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Asymmetric Reprogramming Capacity of Parental Pronuclei in Mouse Zygotes

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SUMMARY

It has been demonstrated that reprogramming factors are sequestered in the pronuclei of zygotes after fertilization, because zygotes enucleated at the M phase instead of interphase of the first mitosis can support the development of cloned embryos. However, the contribution of the parental pronucleus derived from either the sperm or the oocyte in reprogramming remains elusive. Here, we demonstrate that the parental pronuclei have asymmetric reprogramming capacities and that the reprogramming factors reside predominantly in the male pronucleus. As a result, only female pronucleus-depleted (FPD) mouse zygotes can reprogram somatic cells to a pluripotent state and support the full-term development of cloned embryos; male pronucleus-depleted (MPD) zygotes fail to support somatic cell reprogramming. We further demonstrate that fusion of an additional male pronucleus into a zygote greatly enhances reprogramming efficiency. Our data provide a clue to further identify critical reprogramming factors in the male pronucleus.

INTRODUCTION

Somatic cell nuclear transfer (SCNT) experiments have revealed that molecules within oocytes at the metaphase II stage can reprogram somatic cells (Kato et al., 1998; Wakayama et al., 1998; Wilmut et al., 1997). Following fertilization, the reprogramming factors are believed to translocate from the cytoplasm into the pronuclei of zygotes, as zygotes that are enucleated at M phase instead of interphase retain the ability to reprogram somatic cells (Egli et al., 2007).

During fertilization, the parental genomes undergo differential epigenetic reprogramming to form a totipotent zygote. Immediately after fertilization, protamine is quickly released from the paternal genome and the decondensed sperm DNA is repackaged by maternal nucleosomal histones (McLay and Clarke, 2003). The active histone methylation marker H3K4me3 can be detected in the male pronucleus, but repressive histone methylation markers, including H3K9me2-3, H3K27me3, and H4K20me3, are mostly absent (Lepikhov et al., 2010). In contrast, all of these histone methylation markers can be detected in the female pronucleus. In addition to histone modifications, it is widely known that the paternal genome undergoes genome-wide DNA demethylation around the PN3 stage, whereas the DNA methylation state of the maternal genome seems to maintain a constant level (Mayer et al., 2000; Wossidlo et al., 2010).

Although the epigenetic modifications appear to differ between the male and female pronucleus, it remains unknown whether the parental pronuclei play distinct roles in reprogramming of somatic cells. In the present study, we designed serial nuclear transplantation experiments to address whether the parental pronuclei contribute equally to reprogramming. Interestingly, we found that the reprogramming factors seem to sequester asymmetrically in the parental pronuclei and the critical reprogramming factors reside predominantly in the male pronucleus. As a result, cloned offspring and nuclear transfer embryonic stem cell (ntESC) lines could only be generated from female pronucleus-depleted (FPD) zygotes, and male pronucleus-depleted (MPD) zygotes failed to support somatic cell reprogramming. We further demonstrated that the distinct epigenetic modifications of parental pronuclei might contribute directly to the developmental differences observed among somatic cell cloned embryos. More importantly, we found that fusion of an extra male pronucleus can significantly increase the efficiency of zygotic reprogramming, which may be informative for deriving human ntESC lines by using clinically discarded multipronuclei zygotes.

RESULTS

FPD Zygotes Enucleated in Mitosis Can Support Full-Term Development of ESC Cloned Embryos

To determine the reprogramming capacity of the parental pronucleus, we used FPD or MPD zygotes that had been enucleated at M phase as the recipient cytoplasm. Zygotes that had been enucleated at M phase or interphase were used as positive and negative controls, respectively (Figure 1A). As shown in Figure 1B, the female and male pronuclei of the mouse zygote could

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be clearly distinguished based on their distance from the second polar body and their distinct size (Adenot et al., 1997). Immunocytochemistry staining of 5-hydroxymethylcytosine (5hmC) and 5mC further confirmed this observation (Figure 1E) (Gu et al., 2011; Wossidlo et al., 2010). The removal of either pronucleus was performed by piezo-drill-assisted micromanipulation and was further confirmed by 5mC and 5hmC staining (Figures 1C–1G). The preimplantation developmental efficiency of the resultant haploid androgenetic or gynogenetic embryos was similar to previously reported results (Table S1) (Modliński, 1975; Yang et al., 2012).

FPD zygotes, MPD zygotes, and control intact zygotes were cultured in embryo culture medium containing demecolcine (DC) to prevent the progression of mitosis and to limit spindle formation at metaphase (Gasparrini et al., 2003). After the mitotic zygotes were released from DC exposure, they were transferred to medium containing MG-132, a proteasome inhibitor, for 25 min to allow assembly of the mitotic spindle (Riaz et al., 2011), which can be visualized by light microscopy (Figure 1H). Subsequently, the spindle was removed by micromanipulation, and staining with the DNA dye Hoechst 33342 confirmed the successful removal of chromosomes (Figure 1I). To compare the developmental potential of cloned embryos reconstituted with the different recipient cytoplasts, we used both OG2 (Oct4-GFP transgenic) ESCs (Figure S1A) and mouse embryonic fibroblasts (MEFs) arrested at M phase as donor cells for chromosome transfer experiments. The M-phase spindle-chromosome complexes were then microinjected into the aforementioned recipient cytoplasts to reconstitute cloned embryos (Figure 1J). After chromosome transfer and release from MG-132 exposure, segregation of the chromosomes and cytokinesis of the cloned embryos were observed within 2 hr (Figure 1K).

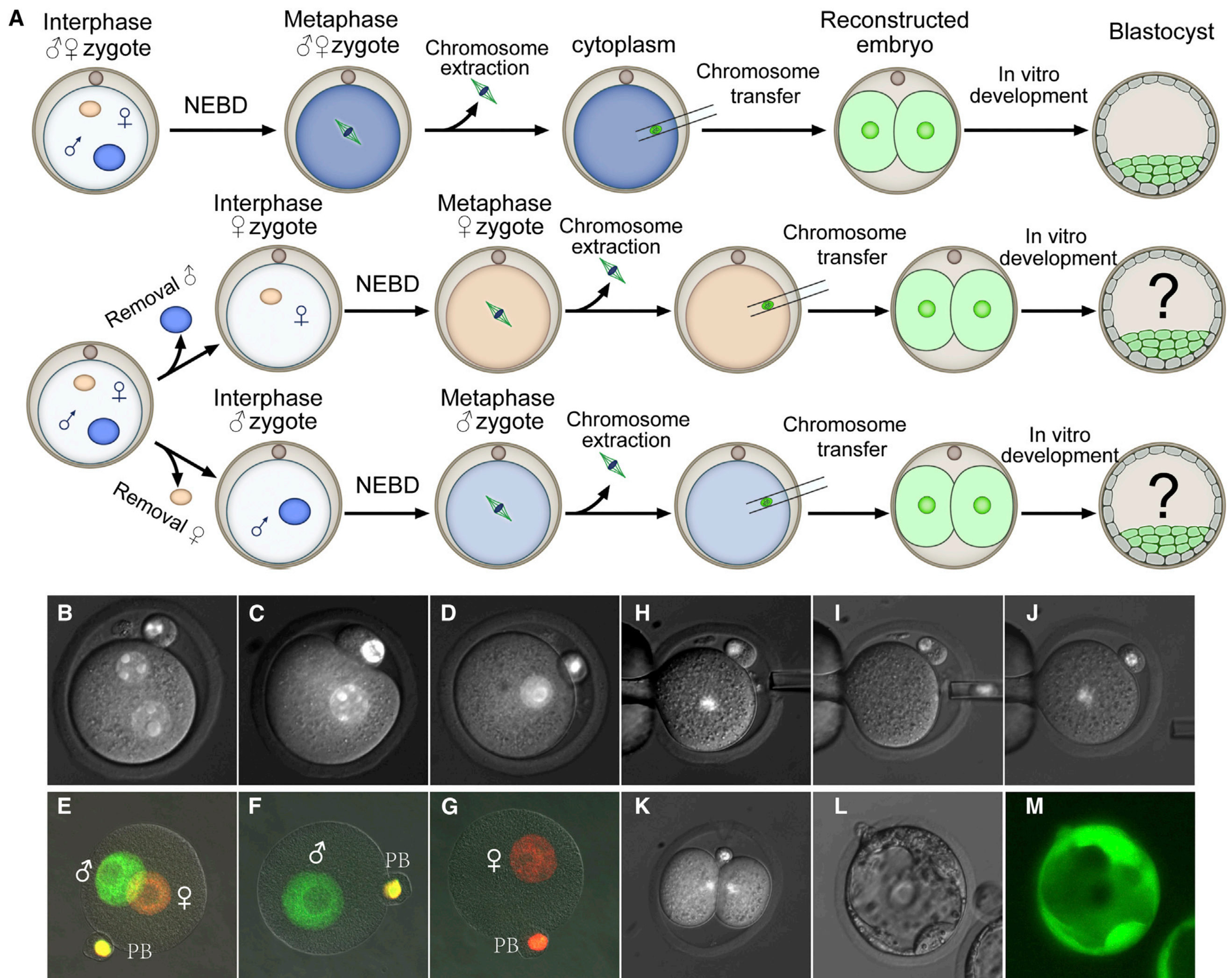
When ESCs were used as donors, 58 of 64 cloned embryos reconstructed using a zygote enucleated at M phase of mitosis cleaved, and 53% of the cleaved embryos developed to the blastocyst stage (Figure 1N), which is consistent with a previous report (Egli et al., 2007). When FPD zygotes were used for ESC chromosome transfer, 16% of the cloned embryos developed to the blastocyst stage. Albeit with low efficiency, 10% of the cloned embryos reconstructed using MPD zygotes developed to the blastocyst stage (Figures 1L–1N). When cloned embryos developed to the 4-cell stage, the differences between the FPD and MPD groups were significant (52% versus 30%) (Figure 1N). Serving as the negative control, nuclear transfer into zygotes at interphase led to development failure (Figure 1N).

We next evaluated the in vivo developmental potential of ESC cloned embryos reconstructed using either FPD or MPD zygotes by transferring 2-cell-stage cloned embryos into the oviducts of pseudo-pregnant females (Figures 2A and S1B). We evaluated the embryonic development of these cloned embryos, which were reconstituted using two different recipient cytoplasts, at embryonic day 10.5 (E10.5) and E14.5 and at full-term development, E19.5 (Table S2). One E10.5 embryo and one E14.5 embryo were recovered from the 215 transferred OG2-ESC cloned embryos reconstructed using FPD zygotes by cesarean section (Figures S1D–S1G). Oct4-GFP-positive cells could be visualized in the gonads of the E10.5 and E14.5 cloned embryos (Figures S1D and S1F). In contrast, no OG2-ESC cloned embryos reconstructed using MPD zygotes developed to the E10.5 stage (Figure S1C). Next, we used CMV-GFP ESCs as donor cells for chromosome transfer to easily observe cell origin. For this purpose, 2-cell-stage cloned embryos with GFP were produced and transferred to pseudo-pregnant females (Figure 2B). Three living pups were successfully recovered by cesarean section at E19.5 from 561 CMV-GFP ESC cloned embryos reconstructed using FPD zygotes (Figures 2C and 2D). All three of the cloned pups exhibited regular respiration, but one was killed because of a midline closure defect and one was rejected by the foster mother. One pup survived to adulthood and proceeded to produce F1 pups after mating with normal ICR mice (Figure 2C). The cloned pup was GFP positive, and PCR analysis of polymorphic markers verified that they were the same as those of the ESCs (Figures 2D and 2E). Consistent with previous reports (Eggan et al., 2001; Gao et al., 2003; Wakayama et al., 1999), the placental weight of the ESC cloned pups was found to be significantly higher than that of control placentas (Figure 2F). In striking contrast, no cloned pups were obtained from the 308 transferred CMV-GFP ESC cloned embryos reconstructed using MPD zygotes.

The experiments described thus far demonstrated that ESC chromosomes can be successfully transferred into either FPD or MPD zygotes to reconstruct cloned embryos. However, the developmental potential of the ESC cloned embryos reconstructed using FPD zygotes appeared to be better than that of the cloned embryos reconstructed using MPD zygotes.

FPD Zygotes, but Not MPD Zygotes, Can Support Preimplantation Development of Somatic Cloned Embryos

We next sought to investigate whether and to what extent the FPD or MPD zygotes could support somatic cell reprogramming. OG2 MEFs were used as donor cells for chromosome transfer, and reactivation of Oct4 in the cloned embryos served as a hallmark of successful reprogramming (Figure 3A). We performed 16 experiments using FPD zygotes (n = 1,030), and consistently obtained somatic cloned blastocysts in 14 of them. In contrast, a total of nine experiments using MPD zygotes were performed, and no cloned blastocyst was obtained. In addition, we also compared the percentages of 4-cell embryo and morulae development between the FPD versus MPD groups, and the difference appeared significant (40% versus 16%) (Figure 1N). Overall, our results demonstrated that only somatic cell cloned embryos reconstructed using FPD zygotes can develop to the blastocyst stage. Moreover, expression of the Oct4-GFP transgene was observed only in the cloned embryos reconstructed using FPD zygotes. Oct4-GFP expression in the cloned embryos was detectable in late-cleavage-stage embryos and was strong in cloned blastocysts (Figures 3B and S2A–S2C), similar to previously reported results (Riaz et al., 2011; Yoshimizu et al., 1999). We further demonstrated that this asymmetric reprogramming capacity is likely not caused by the size difference between the parental pronuclei, because depletion of the male pronucleus at the earlier stage



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Donor cells	Recipient cytoplasm	No. manipulated	No. cleaved	4 cell (%of cleaved)	Morula (%of cleaved)	Blastocyst* (%of cleaved)
OG2-ES	Zyg. (M)	64	58	50(86)^{A}	37(64)^{A}	31(53)^{A}
	♂Zyg. (M)	170	147	76(52)^{B}	30(20)^{B}	24(16)^{B}
	♀Zyg. (M)	263	238	72(30)^{C}	30(13)^{C}	23(10)^{C}
	Zyg. (I)	48	30	0(0)^{D}	0(0)^{D}	0(0)^{D}
OG2-MEF	Zyg. (M)	206	178	138(78)$^{\alpha}$	41(23)$^{\alpha}$	31(17)$^{\alpha}$
	♂Zyg. (M)	1030	718	287(40)$^{\beta}$	49(7)$^{\beta}$	30(4)$^{\beta}$
	♀Zyg. (M)	401	296	46(16)$^{\gamma}$	1(0.7)$^{\gamma}$	0(0)$^{\gamma}$
	Zyg. (I)	42	26	0(0)$^{\delta}$	0(0)$^{\gamma}$	0(0)$^{\gamma}$

Figure 1. Chromosome Transfer into FPD or MPD Zygotes Enucleated at M Phase of Mitosis

(A) Schematics of chromosome transfer into FPD or MPD zygotes. Following fertilization, factors that are required for reprogramming and development are hypothesized to sequester equally or selectively in the male or female pronucleus. After removal of the female or male pronucleus at the PN3-PN4 stage,

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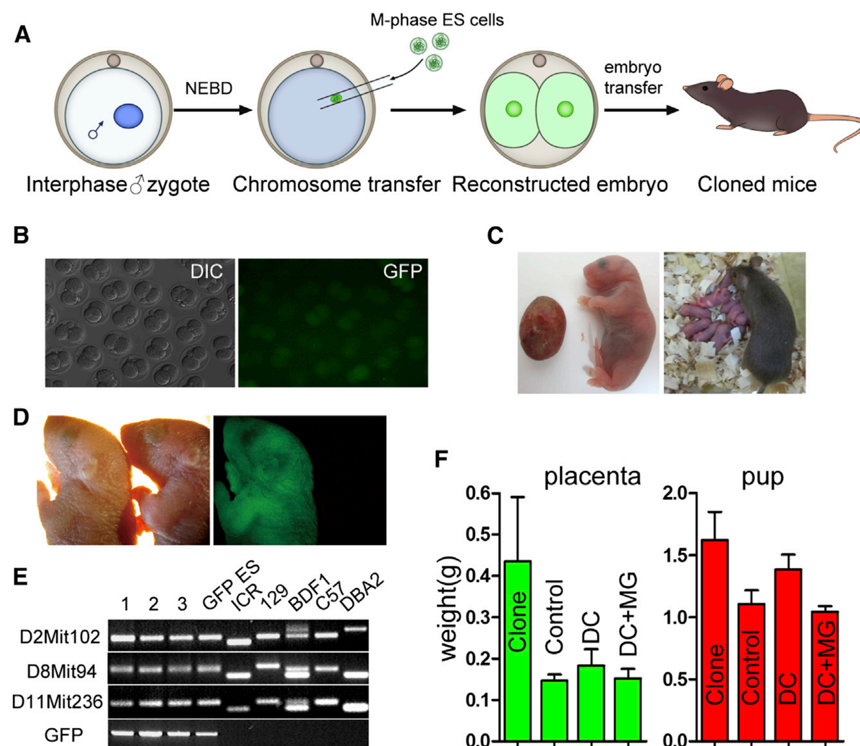


Figure 2. In Vivo Developmental Potential of Cloned Embryos Reconstructed Using ESCs

(A) Diagram of ESC chromosome transfer into an enucleated FPD zygote for mouse cloning.

(B) Two-cell-stage embryos reconstructed using CMV-GFP ESCs. Green fluorescence indicates that the CMV-GFP transgene has been expressed.

(C) A live cloned pup with an enlarged placenta, and an adult cloned mouse with germline transmission ability.

(D) The CMV-GFP ESC-cloned pup exhibits green fluorescence, whereas the control does not.

(E) DNA genotyping of the cloned pups confirmed their ESC origin.

(F) Body and placental weights of the cloned pups at birth. Control pups were from untreated normal embryos. DC, DC treatment 28–33 hr after human chorionic gonadotropin (hCG) injection; MG, 2 μM MG-132 treatment 33–33.5 hr after injection with hCG. Data are mean ± SD.

See also Figure S1 and Table S2.

(PN2), when no size difference was distinguished between parental pronuclei, showed no beneficial effects on somatic cell reprogramming (Table S3). In addition, we also evaluated the postimplantation development of somatic cloned embryos reconstructed with FPD zygotes. Unfortunately, no live cloned mice were obtained, although some degenerated embryos could be observed (Table S4).

Fully Pluripotent ESC Lines Established from Somatic Cloned Embryos Reconstructed by Chromosome Transfer into FPD Zygotes

To investigate whether chromosome transfer ESC (ctESC) lines can be successfully established from somatic cloned embryos reconstructed using FPD zygotes, we individually plated the cloned blastocysts in 96-well plates. Four ctESC lines (referred to as ♂M-1 to ♂M-4) expressing Oct4-GFP were ultimately established from 24 cultured cloned blastocysts (Figure 3C). As a control, one ctESC line exhibiting pluripotency was established from the somatic cloned embryo reconstructed using fibroblast chromosome transfer into a zygote enucleated at M phase (Figure S3).

The characteristics of these four ctESC lines were evaluated. Karyotyping analysis revealed the presence of the normal 40 chromosomes in one female cell line and three male cell lines (Figure 3D). The expression of pluripotent ESC markers, such as Oct4, Sox2, Nanog, and SSEA1, was observed by staining (Figure 3E). Bisulfite sequencing analysis indicated that successful demethylation occurred in the promoters of *Oct4* and *Nanog* in the ctESC lines (Figure 3F). The global gene-expression profile of the ctESC lines clustered closely with normal R1 ESCs (Figure 3G). The in vivo differentiation potential of these ctESC lines was also confirmed by a teratoma assay following

manipulated zygotes underwent nuclear envelope breakdown (NEBD) and entered mitosis. Mitotic donor cells were then transferred into the enucleated FPD or MPD zygotes to reconstitute cloned embryos.

(B) Mouse zygote with two pronuclei at interphase.

(C) FPD zygote in interphase.

(D) MPD zygote in interphase.

(E–G) Immunofluorescent staining of 5hmC (green) and 5mC (red) in a normal zygote (E), FPD zygote (F), and MPD zygote (G). ♂ and ♀ indicate the male and female pronucleus, respectively. PB, polar body.

(H) FPD zygote at M phase in the presence of MG-132.

(I) The spindle was removed from the FPD zygote at M phase.

(J) ESC spindle-chromosome complex was injected into the enucleated FPD zygote.

(K) Chromosome segregation and cytokinesis occurred in the reconstructed embryo within 2 hr after transfer.

(L and M) Blastocyst-stage cloned embryos reconstructed by ESC chromosome transfer into male-pronucleus-depleted zygotes.

(N) Developmental potency of cloned embryos reconstructed using ESCs or somatic cells. Zyg., zygote. ♂Zyg., FPD zygote. ♀Zyg., MPD zygote. M, M phase; I, interphase. Data are mean ± SEM. The values with different superscript letters indicate significant differences in the same column. Different donor cells were compared separately ($p < 0.05$). *Blastocyst rate based on the number of blastocysts at E3.5.

See also Table S1.

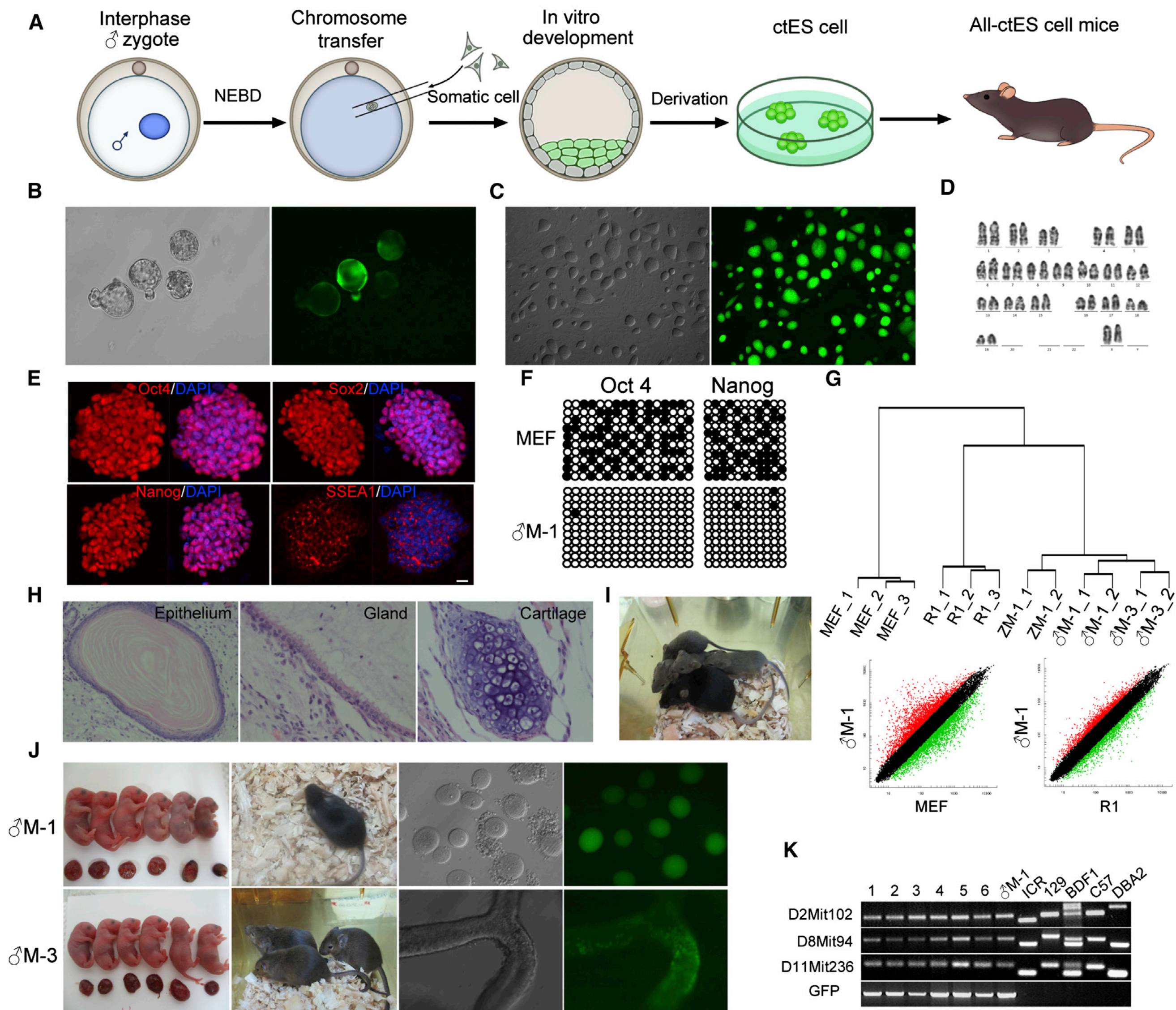


Figure 3. Derivation of ctESC Lines from Somatic Cell Chromosome Transfer-Derived Cloned Blastocysts

(A) Schematics of ctESC derivation from cloned embryos reconstructed by somatic cell chromosome transfer (SCCT) into enucleated FPD zygotes, and clonal mice production through tetraploid complementation.

(B) Cloned blastocysts produced by SCCT into enucleated FPD zygotes.

(C) ctESC line produced from the cloned blastocysts.

(D) Karyotype of the ctESC line.

(E) Immunostaining of the pluripotent markers Oct4, Sox2, Nanog, and SSEA1. Scale bar, 20 μm.

(F) Methylation analysis of MEFs and ctESCs. Open and closed circles indicate unmethylated and methylated CpGs, respectively.

(G) Gene-expression analysis of ctESC lines, zygote-derived ESC lines, MEFs, and R1 cells.

(H) ctESCs possessed multiple-differentiation potential, as shown in teratoma sections.

(I) A female chimera and its germline offspring.

(J) Full-term all-ctESC (clonal) mice produced from the ctESC lines through tetraploid complementation. Green fluorescence indicates that both female (upper row) and male (lower row) clonal mice carry the Oct4-GFP transgene, and the oocytes and seminiferous tubules are GFP positive.

(K) DNA genotyping of the clonal mice confirms their ctESC origin.

See also Figures S2–S4 and Tables S3–S6.

injection into immunocompromised severe combined immunodeficiency (SCID) mice. Cell types representing all three germ layers were observed (Figure 3H).

All four ctESC lines possessed a high degree of germline chimerism, as judged by the green fluorescence of E13.5 chimeric embryos generated by injecting ctESCs into normal

blastocysts (Table S5). When allowed to develop to term, the postnatal chimeric mice also displayed a high degree of agouti coat-color chimerism and germline transmission (Figure 3I).

To further investigate the level of pluripotency of the ctESC lines derived from the somatic cloned embryos reconstructed using FPD zygotes, we performed tetraploid complementation, the most stringent test of pluripotency. In total, 36 full-term all-ctESC mice were obtained from three of the four ctESC lines with high efficiency (Figure 3J; Table S6). Polymorphic marker analysis using PCR verified that the all-ctESC mice were indeed derived from the ctESC lines (Figure 3K). Furthermore, the all-ctESC mice exhibited Oct4-GFP transgene expression in the germinal vesicle (GV) oocytes or seminiferous tubules (Figure 3J). Moreover, the all-ctESC mice produced using tetraploid complementation grew to adulthood and produced F1 pups after mating with normal ICR mice (Figures S4A–S4C). Most importantly, the oocytes collected from the female F1 mice exhibited Oct4-GFP positivity (Figures S4D and S4E).

These results demonstrated that the FPD zygotes could successfully reprogram somatic cells to full pluripotency. In striking contrast, the MPD zygotes were incapable of reprogramming somatic cells, which indicates that the parental pronuclei have asymmetric reprogramming capacities.

Distinct Epigenetic Reprogramming Ability of Parental Pronuclei in Somatic Cloned Embryos

To better understand the underlying mechanism of the reprogramming asymmetry of the parental pronuclei, we analyzed the major epigenetic modifications that occurred in the somatic cloned embryos reconstructed with either the FPD or MPD zygotes. Since most somatic cloned embryos reconstructed using MPD zygotes were arrested at the 2-cell stage, we used 2-cell-stage cloned embryos for the analysis. The intensity of H3K4me3 staining did not differ between the two types of cloned embryos (Figures 4A and 4B). However, staining for H3K9 acetylation, H3K9me3, 5hmC, and 5mC revealed dramatic differences between the two types of cloned embryos (Figures 4A and 4B). The staining intensity of the gene-silencing markers H3K9me3 and 5mC (Bui et al., 2008; Chen et al., 2013; Wang et al., 2007; Wossidlo et al., 2010, 2011) in the cloned embryos reconstructed using MPD zygotes was much higher than that in the cloned embryos reconstructed using FPD zygotes. In contrast, the staining intensity of the putative gene-activating markers H3K9 acetylation and 5hmC (Lennartsson and Ekwall, 2009; Weinberger et al., 2012) in cloned embryos reconstructed using MPD zygotes was significantly lower than that in the cloned embryos reconstructed using FPD zygotes (Figures 4A and 4B). Furthermore, bisulfite sequencing analysis indicated that successful demethylation occurred in the promoter of *Oct4* in the 2-cell-stage cloned embryos reconstructed using FPD zygotes. In contrast, the promoter of *Oct4* remained highly methylated in the 2-cell-stage cloned embryos reconstructed using MPD zygotes (Figure 4C). Taken together, these results indicate that the genes that are important for development might not be properly activated in the cloned embryos reconstructed using MPD zygotes, which would cause their developmental arrest.

Fusion of an Extra Male Pronucleus into the Zygote Can Significantly Improve the Zygotic Reprogramming Ability

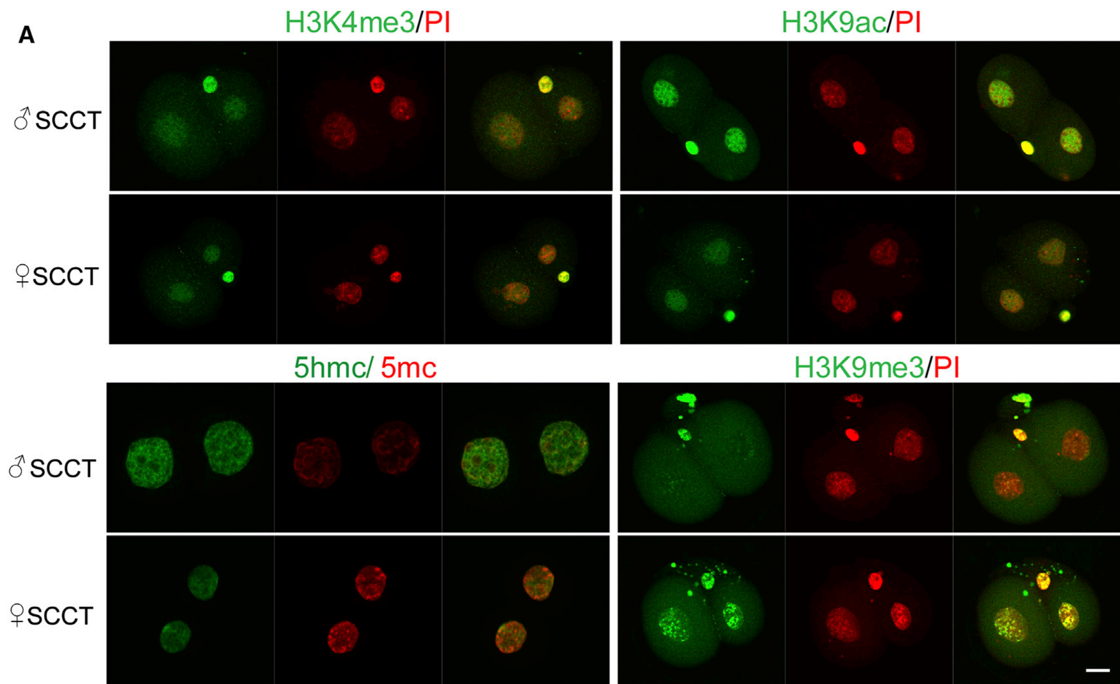
As noted above, although FPD zygotes can reprogram somatic cells to a pluripotent state and produce full-term cloned embryos, the cloned embryos still have a lower developmental efficiency than those reconstructed with zygotes enucleated at M phase. We therefore attempted to ask whether the developmental potential of cloned embryos could be improved by introducing an extra male pronucleus into the FPD zygotes or normal zygotes. We obtained two-male-pronuclei zygotes by fusing an extra male pronucleus with the FPD zygotes (Figure 4D). The fusion of an extra male pronucleus with the FPD zygotes significantly improved the reprogramming process. The cloned embryos reconstructed using two-male-pronuclei zygotes had a significantly higher blastocyst development efficiency than the single-pronucleus zygotes (19% versus 4%; Figure 4E). Most interestingly, the fusion of an extra male pronucleus with mouse zygotes significantly increased the reprogramming efficiency to a level even higher than that observed in oocytes (Figures 4F and 4G; Table S7).

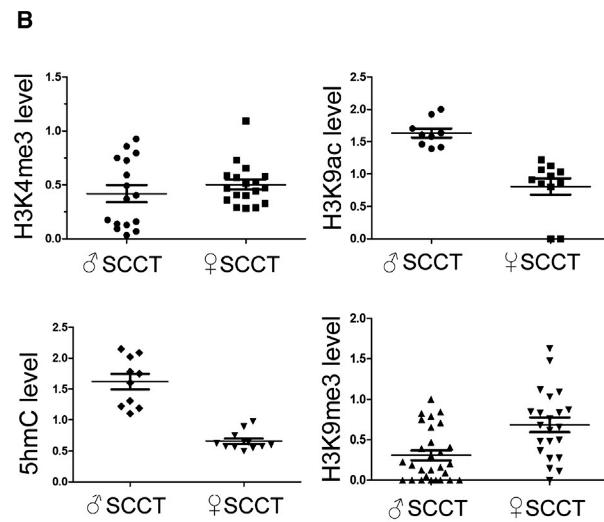
DISCUSSION

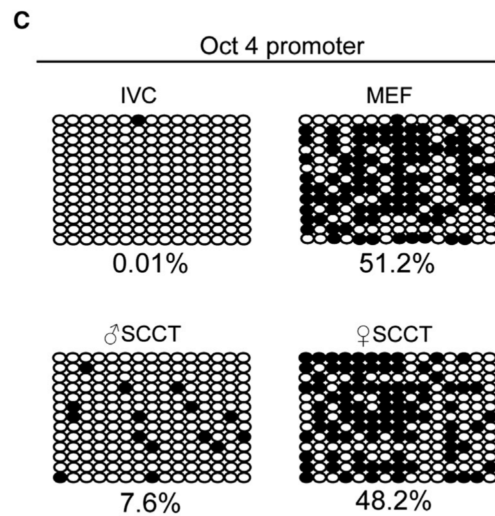
In summary, our present study clearly demonstrates that the parental pronuclei of the mouse zygote have asymmetric reprogramming capacities. Our study indicates that the reprogramming factors preferentially translocate into the male pronucleus following fertilization, and thus provides a fundamental basis for characterizing candidate reprogramming factors in the future. Some well-studied factors might correlate with this reprogramming asymmetry. It was previously shown that Parp1 is localized mainly in the male pronucleus, and the overexpression of Parp1 can effectively promote induced pluripotent stem cell (iPSC) induction (Chiou et al., 2013; Wossidlo et al., 2010). Moreover, we found that Parp1 was localized mainly in the male pronucleus at distinct pronuclear stages (PN1–PN5) and Parp1 dissociated from chromatin during mitosis (Figure S4F). Similarly, DNA dioxygenase Tet3 localizes mainly in the male pronucleus following fertilization, and oocytes lacking Tet3 have a reduced ability to reprogram somatic cells (Gu et al., 2011). Furthermore, H3K9me3 has been found to serve as a barrier during somatic cell reprogramming into iPSCs (Chen et al., 2013), and in our experiments, we noticed that the removal of somatic H3K9me3 could only be observed in cloned embryos reconstructed with FPD zygotes.

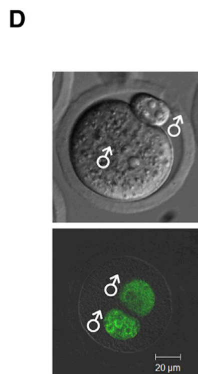
The cytoplasm of an oocyte is evolutionally designed to reprogram the sperm. It is well accepted that the sperm is more differentiated, as the paternal genome of mouse sperm contains higher levels of genome-wide DNA methylation than the oocyte (Smallwood et al., 2011), and therefore more reprogramming factors are required. This might explain why the critical reprogramming factors selectively translocate into the male pronucleus during fertilization.

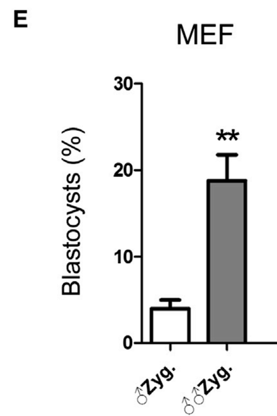
We recently demonstrated that ntESCs exhibit enhanced telomere rejuvenation and improved mitochondrial function relative to iPSCs (Le et al., 2014). However, the cloning procedure needs to be further optimized because the cloning efficiency

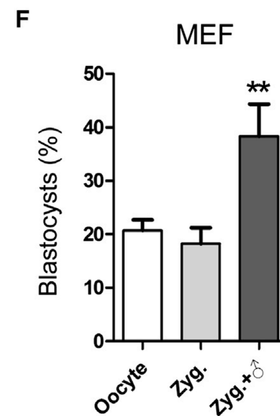


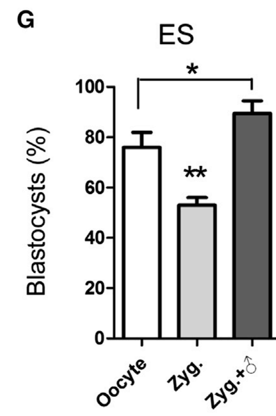












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remains very low at present. One possible explanation for this low efficiency is that the reprogramming factors within an oocyte are insufficient for reprogramming a somatic cell, but attempts to remedy this by adding additional cytoplasm showed no significant effect (Sayaka et al., 2008). Given the fact that the reprogramming capacity of zygotes is inferior to that of oocytes, it is very important to search for a new approach to improve the reprogramming capacity of zygotes. Our study demonstrates that the fusion of an extra male pronucleus can dramatically improve the reprogramming capacity of zygotes. It is informative regarding the derivation of human ntESC lines using multi-pronuclei zygotes instead of oocytes (Tachibana et al., 2013), because multi-pronuclei zygotes are generally discarded in human in vitro fertilization clinics, and the use of these discarded embryos for the derivation of patient-specific ntESC lines no longer poses ethical issues.

EXPERIMENTAL PROCEDURES

Mice

All mice used in this study were housed in the animal facility of the National Institute of Biological Sciences. Our study procedures were consistent with the National Institute of Biological Sciences guidelines.

Chromosome Transfer

Chromosome transfer was conducted as previously described (Egli et al., 2007). Female or male pronuclei were removed using a piezo-drill micromanipulator. After treatment with DC and MG-132, the spindle-chromosome complexes of the mitosis-arrested haploid zygotes were removed, and mitotic donor cell chromosomes were then transferred into the enucleated zygotes. The reconstructed embryos were cultured and allowed to develop to the blastocyst stage.

Immunofluorescence Staining

ESCs and reconstructed and normal early embryos were stained according to previously described protocols (Gao et al., 2013; Wang et al., 2007). Stained cells were observed with an LSM 510 META microscope (Zeiss).

Microarray Analysis

Total RNA was extracted using Trizol reagent (Invitrogen) in two separate experiments. Analysis with the Mouse Gene 1.0 ST array (Affymetrix) was performed at CapitalBio in Beijing.

ACCESSION NUMBERS

The microarray data have been deposited in the GEO database under accession number GSE49148.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, four figures, and seven tables and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2014.02.018.

AUTHOR CONTRIBUTIONS

W.L., Z.H., and S.G. conceived the experiments. W.L., J.Y., and X.K. performed most of the experiments. Y.J., H.G., Y.Z., B.H., W.H., and H.W. contributed reagents and helped with experiments. W.L., Z.H., and S.G. analyzed data and wrote the paper.

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Figure 4. Fusion of an Extra Male Pronucleus into the Zygote Can Significantly Improve the Zygotic Reprogramming Capacity

(A) Immunofluorescence images of reconstructed 2-cell embryos after SCCT into enucleated FPD or MPD zygotes. Embryos approximately 10 hr after SCCT were stained for H3K4me3, H3K9ac, 5hmC/5mC, and H3K9me3. ♂SCCT, SCCT into FPD zygote enucleated at M phase. ♀SCCT, SCCT into MPD zygote enucleated at M phase. Scale bar, 20 μm.

(B) Quantitative analysis of H3K4me3, H3K9ac, 5hmC, and H3K9me3 fluorescence intensity in reconstructed 2-cell-stage embryos. Each data point is based on the level of the green signal relative to the PI (5hmC relative to DAPI) staining intensity of the same pronucleus. Data are mean ± SD.

(C) Bisulfite sequencing analysis of *Oct4* demethylation in 2-cell-stage cloned embryos reconstructed using SCCT into enucleated FPD or MPD zygotes. MEFs and normal 2-cell-stage embryos were used as controls. IVC, in vitro cultured.

(D) Immunofluorescent staining of 5hmC (green) and 5mC (red) in a ♂♂zygote (♂♂zygote indicates that an extra male pronucleus was fused into a FPD zygote).

(E) Developmental potency of cloned embryos reconstructed with or without an extra male pronucleus.

(F and G) Developmental potency of cloned embryos reconstructed with a zygote fused with an extra male pronucleus. Data in (E)–(G) are represented as mean ± SEM. *$p < 0.05$; **$p < 0.01$.

See also Table S7.

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