Establishment of stem cell lines from nuclear transferred and parthenogenetically activated mouse oocytes for therapeutic cloning

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Objective: To establish embryonic stem cell lines from nuclear transfer of somatic cell nuclei isolated from the same oocyte donor and from parthenogenetic activation. The study also evaluated the effect of the micromanipulation procedure on the outcome of somatic cell nuclear transfer in mice.
Design: Randomized, prospective study.
Setting: Hospital-based assisted reproductive technology laboratory.
Animal(s): F(1) (C57BL/6 × 129P3/J) mice.
Intervention(s): Metaphase II–stage oocytes were either parthenogenetically activated or nuclear transferred with cumulus cell nuclei or parthenogenetically activated after a sham-manipulation procedure.
Main Outcome Measure(s): Embryogenesis and embryonic stem cell establishment.
Result(s): The development rate to morula/blastocyst of nuclear transferred oocytes (27.9% ± 5.9%) was significantly lower than that of the sham-manipulated (84.1% ± 5.6%) or parthenogenetic (98.6% ± 1.4%) groups. A sharp decrease in cleavage potential was obvious in the two- to four-cell transition for the nuclear transferred embryos (79.0% ± 4.6% and 43.3% ± 5.0%), implying incomplete nuclear reprogramming in arrested oocytes. However, the cleavage, as well as the development rate, of parthenogenetic and sham-manipulated groups did not differ significantly. The embryonic stem cell line establishment rate was higher from parthenogenetically activated oocytes (15.7%) than nuclear transferred (4.3%) or sham-manipulated oocytes (12.5%). Cell colonies from all groups displayed typical morphology of mice embryonic stem cells and could be maintained successfully with undifferentiated morphology after continuous proliferation for more than 120 passages still maintaining normal karyotype. All these cells were positive for mice embryonic stem cell markers such as Oct-4 and SSEA-1 based on immunocytochemistry and reverse transcriptase–polymerase chain reaction. The clonal origin of the ntES cell line and the parthenogenetic embryonic stem cell lines were confirmed by polymerase chain reaction analysis of the polymorphic markers. Blastocyst injection experiments demonstrated that these lines contributed to resulting chimeras and are germ-line competent.
Conclusion(s): We report the establishment of ntES cell lines from somatic cells isolated from same individual. Our data also suggest that embryo micromanipulation procedure during the nuclear transfer procedure influences the developmental ability and embryonic stem cell establishment rate of nuclear transferred embryos. (Fertil Steril® 2008;89:1314–23. ©2008 by American Society for Reproductive Medicine.)

Key Words: Nuclear transfer, embryonic stem cell, parthenogenetic activation, mouse, chimera

Production of pluripotent stem cells by therapeutic cloning has potential biomedical applications in the treatment of infertility and degenerative diseases in humans (1). However, research attempts to establish human stem cell lines have been hindered severely by religious and ethical considerations. Mice represent a premier model organism for the study of human diseases and development because of their defined genetic background, abundant genetic information, and short gestation period and life span. Several people have produced stem cell lines from nuclear transfer–derived cloned embryos and demonstrated their utility in therapeutic cloning. Successful implementation of these technologies in humans, however, requires avoidance of ethical problems and probable immunorejection after in vivo cell transplantation, which can be achieved by production of stem cells from cloned embryos nuclear transferred with somatic cells (ntES) of the same patient or via parthenogenetic activation of the patient's own oocyte (2). However, to our knowledge, no one has reported the production of ntES from cloned embryos nuclear transferred with somatic cells derived from the same oocyte donor.

Currently, the generation of adult animals by somatic cell nuclear transfer is extremely inefficient, with most clones

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dying soon after implantation or having serious phenotypic and gene expression abnormalities. Aberrant reprogramming of the somatic nuclei in the enucleated oocytes and altered gene expression patterns of the cloned preimplantation embryos have been suggested as the possible cause of lower efficiency of somatic cell nuclear transfer technology (3, 4). These conclusions were drawn mainly by comparison with in vivo or in vitro–fertilized embryos. The differences observed in gene expression in embryos however, do not necessarily reflect the potential of cloned embryos to develop into young (5). We hypothesize that subtle damages and stress to cytoplast and/or donor cells during the embryo micromanipulation procedure may also have a long-term effect on the lower developmental ability and offspring production rate of nuclear transferred embryos. However, this factor has had less attention paid to it in the literature. Furthermore, nuclear transferred embryos have similar potential to develop into blastocysts as in vitro–fertilized embryos (6). Therefore, it is important to determine what occurs after the blastocyst stage in cloned embryos. For this, production and analysis of the ntES cells derived from cloned embryos of different developmental potential could be used as a valuable tool for studying the dynamics of genetic and epigenetic changes during nuclear reprogramming of cloned embryos.

Therefore, this study was designed to establish embryonic stem cell lines from nuclear transfer of somatic cell nuclei isolated from the same oocyte donor and from parthenogenetically activated oocytes. We also analyzed the effect of embryo manipulation during the nuclear transfer procedure on preimplantation embryo development and evaluated these blastocysts for their ability to form embryonic stem cell lines.

MATERIALS AND METHODS

Experimental Design

Oocytes were subjected to one of the three experimental groups: [1] pathenogenetic activation, [2] nuclear transferred, and [3] sham manipulation. The oocytes in the sham-manipulated group were subjected to the sham enucleation of cytoplasm without removal of the metaphase plate and were parthenogenetically activated similar to the nuclear transferred group after sham injection of manipulation medium.

Animals

The 129B6F1 (C57BL/6 × 129P3/J) hybrid female mice aged between 7 and 12 weeks were used as a source of oocytes and cumulus nuclear donor cells. Animals were bred and maintained according to national standards for laboratory animal rearing in Korea. All animal experiments were approved and performed under the guidelines of the Konkuk University Animal Care and Experimentation Committee.

Culture Conditions

All reagents used for the preparation of media were from Sigma-Aldrich (St. Louis, MO) unless stated otherwise. Oocytes and embryos were cultured in M16 medium (7) supplemented with 4 mg/mL bovine serum albumin at 37°C under 5% CO_2 in air. Handling of donor cells and recipient oocytes was carried out in M2 medium at room temperature (25°C–29°C). Ca^{2+}- free M16 containing 10 mmol/L $SrCl_2$ (8) and 5 μg/mL cytochalasin B was used for parthenogenetic activation of oocytes and reconstructed embryos.

Isolation of Donor Cells and Oocytes

Mature 129B6F1 female mice were induced to superovulate by consecutive injections of 5 IU pregnant mare serum gonadotropin (Sigma) and 5 IU hCG (Follimon) 48 hours apart. Between 13 and 14 hours after hCG injection, cumulus-oocyte complexes were recovered from the oviduct, and cumulus cells were removed from the metaphase II–arrested oocytes by treatment with 0.1% (wt/vol) bovine testicular hyaluronidase (300 USP units/mg) in M2 medium. After rinsing several times, apparently normal oocytes showing clear cytoplasm with uniform texture, homogeneous fine granularity, and intact first polar body were selected and kept in an incubator (37°, 5% CO_2 in air) until further processing. The cumulus cells that were to serve as donors of nuclei were washed and suspended in M2 medium containing 10% (wt/vol) polyvinylpyrrolidone (mol wt: 360,000) and kept at room temperature for up to 2 to 3 hours until injection. Both oocytes and cumulus cells isolated from an individual animal were identified and kept separately from those of the other animals.

Oocyte Enucleation and Sham Enucleation

Denuded oocytes were transferred to a droplet of M2 medium supplemented with 5 μg/mL cytochalasin B to minimize oocyte damage and were placed on the microscope stage set to 32° temperature. The oocyte was positioned in the holding pipette (10–15 μm inner diameter) so that the metaphase II chromosome-spindle complex, visualized as a translucent region in the oocyte's cytoplasm under the inverted microscope equipped with differential interference contrast optics, was at the 3 o'clock position. Several piezo pulses were used to drill a small hole in the zona, and the pipette was pushed forward through the hole to touch the spindle. The microinjector was then controlled carefully to exert a negative suction to pinch off the spindle with minimal surrounding ooplasm (9). For sham enucleation, instead of removing the spindle complex, a small volume of ooplasm was removed by using the same procedure. Immediately after enucleation, oocytes were placed in droplets of cytochalasin B–free M16 medium overlaid with warm mineral oil and transferred to the incubator (5% CO_2 in air) at 37° for up to 2 to 3 hours until nucleus injection (10).

Injection of Donor Cell Nuclei Into Enucleated Oocytes and Sham Injection

The injection procedure was similar to that of Kimura and Yanagimachi (11) with partial modifications. The whole

procedure of injection was carried out at room temperature of 25°C to 29°C. Middle-sized cumulus cells (9–13 μm [12]), isolated from the same animal from which oocytes were recovered, were picked individually and aspirated in and out of the injection pipette (inner diameter 5 μm) until the cell's plasma membrane was broken, as evidenced by cytoplasmic dispersal around the needle tip. Then a deep invagination of the oolemma was made with the injection pipette, and the oolemma was broken by gentle suction of the ooplasm before injecting the isolated nucleus into the enucleated oocyte (Fig. 1). Care was taken to keep the injection of manipulation medium to a minimum. In the sham-enucleated oocytes, a minimal amount of injection medium was injected without the nuclei. The whole injection procedure was completed within 15 minutes, after which reconstructed oocytes were washed and cultured in droplets of M16 medium under mineral oil in 5% CO_2 at 37° up to 1 to 2 hours until activation.

Oocyte Activation and In Vitro Culture

For activation, injected oocytes were cultured for 5 to 6 hours in Ca^{2+} -free M16 supplemented with 10 mmol/L $SrCl_2$ to induce activation (8) and 5 μg/mL cytochalasin B to inhibit cytokinesis and extrusion of chromosomes in the form of pseudo-second polar body (12, 13). After activation, embryos with at least one pseudopronucleus were considered activated and were further cultured in droplets of G1.3 medium (Vitrolife, Gothenburg, Sweden) for 2 days and subsequently in G2.3 medium (Vitrolife, Gothenburg, Sweden) under mineral oil in 5% CO_2 at 37°. Embryos were evaluated for cleavages at 24 hours (two cell), 48 hours (four cell), and 72 hours (morula/blastocyst) after activation.

Embryonic Stem Cell Isolation and Maintenance in Culture

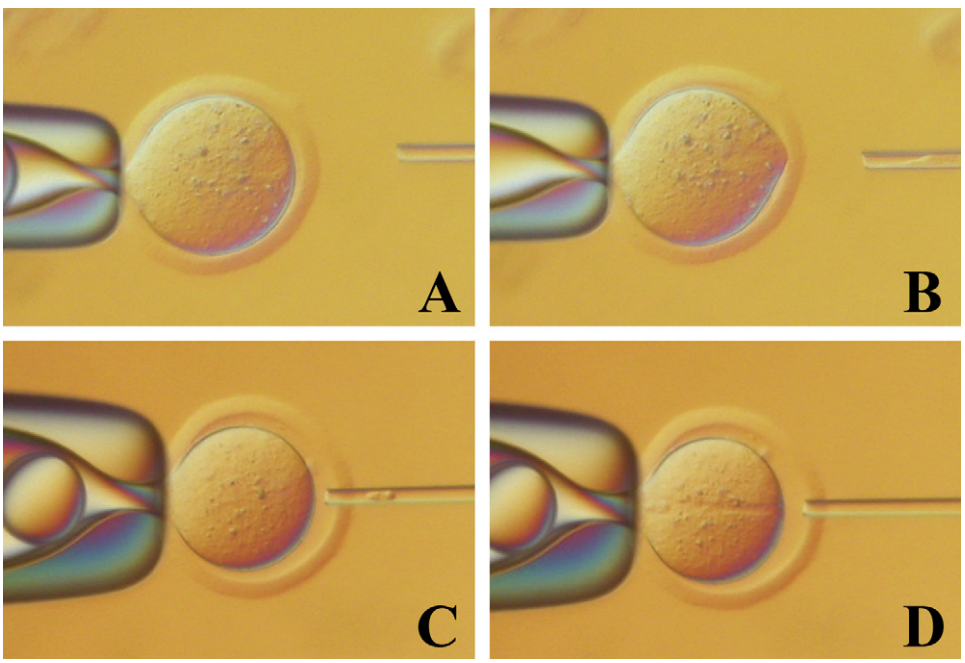
The inner cell mass was isolated by mechanical dissociation and plated on mitomycin C–treated STO cells. Culture medium consisted of knockout Dulbecco's minimum essential medium (GIBCO BRL, Burlington, Ontario, Canada) supplemented with 15% fetal calf serum (Hyclone, Logan, UT), 1,000 units of leukemia inhibitory factor (Chemicon, Temecula, CA), 1% penicillin-streptomycin, 1% L-glutamine, and 1% nonessential amino acids. After 5 to 7 days of culture, inner cell mass–derived outgrowths were disaggregated and reseeded on nonproliferative mitomycin C–treated STO cells in fresh medium. To passage the putative embryonic stem cells, we selected, dissociated, and replated individual colonies with a uniform undifferentiated morphology. Cells were subcultured every 48 hours on a feeder layer of mitomycin C–treated STO cells.

Alkaline Phosphatase Activity

For assessing the alkaline phosphatase activity in the cytoplasm of putative embryonic stem cells, the cells were fixed with 4% paraformaldehyde and stained histochemically

FIGURE 1

Manipulation of mice oocytes. Note the cytoplasmic characteristics and the position of the pipettes in relation to the chromosome-spindle complex at pre-enucleation (**A**), postenucleation (**B**), preinjection (**C**), and postinjection of the donor cell nuclei (**D**).



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with use of an alkaline phosphatase staining kit (Vector Laboratories, Burlingame, CA) following the manufacturer's protocol. Alkaline phosphatase activity was estimated by visual analysis of the stained cells.

Immunocytochemical Staining for the Embryonic Stem Cell–Specific Marker

Immunocytochemical analysis for embryonic stem cell–specific markers was performed essentially as described by Sutherland et al. (14). Cells were fixed with ethanol and incubated with appropriate dilutions of mouse monoclonal antibodies against mouse stage specific embryonic antigen 1 (SSEA-1, catalog No. sc-21702; Santa Cruz Biotechnology, Santa Cruz, CA), laminin, or Oct-3/4 (catalog No. sc-5279; Santa Cruz Biotechnology). Immunoglobulin M (Chemicon, Temecula, CA) and rabbit IgG were used as negative controls. Primary antibodies were localized with biotinylated secondary antibody and an avidin and horseradish peroxidase conjugated complex with use of a Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA).

Deoxyribonucleic Acid Typing

Polymorphisms for microsatellite markers were investigated by the simple sequence length polymorphism method. Polymerase chain reaction (PCR) amplification of the microsatellite markers D1Mit362, D7Mit44, and DXMit136 was performed by using primer pairs synthesized according to the Mouse Genome Database (Table 1). Deoxyribonucleic acids were extracted from embryonic stem colonies, STO cells, and tail tip of C57BL/6, 129B6F1 hybrid, 129P3/J, and BALB/c mouse strains.

Karyotyping for Ploidy Evaluation

For evaluating the ploidy, karyotyping of the cultured embryonic stem cells was performed as described by Nagy et al. (15) and was examined by phase-contrast microscopy. For the karyotype determination, 100 metaphase plates were counted for chromosome number, and five metaphase spreads were analyzed each time.

Production of Chimera

Host embryos at the two-cell stage were recovered from 129P3/J or C57BL/6 females mated with each 129P3/J or C57BL/6 male and cultured in vitro up to the blastocyst stage. Just before blastocyst injection, embryonic stem cells at 50 to 55 passage were trypsinized for 5 minutes, harvested from the supernatant by centrifugation, and resuspended in M2 medium. A blunt-tip microinjection pipette with an inner diameter of 10 to 15 μm was used for embryonic stem cell injection. Up to 10 to 15 embryonic stem cells were picked at the tip of the injection pipette and were injected into the cavity of the blastocyst by piezoactuated microinjection. After injection, the chimeric blastocysts were transferred to the uterine horn of a 2.5-day–postcoital pseudopregnant BALB/c female.

Statistical Analysis

Statistical analysis was carried out with SAS software (Statistical Analysis System Inc., Cary, NC). Data were analyzed by analysis of variance to determine statistical differences among the development rates and by χ^2 test to determine the differences among embryonic stem cell establishment rates. Data are presented as mean $\pm$ SEM. Differences at $P \leq .05$ were considered significant.

RESULTS

Developmental Rate of Parthenogenetic, Nuclear Transferred, and Sham-Manipulated Oocytes

The data on the developmental ability of parthenogenetic, nuclear transferred, and sham-manipulated oocytes are

TABLE 1

Details of primers used for PCR analysis.

Gene name	Primer sequence (5′ to 3′)	Accession No.	Amplicon size
D1Mit362	TGTGTGACTGCTTGGAAGATG CTGAGTCCCTAAAGTTGTCCTTG	MGI704467	120, 148
D7Mit44	TTCTGGCCTCTGTGAAGTAGTG GTGAAAACCATGGTGCAGATG	MGI703938	170, 190
DXMit136	ACGGAAACACTCTTATGTGCG ATTTTGATTACAGCATGTCCCC	MGI707019	186, 194
Oct-4 (1)	GGCGTTCTCTTTGCAAAGGTGTTC CTCGAACCACATCCTTCTCT	GenBank BC068268	312
Oct-4 (2)	GATGGCATACTGTGGACCTCAG CAGATGGTGGTC	GenBank BC068268	333
β-Actin	GGCCCAGAGCAAGAGAGGTATCC ACGCACGATTTCCCTCTCAGC	GenBank NM007393	460

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summarized in Table 2 and Figure 2. A mean of 61.9% ± 6.0% and 100% ± 0 of mice oocytes survived the manipulation in the nuclear transferred group and the sham-manipulation group, respectively. Oocytes nuclear transferred with a cumulus cell nuclei showed a significantly lower morula/blastocyst development rate of 27.9% ± 5.9% against 84.1% ± 5.6% in the sham-manipulated group and 98.6% ± 1.6% in the parthenogenetically activated group ($P<.05$). The morula/blastocyst rate did not differ between the sham-manipulated and the parthenogenetically activated group ($P<.05$). Only 79.0% ± 4.6% and 43.3% ± 5.0% of the nuclear transferred oocytes developed to the two- and four-cell stage, respectively, which is significantly lower ($P<.05$) than the percentage of the sham-manipulated (95.0% ± 3.8% and 93.3% ± 3.7%) or parthenogenetically activated oocytes (100.0% ± 0 and 100.0% ± 0). The same trend was observed with the formation of the pseudopronuclear formation in the parthenogenetically activated, nuclear transferred, and sham-manipulated group (98.6% ± 1.4%, 62.8% ± 8.9%, 93.8% ± 2.9%). No significant difference was observed between the sham-manipulated and parthenogenetically activated groups. Interestingly, a sharp decrease in cleavage potential became obvious in the two- to four-cell transition for the nuclear transferred embryos.

Establishment and Characterization of Embryonic Stem Cell Lines

The embryonic stem cell line establishment rates were 15.7%, 4.3%, and 12.5% for the parthenogenetically activated, nuclear transferred, and sham-manipulated groups. The success rate of the established embryonic stem cells was lower for the nuclear transferred group than for the parthenogenetically activated and sham-manipulated groups (Table 3). The colonies of embryonic stem cells were morphologically similar to the typical mice embryonic stem cells. The embryonic stem cells grew as typical round-shaped colonies with a smooth surface and a distinct colony boundary from the surrounding feeder layers (Fig. 2D). To investigate the pluripotency of embryonic stem cell lines, we immunohistochemically analyzed markers that characterize undifferentiated mice embryonic stem cells, including alkaline phosphatase activity, SSEA-1, and Oct-4 expression. This revealed that these putative embryonic stem cells were similar to the established mice embryonic stem cells, not only in morphology but also in the expression of specific cell markers for totipotency. The clumps stained positive for alkaline phosphatase activity and were presumably undifferentiated (Fig. 3A). Also, the feeder cells showed no signal for either SSEA-1 or Oct-4, but the embryonic stem cells showed a high expression (Fig. 3B and 3C). As shown in Figure 4, we performed reverse transcriptate–PCR assay to analyze the expression patterns of Oct-4 in cumulus clone-derived embryonic stem cells and parthenogenetic embryonic stem cells that were positive for the expression of Oct-4. This activity of Oct-4 (312 and 333 base pairs [bp]) was found in the undifferentiated embryonic stem cells in mammals.

TABLE 2

Development rate of mouse oocytes after parthenogenesis, nuclear transfer, or parthenogenetic activation after sham-manipulation.

Groups	No. of oocytes used	Percentage of oocytes survived							
		Postenucleation	Postinjection	PN formation	Two-cell	Four-cell	Eight-cell	Morula and blastocyst
PA	124	NA	NA	98.6[a] ± 1.4 (121)	100.0[a] ± 0.0 (121)	100.0[a] ± 0.0 (121)	98.6[a] ± 1.4 (118)	98.6[a] ± 1.4 (118)
SA	106	100.0[a] ± 0.0 (106)	NA	93.8[a] ± 2.9 (98)	95.0[a] ± 3.8 (94)	93.3[a] ± 3.7 (91)	92.8[a] ± 3.8 (90)	84.1[a] ± 5.6 (82)
NT	516	91.2[a] ± 0.9 (468)	61.9 ± 6.0 (289)	62.8[b] ± 8.9 (189)	79.0[b] ± 4.6 (144)	43.3[b] ± 5.0 (70)	32.0[b] ± 5.9 (50)	27.9[b] ± 5.9 (45)

Note: PA = parthenogenesis; SA = parthenogenetic activation after sham manipulation; NT = nuclear transfer; NA = not applicable; PN = pronucleus. Values in parentheses indicate the actual numbers. Experiments were replicated four, four, and eight times for PA, SA, and NT groups, respectively. Development rates (mean ± SEM) were calculated from the number of embryos that formed pronucleus (PN formation).

[a] $P<.05$ compared with values with different superscripts within column.

[b] $P<.05$ compared with values with different superscripts within column.

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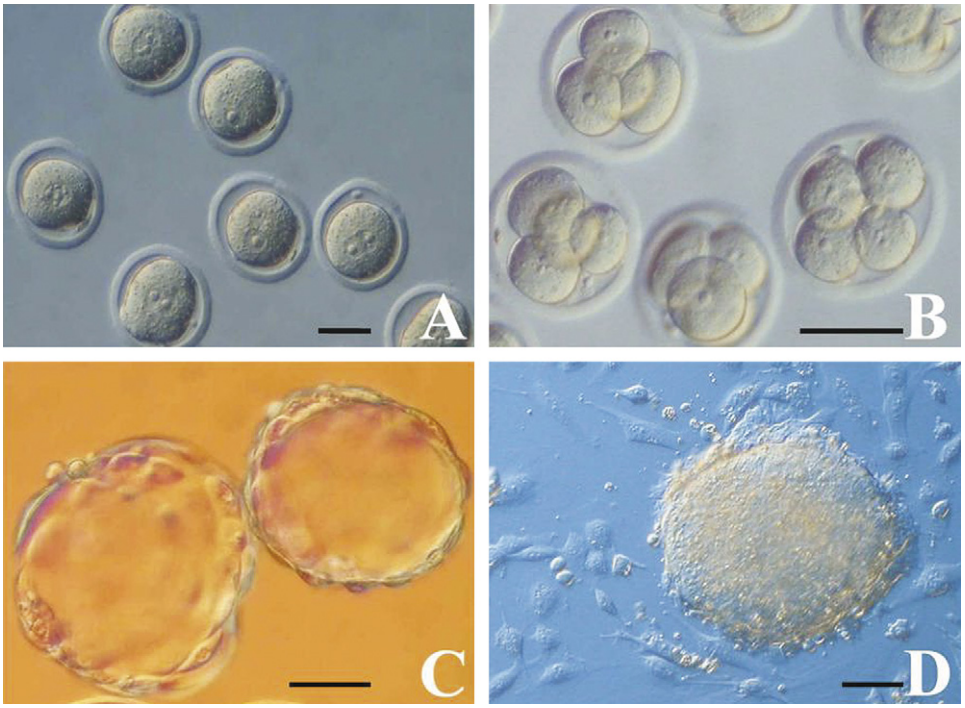
FIGURE 2

Morphology of mice embryos and ntES cells produced by nuclear transfer of cumulus cell nuclei. **(A)** Pseudopronucleus formation, **(B)** four-cell stage, **(C)** blastocyst, **(D)** ntES cell colony. Bar = 50 μm.



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Genotype Analysis of the Pluripotent Embryonic Stem Cells

Clonal origin of ntES cell lines was examined by PCR analysis of polymorphic markers, namely, D1Mit362, D7Mit44, and DXMit136. Genomic analysis of genomic DNA of ntES cells (Fig. 5A, lane 4) showed sharp discrete bands of 148 bp and 120 bp similar to those of 129B6F1 hybrid (Fig. 5A, lane 2), whereas C57BL/6 (Fig. 5A, lane 1), STO cells (Fig. 5A, lane 5), and BALB/c (Fig. 5A, lane 6) showed bands of 120 bp only and 129P3/J (Fig. 5A, lane 3) showed a band of 148 bp only. A similar observation was made in the case of D7Mit44 (Fig. 5B) and DXMit136 (Fig. 5C). The band pattern of ntES cells (lane 4) corresponded to those of 129B6F1 hybrid (lane 2), confirming their clonality.

Karyotype of the Pluripotent Embryonic Stem Cells

Because defective mitotic spindles after somatic cell nuclear transfer can result from the depletion of microtubule motor

TABLE 3

Establishment rate of embryonic stem cells from mice oocytes after parthenogenesis, nuclear transfer, or parthenogenetic activation after sham manipulation.

Groups	No. of zygotes	Percentage of blastocysts formed	Percentage of ES cell attached	Percentage of ES cell lines established
PA	108	97.2[a] (105)	25.9[a] (28)	15.7[a] (17)
SA	8	87.5[a] (7)	12.5[b] (1)	12.5[b] (1)
NT	47	25.5[b] (12)	10.6[b] (5)	4.3[c] (2)

Note: PA = parthenogenesis; SA = parthenogenetic activation after sham manipulation; NT = nuclear transfer. Percentages were calculated from number of zygotes. Values in parentheses indicate the actual numbers.

[a] $P<.05$ compared with values with different superscripts within column.

[b] $P<.05$ compared with values with different superscripts within column.

[c] $P<.05$ compared with values with different superscripts within column.

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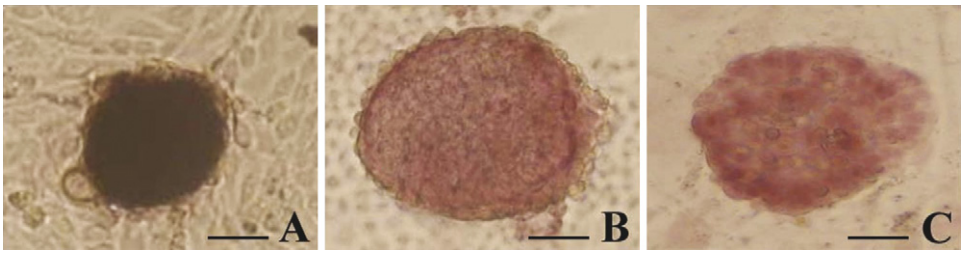
FIGURE 3

Characterization of embryonic stem cells for embryonic stem cell markers. Both parthenogenetic and ntES cells showed strong alkaline phosphase activity (**A**) and expressed epitopes that are recognized by antibodies against SSEA-1 (**B**) and Oct-4 (**C**). Bar = 50 μm.



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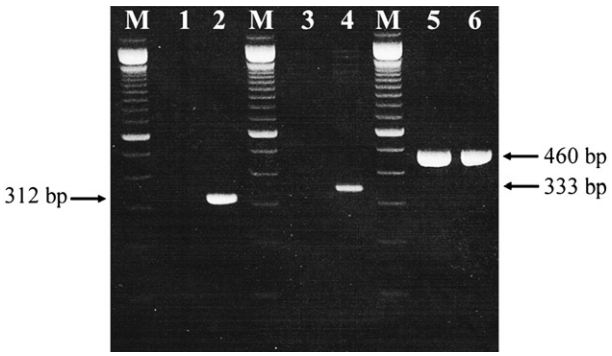
and centrosome proteins lost to the meiotic spindle after the enucleation or during the subsequent passage of embryonic stem cells, we examined the karyotype of the pluripotent embryonic stem cells derived from parthenogenetic, nuclear transferred, and sham-manipulated groups to rule out aneuploidy. The karyotyping results indicated a modal chromosome count of 40 with normal female mouse cells (XX) in accordance with their donor cell or oocyte origin. All embryonic stem cell lines maintained normal karyotypes (Fig. 6).

Chimera Production

The data of chimera production from injection of embryonic stem cells into the blastocyst are presented in Table 4. Of the injected blastocysts that were carried to term, 4 of 10 pups born from the ntES cell line were chimeras and 3 of the chimeras were male, whereas 1 of 7 pups born from the parthenogenetic embryonic stem cell line was a chimera and was male. All chimeras except one produced from blastocyst injections showed >50% donor contribution to coat color (Fig. 7). Interestingly, each chimera had one eye red colored whereas the other was black (Fig. 7A). This finding supports the developmental capacity of ntES and parthenogenetic cell lines.

FIGURE 4

Reverse transcriptase–PCR analysis of STO (*lanes 1* and *3*) and embryonic stem cells produced by nuclear transfer (*lanes 2* and *4*) for the expression of Oct-4. Two sets of Oct-4 primers were used (see Table 1 for details). *Lanes 1* and *2*: Oct-4 primer set 1; *lanes 3* and *4*: Oct-4 primer set 2; *lanes 5* and *6*: β-actin.



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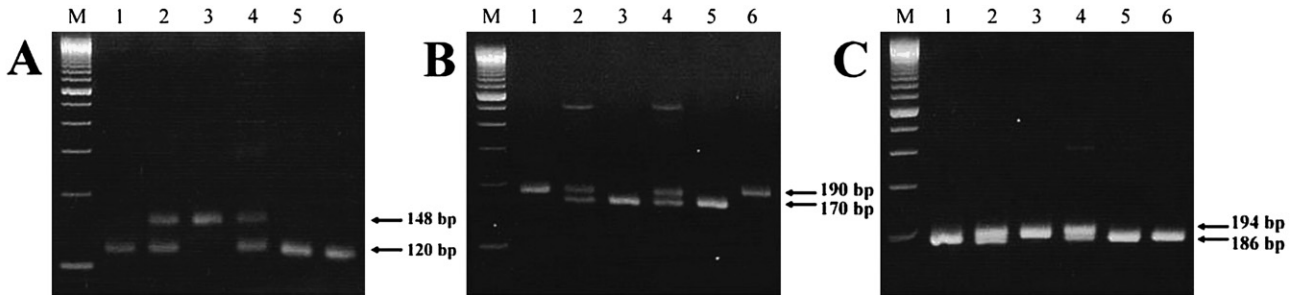
DISCUSSION

This study established embryonic stem cell lines from nuclear transfer of somatic cell nuclei isolated from the same oocyte donor and parthenogenetically activated oocytes for therapeutic cloning. We also analyzed the effect of embryo manipulation during the nuclear transfer procedure on preimplantation embryo development and evaluated these blastocysts for their ability to form embryonic stem cell lines.

With the first report of a mouse ntES cell line in the year 2000, in which a single ntES cell line was developed (16), several reports have documented the establishment, characterization, and germ-line transmission of ntES cells (17–26). The ntES cell lines possessed the same capacities for unlimited differentiation and self-renewal as those of conventional embryonic stem cell lines derived from normal embryos produced by fertilization. However, for applying these technologies for therapeutic purposes in humans it is of the utmost importance to solve associated ethical problems and probable immunorejection after in vivo cell transplantation. Although Lanza et al. (27) have reported that cloned cells and tissues with allogenic mitochondrial DNA can be grafted back into the nuclear donor organism without destruction by the immune system, nonhistocompatibility because of maternally transmitted antigens arising from mitochondrial DNA still poses a threat of immunorejection (28). This best can be achieved by production of stem cells from cloned embryos nuclear transferred with somatic cells (ntES) of the same patient or via parthenogenetic activation of the patient's own oocyte (2). However, to our knowledge, there is no previous report on the production of ntES from cloned embryos nuclear transferred with somatic cells

FIGURE 5

Deoxyribonucleic acid typing for microsatellite markers (D1MIT362, D7MIT44, and DXMIT136) in genomic DNA from the tail tip of mouse strain C57BL/6 (*lane 1*), 129B6F1 (*lane 2*), 129P3/J (*lane 3*), BALB/c (*lane 6*), ntES cell line (*lane 4*), and STO cells (*lane 5*), thus confirming their clonal origin. The polymorphic markers D1MIT362 (**A**), D7MIT44 (**B**), and DXMIT136 (**C**) are conserved in genomic DNA from the 129B6F1 mouse and the ntES cell lines (*lanes 2* and *4*), but they differ from those of the STO cells, C57BL/6, 129P3/J, and the BALB/c mice.



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derived from the same oocyte donor. Furthermore, although the establishment of stem cell lines from metaphase II–stage oocytes by parthenogenetic activation had been studied and documented since 1983 in the mouse (29), the pluripotency of the cells and the efficacy of their derivatives have been examined poorly. In the present study, we established embryonic stem cell lines from blastocysts that were derived from nuclear transfer of somatic cells isolated from the same individual from which oocytes were retrieved and also from parthenogenetic activation of the oocytes. All established cell lines were positive for alkaline phosphatase staining, suggesting that these embryonic stem cell lines are pluripotent. The cell colonies displayed morphology similar to that reported previously for mouse embryonic stem cells. The

FIGURE 6

Karyotype analysis of embryonic stem cells produced by nuclear transfer or parthenogenetic activation. Karyotype showed the same normal diploid chromosomal complementation in both groups.

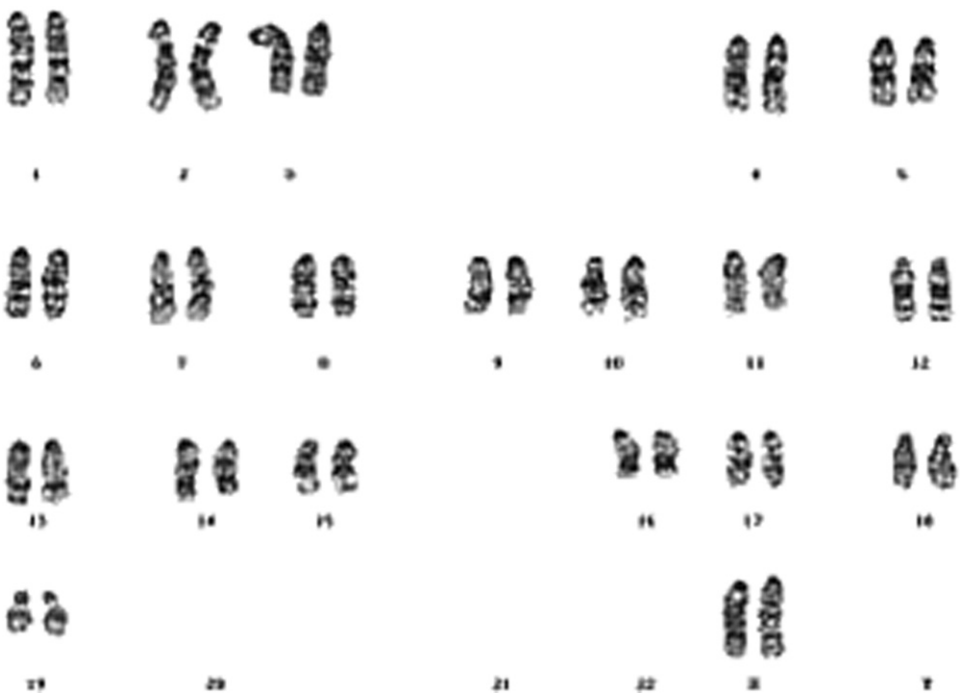

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TABLE 4

Production of chimeric mice by blastocyst injection of ES cells derived from nuclear transfer of somatic cell nuclear transfer or parthenogenetic activation.

Groups	No. of embryos transferred	No. (%) of neonates born	No. (%) of chimera	No. of males and females
NT	135	10 (7.4)	4 (40.0)	3 ♂ and 1 ♀
PA	24	7 (29.0)	1 (14.3)	1 ♂

Note: PA = parthenogenesis; NT = nuclear transfer.

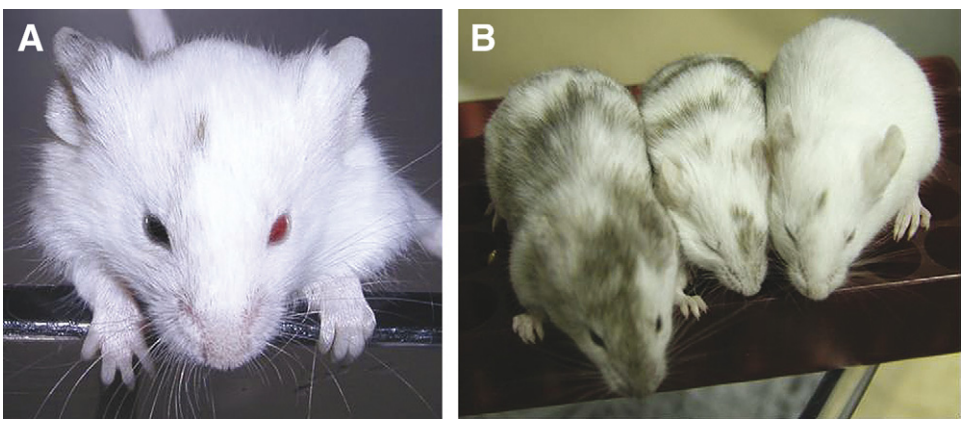
Ju. Establishment of mouse ntES cell line. Fertil Steril 2008.

cell line could be maintained successfully in undifferentiated morphology after continuous proliferation for more than 120 passages, yet maintaining normal karyotype. These cells also expressed mouse embryonic stem cell markers such as Oct-4 and SSEA-1. Reverse transcriptase–PCR further confirmed the Oct-4 expression in ntES cells. The clonal origin of ntES cell lines and parthenogenetic embryonic stem cells was confirmed by PCR analysis of polymorphic markers. Blastocyst injection experiments demonstrated that these lines contributed to resulting chimeras and are germ-line competent. The somatic cells thus have regained pluripotency because of nuclear reprogramming by the recipient cytoplast. These ntES cells with regained potential for development can now be used for therapeutic cloning purposes (30) besides eliminating the need for repeated nuclear remodeling of somatic cells from the donor to produce cloned mice. Thus, the present study documents that mouse embryonic stem cells can be obtained successfully after nuclear transfer of cumulus cells derived from the same oocyte donor, as well as by parthenogenetic activation.

We also analyzed the effect of embryo manipulation during the nuclear transferred procedure on preimplantation embryo development and evaluated these blastocysts for their ability to form embryonic stem cell lines. Our results show and confirm previous findings that blastocyst formation rates are consistently lower for nuclear transferred embryos than for parthenogenetically activated embryos. A sharp decrease in cleavage potential was observed particularly at two- to four-cell transition suggesting a failure of remodeling and reprogramming of foreign nuclei in the arrested oocytes. However, the pronuclear formation rate and development rate did not differ between the sham-manipulated and parthenogenetically activated groups. Furthermore, the embryonic stem cells isolated from all groups did not show any aberrancy in their establishment and maintenance, but the parthenogenetic group showed a higher rate of embryonic stem cell line establishment. The embryonic stem cells could also be established successfully from blastocysts produced by parthenogenetic activation of sham-manipulated oocytes with similar efficiency. This implies that oocyte manipulation and handling during nuclear transfer procedure has no major effect on the blastocyst formation rate but influences the potential of these blastocysts to retain their pluripotency. The lower development rate to morula/blastocyst in

FIGURE 7

Chimeras produced by injection of ntES cells into the blastocysts that were transferred to pseudopregnant BALB/c females. Note the eyes of different color in **A**.



Ju. Establishment of mouse ntES cell line. Fertil Steril 2008.

reconstructed embryos is probably a reflection of incomplete or inappropriate nuclear reprogramming by oocyte cytoplasts. It is also plausible that removal of cytoplasm surrounding metaphase spindles during the enucleation step of nuclear transfer might have reduced their developmental potential (31).

In conclusion, we report the production of embryonic stem cell lines from nuclear transfer of somatic cells from the same oocyte donor and from parthenogenetic activation. Our data also suggest that oocyte handling and manipulation during the nuclear transfer procedure influences the developmental ability and embryonic stem cell establishment rate of nuclear transferred embryos.

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