

High-efficiency somatic reprogramming induced by intact MII oocytes

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Somatic nuclei can be reprogrammed into a pluripotent state by nuclear transfer, cell fusion and expression of transcription factors. However, these reprogramming processes are very inefficient, which has greatly hindered efforts to elucidate the underlying molecular mechanisms. Here, we report a new reprogramming strategy that combines the advantages of all three reprogramming methodologies into one process. We injected nuclei from cumulus cells into intact MII oocytes. Following activation, 80% of the reconstructed embryos developed to the blastocyst stage, and tetraploid (4N) embryonic stem (ES) cell lines were generated at a rate of 30% per reconstructed oocyte. We also generated triploid (3N) ES cells after injection of somatic nuclei into activated oocytes. 4N and 3N ES cells expressed pluripotent markers and differentiated into cell types of three embryonic germ layers *in vivo*. Moreover, all ES cells generated histocompatible, differentiated cells after being engrafted in immunocompetent B6D2F1 mice, showing that ES cells derived from this reprogramming strategy might serve as a source of genetically tailored tissues for transplantation. Thus, we have established a simple and highly efficient reprogramming procedure that provides a system for investigating the molecular mechanisms involved in somatic reprogramming.

Keywords: somatic reprogramming; nuclear transfer; intact oocytes; embryonic stem cell; tetraploid

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Introduction

The cloning of animals from somatic cells demonstrates that uncharacterized maternal factors in MII oocytes have the ability to convert somatic cells into the embryonic state [1, 2]. The fact that pluripotent 4N hybrid cells can be generated from the fusion of somatic cells with embryonic stem (ES) [3, 4], embryonic germ (EG) [5] and embryonic carcinoma [6] cells suggests that nuclear factors or pluripotent genes expressed in these cells are responsible for somatic reprogramming [7]. Derivation of induced pluripotent stem (iPS) cells from somatic cells indicates that overexpression of key transcription factors can drive somatic reprogramming [8, 9]. However, the efficiency of somatic reprogramming

by these processes is very low [10]. We speculate that a combination of different reprogramming strategies into one procedure may improve somatic reprogramming efficiency. Therefore, somatic nuclei could be injected into intact MII oocytes. Following activation by SrCl₂ and cytochalasin B (CB) [1], reconstructed oocytes could develop to blastocysts, from which 4N ES cell lines could be generated. In this procedure, undefined maternal factors and nuclear factors in MII oocytes would act on somatic nuclei in the first cell cycle, after which nuclear factors and transcription factors expressed in the nuclei of parthenogenetic embryos would continue the process of reprogramming the somatic nuclei. However, it is not known whether 4N ES cells can be efficiently derived from 4N blastocysts, since 4N embryos cannot develop to term independently (although they can develop into extraembryonic lineages, as complemented by the introduction of ES cells [11]). Moreover, the low cell number of the inner cell mass (ICM) in 4N blastocysts may impede ES cell derivation *in vitro* [12].

Here, we first demonstrate that 4N ES cells can be efficiently derived from parthenogenetic 4N blastocysts.

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Second, we show that somatic nuclei can be efficiently reprogrammed by intact oocytes and that 4N ES cell lines can be generated at a rate of 30% per oocyte. Third, we demonstrate that all ES cell lines derived here generated histocompatible, differentiated cells after being engrafted into immunocompetent mice. Thus, a simple and highly efficient reprogramming procedure was established that provides a system by which the molecular mechanisms involved in somatic reprogramming can be investigated.

Results

Tetraploid ES cells can be derived efficiently from parthenogenetic 4N blastocysts

Parthenogenetic diploid (2N) embryos can be generated by activation of MII oocytes in the presence of CB, which prevents extrusion of the second polar body [13]. Oocytes from heterozygous transgenic mice (a B6D2F1 background) expressing the enhanced green fluorescence protein (EGFP) controlled by the Oct4 (Pou5f1) promoter (Oct4-EGFP mice) were activated in a culture medium containing SrCl₂ and CB. From 32 activated oocytes, we derived 24 ES cell lines (termed P2N; Table 1). To de-

rive 4N ES cell lines, the two cells in the two-cell stage of parthenogenetic 2N embryos were fused into a single cell (Figure 1A). Of 33 fused embryos, 30 developed to blastocysts (Figure 1B), and 21 ES cell lines (termed P4N) were generated after engraftment in ES culture medium (Table 1). Intense green fluorescence was observed in most cell lines (Figure 1C), reflecting the use of Oct4-EGFP heterozygous mice as oocyte donors. Immunostaining of P4N ES cells revealed the presence of markers that are characteristic of mouse ES cells. These included Sox2 and SSEA-1 (Figure 1D). Karyotype analysis showed that these cell lines predominantly have 80 chromosomes (Figure 1E). Fluorescence-activated cell sorting (FACS) of these cells showed that they contained twice the DNA content of the parthenogenetic 2N ES cells (Figure 1F). To demonstrate the differentiation potency of these P4N ES cells further, we injected ES cells from four cell lines into immunodeficient mice (SCID). In all of these mice, we observed tumor formation. Histology of the teratomas revealed tissue types from all three EG layers: the mesoderm (muscle), endoderm (epithelium) and ectoderm (neural rosettes) (Figure 1G).

Table 1 Establishment of ES cell lines from blastocysts reconstructed by injection of cumulus cells into intact and enucleated oocytes

Oocyte (time after activation)	Donor cell	No. of reconstructed oocytes	No. of blastocysts (% of reconstructed oocytes)	No. of Oct4-EGFP-positive blastocysts (% of reconstructed blastocysts)	ES cell lines (% of reconstructed oocytes) ¹
<i>Intact MII oocyte</i>					
MII (0 h) ²	–	32	28 (88)	–	24 (75)
MII (0 h) ³	–	33	30 (91)	–	21 (64)
MII (0 h)	CC-EGFP	20	18 (90)	–	7 (35)
MII (0 h)	CC-Oct4-EGFP	54	41 (76)	41 (100)	15 (28)
Subtotal		74	59 (80)	–	22 (30) ^A
MII (1 h)	CC-Oct4-EGFP	60	33 (55)	30 (91)	12 (20) ^B
MII (3 h)	CC-Oct4-EGFP	108	9 (8)	6 (67)	2 (2)
MII (6 h)	CC-Oct4-EGFP	85	19 (22)	4 (21)	0
<i>Enucleated MII oocyte</i>					
MII (0 h)	CC	34	14 (41)	–	3 (9) ^a
MII (1 h)	CC	71	7 (10)	–	4 (6) ^b
MII (3 h)	CC	35	0	0	0
MII (6 h)	CC	38	0	0	0

¹The data were analyzed using a χ^2 -test ($P < 0.05$); A vs a and B vs b.

²2N parthenogenetic embryos.

³4N parthenogenetic embryos.

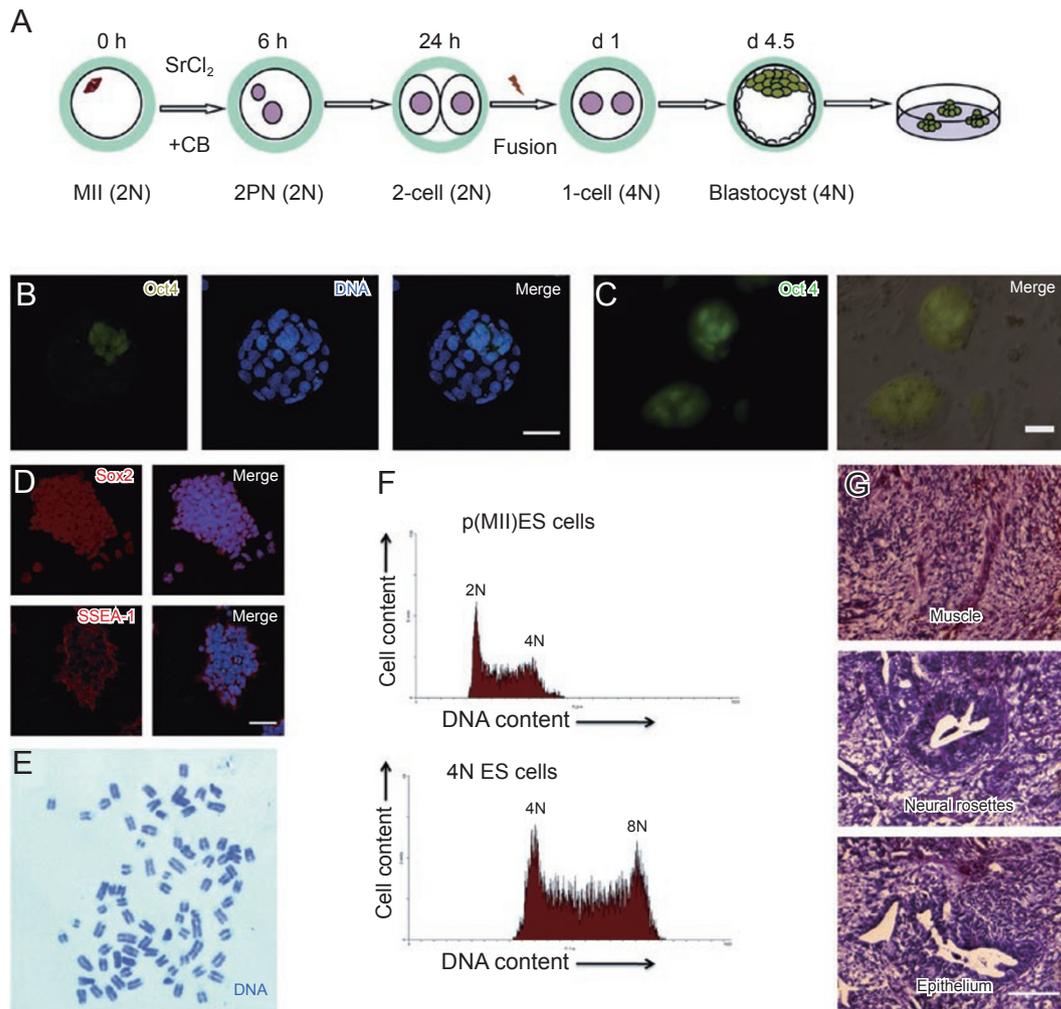


Figure 1 Generation of 4N ES cells from parthenogenetic 4N blastocysts. **(A)** Schema of P4N ES cell derivation. **(B)** Parthenogenetic 4N blastocysts from OCT4-EGFP donor mice. Left, green fluorescent image of ICM; middle, blue fluorescence represents nuclear staining by Hoechst 33342; right, overlay of both. Scale bar, 50 μ m. **(C)** Oct4 expression in P4N ES cells. Left, the green fluorescence represents Oct4 expression and right, the same ES cell colonies in culture; a bright-field image overlay with green fluorescence. Scale bar, 100 μ m. **(D)** Expression of the pluripotency marker gene Sox2 and the embryonic antigen SSEA-1 in P4N ES cells, as detected by immunostaining. Scale bar, 50 μ m. **(E)** Karyotype of P4N ES cells showing a set of 80 mouse chromosomes. **(F)** FACS analysis of P4N ES cells stained with propidium iodide showing twice the relative DNA content of the 2N parthenogenetic ES cells. **(G)** Histology of teratomas. Top, muscle; middle, neural rosettes; bottom, epithelium. Scale bar, 100 μ m.

Intact MII oocytes can efficiently reprogram somatic nuclei

This first set of experiments demonstrated that 4N ES cells can be efficiently derived from 4N parthenogenetic blastocysts. We then injected cumulus cells (CCs) from transgenic mice expressing EGFP controlled by the chicken β -actin promoter (EGFP mice, B6D2F1 background) and from Oct4-EGFP transgenic mice (B6D2F1 background) into intact oocytes from wild-type B6D2F1 mice. The reconstructed oocytes were exposed to acti-

vation medium for 6 h and cultured in KSOM medium until the blastocyst stage (Figure 2A). From 20 oocytes that were injected with CCs from EGFP transgenic mice, we derived 18 reconstructed blastocysts and established 7 ES cell lines (termed NT4N), all of which expressed EGFP (Table 1). From 54 oocytes that were reconstructed with CCs from Oct4-EGFP transgenic mice, we derived a set of 41 reconstructed blastocysts and 15 ES cell lines (Table 1). Oct4 expression was identified in the ICM and ES cells by green fluorescence (Figure 2B and 2C). The

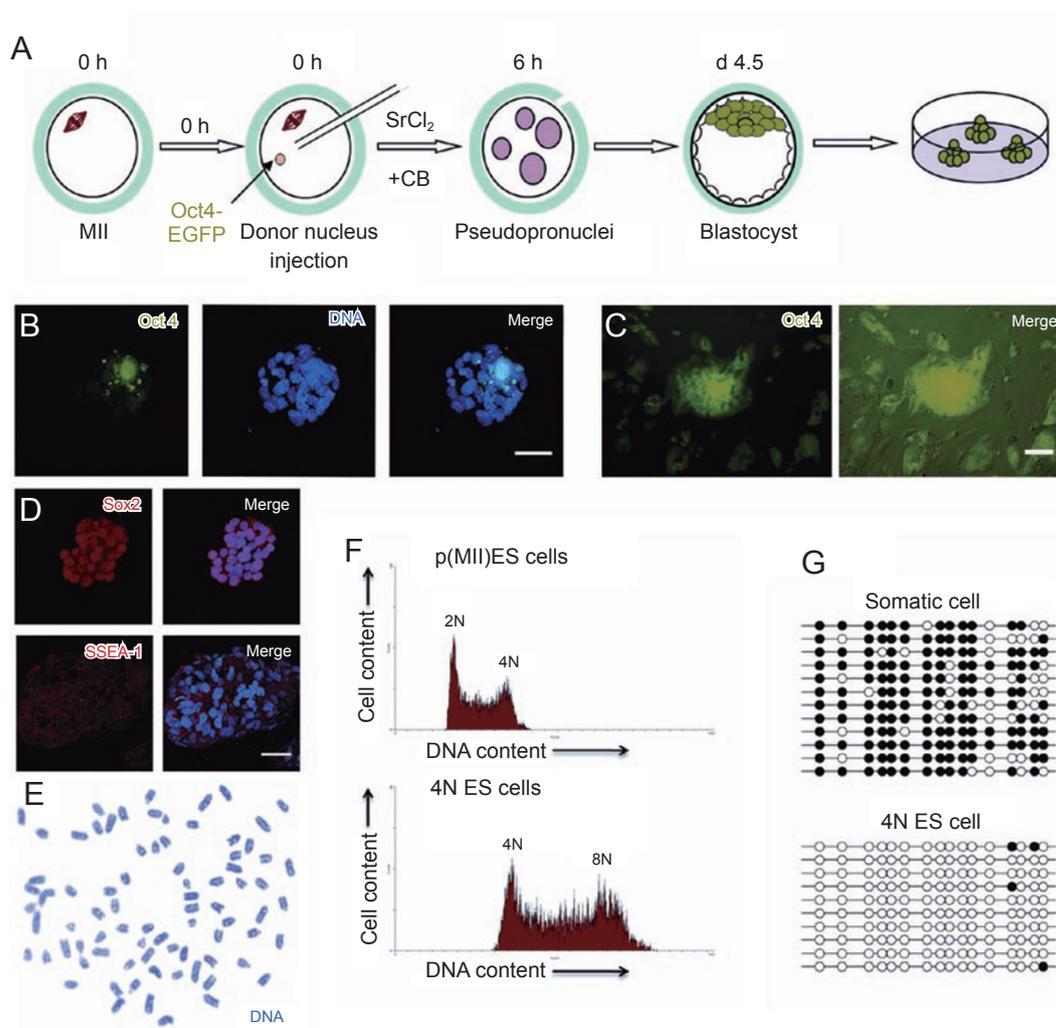


Figure 2 Generation of 4N ES cells from reconstructed blastocysts produced by injection of somatic cells into intact MII oocytes. **(A)** Schema of NT4N ES cell derivation. **(B)** A reconstructed 4N blastocyst derived after injection of a CC with Oct4-EGFP into an intact oocyte. Left, green fluorescent image of ICM; middle, blue fluorescence represents nuclear staining by Hoechst 33342; right, overlay of both. Scale bar, 50 μ m. **(C)** Oct4 expression in NT4N ES cells. Left, the green fluorescence represents Oct4 expression and right, the same ES cell colonies in culture; a bright-field image overlay with green fluorescence. Scale bar, 100 μ m. **(D)** Expression of the pluripotent marker gene Sox2 and the embryonic antigen SSEA-1 in NT4N ES cells, as detected by immunostaining. Scale bar, 50 μ m. **(E)** Karyotype of NT4N ES cells showing a set of 80 mouse chromosomes. **(F)** FACS analysis of NT4N ES cells stained with propidium iodide showing twice the relative DNA content of the 2N parthenogenetic ES cells. **(G)** Methylation analysis via bisulfite sequencing of the somatic Oct4 promoter after reprogramming. Open and filled circles denote unmethylated and methylated CpG islands, respectively.

established cell lines expressed Sox2 and SSEA-1 (Figure 2D) and were all tetraploid (Figure 2E and 2F). Bisulfite genomic sequencing analysis of the endogenous Oct4 promoter of somatic nuclei showed that the epigenetic state of the somatic cells had been reprogrammed (Figure 2G). These results indicate that intact MII oocytes can efficiently reprogram somatic nuclei, and that 4N ES cells can be generated from blastocysts reconstructed by injection of somatic nuclei into intact MII oocytes. The average efficiency of NT4N ES cell establishment in suc-

cessfully injected oocytes is 30%. This value is threefold higher than the efficiency of nuclear transfer ES (NTES) cell derivation from CCs that we observed here (Table 1) and is higher than that reported in the mouse NTES literature using different cell types [14–16]. However, the rate of NT4N ES cell derivation was twofold lower than the derivation of P4N cells (30% vs 64% per oocyte). Previous studies show that cell number in the blastocyst is a very important factor for the further development of embryos [12, 17]. We then counted the total number of

blastocyst nuclei and the number of ICM nuclei using DAPI staining and Oct4-EGFP fluorescence, respectively. We found that the total cell number of 4N blastocysts and ICM was consistently higher for parthenogenetic 4N blastocysts than for NT4N blastocysts (data not shown). Similarly, fewer cells were observed in cloned blastocysts derived from the standard NT procedure, and this might reflect the lower rate of cell proliferation caused by somatic reprogramming [18] during preimplantation development.

Activated intact MII oocytes show enhanced reprogramming ability

The above results suggest that the existence of the MII chromosome-spindle complex (CSC) may promote somatic reprogramming in MII oocytes. To investigate whether nuclear materials at different cell cycle stages can still enhance somatic reprogramming during the first

cell division cycle of embryo development, we activated the MII oocytes and injected CCs from OCT4-EGFP mice into enucleated and intact oocytes at different time periods after activation (Figure 3A). Previous research indicated that a small percentage of embryos reconstructed from oocytes activated 1 h before NT developed to the blastocyst stage, but that none of them developed to term after transplantation into the uterus of a foster mother [19]. Here, we established four ES cell lines from 71 embryos reconstructed with oocytes that were activated 1 h before NT (Table 1). When oocytes that were activated for 3 h or longer before NT were used as recipients, the reconstructed embryos never developed beyond the two-cell stage (Table 1). In contrast, activated intact oocytes showed enhanced reprogramming ability. We established 12 ES cell lines from 60 reconstructed embryos generated from oocytes that were activated 1 h before the injection of CCs (Table 1). When CCs were

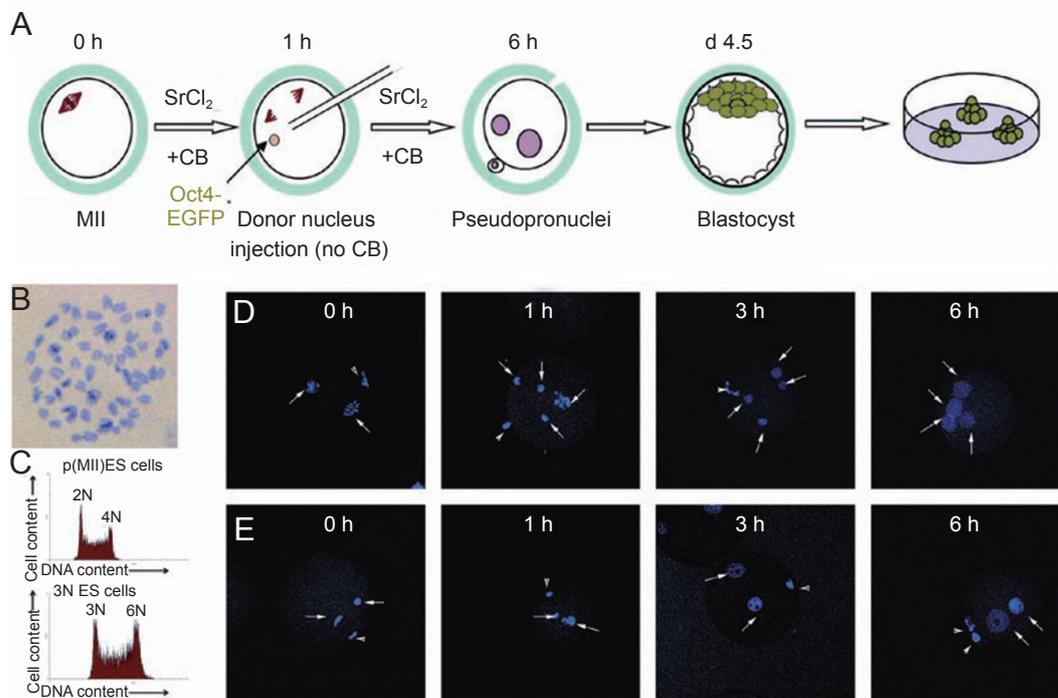


Figure 3 Generation of 3N ES cells from reconstructed blastocysts produced by injection of somatic cells into activated intact MII oocytes. **(A)** Schema of NT3N ES cell derivation. **(B)** Karyotype of NT3N ES cells showing a set of 60 mouse chromosomes. **(C)** FACS analysis of NT3N ES cells stained with propidium iodide, showing 1.5 times the relative DNA content of the 2N parthenogenetic ES cells. **(D)** Formation of four pronuclei following the injection of CCs into intact oocytes. (Arrows indicate nuclei, and arrowheads indicate the first polar bodies). One CC was injected into an intact oocyte in CB-free medium and then activated for 6 h in medium containing SrCl₂ and CB. 0 h, the time at which one CC was injected into an intact oocyte; 1-6 h, different time periods post activation. **(E)** Formation of two pronuclei and the second polar body extrusion after injection of CCs into activated oocytes (Arrows indicate nuclei and arrowheads indicate polar bodies). In CB-free medium, one CC was injected into one oocyte that had been activated for 1 h. After 30 min to 1 h, the reconstructed oocytes were returned to the activation medium for a further 5 h. 0 h, the time at which one CC was injected into an activated intact oocyte; 1-6 h, different time periods after exposure to activation medium.

injected into oocytes 3 h after being activated, two cell lines were derived from 108 reconstructed oocytes (Table 1). Pronuclei formed 5–6 h after oocytes were activated. When CCs were injected into these parthenogenetic embryos, 22% of the reconstructed embryos developed into blastocysts, 21% of which showed green fluorescence. However, none of them formed cell lines after engraftment in ES culture medium (Table 1).

A total of 14 NTES cell lines were generated using activated intact oocytes as recipients and all were GFP-positive, indicating that Oct4 was expressed in these cells. Surprisingly, karyotype and FACS analysis of these cells showed that 13 of the cell lines were triploid (termed NT3N; Figure 3B and 3C). CB was used in the activation procedure to avoid second polar body exclusion, except when somatic nuclei were injected into the oocytes. We reasoned that, after activation of the oocytes, the second polar body would be extruded between 30 min and 1 h when the oocytes were manipulated in CB-free medium. To test our assumption, we incubated oocytes at different cell cycle stages with Hoechst 33342 and observed the formation of pronuclei and polar bodies. When CCs were injected into intact oocytes followed by activation treatment with SrCl₂ and CB, formation of the second polar body was suppressed and four pronuclei were observed (Figure 3D) in most reconstructed oocytes (9/10). Of these, two pronuclei were from the oocyte and two pronuclei were from the somatic nucleus. Interestingly, using the standard mouse NT procedure, two pronuclei can also be formed in the reconstructed oocytes [19] following activation. In contrast, when CCs were injected into oocytes 1 h after activation in CB-free medium, extrusion of the second polar body was observed in most of the reconstructed oocytes (16/18) and two pronuclei were formed (Figure 3E). Of these, one pronucleus was from the oocyte and the other was from the somatic nucleus.

Histocompatibility of differentiated progeny of 4N and 3N ES cells

To determine the differentiation potency of NT4N ES cells generated by injection of somatic cells into intact oocytes, we injected ES cells from five lines into immunodeficient (SCID) mice. Teratoma formation was observed in all of them. Histological examination indicated that the teratomas contained various tissues from all three germ layers, including muscle (mesoderm), epithelial tissues (endoderm) and neural rosettes (ectoderm) (data not shown). Given that MHC-matched parthenogenetic ES cells show immunocompatibility when transplanted into immunocompetent mice with the same genetic background [13], we reasoned that all the cell lines that we generated can engraft in B6D2F1 recipients, because the

derived ES cells contained a whole set of genetic materials from donor cells and a half set of genetic materials from oocytes of the B6D2F1 mice. Therefore, we injected ES cells from all NT4N and NT3N cell lines into B6D2F1 female mice. Teratoma formation was observed in all of them (data not shown). These data demonstrate that the 4N or 3N ES cells generated by our strategies are genetically matched to the oocyte and somatic donors.

Discussion

We have established a simple, highly efficient system for investigating the mechanisms involved in somatic reprogramming. In this system, intact MII oocytes are used as recipients instead of enucleated oocytes in the mouse NT procedure. The rate of ES cell derivation from reconstructed oocytes is 30%. To our knowledge, this represents the highest efficiency reported for somatic reprogramming to date. Several features could be responsible for the enhanced reprogramming capability of intact MII oocytes. First, the overall inefficiency of NT could be attributable to technical difficulties in nuclear isolation and transfer [20], and direct injection of somatic nuclei into intact oocytes could reduce enucleation trauma to the oocytes. Second, the oocyte nucleus, which supports preimplantation development of the parthenogenetic embryo, could help maintain normal cell division of the reconstructed embryo containing the somatic nucleus. In support of this idea, the formation of blastocysts was twofold higher after reconstructed oocytes were cultured for 3.5 days *in vitro* if somatic nuclei were injected into intact oocytes rather than into enucleated oocytes (80% vs 41% per reconstructed oocyte). Third, in addition to maternal factors within the oocytes, some transcription factors (such as *Oct4*) that are expressed during early embryo development may also help reprogram the somatic nucleus [21]. Somatic reprogramming after NT continues as development progresses through cleavage and probably even to gastrulation [22, 23]. Furthermore, *Oct4* expression is the essential factor in iPS cell formation [24–26], which demonstrates that *Oct4* plays an important role in somatic reprogramming. *Oct4* expression begins at the four- to eight-cell stage in mouse parthenogenetic embryos and could start reprogramming the somatic nucleus in the reconstructed embryos. Fourth, undefined nuclear factors present in the CSC of oocytes, in the nuclei of the blastomeres of a parthenogenetic embryo [27, 28] and in the nuclei of parthenogenetic ES cells [7] may have important roles in conferring pluripotency to the somatic nucleus present in the reconstructed oocyte, in the reconstructed embryo and in the 4N or 3N ES cells, respectively. Activated intact oocytes show stronger re-

programming ability than enucleated oocytes, possibly indicating that oocyte nuclei contain extra factors for reprogramming somatic nuclei.

In the standard NT procedure, enucleated MII oocytes could reprogram differentiated donor cells. Here, intact MII oocytes displayed enhanced reprogramming ability. However, intact oocytes that were activated for 5–6 h, which places them in the interphase of the cell cycle, could not reprogram somatic nuclei for successful derivation of ES cell lines. Similarly, enucleated interphase zygotes failed to support the development of reconstructed embryos after NT [29, 30]. However, Egli *et al.* [27, 28] recently demonstrated that mouse zygotes and two-cell-stage blastomeres retain their reprogramming activities when they are arrested in mitosis. Given these collective findings, it seems likely that the reprogramming activities of recipients are determined in the meiotic or mitotic stage. Therefore, we propose that parthenogenetic oocytes in metaphase might be suitable recipients for NT.

Beyond establishing a simple and highly efficient experimental system for investigating the mechanism of somatic reprogramming, the approach outlined here could also provide an alternative route for creating histocompatible ES cells for use in the study and treatment of disease. Although 4N or 3N ES cells cannot sustain full organismal development, our reprogramming strategy could provide completely personalized pluripotent stem cell populations for therapeutic use if oocyte-derived chromosomes can be targeted for elimination [31]. Moreover, high reprogramming efficiency using intact oocytes could make the derivation of genetically tailored human ES cells possible from 4N-reconstructed embryos generated by injection of somatic nuclei into intact oocytes from the same donor.

Materials and Methods

Preparation of donor cells

Female EGFP transgenic B6D2F1 mice, Oct4-EGFP transgenic B6D2F1 mice and wild-type B6D2F1 mice were superovulated with 5 U of PMSG and 5 U of HCG 48 h later. CCs were isolated from cumulus-oocyte complexes 14 h after HCG injection. The cells were washed two times with HEPES-CZB medium and suspended in HEPES-CZB medium containing 3% (w/v) polyvinylpyrrolidone.

Derivation of parthenogenetic 2N and 4N blastocysts

Oct4-EGFP transgenic B6D2F1 mice were used as oocyte donors. Oocytes were obtained 14 h after HCG injection and activated for 6 h in activation medium (calcium-free CZB medium containing 10 mM Sr^{2+} and 5 $\mu\text{g}/\text{ml}$ CB). Parthenogenetic embryos were cultured in KSOM medium with amino acids for 3.5–4 days to reach the blastocyst stage. To generate 4N parthenogenetic embryos, we electrofused two-cell parthenogenetic embryos to

produce one-cell 4N embryos. Two-cell embryos were aligned using an alternating current in 0.3 M mannitol solution, and a single direct current pulse of 1 000 V/cm was applied for 20 μs . After electrofusion, the embryos were returned to KSOM medium with amino acids, and the fused embryos were cultured for 2 days to reach the blastocyst stage.

NT with intact and enucleated oocytes

NT was performed as described [1] with modifications [14]. Briefly, metaphase II-arrested oocytes were collected from superovulated B6D2F1 females (8–10 weeks) and CCs were removed using hyaluronidase. In the standard NT procedure, the oocytes were enucleated in a droplet of HEPES-CZB medium containing 5 $\mu\text{g}/\text{ml}$ CB using a blunt Piezo-driven pipette. After enucleation, the spindle-free oocytes were washed extensively and maintained in CZB medium up to 2 h before nucleus injection. The CCs were aspirated in and out of the injection pipette to remove the cytoplasmic material and then injected into enucleated oocytes. For NT with intact oocytes, we directly injected CCs into freshly isolated MII oocytes. The reconstructed oocytes were cultured in CZB medium for 1 h and then activated for 5–6 h in activation medium. Following activation, all of the reconstructed embryos were cultured in KSOM medium with amino acids at 37 °C under 5% CO_2 in air. For NT with the activated oocytes, oocytes were activated for 1–6 h and used as NT recipients at different time periods after activation.

Derivation of ES cells

Expanded or hatched blastocysts were used to derive ES cells as described [14]. The zona pellucida was removed using acid Tyrode solution. Each blastocyst was transferred into one well of a 96-well plate seeded with ICR embryonic fibroblast feeders in ES medium supplemented with 20% knockout serum replacement, 1 500 U/ml LIF and 50 μM of the MEK1 inhibitor (PD098059). After 4–5 days in culture, the colonies were trypsinized and transferred to a 96-well plate with a fresh feeder layer in fresh medium. Clonal expansion of the NTES cells proceeded from 48-well plates to 6-well plates with feeder cells and then to gelatinized 25- cm^2 flasks for routine culture in ES medium to which 15% fetal calf serum and 1 000 U/ml LIF had been added.

Karyotype analysis

ES cells were incubated with 0.2 mg/ml demecolcine (Sigma) for 3 h. After trypsinization, the ES cells were resuspended in 0.075 M KCl at 37 °C for 30 min. Hypotonic solution-treated cells were fixed in methanol: acetic acid (3:1 in volume) for 30 min and dropped onto precleaned slides and stained with Giemsa for 15 min. More than 10 metaphase spreads were analyzed.

FACS analysis

For DNA analysis, the cells were washed in PBS with 1% glucose, fixed with 75% ethanol, stained with 50 $\mu\text{g}/\text{ml}$ propidium iodide and analyzed by FACS.

Immunostaining

Cells on glass cover slips were fixed in PBS supplemented with 4% paraformaldehyde for 15 min at room temperature (RT). The cells were then permeabilized using 0.1% Triton X-100 in PBS for 15 min at RT. The cells were blocked for 30 min in 3% BSA in

PBS. All primary antibodies against SSEA-1 (mab4301, Millipore) and Sox2 (ab5603, Millipore) were diluted in the same blocking buffer and incubated with the samples overnight at 4 °C. The cells were treated with a fluorescently coupled secondary antibody and then incubated for 1 h at RT. The nuclei were stained with Hoechst 33342 (Sigma) for 3 min at RT.

Bisulfite DNA methylation analysis

Genomic DNA from ES cells was restricted with *EcoRV* and treated with sodium bisulfite as previously described [32]. The treated DNA was amplified using nested PCR. The primers used for the Oct4 promoter and enhancer region were 5'-AGG TGT AAT GGT TGT TTT GTT TTG GTT TTG-3' and 5'-TAA CCC ATC ACC CCC ACC TAA TAA AAA TAA-3' (outer from -515 to +33), and 5'-TAT GGG TTG AAA TAT TGG GTT TAT TTA TAT-3' and 5'-TCT AAA ACC AAA TAT CCA ACC ATA A-3' (inner from -483 to -3). The PCR products were cloned into T-vectors (Promega) and individually sequenced.

Teratoma formation

For teratoma induction, we harvested 1×10^7 cells of each ES cell line in 1 ml DMEM containing 10% FBS. We injected 100 μ l of the cell suspension (1×10^6 cells) intramuscularly into NOD-SCID or B6D2F1 mice with matrigel. After 6-8 weeks, teratomas were harvested. Sections were stained with hematoxylin and eosin staining.

Statistical analysis

We compared the efficiency of ES derivation between groups using χ^2 -analysis.

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