Human cleaving embryos enable efficient mitochondrial base-editing with DdCBE

Yinghui Wei1,2, Chunlong Xu3, Hu Feng4, Kui Xu4, Zhifang Li4, Jing Hu2, Ping Zhou4, Yu Wei2, Zhenrui Zuo4, Erwei Zuo4, Wen Li1, Hui Yang2 and Meiling Zhang1,3

Dear Editor,

Mitochondrial diseases could be caused by heritable mutations in both mtDNA and nuclear DNA. Because mitochondria membrane insulin mtDNAs access from large ribonucleoprotein complex of Cas9 and sgRNA, mtDNAs are intractable for genetic modifications with RNA-guided CRISPR/Cas9 system commonly used for effectively editing nuclear DNA of different species. Although ZFN and TALEN have been previously engineered to successfully cut and eliminate mtDNA, these systems have not been widely used for editing of mitochondrial genome. By injection of DdCBE mRNA into clinically discarded human embryos with three pronuclei (3PN) to check protein expression and cellular localization. Immunostaining results using anti-HA and anti-FLAG antibodies revealed high expression of the DddA-TALE fusion deaminase pairs and their proper localization with mitotracker signal in the blastomere cell of human embryos (Supplementary Fig. S1a). Our previous study with cytosine and adenine base editors in human embryos showed that cleaving embryos enable robust base conversion in nuclear DNA. To determine the optimal embryonic stage for mtDNA editing, we injected ND4-DdCBE mRNA into human 3PN-derivered zygote and performed genotyping analysis 48 h post injection (Fig. 1a). We found that ND4-DdCBE exhibited detectable activity for C4 position, and injection of human embryos at the cleavage stage significantly improved base editing efficiency of ND4-DdCBE, compared with the injection at the zygote stage (Fig. 1b; Supplementary Fig. S1c). To confirm the finding with ND4-DdCBE, we further constructed DdCBE targeting ND6, ND1, ND5.1, and ATP8 genes on mitochondrial genome. By injection of ND6-, ND1-, ND5.1-, or ATP8-DdCBE mRNA into human 3PN-derived zygote and cleaving embryos, we found DdCBE also induced efficient C-to-T base conversions and injection of DdCBE at 8-cell stage showed dramatic increase of the base conversion rate (Supplementary Figs. S1c and S2a–d). In particular, human embryos at 8-cell stage could support up to 60% cytosine conversion compared with less than 10% cytosine conversion at other stages (Supplementary Figs. S1c and S2a–d). Moreover, we showed that the advantage of using...
Fig. 1 (See legend on next page.)
8-cell embryos was not due to the total amount of injected editing agents, since when we increased the concentration of editing agents for 2-cell embryos by 4-fold and 4-cell embryos by 2-fold, no significant improvement of editing efficiency was observed (Supplementary Fig. S1b).

To further verify our finding from 3PN embryo experiments in normal 2PN embryos (derived from immature MI oocyte injected with sperm) and interrogate off-target effects of DdCBE, we performed mitochondrial genome-wide sequencing analysis after injecting ND4-DdCBE/Dead-DdCBE and GFP mRNA or only GFP mRNA into four blastomeres of 2PN 8-cell embryos (Fig. 1c). The other four blastomeres were left un-injected to eliminate the differences in the genetic background between the gene-edited and control embryos. We found only GFP-positive blastomeres (GFP⁺) injected with both ND4-DdCBE and GFP mRNA showed around 25% C>G-to-T>A conversions while dead DdCBE-injected, un.injected (GFP⁻), or GFP only ones remained intact on target loci (Fig. 1d). We then performed mtDNA sequencing of both GFP⁺ and GFP⁻ blastomeres, and used GFP⁻ blastomeres as a negative control to call single nucleotide variations (SNVs) potentially resulted from ND4-DdCBE off-target effects on mitochondrial genome. Most of SNVs identified in 2PN GFP⁺ embryos were centered around on-target loci (Fig. 1e), probably due to dsDNA affinity of deaminase derived from interbacterial toxins and unstable binding of target sequence by the TALE pair. Besides SNVs caused by bystander effects of DdCBE, there were some distal SNVs with less than 1% allelic frequency far from TALE recognition sequences (Fig. 1e), potentially caused by off-target activity of the DdCBE. To further characterize off-target profile of DdCBE, we performed the same experiments at mitochondrial ND6 locus. Similar to the ND4-DdCBE results, ND6-DdCBE showed around 20% on-target C>G-to-T>A conversions (Supplementary Fig. S4a). However, ND6-DdCBE yielded more off-target SNVs than the ND4-DdCBE (Supplementary Fig. S4b), probably due to the difference of TALE recognition sequences. Furthermore, we analyzed all SNVs identified in ND4- and ND6-targeting samples and found significant enrichment of C-to-T/G-to-A conversion (Supplementary Figs. S3b and S4c). Besides, we retrieved the 20 bp regions flanking each off-target SNV, and found a strong 5'TC-TC-3' preference for ND4- and ND6-DdCBE off-target edits (Fig. 1f; Supplementary Figs. S3a and S4d), consistent with the identity of cytosine deaminase for DdCBE. In addition, we did not observe significant off-target editing in nuclear pseudogenes, even though they differ by only 0–1 bp from the target sites on mtDNA (Supplementary Fig. S5). Finally, we checked the morphology and developmental rate of human 2PN embryos after the injection of DdCBE mRNA. We detected no morphological abnormality of 8-cell injected embryos compared with normal ones (Fig. 1g). Development rate also showed non-significant difference between 8-cell injected embryos and non-injected ones, implying high applicability of DdCBE in human embryos for specific mtDNA modifications (Fig. 1h).

Taken together, our results demonstrate that DdCBE is an effective base editor for inducing point mutations in mtDNA of human embryos, and the efficiency is much higher in 8-cell embryos. Our 8-cell injection method could help generate mitochondrial disease models as well as derived embryonic stem cells for functional investigation of disease-associated mutations in mtDNA. The current study is performed on clinically discard 3PN and 2PN embryos from healthy people. Therefore, it warrants further study to test the capability of DdCBE to correct disease-causing mutations in mtDNA of the embryos from donor patients, which may provide the alternative way for prevention of heritable mtDNA mutations leading to the untreatable mitochondrial diseases after birth. Given the bystander and off-target editing profile, DdCBE remains to be further optimized for both basic and therapeutic research in the future.

Acknowledgements
We thank Dr. Z.J. Chen for helpful discussions, insightful comments on this manuscript; Y. Wang, Y. Zhang, and Q. Hu from Optical imaging facility of the Center for Excellence in Brain Science and Intelligence Technology, Chinese Academy of Sciences for their technical support. We thank N. Zhong and Q.F. Wang for technical assistance. This work was supported by Chinese National Science and Technology major project R&D Program of China (2017YFC1001302 and 2018YFC2000101), Strategic Priority Research Program of Chinese Academy of Science (XDB32060000), the National Natural Science Foundation of China.
Author details
1International Peace Maternity and Child Health Hospital, School of Medicine, Shanghai Jiao Tong University, Shanghai, China. 2Institute of Neuroscience, State Key Laboratory of Neuroscience, Key Laboratory of Primate Neurobiology, CAS Center for Excellence in Brain Science and Intelligence Technology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai, China. 3Shanghai Center for Brain Science and Brain-Inspired Intelligence Technology, Shanghai, China. 4Shenzhen Branch, Guangdong Laboratory for Lingnan Modern Agriculture, Genome Analysis Laboratory of the Ministry of Agriculture, Agricultural Genomics Institute at Shenzhen, Chinese Academy of Agricultural Sciences, Shenzhen, China

Author contributions
YW, CX, WL, HY, and MZ jointly conceived the project. YW, CX, HY, and MZ designed and conducted experiments. YW, CX, KX, ZL, JH, LZ, Yu W, and ZZ assisted with plasmids construction, PCR and other molecular experiments. YW, ZL, EZ, and MZ performed embryonic experiments. HF performed analysis of deep-sequencing data. WL, HY, and MZ co-supervised the whole project. YW, CX, WL, HY, and MZ wrote the manuscript.

Conflict of interest
The authors declare no competing interests.

Publisher’s note
Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Supplementary information
The online version contains supplementary material available at https://doi.org/10.1038/s41421-021-00372-0.

Received: 22 September 2021 Accepted: 28 December 2021 Published online: 01 February 2022

References
1. Craven, L., Alston, C. L., Taylor, R. W. & Turnbull, D. M. Recent advances in mitochondrial disease. Annu. Rev. Genomics Hum. Genet. 18, 257–275 (2017).
2. Stewart, J. B. & Chinnery, P. F. The dynamics of mitochondrial DNA heteroplasmy: implications for human health and disease. Nat. Rev. Genet. 16, 530–542 (2015).
3. Gorman, G. S. et al. Mitochondrial diseases. Nat. Rev. Dis. Prim. 2, 16080 (2016).
4. Hopper, R. K. et al. Mitochondrial matrix phosphoproteome: effect of extra mitochondrial calcium. Biochemistry 45, 2524–2536 (2006).
5. Gammage, P. A. et al. Mitochondrially targeted ZFNs for selective degradation of pathogenic mitochondrial genomes bearing large-scale deletions or point mutations. EMBO Mol. Med. 6, 458–466 (2014).
6. Bacin, S. R., Williams, S. L., Pinto, M., Peralta, S. & Moraes, C. T. Specific elimination of mutant mitochondrial genomes in patient-derived cells by mitoTALENs. Nat. Med. 19, 1111–1113 (2013).
7. Gammage, P. A. et al. Genome editing in mitochondria corrects a pathogenic mtDNA mutation in vivo. Nat. Med. 24, 1691–1695 (2018).
8. Bacin, S. R. et al. MitoTALEN reduces mutant mtDNA load and restores tRNA (A) levels in a mouse model of heteroplasmic mtDNA mutation. Nat. Med. 24, 1696–1700 (2018).
9. Reddy, P. et al. Selective elimination of mitochondrial mutations in the germline by genome editing. Cell 161, 459–469 (2015).
10. Mok, B. Y. et al. A bacterial cytidine deaminase toxin enables CRISPR-free mitochondrial base editing. Nature 583, 631–637 (2020).
11. Guo, J. et al. Precision modeling of mitochondrial diseases in zebrafish via DdCBE-mediated mtDNA base editing. Cell Discov. 7, 78 (2021).
12. Lee, H. et al. Mitochondrial DNA editing in mice with DdDA-TALE fusion deaminases. Nat. Commun. 12, 1190 (2021).
13. Zhang, M. et al. Human cleaving embryos enable robust homozygotic nucleotide substitutions by base editors. Genome Biol. 20, 101 (2019).