BIOTECHNOLOGY

Cytosine base editor generates substantial off-target single-nucleotide variants in mouse embryos

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Genome editing holds promise for correcting pathogenic mutations. However, it is difficult to determine off-target effects of editing due to single-nucleotide polymorphism in individuals. Here we developed a method named GOTI (genome-wide off-target analysis by two-cell embryo injection) to detect off-target mutations by editing one blastomere of two-cell mouse embryos using either CRISPR-Cas9 or base editors. Comparison of the whole-genome sequences of progeny cells of edited and nonedited blastomeres at embryonic day 14.5 showed that off-target single-nucleotide variants (SNVs) were rare in embryos edited by CRISPR-Cas9 or adenine base editor, with a frequency close to the spontaneous mutation rate. By contrast, cytosine base editing induced SNVs at more than 20-fold higher frequencies, requiring a solution to address its fidelity.

Genome editing holds great potential for treating genetic diseases induced by pathogenic mutations (*1*). A comprehensive analysis of off-target effects by genome editing is required for their utility (*2*). Multiple methods have been developed to detect genome-wide gene editing of off-target sites (*2*–*5*). However, these approaches are not applicable to detecting single-nucleotide variants (SNVs) in vivo. In this study, we developed a method named GOTI (genome-wide off-target analysis by two-cell embryo injection) to evaluate the off-target effects induced by CRISPR-Cas9, cytosine base editor 3 [BE3, rAPOBEC1-nCas9-UGI; a single protein consisting of the rat APOBEC1 (rAPOBEC1) cytosine deaminase tethered to Cas9 nickase (nCas9), which is covalently linked to uracil DNA glycosylase inhibitor (UGI)], and adenine base editor 7.10 [ABE7.10, TadA-TadA*-nCas9; a wild-type tRNA adenosine deaminase (TadA) and an evolved TadA* heterodimer fused to nCas9], three commonly used gene-editing tools (*6*–*8*). Briefly, we injected CRISPR-Cas9, BE3, or ABE7.10, together with Cre mRNA, into one blastomere of two-cell embryos derived from Ai9 (CAG-LoxP-Stop-LoxP-tdTomato) mice (*9*, *10*) (Fig. 1A). The progeny cells of the edited and nonedited blastomeres were then sorted by fluorescence-activated cell sorting (FACS) on the basis of tdTomato expression in gene-edited cells at embryonic day 14.5 (E14.5) (Fig. 1B), when the whole embryo could be readily digested to obtain enough single cells. Whole-genome sequencing (WGS) was then performed separately on the tdTomato$^+$ and tdTomato$^-$ cells. SNVs and indels were called by three algorithms in the tdTomato$^+$ sample, with the tdTomato$^-$ sample from the same embryo as the reference (Fig. 1A).

We included 12 groups in our study: one Cre group (Cre only), six Cas9 groups with or without single-guide RNA (sgRNA) (Cas9, Cas9-LacZ, Cas9-Pde6b, Cas9-Tyr-A, Cas9-Tyr-B, and Cas9-Tyr-C), three BE3 groups with or without sgRNA (BE3, BE3-Tyr-C, and BE3-Tyr-D) (*11*), and two ABE7.10 groups with or without sgRNA (ABE7.10 and ABE7.10-Tyr-E). First, we validated the on-target efficiency of our approach in embryos at the eight-cell and E14.5 stages by Sanger sequencing (figs. S1 to S3). To further explore the on-target efficiency and potential genome-wide off-target effects, we performed WGS at an average depth of 47× on 46 samples from 23 E14.5 embryos (table S1). The activities of Cas9, BE3, and ABE7.10 in tdTomato$^+$ cells were confirmed by high on-target efficiencies to induce indels and nucleotide substitutions (Fig. 1C, fig. S4, and tables S2 and S3).

For the off-target editing effects, we found only zero to four indels in embryos from all 12 groups (figs. S5 and S6 and tables S2 and S4), and none of them overlapped with the predicted off-target sites (fig. S5 and table S5). For all Cas9-treated embryos, there was no significant difference among different Cas9 groups (12 SNVs per embryo on average) or in comparison with the "Cre-only" group (14 SNVs per embryo on average) (figs. S7 and S8 and tables S2 and S6). The SNVs detected in the Cre- or Cas9-treated samples were likely caused by spontaneous mutations during genome replication during development, because the number of variants was within the range of simulated spontaneous mutations and no sequence similarity was observed between the adjacent sequences of the identified SNVs and the target sites (fig. S8 and methods) (*12*).

Surprisingly, we found, on average, 283 SNVs per embryo in BE3-treated embryos, a level at least 20 times higher than that observed in Cre- or Cas9-treated embryos (Fig. 2A, fig. S7, and tables S2 and S7). By contrast, ABE7.10 generated, on average, 10 SNVs per embryo, with a frequency close to the spontaneous mutation rate (Fig. 2A and table S2). We further compared the off-target sites identified in the "BE3-only" group with those of the BE3-Tyr-C or BE3-Tyr-D groups and found that the presence of sgRNAs did not induce significantly higher SNVs (P = 0.21, Kruskal-Wallis test). In addition, these variants were specifically identified in tdTomato$^+$ cells rather than in tdTomato$^-$ cells (see methods, fig. S9, and table S8). Notably, more than 90% of the SNVs identified in the BE3-edited cells were mutated from G to A or C to T, a mutation bias not observed in Cre-, Cas9-, or ABE7.10-treated cells (Fig. 2, B and C, and fig. S10). This bias was the same as that of cytosine deaminase APOBEC1 itself (*13*), indicating that these mutations were not spontaneous but induced by BE3 editing. Previous studies have shown that the action of several members of the APOBEC family (including APOBEC1) require single-stranded DNA (*14*–*16*). Consistently, our analysis showed that SNVs induced by BE3 were significantly enriched in transcribed regions (Fig. 3A), especially in genes with high expression (Fig. 3B and fig. S11). Interestingly, none of the off-target sites were shared by any of the BE3-treated embryos or overlapped with predicted off-target mutations (Fig. 3, C and D). In addition, no similarity was observed between the off- and on-target sequences, whereas the top predicted off-target sites showed high sequence similarity with BE3 on-target loci (fig. S12). Thus, the BE3 off-target SNVs were sgRNA-independent and likely caused by overexpression of APOBEC1.

Among 1698 SNVs in BE3-treated embryos, 26 were located on exons, 14 of which led to nonsynonymous changes (fig. S13). We successfully amplified 20 of them by polymerase chain reaction (PCR) and confirmed their presence by Sanger sequencing (fig. S14 and table S9). We also found that one SNV was located in a proto-oncogene and 13 SNVs were located in tumor

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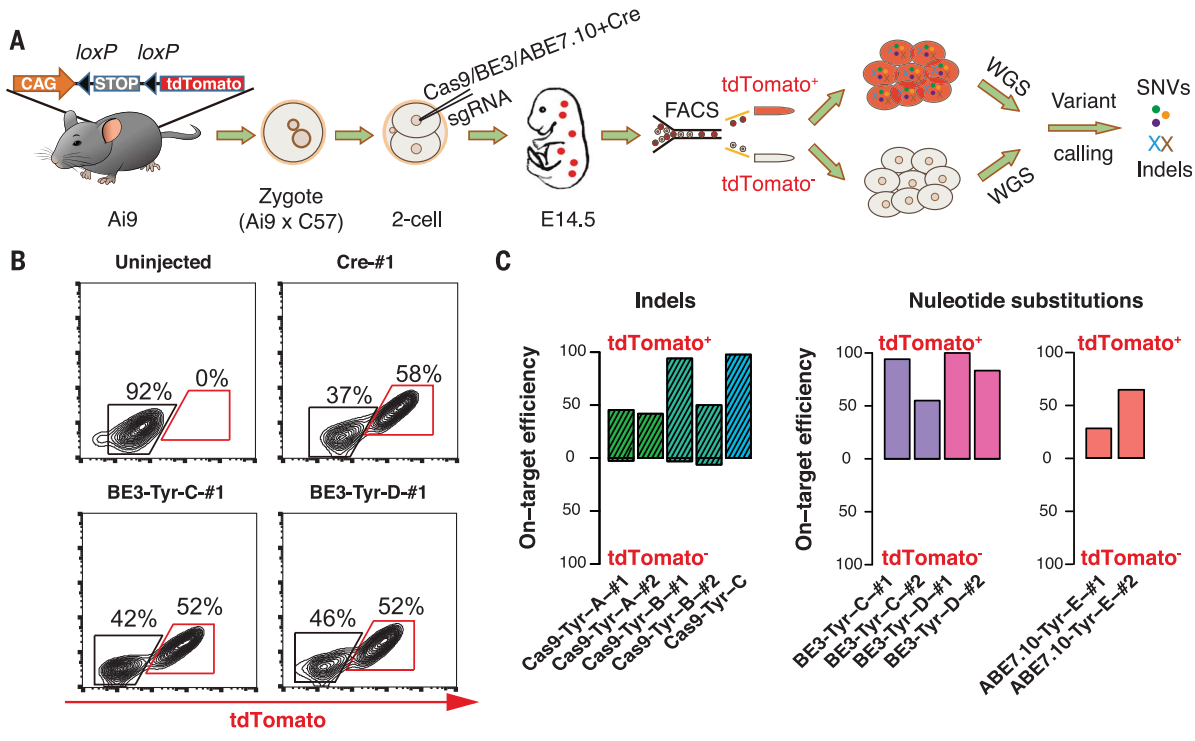

Fig. 1. CRISPR-Cas9–, BE3-, or ABE7.10-mediated gene editing in one blastomere of two-cell embryos. (**A**) Experimental design. C57, an inbred strain of mice. (**B**) FACS analysis in indicated embryos. (**C**) Percentage of on-target efficiency for tdTomato$^+$ and tdTomato$^-$ cells on the basis of WGS. On-target efficiencies of Cas9, BE3, and ABE7.10 in tdTomato$^+$ cells were 66 ± 12% SEM indels (n = 5), 83 ± 10% SEM nucleotide substitutions (n = 4), and 47 ± 18% SEM nucleotide substitutions (n = 2), respectively.

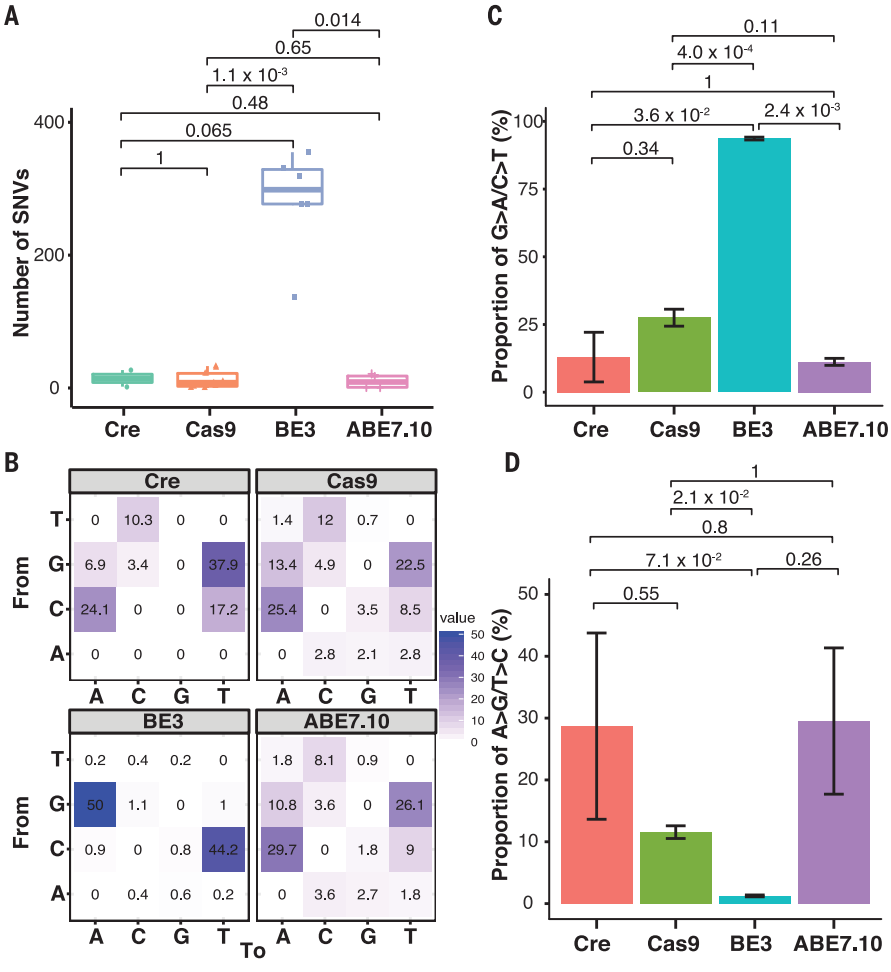

Fig. 2. Substantial off-target SNVs generated in BE3-treated mouse embryos. (**A**) Comparison of the total number of detected off-target SNVs. The number of SNVs for Cre-, Cas9-, BE3-, and ABE7.10-treated embryos were 14 ± 12 SEM (n = 2), 12 ± 4 SEM (n = 11), 283 ± 32 SEM (n = 6), and 10 ± 5 SEM (n = 4) SNVs, respectively. (**B**) Distribution of mutation types. The number in each cell indicates the proportion of a certain type of mutation among all mutations. (**C**) Proportion of G·C to A·T mutations for Cre, Cas9, BE3, and ABE7.10 groups. (**D**) Proportion of A·T to G·C mutations for Cre, Cas9, BE3, and ABE7.10 groups. Two Cre, 11 Cas9, 6 BE3, and 4 ABE7.10 samples were analyzed. In (A), (C), and (D), the P values shown above the horizontal bars were calculated by two-sided Wilcoxon rank sum test, and error bars indicate SEM.

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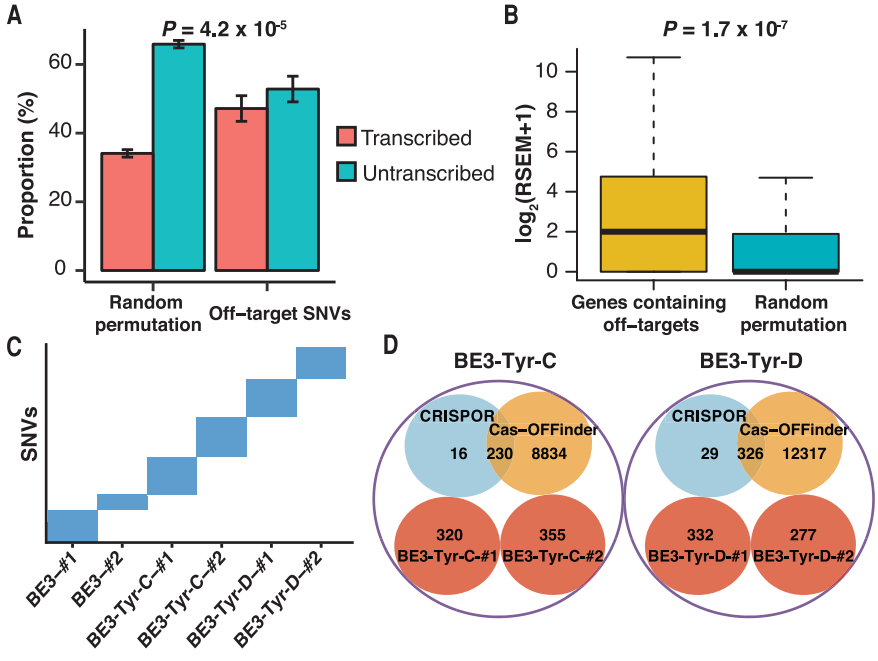

Fig. 3. Characteristics of BE3-induced off-target SNVs. (**A**) Off-target SNVs are enriched in the transcribed regions of the genome compared with random permutation. (**B**) Genes containing off-target SNVs were significantly more highly expressed than random simulated genes in four-cell embryos. RSEM, RNA sequencing by expectation maximization. (**C**) SNVs identified from each embryo were nonoverlapping. (**D**) Overlap among SNVs detected by GOTI with predicted off-targets by Cas-OFFinder and CRISPOR. In (A) and (B), *P* values were calculated by two-sided Wilcoxon rank sum test.

suppressors (fig. S13), raising the concern about the oncogenic risk of BE3 editing. This risk might be reduced by expressing lower amounts of BE3. However, we found that the on-target efficiencies were progressively reduced with the use of lower amounts of BE3 (fig. S15 and table S10).

Intriguingly, we found that numerous de novo SNVs are induced by BE3, which was not reported in previous studies. A possible explanation is that our method, GOTI, examines the cell population derived from a single gene-edited blastomere, whereas previous studies used large pools of cells for which editing is variable, resulting in loss of signal for random off-targets due to population averaging. Unlike BE3, ABE7.10 induced no increase in SNVs, probably owing to the lack of DNA-binding ability of TadA (*17*). These results are consistent with a similar study in rice plants (*18*). The off-target effects of base editors may be reduced by decreasing the DNA-binding ability of APOBEC1 or by using different versions of cytidine deaminase (*19*–*21*). In summary, GOTI could be useful for examining off-target effects of various gene-editing tools without the interference of single-nucleotide polymorphisms present in different individuals.

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ACKNOWLEDGMENTS

We thank M. Poo, D. Li, G. Xu, and K. Roy for helpful discussions and insightful comments on this manuscript, and the FACS facility at the Institute of Neuroscience (ION). **Funding:** R&D Program of China (2018YFC2000100 and 2017YFC1001302 to H.Y., and 2017YFC0908405 to W.W.), CAS Strategic Priority Research Program (XDB32060000), National Natural Science Foundation of China (31871502 and 31522037), Shanghai Municipal Science and Technology Major Project (2018SHZDZX05), Shanghai City Committee of Science and Technology project (18411953700 and 18JC1410100), and NIH P01 Center grant (P01HG00020527 to L.M.S.). **Author contributions:** E.Z. designed and performed experiments. Y.S., W.W., H.S., and L.Y. performed data analysis. T.Y. performed PCR analysis. W.Y. performed mouse embryo transfer. H.Y., Y.L., and L.M.S. supervised the project and designed experiments. All the authors wrote this report. **Competing interests:** The authors declare no competing financial interests. **Data and materials availability:** All the sequencing data were deposited in the NCBI Sequence Read Archive (SRA) under project accession no. SRP119022 and at www.biosino.org/node/project/detail/OEP000195.

SUPPLEMENTARY MATERIALS

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Materials and Methods
Figs. S1 to S15
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8 November 2018; accepted 17 February 2019
Published online 28 February 2019
10.1126/science.aav9973

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Science **364** (6437), 289-292.
DOI: 10.1126/science.aav9973originally published online February 28, 2019

Spotting off-targets from gene editing

Unintended genomic modifications limit the potential therapeutic use of gene-editing tools. Available methods to find off-targets generally do not work in vivo or detect single-nucleotide changes. Three papers in this issue report new methods for monitoring gene-editing tools in vivo (see the Perspective by Kempton and Qi). Wienert *et al.* followed the recruitment of a DNA repair protein to DNA breaks induced by CRISPR-Cas9, enabling unbiased detection of off-target editing in cellular and animal models. Zuo *et al.* identified off-targets without the interference of natural genetic heterogeneity by injecting base editors into one blastomere of a two-cell mouse embryo and leaving the other genetically identical blastomere unedited. Jin *et al.* performed whole-genome sequencing on individual, genome-edited rice plants to identify unintended mutations. Cytosine, but not adenine, base editors induced numerous single-nucleotide variants in both mouse and rice.

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