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Letter to the Editor

Co-participation of paternal and maternal genomes before the blastocyst stage is not required for full-term development of mouse embryos

Dear Editor,

The development of all the mammalian species begins from fertilization of a sperm and an egg into a diploid state. The maternal and paternal genomes are not functionally equivalent in term of their differential contributions to the imprinting gene expressions, and they are complementary and both essential for embryonic and postnatal development (McGrath and Solter, 1984; Surani et al., 1986). It is well known that the nucleus of blastomeres adopt a 'Rabl configuration' by which the paternal and maternal genomes separate spatially within the same nucleus until the 8-cell stage (Mayer et al., 2000; Santenard et al., 2010). Previous studies showed that diploid embryos reconstituted by fusion of androgenetic and parthenogenetic haploid blastomeres at the 4-cell stage can produce living offspring, indicating that the paternal genome is not necessarily associated with the maternal genome within the same nucleus before the 8-cell stage (Renard et al., 1991). However, it has not been fully elucidated whether the participation of both genomes is required in later embryonic stages for full-term development.

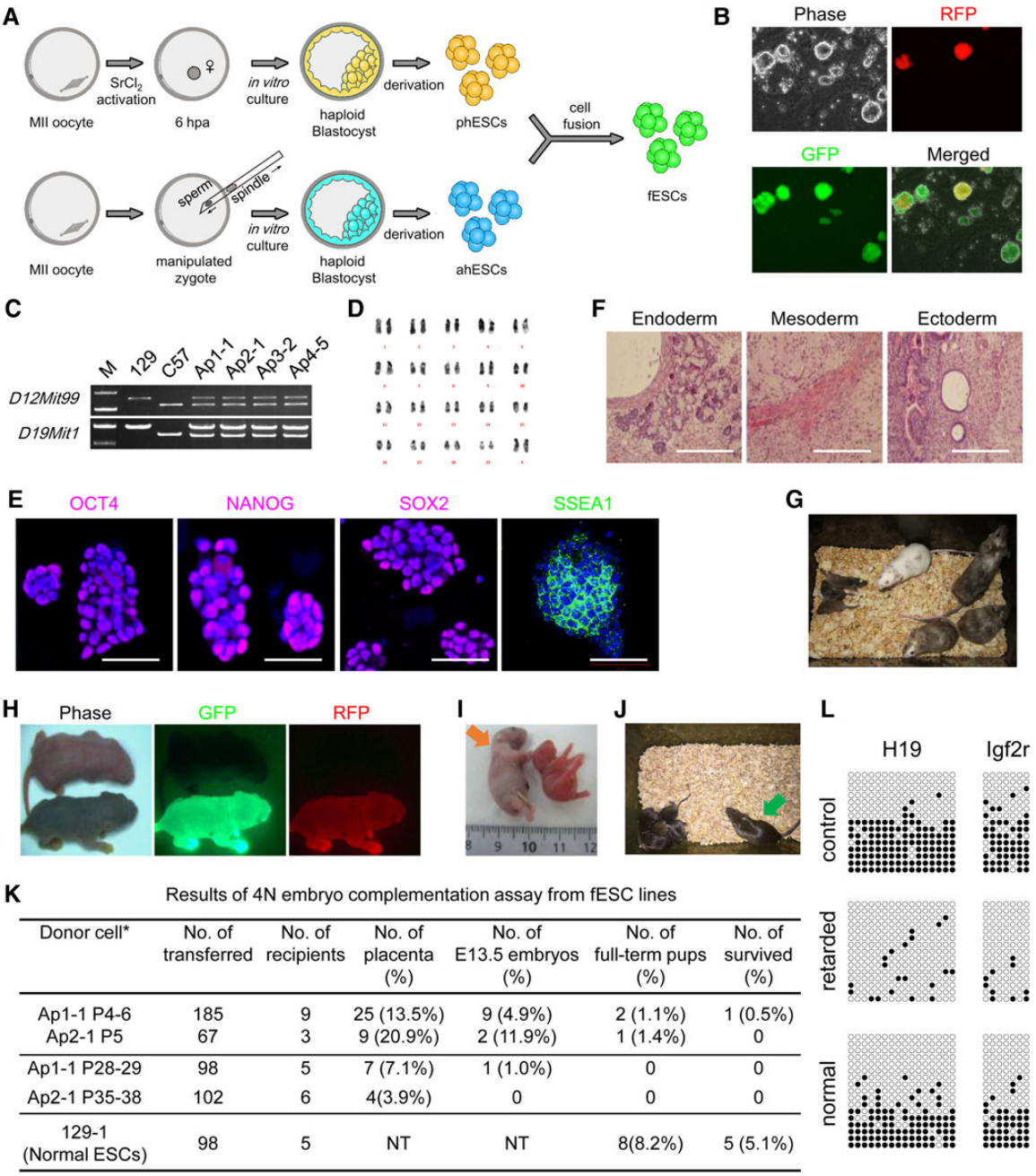
Recently, we and others have derived the mouse androgenetic and parthenogenetic haploid embryonic stem cells (ahESCs and phESCs) from the uniparental haploid blastocysts containing only the sperm or oocyte genome, respectively (Leeb and Wutz, 2011; Li et al., 2012; Yang et al., 2012). We hypothesize that if the fused ESCs (fESCs) from ahESCs and phESCs were established, the interaction between the maternal and paternal genomes would be completely eliminated during the fESC-generation process. Then the developmental potential of the fESCs could reflect whether co-participation of parental genomes before the blastocyst stage is essential for mouse embryonic development. Hence, we sought to establish a new way to exploit the requirement of co-participation of parental genomes in early development for mouse full-term development.

Firstly, we derived mouse ahESC and phESC lines as described, respectively (Leeb and Wutz, 2011; Li et al., 2012). The ahESCs and phESCs stably expressed green florescence protein (GFP) and red florescence protein (RFP), respectively, and maintained high percentage of haploid component through several rounds of fluorescence-activated cell sorting (FACS). To generate diploid fESCs, we sorted the haploid ahESCs and phESCs (both under passage 10) by FACS, fused them with polyethylene glycol (PEG), and then sorted diploid cells with double-positive expression of GFP and RFP to derive sub-cell-lines from single cell colony (assigned as passage 1 for fESC lines) (Figure 1A). We generated ~30 cell lines in three independent experiments, and randomly chose four cell lines, named AP1-1, AP2-1, AP3-2, and AP4-5, for further analyses.

These fESC lines maintained a classical mESC morphology and were positive for both GFP and RFP (Figure 1B). Simple sequence length polymorphism (SSLP) analysis confirmed the presence of the 129 genetic origin of ahESCs and C57 genetic origin of phESCs (Figure 1C). Further, these cell lines kept an intact karyotype with 38 + XX mouse chromosomes (Figure 1D). Similar to normal diploid mESCs, these cells exhibited positive signals for alkaline phosphatase staining (Supplementary Figure S1A), and expressed typical pluripotent markers including Oct4, Nanog, Sox2, and SSEA-1 (Figure 1E and Supplementary Figure S1B). These results demonstrate that we can derive stable mouse fESCs through fusion of mouse ahESCs and phESCs.

Next, we examined the developmental potential of the fESCs by analyzing the differentiation *in vitro* and production of chimeras *in vivo*. The fESCs could form embryoid bodies (EBs) (Supplementary Figure S2A and B), and form teratomas with all three germ layers at ~4 weeks after subcutaneous injection into severely combined immune deficiency mice (Figure 1F). Furthermore, by injecting the fESCs (agouti coat color) into mouse blastocysts (CD1 background with albino coat color), we obtained mouse chimeras with high-rate contribution of the fESCs, measured by coat color chimerism (Figure 1G and Supplementary Table S1). Due to lack of Y chromosomes in the ahESCs and fESCs, these cells could not differentiate into mature sperm (Li et al., 2012; Yang et al., 2012). Therefore, we mated female chimeras with male CD1 mouse for a germline transmission test. The birth of pups with agouti coat color indicated the successful germline transmission of the fESCs (Figure 1G). These results collectively demonstrate that the fESCs were pluripotent stem cells that could differentiate into all three germ layers as well as functional germ cells.

We then performed tetraploid (4N) complementation assay, which is the most stringent test for the developmental potential of ESCs (Jaenisch and Young, 2008), to examine whether the fESCs could support mouse full-term development (Supplementary Figure S3A). Out of 452 manipulated 4N blastocysts for all four fESC lines (passage 3 to passage 38), a total of three live pups (named fAP mice, reflecting mice from



K Results of 4N embryo complementation assay from fESC lines

Donor cell*	No. of transferred	No. of recipients	No. of placenta (%)	No. of E13.5 embryos (%)	No. of full-term pups (%)	No. of survived (%)
Ap1-1 P4-6	185	9	25 (13.5%)	9 (4.9%)	2 (1.1%)	1 (0.5%)
Ap2-1 P5	67	3	9 (20.9%)	2 (11.9%)	1 (1.4%)	0
Ap1-1 P28-29	98	5	7 (7.1%)	1 (1.0%)	0	0
Ap2-1 P35-38	102	6	4(3.9%)	0	0	0
129-1 (Normal ESCs)	98	5	NT	NT	8(8.2%)	5 (5.1%)

Figure 1 The generation and pluripotency of mouse fESCs. (**A**) Schematic overview of the derivation process of fESCs. Mouse androgenetic and parthenogenetic haploid ESC lines were obtained as previously reported. These cells are stably integrated of either GFP or RFP, and maintain high percentage of haploid component. (**B**) The morphology of Ap1-1 shows a classic doomed-colony with both GFP marker for androgenetic and RFP marker for parthenogenetic origin. (**C**) SSLP analysis of Ap1-1, AP2-1, Ap3-2, and Ap4-5 shows that these fESCs have both parental genetic origins with two DNA markers located in two chromosomes, respectively. (**D**) Standard G-binding karyotype of a fESC line (Ap1-1 passage 10), with 38 + XX chromosomal set. (**E**) fESCs express typical pluripotent markers including Oct4, Nanog, Sox2, and SSEA-1, examined by immunofluorescence staining. Scale bar, 100 μm. (**F**) Teratoma formation of Ap1-1(passage 12). Three germ layers are identified in the teratoma dissection slices by staining with hematoxylin and eosin. Scale bar, 500 μm. (**G**) Germline transmission of fESCs in the female chimera from injection of Ap1-1 (agouti) at passage 10 into CD1 blastocysts (white coat color) and litter from mating to CD1 male shows a germline transmission of coat color. (**H**) Live normal newborn fAp pups from 4N complementation using Ap1-1 fESCs. The pups from a pseudopregnant mouse at E19.5 are shown, with the expression of GFP and RFP throughout the pup body. (**I**) The growth-retarded fAp pup (arrow) died within 1 h after birth. (**J**) One Ap1-1-generated 4N complementation mouse survived to adulthood (arrow) and had offsprings. (**K**) Results of 4N complementation assay from fESC lines. (**L**) Methylation status of the DMRs of *H19* and *Igf2r* in a control pup (top), a growth-retarded fAP pup (middle), and a normal fAP pup (bottom).

fusion cells of ahESCs and phESCs) were recovered by cesarean section (C section) at 19.5 days (E19.5) of gestation (Figure 1H and I). All of them were female as expected, and carried both GFP and RFP from the parental fESCs (Figure 1H). One alive fAP mouse grew to adulthood with normal fertility (Figure 1J), while the other two died immediately after birth. Of two died fAP pups, one displayed overgrowth phenotypes frequently observed in 4N complementation assays (Zhao et al., 2009), and the other showed growth retardation (Figure 1K). Since the haploid ESCs often lose imprinting during derivation and culture, we examined the methylation status of the differentially methylated regions (DMRs) of important imprinted genes, including *H19*, *Igf2r*, *Snrpn*, *Kcnq*, and *IG-DMR*, in fAP mouse pups by bisulfate sequencing (Figure 1L and Supplementary Figure S4). Loss of imprinting was frequently observed in growth-retarded pup, whereas the imprinting status in live newborn fAP mouse was nearly normal as in control mouse, indicating that early death of newborn fAP pups could be partially attributed to the loss of imprinting (Figure 1L and Supplementary Figure S4). Consistently, the fESCs at high passages could not produce full-term pups (Figure 1K).

In summary, by utilizing the fused diploid ESCs from ahESCs and phESCs, we established a new approach to determine whether co-participation of parental genomes is needed for successful embryonic development. We showed that these fESCs, generated without interaction of paternal and maternal genomes, could produce viable and fertile progenies by tetraploid complementation. Our results demonstrate that full-term development of mouse embryos does not require co-participation of both paternal and maternal genomes before the blastocyst stage, which allows an *in vitro* functional analysis of the dynamics of DNA methylation first within each haploid parental genome and then in the diploid nucleus after their assembling. Given the important applications of haploid ESCs in genetic screening and genetic modification, our research also provides a new approach for generation of genetic models for recessive traits.

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