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LETTER TO THE EDITOR

Mitochondrial replacement in macaque monkey offspring by first polar body transfer

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Dear Editor,

Mitochondrial DNA (mtDNA) mutations are maternally inherited to the offspring and mitochondrial replacement therapy (MRT) provides a promising approach for preventing mtDNA-related diseases.[1,2] First polar body transfer (PB1T) has been used for mitochondrial replacement to generate mouse offspring and normal human embryos that yield embryonic stem cells.[3,4] However, generation of primate offspring using PB1T has not been reported. Here, we show that PB1T-based mitochondrial replacement could generate healthy macaque monkey (*Macaca fascicularis*), a useful non-human primate model for evaluating methods that may improve the efficiency and safety of PB1T for MRT.

We first investigated the genomic integrity of the first polar body transfer (PB1) of monkey (*Macaca fascicularis*) (Fig. 1a). Single-cell array comparative genomic hybridization (aCGH)[3] was performed to examine genome integrity of the isolated monkey PB1 in comparison with spindle–chromosome complex (SCC) from the same freshly collected MII oocyte. Results from four pairs of PB1 and SCC showed that they had the same chromosome number and exhibited no aneuploidy and major deletion (Fig. 1b and Supplementary information, Fig. S1a–c). Further, single-cell whole-genome sequencing was performed using freshly isolated PB1s and SCCs. We detected some structural variations in both PB1 and SCC. However, there were no significant differences in the numbers of structural variations in these two types of samples, indicating that the genome integrity of PB1 is not degenerated in our study (Fig. 1c and Supplementary information, Table S1).

Immunostaining with an antibody against the DNA damage marker phospho-H2A.X showed no immune-reactivity in both PB1 and SCC (Supplementary information, Fig. S1d–f). Further immunostaining of 5-methylcytosine (5mC), histone H3 tri-methyl K9 (H3K9me3) and histone H3 phospho-S10 (phospho H3) showed that monkey PB1 and SCC exhibited similar epigenetic modifications (Fig. 1d–f; Supplementary information, Fig. S1g–x). To further address whether SCC and PB1 genomes have similar DNA methylation levels, we applied single-cell bisulfite sequencing to map genome-wide base-resolution DNA methylation of SCC and PB1 ($n = 3$). Our data indicate that DNA methylation levels are similar in PB1 and SCC genomes (Fig. 1g). All these results implied that the monkey PB1 could functionally replace the SCC of the oocyte.

Mitochondria associated with PB1 and SCC could be visualized by staining of monkey oocytes with the fluorescent dye MitoTracker,[5] together with DNA dye Hoechst. We found that within the intact oocyte mitochondria associated with PB1 were concentrated into high-density clusters, whereas those associated with SCC were distributed evenly as other mitochondria in the ooplasm (Fig. 1h; Supplementary information, Fig. S2a–f). For monkey PB1s and SCCs isolated from freshly collected MII oocytes by micropipette suction, the volume of PB1 was slightly larger than that of SCC (Fig. 1i, j; Supplementary information, Table S2), and MitoTracker staining intensity for the individual PB1 was higher than that of SCC (Fig. 1k, l), indicating that the isolated PB1 contained more mitochondria than the isolated SCC. According to the chip-based digital PCR[6] analysis, the average numbers of mtDNA copies associated with the isolated PB1s and SCCs were 1690 ± 984 and 886 ± 545 ($n = 9$ for each), respectively. This is different from the finding in a previous mouse study that isolated PB1s carried less mtDNAs than SCCs.[3] Quantification of the mtDNA copy number in whole monkey oocytes was also performed by chip-based digital PCR, revealing an average of $1{,}551{,}433 \pm 244{,}981$ ($n = 9$) mtDNA copy number in each MII oocyte (Fig. 1m; Supplementary information, Fig. S2g–j and Table S3). Although PB1 carried more mtDNA copies than SCC, the amount of mtDNA in isolated PB1 was about 1/1000 of the whole oocyte. This indicated that without carrying a large amount of mtDNAs, PB1T could be used for MRT.

Immunostaining of microtubule with α-tubulin antibody together with DAPI staining of intact monkey MII oocytes showed no detectable metaphase spindle, and scattered chromosomes in the PB1 (Supplementary information, Fig. S3a–f). This is consistent with the absence of spindle in human PB1,[7] but differs from that found in mouse oocytes[3] where spindle microtubule in PB1 is similar to that of SCC. We then performed PB1 transfer by the procedure as follows. The SCC was first removed by suction with a micropipette, under polarized light illumination.[8] The PB1 was obtained from a donor oocyte by suction with a micropipette, and the isolated PB1 was then incubated in a solution containing the viral envelope of the Hemagglutinin Virus of Japan (HVJ-E) for about 10–20 s, followed by injection of the PB1 into the perivitelline space of an enucleated oocyte to allow spontaneous fusion of the PB1 with the oocyte (Fig. 1n–q). As shown in an example of reconstructed oocyte at 1 h after the fusion, a de novo formed normal SCC can be visualized with polarized light illumination (Fig. 1r), and similar results were observed in 3 reconstructed oocytes with α-tubulin immunostaining (Supplementary information, Fig. S3g–r).

We next examined the embryonic development viability of reconstructed oocytes by PB1T using PB1s from one female monkey donor and enucleated oocytes from another donor. In total, we obtained 30 reconstructed oocytes for which the PB1 and host oocyte came from different monkeys. The PB1T-reconstructed oocytes were fertilized with intracytoplasmic sperm injection (ICSI) and 25 zygotes were obtained, including 21 normal zygotes that had two pronuclei (Fig. 1s) and 4 abnormal zygotes with one or three pronuclei (Supplementary information, Table S4). This fertility rate was similar to that found for ICSI of non-manipulated oocytes.[9] Subsequent transfer of 20 reconstructed normal embryos into 6 female surrogates yielded two pregnancies with singletons, as confirmed by ultrasound examination (Supplementary information, Table S4). This pregnancy rate is comparable to that found for ICSI using non-manipulated oocytes.[9] Two healthy monkey offspring, we named PB1T infant A and PB1T infant B, were successfully delivered by cesarean section at 162

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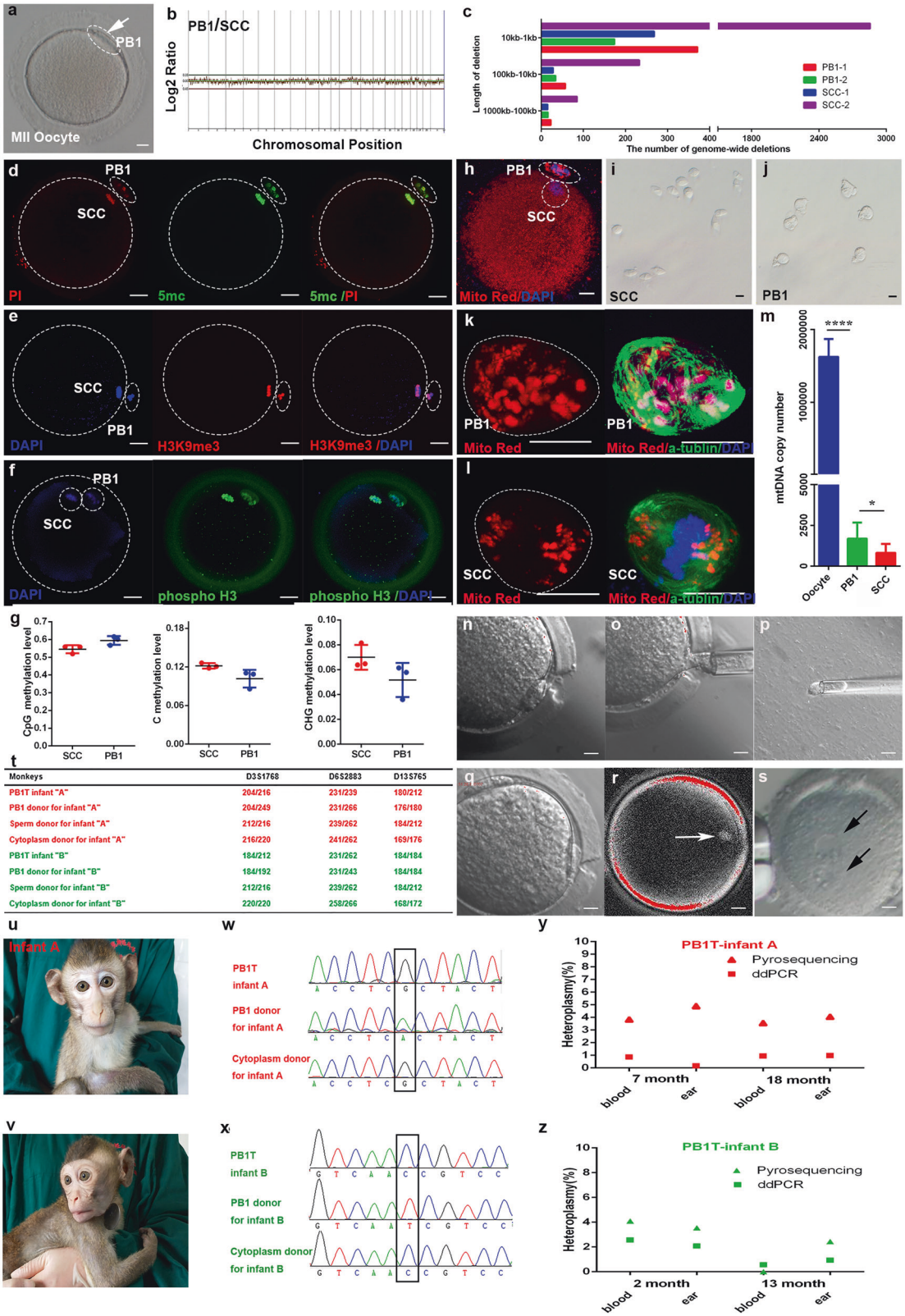

and 145 days, respectively (Fig. 1u, v). To our knowledge, this is the first two live primate offspring generated from reconstructed oocytes by PB1T. These two monkeys are now 27- and 22-month old, respectively, in good health at the time of manuscript submission. We have performed genotyping analysis using short tandem repeats (STRs) on the nuclear genome of the ear tissues of PB1T infant A and PB1T infant B in order to identify their parental origin. The results on 30 STR loci showed that both monkeys inherited the maternal genome of their PB1 donor monkey (Fig. 1t and Supplementary information, Table S5). To identify the origin

Fig. 1 Generation of live monkey offspring using oocytes reconstructed by PB1T. a A freshly collected monkey MII oocyte with intact PB1 (as shown by the white arrow). Scale bar, 10 μm. **b** Single PB1 and SSC of monkey oocyte shared the same genomic landscape by aCGH. No major alterations (amplifications and losses) were detected in PB1. **c** The numbers of genome-wide deletions in PB1 and SCC. **d–f** Immunofluorescent images of epigenetic analysis (5mC, H3K9me3 and phospho H3) of monkey PB1 and SCC. Scale bar, 10 μm. **g** Methylation levels of single SCC and PB1 by genome-wide methylation sequencing ($n = 3$), mC ($P = 0.073$), mCpG ($P = 0.062$), mCHG ($P = 0.136$). **h** MitoTracker staining showed that monkey PB1 contained high-density mtDNA. Scale bar, 10 μm. **i, j** Images of monkey SCC and PB1 isolated by micromanipulation. Scale bar, 10 μm. **k, l** MitoTracker staining of individual monkey PB1 and SCC. Scale bar, 10 μm. **m** Quantification of mtDNA copy numbers of monkey PB1, SCC and oocyte by digital PCR. ****$P < 0.0001$, *$P < 0.05$. Error bars indicate SD; $n = 9, 9, 9$ oocytes, PB1s and SCCs, respectively. **n–q** Procedure of monkey PB1 isolation by micromanipulation. PB1 was transferred to SCC-removed monkey oocytes. **r** Monkey MII SCC was de novo formed (as shown by the white arrow). **s** Two-pronucleus zygotes generated from PB1T-reconstructed oocytes by ICSI (as shown by the black arrows). Scale bar, 10 μm. **t** Three examples of STRs from ear tissues of "PB1T infant A" and "PB1T infant B" showing that their maternal DNAs were originated from PB1 donor monkeys instead of their cytoplasm donor monkeys. **u, v** Images of two live monkey offspring at 19-month old and 14-month old**. w, x** Examples of SNPs for "PB1T infant A" and "PB1T infant B", respectively, showing that their mtDNA SNPs were identical to those of the cytoplasm donor monkey. **y, z** Pyrosequencing analysis in two samples of monkey offspring at two time points showed <5% mtDNA heteroplasmy levels carried by the two monkeys.

of mtDNA in the two PB1T-derived monkeys, we sequenced the mitochondrial D-loop regions or the mitochondria gene ND3 of these monkeys and the PB1 and host oocyte donor monkeys. Comparison of single nucleotide polymorphisms (SNPs) showed that mtDNA of two offspring mainly originated from the host oocyte donor monkey (Fig. 1w, x; Supplementary information, Fig. S4a, b).

One critical issue is the prevalence of mtDNA in the PB1T-derived offspring that was due to mitochondria carryover during PB1 transfer. This is highly relevant for the potential use of PB1T method in MRT. We first performed TA clone analysis[5] on the mtDNA heteroplasmy using mtDNAs from the ear tissues of PB1T-derived monkeys. We amplified the D-loop region of mitochondria, and sequenced a total of 36 TA clones in each monkey. We found that all TA clones had the same sequence as those found in the host oocyte donor monkey, suggesting that host oocyte mtDNA dominated the mtDNA of PB1T-derived offspring and the fraction of carryover mtDNA associated with PB1 transfer was too small to be detected by this method. To determine the precise fraction of the carryover mtDNA, we then performed high-throughput pyrosequencing and digital PCR analysis on both the ear and blood samples from the two PB1T-derived monkeys, at two different time points after birth. By pyrosequencing, we found a stable low-level mtDNA heteroplasmy (< 5%) in the ear and blood samples over the 1.5-year period, indicating that mtDNA carryover with PB1 transfer remained a small fraction of the total mtDNA. By digital PCR analysis, we found a lower mtDNA heteroplasmy than the pyrosequencing method (Fig. 1y, z; Supplementary information, Fig. S4c–h and Table S6). Because we used the same locus for analysis, we think that the difference was caused by the different analysis methods. This indicated that a stricter mtDNA heteroplasmy analysis should be performed in the MRT studies. There was no apparent upward mtDNA drift in the PB1T-derived offspring using both methods. MtDNA heteroplasmy level of our PB1T-derived monkeys was higher than that found in monkeys derived from SSC transfer,[5] consistent with our earlier observation on the mitochondial carryover with isolated PB1 and SCC (Fig. 1h). It has been proposed that 15% is generally the level of mutant mtDNA that produces deleterious effects on cellular functions.[6] With heteroplasmy less than 5% and the absence of upward drift in carryover mtDNA found in our PB1T-derived monkey, PB1 transfer method may provide an acceptable approach for mitochondrial replacement.[10,11] The mtDNA heteroplasmy level of more accessible tissues in PB1T infants A and B will be further monitored in the future.

Health condition of the two PB1T monkey infants was monitored carefully and physical examination was performed every month to assess the body temperature, heart and respiratory rates, head-truck length, body weight, abdominal and head circumferences.[12] No abnormality was detected in the body temperature, heart and respiratory rates of PB1T monkeys. We performed statistical comparison of four physical parameters (head-truck length, body weight, abdominal and head circumferences) and found no significant difference in the body growth between 2 PB1T monkeys and 5 wild-type control monkeys (Supplementary information, Fig. S4i–l). Further detection and monitoring of body growth should be continued along with the growth of PB1T monkeys.

SCC transfer, PB1 transfer and pronuclear transfer, which have been performed in human embryos, showed great prospect in MRT for mtDNA mutation diseases. All these methods should be used for generating non-human primate offspring to test the safety and efficiency before they are applied in clinical practice. In our study, we first demonstrated that PB1 could functionally replace SCC in generating healthy primate offspring by PB1T. The efficiencies of ICSI and pregnancy using PB1T-derived oocytes are comparable to those using non-manipulated oocytes. Importantly, both PB1T-derived offspring showed <5% mtDNA heteroplasmy level. These results indicate that PB1T is a potential and promising mitochondrial replacement method to prevent mtDNA mutation diseases. Given the fact that the quality of oocyte is not affected by removing PB1, PB1 could also be used to increase the oocyte source of infertility treatment,[7] which will benefit patients with limited ovarian reserve. Our result also showed that the monkey PB1 contained more mtDNAs than SSC, which is reverse for that in mouse. We think that the monkey result is more representative than that of mouse in preclinical animal experiment. Besides PB1T, mouse offspring or human embryonic stem cells have been derived using embryos generated by second polar body transfer (PB2T),[4] pre-pronuclear transfer[13] and pronuclear transfer.[14,15] The efficiency and safety study of these methods should also be further performed using macaca monkeys.

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AUTHOR CONTRIBUTIONS

Z.L. and Q.S. designed and supervised the project. Z.L. performed the embryo manipulation experiments. Z.L. and Z.W. performed mtDNA copy number, immunostaining, mitochondrial distribution, mtDNA carryover experiments. Y.W., Y.N., X.Z., and Y.X. performed monkey assisted reproductive experiments. Y.W. and Q.L. performed monkey physical examination. X.Y. and Z.W. performed single-cell DNA methylation analysis. Z.W. performed single-cell whole-genome sequencing analysis. Z.L., Z.W., and Q.S. prepared the manuscript with contributions from all other authors.

ADDITIONAL INFORMATION

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