Nuclear reprogramming by interphase cytoplasm of two-cell mouse embryos

Eunju Kang1, Guangming Wu2, Hong Ma1, Ying Li1, Rebecca Tippner-Hedges1, Masahito Tachibana4, Michelle Sparman1, Don P. Wolf1, Hans R. Schöler2 & Shoukhrat Mitalipov1

Successful mammalian cloning using somatic cell nuclear transfer (SCNT) into unfertilized, metaphase II (MII)-arrested oocytes attests to the cytoplasmic presence of reprogramming factors capable of inducing totipotency in somatic cell nuclei1-6. However, these poorly defined maternal factors presumably decline sharply after fertilization, as the cytoplasm of pronuclear-stage zygotes is reportedly inactive1-3. Recent evidence suggests that zygotic cytoplasm, if maintained at metaphase, can also support derivation of embryonic stem (ES) cells after SCNT2-4, albeit at low efficiency. This led to the conclusion that critical oocyte reprogramming factors present in the metaphase but not in the interphase cytoplasm are ‘trapped’ inside the nucleus during interphase and effectively removed duringenucleation7. Here we investigated the presence of reprogramming activity in the cytoplasm of interphase two-cell mouse embryos (I2C). First, the presence of candidate reprogramming factors was documented in both intact and enucleated metaphase and interphase zygotes and two-cell embryos. Consequently, enucleation did not provide a likely explanation for the inability of interphase cytoplasm to induce reprogramming. Second, when we carefully synchronized the cell cycle stage between the transplanted nucleus (ES cell, fetal fibroblast or terminally differentiated cumulus cell) and the recipient I2C cytoplasm, the reconstructed SCNT embryos developed into blastocysts and ES cells capable of contributing to traditional germline and tetraploid chimaeras. Last, direct transfer of cloned embryos, reconstructed with ES cell nuclei, into recipients resulted in live offspring. Thus, the cytoplasm of I2C supports efficient reprogramming, with the ability to use interphase cytoplasm in SCNT could aid efforts to generate autologous human ES cells for regenerative applications, as donated or discarded embryos are more accessible than unfertilized MII oocytes.

We studied messenger RNA expression levels and cellular localization of several maternal and embryonic factors in unfertilized oocytes and pre-implantation-stage embryos8-13, namely, Hsf1, Brg1 (also known as Smarca4), Bmi1, Oct4 (also known as Pou5f1), Sall4, Esrrb, Apobec1, Aid (also known as Aicda) and Tet1. We initially normalized Gapdh expression and confirmed that mRNA levels were statistically similar in intact and enucleated embryos and that protein was equally distributed throughout the cells and, therefore, enucleation does not seem to deplete these factors in the cytoplasm. (Extended Data Fig. 2a, b).

Success in mammalian SCNT has been attributed to the use of G0/G1-arrested donor nuclei with mature, unfertilized oocytes naturally arrested at MII as the recipient cytoplasm8-13. The slight cell cycle mismatch in this case could presumably be corrected shortly after SCNT by nuclear envelope breakdown followed by premature chromosome condensation induced by metaphase-specific factors present in the cytoplasm14. Thus, both the donor nucleus and recipient cytoplasm resume coordinated embryonic cell divisions after artificial activation of SCNT embryos. In clarifying the importance of cell cycle matching to reprogramming success, we established the timing of cleavage initiation, from which the cell cycle of the recipient I2C cytoplasm could be assessed. Then, we carefully timed the onset and progression of the mitotic cell cycle during the transition from zygote to two-cell-stage embryo. Most zygotes entered first mitosis between 29 and 35 h after human chorionic gonadotropin (hCG) administration and formed centrally localized metaphase spindles detectable with polarizing microscopy. Zygotes progressed quickly through anaphase and telophase, culminating in cell division and formation of the two-cell embryo. Approximately 30 min after the onset of cleavage, two-cell embryos formed nuclei that were visible microscopically, corroborated by nuclear envelope detection using lamin B immunocytochemistry. Nuclei became more prominent by 60 min after cleavage and increased in size during the next 10 h (Fig. 1a-c). Incorporation of 5-bromodeoxyuridine (BrdU), an indicator of S phase, was first detected approximately 3–4 h after cleavage onset and was evident in embryos up to 7–8 h after cleavage. Embryos labelled from 8 h after cleavage did not incorporate BrdU, suggesting their transition into G2 phase (Fig. 1b). The majority of mouse two-cell embryos completed the full cell cycle and entered into the next metaphase approximately 18–20 h after first cleavage. Thus, we determined the complete cell cycle of a mouse two-cell embryo, starting with the initial cleavage (0 h) and onset of G1 phase. G1 phase concluded and DNA synthesis and S phase were initiated approximately 3 h after the end of the previous metaphase. S phase ended by 8 h and blastomeres entered G2 phase, which lasted a minimum of 9 h (Fig. 1d). We next determined the cell cycle characteristics of two nuclear donor cell types—fetal fibroblasts (FFs) and ES cells—and sorted populations for G0/G1-, G2/M- and S-phase cells (Extended Data Fig. 3).

We then examined SCNT into enucleated I2C (Fig. 2a). When G0/G1-phase FFs were introduced into early G1 (0.5–1 h) enucleated blastomeres, 38% of reconstructed SCNT embryos progressed to blastocysts. This rate was comparable with conventional SCNT of G0/G1 FFs into enucleated MII oocytes (40%), although significantly reduced compared with fertilized controls (94%; Table 1). When G0/G1 FFs were fused into intermediate (1–2 h) or late (2–3 h) G1 enucleated blastomeres, blastocyst development significantly declined (Table 1) and transfer of G0/G1 FFs into S-phase I2C cytoplasm (4–6 h) or metaphase zygotes (0 h) resulted in developmental arrest. When actively proliferating FFs in S phase were introduced into S-phase blastomeres, 24% of the resultant SCNT embryos reached the blastocyst stage. We also used ES cells and cumulus cells for nuclear transfer and observed considerable blastocyst development when our matching criteria were satisfied (Table 1). To examine a possible role for sperm-activated cytoplasm in interphase SCNT success, we generated enucleated parthenogenetic I2C and carried out SCNT with FFs, observing blastocyst development comparable with SCNT into fertilized counterparts or into
conventional MII oocytes (Table 1). Thus, both artificial and sperm-activated cytoplasm of I2C support reprogramming. The arrest of cell-cycle-mismatched SCNT embryos could be secondary to nuclear arrest or apoptosis at the G1/S or S/G2 cell cycle checkpoints17 (Extended Data Fig. 4).

We used FFs carrying the green fluorescent protein (GFP) transgene under the control of the Oct4 promoter and β-galactosidase19 to confirm the origin of the nucleus in cloned embryos. The GFP signal was not detectable after fusion with I2C cytoplasm (Fig. 2b). Re-expression of the GFP transgene was observed in late four-cell embryos that continued into the compact morula. As expected, GFP expression was confined to the inner cell mass (ICM), having disappeared in the trophectoderm (Fig. 2b). Nuclear donor FFs weakly expressed Brg1 (Extended Data Fig. 2b, c) and a similar signal was found in the nuclei of two-cell SCNT embryos shortly after introduction of FF nuclei into I2C cytoplasm (Fig. 2c). However, Brg1 protein became increasingly detectable in the nuclei of SCNT embryos, 3 and 12 h after transfer, and in the four- and eight-cell stages (Fig. 2c and Extended Data Fig. 2d). Whereas Hsf1 was not detected in FFs, it was expressed in SCNT embryos (Extended Data Fig. 2d).

Figure 2d). To examine expression of candidate reprogramming factors in SCNT embryos generated with I2C cytoplasm, we also measured mRNA levels for Apobec1, Aid and Tet1 after 12 h. Expression patterns and levels of Apobec1 and Tet1 were comparable with fertilized controls (P < 0.05), with the exception of the eight-cell stage, at which Tet1 was upregulated in SCNT embryos. Aid transcript levels were significantly lower in SCNT embryos compared with controls (Fig. 2d).

The developmental potential of embryos produced by SCNT with FFs into enucleated I2C was evaluated further by plating blastocysts (n = 26) onto feeder layers. All adhered, eight-formed typical ICM outgrowths and, after subsequent passaging, seven stable nuclear transfer (nt)ES cell lines (27%) with typical colony morphology and expression of Oct4 and SSEA-1 (also known as Fut4) were established (Fig. 3a and 3b). Two-cell embryo Enucleation SCNT 14 h after 3 h mRNA abundance before after 12 h after 3 h after 4 to 5 h after 10 to 12 h in control and SCNT embryos (*P < 0.05).

Data show average ± s.d.
Extended Data Table 1). Similarly, three ntES cell lines were generated from 14 blastocysts (21%) after interphase SCNT with cumulus cells (Extended Data Table 1). With FFs carrying Oct4-GFP as nuclear donor cells, the resulting ntES cells re-expressed GFP at high levels (Fig. 3a). We confirmed normal karyotype and gender in all ntES cell lines (Extended Data Table 1) and corroborated their nuclear DNA origin from somatic cells and mitochondrial (mt)DNA inheritance from I2C cytoplasm (Fig. 3b, Extended Data Fig. 5a, b and Extended Data Table 1). The ability to form chimaeric offspring was evaluated by random selection of female ntES cells derived from FFs (ntES2) and injection into diploid eight-cell embryos. After transfer into recipients, 14 pups were born, 10 of which were chimaeric on the basis of the coat colour contribution of ntES cells on an albino background of host ICR embryos (Extended Data Table 2). The chimaera rate varied from 5% to more than 90% as determined by visual evaluation. Pups produced by breeding chimaeric females with control males showed germline transmission of the ntES cell genotype (Extended Data Fig. 5c).

We tested ntES cells for the ability to generate all-ES-cell offspring by tetraploid complementation20. After transfer of 165 tetraploid embryos injected with ntES2 cells, five live pups were recovered, of which four survived to adulthood and exhibited exclusively the ntES-cell coat colour phenotype (Extended Data Fig. 5d and Extended Data Table 2). We corroborated the all-ntES-cell origin of these mice by microsatellite and mtDNA genotyping (Extended Data Fig. 5a, b, e). Quantitative mtDNA analysis was characterized by the absence of any detectable contribution from host tetraploid embryos (Extended Data Fig. 5b). Additionally, two ntES cell lines derived from adult cumulus cells contributed to chimaeras using both diploid and tetraploid complementation assays (Fig. 3c, d and Extended Data Tables 1, 2). These stringent pluripotency tests confirm that ntES cells derived by SCNT into enucleated I2C blastomeres are the equivalent of embryo-derived ES cells.

Embryos produced by nuclear transfer into I2C cytoplasm were evaluated for totipotency by direct transfer into uteri of pseudopregnant females. When 115 SCNT blastocysts generated with FFs were transferred into ten recipients, four became pregnant and showed implantation sites, but none contained viable fetuses (Extended Data Table 3). An explanation for failed pregnancy may be the absence of a histone deacetylase inhibitor, which is essential for efficient live offspring production in conventional SCNT with MII oocytes21. However, when 89 nuclear transfer blastocysts generated with ES cell nuclei carrying a lacZ transgene were transferred, four live pups were recovered by caesarean section (Extended Data Table 2 and Extended Data Table 3). Their body and placental weights varied significantly (Extended Data Fig. 6b). Whole-body 5-bromo-4-chloro-3-indoly-β-D-galactoside (X-gal) staining confirmed the nuclear transfer origin of the offspring (Fig. 3f) and placenta

Table 1 | In vitro development of embryos after nuclear transfer into enucleated I2C

| Recipient cytoplasm type | Recipient cytoplasm cell cycle (hours after cleavage) | Nuclear donor cell cycle | Donor cell type | N | Cleaved embryos (%) | Blastocysts (%) |
|--------------------------|-----------------------------------------------|------------------------|----------------|---|---------------------|----------------|
| In vivo fertilized two-cell embryos | Metaphase | G0/G1 | FFs | 55 | 2 (4) | 0 |
| Early G1 (0.5–1) | G0/G1 | FFs | 227 | 11.0 (48) | 86 (38) |
| Intermediate G1 (1–2) | G0/G1 | FFs | 56 | 12.21 | 8 (14) |
| Late (2–3) | G0/G1 | FFs | 37 | 5.14 | 4 (11) |
| S (4–6) | S | FFs | 72 | 2.38 | 17 (24) |
| Early G1 (0.5–1) | G1 | ES cells | 161 | 63.39 | 53 (33) |
| Early G1 (0.5–1) | S | ES cells | 43 | 0 | 0 |
| S (4–6) | S | ES cells | 744 | 335 (45) | 293 (39) |
| Early G1 (0.5–1) | G0/G1 | CCs | 257 | 58.23 | 39 (15) |
| Intact controls | N/A | N/A | 135 | 133 (99) | 127 (94)* |
| Parthenogenetically activated two-cell embryos | Early G1 (0.5–1) | G0/G1 | FFs | 32 | 15.47 | 11 (34) |
| Intact parthenote controls | N/A | N/A | 86 | 84 (98) | 78 (91)* |
| MII oocytes | Lift | G0/G1 | FFs | 289 | 161 (56) | 116 (40) |
| MI | G1 | ES cells | 113 | 46 (41) | 25 (22) |
| MI | G0/G1 | CCs | 136 | 95 (70) | 23 (17) |
| S | G0/G1 | ES cells | 70 | 8 (11) | 2 (3) |

Control versus experimental treatment within each group. CCs, cumulus cells; N/A, not applicable. *P < 0.05.

Figure 3 | Characterization of ntES cells and live offspring born after interphase SCNT. a, Morphology of ntES cells and expression of the pluripotency markers Oct4 and SSEA-1 by immunostaining. Second from the left, re-expression of GFP in ntES cells derived from FFs carrying Oct4–GFP (original magnification, ×100). b, Genotyping of ntES cells using chromosomal markers D1Mit26, D10Mit3 and D14Mit10. Numbers indicate the position of the chromosome. c, Diploid chimaeric pups generated by injection of ntES cells from cumulus cells (black) into ICR host embryos (albino). Note that two of the three pups contained exclusively the ntES cell phenotype. d, Tetraploid complementation pups produced after injection of ntES cells (black) into tetraploid host embryo (agouti). e, Genotyping of chimaeric offspring generated with ntES cells derived from cumulus cells by mtDNA profiling. All tetraploid and two of the three diploid chimaeras contained ntES cell mtDNA only. PWD is mtDNA haplotype derived from PWD/ph mice (GenBank accession EF108343.1). f, X-gal staining of control (non-stained, left) and cloned pup carrying the lacZ gene (blue stained, right). Scale bar, 0.5 cm. g, X-gal detection in placental tissue section recovered from a cloned pup. Scale bar, 0.5 cm.
Interphase nuclear transfer into fertilized embryos has resulted in live offspring when pronuclei were exchanged between two zygotes and when four- to eight-cell blastomere nuclei were transferred into enucleated two-cell embryos. However, nuclear transfer of more advanced ICM cells, ES cells or somatic cells into interphase cytoplasm resulted in early developmental arrest, probably reflecting mismatched cell cycles. Nuclear transfer of 4–16-cell blastomeres into enucleated MII oocytes has also resulted in live offspring in several species, including humans. SCNT embryos were placed into droplets of the recombinant envelope (HVJ-E; GenomONE-CF HVJ Envelope Cell Fusion Kit) and kept on ice until use. Modified I2C enucleation and nuclear transfer were done with spindle observation using an optical birefringence system (Oosight; Otsuka). The present results suggest that the interphase cytoplasm has reprogramming capacity and that precise cell cycle synchrony between donor and recipient can lead to the development of functional ES cells or live offspring. This supposition would undermine the premise that SCNT failures involving interphase cytoplasm are secondary to the absence of critical maternal factors removed during enucleation. In view of the present results in mice and the unsuccessful attempt to conduct SCNT into human metaphase zygotes, SCNT into human two-cell embryos with proper cell cycle matching should be reconsidered.

 METHODS SUMMARY

All animal procedures were approved by the Institutional Animal Care and Use Committee at Oregon Health & Science University. Two-to-three-month-old B6D2F1 (C57BL/6J female × DBA/2 male) or B6D2F1-mtW2ph (C57BL/6J-mtW2ph/Fore) female × DBA/2 male) females were superovulated with 5–10 international units of (IU) pregnant mare’s serum gonadotropin (PMSG) and hCG and mated to B6D2F1-mtW2ph/Fore males. Zygotes were collected from the excised oviducts of plugged females at 24–26 h after hCG injection and monitored for cleavage. Nuclear transfer was carried out based on timing after the first cleavage division, 30–38 h after hCG treatment. Nuclear donor FFs, cumulus cells or ES cells suspended in Dulbecco’s phosphate-buffered saline (DPBS) (10 μl) were mixed with 5 μl haemagglutinating virus of Japan envelope (HVJ-E; GenomONE-CF HVJ Envelope Cell Fusion Kit) and kept on ice until use. Modified I2C enucleation and nuclear transfer were done as shown in Supplementary Video 1. SCNT embryos were placed into droplets of KSOM medium covered with mineral oil and cultured to the blastocyst stage. Conventional MII SCNT was performed as described except that enucleation was done with spindle observation using an optical birefringence system (OosightTM; http://www.cri-inc.com) in a glass-bottom dish. All manipulations were completed on the heated stage (37°C) of an Olympus microscope equipped with a XYClone laser (http://www.hamiltonthorne.com) for zona drilling.

Online Content Methods, along with any additional Extended Data display items and Source Data, are available in the online version of the paper; references unique to these sections appear only in the online paper.

Received 11 November 2013; accepted 6 February 2014.

Published online 26 March 2014.

1. Wilmut, I., Schnieke, A. E., McWhir, J., Kind, A. J. & Campbell, K. H. Viable offspring derived from fetal and adult mammalian cells. Nature 385, 810–813 (1997).
2. Gurdon, J. B. & Wilmut, I. Nuclear transfer to eggs and oocytes. Cold Spring Harb. Perspect. Biol. 3, a002659 (2011).
3. Tachibana, M., et al. Human embryonic stem cells derived by somatic cell nuclear transfer. Cell 153, 1228–1238 (2013).
4. McGrath, J. & Softer, D. Inability of mouse blastomere nuclei transferred to enucleated zygotes to support development in vitro. Science 226, 1317–1318 (1985).
5. Wakahayama, T., Tateno, H., Mombaerts, P. & Yanagimachi, R. Nuclear transfer into enucleated zygotes. Nature Genet. 24, 108–109 (2000).
6. Egli, D., Sandler, V. M., Shinohara, M. L., Cantor, H. & Eggan, K. Reprogramming after chromosome transfer into mouse blastomeres. Curr. Biol. 19, 1403–1409 (2009).
7. Egli, D., Rosains, J., Birkhoff, G. & Eggan, K. Developmental reprogramming after chromosome transfer into mitotic mouse zygotes. Nature 447, 679–685 (2007).
8. Tsunoda, Y. et al. Mouse cloning and somatic cell reprogramming using electrofused blastomeres. Cell Res. 21, 770–778 (2011).
9. Egli, D., Birkhoff, G. & Eggan, K. Mediators of reprogramming: transcription factors and transitions through mitosis. Nature Rev. Mol. Cell Biol. 9, 505–516 (2008).
10. Nichols, J. et al. Formation of pluripotent stem cells in the mammalian embryo depends on the POU transcription factor Oct4. Cell 95, 379–391 (1998).
11. Zhang, J. et al. Sall4 modulates embryonic stem cell pluripotency and early embryonic development by the transcriptional regulation of Pou5f1. Nature Cell Biol. 8, 1114–1123 (2006).
12. Festuccia, N. et al. Esrb is a direct Nanog target gene that can substitute for Nanog function in pluripotent cell. Cell Stem Cell 11, 477–490 (2012).
13. Jullien, J., Pasque, V., Hailey-Stott, R. P., Miyamoto, K. & Gurdon, J. B. Mechanisms of nuclear reprogramming by eggs and oocytes: a deterministic process? Nature Rev. Mol. Cell Biol. 12, 453–459 (2011).
14. Zheng, L., Roeder, R. G. & Luo, Y. S phase activation of the histone H2B promoter by OCTA-3, a coactivator complex that contains GAPDH as a key component. Cell 114, 255–266 (2003).
15. Wakayama, T., Rodriguez, I., Perry, A. C., Yanagimachi, R. & Mombaerts, P. Mice cloned from embryonic stem cells. Proc. Natl Acad. Sci. USA 96, 14984–14989 (1999).
16. Mitalipov, S. M. et al. Reprogramming following somatic cell nuclear transfer in primates is dependent upon nuclear remodeling. Hum. Reprod. 22, 2232–2242 (2007).
17. Jaroudi, S. & SenGupta, S. DNA repair in mammalian embryos. Mutat. Res. 635, 53–77 (2007).
18. Scholer, H. R., Dressler, G. R., Balling, R., Rohdewohld, H. & Gruss, P. Oct-4: a genome-specific transcription factor mapping to the mouse t-complex. EMBO J. 9, 2185–2195 (1990).
19. Zambrowicz, B. P. et al. Disruption of overlapping transcripts in the ROSA l2g6 gene trap strain leads to widespread expression of l2g-galatosidase in mouse embryos and hematopoietic cells. Proc. Natl Acad. Sci. USA 94, 3789–3794 (1997).
20. Wu, G. et al. Generation of healthy mice from gene-corrected disease-specific induced pluripotent stem cells. PLoS Biol. 9, e1001099 (2011).
21. Kishigami, S. et al. Significant improvement of mouse cloning technique by treatment with trichostatin A after somatic nuclear transfer. Biochem. Biophys. Res. Commun. 340, 183–189 (2006).
22. Tsunoda, Y. et al. Full-term development of mouse blastomere nuclei transplanted into enucleated two-cell embryos. J. Exp. Zool. 242, 147–151 (1987).
23. Eckardt, S., Leu, N. A., Kurossaka, S. & McLaughlin, K. J. Differential reprogramming of somatic cell nuclei after transfer into mouse cleavage stage blastomeres. Reproduction 129, 547–556 (2005).
24. Mitalipov, S. M., Yeoman, R. R., Nusser, K. D. & Wolf, D. P. Rhesus monkey embryos produced by nuclear transfer from embryonic blastomeres or somatic cells. Biol. Reprod. 66, 1367–1372 (2002).
25. Stice, S. L. & Robl, J. M. Nuclear reprogramming in nuclear transplant rabbit embryos. Biol. Reprod. 39, 657–664 (1988).
26. Willadsen, S. M. Nuclear transplantation in sheep embryos. Nature 320, 63–65 (1985).
27. Cheong, H. T., Takahashi, Y. & Kanagawa, H. Birth of mice after transplantation of early cell-cycle-stage embryonic nuclei into enucleated oocytes. Biol. Reprod. 48, 958–963 (1993).
28. Stice, S. L., Keefer, C. L. & Matthews, L. Bovine nuclear transfer embryos: oocyte activation prior to blastomere fusion. Mol. Reprod. Dev. 38, 61–68 (1994).
29. Egli, D. et al. Reprogramming within hours following nuclear transfer into mouse but not human zygotes. Nature Commun. 2, 488 (2011).

Supplementary Information is available in the online version of the paper.

Acknowledgements The authors would like to acknowledge the Small Lab Animal Unit at Oregon National Primate Research Center for providing expertise and services that contributed to this project. We are indebted to E. Wolf, A. Sugawara, C. Ramsey, H.-S. Lee, J. Woodward, D. Meluzino Sanchis, C. Van Dyken and B. Manson for their technical support. The study was supported by grants from the National Institutes of Health (R01HD052376, R01HD057121, R01HD059946, R01EY021214, P51OD01192) and funds from the Leducq Fondation and the Collins Medical Trust.

Author Contributions S.M. and E.K. conceived and planned the study. E.K. carried out SCNT experiments, G.W. conducted tetraploid chimera experiments, H.M., M.T. and M.S. assisted with colony management, oocyte and embryo collections, embryo transfers, ES cell isolation and culture and expression profiling. E.K., D.P.W., H.P.S. and S.M. analysed data and wrote the manuscript.

Author Information Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to S.M. (mtalipo@ohsu.edu).
METHODS
Preparation of nuclear donor cells. The carcass of an embryonic day 14 fetus was collected and treated with 0.1% collagenase for 30 min. The digested tissue was cultured with 10% FBS and 100 μg ml⁻¹ penicillin/streptomycin in DMEM. Early passage (2–4) FFs were used for SCNT. For G0/G1 phase, cells were seeded in 4-well plates, and cultured for 1 week with 0.5% FBS medium. For S phase, G0/G1-arrested FFs were seeded at low density in ESC growth medium (see later), and cultured for 20 h. The cumulus–oocyte complexes were collected from superovulated females 18–20 h after hCG injection (see later) and briefly exposed to a medium containing 0.1% hyaluronidase for dispersion. Dispersed cumulus cells were rinsed and kept in KSO medium (Millipore) before use. ES cells and FFs were fixed overnight in 70% ethanol, washed in DPBS and stained with 10 μg ml⁻¹ propidium iodide (Sigma) in DPBS for 1 h on ice for flow cytometric analysis (BD Biosciences, LSRII flow cytometer). FFs were either arrested at G0/G1 by culture to confluency under low serum conditions or forced to proliferate and enter S phase after re-plating G0/G1-arrested cells at low density in medium containing serum. In confluent FFs, nearly 79% of the cells were at G0/G1, 3% were at S and 18% were at G2/M phase. However, after sorting by size (small ≤ 12 μm, large ≥ 16 μm), small-sized FFs were enriched for G0/G1 cells (91%). In proliferating FFs, the proportion of S-phase cells was significantly increased (70%). We also confirmed S phase by incorporation of BrdU (BD PharmingenTM; 30 min incubation). G0/G1-arrested cells were then re-plated in 0.5% FBS medium: KO DMEM, 1 mM-glutamine, 100 units ml⁻¹ penicillin/streptomycin, 100 μg ml⁻¹ LIF, 10% FBS and 10% KOSR.

Derivation and culture of ES cells. Defined as S phase. Both blastomeres were enucleated under HMC, and an intact cytoplasm and the transplanted nucleus.

Cell cycle determination in recipient embryos. Pronuclear-stage zygotes were collected from superovulated, mated females and continuously examined before and after the first cleavage division by HMC and polarized microscopy. Selected embryos were also fixed and labelled with antibodies against lamin B and DAPI to follow nuclear membrane and chromatin dynamics, respectively. To define the onset of S phase, we sampled every hour after the onset of cleavage into the two-cell stage with BrdU, which incorporates into newly synthesized DNA during replication. The embryos were incubated in 100 μM BrdU for 20 min with the remaining steps carried out according to the manufacturer’s instructions before embryo observation by fluorescence microscopy (×400).

Interphase two-cell SCNT. Nuclear transfer of FFs, ES cells or cumulus cells into enucleated I2C was undertaken, ensuring cell cycle match between the recipient cytoplasm and the transplanted nucleus. In vivo fertilized, pronuclear-stage zygotes were recovered and continuously monitored for the onset of cleavage. Two-cell blastomeres enucleated within 0–5 h after the onset of cleavage were considered to be G1 blastomeres whereas enucleations conducted 4–6 h after the first mitosis were defined as S phase. Both blastomeres were enucleated under HMC, and an intact nuclear donor cell was introduced into each enucleated blastomere using the fusogenic activity of HVJ-E (Fig. 2b and Supplementary Video 1).

Chimaera assay and embryo transfer. Approximately 10 ES cells were injected into 4–8-cell embryos recovered from B6D2F1 females mated with C57 males for tetraploid embryo complementation or from ICR for diploid chimaeras. Injected embryos were cultured for 1 day in KSO. SCNT or chimaeric blastocysts were transferred into the uteri of pseudopregnant (E2.5) ICR females. Caesarean section was performed from embryonic day 18.5 to 20.5.

Genotyping analyses. Genotyping primers, D1Mit126, D10Mit3 and D14Mit10 were obtained from Mouse Genome Informatics (http://informatics.jax.org). GAP, lacZ and mitochondrial analysis primers are provided later. DNA was extracted from tail tips using Arcturus Microengineers reagent kit (Applied Biosystems) or from cultured cells using a DNA kit (Genta SYSTEMS). PCR products were separated by 3% agarose gels and visualized by ethidium bromide staining. PCR products were sequenced and the informative single nucleotide polymorphic (SNP) sites were identified using Sequencher v.4.7 software (GeneCodes). Allele refractory mutation system quantitative PCR (ARMS-qPCR) assays were performed to measure mtDNA heteroplasmy levels in chimaeric offspring as previously described.16 For the discriminative assay, primers and TaqMan probes (FAM) were designed to target to the unique mtDNA region in ND5 gene for the wild-type and PWD/ph mtDNA. For the consensus assay, primers and probes (TET) were targeted to the common region of the COXIII gene. Multiplex PCR reactions (15 μl) containing 1× PCR Master Mix, 100–250 nM of each of the primers, 150 nM of each TaqMan probe and 4 ng of total genomic DNA at a 1:8 dilution were run using ABI 7900HT (Applied Biosystems).

X-gal staining. Fetuses and placenta (embryonic day 18.5) were removed by caesarean section and fixed overnight in 3% formaldehyde at 4 °C, washed three times in PBS, cleared in 70% ethanol followed by incubation in 30% sucrose at 4 °C overnight, embedding (Tissue-Tek OCT compound, Sakura Finetechology) and freezing on dry ice. Sagittal sections (10 μm) were cut at −18 °C with a cryostat (Leica Instrument). The specimen was stained overnight at 37 °C in X-gal (1 mg ml⁻¹), potassium ferricyanide (5 mM), potassium ferrocyanide (5 mM), MgCl₂ (2 mM) in PBS.

Analysis of gene expression. Total RNA was isolated and pooled from ten embryos for each experiment (MII oocytes (18h), interphase (one cell, 24–26h), metaphase (one cell, 28–30h), two cell (31–33h), two-cell cytoplasm (nuclei removed, 31–33h), four cell (50–52h), eight cell (66–68h), 16 cell (74–76h) and blastocyst (98–100h) with an Arcturus pico pure RNA isolation kit (Life Technologies). RNA was immediately treated with Dnase I (Invitrogen) and reversed into cDNA using the SuperScript III first-strand synthesis system (Invitrogen) according to the manufacturer’s instructions. RT–PCR was performed using an ABI 7900HT Real time PCR system and the SYBR Green PCR mix (Applied Biosystems). The samples were heated to 95 °C for 10 min and run for 40 cycles of: 94 °C for 15 s, 58 °C for 20 s, 72 °C for 30 s. Gene expression was normalized to GAPDH.

In Ab iomas staining. Embryos were labelled with antibodies for Br gå (SC-10768), lamin B (SC-6217), Bnti (SC-30943) and Hsf1 (SC-9144). ES cells were stained with antibodies for Oct4 (SC-30943) and SSEA-1 (SC-8061) and DAPI was used for counterstaining nuclei.

All antibodies were purchased from Santa Cruz.

Western blot. Forty embryos were collected and washed three times in TBS and transferred into 7 μl of RIPA extraction buffer supplemented with protease inhibitors. Protein samples were diluted with 2.5 μl sample buffer and 1 μl reducing agent, incubated for 10 min at 70 °C, and size separated using 4–12% Bis-Tris Mini Gel (Novex, Life Technologies). Proteins were blotted to polyvinylidene fluoride (PVDF) membranes (Hybond-P; GE Healthcare). Nonspecific binding was blocked by blocking solution (Invitrogen) at room temperature for 1 h. Membranes were probed for 1 h at room temperature with primary antibody (1:200 rabbit anti-Brg1, SC-10768; 1:5,000 rabbit anti-Gapdh, G9545; Sigma). Primary antibodies were detected with an HRP-conjugated secondary antibody (1:10,000 goat anti-rabbit) applied for 1 h at room temperature and developed with Femto Maximum Chemiluminescent Substrates (SuperSignal 34095; Thermo Scientific).

Statistical analysis. All data are presented as average ± s.d. One-way analysis of variance was used to compare differences among groups followed by post hoc testing using the Bonferroni method.

Primer information. All primers are given as 5′–3′. Acb, F-AGCCATGTACG TAGCCATCC, R-CTTCTACGTTGTTGTTGAA; Gapdh, F-CACCACCAGA GATGCTAGAAGTACTGA (ARMS qPCR); mtPWD/ph-ND2, F-ACTGCACA CTTCTGCAACCA, R-CAAGACCAACCATCTCGGTCTT; Hsf1, F-CACCGACCA AAAAGGTGTTCA, R-RTAGGGCTGAGATGAGACCTG; Gd9545; Sigma). Primary antibodies were detected with an HRP-conjugated secondary antibody (1:10,000 goat anti-rabbit) applied for 1 h at room temperature and developed with Femto Maximum Chemiluminescent Substrates (SuperSignal 34095; Thermo Scientific).

30. Lee, H. S. et al. Rapid mitochondrial DNA segregation in primate preimplantation embryos precedes somatic and germline bottlenecks. Cell. Rep. 1, 506–515 (2012).
Extended Data Figure 1 | Maternal and embryonic gene expression patterns. a, Immunocytochemical detection of Gapdh signal demonstrated even distribution in nuclei and cytoplasm in MII oocytes and interphase zygotes and two-cell embryos (original magnification, ×400). b, Expression of Gapdh normalized to Actb. No significant differences were seen between intact and enucleated two-cell embryos (four replicates each containing pooled RNA from five embryos, \( P > 0.05 \)). c, Gapdh expression was relatively low and constant with no significant differences seen until the eight-cell stage. Expression was increased in 16-cell embryos and blastocysts (three replicates each containing pooled RNA from ten embryos, \( * P < 0.05; ** P < 0.01 \)).

d, Gene expression of the transcriptional regulators Bmi1, Hsf1, Brg1, Sall4 and Esrrb, the transcription factor Oct4, and epigenetic factors Apobec1, Aid and Tet1 during mouse pre-implantation embryo development. The level of expression of Brg1, Apobec1, Oct4, Sall4, Aid, Tet1 and Esrrb underwent marked increases at or after the four-cell stage. The red bars indicate enucleated oocytes, zygotes or two-cell embryos. No significant differences were observed between intact and enucleated counterparts (\( n = 3 \) biological replicates, \( P > 0.05 \)). Gene expression was normalized to Gapdh. Error bars indicate average ± s.d. cyto, cytoplast; I, interphase; M, metaphase; MII, metaphase II-arrested oocyte; Z, zygote.
Extended Data Figure 2 | Protein expression of candidate reprogramming factors. a, Immunocytochemical detection of Bmi1, Hsf1 and Brg1 in zygotic interphase, metaphase and two-cell interphase embryos. The expression pattern of these proteins was similar to that observed for mRNA expression (Extended Data Fig. 1d). I2C embryos were fixed 4 h after cleavage. 1, interphase; M, metaphase; Z, zygote. b, Western blot detection of Brg1 in nuclear donor cells and in intact and enucleated two-cell embryos (n = 40). c, Immunocytochemical detection of Brg1 and Hsf1 in nuclear donor FFs and ES cells. Scale bars, 400 μm. d, Immunocytochemical detection of Brg1 and Hsf1 in SCNT embryos (original magnification, ×400). MII, MII oocyte.
Extended Data Figure 3 | Cell cycle determination in nuclear donor cells.

a, Confluent FF populations subjected to flow cytometric processing. After sorting by size and density, 91% of small-sized cells were in the G0/G1 phase of the cell cycle.
b, Proliferating FF populations were mostly at S phase.
c, ES cell populations by flow cytometry. The size was correlated with the cell cycle, 70% of the small-, medium- and large-sized cells were at G0/G1, S and G2/M, respectively.
d, FITC-conjugated BrdU was used to define S-phase FFs and ES cells. PI, propidium iodide. Scale bars, 100 μm.
e, Correlation of cell size and cell cycle in FFs and ES cells. Cell size was measured photographically after 3.7% formaldehyde fixation. S-phase cells were separated by BrdU integration (green). The assignments of cell size provided in panels a, b and c were defined from the results in panel e (*P < 0.05). pFFs, proliferating fetal fibroblasts.
Extended Data Figure 4 | Nuclear staining of cell-cycle-matched and mismatched SCNT embryos. Left, expanded nuclei of developmentally competent two-cell SCNT embryos generated by transfer of S-phase FF nuclei into enucleated S-phase two-cell embryo. Middle and right panels, condensed or dispersed nuclei of arrested SCNT embryos generated after cell cycle mismatch between donor nucleus and recipient cytoplasm. Embryos were arrested and apoptotic by the G1/S or S/G2 cell cycle checkpoints, respectively. SCNT embryos were fixed 15 h after cleavage (original magnification, ×400).
Extended Data Figure 5 | Genotyping of chimaeric mice. a, Chromatogram of mtDNA depicting sequence differences at 11,939, 12,062 and 12,122 base pairs (bp) and demonstrating that mtDNA in ntES cells was derived from recipient embryo cytoplasm. Sequence differences between chimaera host embryos and ntES cells at 11,939, 12,199, 12,002, 12,005, 12,008 and 12,062 bp allowed quantification of chimaerism. The mtDNA from a diploid chimaeric mouse appeared as a double peak. The tetraploid chimaera showed only ntES cell mtDNA (GeneBank accession EF108343.1). b, Allele refractory mutation system quantitative PCR (ARMS-qPCR) assay in diploid and tetraploid chimaeras. Similar colours indicate similar origin. Distances between colour plots represent the contribution of chimaeric host embryos. Amplification plot (left) and bar graph (right). Detection limit of this assay is 0.01%. c, High ntES cell contribution chimaeric female and her pups demonstrating germline transmission. White spot on the head of the dam originated from the ICR host embryo. d, Tetraploid embryo complementation pups born after ntES cell (black) injection into host eight-cell-stage tetraploid embryos. e, Genotyping of tetraploid complementation pups.
Extended Data Figure 6 | Cloned offspring and genotyping. a, Body and placenta of cloned and control pups delivered by caesarean section at 20 days. Clones and placentas were larger or smaller than controls. Scale bars, 0.5 cm. b, Fetal and placental weights (g) of cloned and control pups. c, Genotyping of nuclear donor cells and cloned pups carrying ROSA26^{S^G}_{OG2}.
Extended Data Table 1 | Origin of ntES cell lines generated by SCNT into enucleated I2C

| ntES cells | Gender | Nuclear donor cells | Genotype of donor cells | mtDNA of donor cells | 2-cell embryo mtDNA | Diploid chimaera | Tetraploid chimaera |
|------------|--------|---------------------|-------------------------|----------------------|---------------------|------------------|--------------------|
| ntES1      | M      | FFs                 | B6D2F1                  | common               | PWD                 | n/t              | n/t                |
| ntES2      | F      | FFs                 | 129S1/SvImJ            | MOLF                 | PWD                 | yes              | yes                |
| ntES3      | M      | FFs                 | B6D2F1                  | common               | PWD                 | yes              | n/t                |
| ntES4      | M      | FFs                 | B6D2F1                  | common               | PWD                 | n/t              | n/t                |
| ntES5      | M      | FFs                 | B6D2F1                  | common               | common              | n/t              | n/t                |
| ntES6      | F      | FFs                 | Oct4-GFP               | common               | common              | n/t              | n/t                |
| ntES7      | F      | FFs                 | Oct4-GFP               | common               | PWD                 | n/t              | n/t                |
| ntES8      | F      | CCs                 | B6D2F1                  | PWD                  | PWD                 | yes              | yes                |
| ntES9      | F      | CCs                 | B6D2F1                  | PWD                  | PWD                 | n/t              | yes                |
| ntES10     | F      | CCs                 | B6D2F1                  | PWD                  | PWD                 | n/t              | n/t                |

CCs, cumulus cells; n/t, not tested.
Extended Data Table 2 | Ability of ntES cells to contribute to chimaeras

| Cell line | Host embryo type | N   | N recipients (pregnant) | N Pups born /recipients | N chimaeras | >90% chimaerism | Survived >90% chimaerism |
|-----------|------------------|-----|-------------------------|-------------------------|-------------|----------------|------------------------|
| ntES2     | diploid          | 217 | 12 (4)                  | 14/4                    | 10          | 5             | 3                      |
| ntES2     | tetraploid       | 165 | 14 (6)                  | 5/3                     | 5           | 5             | 4                      |
| ntES3     | diploid          | 250 | 13 (6)                  | 12/4                    | 12          | 3             | 1                      |
| ntES8     | diploid          | 90  | 6 (3)                   | 9/3                     | 9           | 8             | 7                      |
| ntES8     | tetraploid       | 180 | 15 (6)                  | 7/3                     | 7           | 7             | 6                      |
| ntES9     | tetraploid       | 137 | 9 (5)                   | 5/3                     | 5           | 5             | 4                      |
| Control ES cells | tetraploid | 313 | 22 (11)                 | 19/6                    | 19          | 19            | 4                      |
Extended Data Table 3 | *In vivo* development of embryos derived by nuclear transfer into enucleated I2C or MII oocytes

| Nuclear donor cells | Recipient cytoplasm | N embryos transferred/recipient | N pregnant recipients | Live pups born (%) |
|--------------------|---------------------|--------------------------------|-----------------------|-------------------|
| FFs                | Interphase 2-cell embryo | 115/10                         | 4                     | 0                 |
|                    | MII oocyte           | 170/10                         | 4                     | 1 (0.6%)          |
| ES cells           | Interphase 2-cell embryo | 89/8                           | 3                     | 4 (4.5%)          |
Corrigendum: Nuclear reprogramming by interphase cytoplasm of two-cell mouse embryos
Eunju Kang, Guangming Wu, Hong Ma, Ying Li, Rebecca Tippner-Hedges, Masahito Tachibana, Michelle Sparman, Don P. Wolf, Hans R. Schöler & Shoukhrat Mitalipov

Nature 509, 101–104 (2014); doi:10.1038/nature13134

In the first sentence of the main text of this Letter, the words ‘...Brg1, Bmi1 (also known as Smarca4)...’ should have read ‘...Brg1 (also known as Smarca4), Bmi1...’. This has been corrected in the PDF and HTML versions of the article.