Comparison of gene disruption induced by cytosine base editing-mediated iSTOP with CRISPR/Cas9-mediated frameshift

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Abstract
Objectives: Recently developed CRISPR-dependent cytosine base editor (CBE), converting four codons (CAA, CAG, CGA and TGG) into stop codons without DNA double-strand breaks (DSB), serves as an efficient gene disruption strategy besides uncontrollable CRISPR-mediated frameshift. However, the detailed difference of gene knockout between the two systems has not been clarified.

Materials and methods: Here, we selected some sgRNAs with different position background, then HEK293T cells were transfected with CBE/Cas9 plasmids together with sgRNAs. GFP-positive cells were harvested by fluorescence-activated cell sorting (FACS) 48 hours after transfection. Genomic DNA was collected for deep sequencing to analyse editing efficiency and genotype. RNA and protein were extracted to analyse gene mRNA level using qPCR analysis and Western blot.

Results: Here, we compared the gene disruption by CBE-mediated iSTOP with CRISPR/Cas9-mediated frameshift. We found BE-mediated gene knockout yielded fewer genotypes. BE-mediated gene editing precisely achieved silencing of two neighbours genes, while CRISPR/Cas9 may delete the large fragment between two target sites. All of three stop codons could efficiently disrupt the target genes. It is worth notifying, Cas9-mediated gene knockout showed a more impact on neighbouring genes mRNA level than the BE editor.

Conclusions: Our results reveal the differences between the two gene knockout strategies and provide useful information for choosing the appropriate gene disruption strategy.

1 | INTRODUCTION

CRISPR/Cas9 is now a powerful genome editing toolkit in gene modification for cells,1-4 animals1,4 and plants.1-4 Non-homologous end joining (NHEJ) following with CRISPR/Cas9-mediated double-strand break (DSB) can lead to the frameshifts, including introduction of insertions, deletions, translocations or other DNA rearrangements at the site of a DSB, and then result in gene knockout.5-7 But CRISPR/Cas9 gave rise to a significant increase in apoptosis,8 possibly owing to DSB-induced toxicity.9-11 Furthermore, Grégoire Cullot and
co-workers found unexpected chromosomal truncations resulting from only one Cas9 nuclease-induced DSB in cell lines and primary cells by a p53-dependent mechanism. By analysing post-transcriptional and post-translational effects of frameshift-inducing insertions or deletions (indels) in a panel of CRISPR-edited cell lines, Lum, L. and his colleague observed changes in the array of transcripts or proteins expressed from CRISPR-targeted genes in ~50% of the cell lines studied. Recently, cytosine base editor (CBE) mediates the direct conversion of a C•G base pair to T•A base pair, providing a new technique to precisely edit target genes. Moreover, CBE can install premature stop codons to disrupt genes by precisely converting four codons (CAA, CAG, CGA or TGG) into stop codons, which provides a new method for gene knockout without the potential side effects resulting from double-strand DNA cleavage.

Although gene disruption has been achieved by CBE-mediated iSTOP and CRISPR/Cas9-mediated frameshift, the detailed difference of gene knockout between the two systems has not been clarified. Here, we selected 13 sgRNAs with different position background to thoroughly compare the two systems by analysing the genotype, gene expression using deep sequencing, qPCR and Western blot. We also detected the editing results using two adjacent sgRNAs.

### 2 | MATERIALS AND METHODS

#### 2.1 | Cell culture and transfection

HEK293T cells were cultured in Dulbecco’s Modified Eagle Medium (DMEM) (HyClone, SH30243.01) supplemented with 10% foetal bovine serum (FBS) (v/v) (Gemini, 900-108) and 1% penicillin streptomycin (v/v) (Gibco, 15140122). BE3 plasmid was obtained from Addgene (Addgene, 73021). sgRNA oligos were annealed into pGL3-sgRNA-EGFP expression vector with U6 promoter (Addgene, 107721). Transfection was performed according to the manufacturer’s protocols (Thermo Fisher Scientific, 11668019). In brief, HEK293T cells were seeded on Poly-L-lysine solution (Sigma, P4707) coated 24-well plates (JETBIOFIL, TCP010012), and transfection was performed at approximately 70% density about 14 hours after seeding. 333 ng sgRNA plasmids and 666 ng BE3/Cas9 plasmids were transfected with 2 μL Lipofectamine 2000 (Thermo Fisher Scientific, 11668019). The medium was replaced with fresh medium 6 hours after transfection, and GFP-positive cells were harvested by fluorescence-activated cell sorting (FACS) 48 hours after transfection. Cells were incubated at 37°C with 5% CO₂. sgRNAs used are listed in Table S1.
FIGURE 2  CRISPR/Cas9-mediated frameshift yields more genotypes than BE-mediated iSTOP. (A) The used genes and related sgRNA sequences. PAM sequences are highlighted in green. The targeting sequences are underlined. (B) The editing efficiency of BE3-edited cell populations. Cell populations were the top 25% of GFP-positive collected by FACS. (C) The indel frequency of Cas9-edited cell populations. Cell populations were the top 25% of GFP-positive collected by FACS. (D) The number of genotypes is obtained from BE3-mediated iSTOP or CRISPR/Cas9-mediated gene editing. The genotypes were counted after filtering genotypes counted less than 0.1%. Data were analysed by Student’s t test (**P < .01, ***P < .001, ****P < .0001, ns-not significant) and shown as mean ± SEM (n = 3 from three independent experiments). (E) Summary of identified monoclonal cells and homozygous cells edited by BE or CRISPR/Cas9. (F) The kinds of genotype for CRISPR/Cas9 edited cells.
2.2 | Genomic DNA extraction and PCR amplification

Genomic DNA of cells collected by FACS was extracted using QuickExtract™ DNA Extraction Solution (Lucigen, QE09050) according to the manufacturer’s protocols. All the primers used for PCR amplification can be found in Table S2.

2.3 | Real-time quantitative PCR (qPCR) analysis

Total RNA from knockout cell lines was isolated using the TRIzol reagent (Invitrogen, 15596-018) and 1 μg of total RNA, in 20 μL mixed reverse-transcription reagent, was reverse transcribed using HiScript Q RT SuperMix with gDNA wiper (Vazyme, R223-01). The specific primer pairs (Table S3) were used for qPCR amplification utilizing the Viia™ 7 Real-Time PCR System (Applied Biosystems). The qPCR reaction includes 1 μL of cDNA templates, 10 μmol/L of each specific primer and 10 μL 2 × ChamQ SYBR qPCR Master Mix (Vazyme, Q331-02). The reaction procedure was set as follows: 95°C for 3 minutes and 40 cycles of 95°C for 15 seconds followed by 60°C for 1 minute. Relative quantification of the target gene expression was calculated using 2-ΔΔct method. Each reaction was performed in triplicate, and the data were calculated as M ± SD.

2.4 | Deep sequencing analysis

The primers for deep sequencing were listed in Table S4. The touchdown PCR reaction procedure was set as follows: 95°C for 3 minutes and 10 cycles of 95°C for 15 seconds, 68°C for 30 seconds followed by 72°C for 30 seconds; then 25 cycles of 95°C for 15 seconds, 58°C for 30 seconds followed by 72°C for 30 seconds.16 The purified PCR products were sequenced using Hiseq X-10 (2 × 150) platform. The deep sequencing data were processed using BWA-MEM algorithm. All the sequencing data were deposited in the National Omics Data Encyclopedia (NODE) under the project accession OEP000254.

2.5 | Western blot analysis

Western blot analysis was performed according to the manufacturer’s protocols. The used antibodies include anti-p53 (Santa

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**FIGURE 3** Genotype analysis of target genes edited by CRISPR/Cas9. The top five genotypes were shown. The sgRNA targeting sites are underlined. The PAM sequences are highlighted in green, the insertions and deletions in red.
Both BE3 and CRISPR/Cas9 can effectively knock out the targeting genes

Firstly, two sgRNAs targeting TP53 loci, a well-established tumour suppressor, were selected and both of the two sgRNAs contain the potential stop codon. Then, sgRNA expression plasmids were transfected into HEK293T cells together with BE3 or Cas9 plasmids. The GFP-positive cells were harvested 48 hours later using FACS and genomic DNA was extracted to detect the editing efficiency by Sanger sequencing. The results showed both BE3 and CRISPR/Cas9 could edit the target sites. BE3 induced the stop codon while CRISPR/Cas9 produced the indels at the target sites (Figure 1A). To further analyse the knockout effects, we sorted single cells for TP53-sg1 and got a pure clone (#16) for BE3, and two clones for CRISPR/Cas9 edited cells (#3, #4) (Figure 1B). Then qPCR was performed to determine the mRNA level of TP53. Using three pairs of specific primers (termed front, middle and back related to the position of sgRNA), the mRNA level of TP53, respectively, showed more than 90 per cent decline \((P < .001)\) in BE3-edited cell lines compared with the wild-type cells. While in Cas9-edited cell lines, the gene mRNA level did not show a significant difference compared with the wild-type cells (Figure 1C). Considering the different mechanism of gene knockout induced by the two systems, we performed Western blot analysis of the three obtained cell lines to demonstrate the knock out effects at protein level, and the results showed no p53 protein was detected, indicating both BE3 and CRISPR/Cas9 could thoroughly knock out the target genes (Figure 1D).
3.2 CRISPR/Cas9 results in more genotypes than BE3

Considering the uncontrollability of Cas9-mediated genotypes, we analyse the genotypes obtained from BE3 or Cas9 edited cells. Three sgRNAs for TGFβ1, one sgRNA for TP53, one sgRNA for AKT1 and three sgRNAs for STAT1 (Figure 2A) were selected to co-transfect HEK293T cells with BE3 or Cas9 plasmids. GFP-positive cells were harvested using FACS, genomic DNA was extracted, and the target sequences were amplified for deep sequencing. Both the BE3-mediated iSTOP mutation (Figure 2B) and Cas9-mediated indel (Figure 2C) showed a high efficiency with the specific sgRNAs. The genotypes were counted after filtering genotypes which counted less than 0.1%. The results showed mean number of triplicates in BE3-edited groups was 27, 33, 23, 48, 14, 52, 41 and 35, respectively. While in Cas9-mediated editing groups, the paralleled quantities were 72, 86, 42, 50, 53, 118, 116 and 58, respectively (Figure 2D). We summarized the number of detected monoclonal cells, homozygous cells and found the average ratio of homozygote in BE3-edited groups was 32.4%, while it was about 16.9% in Cas9 groups, suggesting it is easier to get homozygous genotype for BE3 edited cells (Figure 2E). Furthermore, almost all the genotypes induced by BE3 were base substitution (Figure S1). While the insertions or deletions were observed in CRISPR/Cas9 group (Figure 3). Considering the 3n indels may not thoroughly disrupt targeting genes, we divided genotypes into 3n, 3n + 1 and 3n + 2. It is interesting to find the ratio for these three kinds of genotype is distributed randomly. The 3n genotypes counted about 30% in STAT-1 and STAT-2 groups (Figure 2F).

3.3 Knockout of two neighbouring genes

Considering Cas9/dual sgRNAs may induce fragment deletion, which may affect simultaneous multiple gene disruption, we tried to disrupt two neighbouring genes simultaneously by BE3-mediated iSTOP. We selected two sgRNAs, respectively, targeting SPNS1 and LAT loci, which are 57 bp away, and the distance of two used sgRNAs is 2619 bp (Figure 4A). When BE3 plasmid was co-transfected with SPNS1 sgRNA or LAT sgRNA, the amplified products were 3274 bp, as the same size as the control group (Figure 4B). Then, the products were gel extracted for DNA sequencing. The Sanger sequencing results showed that
FIGURE 6  BE-mediated gene disruption affected neighbouring genes less than CRISPR/Cas9. (A) The relative expression of neighbouring genes around STAT1 gene. (B) The relative expression of neighbouring genes around TGFβ1 gene. Three sgRNAs for each target gene and three clones for each sgRNA were detected by qPCR. Error bar shown as mean ± SEM (n = 3 from three independent experiments)
BE3 produced the stop codon for each target site (Figure 4C). The similar size of PCR products was also observed when Cas9 plasmid co-transfected with SPNS1 sgRNA or LAT sgRNA, respectively (Figure 4B). From the sequencing results, Cas9 produced the indels for each target site (Figure 4C). When SPNS1 sgRNA and LAT sgRNA were co-transfected with BE3 plasmid, we also obtained a PCR product with 3274 bp. The sequencing results showed both two sites were also efficiently edited (Figure 4C), indicating co-transfection of BE3 plasmid with two sgRNAs simultaneously produced stop codons for two neighbouring genes. In contrast, for the CRISPR/Cas9 group, a 750 bp fragment was observed instead of the 3274 bp fragment when transfected with SPNS1 sgRNA and LAT sgRNA simultaneously (Figure 4B). It is worth noting that there were also exist other fragments (Figure 4B), implying the knock-out efficiency did not reach 100%. Then, we recycled the PCR product and analysed the genotype using TA cloning. The knock-out efficiency did not reach 100%. Then, we recycled the PCR product and analysed the genotype using TA cloning. The results revealed that co-transfection of Cas9 with SPNS1 sgRNA and LAT sgRNA gave rise to long fragment deletion between the two target sites as expected (Figure 4D). Taken together, BE3-mediated iSTOP instead CRISPR/Cas9-mediated frameshift is a good strategy for simultaneously targeting two close loci.

3.4 | No efficiency difference between stop codons

Since there may have different potential stop codons over a gene, we then asked if there is efficiency difference by different iSTOPs on the same target gene. To this end, we designed four sgRNAs to introduce different stop codons for APEX1 gene (Figure 5A). As expected, the APEX1 protein was successfully knockout in all four kinds of stop codon induced cell lines (Figure 5B-D), demonstrating three stop codons act identically to disrupt a gene without efficiency difference.

3.5 | CRISPR/Cas9 has more influence on neighbouring genes than BE3

To evaluate the influence on neighbouring genes of these two strategies. Three sgRNAs for STAT1 and TGFB1gene and four neighbouring genes for each gene were chosen for the test. The BE3 plasmids or Cas9 plasmids were co-transfected with corresponding sgRNAs, and three single-cell clones for each group were detected. Using these modified cell lines, the qPCR analysis was performed to detect the mRNA level of neighbouring and non-neighbouring genes. Fold change of 1 ± 0.3 was set as threshold. For STAT1 knockout cell lines, the mRNA level of gene GLS, NAB1, MFSD6 and INPP1 in BE3- and Cas9-edited cells were both affected compared with the WT group, but CRISPR/Cas9 caused much more fluctuations (Figure 6A). For example, in gene of INPP1 most of the Cas9-edited clones showed variation out of the scope. Similarly, CRISPR/Cas9 editing had more impact on the mRNA level of gene HNRNPUL1, CCDC97, EXOSC5 and B9D2 (Figure 6B). For non-neighbouring genes, their mRNA levels had no obvious variation trend, which changed within 1.5-fold in both STAT1- and TGFB1-knockout cell lines (Figure S2). In summary, BE-mediated gene knockout showed less side effects on neighbouring genes compared with CRISPR/Cas9 strategy.

4 | DISCUSSION

Both CBE-mediated iSTOP and CRISPR/Cas9-mediated frameshift have been widely used for gene disruption.22-25 Base editing systems provide a precise substitution of the C-to-T and A-to-G without DSB, thus give less deleterious effects to the genome.8,15 Here, we compared the gene disruption mediated by these two strategies carefully. As expected, BE mediated precise base substitution, then yielded fewer genotypes than Cas9-mediated frameshift, which edits genome uncontrollably, including Cas9-mediated 3n indels affect the gene disruption. Therefore, significantly fewer genotypes were obtained in BE3 groups compared with CRISPR/Cas9 group. Consequently, it is easy to get isogenic homozygous mutant cell colonies by BE-mediated iSTOP.

Dual sgRNAs have been used for fragment deletion by Cas9,18,19 we tested double knockout of two neighbouring genes and found CRISPR/Cas9 easily delete the large fragment between the two sgRNAs. In contrast BE only induced the stop codon at the target sites, resulting in precise and safe simultaneous disruption of two genes, suggesting BE-mediated iSTOP is the best choice for multiple genes knockout. Kuscu et al identified that iSTOP strategy had a significant reduction in apoptosis when compared with Cas9 nuclease treatment.8 Interestingly, we also found that BE-mediated knockout had less influence on the relative expression level of neighbouring genes. This may be caused by different level changes by BE- or Cas9-mediated knockout strategies. The BE3 editing cause genotype changing, similarly to single nucleotide polymorphism (SNP), having little effect on the chromosomal structure and neighbouring genes. CRISPR/Cas9 cause significant on-target mutagenesis, such as large deletions and more complex genomic rearrangements.6 Fluctuations in the mRNA level of neighbouring genes may be one of its manifestations.

In summary, we demonstrated the BE-mediated iSTOP disrupts gene more controllable with fewer side effects.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

AUTHOR CONTRIBUTIONS

Huang X and Cui S conceived and designed the project. Dang L, Li G, Wang X, Huang S, Zhang Y, Miao Y and Zeng L performed the
experiments. Huang X and Cui S supervised the project. Dang L and Li G analysed the data and wrote the paper.

DATA AVAILABILITY STATEMENT
All the sequencing data were deposited in the National Omics Data Encyclopedia (NODE) under the project accession OEP000254. The data that support the findings of this study are available on request from the corresponding author.

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SUPPORTING INFORMATION
Additional supporting information may be found in the Supporting Information section.

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