Molecular Therapy
Nucleic Acids
Original Article

Large-Fragment Deletions Induced by Cas9 Cleavage while Not in the BEs System

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CRISPR-Cas9 and base editors (BEs) systems are poised to become the gene-editing tool of choice in clinical contexts; however, large-fragment deletion was found in Cas9-mediated mutation cells and mice. In this study, by analyzing 16 gene-edited rabbit lines (including 112 rabbits) generated using SpCas9, BEs, xCas9, and xCas9-BEs with long-range PCR genotyping and long-read sequencing by the PacBio platform, we show the extension of thousands of base fragment deletions in single-guide RNA/Cas9 and xCas9 system mutation rabbits, but no deletions were found in BE-induced mutation rabbits. Thus, we first validated that no large-fragment deletion was induced by the BEs system, suggesting that BE systems can be beneficial tools for the further development of highly accurate and secure gene therapy for the clinical treatment of human genetic disorders.

INTRODUCTION

CRISPR-Cas9 is an RNA-guided DNA endonuclease system that targets specific genomic sequences and induces site-specific DNA double-strand breaks (DSBs), which can lead to the activation of the non-conservative non-homologous end joining (NHEJ) repair pathway.[1] However, although numerous studies have demonstrated the efficacy of engineered Cas9 nucleases in inducing DSBs at sites of interest, CRISPR-Cas9-mediated genome editing may generate unintended alterations.[2] Recent studies have confirmed that the CRISPR-Cas9 system can lead to significant on-target mutagenesis, such as frequent large-fragment deletions (kilobase scale) and even complex genomic rearrangements at target sites in gene-editing cells and mice.[3–6] In contrast, base editors (BEs), an ideal genome editing tool for genome modification and genetic disease therapies, can edit target genes and repair pathogenic mutations without generating DSBs or relying on template donor DNA.[7–9] Although Zuo et al.[10] showed that cytosine base editing induced SNVs at more than 20-fold higher frequencies by embryonic day 14.5, it was still unknown whether BE-mediated genome editing can cause fragment deletion until now. In this study, based on combined analyses of gene-edited rabbits that have been successfully created by our group,[11–14] we present the first evidence showing the frequency of large-fragment deletion in CRISPR-mediated, but not in BE-mediated, genome editing in animals.

RESULTS AND DISCUSSION

To investigate whether CRISPR-Cas9-mediated genome editing induces unexpected large-fragment deletion, 112 individual rabbits from 16 mutant rabbit lines were utilized in this study (Table 1). We categorized these rabbits as follows: (1) SpCas9-mediated mutant lines: F1, F2, S1, F3, G1 and S2; (2) xCas9-mediated mutant lines: T1 and T2; (3) BE-mediated mutant lines (BE4 and ABE systems): D1, M1, T3, T4, D2, and S3; and (4) xCas9/BE-mediated (xBE) mutant lines: T5 and T6. Negative control individuals were also selected for each group.

To examine whether the SpCas9-mediated gene-edited rabbit lines were carrying unexpected large-fragment deletions (Table 1), we selected a 6- to 6.5-kb region with primers that were equidistant from the guide RNA (gRNA) protospacer adjacent motif (PAM) sequences in 37 mutant rabbits and sequenced the PCR products via the PacBio platform, which is the newest single molecule, real-time (SMRT) sequencing platform that provides ultra-long sequencing reads with high consensus accuracy.[15] The results showed that multiple kilobase-scale depletions of read coverage were detected around the cut sites in most SpCas9-mediated mutant rabbits, consistent with the presence of large deletions (Figure 1A; F1, Figure S1; F2, Figure S2; S1, Figure S3; F3, Figure S4; G1, Figure S5; S2, Figure S6). Duplications ranging from 11 to 4.5 kb and large-fragment deletions (>100 bp) were detected in 24 out of 37 mutant samples (64.9%, Figure 1C; Table 1), suggesting that SpCas9-mediated gene mutation induces large-fragment deletions in rabbits.

Received 18 February 2020; accepted 22 June 2020;
https://doi.org/10.1016/j.omtn.2020.06.019.

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Table 1. Summary of the CRISPR-Induced Mutant Rabbit Lines in This Study

Method	System	Rabbits Lines	Gene	Positive Mutation Rabbits	Rabbit with Large Fragment Mutation (% Positive Rabbits)	Rabbits without Large Fragment Mutation (% Positive Rabbits)
Cas9	SpCas9	F1 (Figure S1)	*F8*	7	7 (100)	0
		F2 (Figure S2)	*F9*	6	2 (33.3)	4 (67.7)
		S1 (Figure S3)	*Sost*	5	3 (80.0)	2 (20.0)
		F3 (Figure S4)	*Fbn1*	7	6 (85.7)	1 (14.3)
		G1 (Figure S5)	*Ggta*	7	2 (28.6)	5 (71.4)
		S2 (Figure S6)	*Sry*	5	4 (80.0)	1 (20.0)
	xCas9	T1 (Figure S7)	*Tyr*	4	2 (50.0)	2 (50.0)
		T2 (Figure S8)	*Tyr*	7	4 (57.1)	3 (42.9)
BEs	BE4	D1 (Figure S9)	*Dmd*	7	0	7 (100)
		M1 (Figure S10)	*Mstn*	7	0	7 (100)
		T3 (Figure S11)	*Tia1*	7	0	7 (100)
		T4 (Figure S12)	*Tyr*	7	0	7 (100)
	ABE	D2 (Figure S13)	*Dmd*	7	0	7 (100)
		S3 (Figure S14)	*Sod1*	7	0	7 (100)
	xCas9-BE4	T5 (Figure S15)	*Tyr*	7	0	7 (100)
	xCas9-ABE	T6 (Figure S16)	*Tyr*	7	0	7 (100)

Moreover, Liu's group has published a new version of the SpCas9 variant, termed xCas9(3.7), which exhibited the broadest PAM compatibility and outstanding accuracy.[16] To test whether xCas9 induces large-fragment deletions in mutant rabbits, 14 rabbits from T1 and T2 lines were examined by PCR amplification and PacBio deep-sequencing analysis (Figures 1A and 1C). The results showed that 6 out of 11 rabbits had at least one 100-bp to 3-kb fragment deletion (54.5%, Table 1), indicating that large-fragment deletions were also observed in mutant rabbits generated via xCas9.

BE systems enable single-nucleotide substitutions efficiently without generating DSBs.[7–9] Because DSBs may induce complex chromosomal rearrangements, we speculated that BE systems could lead to a reduced frequency of large-fragment deletion. To confirm this hypothesis, 56 rabbits with BE4-, ABE-, xBE4- and xABE-mediated mutations were analyzed. The categorization of the experimental animals is listed in Table 1. After PCR amplification, the PacBio sequencing results confirmed that no fragment deletions were found in BE-mediated and xBE-mediated mutant rabbits (Figures 1B and 1D; D1, Figure S9; M1, Figure S10; T3, Figure S11; T4, Figure S12; D2, Figure S13; S3, Figure S14; T5, Figure S14; T6, Figure S16).

In summary, our data demonstrated that, in comparison with the CRISPR-Cas9 system, BEs do not induce DSBs and large-fragment deletions when mediating animal genome modification. Thus, BE systems can be beneficial tools for the further development of highly accurate and secure gene therapy for the clinical treatment of human genetic disorders.

MATERIALS AND METHODS

Ethics Statement

The rabbits used in this study were New Zealand White. All animal studies were conducted according to the experimental practices and standards approved by the Animal Welfare and Research Ethics Committee at Jilin University.

Mutation Screening in Rabbits

The gene-edited rabbits were successfully created in previous research by our group.[11–14] The protocol for mutation detection has been described previously.[17] DNA from knockout rabbits was isolated using the TIANamp genomic DNA kit (Tiangen, Beijing, China) according to the manufacturer's instructions. The PCR products were gel-purified and cloned into pGM-T (Tiangen, Beijing, China). At least 30 positive plasmid clones were sequenced by Comate Bioscience (Changchun, China) with Sanger sequencing and analyzed using commercial software (DNAMAN, Lynnon Biosoft, San Ramon, CA, USA, and the basic local alignment search tool, National Center for Biotechnology Information, Bethesda, MD, USA).

Large-Fragment Mutation Detection by PCR Assay

Genomic DNA was extracted from mutation and control rabbits. The DNA was amplified with Q5 high-fidelity 2× master mix (#E0555L, NEB, USA). Primers for large-fragment mutation detection were designed using Primer3-BLAST (Table S1). The PCR products were purified using a universal DNA purification kit (Tiangen, Beijing, China) according to the manufacturer's instructions and then analyzed by PacBio Sanger sequencing.

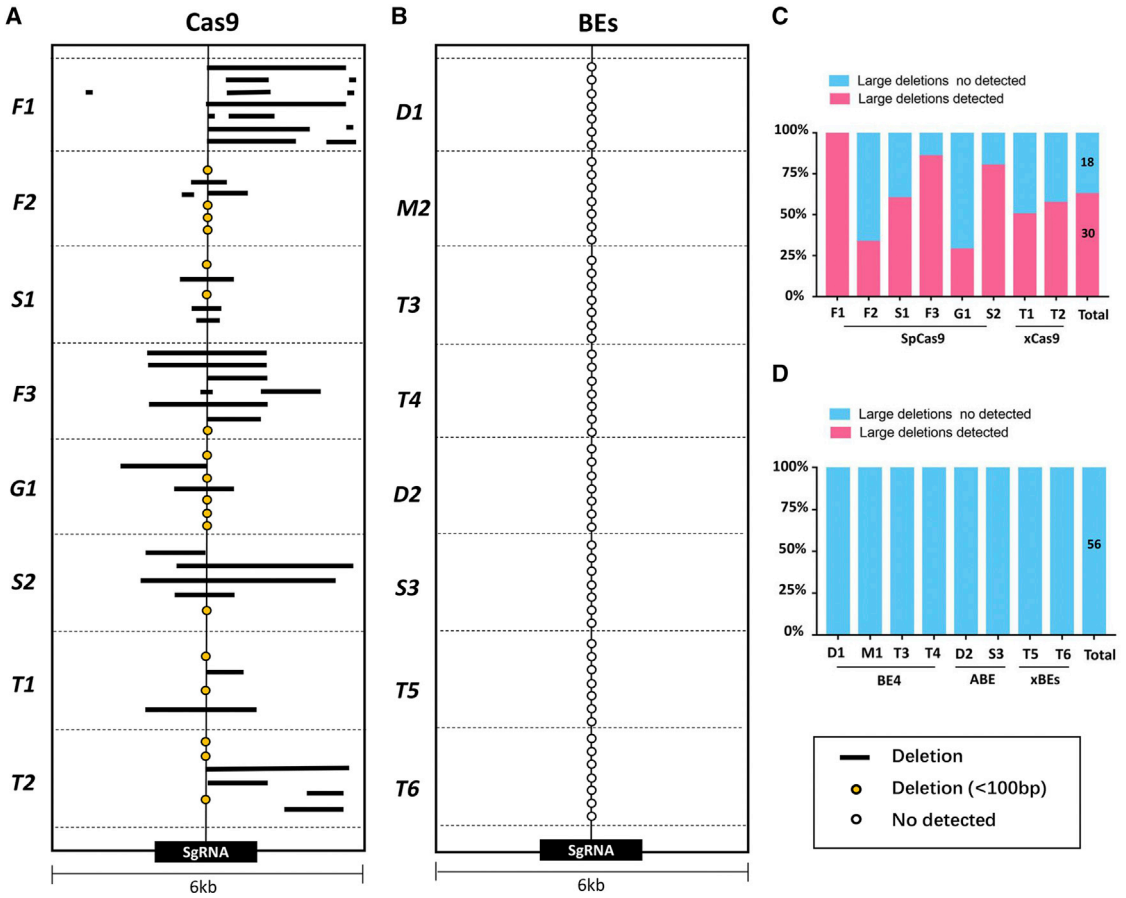

Figure 1. Analysis of Large Deletions in Rabbits after Cas9- and BE-Mediated Genome Editing

(A and B) Analysis of fragment deletions in rabbits after Cas9-mediated genome editing (A) and BE-mediated genome editing (B). The position of the gRNA is shown as a vertical line with intersections. Intended deletions (<100 bp) are indicated with orange circles. Black lines represent deletions (>100 bp). White circles represent individuals without deletions. The dotted horizontal lines separate the various rabbit lines. (C and D) The percentages of founders that contained large deletions detected by long-range PCR (6 kb) in rabbits after Cas9-mediated genome editing (C) and BE-mediated genome editing (D).

Bioinformatics

Alignment of Sanger-sequenced PCR products was performed using BLAT (v36) and converted into BAM format using a customized script from T. Marschall (https://github.com/ALLBio/allbiotc2/tree/master/synthetic-benchmark). Mixed traces were resolved using Poly Peak Parser.[18] Analysis of PacBio data was performed using the command line version of SMRT Link software (pbtranscript 1.0.1.TAG-1470) and the "Running Iso Seq using SMRT Link" tutorial on GitHub. The resulting alleles were mapped to the reference genome using BWA-MEM (v0.7.17-r1188). The mapped reads were clustered furthered using a custom script. Genome coverage was calculated with "bedtools genomecovdz" (v2.27.1).

Data Availability

The sequence data from this work have been deposited in the Sequence Read Archive under accession code SRP192942. The authors state that all data necessary for confirming the conclusions presented in the article are represented fully within the article or available from authors upon request.

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.omtn.2020.06.019.

AUTHOR CONTRIBUTIONS

Y.S., Y.Z., M.C., and T.S. performed the experiments. Y.S. and Z.L. conceived the idea and provided funding support. Y.S. and Y.Z. wrote the manuscript. All authors reviewed the manuscript.

CONFLICTS OF INTEREST

The authors declare no competing interests.

ACKNOWLEDGMENTS

The authors thank Peiran Hu for excellent technical assistance at the Embryo Engineering Center. This study was supported by the

National Key Research and Development Program of China Stem Cell and Translational Research (2019YFA0110702 and 2017YFA0105101) and by the Strategic Priority Research Program of the Chinese Academy of Sciences (XDA16030501 and XDA16030503).

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