

Optimization of the base editor BE4max in chicken somatic cells

Tianpeng Xu,¹ Jing Zhong,¹ Zhenwen Huang, Lintian Yu, Jitan Zheng, Long Xie, Lingling Sun, Xingting Liu, and Yangqing Lu²

State Key Laboratory for Conservation and Utilization of Subtropical Agro-bioresources, College of Animal Science and Technology, Guangxi University, Nanning, 530004, China

ABSTRACT Advanced animal reproductive and breeding biotechnology has made it possible to alter traits or create new genetic resources by the direct knock-in or knock-out of target genes. Base editing technology can achieve single-base mutations without double-stranded DNA breaks, and is a promising tool for use in the genetic modification and breeding of livestock. However, the application of base editors (BEs) in chicken has not been optimized. We evaluated the efficacy of BE4max in chicken somatic cells (DF-1). The

key element of BE4max, cytosine deaminase (APO-BEC), was optimized for chicken. The base editing efficiency of the optimized chBE4max editor, compared with the original BE4max editor, was improved by $10.4\% \pm 4.6$. By inhibiting the expression of the uracil DNA glycosylase-related gene methyl binding domain protein 4 (*MBD4*) by siRNA in chicken DF-1 cells, the editing efficiency was enhanced by $4.43\% \pm 1.4$ compared to the control. These results suggest that this editor may have applications in poultry breeding studies.

Key words: base editing, BE4max, codon optimization, *MBD4*, chicken

2022 Poultry Science 101:102174

<https://doi.org/10.1016/j.psj.2022.102174>

INTRODUCTION

Chicken is an important agricultural animal that provides meat and eggs. The production efficiency of the chicken industry has improved in recent decades. Traditionally, chicken breeding relied on natural and human selection. The genetic improvement in livestock is driven by the selection of favorable traits. With the continuous development of livestock industry, the contribution of animal varieties to animal husbandry production has been recognized. Breeding has been facilitated by innovations in genome-editing technology (Tyagi et al., 2020). Gene-edited plants and animals with improved productivity are also increasingly gaining acceptance (Frewer et al., 1997).

Base editing techniques that enable precise single-base editing without double-strand DNA breaks and homologous templates were first reported in 2016 (Komor et al., 2016). Several base editors with high safety and specificity have since been developed, including BE2, BE3, and BE4. The fourth-generation cytosine base editor BE4max can precisely convert C-G to T-A (Rees and Liu, 2018). More recently, Lee et al. (2020) introduced a

premature terminal codon into ovotransferrin (*TF*) and myostatin (*MSTN*) gene using a base editor and produced heritable progeny. This result demonstrated the application potential of base editing technology in chicken breeding. BE4max was initially developed and validated on a murine model and it was then optimized for mammals. BE4max induced 1.8-fold higher editing than BE4, which corrected pathogenic single-nucleotide polymorphisms (SNPs) in many mammalian cell types with improved efficiency (Koblan et al., 2018).

However, the efficacy and efficiency of BE4max in chicken is unexplored to determine the application of this base editor in avian species. In this study, we demonstrated that BE4max can convert C-G to T-A in the genome of chicken somatic cells. Improved gene editing efficiency was achieved after codon optimization according to the chicken bias. Inhibition of the expression of *MBD4*, a DNA glycosylase in the base excision repair (BER) pathway, improved the efficiency of base editing. The exploration of chBE4max in this study is beneficial to advance the application of base editing technology in chicken biological research and breeding in avian species.

MATERIALS AND METHODS

Plasmid Construction

Codon optimization of the base editor chBE4max was generated by replacing APOBEC1 in BE4max (Addgene

© 2022 Published by Elsevier Inc. on behalf of Poultry Science Association Inc. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

Received April 16, 2022.

Accepted September 7, 2022.

¹These authors have contributed equally.

²Corresponding author: lyq@gxu.edu.cn

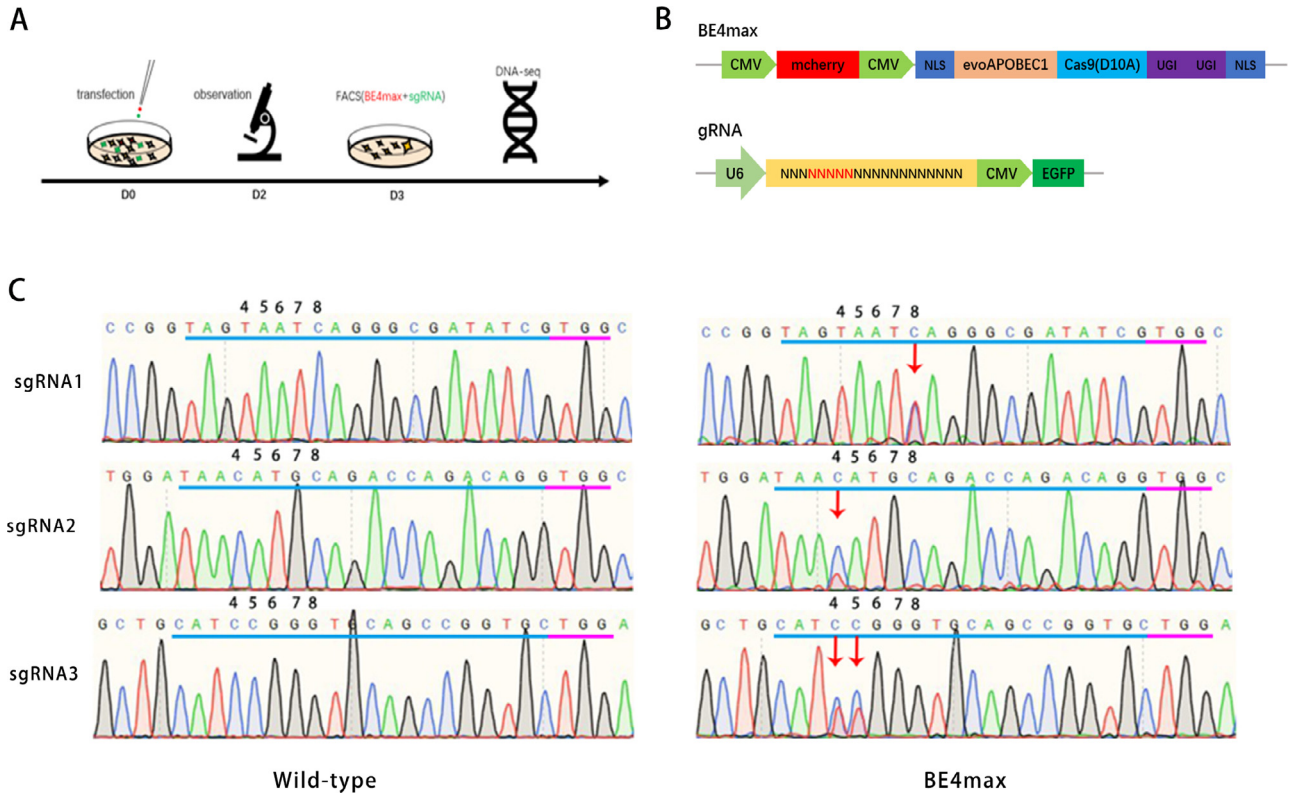


Figure 1. BE4max-mediated gene editing in chicken DF-1 Cells. (A) Flow chart of double positive cell acquisition. (B) Schematic of the BE4max and gRNA vectors. (C) Sanger sequencing results of chicken somatic cells co-transfected with BE4max and related gRNAs. Pink lines indicate the protospacer adjacent motif (PAMs) and blue lines indicate sgRNA sequences. Red arrows indicate substituted nucleotides.

plasmid # 112093) with optimized codons according to the chicken bias. Original APOBEC1 was removed from BE4max by double digestion with Not I and Bgl II, and codon-optimized evoAPOBEC1 (**chevoAPOBEC1**) was obtained from the synthetic plasmid. The optimized APOBEC1 was synthesized by Shenzhen BGI Genomics Company Limited., (Shenzhen, China).

For construction of sgRNAs, oligos were synthesized, annealed, and cloned into the BpiI site of the sgRNA-expressing vector, U6-sgRNA-CMV-EGFP (Figure 1B and Supplementary Table S1).

Cell Culture, Transfection, and Genotyping

DF-1 cells were cultured in DMEM/F12 medium (Biological Industries, Israel) containing 10% fetal bovine serum (Biological Industries). DF-1 cells were cultured at 37°C in a 5% CO₂ incubator. Transfection were performed in the presence of Lipofectamine 3000 Reagent according to manufacturer instructions. Briefly, a total of 2μg of plasmids (BE4max or chBE4max: sgRNA = 1: 1) were used for each well of a 24-well plate, and the transfection solution was removed 12 h after transfection. Positive cells were sorted 3 d after transfection by flow cytometers.

Genomic DNA of the sorted DF-1 cells was extracted using TIANamp Genomic DNA Kit (Tiangen Biotech, Beijing, China). Genotypes of all cells were determined by Sanger sequencing. PCR fragments for Sanger sequencing were generated in a one-step PCR reaction.

The primers are listed in Supplementary Table S2. The editing efficiency of each target site was determined by ImageJ analysis.

siRNA Transfection and Q-PCR Analysis

Small interfering RNAs (siRNAs) targeting *MBD4* were designed and synthesized by Shanghai GenePharma Company Limited., (Shanghai China). The sequences of *MBD4*-siRNAs were as follows: *MBD4*-siRNA1, 5'-CGACCAUAAUUCUCAUAATT-3'; *MBD4*-siRNA2, 5'-CCACGAGGUACAGAAGAAUTT-3'; and *MBD4*-siRNA3, 5'-GUGCUGAACAUACAGUUCUTT-3'. Negative control-siRNA (NC-siRNA) or *MBD4*-siRNA were transfected to DF-1 cells using the Lipofectamine RNAiMAX Transfection Reagent (Invitrogen | Thermo Fisher Scientific – CN, Shanghai, China).

Total RNA was extracted from DF-1 cells using the Omega RNA kit. The first-strand cDNA was synthesized using a Reverse Transcription Reagent Kit and gDNA Eraser (Takara Biomedical Technology, Dalian, China) was used to remove contaminating genomic DNA. Q-PCR reactions were performed using TB Green Premix Ex Taq II (Takara Biomedical Technology). All the primer sequences are shown in Supplementary Table S3.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism (GraphPad Software, La Jolla, CA, USA).

Significant differences in base editing efficiency were evaluated by Student's *t* test. A value of $P < 0.05$ indicated statistical significance.

RESULTS

BE4max-Mediated Gene Editing in Chicken DF-1 Cells

To determine the efficacy of the base-editing system consisting of BE4max and gRNA in the chicken genome (Figure 1B), we selected gRNAs (sgRNA1, sgRNA2, sgRNA3) at three target loci based on the reported base editing activity window (Supplementary Table S1). The GFP and mCherry double positive cells were sorted by flow cytometry for genotype analysis in DF-1 cells 72 h after co-transfection of BE4max and gRNA (Figure 1A and Supplementary Figure S1A). PCR amplification of the region covering the target site was performed, and the purified PCR products were analyzed by Sanger sequencing (Supplementary Figure S1B). Sequencing analysis indicated that the BE4max system successfully induced base conversion of C→T at all selected loci (Figure 1C).

Among the 3 gRNAs we selected, a total of 4 sites located within the base editing activity window achieved C → T mutations (Figure 1C). These sites are named gRNA1-8, gRNA2-4, gRNA3-4, and gRNA3-5, according to their specific positions. By calculating the ratio of T/C+T, the corresponding base editing efficiency was 51.35, 26.79, 44.50, and 39.97% (Supplementary Figures S1C and D). This finding suggested that BE4max system can induce C→T base editing in chicken, but it also presents sequence preference over different target sites.

Codon Optimization of APOBEC1 Improves the Base Editing Efficiency of BE4max

Codon optimization is a common strategy to improve the efficiency of base editing. The efficiency of the base editor has been successfully optimized using codon optimization on different species and different cell types (Koblan et al., 2018; Qin et al., 2018; Zafra et al., 2018). Therefore, we conjectured that specific modifications of BE4max would facilitate its action in chicken. We synthesized the chicken codon-optimized APOBEC1 and designated this new construct as chBE4max (Supplementary Sequences).

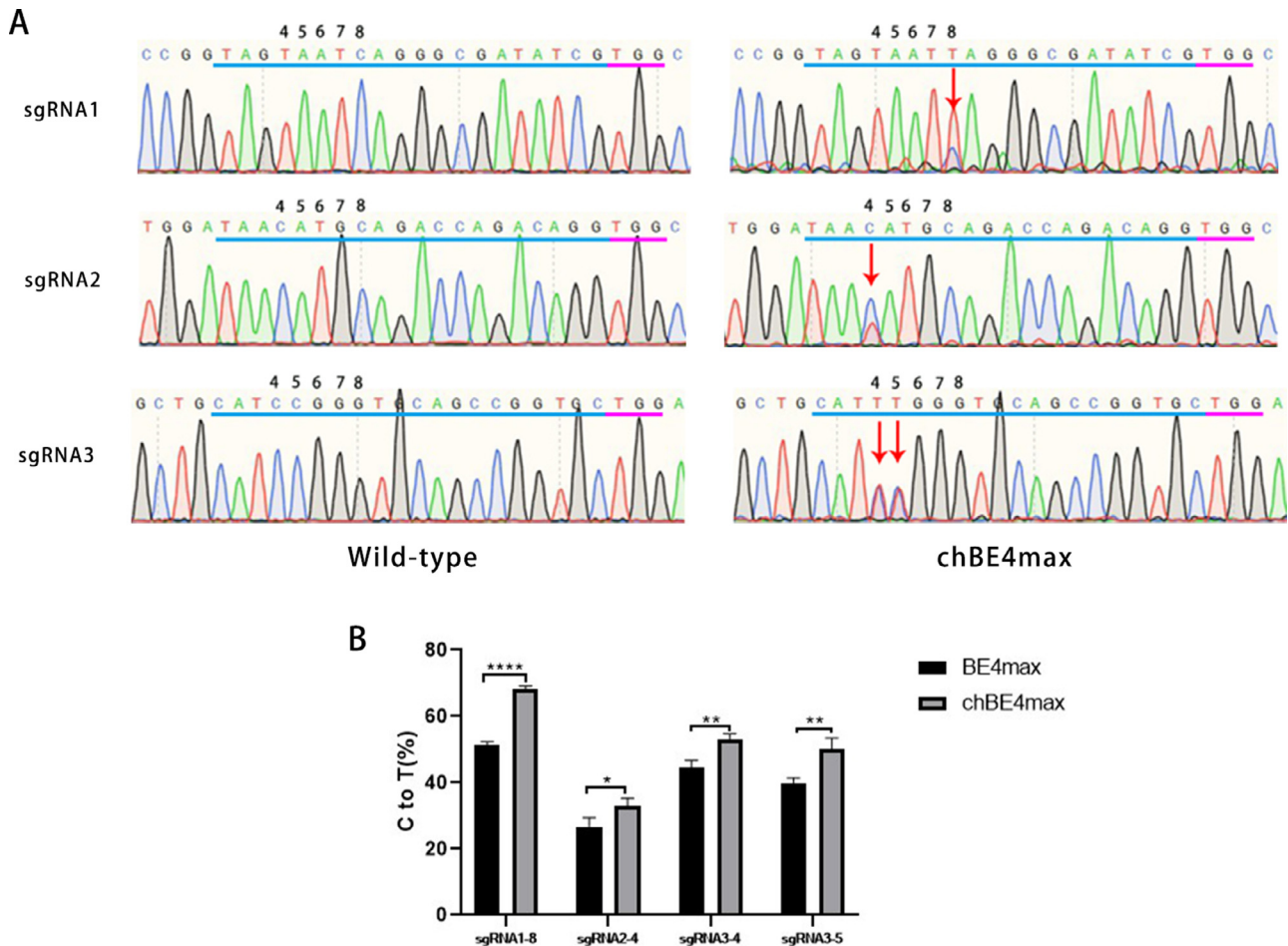


Figure 2. Codon optimization of APOBEC1 further improves the base editing efficiency of BE4max in DF-1 cells. (A) Sanger sequencing results of chicken somatic cells co-transfected with chBE4max and gRNAs. Pink lines indicate the PAMs and blue lines indicate sgRNA sequences. Red arrows indicate substituted nucleotides. (B) Base conversion efficiency of BE4max and chBE4max at each site. Values were shown as mean \pm SEM ($n = 3$); **** $P < 0.001$, ** $P < 0.01$ and * $P < 0.05$.

The chevoAPOBEC1 (1,123 bp) was ligated to BE4max without evoAPOBEC1 (11,141 bp) by T4 ligase to generate a new BE named chBE4max (Supplementary Figure S2A). The successful construction of the vector was confirmed by restriction enzyme digestion, gel electrophoresis, and Sanger sequencing (Supplementary Figures S2B and C).

To investigate whether the strategy of codon optimization is effective in DF-1 cells, the editing efficiency of BE4max and chBE4max was compared at the same sites. The chBE4max and gRNA were co-transfected into DF-1 cells for 72 h and double positive cells were collected by flow cytometry for genotype analysis. The base editing efficiency of chBE4max in gRNA1-8, gRNA2-4, gRNA3-4, and gRNA3-5 was 68.23, 32.80, 53.17, and 50.11%, respectively, by analysis of Sanger sequencing results (Figure 2A and Supplementary Figure S3B). Results demonstrated that base editing efficiency of chBE4max was improved by $10.4\% \pm 4.6$ compared with that of BE4max (Figure 2B).

Inhibition of the Expression of *MBD4* Improves the Efficiency of chBE4max in DF-1 Cells

To test the effect of *MBD4* on gene editing in chicken cells, the siRNA approach was used to knock-down the expression of *MBD4* in DF-1 cells. Expression of *MBD4* was reduced to 40% after transfection of siRNA in DF-1 cells (Figure 3A). Three gRNAs (sgRNA4, sgRNA5, and sgRNA6) targeting to 4-6, 5-4, 5-5, 6-5, 6-8, and 6-9 sites were used to examine base editing efficiency (Supplementary Table S1). DF-1 cells were first pretreated with NC-siRNA and *MBD4*-siRNA for 48 h and then subjected to co-transfection of sgRNA and chBE4max plasmids. The sequencing results after 72 h of transfection showed that 5 of the 6 induced C→T transition sites in the *MBD4*-siRNA group showed significantly higher base editing efficiency than the NC-siRNA group did, with an average improvement of $4.43\% \pm 1.4$ (Figures 3B and 3C and Supplementary Figures S4 A and B).

DISCUSSION

In contrast to traditional crossbreeding and selection based on livestock phenotype, molecular breeding provides precise modification of specific genes and allows for rapid achievement of desired traits with significant less time and lower cost. Genome editing technology is now widely used to explore molecular functions in various organisms. Yet, the use of genome editing technology in birds has been limited.

Conventional CRISPR/Cas systems can edit animal genomes by cutting or inserting. However, this usually causes double strand breaks (DSBs) in the DNA, disrupts the structure of chromosomes and affects genome stability. Functional genomics and bioinformatics has revealed a large number of genes correlated to chicken disease resistance and economic traits (Ouyang et al.,

2008; Chen et al., 2014; Long et al., 2019). The difference in phenotypic characteristics usually results from single nucleotide polymorphism (SNP) in gene loci. This suggests potential targets for genetic improvement using base-editing techniques (Wang et al., 2018; Kim et al., 2019). The BEs provide a safe and efficient strategy to edit individual base loci without DSBs. In the current study, BE4max was active in chicken somatic cells. The editing activity window was recognized to be 4–8 bases away from the PAM locus, which is consistent with data reported on other species (Zong et al., 2017; Liu et al., 2018; Shi et al., 2019).

Because of the limitation on the editing window, the base editing system is more stringent in its selection of editing sites than the traditional CRISPR/Cas9 system (Rees and Liu, 2018). In mammals such as mice, a large amount of optimization work has been used to develop base editing technologies that apply to different conditions. However, these optimization efforts have not been reported in chicken breeding. The lower editing efficiency, editing precision and small number of editable targets are all key factors limiting the application of base editing technology in chicken breeding.

The cytosine deaminase evoAPOBEC1 in BE4max used in this study was modified from rat-derived APOBEC1 (Koblan et al., 2018). Although genes from different organisms usually share a common set of genetic codons, the codon preference varies in different organisms. Mammalian-derived cytosine deaminase presented lower efficiency in chicken cells due to the effect of codon preference. In addition, synonymous codon substitutions may also disrupt co-translational protein folding, which in turn affects protein expression (Walsh et al., 2020). Our results indicated that the gene editing efficiency of BE4max is higher than evoAPOBEC1 in chicken somatic cells after optimization of the codon of cytosine deaminase as per chicken bias.

In mammalian cells, the BER pathway plays an important role in maintaining genomic stability via a DNA repair mechanism (Zanotti and Gearhart, 2016). Co-expression of Uracil Glycosylase Inhibitor (UGI) with the base editor can increase the activity of the base editor by adding UGI to the base editing complexes (Wang et al., 2017; Winter et al., 2019). Similar findings have been reported in avian species. Lee et al. (2020) showed that the high expression of uracil N-glycosylase (*UNG*) was the main reason for inhibiting the editing activity of the BEs in chicken primary germ cells. *MBD4* is a DNA glycosylase with a similar function to *UNG* to recognize and repair mismatches of T:G or U:G in the DNA strand in the BER pathway (Krokan et al., 2002; Visnes et al., 2009). DT40 B cells can significantly increase the frequency of somatic hypermutation after the knockdown of the *MBD4* gene in chicken (Costello et al., 2019). Therefore, we conjectured that *MBD4* might affect the activity of BE4max in DF-1 cells. Similar results were found in this study that the editing efficiency of the BE4max was significantly improved by the inhibition of *MBD4*. The base editor needs to generate G:C mismatches in order to function, which can be

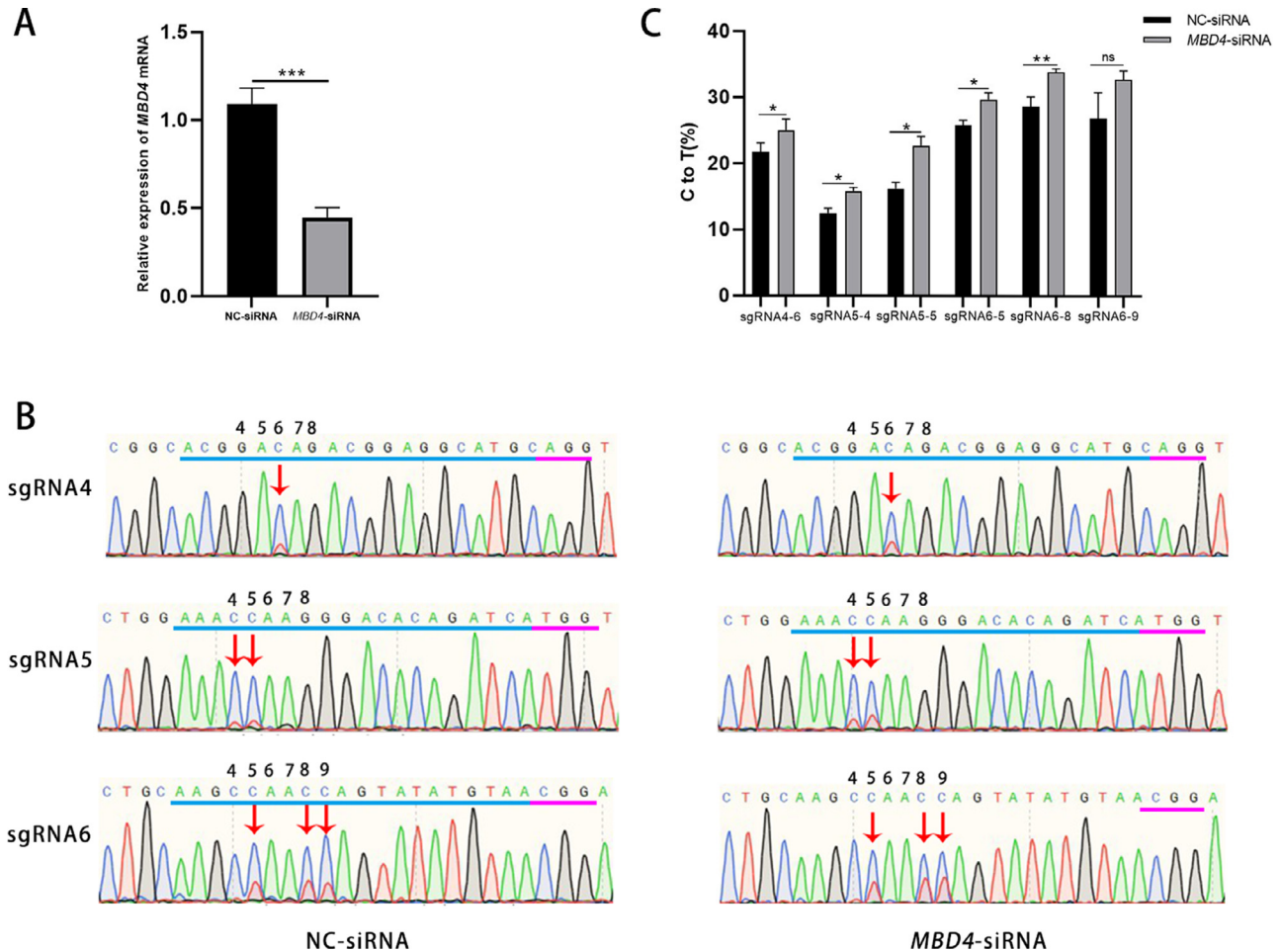


Figure 3. Inhibition of the expression of MBD4 improves the efficiency of chBE4max in DF-1 cells. (A) Detection of interference efficiency of *MBD4*-siRNA. (B) Sanger sequencing results of NC-siRNA group and *MBD4*-siRNA chicken somatic cells co-transfected with chBE4max and gRNAs. Pink lines indicate the PAMs and blue lines indicate sgRNA sequences. Red arrows indicate substituted nucleotides. (C) Comparison of chBE4max editing activity in the NC-siRNA group and *MBD4*-siRNA group.

recognized and repaired by *MBD4*, thereby inhibiting editing activity. Therefore, this study provides data that supports the practical application of base editing technology in chickens.

In conclusion, this research demonstrated that the BE4max editing system can be used to induce single-base mutations in chicken somatic cells. The editing efficiency of BE4max was improved by codon optimization and repression of *MBD4* gene expression. This study provides a reference for gene function research and the generation of gene-edited chickens.

ACKNOWLEDGMENTS

This study was jointly supported by National Key R&D Program of China (2021YFD1300100) and National Natural Science Foundation of China (31960157), Guangxi Key Research & Development Program (AB21220005) the Guangxi Key Laboratory of Veterinary Biotechnology Research Fund (No.19-50-40-B-04) and the Talent and Science Project of Bama County (20210004). The authors thank Zhiqiang Wang for his assistance in flow cytometry analysis. We would also like to thank all participants enrolled in the present study

DISCLOSURES

All authors disclosed no relevant relationships.

SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found in the online version at [doi:10.1016/j.psj.2022.102174](https://doi.org/10.1016/j.psj.2022.102174).

REFERENCES

Chen, B., W. Pan, L. Zhang, J. Liu, H. Ouyang, Q. Nie, and X. Zhang. 2014. NHE1 gene associated with avian leukosis virus subgroup J infection in chicken. *Mol. Biol. Rep.* 41:6519–6524.

Costello, R., J. F. Cantillo, and A. L. Kenter. 2019. Chicken MBD4 regulates immunoglobulin diversification by somatic hypermutation. *Front. Immunol.* 10:2540.

Frewer, L. J., C. Howard, and R. Shepherd. 1997. Public concerns in the United Kingdom about general and specific applications of genetic engineering: risk, benefit, and ethics. *Sci. Technol. Human Values* 22:98–124.

Kim, Y. C., M. J. Jeong, and B. H. Jeong. 2019. Genetic characteristics and polymorphisms in the chicken interferon-induced transmembrane protein (IFITM3) gene. *Vet. Res. Commun.* 43:203–214.

Koblan, L. W., J. L. Doman, C. Wilson, J. M. Levy, T. Tay, G. A. Newby, J. P. Maianti, A. Raguram, and D. R. Liu. 2018.

- Improving cytidine and adenine base editors by expression optimization and ancestral reconstruction. *Nat. Biotechnol.* 36:843–846.
- Komor, A. C., Y. B. Kim, M. S. Packer, J. A. Zuris, and D. R. Liu. 2016. Programmable editing of a target base in genomic DNA without double-stranded DNA cleavage. *Nature* 533:420–424.
- Krokan, H. E., F. Drablos, and G. Slupphaug. 2002. Uracil in DNA—occurrence, consequences and repair. *Oncogene* 21:8935–8948.
- Lee, K. Y., H. J. Lee, H. J. Choi, S. T. Han, K. H. Lee, K. J. Park, J. S. Park, K. M. Jung, Y. M. Kim, H. J. Han, and J. Y. Han. 2020. Highly elevated base excision repair pathway in primordial germ cells causes low base editing activity in chickens. *FASEB J.* 34:15907–15921.
- Liu, Z., M. Chen, S. Chen, J. Deng, Y. Song, L. Lai, and Z. Li. 2018. Highly efficient RNA-guided base editing in rabbit. *Nat. Commun.* 9:2717.
- Long, J. S., A. Idoko-Akoh, B. Mistry, D. Goldhill, E. Staller, J. Schreyer, C. Ross, S. Goodbourn, H. Shelton, M. A. Skinner, H. Sang, M. J. McGrew, and W. Barclay. 2019. Species specific differences in use of ANP32 proteins by influenza A virus. *Elife* 8:e45066.
- Ouyang, J. H., L. Xie, Q. Nie, C. Luo, Y. Liang, H. Zeng, and X. Zhang. 2008. Single nucleotide polymorphism (SNP) at the GHR gene and its associations with chicken growth and fat deposition traits. *Br. Poult. Sci.* 49:87–95.
- Qin, W., X. Lu, Y. Liu, H. Bai, S. Li, and S. Lin. 2018. Precise A*T to G*C base editing in the zebrafish genome. *BMC Biol.* 16:139.
- Rees, H. A., and D. R. Liu. 2018. Base editing: precision chemistry on the genome and transcriptome of living cells. *Nat. Rev. Genet.* 19:770–788.
- Shi, Z., H. Xin, D. Tian, J. Lian, J. Wang, G. Liu, R. Ran, S. Shi, Z. Zhang, Y. Shi, Y. Deng, C. Hou, and Y. Chen. 2019. Modeling human point mutation diseases in *Xenopus tropicalis* with a modified CRISPR/Cas9 system. *FASEB J.* 33:6962–6968.
- Tyagi, S., R. Choudhary, A. Das, S. Won, and P. Shukla. 2020. CRISPR-Cas9 system: a genome-editing tool with endless possibilities. *J. Biotechnol.* 319:36–53.
- Visnes, T., B. Doseeth, H. S. Pettersen, L. Hagen, M. M. Sousa, M. Akbari, M. Otterlei, B. Kavli, G. Slupphaug, and H. E. Krokan. 2009. Uracil in DNA and its processing by different DNA glycosylases. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 364:563–568.
- Walsh, I. M., M. A. Bowman, I. F. Soto Santarriaga, A. Rodriguez, and P. L. Clark. 2020. Synonymous codon substitutions perturb cotranslational protein folding in vivo and impair cell fitness. *Proc. Natl. Acad. Sci. U. S. A.* 117:3528–3534.
- Wang, L., W. Xue, L. Yan, X. Li, J. Wei, M. Chen, J. Wu, B. Yang, L. Yang, and J. Chen. 2017. Enhanced base editing by co-expression of free uracil DNA glycosylase inhibitor. *Cell Res.* 27:1289–1292.
- Wang, Y., F. Guo, H. Qu, C. Luo, J. Wang, and D. Shu. 2018. Associations between variants of bone morphogenetic protein 7 gene and growth traits in chickens. *Br. Poult. Sci.* 59:264–269.
- Winter, J., A. Luu, M. Gapinske, S. Manandhar, S. Shirguppe, W. S. Woods, J. S. Song, and P. Perez-Pinera. 2019. Targeted exon skipping with AAV-mediated split adenine base editors. *Cell Discov.* 5:41.
- Zafra, M. P., E. M. Schatoff, A. Katti, M. Foronda, M. Breinig, A. Y. Schweitzer, A. Simon, T. Han, S. Goswami, E. Montgomery, J. Thibado, E. R. Kastenhuber, F. J. Sánchez-Rivera, J. Shi, C. R. Vakoc, S. W. Lowe, D. F. Tschaharganeh, and L. E. Dow. 2018. Optimized base editors enable efficient editing in cells, organoids and mice. *Nat. Biotechnol.* 36:888–893.
- Zanotti, K. J., and P. J. Gearhart. 2016. Antibody diversification caused by disrupted mismatch repair and promiscuous DNA polymerases. *DNA Repair (Amst.)* 38:110–116.
- Zong, Y., Y. Wang, C. Li, R. Zhang, K. Chen, Y. Ran, J. L. Qiu, D. Wang, and C. Gao. 2017. Precise base editing in rice, wheat and maize with a Cas9-cytidine deaminase fusion. *Nat. Biotechnol.* 35:438–440.