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Differential Developmental Ability of Embryos Cloned from Tissue-Specific Stem Cells

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Key Words. Cloning • Stem cell • Genotype • Chromosome • Gene activation

ABSTRACT

Although cloning animals by somatic cell nuclear transfer is generally inefficient, the use of certain nuclear donor cell types may significantly improve or deteriorate outcomes. We evaluated whether two multipotent stem cell lines produced in vitro—neural stem cells (NSCs) and mesenchymal stem cells (MSCs)—could serve as nuclear donors for nuclear transfer cloning. Most (76%) NSC-derived embryos survived the two-cell–to–four-cell transition, the stage when the major zygotic gene activation occurs. Consistent with this observation, the expression patterns of zygotically active genes were better in NSC-derived embryos than in fibroblast clone embryos, which arrested at the two-cell stage more frequently. Embryo transfer experiments demonstrated that at least some of these NSC embryos had the ability to develop to term fetuses (1.6%, 3/189). In contrast, embryos reconstructed using MSCs showed a low rate of in vitro development and never underwent implantation in vivo. Chromosomal analysis of the donor MSCs revealed very frequent aneuploidy, which probably impaired the potential for development of their derived clones. This is the first demonstration that tissue-specific multipotent stem cells produced in vitro can serve as donors of nuclei for cloning mice; however, these cells may be prone to chromosomal aberrations, leading to high embryonic death rates. We found previously that hematopoietic stem cells (HSCs) are very inefficient donor cells because of their failure to activate the genes essential for embryonic development. Taken together, our data led us to conclude that tissue-specific stem cells in mice, namely NSCs, MSCs, and HSCs, exhibited marked variations in the ability to produce cloned offspring and that this ability varies according to both the epigenetic and genetic status of the original genomes. STEM CELLS *2007;25:1279–1285*

Disclosure of potential conflicts of interest is found at the end of this article.

INTRODUCTION

Cloning animals by somatic cell nuclear transfer depends on many factors, most of which remain unknown. Cloning studies in different animal species have shown that the donor cell type is one of the most important factors determining the success of cloning [1, 2]. Laboratory mouse strains provide the best models for this kind of study, because they allow rigorously controlled experiments using genetically defined animals. Our previous statistical analysis revealed that development of embryos in vitro and in vivo was better in nuclei from neonatal Sertoli cells than in embryos produced from adult cumulus oophorus cells [3, 4]. This donor cell-dependent difference may arise because of the undifferentiated status of the donor genome; neonatal Sertoli cells are small, round, immature cells, unlike the large cells in the mature testis. This assumption is consistent with evidence showing that undifferentiated embryonic stem (ES) cells are the best donor cells for mouse cloning, leading to approximately 20% birth rates per embryo transfer in optimal conditions [5–7]. However, we found previously that hematopoietic stem cells (HSCs), the most undifferentiated cells of the hematopoietic lineage, are very inefficient donor cells compared with other differentiated cells of the same lineage [8, 9]. Development of HSC-derived cloned embryos is characterized by frequent developmental arrest at the two-cell stage. This is caused at least partly by failure to activate the gene for histone deacetylase 1 (*Hdac1*), the key to regulating subsequent zygotic gene activation [10]. Because low *Hdac1* expression level is an inherent characteristic of HSCs and is assumed to be related to their stem cell characters, the poor development of HSC-derived cloned embryos may be unique and not common to other stem cell clones. We were interested in investigating the developmental ability of embryos cloned from other stem cell types.

For reliable nuclear transfer experiments, the donor cells for cloning must be identified precisely by their morphology or should be prepared as a suspension with nearly 100% purity. At present, the mouse stem cells that fulfill this requirement are neural stem cells (NSCs) and mesenchymal stem cells (MSCs), both of which can be established by selective culture in vitro and are fully capable of differentiating in vitro. In this study, we used NSCs and MSCs as nuclear donors for cloning experiments and examined the developmental potential of the resultant embryos in vitro and in vivo. We also performed gene expression analysis of the cloned embryos and karyotyped the donor cells to clarify the results.

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1066-5099/2007/$30.00/0 doi: 10.1634/stemcells.2006-0747

MATERIALS AND METHODS

Preparation of Donor Cells

We used male (C57BL/6 × 129/Sv-ter) F1 strain mice (called B6 × 129F1 for brevity) to prepare the donor cells. NSCs were obtained from the brains of fetuses at 12.5 days postcoitum as described previously [11–13]. In brief, cells were dispersed by repeated pipetting in phosphate-buffered saline (PBS; pH 7.6), and were cultured in Dulbecco's modified Eagle's medium/Ham's F12 medium (Invitrogen, Carlsbad, CA, http://www.invitrogen.com) containing 0.6% glucose, 100 μg/ml bovine transferrin (Invitrogen), 25 μg/ml bovine insulin (Sigma-Aldrich, St. Louis, http://www.sigmaaldrich.com), 10 μg/ml putrescine (Sigma), 30 nM sodium selenite (Sigma), 20 nM progesterone (Sigma), 20 ng/ml human epidermal growth factor (EGF; Sigma), and 20 ng/ml human fibroblast growth factor (FGF; Peprotech, Rocky Hill, NJ, http://www.peprotech.com). Cells were cultured for 1 month by changing the medium every week until neurospheres formed. They were further cultured for more than 1 month until other contaminating cells were depleted from neurospheres.

MSCs were obtained from bone marrow cells according to the method of Sun et al. [14] with slight modifications. Approximately 7.6×10^7 bone marrow cells were collected from four-week-old male mice and cultured in α-minimal essential medium (Invitrogen) containing 10% fetal bovine serum. The medium was changed every 3 days. After four passages, nonhematopoietic cells were collected using a fluorescence-activated cell sorter Vantage SE (BD Biosciences, San Jose, CA, http://www.bdbiosciences.com) as a cell population that was negative for an anti-CD45.2 antibody (eBioscience, San Diego, http://www.ebioscience.com/). Single cells were seeded onto wells of a 96-well plate, and putative MSCs were allowed to proliferate clonally. The cells were used for nuclear transfer shortly after cell line establishment (<2 weeks in culture).

The ability of the NSCs and MSCs to differentiate was tested in vitro before they were used for nuclear transfer experiments. For NSC differentiation, neurospheres were allowed to adhere to poly(L-ornithine) (Sigma)-coated plates (Lab-Tec chamber slides; Nunc, Roskilde, Denmark, http://www.nuncbrand.com) in EGF/FGF-free medium containing 2% bovine calf serum for 4 days [11, 13]. The NSCs proliferated, extended their neurites, and differentiated into neurons. Differentiated cell types were identified by staining using specific antibodies. NSC-derived differentiated cells were fixed in 4% paraformaldehyde in PBS at 25°C for 30 minutes and washed thoroughly with PBS. The cells were permeabilized in 0.3% Triton X-100 in PBS for 5 minutes, washed in PBS, and treated with 10% normal goat serum in PBS for 1 hour. The primary antibodies used were as follows (dilutions in parentheses): rabbit anti-mouse MAP-2 polyclonal antibody (1:500–1:1,000; Chemicon, Temecula, CA, http://www.chemicon.com); mouse anti-GFAP monoclonal IgG_1 (1:500; Chemicon); and mouse anti-O4 monoclonal IgM (1:73; Chemicon). After washing in PBS, the cells were treated with secondary antibodies as follows: Alexa Fluor 488-anti-rabbit IgG (1:400; Invitrogen); Alexa Fluor 594-anti-mouse IgG_1 (1:400; Invitrogen); and Alexa Fluor 350-anti-mouse IgM (1:400; Invitrogen). After washing in PBS, the cells were observed under a fluorescence microscope.

MSCs were induced to differentiate in vitro using methods reported previously (osteoblasts, adipocytes [15], and chondrocytes [14]). To identify the specific cell types, the differentiated cells were stained with a reaction mixture for alkaline phosphatase for osteoblasts (Nichirei Biosciences Inc, Tokyo, Japan, http://www.nichirei.co.jp/bio/english/index.html), Oil red O for adipocytes (Sigma), and Alcian blue (Sigma) for chondrocytes.

Fibroblasts as sources of control nuclei were obtained from the tail tips of adult (2–3 months old) male mice by confluent culture as described previously [16].

Oocyte Collection

Female B6D2F1 strain mice, 7–10 weeks old, were superovulated with 7.5 IU of pregnant mare serum gonadotropin and 7.5 IU of human chorionic gonadotropin (hCG) at 48-hour intervals and killed 16 hours after hCG injection. Mature meiosis stage II (MII) oocytes were collected from their oviducts. Cumulus cells were released in potassium-modified simplex-optimized medium (KSOM) [17] containing 0.1% hyaluronidase and washed several times with fresh medium. Oocytes were cultured in KSOM at 37.5°C in an atmosphere of 5.5% CO_2 in air until enucleation.

Nuclear Transfer

Nuclear transfer was carried out as described previously [4, 8, 18]. MII oocytes were placed in HEPES-buffered KSOM including 7.5 μg/ml cytochalasin B (Calbiochem, San Diego, http://www.emdbiosciences.com), and nuclei were removed with a small amount of cytoplasm. Enucleated oocytes were cultured in KSOM in an incubator (as above) for 30–60 minutes to allow the cell membrane to recover. NSCs and MSCs were enucleated using glass micropipettes and the nuclei of donor cells were injected into the ooplasm using a Piezo-driven micromanipulator (PrimeTech, Tsuchiura, Japan).

Adult fibroblasts were prepared from tail tips as reported [16]. Their nuclei were transferred into enucleated oocytes by electrofusion [19]. After nuclear transfer, reconstructed oocytes were cultured with KSOM for 1–2 hours and transferred into Ca^{2+}-free KSOM, including 3 mM $SrCl_2$ and 5 μg/ml cytochalasin B. One hour later, activated oocytes were transferred into KSOM containing only 5 μg of cytochalasin B and cultured further for 5 hours. After washing, the oocytes were cultured in fresh KSOM at 37.5°C in an atmosphere of 5.5% CO_2 for 48 hours.

Embryo Transfer

Reconstructed embryos that reached the 4–8-cell stage after 48 hours of culture in KSOM were transferred into the oviducts of pseudopregnant ICR strain female mice mated with vasectomized male mice the day before. On day 20, the recipient female mice were examined for the presence of fetuses, and live pups were nursed by lactating ICR female mice.

Chromosomal Analysis

NSC and MSC cell lines established as described above were subjected to chromosomal analysis. NSCs and MSCs in culture dishes were treated with 25 ng/ml colcemide for 30 minutes, and the round cells (composed mostly of cells in metaphase) were collected, spread onto clean glass slides, and allowed to dry in air. Q-banding staining was performed by a combined quinacrine-33258 Hoechst

Table 1. Primer sequence for gene expression analysis

	Primers (5′→3′)	Product size (bp)
Dppa2		83
Forward	gcccagcctgcctccta	
Reverse	tgccatccgtactcaagttatgat	
Dppa3 (Stella/PGC7)		82
Forward	atcgggaagaattaggagcttaca	
Reverse	caaaaaatagctttcacatctgctgt	
Dppa4		263
Forward	aggaagtcagcaccaccgtagt	
Reverse	aagctggaagagggcaatggct	
ERV-L		67
Forward	ggaatgaaggtatgggtcaatcc	
Reverse	ccttcaccttcagccagcac	
HDAC1		91
Forward	gctgccctacaatgactactttga	
Reverse	ctcgttagtgttctggttggtcat	
eIF-1A		89
Forward	caatgaaacggacacatttggt	
Reverse	agatgtcatcaatgtcttcatcatca	
Hprt		
Forward	ABI Mm00446968_m1	

Abbreviation: bp, base pair(s).

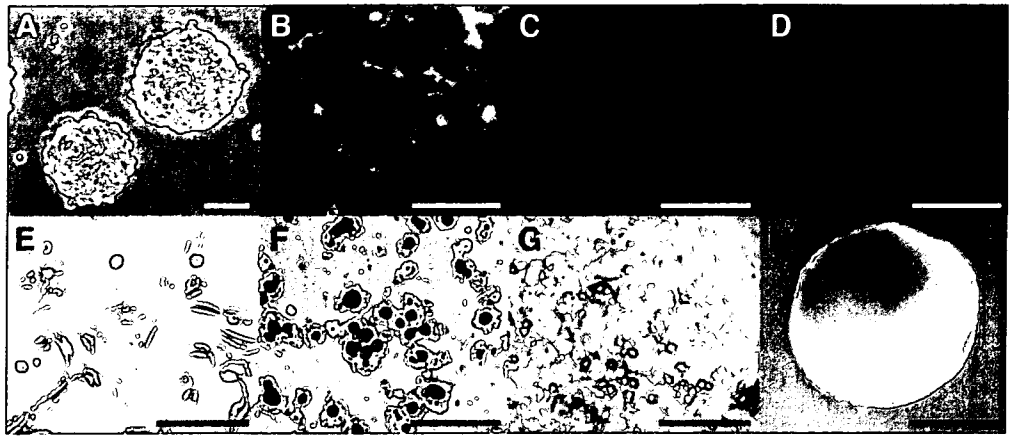

Figure 1. In vitro differentiation of neural stem cells (NSCs) and mesenchymal stem cells (MSCs) used as nuclear donors in this study. **(A):** Undifferentiated NSC neurospheres. **(B):** NSC-derived neurons (anti-MAP2 staining). **(C):** NSC-derived astrocytes (anti-glial fibrillary acidic protein staining). **(D):** NSC-derived oligodendrocytes (anti-O4 staining). **(E):** Undifferentiated MSC cells. **(F):** MSC-derived adipocytes (oil-red O staining). **(G):** MSC-derived osteoblasts (alkaline phosphatase staining). **(H):** MSC-derived chondrocytes (Alcian blue staining). Scale bar, 100 μm.

Table 2. In vitro and in vivo development of embryos cloned from NSCs, MSCs, and adult fibroblasts

Cell type	Embryos cultured	2-Cell (%)	4-Cell (% per 2-cells)	Embryos transferred	Embryos implanted (% per ET)	Term conceptuses (% per ET)	Offspring (% per ET)	Placenta-only conceptuses (% per ET)
NSC	488	328 (67.2)	249 (75.9)[a]	189	82 (43.4)[b]	10 (5.3)	3 (1.6)	7 (3.7)
MSC	232	172 (74.1)	79 (45.9)[a']	78	0 (0.0)[b']	0 (0.0)	0 (0.0)	0 (0.0)
Fibroblast	173	163 (94.2)	68 (41.7)[a']	33	19 (57.6)[b]	1 (3.0)	1 (3.0)	0 (0.0)

[a,a';b,b'] $p < .05$ (Fisher exact probability test).
Abbreviations: ET, embryos transferred; MSC, mesenchymal stem cell; NSC, neural stem cell.

method [20]. Metaphase images were observed under a fluorescent microscope (Axio Photo 2; Carl Zeiss, Jena, Germany, http://www.zeiss.com) and karyotype analysis was performed using an Ikaros karyotyping system (Carl Zeiss).

Gene Expression Analysis

We selected six zygotic genes, *Dppa2*, *Dppa3* (*Stella* or *PGC7*), *Dppa4*, *ERV-L*, *Hdac1*, and *eIF-1A*, based on previous studies on global or specific gene expression [21–23]. Embryos were individually analyzed by a quantitative reverse transcriptase-polymerase chain reaction (PCR) technique. Cloned or in vitro-fertilized (IVF) two-cell embryos 24–26 hours after activation or 26–29 hours after insemination [24] were treated with acid Tyrode's solution to remove the zona pellucida, and cDNA was extracted using Cell-to-cDNA II kits (Ambion, Austin, TX, http://www.ambion.com). PCR products amplified with the primers in Table 1 were diluted serially and used as external standards for quantitative real-time PCR. Measurements of gene expression levels were carried out using an ABI7900HT Sequence Detection system (Applied Biosystems, Foster City, CA, http://www.appledbiosystems.com) with QuantiTect Syber Green or QuantiTect Probe PCR kits (QIAGEN, Hilden, Germany, http://www.qiagen.com).

Statistical Analysis

Development rates of embryos in vitro and in vivo were compared between groups using Fisher's exact probability test. The relative transcription levels of embryos or donor cells determined by quantitative real-time PCR were analyzed by one-way analysis of variance followed by a post hoc procedure using Scheffé's *F* test for multiple comparisons between groups where appropriate. All animals were maintained and used for experiments in accordance with the guidelines of the RIKEN Institute, Japan.

Figure 2. Cloned mouse pups born after nuclear transfer using neural stem cells (NSCs) as donors. Shortly after Caesarian section at full term, two pups recovered their movement and respiration.

RESULTS

Determination of Pluripotency of the Donor NSCs and MSCs

Before the cloning experiments, we characterized the donor NSC and MSC lines for their ability to differentiate in vitro. Under appropriate culture conditions, NSCs differentiated into neurons, astrocytes, and oligodendrocytes, and MSCs differentiated into adipocytes, osteoblasts, and chondrocytes

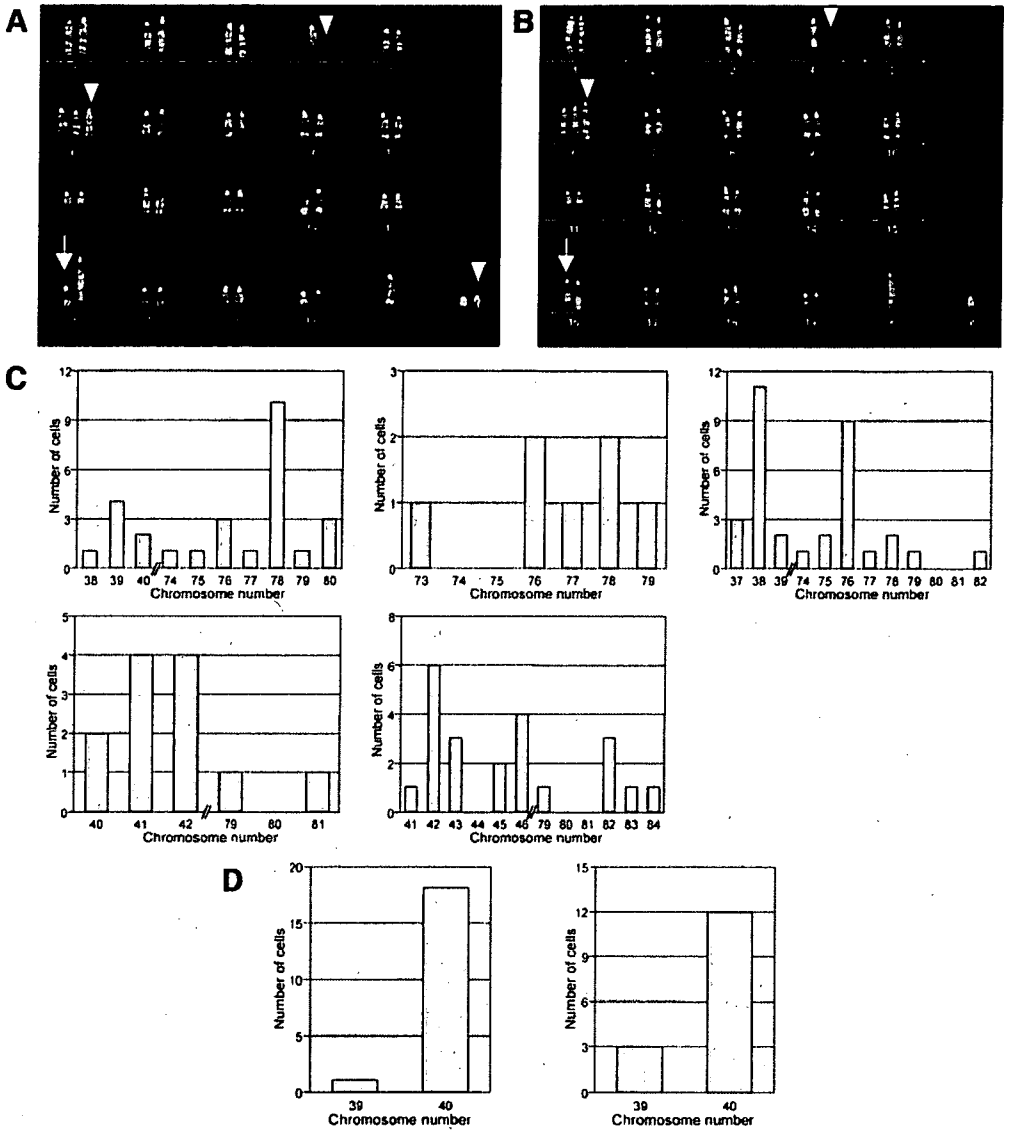

Figure 3. Cytogenetic analysis of neural stem cell (NSC) and mesenchymal stem cell (MSC) lines. **(A, B):** The two chromosome types found in the MSC line used for nuclear transfer. Some MSCs had 41 chromosomes with monosomy 4, trisomy 6, and two Y chromosomes (arrowheads in A). Others had the normal number of chromosomes (2n = 40), but they also had the same monosomy 4 and trisomy 6 (arrowheads in B). Heteromorphism was observed on chromosome 16 in both types (arrows in A and B). The chromosomes of MSCs were especially prone to morphological and numerical abnormalities. **(C):** The distribution of the cells classified according to the chromosome numbers in different MSC lines. All MSC lines comprise cells with abnormal chromosome numbers. **(D):** The distribution of the cells classified according to the chromosome numbers in two NSC lines. In contrast to the MSC lines, NSC lines comprise predominantly cells with the normal ploidy (2n = 40).

(Fig. 1). Thus, the stem cell lineages used here had differentiation potentials similar to those reported elsewhere [11–15].

Development of NSC and MSC Cloned Embryos

Cloned embryos reconstructed with NSC or MSC nuclei were cultured in vitro for 48 hours until they should have reached the four-cell stage. As shown in Table 2, more than half of the reconstructed embryos developed to the two-cell stage, whereas the remaining embryos did not divide. Because these one-cell-arrested embryos had formed pseudopronuclei successfully from the donor nuclei, the likely cause of their arrest was cell cycle asynchrony between the donor cell nucleus and the recipient ooplasm, as reported for cloning with ES cells and immature Sertoli cells [3–5].

Among the two-cell embryos, those derived from NSCs showed a higher rate of growth to the four-cell stage (75.9%) than those from MSCs (45.9%) or fibroblasts (41.7%) ($p < .05$) (Table 2). After transfer of these four-cell embryos into recipient female mice, 5.3% (10/189) reached term in the NCS group. Two normal-looking pups and one dead pup were obtained, whereas the remaining seven were placenta-only conceptuses (Fig. 2). The two offspring lived for 3 weeks and 1 year, respectively, and the latter was proved to be fertile. One pup (3%, 1/33) was obtained in the fibroblast group. In contrast, no MSC embryos developed to term because of complete implantation failure as revealed by examination of the recipient female mice at the time of the Caesarian delivery.

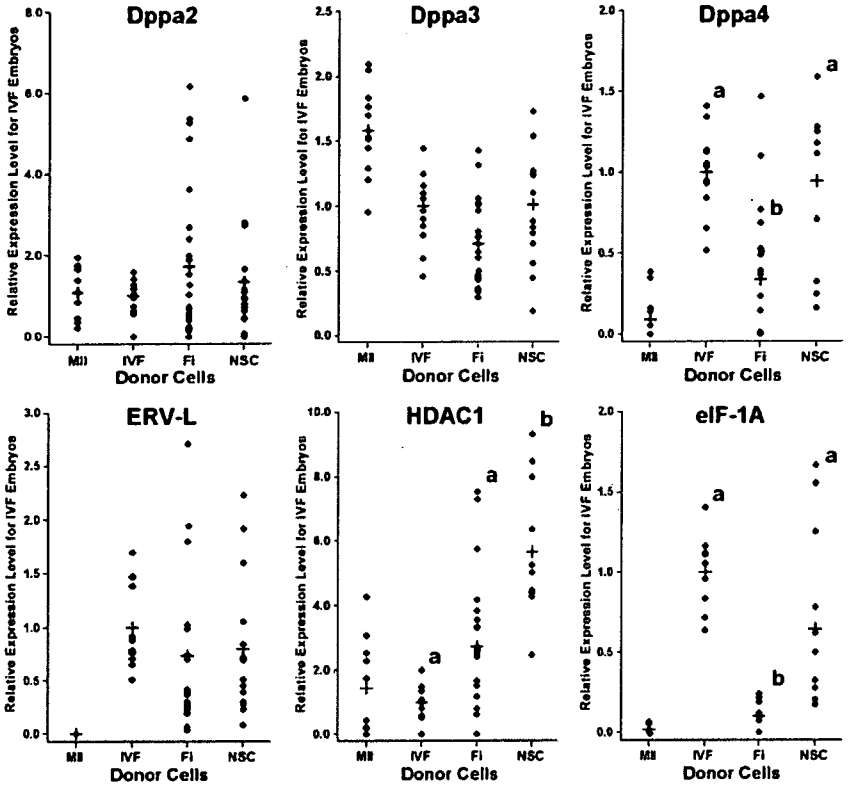

Figure 4. Quantification by real-time reverse transcriptase-polymerase chain reaction of mRNA expression of various zygotic-activated genes in single oocytes and embryos. Genotype (B6 × 129F1)-matched two-cell IVF embryos, two-cell Fi embryos, and two-cell NSC-derived clone embryos were analyzed. MII oocytes were derived from B6D2F1 females, as in the nuclear transfer experiments. Each dot represents a single embryo. Values are expressed relative to those in the IVF group (value = 1). Values with different letters differ significantly ($p < .05$, Scheffé's F test). Abbreviations: Fi, fibroblast-derived clone; MII, meiosis stage II; IVF, in vitro fertilization; NSC, neural stem cell.

Table 3. Efficiencies of cloning male mice from different cell types with the B6 × 129F1 genotype

Cell type	Embryos cultured	2-Cell (%)	4-Cell (% per 2-cells)	Embryos transferred	Embryos implanted (% per ET)	Term conceptuses (% per ET)	Offspring (% per ET)	References
Immature Sertoli	380	290 (76.3)	228 (78.6)	191	98 (51.3)	19 (9.9)	18 (9.4)	[4]
Adult fibroblast	355	334 (94.4)	194 (57.9)	126	82 (65.1)	4 (3.2)	3 (2.4)	[4, 8]
Neural stem	488	328 (67.2)	249 (75.9)	189	82 (43.4)	10 (5.3)	3 (1.6)	This study
NKT lymphocyte	572	534 (93.4)	482 (90.3)	185	112 (60.5)	8 (4.3)	3 (1.6)	[9]
Hematopoietic stem	637	563 (88.4)	302 (53.6)	252	90 (35.7)	2 (0.8)	2 (0.8)	[8]
Primordial germ[a]	2,018	1,011 (55.4)	611 (60.4)	441	252 (57.1)	17 (3.8)	4 (0.9)	[35]
T lymphocyte	385	236 (61.3)	102 (43.2)	44	0 (0.0)	0 (0.0)	0 (0.0)	[9]
Mesenchymal stem	232	172 (74.1)	79 (45.9)	78	0 (0.0)	0 (0.0)	0 (0.0)	This study

Cell types are listed in the order of the offspring rate.
[a] (B6D2F1 × 129)F1 female and male mice.
Abbreviations: ET, embryos transferred; NKT, natural killer T.

Chromosomal Analysis of Donor Cells

We examined the chromosome constitutions of the MSC line used in this study. Fifty-two (67%) of the 78 metaphase chromosome spreads examined had 41 chromosomes because of monosomy 4, trisomy 6, and two Y chromosomes (Fig. 3A). The remaining 26 spreads showed the normal number of chromosomes (2n = 40 in the mouse), but they also had aneuploidy of monosomy four and trisomy six (Fig. 3B). Heteromorphisms were often observed on chromosome 16.

We investigated five other MSC lines and found highly frequent abnormal ploidy in all (Fig. 3C). Their chromosomal patterns were more severely affected compared with that of the donor MSC line (Fig. 3A, 3B), probably because of their longer culture in vitro (additional 1 to 2 months in culture). In contrast, the NSC line had relatively normal ploidy levels (Fig. 3D).

Gene Expression Patterns in NSC Embryos

Because zygotic genes are programmed to activate at specific stages during preimplantation development, their expression pattern is a good indicator of the success of genomic reprogramming by nuclear transfer. We analyzed the expression levels of six genes by real-time quantitative PCR using two-cell NSC-derived cloned embryos, control fibroblast-derived cloned embryos, control IVF embryos, and MII oocytes. As shown in Figure 4, the gene expression patterns of NSC-derived embryos were similar to those of IVF embryos in all genes examined except *Hdac1*, which was more actively expressed in the NSC-derived embryos than in IVF embryos ($p < .001$). By contrast, fibroblast-derived clones tended to show lower expression patterns for *eIF-1A*, *Dppa3*, and *Dppa4* than IVF embryos, although the trend was not significant for *Dppa3* ($p = 2.9 \times 10^{-6}$ for *eIF-1A*; $p = .172$ for *Dppa3*; and $p = .00035$ for *Dppa4*).

DISCUSSION

We aimed to evaluate whether tissue-specific stem cells, NSCs and MSCs, could be used as nuclear donor cells for cloning mice. Because we undertook detailed analysis for the effects of the donor cell type and genotype on cloning efficiency [4], we have assumed that a male genotype produced by hybridization between C57BL/6 and 129 strains (B6 × 129F1) might give us relatively high birthrates for clones. Based on this assumption, we recently cloned hematopoietic lineage cells using B6 × 129F1 male mice as donors and successfully obtained cloned pups derived from the nuclei of HSCs and natural killer T lymphocytes [8, 9]. It is noteworthy that HSCs were inherently inefficient donor cells, and no HSC-derived pups were obtained when we used B6D2F1 female mice [8], the standard oocyte donors for mouse cloning experiments. In this study, therefore, we used B6 × 129F1 male mice as the source of NSCs and MSCs, and we now add the former to the clonable cell type list in mice. Table 3 shows the efficiencies of cloning mice using seven cell types with the male B6 × 129F1 genotype. These data show apparent cell type-specific differences in cloning efficiency ranging from 0% to 9.4% per embryo transferred. In view of the rates of four-cell embryos (per two cells) and offspring (per transfer), NSCs seem to be moderately efficient sources of nuclei for transfer.

After nuclear transfer into the ooplasm, the donor somatic cell genome should be reprogrammed to a state equivalent to that of a fertilized embryo for further development. During the first and second cell cycles in cloned mouse embryos, this reprogramming is manifested in the structural remodeling of the donor nucleus into pseudopronuclei and the initiation of embryo-specific transcription, termed zygotic gene activation (ZGA) [25]. Provided that nuclear transfer is performed a few hours before oocyte activation, nuclear structural remodeling, the timing of ZGA and of whole-genome ZGA activity in somatic cell-derived cloned embryos are indistinguishable from those in normally fertilized embryos [26, 27]. In contrast, analysis of the transcriptional levels of individual zygotic genes reveals that some are repressed in cloned embryos [8, 28], probably reflecting incomplete reprogramming of the donor cell genome. It is noteworthy that the degree of this repression or the genes affected by nuclear transfer may vary with the donor cell type used [8]. Therefore, we hypothesize that the expression patterns of specific ZGA genes—especially those not expressed in unfertilized oocytes—may be good indicators of how effectively the donor cell genome is reprogrammed by nuclear transfer. In our current study, we analyzed six ZGA genes; of these, *Dppa4*, *ERV-L*, and *eIF-1A* seemed to give better information because their expression levels increased significantly from the basal levels at MII to significantly higher levels in the control two-cell IVF embryos (Fig. 4). The expression levels of these three genes did not differ significantly between NSC clones and IVF embryos. In contrast, the expression patterns for *eIF-1A* and *Dppa4* were significantly lower in fibroblast-derived clones than in IVF embryos ($p < .05$). These results, along with the rates of four-cell embryos, indicate that reprogramming was more efficient for the NSC genome than the fibroblast genome. This is consistent with the report of Blelloch et al. [29], who found more frequent generation of nuclear transfer ES cells from NSCs than from fibroblasts using the same B6 × 129F1 genetic background . However, the birth rates in our study did not differ significantly between NSC and fibroblast clones, probably because of technical variations between the embryo transfer experiments and because of the low numbers of pups born in the cloning experiments. Our gene expression analysis using the two-cell embryos also suggests the importance of the donor genotype, because the overall gene expression patterns in our current study were better than those in our previous report using B6D2F1 female donors [8]. These findings show clear associations between the expression levels of certain genes and subsequent embryonic development. Taken together, we expect that the reprogrammability of the different donor cells can be assessed as early as at the two-cell stage by analyzing the expression of appropriate genes as indicators.

In general, genetic factors as well as epigenetic factors may considerably affect the development of cloned embryos . The implantation failure found here for MSC-derived embryos is strongly suggestive of chromosomal abnormalities, as documented by Bosch et al. [30]. Chromosomal abnormalities of cloned embryos may occur because of abnormal behavior of the donor chromosomes or chromosomal abnormalities pre-existing in the donor cells. In the mouse, the chromosomal constitutions of cloned embryos are generally stable because of the presence of cytoplasmic asters that act as microtubule-organizing centers at fertilization [31]. During nuclear transfer, these asters gather together to form a spindle that anchors the donor chromosomes and contributes to the genetic stability of reconstructed embryos [32]. Therefore, chromosomal abnormalities in MSC-derived cloned embryos are likely to derive from those in the original donor cells. Our chromosomal analysis of the donor cells supports this assumption. The rates of MSCs with abnormal chromosomal numbers or morphology were extremely high. According to Miura et al. [33], repeated passages of mouse MSCs lead to spontaneous immortalization, which is very closely associated with chromosomal aberrations. This should be considered when one clones mice from donor cells that have been passaged many times in vitro. By contrast, in bovines, MSCs were cloned successfully and normal offspring were born at the usual efficiency (7%, 1/13), probably because of more stable chromosomal constitutions of bovine MSC lines [34].

We conclude that tissue-specific stem cells in mice, namely NSCs, MSCs, and HSCs, can show marked variations in their ability to produce cloned offspring, according to both the epigenetic and genetic status of their original genomes.

ACKNOWLEDGMENTS

This research was supported by grants from MEXT, MHLW, CREST, and the Human Science Foundation in Japan. K.K. is currently affiliated with The Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan.

NOTE ADDED IN PROOF

Very recently, cloning mice from neonatal neural stem cells has been reported by Mizutani et al. [36].

DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors indicate no potential conflicts of interest.

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Isolation, characterization, and *in vitro* and *in vivo* differentiation of putative thecal stem cells

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Edited by R. Michael Roberts, University of Missouri, Columbia, MO, and approved June 7, 2007 (received for review April 26, 2007)

Although ovarian theca cells play an indispensable role in folliculogenesis by providing follicular structural integrity and steroid substrates for estrogen production, little information is available about their recruitment, growth, and differentiation because their immature forms have not been identified. We have isolated putative thecal stem cells with the ability to self-renew and differentiate *in vivo* and *in vitro*. They are similar to fibroblasts in morphology and proliferate *in vitro* as round colonies with a homogenous cell population. They were induced to differentiate into early precursors and steroidogenic cells in a stepwise manner after treatment with serum, luteinizing hormone, and paracrine factors from granulosa cells. At each differentiation step, these cells displayed appropriate gene expression and morphological markers and later secreted androstenedione. The fully mature morphology was achieved by coculture with isolated granulosa cells. When transplanted into the ovaries, the putative thecal stem cells colonized exclusively in the ovarian interstitium and the thecal layer of follicles as differentiated cells. Thus, thecal stem cells appear to be present in neonatal ovaries and can be isolated, purified, and induced to differentiate *in vitro*. Thecal stem cells could provide an invaluable *in vitro* experimental system to study interactions among the oocytes, granulosa cells, and theca cells during normal folliculogenesis and to study ovarian pathology caused by theca cell dysfunction.

follicle | oocyte | ovary

During folliculogenesis in mammals, oocytes grow while surrounded by an increasing number of granulosa cell layers. From the preantral stage onward, theca cells differentiate as the outer layer of the follicle. These elements provide structural integrity and the androgen substrate for granulosa cell estrogen production, thus playing an indispensable role in follicular development (1). Mature theca cells can be distinguished by their morphology and location *in situ* and can be isolated easily in a crude suspension for *in vitro* analysis. Much information has been reported about the factors and mechanisms regulating theca cell steroidogenesis in several mammalian species (2). However, our knowledge about theca cell recruitment and growth is limited because of the lack of *in vitro* experimental systems to isolate, culture, and differentiate immature theca cells. Generally, it is difficult to distinguish immature theca cells from ovarian stroma cells based on their morphology (3).

In contrast to theca cells, their male counterparts, Leydig cells, have been studied in detail because their putative stem cells can be isolated from mouse and rat testes by flow cytometric sorting (4, 5). Leydig cells can remain undifferentiated *in vitro* for months and can respond to growth factors to differentiate into steroidogenic precursor cells (5). After transplantation into the testes of Leydig cell-deficient mice, these stem cells recolonize normally in the host testicular interstitium and restore the serum testosterone concentration (4). These *in vitro* and *in vivo* approaches using isolated Leydig stem cells may provide further clues about the mechanisms of formation and maintenance of the Leydig cell population and their biochemical characteristics.

Initially, we undertook this study to isolate putative "female germ-line stem (GS) cells," the presence of which is still highly controversial among reproductive biologists and stem cell researchers (6–8). We initially expected that the isolation procedure optimized to male GS cells from neonatal testes (9, 10) might be applicable to putative female counterparts in neonatal ovaries. Under our experimental conditions, we were able to generate round spherical colonies that produced a number of small primitive oocytes as if oogenesis had proceeded inside. However, detailed biochemical analysis revealed that most of the cell types comprising the colonies were somatic cells and not germ-line cells. These cells were later identified as putative ovarian thecal stem cells.

A fundamental property of stem and progenitor cell division is the capacity to retain the proliferative state or to generate differentiated daughter cells. These putative thecal stem cells were purified easily *in vitro*, formed characteristic anchor-independent round colonies, and, after stimulation, started to differentiate and show characteristic signs of steroidogenesis. They also colonized the host ovarian tissue after transplantation in a manner similar to that of Leydig stem cells in mice and rats (4, 5). Our study provides evidence for the presence of putative thecal stem cells in the neonatal mouse ovary. Interestingly, unlike Leydig stem cells, putative thecal stem cells can be purified *in vitro* simply by optimizing the culture conditions without any cell-sorting procedures. Thus, these isolated putative thecal stem cells may provide an invaluable and reproducible experimental system to study their biochemical characteristics. This system would include gene expression and signaling cascades, which are essential for normal folliculogenesis in mammals.

Results

Isolation of Neonatal Ovarian Cells by Selective Culture. Because the original purpose of this study was to establish cell lines of female GS cells (6), cells were cultured from newborn mouse ovaries according to a protocol for male GS cells with slight modifications (9, 10). The culture medium was essentially a serum-free GS medium (GSM-K) containing growth factors [see *Materials*

Author contributions: A.H., M.H., T.K., and A.O. designed research; A.H., M.H., K.H., S.M., K.I., H.M., H.H., M.K.-S., Y.K., T.K., T.S., and A.O. performed research; M.K.-S. and T.S. contributed new reagents/analytic tools; A.H., M.H., K.H., S.M., K.I., and Y.K. analyzed data; and A.H., Y.K., and A.O. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

Abbreviations: GS, germ-line stem; GSM, GS medium; GSM-K, serum-free GSM; GSM-S, serum-containing GSM; ER, endoplasmic reticulum; LH, luteinizing hormone; MVH, mouse vasa homologue.

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This article contains supporting information online at www.pnas.org/cgi/content/full/0703787104/DC1.

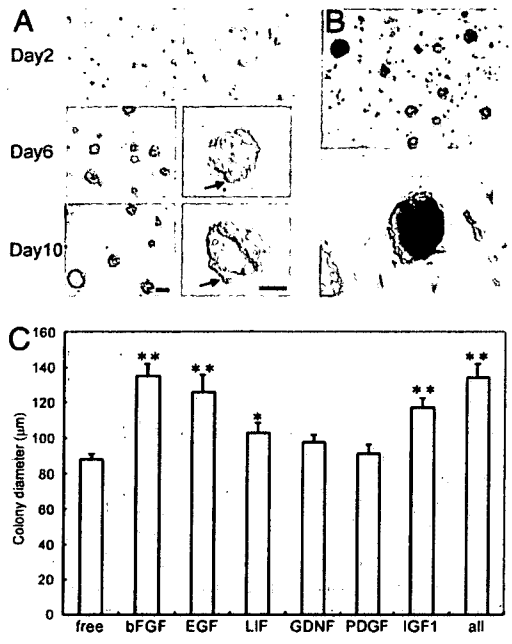

Fig. 1. Development of thecal stem cell colonies *in vitro*. (*A*) The size of the colonies increased rapidly during the first several days and gradually reached the maximum size of ≈120 μm in diameter. Many oocytes (≈20 μm in diameter) continued to protrude from the surface of the colonies (arrows). (Scale bars: *Left*, 100 μm; *Right*, 50 μm.) (*B*) The colonies are weakly positive for alkaline phosphatase staining. (*C*) Effect of growth factors on colony size during the first 14 days of culture. Single growth factors, or some combination thereof, were added to the basic GSM. *, $P < 0.05$; **, $P < 0.01$ [compared with growth-factor-free GSM (three replicates, one-way ANOVA)].

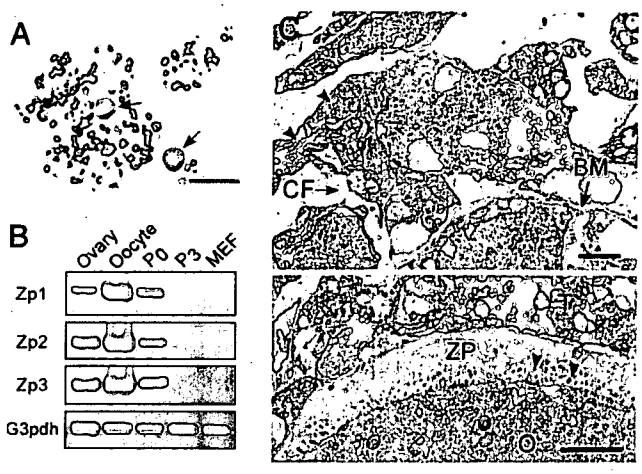

Fig. 2. Cytological and gene expression analysis of thecal stem cell colonies. (*A*) Paraffin-embedded section of colonies stained for BrdU (blue) and MVH (brown). Small cells were generally positive for BrdU but not for MVH. Growing oocytes (arrows) were positive for MVH but not for BrdU, indicating that the oocytes came from preexisting cells in the colonies. (Scale bar: 50 μm.) (*B*) Gene expression analysis (RT-PCR) of the colonies. After the third passage (P3), oocytes disappeared from the colonies, as demonstrated by the absence of oocyte-specific markers (zona pellucida proteins Zp1, Zp2, and Zp3). MEF, mouse embryonic fibroblast. (*C*) Electron micrographs of the colonies. Each cell in the colonies had a relatively small cytoplasm that was rich in ribosomes and rough ER (arrowheads) (*Upper*). Cell-to-cell connections were sparse, and their intercellular space was filled with basement membrane (BM) and collagen fibers (CF) (*Upper*). A thecal stem cell (T) directly surrounds the zona pellucida (ZP) of an oocyte (O), but unlike typical granulosa cells, it does not protrude foot processes to the oocyte surface through the zona (*Lower*). Arrowheads indicate microvilli from the oocyte. (Scale bars: 2 μm.)

and Methods and supporting information (SI) Table 1]. For the primary cell preparation, fibroblast cells were allowed to attach onto the bottom of the gelatin-coated culture plate, but only floating cells were passaged to the secondary culture plate. These replated cells attached weakly on the bottom of the culture wells during an overnight culture and formed round colonies comprising compact clusters of cells with unclear borders. Colony formation was observed 34 times among 44 replicated experiments (77% formation rate). The colonies were consistently positive for alkaline phosphatase staining (five replicated experiments), as are embryonic stem (ES) cells (11) and male GS cell colonies (9) (Fig. 1 *A* and *B*). However, the appearance of these colonies differed from the established stem cell lines, and the intensity of alkaline phosphatase staining was apparently weaker than that of ES cell colonies. The colonies were easily detached from the bottom of the plate by mechanical treatment, and these freed colonies tended to aggregate with each other. The colonies were somewhat difficult to disperse to single cells by enzyme treatment, especially when they grew into large sizes (>50 μm in diameter); therefore, we could not count the exact cell numbers in the colonies. When the first-appearing colonies (≈10 μm in diameter) were allowed to grow singly in culture, they rapidly increased in size for the first several days and then gradually reached a maximum size of ≈120 μm in diameter before the first passage at ≈14 days (Fig. 1*A*). Because the size of cells composing colonies did not change throughout the culture period, the cell proliferation rate is estimated to be $\approx 1.7 \times 10^3$-fold during the 14 days. Their active proliferation *in vitro* is consistent with the intense incorporation of BrdU by the cells comprising the colonies in our cell proliferation assay (below). We examined the effect of six growth factors in the basal GS medium (GSM) (SI Table 1) on the growth of colonies during a 14-day culture period. Four growth factors, bFGF, EGF, LIF, and IGF1, exhibited significant effects on colony growth, although they did not seem to prolong the cell proliferation phase. PDGF and glial cell line-derived neurotrophic factor, which has a critical effect on male GS cell proliferation, had no effect (Fig. 1*C*).

Characterization of Isolated Ovarian Cells. During the first round of culture (14 days), many oocytes (≈20 μm in diameter) continued to protrude from the surface of the colonies and were released into the medium as single oocytes with or without a zona pellucida (Fig. 1*A*). To determine whether these oocytes had been derived from mitotically active cells (presumptive female GS cells) or from preexisting prophase-I oocytes, we performed a cell proliferation assay by adding BrdU to the culture medium. The BrdU-treated colonies were sectioned and analyzed immunochemically. Most of the cells comprising the colony were <10 μm in diameter and stained positively for BrdU, but none were positive for mouse vasa homologue (MVH, a germ-cell marker). In contrast, oocytes >15 μm in diameter that were positive for MVH were all negative for BrdU (Fig. 2*A*), indicating that they were derived from preexisting small oocytes that had already entered prophase I. Consistent with this conclusion, the imprinting status of cells comprising the colonies was the somatic type (parent-of-origin-specific methylation), not the germ-cell type (complete demethylation or biallelic methylation) (SI Fig. 6). After a few passages, the colonies ceased producing oocytes, probably because their precursor oocyte stock had been depleted. This finding was confirmed by the disappearance of mRNA for oocyte-specific genes in the colonies after the third passage (Fig. 2*B*).

Thus, the small cells in the colonies were most likely somatic cells, but we did not know which ovarian cell type(s) was being

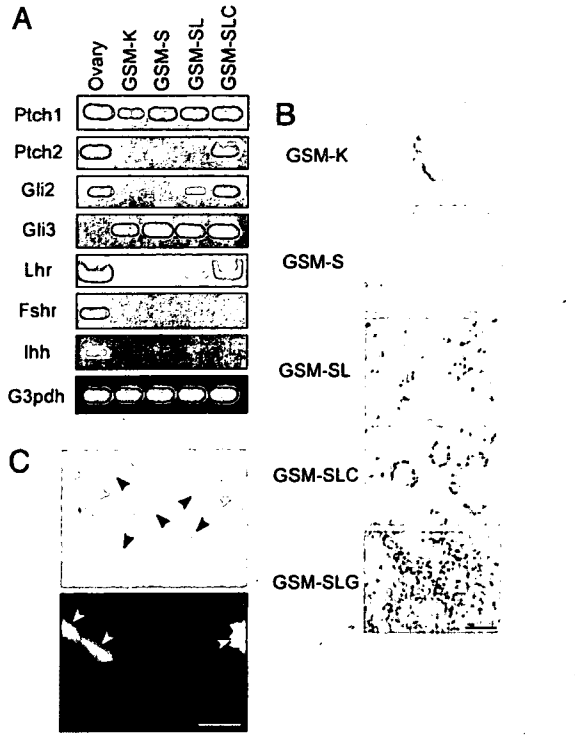

Fig. 3. The stepwise differentiation of thecal stem cells as indicated by gene expression patterns and cytoplasmic lipid droplet development. (*A*) Gene expression analysis (RT-PCR) of differentiating theca cells under different culture conditions. The *Lhr*, *Gli2*, and *Ptch2* genes were expressed as the thecal cells differentiated *in vitro*. The *Fshr* and *Ihh* genes are markers for granulosa cells. GSM-SL, GSM supplemented with LH, IGF1, and stem cell factor. (*B*) Cytoplasmic lipid droplets stained with Oil red O. Initially, very few lipid droplets were visible in the thecal stem cell colonies (GSM-K), but these increased in number progressively as the theca cells differentiated to their fully mature state (GSM-SLG). (Scale bar: 50 μm.) (*C*) Thecal stem cells were cocultured with isolated granulosa cells (GSM-SLG). To identify the cells differentiating from the thecal stem cells correctly, granulosa cells were prepared from EGFP-expressing mice. As expected, only fluorescence-negative cells had lipid droplets (granular inclusions under Hoffman optics) (black arrowheads). White arrowheads indicate EGFP-expressing granulosa cells. (Scale bar: 50 μm.)

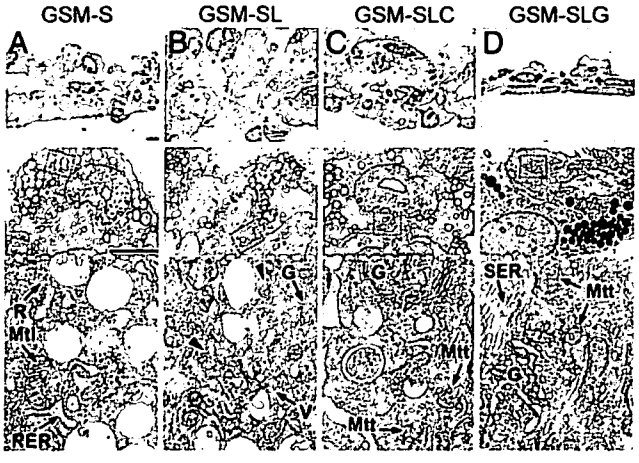

Fig. 4. Light and electron micrographs of cells that differentiated from thecal stem cells *in vitro*. The lower images are the higher magnifications of the rectangular areas of the middle images. Cells in GSM-S (*A*) retained the original undifferentiated features, such as the presence of rough ER (RER), ribosomes (R), and mitochondria with lamellar cristae (Mtl), whereas differentiating cells in GSM-SL (*B*) accumulated Golgi apparatuses (G), vacuoles (V), and mitochondria with cristae intermediate between the lamellar and tubular type (arrowheads). Cells in GSM conditioned with granulosa cells (*C*) and GSM-SLG (*D*) showed more differentiated steroidogenic features. Mtt, mitochondria with typical tubular cristae; SER, smooth ER; G, Golgi apparatuses; GSM-SL, GSM supplemented with LH, IGF1, and stem cell factor; GSM-SLC, GSM conditioned with granulosa cells. (Scale bars: 10 μm.)

purified during culture and passage. Electron microscopic observations revealed that these cells had cytological features common to undifferentiated interstitial cells, and that their characteristics were essentially unchanged throughout the culture period. They were oval or irregular in shape and had a relatively small cytoplasm that was rich in rough endoplasmic reticulum (ER) and ribosomes, whereas smooth ER, Golgi membranes, and lipid droplets were scarce (Fig. 2*C*). Cell-to-cell connections were sparse, and the intercellular space was filled with basement membranes and collagen fibers. Some cells surrounded the zonae pellucidae of the oocytes directly, but unlike typical granulosa cells, they never protruded foot processes to the oocyte surface through the zona (Fig. 2*C*). Some of the round colonies had a weak alkaline phosphatase reaction, which usually localizes specifically in the thecal layers of growing follicles and not in granulosa cells (11) (Fig. 1*B*). Gene expression analysis demonstrated that the cells expressed markers for theca cells (*Ptch1* and *Gli3*), but not for granulosa cells (*Fshr* and *Ihh*) (Fig. 3*A*). Taken together, these findings suggest that the cells isolated from the neonatal ovarian tissue with GSM-K were thecal stem-like cells (hereafter called "putative thecal stem cells" or simply "thecal stem cells"). This finding was supported by their differentiation *in vivo* and *in vitro* in the subsequent experiments, which are described below.

***In Vitro* Differentiation of Putative Thecal Stem Cells.** The cells remained in their undifferentiated state and showed no signs of steroidogenic activity when the colonies were cultured in GSM-K. Adding serum to the media (GSM-S) caused the cells to spread rapidly as a monolayer onto the bottom of the dish (SI Fig. 7*A* and also see the cell shape in Figs. 3*B* and 4) and to accumulate fine lipid droplets in their cytoplasm (Fig. 3*B*). The cell numbers increased approximately four times faster when cells were cultured in GSM-S than in GSM-K (estimated from the size of the colonies for GSM-K). Although they did not stain intensely, the cells appeared to start steroidogenesis because many of the cells stained positively for 3β-hydroxysteroid dehydrogenase (3β-HSD), as shown by blue formazan precipitates in their cytoplasm (SI Fig. 7*B*). However, at the ultrastructural level, they generally kept their undifferentiated morphology and showed few organelles specific to steroidogenesis (Fig. 4*A*). At this stage, they first showed very weak expression of the luteinizing hormone receptor gene, *Lhr* (Fig. 3*A*).

Supplementing the medium with luteinizing hormone (LH), IGF1, and stem cell factor caused the theca cells to differentiate further and to develop more cytoplasmic lipid droplets (Fig. 3*B*). Electron microscopy revealed that the cells developed low electron-dense areas containing many Golgi apparatuses, small vacuoles, and few ribosomes (Fig. 4*B*). They also contained many mitochondria with cristae intermediate between the lamellar and tubular types (Fig. 4*B*). The cells expressed the *Gli2* gene, a theca-specific effector gene of Hedgehog signaling in granulosa cells (13). The androstenedione concentration was significantly higher in the culture supernatant (87.6 and 80.8 pg/ml in two replicated experiments) than in the control medium (GSM supplemented with LH, IGF1, and stem cell factor) containing no cells (10.7 and 11.6 pg/ml in two replicated experiments).

Further differentiation of theca cells could be achieved by supplementation with granulosa cell-conditioned medium.

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Many cells showed characteristic features of steroidogenic cells: many large lipid droplets, smooth ER, Golgi apparatuses, and mitochondria with tubular cristae (Fig. 4*C*). They expressed all of the genes examined including *Ptch2*, which is thought to be expressed in mature theca cells (13) (Fig. 3*A*). A high concentration of androstenedione (116.6 and 150.2 pg/ml in two replicated experiments) was detected in the medium after 7 days of culture.

Fully mature forms of theca cells were observed when they were cocultured with granulosa cells (GSM-SLG). To accurately identify the cells that differentiated from stem cells *in vitro*, a granulosa cell suspension was prepared from EGFP-expressing mice ("green mice"). As shown in Fig. 3*C*, only the fluorescence-negative, stem cell-originating cells had lipid droplets (granular cytoplasmic inclusions under Hoffman optics). Their shape was more oval than that of cells from the other culture groups. At the ultrastructural level, all lipid-including cells were rich in smooth ER and the mitochondria had tubular cristae. Eventually, no cells retained the features of the undifferentiated state (Fig. 4*D*). The androstenedione level in their medium reached 185.8 pg/ml.

Intraovarian Transplantation of Putative Thecal Stem Cells. Finally, to test whether the putative thecal stem cells isolated from the neonatal ovaries retained their stem cell-like properties through isolation and culture *in vitro*, we transplanted GSM-K-cultured stem cells into host ovarian tissues (Fig. 5*A*). After a 7-week culture period with four passages, stem cell colonies expressing green fluorescence were transplanted into the ovaries of two nontransgenic C57BL/6 strain female mice. Two weeks after transplantation, recipient females were stimulated with equine chorionic gonadotropin to induce follicular development, and their ovaries were removed for observation by fluorescence microscopy. The donor cells had proliferated actively and had surrounded fully developed follicles (Fig. 5*B*). In frozen sections, EGFP-positive cells were distributed in the inner and outer theca cell layers of large cells around fully grown follicles, or in the cortical interstitial areas of small individual cells (Fig. 5*C*). A few small, probably less differentiated, theca cells were present around small follicles (Fig. 5*D*). No fluorescence-positive cells were localized in the follicular epithelium.

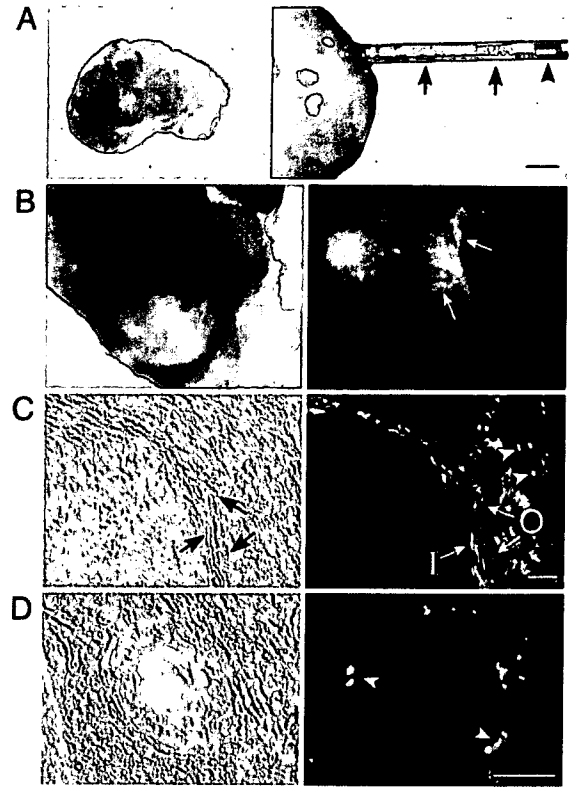

Fig. 5. Intraovarian transplantation of thecal stem cells (*A*) and ovaries 2 weeks after transplantation of thecal stem cells, which colonized exclusively in the ovarian interstitium and the thecal layer of follicles (*B–D*). (*A*) A host ovary removed from a mature female mouse (*Left*). The blood was wiped from the ovary on sterilized filter paper. Thecal stem cell colonies (arrows in *Right*) were transplanted into the ovary by a glass pipette. Arrowhead indicates an air bubble placed for controllable transfer. After transfer, the ovary was inserted into the empty ovarian bursa of another female mouse. (Scale bar: 2 μm.) (*B*) The donor thecal cells (EGFP-positive) surrounding two large follicles were clearly visible by fluorescence microscopy (arrows). (*C*) A frozen section of a large follicular area. The donor thecal stem cells differentiated into large cells and were located in both the inner (I) and outer (O) thecal layers. They were also present in the interstitial area of small cells (arrowheads). (Scale bar: 50 μm.) (*D*) A frozen section of a small primary follicle. A few small, probably less differentiated, theca cells were present around the follicle (arrowheads). (Scale bar: 50 μm.) (*B–D Right*) Corresponding fields observed by fluorescent microscopy are shown.

Discussion

Previously published experiments support the notion that interstitial pretheca cells are located within the ovarian stroma and may have stem cell properties (3), but theca stem cells have not been identified to date. In this study, we provide a clear demonstration that putative thecal stem cells exist in neonatal ovaries, and that they can be isolated *in vitro* as round spherical colonies. By applying appropriate stimuli to the colonies in our *in vitro* experimental system, we were able to fully reproduce the differentiation of theca cells from undifferentiated stem cells to mature steroidogenic theca cells, in a stepwise manner. The differentiation steps represented in this study are illustrated in SI Fig. 8 together with the presumed corresponding sequence of follicular development.

Purification of Putative Thecal Stem Cells in Selective Culture. One of the most intriguing findings was that putative thecal stem cells were readily isolated by culturing neonatal ovarian cells in GSM containing no serum. Generally, serum is added to the *in vitro* culture of a variety of cells including fibroblasts and granulosa cells (14), which are abundant in ovarian tissue. The requirements of thecal stem cells to proliferate *in vitro* probably differ from those of ovarian fibroblasts and granulosa cells. When we examined the effects of several growth factors on the growth of the colonies, only those thecal stem cells in colonies responded and proliferated. This finding suggests that the serum-free condition promoted selection of thecal stem cells in culture. In addition, their proliferating ability in a nonanchored situation might have accelerated the purification. At the first cell preparation step and during subsequent passages, cells having the ability to spread onto the dish were eliminated gradually, and finally only those proliferating in a nonanchoring manner remained in the culture. The purity of the final cell suspension was confirmed by the RT-PCR assay and exclusive perifollicular and interstitial colonization after intraovarian transplantation. Thus, the unique nutritional requirements and nonanchoring proliferation of thecal stem cells most likely contributed to their purification from the neonatal ovarian tissues.

The first aim of our study was to isolate and characterize putative oocyte stem cells (female GS cells), which were first reported by Johnson *et al.* in 2004 (6). To address this aim, we used the GS cell medium to culture the neonatal ovarian cells. During the first several passages, small oocytes could be identified on the surface of colonies, as if they had been growing from the stem cells inside the colonies. However, our detailed histochemical examination identified no MVH-and-BrdU double-

positive oocytes, indicating that only preexisting postmeiotic oocytes grew out from the colonies. This result by itself does not negate the presence of oocyte stem cells in the neonatal ovary, but these stem cells, if any, did not seem to respond to GS cell medium, which has been used in experiments on their male counterparts.

Proliferation Ability of Putative Thecal Stem Cells *in Vitro*. bFGF, EGF, LIF, and IGF1 significantly promoted the growth of stem cell colonies, but the culture conditions seemed to be suboptimal because they did not proliferate indefinitely. Although the cells continued to divide for ≈2 months, as shown by the increases in colony size, the proliferation rate decreased after ≈10 days, and the cell number seemed to reach a plateau after 1 month of culture. However, we cannot exclude the possibility that thecal stem cells have an inherently limited proliferation potential and that this feature was correctly reflected *in vitro*. Unlike male germ cells, which produce spermatozoa continuously throughout life (15), the size of the primordial oocyte reserve appears to be fixed during the prenatal period. Consistent with the nondividing character of oocytes, it is possible that thecal stem cells, which localize in the ovarian interstitium, do not divide actively but gradually acquire a proliferation ability as they differentiate to support the growing follicle. Generally, the tissue-specific stem cells studied so far are thought to depend on specialized cellular microenvironments and the *ex vivo* expansion of pure populations of tissue stem cells has proven elusive (16). We also observed that induction of differentiation by serum simultaneously promoted proliferation of differentiating theca cells *in vitro*.

Differentiation of Putative Thecal Stem Cells *in Vitro* and *in Vivo*. Thecal stem cells in round colonies had morphological characteristics common to undifferentiated interstitial cells such as small fibroblasts: they were oval and contained many organelles including mitochondria with laminar cristae, rough ER, and ribosomes. These thecal stem cells maintained these undifferentiated features for as long as they proliferated within round colonies. Stimulation with serum caused these cells to spread onto the bottom of the dish and to start to accumulate small lipid droplets in their cytoplasm. Although they seemed to stay in a relatively undifferentiated state, they expressed a small amount of *Lhr* mRNA. Treatment with LH, IGF1, and stem cell factor or paracrine factors from granulosa cells caused further significant differentiation of theca cells, leading to accumulation of many lipid droplets, formation of smooth ER, and the emergence of mitochondria with tubular cristae. All these cytological features are indicative of steroidogenic ability (17), a conclusion supported by the detection of androstenedione at a high concentration in the supernatant. These differentiation patterns of theca cells are consistent with previous findings showing that their proliferation and differentiation are independent of LH receptors in the early stages, but become LH receptor-dependent at a later stage (18), and that IGF1 synergistically augments the LH stimulation of androstenedione production (19). The stepwise emergence of several theca cell markers is also consistent with a previous *in situ* hybridization study that suggested that granulosa-derived factors, *Ihh* and *Dhh*, are candidate initiators of early thecal differentiation (13). We conclude that our culture system induced the differentiation of theca cells from their stem cells in a stepwise manner similar to that occurring *in vivo*, without the presence of other cell types. However, their full differentiation as shown by cells in the inner thecal layer of follicles required the presence of granulosa cells. Granulosa cells from adult bovine ovaries also stimulate androstenedione production by stromal cells (20). It is unlikely that direct cell-to-cell contact with granulosa cells was required for such terminal differentiation because of the intervening basement membrane between the granulosa and the inner thecal layers in normal follicles. Instead, close proximity probably promoted a paracrine effect of the granulosa cells. The presence of such paracrine mechanisms has been shown experimentally in an *in vivo* assay in hypophysectomized rats (21).

A fundamental question that remains unanswered about the differentiation of the theca cell lineage is the mechanisms by which the inner and outer thecal layers are formed from the common stem cells (3). The cells comprising the inner layer are steroidogenic cells and blood vessel cells, whereas cells comprising the outer layer are unidentified fibroblast-like cells and smooth muscle cells (22, 23). Our transplantation experiments revealed that the isolated thecal stem cells had the capacity to contribute to the formation of both inner and outer thecal layer cells, although the latter seemed to be more heterogeneous in composition. It is possible that the difference between cells in the two thecal layers represents the different differentiation status of these theca cells' lineage. This assumption agrees with the results of our *in vitro* differentiation experiments, in which coculture with granulosa cells led to the terminal thecal differentiation from the relatively undifferentiated, fibroblast-like cells. It is also interesting that, only 2 weeks after transplantation, the donor theca cells were observed in fully grown follicles. Because follicular development in mature rodents is estimated to take ≈6 weeks (24), it is probable that the transplanted thecal cells were able to invade partially grown preexisting follicles.

As far as we know, this is the first demonstration of successful intraovarian transplantation of cells isolated *in vitro*. We found that direct transplantation of cells into intact ovaries *in situ* caused heavy bleeding, and these transplanted cells never survived. In combination with an ovary transfer (removal and replacement) technique, the transplantation was very successful, and we could show that the putative thecal cells we purified were transplantable, as are other tissue-specific stem cells (25). Our intraovarian transplantation technique should enable us to assess the ability of a variety of cells to differentiate and function in the ovarian tissue.

Theca Cell Culture as an *in Vitro* Model for Soma–Germ and Soma–Soma Interactions. In mice, assembly of oocytes and somatic cells into follicles occurs within the first week after birth, and fully expanded antral follicles appear shortly before puberty (26). The ovaries of 2-day-old mice are devoid of distinct follicular structures, and when the follicles grow to enclose two to three layers of granulosa cells, theca cells start to surround the follicles and acquire the steroidogenic ability regulated primarily by LH. Androstenedione, a steroid hormone produced by theca cells, is converted into estradiol by granulosa cells (1, 2). Thus, folliculogenesis is a complex process in which germ cells (oocytes) and two types of somatic cells (theca cells and granulosa cells) interact through paracrine or direct mechanisms. In this study, we first demonstrated that putative thecal stem cells can be isolated and purified *in vitro* and induced to differentiate to acquire active steroidogenic ability. These isolated theca cells may provide invaluable experimental systems to study theca–granulosa and theca–oocyte interactions; e.g., kit–kit ligand (27) and Hedgehog signaling systems (13). We have also recently found that theca cells can promote the growth of granulosa-free oocytes in a paracrine fashion, indicating the presence of theca-derived oocyte trophic factors (unpublished data). Other than the factors involved in normal folliculogenesis, the mechanisms underlying ovarian pathology caused by theca cell dysfunction, including hyperandrogenism and polycystic ovary syndrome (1), could be unraveled by studying thecal stem cells in culture.

Materials and Methods

Cell Preparation and Culture. To prepare thecal stem cells, ovaries were collected from newborn (2–4 days after birth) ICR or

C57BL/6-Tg(CAG-EGFP)C14-Y01-FM131Osb female mice. The latter are the so-called "green mice" that show green fluorescence in the whole body (28). Cell culture was performed according to the protocol for establishment of male GS cells with slight modifications (9, 10). In brief, isolated ovaries were treated with 1 mg/ml collagenase in Hanks's solution at 37°C for 15 min and then treated with 0.2% trypsin and 1.4 mg/ml DNase for 10 min. Loosened ovarian tissues were dissected into single cells by gentle pipetting and were allocated into wells of a 0.1% (wt/vol) gelatin-coated 24-well tissue-culture plate ($2.0–2.5 \times 10^5$ cells per 2.0-cm^2 well). After incubation for 4 h, floating cells were recovered and placed into another culture well containing GSM-K (SI Table 1). The cells were maintained at 37°C under 5% CO_2 in air. A half-volume of the medium was changed every 2–3 days. After 14 days of culture, colonies were dispersed by trypsin treatment and replated into culture wells containing fresh medium (2× dilution). Colonies grew to the original size in ≈10–14 days, and passages were repeated until the cells stopped proliferating (at four to five passages).

Granulosa cells were prepared from follicles in the ovaries from 7.5 units of equine chorionic gonadotropin-stimulated TgX(act-EGFP)Osb#50 female mice (another green mouse strain). To remove the thecal layers from the isolated follicles, they were incubated in StemPro-34 serum-free medium containing 0.25% trypsin and 0.02% EDTA for 10 min at 37°C. Oocytes were washed and stripped of the enclosing follicular cells by pipetting. Groups of granulosa cells from 50 follicles were transferred to a four-well dish (Nunc, Roskilde, Denmark). Cultures were carried out in GSM supplemented with 15% FBS (GSM-S) (SI Table 1) at 37°C with 5% CO_2 in air. Two days later, granulosa cell clumps were dispersed by trypsin treatment and replated to a fresh culture plate (5× dilution). The lack of contamination by thecal cells in the granulosa cell suspension was confirmed by RT-PCR with specific primers (data not shown). Granulosa-conditioned medium was prepared from wells after 24 h of incubation of 80–100% confluent granulosa cells.

***In Vitro* Differentiation.** Putative theca stem cells (1.1×10^5 cells) were cultured for 4–5 weeks with three passages and were then replated and cultured under one of the five conditions indicated in SI Table 1. After 6 days of culture, these cells were subjected to RT-PCR, Oil red O staining, or electron microscopic observation. In some experiments, the androstenedione concentration in the supernatant was measured by liquid chromatography-tandem mass spectrometric analysis (29). This analysis was performed by Teikoku Hormone (Kawasaki, Japan).

Intraovarian Transplantation. Thecal stem cells (3.5×10^5) derived from C57BL/6-Tg(CAG-EGFP)C14-Y01-FM131Osb were cultured in GSM-K for 7 weeks with four passages. In a preliminary study, we found that direct transplantation of cells into intact ovaries *in situ* caused heavy bleeding, and no cells survived after the operation. Therefore, we first removed the host ovaries from mature C57BL/6 females, and the blood was wiped from the ovaries on sterilized filter paper (Fig. 5*A*). Approximately 20 colonies were picked up by a glass pipette (≈100 μm inner diameter) together with a minimal amount of medium. The tip of the pipette was inserted into the host ovary along the longer axis (Fig. 5*A*). The cell colonies were transplanted in the opposite side of the ovarian cortex by applying gentle positive pressure, and the pipette was slowly pulled out of the ovary. Each ovary that received donor cells was inserted into the empty ovarian bursa of another host C57BL/6 female mouse under anesthesia as described (30). Two weeks after transplantation, the host females were administered 5 units of equine chorionic gonadotropin, and the ovaries were retrieved 48 h later. These transplantation experiments were replicated twice.

Cytological, Histological, and Gene Expression Analyses. Cytological, histological, and gene expression analyses were performed according to established protocols (see *SI Materials and Methods*).

Animals. All animals were maintained and used for experiments in accordance with the guidelines of the RIKEN Institute.

EGFP-expressing strains of mice (green mice) used in this study belong to Dr. M. Okabe (Osaka University, Osaka, Japan) and were provided by RIKEN BioResource Center with the support of the National BioResources Project of the Ministry of Education, Culture, Sports, Science, and Technology of Japan. The anti-MVH antibody was kindly provided by Dr. T. Noce (Mitsubishi Kagaku Institute of Life Science, Tokyo, Japan). This research was supported by grants from the Ministry of Education, Culture, Sports, Science, and Technology (to A.H. and A.O.); the Ministry of Health, Labour, and Welfare (A.O.); and Core Research for Evolutional Science and Technology (A.O.). A.H. is the recipient of a research fellowship from the RIKEN Special Postdoctoral Researchers Program.

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Journal of Reproduction and Development, Vol. 53, No. 6, 2007

—Full Paper—

The Developmental Ability of Vitrified Oocytes from Different Mouse Strains Assessed by Parthenogenetic Activation and Intracytoplasmic Sperm Injection

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Abstract. Assessment of the developmental ability of oocytes following freezing and thawing is an important step for optimizing oocyte cryopreservation techniques. However, the *in vitro* fertilization of frozen-thawed mouse oocytes is often inefficient because of incomplete capacitation of spermatozoa in the absence of surrounding cumulus cells. This study was undertaken to determine whether the oocyte cryopreservation efficiency of different strains of mice could be assessed from the development of oocytes following parthenogenetic activation and intracytoplasmic sperm injection (ICSI). Oocytes were collected from hybrid (C57BL/6 DBA/2) F1 or inbred (C57BL/6J, C3H/HeN, DBA/2J and BALB/cA) strains and were vitrified in a solution containing ethylene glycol, DMSO, Ficoll and sucrose. In the first series of experiments, oocytes were activated parthenogenetically by Sr^{2+} treatment after warming. The oocytes from the inbred strains, but not those of the F1 hybrid, were diploidized by cytochalasin treatment to obtain a sufficient number of blastocysts. In all strains tested, parthenogenetic embryos derived from vitrified oocytes developed into blastocysts at rates between 23 and 68%. In the second series of experiments, vitrified oocytes from each strain were injected with homologous spermatozoa after warming. Normal offspring were obtained from all strains at rates between 5 and 26% per embryo transferred. Thus, the feasibility of oocyte cryopreservation protocols can be assessed easily by *in vitro* development of parthenogenetic embryos or by *in vivo* development of ICSI embryos. Moreover, the oocytes of these four major inbred strains of mice can be cryopreserved safely for production of offspring.

Key words: Intracytoplasmic sperm injection (ICSI), Mouse, Oocyte, Parthenogenetic development, Vitrification

(J. Reprod. Dev. 53: 1199–1206, 2007)

Cryopreservation of embryos and gametes is a major strategy for genetic conservation of mammalian species. In laboratory mice, this technique is also important to preserve invaluable genetic resources from naturally occurring mutant mice and to save costs and space for storage of genetically engineered mice, the number of which is expanding very rapidly. Thanks to intensive technical development during recent decades, mouse oocytes [1, 2], spermatozoa [3, 4] and embryos [5–7] can now be cryopreserved successfully using appropriate methods. However, as most of these

Accepted for publication: August 10, 2007
Published online: September 4, 2007
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cryopreservation techniques have been devised using F1 hybrid and outbred ICR mice, it remains uncertain whether they are applicable to inbred strains. For example, spermatozoa from the C57BL/6 strain were found to be very sensitive to cryoinjury and were often unable to fertilize oocytes after freezing and thawing by conventional procedures [8, 9]. Therefore, it is desirable to optimize the cryopreservation methods for each inbred strain of mice.

The efficiency of cryopreserving gametes and embryos is usually assessed by the ability of the resultant embryos to develop into blastocysts or term offspring. However, in the case of mouse oocytes, removal of cumulus cells and the freezing procedure itself often cause significant reduction of the fertilization rate. This is because of changes in biochemical moieties in the zona pellucida (zona hardening) and the lack of cumulus cells, which are known to accelerate capacitation of spermatozoa [10, 11]. To circumvent these obstacles, partial zona dissection or intracytoplasmic sperm injection (ICSI) have been employed [8, 12, 13]. Even whole organ or body cryopreservation has enabled normal birth of mice when combined with ICSI [14]. However, these techniques require extensive micromanipulation skills, and their outcomes often fluctuate according to timing of the experiment and the operator.

Another strategy to assess the feasibility of cryopreserving oocytes would be parthenogenetic activation of oocytes, which has been extensively employed in reproductive engineering studies in the mouse. As this technique requires no special skills, the results are very consistent and reliable, although the resultant embryos never develop to term because of absence of the paternally imprinted genome [15]. Wakayama *et al.* [16] optimized the conditions for nonfrozen storage of oocytes by analyzing the developmental ability of parthenogenetic embryos derived from them.

This study was undertaken to determine whether the developmental ability of cryopreserved oocytes could be assessed by parthenogenetic activation and ICSI in different strains of mice. For cryopreservation of mouse oocytes, we employed a vitrification technique of "minimal volume approaches", which is considered broadly applicable to oocytes and embryos from a variety of species [17].

Materials and Methods

Animals

For collection of oocytes and spermatozoa, B6D2F1 (C57BL/6 DBA/2) mice (Japan SLC, Shizuoka, Japan) and four inbred strains (C57BL/6J, C3H/HeN, DBA/2J and BALB/cA; Clea Japan, Tokyo, Japan) of mice were used. For embryo transfer experiments, pseudopregnant female ICR mice (Clea Japan) were used as recipients. The mice were maintained under specific pathogen-free conditions at the RIKEN Bioresource Center, Ibaraki, Japan. They were provided with water and commercial laboratory mouse chow *ad libitum* and housed under controlled lighting conditions (daily light period of 0700–2100 h). All animals were maintained and used for experiments in accordance with the guidelines of the RIKEN Bioresource Center.

Oocyte collection and cryopreservation

Female mice were induced to superovulate at 8–16 weeks of age by the injection of 7.5 IU of equine chorionic gonadotropin followed by 7.5 IU of human chorionic gonadotropin (hCG) 48 h later. Mature MII oocytes were collected from the oviducts 15–16 h after the hCG injection and were released from the cumulus cells by treatment with 0.1% bovine testicular hyaluronidase (Sigma-Aldrich, St. Louis, MO, USA) in CZB medium [18]. Only those oocytes with a normal appearance were incubated further in fresh CZB medium at 37 C in an atmosphere of 5% CO_2 in air.

The oocytes were vitrified by the method developed by Lane *et al.* [19] and Lane and Gardner [20], with slight modifications. Cumulus-free oocytes were first pretreated (equilibrated) in modified Dulbecco's phosphate buffered saline (PB1 [21]) containing 8% (v/v) ethylene glycol (EG) and 8% (v/v) dimethylsulfoxide (DMSO) for 1–5 min and were then transferred into vitrification medium [PB1 containing 16% (v/v) EG, 16% (v/v) DMSO, 10 mg/ml Ficoll and 0.65 M sucrose]. The oocytes were loaded into vitrification carrier tools (Cryotops, Kitazato Supply, Shizuoka, Japan) and immersed in liquid nitrogen. The time from immersion into the vitrification medium to plunging into liquid nitrogen was adjusted to be as close to 1 min as possible. All procedures were undertaken at room temperature (23–25 C).

After storage for more than 6 days, the oocytes

were warmed and retrieved as follows. The Cryotops were directly placed into the warming solution (PB1 containing 0.5 M sucrose at 37 C), and the oocytes were picked up 2 min later. The oocytes were then transferred to a second warming solution containing 0.25 M sucrose. After 3 min , they were washed in fresh PB1 three times and incubated in CZB medium at 37 C in an atmosphere of 5% CO_2 in air until artificial activation or ICSI. The warmed oocytes were considered to have "survived" if they lacked dark contracted cytoplasm, vacuolization or a cracked zona pellucida.

Oocyte activation and embryo culture

The developmental ability of the vitrified oocytes was assessed by parthenogenetic activation. Haploid parthenogenetic embryos were produced by activating oocytes for 0.5–2.5 h with Ca^{2+}-free CZB medium containing 2.5–10 mM $SrCl_2$. When diploid parthenogenetic oocytes were constructed, oocytes were activated in $SrCl_2$ solutions containing 5 g/ml cytochalasin B and then cultured for up to 6 h in CZB medium containing 5 g/ml cytochalasin B. The cytochalasin B was removed by washing with fresh CZB medium. The haploid and diploid parthenogenetic embryos were cultured in CZB medium at 37 C in an atmosphere of 5% CO_2 in air for 120 h.

ICSI and embryo transfer

ICSI was performed using a Piezo-driven micromanipulator (Prime Tech Ltd., Ibaraki, Japan) as described previously [22, 23]. Spermatozoa were prepared from frozen stocks collected from the cauda epididymidis of mature male mice (8–16 weeks of age) of the same strain as the females supplying the oocytes. Freezing and thawing of spermatozoa were performed as described previously [9]. Thawed spermatozoa were suspended in a drop containing 12% polyvinylpyrrolidone in nucleus isolation medium [24]. A single spermatozoon was aspirated into an injection pipette. The sperm head was separated from the tail by applying a few Piezo pulses to the neck and was then injected into an oocyte in a Hepes–CZB drop. After injection, the oocytes were kept at room temperature for about 10 min before incubation at 37 C. The surviving oocytes were cultured in CZB medium at 37 C under 5% CO_2 in air for 24 h.

Fertilized embryos that reached the 2-cell stage by 24 h in culture were transferred into pseudopregnant female ICR mice (8–12 weeks old) on day 1 (the day following sterile mating with a vasectomized male). Six to 10 embryos were transferred into each oviduct (12 to 20 per recipient). On day 20, the recipient females were sacrificed to examine them for the presence of fetuses by Caesarean section. Live pups were nursed by lactating female ICR or BALB/cA mice. The females that received embryos from the fresh oocyte groups were subcutaneously injected with progesterone (2 mg per animal) on day 19 to prevent natural delivery.

Statistical analysis

Each group consisted of at least 58 reconstructed embryos, with a minimum of three replicates per run. The percentages of embryos that developed to the 2-cell or blastocyst stages were analyzed using arcsine transformation followed by ANOVA. A post hoc procedure using Scheffé's test was adopted for multiple comparisons between groups where appropriate. In some experiments with incomplete factorial designs, Fisher's exact probability test was used for analysis. $P<0.05$ was considered statistically significant.

Results

Optimization of protocols for parthenogenetic activation

For precise assessment of the developmental ability of vitrified oocytes *in vitro*, we first optimized the protocols for parthenogenetic activation using freshly retrieved oocytes from different strains of mice. The oocytes from the B6D2F1 mice exhibited high rates of development into blastocysts even after a minimal activation stimulus (2.5 mM $SrCl_2$ for 0.5 h) without diploidization using cytochalasin (Fig. 1). Therefore, we applied this activation protocol to the evaluation in the subsequent experiments using B6D2F1 oocytes. By contrast, very few (0–19%) oocytes from the inbred strains developed into blastocysts after the same activation stimulus or stronger activation treatments, up to 10 mM $SrCl_2$ for 2.5 h. Therefore, we activated these oocytes in the presence of cytochalasin to produce diploid parthenogenetic embryos while applying the maximum activation stimulus (10 mM $SrCl_2$ for 2.5 h). We found that these diploid parthenogenetic embryos developed into

blastocysts at higher rates in all strains, ranging between 41 and 79% (incorporated into Table 1 as "fresh oocytes").

Development of vitrified oocytes after parthenogenetic activation

To see the efficiency of our oocyte vitrification protocol, we examined the rates of survival after warming and development into blastocysts after parthenogenetic activation. We also examined whether the equilibration time before vitrification might affect these rates. In all strains tested, blastocysts were obtained from vitrified oocytes at rates between 21 and 68% (Table 1). However, there was a strain-dependent difference in sensitivity to vitrification; BALB/cA strain oocytes had significantly less ability to develop into blastocysts than the corresponding non-vitrified control oocytes (Table 1). The equilibration time had no significant effects on survival or *in vitro* development except for the C3H/HeN strain (Table 1).

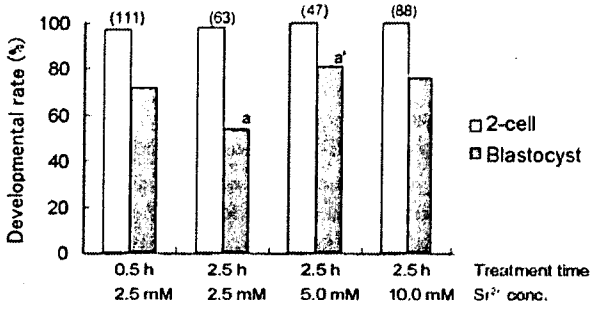

Fig. 1. Development of haploid parthenogenetic B6D2F1 embryos produced by different activation treatments with $SrCl_2$ (varied exposure time and concentration). Each group consisted of 47–111 oocytes (numbers in parentheses). $^{a, a'}P>0.05$ (Fisher's exact probability test).

Development of vitrified oocytes following ICSI

To test whether the vitrified oocytes from the different strains of mice retained their ability to develop into offspring, we undertook ICSI experiments using epididymal spermatozoa obtained from male mice of the same strain. The oocytes used for the ICSI experiments were vitrified and warmed as above, with a constant equilibration time (1 min). Most oocytes survived injection (>75%) and developed to the 2-cell stage (>85%) irrespective of the strain used. After embryo trans-

Table 1. Survival and development of vitrified oocytes from different strains of mice following parthenogenetic activation

Strain	Equilibration time before vitrification	No. of oocytes retrieved	% Survival	No. of oocytes cultured	% Developed to 2-cell	% Developed to Blastocyst
B6D2F1	1 min	89	99 1	88	92 4	60 13
(Haploid)	3 min	72	99 6	69	91 4	58 25
	5 min	60	100 0	60	92 2	62 8
	Fresh			111	97 1	72 13
C57BL/6J	1 min	125	98 1	116	99 1	44 8
(Diploid)	3 min	101	98 1	95	99 1	44 6
	5 min	118	97 2	108	95 4	43 4
	Fresh			119	96 1	47 9
DBA/2J	1 min	152	93 9	111	88 4	21 5
(Diploid)	3 min	178	100 0	138	92 3	23 5
	5 min	140	99 1	97	89 4	27 6
	Fresh			167	97 2	41 6
C3H/HeN	1 min	100	99 1	65	92 1^a	58 7
(Diploid)	3 min	117	98 1	55	96 2^b	60 3^c
	5 min	149	99 1	79	100 0^b	68 7
	Fresh			180	99 0^b	79 7^d
BALB/cA	1 min	98	100 0	79	63 12	23 7^e
(Diploid)	3 min	103	100 0	76	89 4	21 5^e
	5 min	98	100 0	80	73 9	21 6^e
	Fresh			151	84 4	41 6^f

Summarized from three to nine replicated experiments. Diploid parthenogenetic embryos were generated in the four inbred strains by cytochalasin treatment at the time of activation. The values are percents SE. $^{a, b; c, d; e, f}P<0.05$ (One-way ANOVA within each strain).

Table 2. *In vivo* development of embryos following ICSI using vitrified or fresh oocytes in different strains of mice

Strain	Oocyte	No. of embryos Transferred	No. (%) of embryos implanted	No. (%) of offspring
B6D2F1	Vitrified	58	33 (57)	15 (26)[a]
	Fresh	80	61 (76)	45 (56)[a']
C57BL/6J	Vitrified	48	15 (31)	7 (15)[b]
	Fresh	71	52 (71)	21 (30)[b']
DBA/2J	Vitrified	78	11 (14)	7 (9)
	Fresh	116	37 (32)	19 (16)
C3H/HeN	Vitrified	50	11 (22)	3 (6)[c]
	Fresh	82	40 (49)	15 (18)[c']
BALB/cA	Vitrified	76	18 (24)	4 (5)[d]
	Fresh	79	28 (35)	12 (15)[d']

Vitrified-warmed or fresh oocytes were injected with frozen-thawed spermatozoa from the homologous strain. [a, a']$P<0.001$ and [b, b'; c, c'; d, d']$P< 0.05$ (Fisher's exact probability test).

Fig. 2. BALB/cA pups born after ICSI using vitrified oocytes and frozen spermatozoa. The pups looked normal and recovered active movement shortly after delivery by Caesarian section.

fer, we obtained normal pups from all strains examined (Table 2 and Fig. 2). When compared with the birth rates from the corresponding experiments using fresh oocytes, the birth rates from in experiments using vitrified oocytes were about half or one-third lower (statistically significant in four strains).

Discussion

This study shows that mouse oocytes from different strains can be safely cryopreserved by vitrification protocols using a minimal volume carrier (Cryotop). Oocytes vitrified and then warmed developed into blastocysts *in vitro* following parthenogenetic activation, although the rates were slightly or significantly lower than those from fresh oocytes. Since the first successes of mammalian embryo cryopreservation, slow freezing and vitrification have been the two major technologies for preservation of mammalian genetic resources. Vitrification was first introduced for the cryopreservation of mouse embryos in 1985 [7]. The benefits of vitrification are the rapidity, simplicity and inexpensiveness of the technique; however, the cryoprotectants used are usually more embryotoxic than those used for slow freezing. To circumvent this problem, considerable efforts have been made to determine less toxic cryoprotectants. One of the candidate agents was ethylene glycol, which was first successfully introduced by Kasai *et al.* for mouse embryos [25] and then by Lane *et al.* for hamster embryos [19]. In the present study, we also employed an ethylene glycol-based cryoprotectant solution and found it to be very effective for vitrification of mouse oocytes.

Another problem inherent to vitrification is the requirement of very rapid cooling [26]. Theoretically, this can be achieved by employing containers that carry a very small volume of cryoprotectant solution. In our preliminary experiments, Cryotops gave the best oocyte survival and subsequent development among the containers tested (cryotubes, Cryotops and straws), probably because of the small volume they carry, which produces the highest cooling and warming rates. Different carrier tools have also been applied successfully to minimize the volume of the cryoprotectant solution, including open pulled straws and cryoloops

(for review, see [17]). Rabbit [27], bovine [28, 29] and human [30] oocytes have been cryopreserved using both methods, and healthy offspring have been reported following embryo transfer in the latter two species [29, 30].

In addition to the composition of the cryoprotectant solution and the type of container, several factors may affect the viability of vitrified oocytes. Permeation by a cryoprotectant is essential to prevent intracellular ice crystal formation, but long exposure to a cryoprotectant increases toxic damage to oocytes. In this study, therefore, we tested whether the pretreatment (or equilibration) time before cooling might affect the viability and developmental ability of oocytes from different strains of mice. However, as far as we could determine, equilibration times between 1 min and 5 min had no significant effects on these parameters. Nevertheless, since the results were very consistent within each strain, this may indicate that the oocyte cryopreservation protocol can be optimized based on the parthenogenetic development of oocytes. Assessment of the developmental ability of vitrified oocytes by conventional *in vitro* fertilization (IVF) is difficult because IVF rates can vary greatly vary in the mouse when their oocytes are freed from cumulus cells and frozen-thawed [12].

As expected from previous embryo culture and ICSI studies [31], the oocytes from F1 hybrids exhibited the highest tolerance to damage associated with vitrification, irrespective of the experimental conditions employed. Even being haploid, most of these vitrified and activated oocytes developed into blastocysts. In contrast, oocytes from the inbred strains of mice seemed more sensitive to the vitrification procedure, with lower developmental rates than the non-vitrified controls. However, we found that at least 40% of these diploid parthenogenetic embryos developed into blastocysts, although there was an apparent strain-dependent difference in their *in vitro* developmental ability.

In the final part of this study, we undertook ICSI experiments to determine whether the vitrified oocytes retained the ability to support full-term development. As mentioned above, *in vitro* development of parthenogenetic embryos is a good indicator of the viability of vitrified oocytes. However, oocytes are generally prone to disruption of the metaphase spindle during cooling, which may lead to chromosomal segregation aberrations and aneuploidy in embryos, although such disruption is more reversible in mice than in bovine or human oocytes [32–34]. According to previous studies, embryonic aneuploidy does not always compromise preimplantation development, but does impair postimplantation development [35, 36]. Therefore, ICSI followed by embryo transfer may provide accurate information concerning the quality of genomic and cytological integrity after oocyte cryopreservation. Our ICSI experiments clearly indicated that at least some of the vitrified oocytes were genetically normal for all strains examined. However, the efficiencies of producing ICSI offspring in our study were two to three times lower than those from ICSI using non-vitrified oocytes (Table 2), and no correlations were found between the results of parthenogenetic development and ICSI. The possibility of cytogenetic errors causing this decreased efficiency should be clarified by detailed chromosomal examinations.

The strains we used included C57BL/6, DBA/2, BALB/c and C3H/He mice. These strains are widely used as genetic backgrounds for recombinant inbred or congenic strains and for genetically modified strains (see RIKEN Bioresource Center, http://www.brc.riken.go.jp/lab/animal/en/). Our vitrification protocol for mature oocytes of different strains, combined with the ICSI technique, may enable preservation of genetically important stocks of mice using a reliable, convenient and space-saving method.

Acknowledgments

This work was supported by grants from the Ministry of Education, Culture, Sports, Science and Technology of Japan, Ministry of Health, Labor and Welfare of Japan and CREST of the Japan Science and Technology Agency to A. O. and by the Promotion and Mutual Aid Corporation for Private Schools of Japan and a Matching Fund Subsidy for Private Universities to N. K.

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