

## LETTER TO THE EDITOR

# Parthenogenetic haploid embryonic stem cells produce fertile mice

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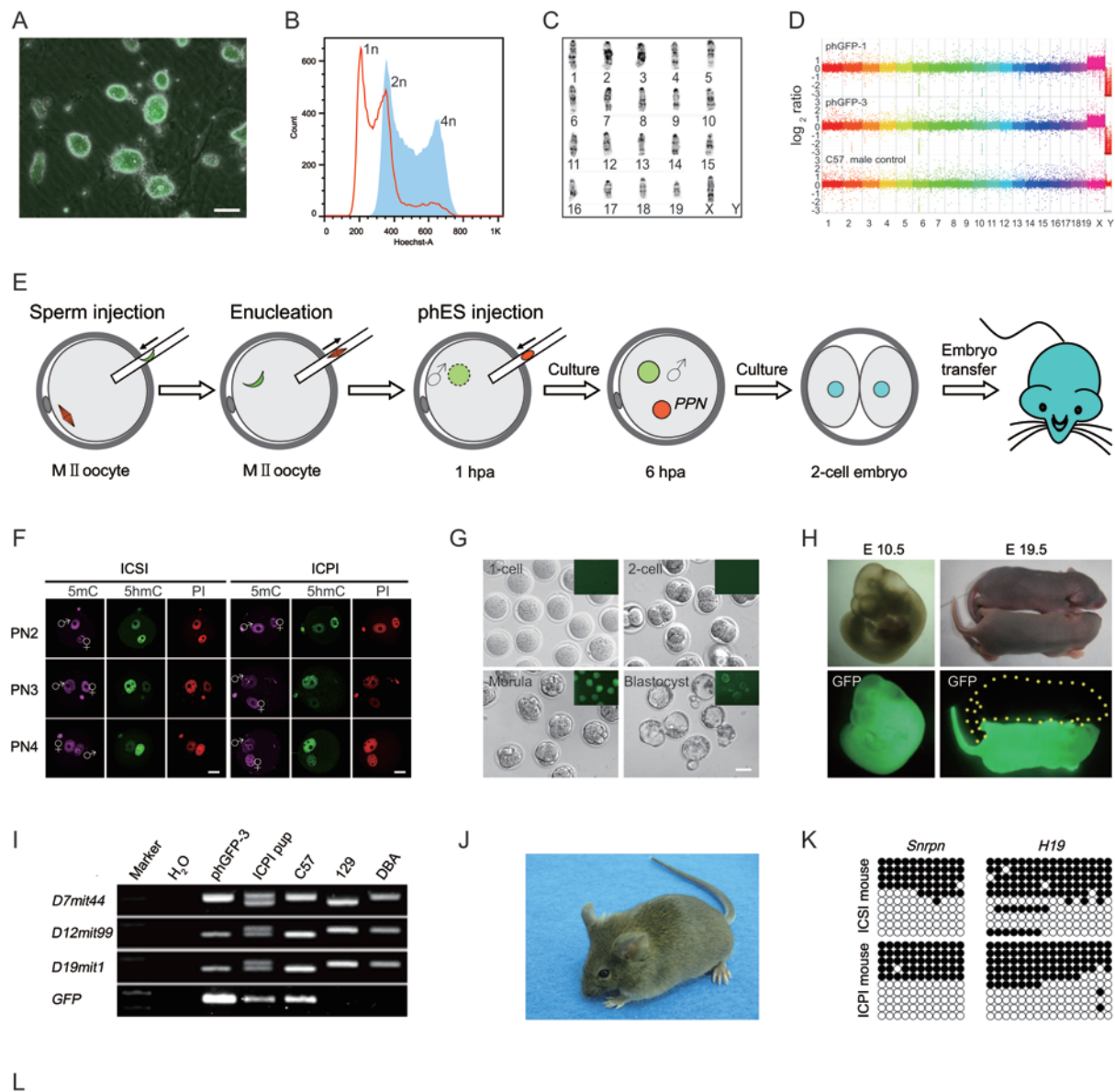
**Dear Editor,**

Abnormal oocytes are one of the major causes of reproductive failure, and also limit the efficiency of assisted reproduction [1]. The generation of functional oocytes either by differentiation of pluripotent stem cells [2] or *in vitro* culturing of the embryonic genital ridges [3] has not yet been achieved. Recently, we and other groups have reported that live fertile mice could be successfully produced by injecting androgenetic haploid embryonic stem (ahES) cells that carry some paternal imprints into oocytes in place of sperm [4, 5]. Considering their parthenogenetic origin, it would be interesting to know whether parthenogenetic haploid ES (phES) cells [6–8] could also support embryonic development when substituted for the maternal genome, and whether they could directly deliver their genomes at animal level. Here we established several phES cell lines and characterized their pluripotency and diploidization progression *in vivo*. We also demonstrated that phES cells could reconstruct embryos and produce fertile mice when injected into oocytes in place of maternal genome.

Haploid parthenogenetic embryos were generated from oocytes carrying a chicken  $\beta$ -actin [4] or *Oct4* [9] gene promoter-driven enhanced green fluorescent protein transgene (*EGFP* or *Oct4-EGFP* in short). We generated a total of 13 ES cell lines (indicated as phGFP cell lines, Supplementary information, Figure S1 and Table S1) from 40 haploid parthenogenetic morulas (from EGFP oocytes) and all of the cell lines inherited the EGFP expression (Figure 1A). In addition, nine cell lines were maintained in a haploid state after three or more rounds of purification via fluorescence-activated cell sorting (FACS) and subsequent expansion of the haploid cells (Figure 1B). Interestingly, these cell lines retained their haploid genome even after being passaged for more than 30 times (Figure 1C). We also obtained two *Oct4-EGFP* haploid cell lines from 30 activated oocytes of *Oct4-EGFP* mice (indicated as phES OG cell lines, Supple-

mentary information, Table S1). We assessed the genomic integrity of all the phES cell lines by comparative genomic hybridization (CGH) analysis, and did not observe any copy-number variation between the phES cell lines (Figure 1D). These results indicated that the phES cells could stably maintain an intact haploid genome as previously reported [8].

Pluripotency is an important feature of haploid ES cells. Interestingly, the phES cells showed expression of key pluripotent marker genes such as *Oct4*, *Sox2*, *Nanog*, *SSEA-1* and *Klf4* (Supplementary information, Figure S2A and S2B), very similar to diploid ES cells. Furthermore, the global gene expression profile of phES cells (phGFP-3) showed high correlation ( $r = 0.955$ ) with diploid ES cells, but less correlation ( $r = 0.779$  or  $0.799$ ) with mouse embryonic fibroblasts (MEFs) (Supplementary information, Figure S2C). Interestingly, the phES cells formed teratomas with all 3 germ layers when injected subcutaneously into SCID (severe combined immune deficiency) mice (Supplementary information, Figure S2D). We further used chimera formation assay to analyze the differentiation potential and the progression of the diploidization process of the phES cells *in vivo*. FACS-sorted EGFP-carrying G0- or G1-phase phES cells were obtained from mice with black-colored coats and injected into CD-1 blastocysts (obtained from mice with white-colored coats). We found that these cells properly chimerized the embryos at the E6.5, E8.5, E10.5 and E13.5 stages as reflected by their EGFP expression (Supplementary information, Figure S3). However, only 1.8% of the haploid EGFP-positive cell population was detected at the E6.5 embryonic stage (Supplementary information, Figure S3A), suggesting that the phES cells rapidly diploidize after differentiation, which was similar to the previous results [7]. Out of 38 E13.5-chimeric embryos generated from *Oct4-EGFP*-transgenic phES cells (Supplementary information, Table S2), 2 embryos expressed EGFP in their genital ridges (Supplementary information, Figure S2E), indicating successful germline



*In vivo* development of ICPI embryos.

Donor cell	No. of embryos reconstructed	No. of cleavage embryos	No. of embryos transferred	No. of implantations ( % )	No. of full-term pups ( % )	No. of survived pups
phGFP-1	125	91 (72.8)	91	16 (17.6)	0	0
phGFP-3	149	106 (71.1)	106	33 (31.1)	1 (0.9)	1
phGFP-5	118	93 (78.8)	93	22 (23.7)	1 (1.1)	0
Total	392	290 (74.0)	290	71 (24.5)	2 (0.7)	1
ICSI	18	16 (88.9)	16	14 (87.5)	10 (62.5)	10

transmission of the phES cells (phES OG-1). Finally, a total of six full-term chimeric pups (Supplementary information, Table S2) were obtained. However, only four survived to adulthood (Supplementary information, Figure S2F). The adult chimeric mouse produced from the phGFP cell line showed EGFP expression in multiple organs including kidney (7.7%), spleen (3.9%), heart (14.6%) and liver (12.6%), which reflected the broad differentiation ability of phES cells *in vivo* (Supplementary information, Figure S4). Furthermore, all the EGFP-positive cells were found to be diploid. Taken together, our results confirmed that the phES cells were pluripotent, although they were in diploid status.

To test whether the genome of a phES cell could take place of the maternal genome, first we performed intracytoplasmic sperm injection (ICSI), followed by removal of spindle to generate an androgenetic haploid embryo [4]. One h later, we performed intracytoplasmic phES cell (at G0/G1 stage) injection (ICPI) to generate diploid reconstructed embryos (Figure 1E). The phES cells (phGFP cell lines) formed 'maternal' pseudo-pronuclei and exhibited the genomic methylation pattern very similar to the ICSI embryos (Figure 1F). The ICPI embryos further developed to morula and blastocyst *in vitro* (Figure 1G), and inherited the *EGFP* transgene derived from phES cells. To determine the capacity for *in vivo* development of ICPI embryos, a total of 290 2-cell stage embryos were transferred into pseudo-pregnant mice. From this, two full-term pups were finally obtained. Both EGFP expression (Figure 1H) and simple sequence length polymorphism (SSLP) analysis (Figure 1I) confirmed that the pups were indeed produced from phES cells. Interestingly, one of these two pups survived to adult-

hood (Figure 1J), and showed normal methylation pattern of differentially methylated regions (DMRs) of *Snrpn* and *H19* (Figure 1K). This adult male mouse was fertile and delivered normal pups that inherited its *EGFP* transgene (Figure 1L). This indicated the Mendelian separation theory (Supplementary information, Figure S5). Taken together, our results demonstrate that phES cells could functionally replace the maternal genome and support full-term embryonic development, albeit with low efficiency.

Our data showed that phES cells were pluripotent, contributing to the germline of chimeric mice, and could produce a live mouse by ICPI. However, the efficiency was very low, which may have been caused by two reasons: 1) the ICPI embryo manipulations, especially the spindle removal procedure may have harmed the embryo's subsequent development [10]; 2) the epigenetic state of the phES cells may differ from that of the spindle and thereby compromised the development. Future efforts in modification of the manipulation process, utilization of the small compounds to reduce the negative effects caused by the spindle removal, and stabilization of the epigenetic status of the phES cells, especially the maternal imprinting, may improve the developmental efficiency of ICPI embryos. When women survive conditions such as ovarian cancer, phES cells could provide an alternative means of maintaining their fertility, and with ICPI procedure, a woman could give birth to a child who inherited her DNA. Compared to oocyte cryopreservation, cryopreservation of phES cells is easier and more convenient, and these cells are known to survive much longer in liquid nitrogen. Administration of phES cells also provides a novel way of gene therapy for the treatment of severe human

**Figure 1** Parthenogenetic haploid ES cells produce fertile mice. **(A)** Fluorescence detection of phES cells that carry an *EGFP* transgene. Scale bar, 50  $\mu$ m. **(B)** DNA content of phES cells (phGFP-3, passage 11, red) analyzed by FACS. Cells were purified three times by FACS selection of G0/G1 phase haploid cells, followed by culturing for three more passages. Diploid ES cells (blue) with 2n chromosome sets were used as control. **(C)** Karyotype analysis of phES cell lines. Shown is the standard G-banding karyotype of phGFP-3 cells (passage 12) with 19 + X chromosomal set. **(D)** Comparative genomic hybridization (CGH) analysis of two phES cell lines (phGFP-1 Passage 16, and phGFP-3 Passage 16). Comparative results of the genomic DNA of the phES cells and the control C57BL/6 male mouse kidney were shown as the y axis, on a log<sub>2</sub> base scale. **(E)** Diagram showing the generation of phES pups. hpa, hours post activation. **(F)** 5mC and 5hmC staining of the reconstructed embryos (6-7 hpa) with pronucleus after sperm injection. The ICSI embryos were shown in the left panel, whereas reconstructed ICPI embryos were shown in the right panel. **(G)** Pre-implantation development of ICPI embryos produced by phES cell injection. Scale bar, 50  $\mu$ m. **(H)** Left panel shows an E10.5 ICPI fetus with EGFP expression that was produced by phGFP-3. Right panel shows one P2 ICPI pup (phGFP-3) with EGFP expression, and one P2 wild-type ICSI pup. **(I)** Genetic background analysis of ICPI pup. Three simple sequence length polymorphism (SSLP) DNA markers from different chromosomes and the EGFP positive analysis showed that the pup was originated from hybrid genetic background, i.e., the C57BL/6 strain (phGFP-3 origin) and the 129Sv strain (sperm origin). Primer sequences were cited from the Mouse Genome Informatics website (<http://www.informatics.jax.org/>). **(J)** An adult male mouse produced by phES cell (phGFP-3) injection. **(K)** Bisulfite sequencing analysis of DMRs of imprinted genes *Snrpn* and *H19*. Mouse tail DNAs isolated from ICSI and phES adult off-springs were used. Filled circles represent methylated CpG sites, whereas open circles represent unmethylated CpG sites. Primer sequences were listed in Supplementary information, Table S3. **(L)** *In vivo* development of ICPI embryos.

diseases caused by cells with mitochondrial defects inherited through the egg's cytoplasm. ICPI injection could transfer the maternal DNA and fewer mtDNA than spindle transfer procedure, and thus provide the best clear mtDNA replacement in oocytes. Nevertheless, our study demonstrated that the pHES cells could support embryo development via substitution of maternal genome, which further provides a new model for studying epigenetic regulation of embryonic development, and may shed new light on assisted reproduction.

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## References

- 1 Serhal PF, Ranieri DM, Kinis A, et al. *Hum Reprod* 1997; **12**:1267-1270.
- 2 Hayashi K, Ogushi S, Kurimoto K, et al. *Science* 2012; **338**:971-975.
- 3 Obata Y, Kono T, Hatada I. *Nature* 2002; **418**:497.
- 4 Li W, Shuai L, Wan H, et al. *Nature* 2012; **490**:407-411.
- 5 Yang H, Shi L, Wang BA, et al. *Cell* 2012; **149**:605-617.
- 6 Leeb M, Walker R, Mansfield B, et al. *Development* 2012; **139**:3301-3305.
- 7 Elling U, Taubenschmid J, Wirsberger G, et al. *Cell Stem Cell* 2011; **9**:563-574.
- 8 Leeb M, Wutz A. *Nature* 2011; **479**:131-134.
- 9 Yoshimizu T, Sugiyama N, De Felice M, et al. *Dev Growth Differ* 1999; **41**:675-684.
- 10 Noggle S, Fung HL, Gore A, et al. *Nature* 2011; **478**:70-75.

(Supplementary information is linked to the online version of the paper on the *Cell Research* website.)