***Review article***

# BASE EDITING: A PROMISING TOOL FOR GENETIC MANIPULATION IN MAMMALIAN SOMATIC AND STEM CELL LINES

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**ABSTRACT:** The CRISPR system enables precise editing in genomic DNA but relies on intracellular homology-directed recombination (HDR) repair pathways and is extremely inefficient. Base editing technology developed based on the CRISPR/Cas9 system builds three Base editors (BE) by fusing nucleases that have lost cutting activity with different base deaminases: Cytosine base editor (CBE) and Adenine base editor (ABE) and Glycosylase base editors (GBE). These two types of editors can complete the substitution of C > T (G > A) or A > G (T > C) at gene target sites without producing DNA double-strand breaks, and finally achieve accurate base editing. At present, base editing technology has been widely used in gene therapy, animal model construction, precision animal breeding, gene function analysis, and other fields, providing a powerful technical tool for basic and applied research. This paper summarizes the development and optimization process of base editing technology, and its application in livestock and poultry, to provide a reference for researchers in related fields to use base editing systems.

**Keywords:** CRISPR/Cas9；base editing；cytosine base editor (CBE)；adenine base editor (ABE)；Glycosylase base editors (GBE).

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## INTRODUCTION

Gene editing is a revolutionary biotechnology that is used to modify the genome of an organism. It is based on a variety of tools that allow scientists to make precise edits and changes to specific genetic sequences. In recent years, with the emergence of a variety of artificial endonuclease technologies, gene editing technology has been rapidly developed and widely applied. Artificial endonuclease technology mainly includes four types: mega-nuclease, Zinc Finger Nuclease (ZFN), transcription activator-like effector nuclease, clustered regularly interspaced short palindromic repeats, CRISPR/CRISPR-Associated proteins (Cas) system. Artificial nucleases can precisely target double-stranded DNA to produce double-strand breaks (DSBS). DSB induces cells to initiate two major DNA damage repair mechanisms: non-homologous end joining (NHEJ) [1], and homology-directed repair (HDR)[2]. NHEJ can occur at any stage of the cell cycle and is the most efficient repair mode, but because the canonical non-homologous end joining (C-NHEJ) DNA sequence that is refined by NHEJ is again cleaved by nuclease. So eventually there will be a different length fragment (mainly less number several bases) insert, delete, or replace (insertion/deletion/substitution, indel), NHEJ can occur at any stage of the cell cycle and is the most efficient repair mode. Canonical non-homologous end joining (C-NHEJ) DNA sequence is again cleaved by nucleases. So eventually there will be a different length fragment (mainly less number several bases) insert, delete, or replace (insertion/deletion/substitution, indel), which results in the inactivation of the alternative-non homologous end joining (A-NHEJ)[3, 4]. HDR is an accurate but inefficient repair method that occurs only in the S/G2 phase of the cell cycle when homologous sequences are used as a recombination template[5]. CRISPR/Cas is a gene-manipulation technique in which RNA-guided engineered nucleases cut DNA at specific locations in the genome, capable of altering DNA sequences or making RNA transcripts absent in living cells. With the deepening of research, more and more studies have shown that Cas9 nuclease-mediated gene

editing technology has some shortcomings, such as: (1) excessive DSB production in cells may lead to cell death[6, 7], (2) The repair efficiency by HDR pathway after DSB production is low, especially in non-dividing cells and adult animals, how to reduce the NHEJ-mediated indels and improve the HDR-mediated precise modification are still interesting issues[8, 9], and (3) A major concern with the application of this system is off-target mutagenesis[8]. It is not conducive to the wide application of this technology. Base editing technology emerged as a more accurate gene editing tool.

Cas nucleases can bind to other protein domains, allowing their DNA recognition sites to bind to new enzyme active sites. Fusion with the nucleoside deaminase or reverse transcriptase domain produces the base editor[10]. The catalytic inactivation (deactivation) of the Cas9 protein (dCas9) or Cas9 notch enzyme, which does not cause the DNA double-strand to be cut but the single strand to be cut, introduces the DNA single strand, largely preventing the introduction of indel while fully retaining the DNA recognition properties conferred by sgRNA[11, 12]. Emerging base editing techniques do not require DSB generation and homologous repair templates, do not rely on HDR repair pathways, and can more accurately rewrite the genome to produce specific types of point mutations[13].

At present, the types of base editors are mainly base editors that rely on deaminase, including cytosine base editor CBE and adenine base editor ABE. Another non-deaminase-dependent glycosylase-based base editing tool Along with base editors, such as the gBE developed by Yang's team, the base editing tools developed above enable direct editing of adenine (A), cytosine (C) or guanine (G). In June 2024, Yang Hui's team published a paper in Nature Communications entitled: Development of deaminase-free T-to-S base editor and C-to-G base editor by engineered human uracil DNA glycosylase[14], The research described in this paper shows that direct and efficient editing of T and C is achieved using novel glycosylase base editing tools. With the continuous enrichment of base editing tools, genome manipulation technology has developed from the original "scalpel" of Cas9 to the "correction pen" of the base editor, and has become another sharp tool for life science research in the 21st century.

Since 2006, the preparation and application of induced pluripotent stem cells (iPSCs) have ushered in a new era of stem cell therapy. The use of stem cells for gene therapy is to isolate and culture the patient's cells (usually adult stem/progenitor cells, immune cells represented by CAR-T cell therapy, or iPSCs cells with promising applications), and perform genetic manipulation to repair the genetic defects of the patient's cells, and then treat them by autotransfusion. In vitro, genetic manipulation of stem cells has the advantage of culture and expansion, can further enrich the required positive cells, and effectively improve the efficiency of treatment. With the continuous development of gene editing technology, especially base editing technology, the use of base editing tools in the editing process is not prone to large fragment deletion, and the average efficiency is higher than HDR: it promotes the precise editing of stem cells and provides a new idea for the accurate treatment of diseases.

**Base editor methodology:** Base editing technology is based on the development and evolution of CRISPR/Cas9 technology, and is a new gene editing tool developed without relying on double-strand break (Table 1). Can be a more accurate, efficient, safe, and extensive genetic modification. The base editor consists of three main components: base deaminases, Cas9 variants, and sgRNAs. The medium base deaminase is responsible for the deamination of the base, the Cas9 variant is responsible for binding the DNA target site without cutting or only cutting one DNA strand, and the sgRNA guides the complex formed by the Cas9 protein and the base deaminase to target the base sequence. With the improvement of the base editor, not only can the current pyrimidine to pyrimidine and purine to purine change, that is, the change of C·T, T·C, G·A and A·G, but also gradually can realize the transmutation between bases.

**CBE base editing system**

**Development and establishment of CBE system:** The CBE system is mainly composed of a Cas9 nuclease (dCas9 or Cas9n) with impaired cleavage activity fusing with cytosine deaminase, binding to the targeted DNA sequence under the guidance of sgRNA, and then the DNA double-stranded declining to form R-loop structure, exposing single-stranded DNA. Cytosine deaminase then deaminates the single strand DNA C in the active window into U and then generates the conversion of C·G base pairs to T·A base pairs through the DNA repair mechanism of the cell (Figure 1). Cytosine deaminases that occur in nature mostly act on RNA, The APOBEC (apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like) family is a few reported cytosine deaminases that can act on single-stranded DNA. The common feature of this class of deaminases is that they all have a CDA (cytidine deaminase) conserved domain that can deamination base C. By combining with the CRISPR system, this class of cytosine deaminase is developed into a new type of "editor" from the traditional "mutator"[11].

Taking advantage of the characteristic that APOBEC1 acts on single-stranded DNA but not double-stranded DNA, in 2016, the laboratory of David R. Liu at Harvard University in the United States named the CBE produced by the fusion of dCas9 and APOBEC1 as BE1[11]. BE1 can efficiently deaminate cytosine, but the deamination activity is limited to the 5 bp window of

single-stranded DNA, and the efficiency varies at different locations. Although BE1 can effectively target cytosine for deamination *in-vitro*, deamination efficiency in mammalian cells is greatly reduced, possibly due to cell repair of U·G intermediates [4]. Uracil n-glycosylase (UNG) recognizes the U·G mismatch and then breaks the glycosidic bond between uracil and the DNA deoxyribose skeleton, thereby restoring the U·G intermediate produced by BE1 to the original C·G base pair. To inhibit UNG activity, David Liu et al. developed BE2 by fusing uracil glycosidase inhibitors (UGI) from phages to the C-terminal of BE1. BE2 is capable of converting C·G base pairs to T·A base pairs, but the editing efficiency in mammalian cells is still low [4]. The editing efficiency of BE2 is low because it can only edit one DNA strand. To achieve the conversion of G to A in non-deamination DNA strands, David Liu and his collaborators designed BE3, which replaced dCas9 with Cas9 single-incision enzyme (Cas9n, D10A). It can specifically cut the unedited strand of DNA and stimulate cells to use the edited strand as a template to repair the unedited strand, which improves the efficiency of base editing in mammalian cells. Although BE3 produces indels, it is much lower than that produced by DNA double-strand breaks, and the conversion efficiency of C·G to T·A is significantly higher than that of homologous recombination (HDR), enabling efficient and precise base conversion in eukaryotic cells[11].

In the same year, Chang's team reported the activation-induced fusion of cytidine deaminase (AID) with nuclease inactive aggregation regularly intervaled short palindromic repeat (CRISPR) associated protein 9 (dCas9), DCas9-AID-P182X (AIDx) under the guidance of single guide sgRNA. Directly converts cytidine or guanine to the other three bases independent of the AID motif, producing a large number of variants at the desired site. In combination with uracil-DNA glycosylase inhibitors, dCas9-AIDx specifically converts the targeted cytidine to thymine, producing specific point mutations. We developed a targeted AID-mediated nucleotide mutation (TAM) method and demonstrated that TAM can effectively regulate various forms of m RNA splicing[15].

In 2016, Nishida and collaborators combined dCas9 with PmCDA1, a direct homolog of AID from lampreys, to form the complex Target-AID, which enables the C-to-T base switch[16]. In addition, Nature Methods also reported on the base editing technology CRISPR-X, which combines MS2 with AID and uses MS2 to recruit AID to the target gene fragment paired with g RNA to achieve base editing. The technique can target about 20 bases using g RNA, greatly increasing the range and number of base edits[17].

**Optimization of CBE system:** In the subsequent research and development, David Liu's team reformed the base editor BE in many aspects. To make Cas9 more widely targeted, the researchers used Staphylococcus aureus Cas9 (SaCas9), SaCas9 mutants, and saCas9 mutants to replace the previous SpCas9. SaCas9 can recognize a variety of PAM sequences. To expand the editing range of the clip editor, Young and colleagues recently reported three SpCas9 mutants that accept either NGA (VQR-Cas9), NGAG (EQR-Cas9), or NGCG (over-cas9) PAM sequence 15, And an engineered SaCas9 mutant with three mutations (SaKKH-Cas9), which liberalizes its PAM requirements to NNNRRT[18]. To reduce the editing window without greatly reducing base editing activity, a base editor (YE1-BE3) was produced, which has a similar maximum editing efficiency to be3, but greatly reduces the editing window width by about 2 nucleotides at both sites A and B. YE2-BE3 showed lower editing efficiency, but also reduced the editing window width of sites A and B to about 2 nucleotides. The maximum editing efficiency and editing window width of EE-BE3 are similar to those of YE2-BE3.YEE-BE3) had an average maximum edit yield 2.9 times lowe[r][18]. To engineer BEs with greater precision within the editing window， the researchers replaced APOBEC1 in BE3 with APOBEC3A (A3A) cytidine deaminase to produce A3A-BE3, they conclude that mutation of A3A can restore its cytidine deaminase sequence preference in the context of a BE fusion[18].

In an attempt to more broadly assess the accuracy of A3A-BE3 fusion on more endogenous human loci, the researchers tested 12 different gRNAs for three different human genes and directly compared the editing activity of seven base editor fusions, ultimately selecting eA3A-BE3 to show the highest activity on homologous MOBS. Bystander cytidine editing was also minimized at all sites tested[19].

Because CRISPR endonucleases require a specific proto-spacer adjacent motif (PAM) on either side of the target site, their target sequence space is limited. In this study, Pranam Chatterjee et al. demonstrated the natural PAM plasticity of highly similar but previously uncharacterized Cas9 (ScCas9) from Streptococcus Canis (ScCas9) through rational manipulation of differentiating motif insertion (Table 1). Not only does it have significant sequence homology with SpCas9 (89.2%), but it also inserts 10 positively charged amino acids at positions 367 to 376 of the highly conserved REC3 domain. To this end, we report affinity for the smallest 5' -ng-3 'PAM sequence and demonstrate the precise editing ability of this homology in bacterial and human cells[20].

Researchers noted that the Cas9n DNA sequence in BE3 was not optimized for expression in mammalian cells, containing a large number of non-favored codons and 6 potential polyadenylation sites (AATAAA or ATTAAA) throughout the cDNA; we therefore reconstructed the BE3 enzyme using an extensively optimized Cas9n sequence, named RA-BE3[21]. To improve the efficiency of gene targeting, NFLS-BE3 was

designed[21]. In 2019, Zhang et al. obtained the best base editor MS2 - BE - rAPOBEC1 (SGRNA-2X MS2, MCP - rAPOBEC1, and dCas9) based on the MS2 system. The tool can mutate multiple bases at target sites simultaneously, providing a new way to study genomic function[22].

In August 2017, David Liu et al. fused cytosine deaminase APOBEC1 and uracil glycosylase inhibitor UGI on Cas9n(D10A) protein, and inhibited endogenous uracil glycosidase activity by changing the expression level of UGI. The fourth-generation base editor BE4 is obtained. The BE4 system can convert cytosine C hydroamination at the target site into uracil U, and then further convert the edited uracil U into thymine T through the cell's DNA repair mechanism, thus realizing the single base conversion process from C to T. BE4 can improve the editing efficiency of C·G to T·A by about 50%, halve the probability of unwanted by-products compared to BE3, and greatly reduce the frequency of indels and unintended mutations. The base editing system BE4 is the most advanced technology that can realize single-base editing. In the editing process, there is no need for a double-strand break in the system, and only a single DNA strand incision can be used to accurately edit a single base by using related enzymes. This technology effectively avoids genome damage and other adverse effects in the editing process[23-25].

To investigate and solve the problem that the efficiency of base editors at certain target sites or specific cell types limits their utility, researchers et al., test whether base editing in cells is limited by the plasmid transfection efficiency of base editors or the expression of base editors, transfected HEK293T cells with a mixture of three plasmids, and found that, The number of base editor cells and/or the number of functional editor proteins produced by each cell is a major bottleneck for editing efficiency. Subsequently, to optimize nuclear localization, the researchers continued to test the fusion of the N and c terminals of BE4 with the SV40 NLS or NLS (bpNLS) used in BE4 and found that bpNLS at the N and C terminals (bis-bpNLS) performed best. The editing efficiency of C·G-to-T·A at the five genomic loci mediated was increased by an average of 1.3 times, followed by codon optimization to further improve the base editing efficiency, and finally found that AncBE4max and BE4max were significantly improved compared with BE4[25].

To improve the editing efficiency and target sequence compatibility of phage-assisted base editors, Nat Biotechnol et al. developed a Continuous evolution technique for phage-assisted base editors (BE-PACE). We use BE-PACE to evolve a new cytosine base editor (cbe) that overcomes the target sequence context constraints of standard cbe. One evolved CBE, evoAPOBEC1-BE4max, was 26 times more efficient at editing GC (an environment disliked by wild-type APOBEC1 deaminase) while maintaining efficient editing in all other sequence environments tested. Another evolved deaminase, evoFERNY, was 29 percent smaller than APOBEC1 and edited efficiently in all sequence contexts tested. We have also evolved a CBE based on CDA1 deaminase with higher editing efficiency at difficult target sites. Finally, we use evolutionary cbe data to elucidate the relationship between deaminase activity, base editing efficiency, editing window width, and by-product formation. These findings set up a system for the rapid evolution of base editors and inform their use and improvement[26].

Pranam Chatterjee et al. demonstrated a key expansion of the targetable sequence space for type II-A CRISPR-related enzymes by identifying a natural 5 '-NAAN-3' PAM preference for Cas9 in Streptococcus macacae (SmacCas9). To achieve high editing activity, the researchers grafted SmacCas9's pam interaction domain onto its homology from Streptococcus pyogenes (SpyCas9) and further engineered a more efficient variant (iSpyMac) to achieve strong genome editing activity. It was finally determined that the resulting hybrid could target all adenine dinucleotide PAM sequences and had powerful and accurate editing capabilities in human cells[27].

To eliminate the restriction that CRISPR-Cas enzymes need to recognize PAM sites, we designed a variant of Streptococcus pyogenes Cas9 (SpCas9) to eliminate the NGG PAM requirement. Russell T Walton et al. developed a variant called SpG that can target an extended set of NGN PAMs. To further optimize this enzyme, a nearly PAM-free variant of SpCas9 (NRN and to a lesser extent NYN PAMs) called SpRY was developed. The SpRY nuclease and base editor variants can target almost all PAMs, showing strong activity at a wide range of sites in human cells with NRN PAMs and lower but substantial activity at sites with NYN PAMs[28].

Cytosine base editors (CBEs) are efficient at generating precise C·G to T·A base transitions, but activation of induced cytosine deaminase/APolipoprotein B mRNA editing enzymes catalyzed polypeptide-like (AID/APOBEC) protein family deaminase components can cause considerable off-target effects and induction. To explore unnatural cytosine deaminase, the researchers repurpurated adenine deaminase TadA-8e for cytosine conversion[29]. The introduction of the N46L variant into TadA-8e eliminated its adenine deaminase activity, resulting in a TADa-8E-derived C-to-G base editor (Td-CGBE) capable of efficient and precise editing of C·G-to-G·C. By fusing with uracil glycosylase inhibitors and further introducing other variants, a series of Td-CBEs with high activity similar to BE4max or higher accuracy than other reported precise CBEs were obtained. Td-CGBE/Td-CBEs showed very low indel effects and background level Cas9-dependent or Cas9-independent off-target editing of DNA/RNA. In addition,

Td-CGBE/Td-CBEs were more effective at producing accurate edits at homopytosine sites in cells or mouse embryos, indicating their accuracy and safety in gene therapy and other applications.

TAM-CBE (cytidyl base editor) induces almost complete skipping of the respective exons by targeting these ESEs in patient-induced pluripotent stem cell (iPSC)-derived cardiomyocytes. Combined with a strategy to disrupt splicing sites, the researchers used TAM-CBE to identify suitable single-conducting RNAs (sgRNAs) to effectively skip most DMD hot spot exons without numerous double-strand breaks. Our study therefore expands the potential target of CBE-mediated exon hops for the treatment of Duchenne muscular dystrophy (DMD) DMD and other RNA mis-splicing disorders[30].

Base editors show promise for treating genetic diseases in humans, but most current systems use deaminase, which leads to off-target effects and is limited in the type of editing. In this study, Ye et al. constructed base-free editors of deaminase for cytosine (DAF-CBE) and thymine (DAF-TBE), which contain only cytosine-DNA or thymidine-DNA glycosylase (CDG/TDG) variants, respectively, linked to the Cas9 enzyme. Two variants with enhanced base conversion activity - cdg-nCAS9 and tdg-nCAS9 - were produced by multiple rounds of mutagenesis through directed evolution in *E. coli*, and the efficiency of C-to-A was as high as 58.7%, and that of T-to-A was as high as 54.3% (Figure 1). DAF-BEs achieve C-to-G/T-to-G editing in mammalian cells with minimal Cas9-dependent and non-Cas9-dependent off-target effects and minimal RNA off-target effects. The additional engineering produced the DAF-CBE2/DAF-TBE2 (Table 3), which changed from the 5' end of the original spacer to the middle editing window, increasing the C-to-G/T-to-G editing efficiency by 3.5X and 1.2X, respectively. Compared to the basic editor or CGBE, DAF-BEs extend the conversion types of base editors with similar efficiency, smaller size, and lower off-target effects[31].

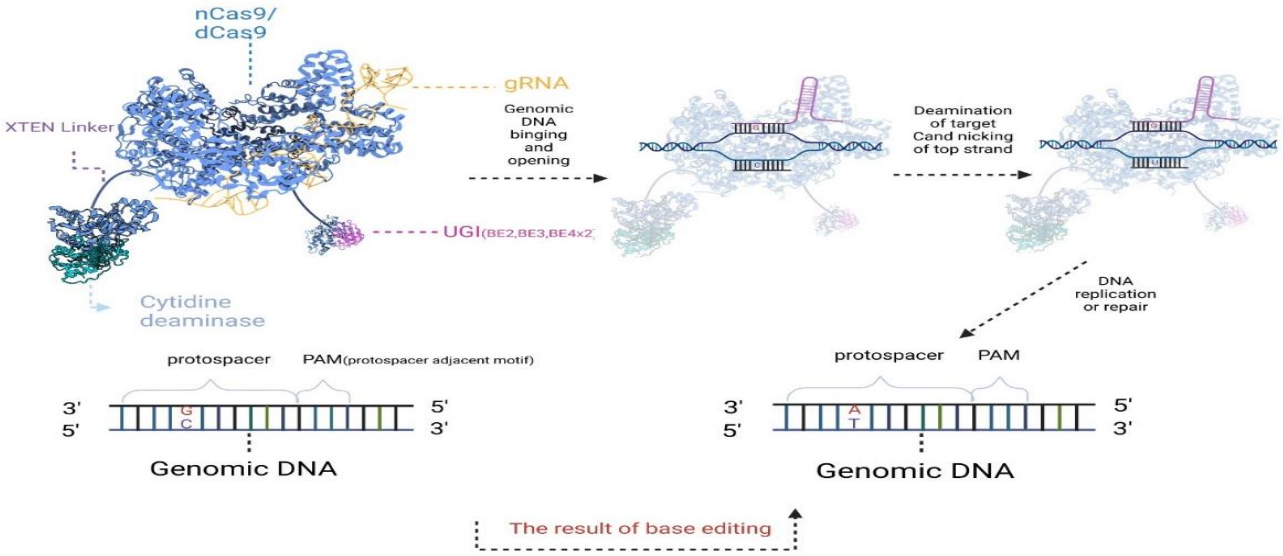


**Figure 1. Base Editing Mechanisms for Precise Gene Modification.** Mechanisms of adenosine base editors (ABE) and cytosine base editors (CBE) for targeted single-nucleotide modifications within genomic DNA. On the left, CBE uses a catalytically dead or nickase Cas9 (dCas9/nCas9) fused to a cytidine deaminase, which converts cytosine (C) to thymine (T) within a specific DNA sequence defined by the guide RNA (gRNA). The uracil glycosylase inhibitor (UGI) prevents unwanted uracil excision. On the right, ABE facilitates the conversion of adenine (A) to guanine (G) using an adenosine deaminase fused to nCas9/dCas9. Both systems induce targeted single-strand nicks to promote replication or repair of the newly edited DNA sequence.

**Table 1. Available base editors are widely used in genetic engineering.**

| Base-Editor | Architecture | Efficiency | Characteristic | Reference |
|---|---|---|---|---|
| BE1 | rAPOBEC1-dCas9 | 0.8–7.7% in human cells | First-generation BE | [11] |
| BE2 | rAPOBEC1-dCas9-UGI | Up to 20% | fused UGI to the C-terminus of BE1 | [11] |
| BE3 | rAPOBEC1-SpnCas9-UGI | Varies widely by cell type & target genes | Prefers TC motifs | [11, 23] |
| saBE3 | rAPOBEC1-HFnCas9-UGI | 50%-75% | Reduced off-target editing | [18, 32] |
| VQR-BE3 | rAPOBEC1-VQR SpnCas9-UGI | >50% | Expanded PAM targeting | [18] |
| EQR-BE3 | rAPOBEC1-EQR SpnCas9-UGI | >50% | Expanded PAM targeting | [18] |
| VRER-BE3 | rAPOBEC1-VRER SpnCas9-UGI | >50% | Expanded PAM targeting | [18] |
| SaKKHBE3 | rAPOBEC1-KKH SanCas9-UGI | >50% | Expanded PAM targeting | [18] |
| YE1-BE3 | rAPOBEC1 (W90Y, R126E) SpnCas9-UGI | Comparable to BE3 | Narrowed editing window | [18, 19] |
| YE2-BE3 | rAPOBEC1 (W90Y, R126E) SpnCas9-UGI | Comparable to BE3 | Narrowed editing window； loss of activity | [18, 19] |
| EE-BE3 | rAPOBEC1 (R126E, R132E) SpnCas9-UGI | Comparable to BE3 | Narrowed editing window | [18, 19] |
| YEE-BE3 | rAPOBEC1 (W90Y, R126E, R132E)-SpnCas9-UGI | Comparable to BE3 | Narrowed editing window | [18, 19] |
| RA-BE3 | rAPOBEC1 (RA)-SpnCas9-UGI | 30–58% | Increased editing efficiency | [21] |
| | rAPOBEC1-SpnCas9-UGI | 41–93% | Additional N-terminus NLS; Increased editing efficiency | [21] |
| A3A-BE3 | hAPOBEC3A-SpnCas9-UGI | 22.50% | Preferential deamination of cytidines in a TCR motif | [19] |
| xCas9-BE3 | rAPOBEC1-xnCas9-UGI | 37±10% (NGG PAM) | Recognize a broad range of PAM sequences | [19] |
| ScCas9-BE3 | rAPOBEC1-ScnCas9-UGI | 19–41% | Affinity to minimal 5′-NNG-3′ PAM sequences | [20] |
| TAM | SpdCas9-hAID (P182X) | N/A | High activity; used for random mutagenesis | [15] |
| Target-AID | SpnCas9-CDA1-UGI | 17–55% | First-generation base-editor | [16] |
| CRISPR-X | SpdCas9-MS2-hAID | N/A | High activity; used for random mutagenesis | [17] |
| MS2- be - rapobec1 | nCas9 (D10A)-nCas9 (D10A)-MCP-AID-UGI | N/A | simultaneously mutate multiple bases at the target site | [22] |
| BE4 | rAPOBEC1-SpnCas9-UGI-UGI | Varies widely by cell type & target genes | Increased editing efficiency | [23-25] |
| BE4-Gam | Gam-rAPOBEC1-SpnCas9-UGI-UGI | 17–58% | Increased editing efficiency and product purity | [24, 25] |
| SaCas9-BE4 | rAPOBEC1-SanCas9-UGI-UGI | 25–60% | Expanded PAM targeting | [25] |
| SaCas9-BE4-Gam | Gam-rAPOBEC1-SanCas9-UGI-UGI | 42–67% | Increased editing efficiency and product purity | [25] |
| BE4-Max | rAPOBEC1-SpnCas9-UGI-UGI | 69–77% | Codon optimized for mammalian cells | [25] |
| AncBE4-Max | rAPOBEC1-SpnCas9-UGI-UGI | 75–84% | Ancestral reconstruction of the deaminase component | [25] |
| evoAPOBEC1-BE4max | rAPOBEC1-SpnCas9-UGI-UGI | 60%-80% | Edited efficiently GC target positioned in the center of the editing window | [26] |
| evoFERNY-BE4max | rAPOBEC1-SpnCas9-UGI-UGI | 60%-80% | useful for viral delivery applications constrained by payload size | [26] |
| evoCDA1-BE4max | rAPOBEC1-SpnCas9-UGI-UGI | 60%-80% | applied when off-target and bystander editing are not concerns and high efficiency is paramount. | [26] |
| iSpyMac-BE3 | rAPOBEC1-iSpyMacnCas9-UGI | 50% | Elevated editing efficiencies on 5′-NAAN-3′ targets | [27] |
| SpG -BES | rAPOBEC1-VQR SpnCas9-UGI | N/A | a highly enzymatically active NGN PAM variant | [28] |
| SpRY-BES | rAPOBEC1-VQR SpnCas9-UGI | N/A | editing nearly all PAMs | [28] |
| TadA-8e-CBE | rAPOBEC1-SpnCas9-UGI-UGI | N/A | precise C • G-to-G • C editing | [29] |
| TAM-CBE | SpdCas9-hAID (P182X) | | leading to targeted exon skipping | [30] |
| DAF-CBE | CDG-nCas9 | 58.70% | minimal Cas9-dependent; Cas9-independent off-target effects; minimalRNA off-target effects | [31] |

**ABE base editing system:** In addition to changes in the C·G to T·A base pairs, there are five other mutation patterns (A·T to G·C, A·T to C·G, A·T to T·A, C·G to A·T, C·G to G·C) in the four bases of DNA in an organism (A, T, C, C, G to G·C) (Figure 2). Among them, the mutation of C·G to T·A base pair accounts for about 47% of all point mutation-related diseases in clinical cases, which is related to the high spontaneous deamination of cytosine in organisms, about 100-500 deamination occurs in A cell every day, so scientists are committed to studying the molecular mechanism of the transformation of A·T into G·C. Because that would correct about 47 percent of diseases associated with point mutations[33, 34]. Similar to cytosine, adenine (A) contains an exocyclic amine that is de-ammoniated to become inosine (I), which prefers complementary pairing with guanine (G), providing an idea for the development of base editors targeting adenine[35]. David Liu's team used the PACE bacterial evolution system to evolve the t RNA adenosine deaminase TadA of *E. coli* into deoxyadenosine deaminase that can act on single-stranded DNA and fused with dCas9 to obtain the TadA*-dCas9 fusion body. Figure 2 shows a highly efficient A-I mutation was achieved in *E. coli*[36]. Unfortunately, the editing efficiency of the TadA*-dCas9 fusion in mammalian cells is not very high, possibly because TadA tends to function as a dimer in nature. Therefore, David Liu's team combined wild non-catalytic TadA monomer with evolved TadA* to form a heterodimer protein, which fused with the amino-terminal of Cas9n (D10A) to obtain a single-chain heterodimer structure (TADa-Tada *-Cas9n, i.e. ABE7.10). This single-chain heterodimer structure greatly improves the editing efficiency of adenine in mammalian cells[36].

For the most commonly used Cas9 from Streptococcus pyogenes (SpCas9), the required PAM sequence is NGG. None of the natural or engineered Cas9 variants function effectively in mammalian cells, providing fewer PAM restrictions than NGG. Therefore, the researchers further used phage-assisted continuous evolution to evolve an extended PAM SpCas9 variant (xCas9) that recognizes a wide range of PAM sequences including NG, GAA, and GAT. To the knowledge of the research, PAM compatibility of xCas9 is the most widely reported among Cas9 proteins active in mammalian cells and supports applications in human cells, including targeted transcriptional activation, nuclease-mediated gene disruption, cytidine, and adenine base editing. The results showed that although xCas9 has broader PAM compatibility, it has greater DNA specificity than SpCas9, lower genome-wide off-target activity at all NGG target sites tested, and minimal off-target activity when targeting genomic sites that are not NGG PAM[37]. The researchers efficiently generated base-edited mice and rats with ABEs, with an efficiency of up to 100 percent. The researchers demonstrated increased ABE activity by injecting chemically modified tracrRNA and crRNA into mouse-fertilized eggs and expanded the editing range by fusing ecTadA [38]mutants with SaCas9n-KKH and Cas9n-VQR variants in cells and embryos. The final study shows that the ABE system is a powerful and convenient tool for introducing precise base switching in rodents. The researchers modified the nuclear localization signal and codon using the adenine (ABE7.10) base editor. The resulting ABEmax editor corrects pathogenic SNPS in a variety of mammalian cell types with significantly improved efficiency[25]. After codon optimization, the researchers found that a single zABE7.10 variant could induce targeted conversion of adenine to guanine at multiple genomic sites in zebrafish, and all of the targets showed high germline targeting efficiency[39].

Michelle F Richter et al. used phage-assisted discontinuous evolution and continuous evolution (PANCE and PACE) to evolve the deaminase component of ABE7.10 to obtain ABE8e. ABE8e contains eight additional mutations that increase activity (kapp) by a factor of 590 compared to ABE7.10. When paired with multiple Cas9 or Cas12 homologues, ABE8e offers significantly improved editing efficiency. ABE8e is more progressive than ABE7.10 and is beneficial for screening, destruction of regulatory regions and multi-base editing applications. By introducing additional mutations in the TadA-8e domain, Cas9-dependent and independent DNA off-target editing, as well as a modest increase in transcriptome-wide RNA off-target editing, can be improved. Finally, the experimental results show that ABE8e can effectively install natural mutations that up-regulate fetal hemoglobin expression in BCL11A enhancers or HBG promoters in human cells, which are targets of poor editing of ABE7.10. ABE8e enhances the effectiveness and applicability of adenine base editing[40].

The deamination of adenine produces inosine, which is treated by polymerase as guanine, but there is no known enzyme that deaminates adenine in DNA. The researchers discovered adenine base editors (ABEs) that mediate the A·T to G·C transformation in genomic DNA. Further optimization resulted in a transfer RNA adenosine deaminase that, when fused to a catalytically damaged CRISPR-Cas9 mutant, could act on DNA. Extensive directed evolution and protein engineering have led to 7th generation ABEs that efficiently convert the target A·T base pair to G·C (about 50% efficiency in human cells), have high product purity (usually at least 99.9%), and have low indel rates (usually no more than 0.1%). Compared to current Cas9 nuclease-based approaches, ABEs introduce point mutations more efficiently and cleanly, make fewer off-targs set genomic modifications, and can install disease-correcting or disease-suppressing mutations in human cells. Along with previous base editors, ABEs can directly, and programmatically introduce all four transition mutations

without double-stranded DNA cutting[36].

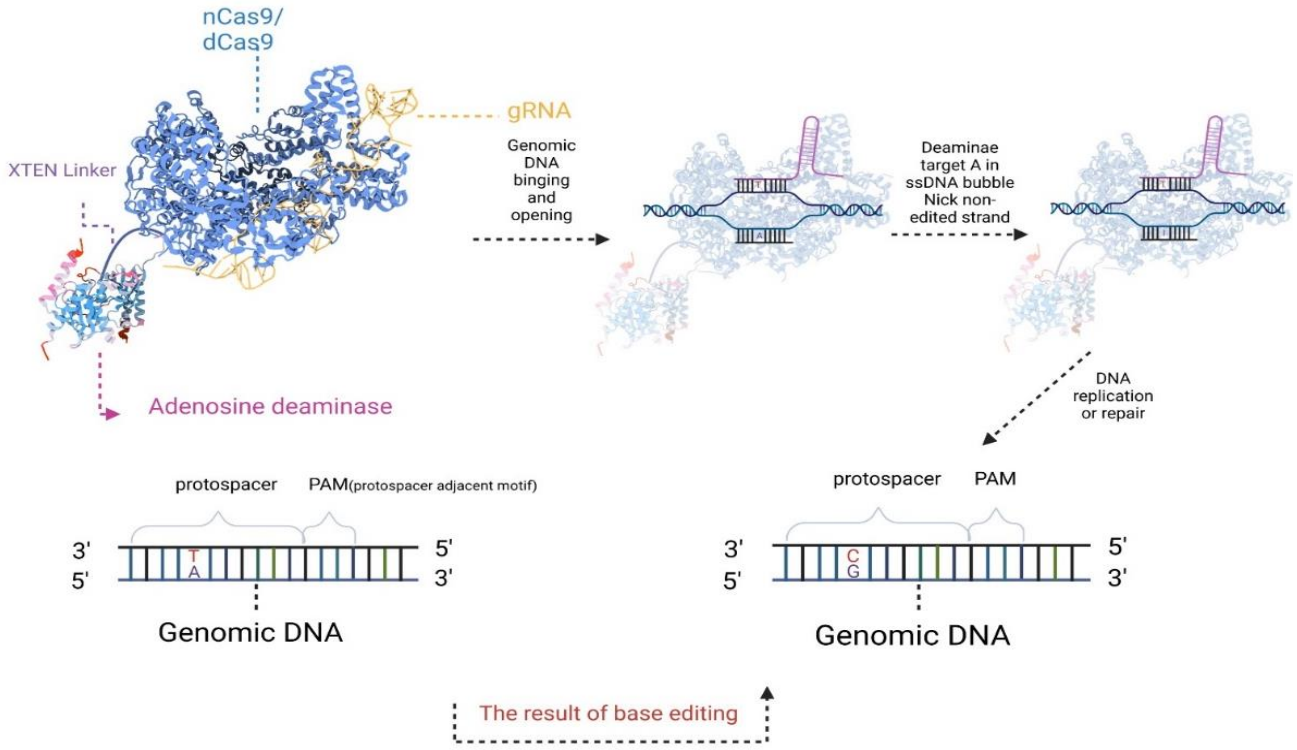


**Figure 2. Precision Base Editing via Cas9 Variants.** Mechanism of adenosine-to-inosine (A-to-I) base editing facilitated by a Cas9 variant fused to an adenosine deaminase. The complex, guided by gRNA, binds and opens the target genomic DNA. The deaminase modifies adenine (A) within a single-stranded DNA bubble, converting it to inosine (interpreted as guanine during replication). A nick on the non-edited strand facilitates DNA repair and replication, ensuring a permanent A-to-G transition. This approach enhances precise gene editing without inducing double-strand breaks, as shown by the conversion of targeted bases within the protospacer adjacent motif (PAM) region.

**GBEs base editing system:** Since the function of deaminase lies in the deamination of bases, ABE and CBE can only achieve the conversion within purine and pyrimidine, respectively, and achieving the conversion between purine and pyrimidine (also known as "transmutation") requires the help of new tools. The discovery of the new tool GBE is due to the in-depth exploration of the by-products of CBE editing (Figure 3). The main principle of GBE is to replace UGI in the CBE system with uracil-n-glycosylase (UNG). UNG can hydrolyze and break the uracil glycosidic bonds infiltrated into DNA, and mediate the transversion of C to G/A by generating baseless intermediates. Zhao et al. developed AId-Cas-ung (GBE), which realizes C·A translocations in *E. coli*, and APOBEC-nCas9-Ung, which realizes C·G translocations in mammalian cells[41, 42]. Soon after, Kurt et al. developed two base editors that could achieve base translocations[43]. The first type was modified on the BE4max system and consisted of nCas9, rAPOBEC1 (R33A), and *E. coli* uracil DNA n-glycosylase eUNG. Remove the two UGIs and increase the eUNG to remove the inhibitory effect of UGI on UDG to obtain a higher probability of C·A or C·G translocations. The second is the modification on CGBE1. The removal of eUNG on CGBE1 to form miniCGBE1 also has a considerable editing efficiency (slightly lower than that of CGBE1), but the probability of double-chain break is significantly lower than that of CGBE1. Both base editors enable target sequence C·G base translocations, reducing the occurrence of non-target C to A, C, T, and double-strand breaks. Yuan et al. optimized the codon and developed OPTI-CGBEs by changing the type of deaminase[44]. To explore the unnatural cytosine deaminase, we repurpose adenine deaminase TadA-8e for cytosine transformation. The introduction of the N46L variant in TadA-8e eliminated its adenine deaminase activity, resulting in a TADA-8E-derived C to G base editor (Td-CGBE)[29].

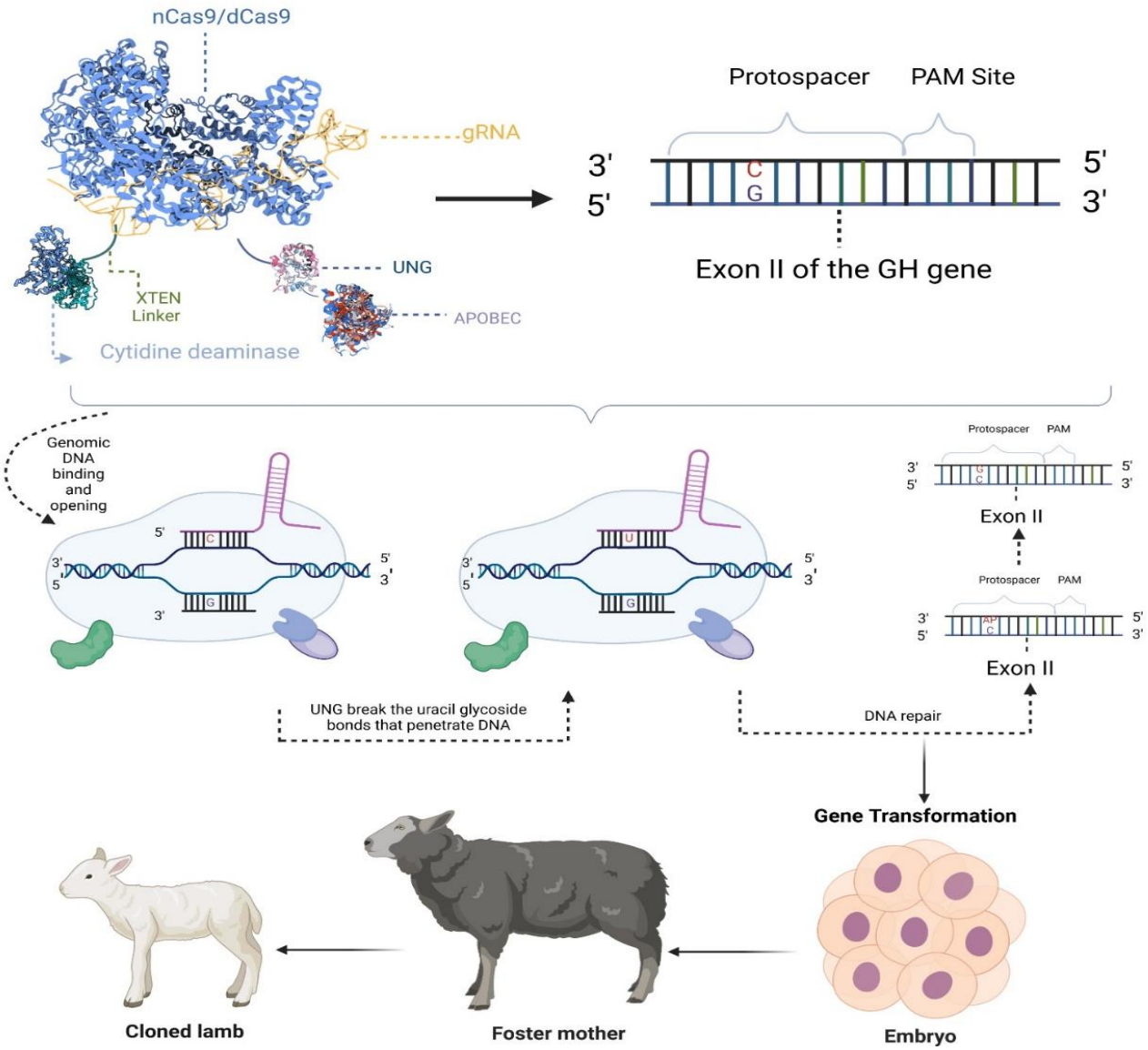


**Figure 3.** Precision gene editing harnessing cytidine deaminase and uracil-DNA glycosylase (UNG) within the nCas9/dCas9 framework facilitates targeted base substitution in the growth hormone (GH) gene. Through a carefully orchestrated DNA binding and repair mechanism, this transformative approach culminates in genome-modified embryos, ultimately yielding genetically tailored cloned lambs.

**Table 2. Architecture and efficiency of available ABEs.**

| Base-Editor | Architecture | Efficiency | Notes | Reference |
|---|---|---|---|---|
| ABE7.8/9/10 | ecTadA-ecTadA *-SpnCas9 | 1.7–20% | First generation ABE | [36] |
| xCas9-ABE7.10 | ecTadA-ecTadA *-nxCas9 | 69% (NGG PAM) | Recognize a broad range of PAM sequences | [37] |
| VQR-ABE | ecTadA-ecTadA *-Sp VQR nCas9 | 20% | Expanded PAM targeting | [38] |
| Sa(KKH)-ABE | ecTadA-ecTadA *-Sa KKH nCas9 | 16% | Expanded PAM targeting | [38] |
| ABEmax | ecTadA-ecTadA *-SpnCas9 | 27–52% | Improved editing efficiency | [25] |
| ABE7.10max | ecTadA-ecTadA *-SpnCas9 | 19.2–40.7% | Improved editing efficiency | [39] |
| ABE8e | ecTadA-ecTadA *-SpnCas9 | 18%–86% | Improved editing efficiency | [40] |

## APPLICATIONS OF BASE EDITING

**Application in cattle:** Among the common genetic defects in the bull pedigree, there are pathogenic mutations caused by multiple base mutations. These genetic defects result in the death of cattle embryos, the miscarriage of cows, or the deformities of calves, resulting in huge economic losses to the industry every year. Wang et al. obtained bovine fertilized eggs through in vitro culture and in vitro fertilization, and then used cytosine base editor BE3 and adenine base editor ABE7.10 by microinjection to achieve efficient gene editing in bovine embryos for the first time, confirming the feasibility of using base editors to directly perform gene editing in bovine embryos. At the same time, the members of the research group also used targeted second-generation sequencing to explore the off-target situation of base editing in bovine embryos using BE3 and ABE7.10 systems. The results showed that there was a miss phenomenon near the target site, but no obvious miss phenomenon was found in the 6 predicted potential miss sites. After that, the team members used the BE3 system and ABE7.10 system to perform multi-gene editing in bovine embryos by microinjection, confirming the feasibility of using the base editor to perform simultaneous multi-gene editing in bovine embryos. Finally, the team again used the BE3 system to knock out the CDX2 gene in bovine embryos by microinjection, and the results of immunofluorescence experiments showed that the knockout was successful. Through this study, efficient base editing, multi-gene editing, and gene knockout were realized in bovine embryos, which confirmed the huge potential of base editor for precision gene editing in bovine embryos, which is of great significance for efficient genetic defect repair and trait improvement in breeding cattle, to reduce embryo death, abortion, and calf deformity caused by genetic defects.

The embryonic development of mammals before implantation is mostly studied based on mouse models. To explore whether there are differences in mammalian mechanisms, Lei Luo et al. developed a gene function loss system using a cytosine base editor in early bovine embryos. Here, our study reports that BE3 and ABE7.10 promote gene editing with over 79% efficiency in bovine embryos. Importantly, we did not find significant off-target editing at potential sites. To improve editing efficiency, the experimental team microinjected 2 to 3 sgRNAs together and found that in about 80% of the embryos, the target genes could be completely deleted in all blastomes, and only less than 10% of the embryos showed mosaics. Three key lineage-specific genes (SOX2, OCT4, and CDX2) were identified. Among them, the knockout of SOX2 led to the failure of blastocyst pluripotency establishment, and the experimental results confirmed that SOX2 was essential for the expression of OCT4 and NANOG, and the knockout of SOX2 (KO) resulted in the significant reduction of OCT4 and NANOG expression in bovine blastocysts and the expression dysregulation of more than 2000 genes. A recent study has shown that OCT4 is required for NANOG expression in bovine blastocysts[45]This suggests that SOX2 may indirectly regulate NANOG. The results showed that CDX2 inhibited the expression of SOX2 in bovine trophoblast ectoderm. These results differ from those in mouse studies and highlight the species-specific role and regulation of SOX2 in mammals[46].

**Application in sheep:** The efficient introduction of multiple pathogenic SNPs in livestock breeding holds great promise for the development of better human disease models[47]. Most production traits of livestock are caused by point mutations. Based on this, Li et al designed sgRNAs for four target-induced nonsense codons (C-to-T conversion) of FGF5, a key regulator of goat hair length, and when introduced into single-cell embryos by microinjection, the BE3 system can achieve efficient single-base substitution in FGF5. It was also found that the BE3-mediated change of single base to nonsense codon does not change the transcription level, but may lead to reduced protein expression through post-transcriptional regulation of FGF5. Taken together, we conclude that the observed phenotype is caused by nonsense mutations in FGF5. Taken together, this study provides the first evidence of base editing in large mammals produced from microinjected single-cell embryos[48].

**Table 3. Architecture, efficiency, and characteristics of other base editors.**

| Base-Editor | Architecture | Efficiency | Characteristics | Notes |
|---|---|---|---|---|
| DAF-TBE | TDG-nCas9 | 54.3% | Increased efficiency of 1.2-fold | Glycosylase-based base editors for efficient T-to-G and C-to-G editing in mammalian cells |
| TSBE3 | UNG(CGBE-CDG )-SpnCas9 | >50% | Using the PLMs, an efficient T>S (G or C) base editor, TSBE3, | Protein language models-assisted optimization of a uracil-N-glycosylase variant enables programmable T-to-G and T-to-C base editing |
| AYBE | ecTadA-ecTadA *-SpnCas9-MPG | 72% | First efficient adenine base transmutation | Programmable A-to-Y base editing by fusing an adenine base editor with an N-methylpurine DNA glycosylase |

Genome editing with BEs without homologous directed repair of double-strand breaks can directly alter single nucleotides. Studies have shown that the p.96R>C variant that inhibits cytokine signaling 2 (SOCS2) has a profound effect on body weight, body size, and milk production in sheep[49, 50]. Zhou et al. developed BE3-mediated lambs using the BE3 system to replace C in SOCS2 by co-injecting BE3 mRNA and single-guided RNA (sgRNA) into sheep-fertilized eggs. The exchange efficiency of single nucleotides reached 25%. In addition, no off-target mutations were detected by parental whole genome sequencing (WGS). At the same time, the phenotypic identification of the edited sheep was carried out, and the results showed that the production traits of sheep were significantly improved by gene editing[51].

BMPR1B is the first major gene of litter size identified in sheep[52]. Zhou et al. used ABE (ABEmax) technology to introduce FecBB mutations into the genome of the Chinese local breed Tan sheep. ABEmax mRNA and sgRNA were co-injected into sheep single-celled fertilized eggs, and the developing embryos were then transferred into surrogate ewes. We successfully obtained lambs with a site-specific mutation (P.LN249ARG) leading to amino acid substitution. In newborn lambs, site-specific mutation efficiency was 75% because six lambs were heterozygous at the FecBB mutation site (g.A746G, p.Q249R) and two were wild-type. We did not detect off-target mutations in the eight edited lambs. Here, we report the validation of the first gene-edited sheep produced by ABE and highlight its potential to improve important economic traits in livestock[53].

To explore the editing efficiency of different versions of base editors on sheep fibroblasts, Sun et al. selected fecundity booroola (FecB) from Ovis aries and fibroblast growth factor 5 (FECB) from *Capra hircus*. FGF5 gene, using four new single base editors, namely xCas9-ABE (adenine base editor), ABEmax4, x Cas9-BE4, and BE4max, was used to make single base spot editing on the fetal fibroblasts of Tan sheep and Shanbei white cashmere goat. In this study, the optimal base editor was selected in the base editing application of sheep fetal fibroblasts, which proved the feasibility of efficient fixed-point editing of sheep genome, and also provided technical support for the application of base editors in gene editing of large mammals.

**Application in pig's genetic modifications:** Pan et al. constructed and used modified CBE plasmids to successfully prepare Bama mini pig single-cell colonies with premature termination of MSTN and no genomic off-target effects. This study lays a foundation for the further application of somatic cell cloning technology to construct MSTN-edited Bama mini pigs with only single base mutations, and avoids biosafety risks to a large extent, to provide a reference for base editing of other gene loci in Bama mini pigs[54].

Because knockout pigs are widely used in agriculture and biomedicine, with the improvement of single-base editing technology, pig et al. first constructed a modified "all-in-one" ABE vector suitable for transfection of pig cells, including ABE system and sgRNA. The results showed that this vector could perform single-base editing on multiple endogenous gene loci in pig cells. Complete sgRNA dependent A·T to G·C conversion. The GHR in the study is a membrane-binding receptor for growth hormone that triggers intracellular signals by binding to GHR to stimulate cell growth and division[55]. Loss-of-function mutations in human GHR trigger Laron syndrome, resulting in short stature and stunted growth[56]. Zhu et al. designed an "all-in-one" ABE to edit a single adenine residue at two sites in the GHR gene. The results showed that ABE-mediated exon jumping leads to gene knockout in pig cells[57].

In this study, the PX-ABEmaxAW vector was constructed based on the PX459 plasmid. We then compared and found no site-specific editing of CD163(differentiation cluster 163) intron 6 receptor, MSTN intron 2 donor, and IGF2(insulin-like growth factor 2) intron 3 in small-ear spotted pig cells. Then, to solve this problem, the research group constructed a new plasmid PXABE8eV106W. Sanger sequencing showed no detection of genomic SGRNA-dependent targeting effects. In summary, compared with PX-ABEmaxAW, PX-ABE8eV106W has higher editing efficiency on CD163, MSTN, and IGF2 genes, and achieves efficient base editing in pig cells, further expanding the application range of this base editing system. However, PX-ABEmaxAW and sgRNA were co-transfected into Guangdong small ear spotted pig renal fibroblasts, and qPCR identification confirmed that MSTN was not expressed in Guangdong small ear spotted pig fetal renal fibroblasts[58].

Jing et al. used AB7.10, ABEmax, NG-ABEmax, ABE8e, and NG-ABE8e to achieve A-to-G (T-to-C) transformation at five genomic sites in porcine fetal fibroblasts (pff). With these five editors, variable but considerable editing efficiency and variable active Windows are observed in these target areas. The strategy of using two sgRNAs in one vector showed higher editing efficiency than using two separate sgRNA expression vectors. Abe-mediated mutations in APOE's start codon silenced its protein expression and unexpectedly eliminated the vast majority of its mRNA. No off-target DNA sites for these editors were detected. There were a large number of off-target RNA events in ABE-edited cells, but no significant enrichment of the KEGG pathway was found. Our study supports ABEs as A powerful tool for A-to-G (T-to-C) point mutation modification in porcine cells[59].

**Applications of mammalian stem cells:** Sickle cell

disease (SCD) is caused by mutations in the beta-globin gene HBB1[60]. The researchers used (ABE8e-NRCH) to convert the SCD allele (HBBS) to Makassar beta-globin (HBBG), a non-pathogenic variant[40, 61-63]. In vitro, delivery of mRNA and targeted guide RNA encoding the base editor into hematopoietic stem cells and progenitor cells (HSPCs) of patients with SCD resulted in 80% of HBBS being converted to HBBG. Gregory A. Newby et al. showed that one-time autotherapy with SCD eliminates pathogenic HBBS, produces benign HBBG, and minimizes the adverse consequences of double-stranded DNA breaks[64]. Delivering a programmed endo-nucleotide enzyme, such as the Cas9-sgRNA RNP complex, into hematopoietic stem cells allows for efficient genome editing, particularly through non-homologous end junction (NHEJ) repair, which may help cure blood diseases[65-69].

Delivering a programmed endo-nucleotide enzyme, such as the Cas9:sgRNA RNP complex, to hematopoietic stem cells enables efficient genome editing, particularly through nonhomologous end junction (NHEJ) repair, which may help cure blood diseases, The researchers focused on purifying the A3A (N57Q)-BE3 protein for electroporation of human peripheral blood (PB) ribonucleoprotein (RNP) for mobilization of CD34+ hematopoietic stem cells (HSPCs). Frequently targeted cytosine base editing was observed on the BCL11A +58 red cell enhancer, and fetal hemoglobin (HbF) induction in erythroid offspring was similar after base editing or nuclease editing. Single therapeutic base editing of the BCL11A enhancer prevented sickle cells and improved globin chain imbalances in erythroid descendants of HSPCs derived from patients with sickle cell disease (SCD) and beta-thalassemia, respectively. In addition, efficient multi-editing can be achieved by destroying the BCL11A red enhancer and correcting the HBB-28A >G promoter mutation. Finally, base editing can be produced in multiline regenerative self-renewing human hematopoietic stem cells with a high frequency, producing an effective induction of HbF *in vivo*. The results demonstrate for the first time the potential of RNP base editing in human hematopoietic stem cells as a viable alternative to nuclease editing for targeted therapeutic genome modification in hematopoietic stem cells[70].

Duchenne muscular dystrophy (DMD) is a fatal X-linked recessive disease caused by mutations in the DMD gene encoding the dystrophin protein[71]. Most of the mutations that cause DMD occur in the "hot spot" region [72] that contains exons 45-55 of the DMD gene that code for the central rod-like domain of the protein. Mutations in the DMD gene are most commonly single or multiple exon deletions, which disrupt the open reading frame (ORF) and introduce premature stop codons, resulting in the production of dystrophin without functional truncation and resulting in a severe muscular degeneration phenotype[73].In human induced pluripotent stem cell (iPSC) -derived cardiomyocytes, mouse models, and large animal models with DMD mutations, muscle editing restored the production of a truncated but functional dystrophin protein. These muscle editing strategies aim to "reconstruct" the correct ORF of dystrophin transcripts by introducing small insertions and deletions (INDELs) through non-homologous end junctions (NHEJ) of double-stranded DNA breaks (DSBS) produced by CRISPR-Cas9. Recovery of ORF can also be accomplished by exon jumping, by "single cutting" the introduction of a single guide RNA (sgRNA) of a large INDELs at the splicing receptor site (SAS) or splicing donor site (SDS), or by introducing a "double cut" using two sgRNAs and removing one or more exons[74-78].

**Discussion and prospects:** Genetic improvement and genetic manipulation are essential tools in advancing agriculture, medicine, and biotechnology[96;97]. Genetic improvement causes the improvement of desirable qualities/traits in animals, plants, and microorganisms by selective breeding or advanced genomic techniques to improve productivity, resistance to disease, and adaptability to the environment. Genetic manipulation, which entails precise changes at the DNA level, enables targetedimprovements, i.e. pest-resistant cr ops and gene therapies for the treatment of genetic disorders [98]. These technologies contribute to food security, sustainable development, and medical breakthroughs, which are solutions to some of the world's biggest health, agricultural, and environmental conservation challenges [99].

In the past, it seemed impossible to efficiently change genetic information at the level of a single base of an organism, but base editors have made this idea a reality. Among the currently known pathogenic mutations in humans, the largest class is point mutation, also known as Single-nucleotide variant (SNV)[79, 80], which is associated with about 2/3 of human diseases. At the same time, SNV is also a major genetic variation affecting livestock traits (such as growth, development, and fertility). Therefore, it is important for human health and animal genetic breeding as well as genetic basic research to continue to develop technologies that optimize, accurately, and efficiently realize base mutations.

With the development of base editing technology, there are also many problems. First, the purity of editing products. The results of the first few studies of CBE showed that the C > R (G or A) transition was observed at certain sites in the genome, and the C > R base transition reduced the purity of base editing products[81-83]. The purity of adenine-based editing products in the ABE system is very high. To date, no A > Y (T or C) editing events have been reported [84], possibly because the cell's ability to remove inosine (I)

from genomic DNA is much weaker than that of uracil (U)[85].

The second is that base editing also produces Indels. Low-frequency Indels can be generated by CBE base editing because UNG in the cell can cut U to form an Apyrimidinic site (AP), and the AP site will create a gap in the editing chain under the action of AP lyase [43]. It then forms a DSB with the nCas9 incision in the non-editing chain and then enters the NHEJ repair pathway that produces Indels easily. Komor et al. found that in addition to increasing the purity of the edited products, the incidence of Indels was also reduced in UNG knockout cells. Komor et al. fused phage MU-derived Gam protein (Mu-GAM) with BE4 to generate BE4-GAM. Compared with BE4, BE4-GAM can further reduce the incidence of Indels in HEK293T cells, because Gam protein can bind to the end of DSB to prevent its degradation and thus prevent the occurrence of NHEJ [25]. In rabbit embryo experiments, BE4-Gam significantly reduced the incidence of Indels and increased the purity of edited products compared with BE3[24, 86].

In the experiments of cell lines[87] and mice [88], the incidence of ABE system Indels was very low, generally less than 1%, and Indels were not even detected in some experiments. ABE produces fewer Indels because it lacks the glycosidase needed for DNA repair, so it does not cut out I and creates incisions in the DNA editing chain [12]. Because intracellular I is removed much less efficiently than U, fewer incisions are made in the editing chain, so ABE has a lower incidence of Indels than CBE.

The third is PAM sequence restriction. Since CRISPR endonucleases require a specific proto-spacer adjacent motif (PAM) on either side of the target site, their target sequence space is limited. As a result, many derivative base editors have been developed. Pranam Chatterjee et al. demonstrated the natural PAM plasticity of highly similar but previously uncharacterized Cas9 (ScCas9) from Streptococcus Canis (ScCas9) through rational manipulation of differentiating motif insertion[18]. Chen's lab used Cas12a, or Cpf1, to develop A CBE system that recognizes the PAM sequence as TTTV (V can be A, C, or G), which works for T-rich genomic DNA[89]. In addition, Cas9 and mutants that recognize different PAM (SaCas9[48], Sa(KKH)Cas9, Sp(VQR)Cas9, and Sp(VRER)Cas9[90, 91], have been developed for use in different ABE base editors. The creation of these editors greatly increased the scope of ABE's editing. The fourth is the problem of editing windows and adjacent sites. The presence of more than one editable C or A in the editing window causes editing of bases other than the target base. The term "Bystander editing" is used to describe base editing events that occur in the sgRNA region other than the target site. When the purpose of base editing is to disrupt promoters, mRNA splicing sites, or other regulatory sequences, or to introduce early termination codons, the occurrence of adjacent base editing in non-CDS regions may be irrelevant. However, when editing the CDS region of functional protein genes, the editing of non-target bases in the editing window will lead to changes in the structure and function of target proteins, especially for gene therapy. The fifth is the background dependence of editing sequences: at some sites that contain GC sequences, rAPOBEC1 is less efficient at deamination[11, 92] DNA methylation at CpG can reduce the efficiency of RapoBEC1-mediated base editing, but human APOBEC3A (hA3A) can edit the C in CpG more effectively, and the editing efficiency is higher than rAPOBEC1[93]. So far, the editing results of ABE7.10 in mammalian cells show that there is no sequence background dependence when ABE7.10 is edited in human cells, but the research results of Kim and his colleagues show that ABE7.10 in *Arabidopsis thaliana* shows a certain editing preference, compared with GA, CA or AA sequences. ABE7.10 preferentially selects A in the edit TA[36].

The application of base editing in livestock breeding mainly focuses on solving genetic diseases caused by base mutation, or using base editing technology to build animal models of animal diseases in livestock and poultry, verifying gene interaction and signaling pathway correlation, and mainly focuses on solving economic traits such as milk production, meat production and hair production of various livestock and poultry such as pigs, cattle, sheep and chicken[94]. Using SCNT to study the ability of these Be3-edited cells to develop *in-vivo*, it was demonstrated that all TWIST2 piglets were confirmed by sequencing to contain the same base switch and that they exhibited similar expected phenotypes as human patients, including eyelid loss, microstomy, macrostomy, hypotrichosis, and abnormal hooves. Zhou et al and Li et al used BE3 to edit SOCS2 and FGF5 genes and successfully obtained mutant sheep with increased growth indicators such as body weight, body size, and hair length[48, 51]. The researchers used the TWIST2 and TYR genes to measure the efficiency of BE3 base conversion; The main direction of research based on mice and humans is to work on human gene therapy. Chadwick et al. used BE3 to generate a W159X stop codon mutation in the mouse Pcsk9 gene and found that the editing efficiency of liver cells was about 25%, and observed a significant reduction in plasma PCSK9 protein levels and plasma cholesterol after 4 weeks[95]. Delivery of codon-optimized CBE into patient-derived fibroblasts in the form of a plasmid can correct the L119P mutation in the MPDU1 gene that causes congenital glycosylation disorder of type 1f[25].

The goal of base editing technology development is to minimize off-target while maximizing base editing efficiency and target range so that it can be applied to more complex research. For most base editing,

the target sequence is fixed, and the future development of base editing focuses on how to improve the specificity and accuracy of base editing. Because of the off-target effect of the whole genome of CBE, more new CBE systems need to BE developed to reduce the off-target editing efficiency of the whole genome as much as possible and improve the safety of the BE system without affecting the editing efficiency of the target site. In short, base editing technology has broad application prospects in the fields of life science basic research, human disease treatment, and biological breeding, and the continuous innovation of this technology will promote the rapid development of various fields.

**Competing interests:** The authors declare that they have no competing interests.

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