE3 (middle left panel), the additional nicking sgRNA is designed to fit only the edited sequences in PE3b (middle right panel), which separates two nicking steps temporally and therefore improves the editing product purity.

(C) Types of genomic changes that can be induced by prime editors: all 12 possible base changes or small indels or some combination of all of these.

animals and plants awaits to be tested, the advent of PE may lead genome editing to new heights.

The PE system features versatility and accuracy across a broader editing region than previous approaches. Compared to currently available BEs, which only induce four types of targeted base transitions (Gehrke et al., 2018; Koblan et al., 2018; Wang et al., 2018), PEs are able to fulfill all 12 types of base changes (Figure 1C) with complementary strengths and weakness. BEs offer higher efficiencies within their well-defined editing windows than PEs; however, PEs can induce both base transitions and transversions in more extended regions (Anzalone et al., 2019). Importantly, when multiple editable bases (bystanders) are within a defined editing window, PEs induced much more accurate on-target editing at the single base than BEs, as the latter suffer from bystander edits. Moreover, PEs outcompete BEs when multiple types of base changes are required simultaneously or desired base changes are out of BEs' editing windows. Finally, although HDR has long been credited with achieving any type of intended edits, HDR depends on DSBs, which inevitably trigger NHEJ to generate high levels of unwanted indels. Thus, PEs yield much higher product purity (i.e., the ratio of intended edits to random indels) than HDR (Anzalone et al., 2019).

The PE system is still in its infancy with plenty of room to improve. For instance, PEs may suffer similar concerns to other editors that are derived from CRISPR/Cas9, including off-target (OT) effects, due to the non-specific binding of sgRNA-Cas9 complex to genome. However, as PE3 and PE3b use two guide RNAs (a pegRNA and a sgRNA) to achieve efficient editing, guide-RNA-dependent OT effects are less likely to happen due to the low likelihood that both guide RNAs bind to the same OT site (Anzalone et al., 2019). Meanwhile, as the excess activity of fused nucleotide deaminase in

BEs may result in guide-RNA-independent OT mutations (Chen et al., 2019), whether PEs also exhibit guide-RNA-independent OT effects by the fused RTase requires further investigation. Moreover, the use of pegRNA in the PE system requires comprehensive design, and therefore, multiple parameters need to be considered, such as the length of the PBS, the sequence of RT template, and the location of the edit. Furthermore, to achieve better editing outcomes, an additional sgRNA for the second nick in PE3 and PE3b should be designed with delicacy. Finally, toward the application of PEs to correct genetic variants associated with diseases, one of the biggest challenges could be how to pack it for delivery. Adeno-associated virus, a commonly used viral vector for gene therapy, is likely too small for PE. The delivery of PE via non-viral systems (Yin et al., 2014) is probably practical, albeit awaiting testing.

To summarize, one can foresee the continuous evolution of PEs and their applications. No doubt, this "one-for-all" prime editing system will empower biomedical research and has clinical potential for human health.

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