Human Embryos Derived by Somatic Cell Nuclear Transfer Using an Alternative Enucleation Approach

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Abstract

Somatic cell nuclear transfer (SCNT) was used to generate patient-specific embryonic stem cells (ESCs) from blastocysts cloned by nuclear transfer (ntESCs). In this study, a total of 135 oocytes were obtained from 12 healthy donors (30–35 years). Human oocytes, obtained within 2 h following transvaginal aspiration, were enucleated using a Spindle Imaging System to position the spindle and chromosomes that lay on the metaphase plate, and a Zona Infrared Laser Optical System was used to open a single hole in the zona pellucida at the ~2 o'clock position. Human fibroblasts and lymphocytes were used to construct SCNT embryos. Nearly half (26 of 58) of the oocytes were fused after electrofusion and embryo development rates were 96.2% (two-cell, 25 of 26), 92.3% (four-cell, 24 of 26), 61.5% (eight-cell, 16 of 26), 34.6% (16-cell, 9 of 26), 26.9% (morula, 7 of 26), and 19.2% (blastocyst, 5 of 26), respectively, following incubation in improved G-series sequential medium. One cloned blastocyst was used for STR-DNA identification and genetic polymorphism analysis of mtDNA, and STR-DNA analysis of all cloned blastocysts indicated they were derived from SCNT. Quantitative analysis showed that mtDNA of cloned embryos reflected the change tendency of those observed in human IVF embryos. Our research provides an alternative enucleation approach for producing human SCNT-derived blastocysts, and may aid in providing a new method for human therapeutic cloning.

Introduction

ABNORMALITIES OR DEFECTS in a single cell type ultimately may affect an entire tissue or organ and lead to disease. In many cases, traditional therapies fail to eliminate diseases or cure patients. The next generation of treatment alternatives will come with the development of embryonic stem cell (ESC)-based therapies. ESCs have immense proliferative capacity, and can differentiate into all kinds of cells of the body under given conditions (Assady et al., 2001; Evans and Kaufman, 1981; Odorico et al., 2001; Reubinoff et al., 2000; Thomson et al., 1998), and eventually will be used to replace diseased cells, tissues, or organs (Jiang et al., 2007). One approach to overcome transplant rejection of human ES cells is to derive them by nuclear transfer of the patient's own cells.

The isolation of pluripotent human ES cells (Thomson et al., 1998) and the breakthrough in somatic cell nuclear transfer (SCNT) in mammals (Wilmut et al., 1997) have raised the possibility of performing human SCNT to generate virtually unlimited supplies of undifferentiated cells for research, with

potential applications in tissue repair and transplantation. This concept, known as "therapeutic cloning," refers to the transfer of the nucleus of a somatic cell into an enucleated donor oocyte (Lanza et al., 1999). Recently, Byrne et al. (2007) produced primate ES cells by SCNT, and there have been other attempts to produce human ES cells by SCNT (Cibell et al., 2001; Heindryckx et al., 2007; Lavoit et al., 2005; Lu et al., 2003; Stojkovic et al., 2005; Trivedi et al., 2006). Some of these attempts (Cibell et al., 2001; Heindryckx et al., 2007; Lavoit et al., 2005) failed to produce blastocysts due to poor oocyte quality. Stojkovic et al. (2005) obtained a cloned blastocyst using undifferentiated human ES cells as donors rather than an adult cell. One report (Lu et al., 2003) failed to prove the genetic origin of the cloned blastocyst. Another report by Trivedi et al. (2006), described successful production of human ES cells by SCNT using a cumulus cell from the same oocyte donor as the donor cell. Cells were infused into the peripheral blood of the recipient, who was the oocyte donor's elder brother (a man afflicted with chronic glomerulonephritis, chronic renal failure, presenting 100% G6PD enzyme deficiency) who matched 6 of 6 HLA with his sister,

the donor. However, in most cases, people who need cell replacement therapy may not be matched to such a donor. French et al. (2008) reported, for the first time, the generation of heterologous SCNT blastocysts. When applied in a therapeutic setting, ES cells, which are isolated from the inner cell masses (ICMs) of cloned preimplantation embryos, carry the nuclear genome of the patient; therefore, following directed cell differentiation, the cells could be transplanted without immune rejection for treatment of degenerative disorders. The aim of the present study was to produce human cloned blastocysts by SCNT using an alternative enucleation approach, which may provide new information on methods for human therapeutic cloning.

Materials and Methods

Materials

All chemicals were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO) unless otherwise stated. All embryo manipulations were performed at 37°C except for the process of electrical fusion, which was performed at room temperature. Cells were cultured at 37°C in 5% CO₂ except for the G-series media, which required 37°C and 6% CO₂ culture conditions.

Fibroblasts were derived from aborted fetuses or males who experienced foreskin resection in our hospital's Outpatient Operating Room. Human lymphocytes were donated by a male patient with Parkinson's disease. Human uterine endometrial epithelium was donated by patients who underwent an abortion in our hospital's Outpatient Operating Room. All materials were obtained from volunteers with informed consent for scientific research. Culture sera were derived from human umbilical cord blood obtained from our hospital.

Methods

Human fibroblast preparation. Primary fibroblast cell lines were isolated from a 2–3-mm skin biopsy sample (F1 and AF2). Skin biopsies were washed three times in Ca²⁺, Mg²⁺-free DPBS (GIBCO, Invitrogen Corporation, Grand Island, NY), containing 100 IU/mL penicillin G and streptomycin, and then minced into 1-mm³ pieces. Pieces were washed three times in Ca²⁺, Mg²⁺-free DPBS and adhered to the flask surface. After 10 min, 300 µL of umbilical cord blood serum were added to prevent drying. After culturing for 2 h, 3 mL of culture medium was added. Medium was replaced every 2–3 days. Fibroblasts were passaged two more times after the culture had grown to full confluency. A few cells were identified by short tandem repeat (STR)-DNA analysis, and the rest were used for further culture.

Giemsa-banding (G-banding) for determining karyotypes of the F1 and AF2 cell lines was conducted by the Genetic Laboratory of Yuhuangding Hospital.

Human lymphocyte isolation. Human peripheral blood mononuclear cells were obtained from a Parkinson's disease patient. Before isolation, Lympholyte solution (Haoyang, Tianjin, China) was brought to room temperature and 6 mL were added to a round-bottom test tube for subsequent use. Blood sample (2 mL) was taken from the Parkinson's disease patient and treated with heparin. The anticoagulated blood

then was added slowly to the top of preheated Lympholyte solution and centrifuged at 1500 rpm for 15 min at 23°C. After centrifugation, lymphocytes were aspirated, transferred to another test tube, and mixed with Hank's medium. After centrifugation at 1000 rpm for 5 min, the supernatant was removed and the pellet was washed three times with Hank's medium. Finally, the pellet was resuspended in RPMI 1640 medium (GIBCO, Invitrogen Corporation) containing 5% fetal calf serum. A few isolated cells were used for immunostaining for CD45 expression, and the remaining cells were cultured for SCNT.

G-banding for determining karyotypes of the F1 and AF2 cell lines was conducted by the Genetic Laboratory of Yuhuangding Hospital.

Feeder cells. A culture of human uterine endometrial epithelium was generated as previously described (Spandorfer et al., 2001). Three days before recovery of the oocytes, epithelial cells were seeded into four-well plates in DMEM/F12 medium containing 10% fetal umbilical cord serum and incubated at 37°C in 5% CO₂.

Ovarian stimulation and oocyte recovery. Oocyte donors underwent routine controlled ovarian hyperstimulation. Each donor was stimulated with one tablet/day of Marvelon (Schering-Plough, Inc., Chatham, NJ) from the 3rd to the 21st day of their menstrual cycle. From the 21st to the 28th days, donors were injected with 0.1 mg/day of Triptorelin (Ferring Pharmaceuticals, Ltd., The Netherlands); the injection dose was changed to 0.05 mg/day until human chorionic gonadotropin (hCG, American Pharmaceutical Partners, Inc., East Schaumburg, IL) was given. From the third to the fifth day (according to donor's condition) a daily injection of 150–225 IU Gonadotropin (Serono, Inc. Rockland, MA) was given until hCG injection. If ultrasound examination showed one to two follicles of 16–18 mm in diameter, a single 10,000 IU dose of hCG was administered. Cumulus-oocyte complexes (COCs) were collected from follicles of anesthetized donors 36 h after hCG administration by ultrasound-guided needle aspiration into sterile test tubes (day 0).

Culture of in vitro fertilization (IVF)-derived embryos. A total of 20 human COCs were generated by the IVF procedure and were cultured in G-series sequential medium (Vitrolife, Gothenburg, Sweden) to the blastocyst stage at 37°C in 6% CO₂. Medium was replaced every 2 days. Developmental efficiency was calculated and the embryo quantities were recorded.

Oocyte treatment. COCs used for parthenogenesis and SCNT were washed in G-MOPS (Vitrolife) three times, treated with 1 × HYASE (Vitrolife) to remove cumulus cells, then washed three times in G-MOPS, followed by incubation in G-1 medium at 37°C in 6% CO₂ in preparation for further use. Oocytes were divided by stage according to the extrusion of the first polar body or lack thereof (Combelles et al., 2002).

Optimized protocols for parthenogenetic embryos. In Experiment 1, 20 randomly selected cumulus-free oocytes were divided equally into groups A and B. After equilibrating in 0.3 M D-mannitol fusion fluid (0.3 M D-mannitol, 0.05 mM

CaCl₂, and 0.1 mM MgCl₂) for 5 min, oocytes were activated by consecutive exposure to two direct current (DC) 1.3 kV/cm for 80 μ sec $\times$ two pulses, 0.1 sec apart, generated from an ECM 2001 (BTX, Boston, MA) and addition of 2 mM 6-dimethylaminopurine in G-1 medium for 4 h with careful washing of oocytes in G-MOPS. A cluster of five oocytes then was added to balanced 50 μ L G-1 medium with a paraffin overlay following culture. Two days later, cleaved embryos were transferred into G-2 medium (group A, three to five embryos/0.8 mL G-2 medium) and G-2 medium cocultured with 2×10^5 /mL human uterine endometrial epithelium cells (group B, three to five embryos/0.8 mL G-2 medium). The total culture period in G-series medium was about 6–7 days. The oocytes were observed every day and daily evaluation of development was performed. In our later work, we selected SCNT-derived embryos culture protocols based on human parthenogenetic embryo studies

In Experiment 2, a total of 27 oocytes was randomly divided into groups A, B, and C and subjected to sequential activation treatments. First, after equilibrating in 0.3 M D-mannitol fusion fluid (0.3 M D-mannitol, 0.05 mM CaCl₂, and 0.1 mM MgCl₂) for 5 min, oocytes were activated by the following conditions: A—1.1 kV/cm for 80 μ sec $\times$ two pulses, 0.1 sec apart; B—1.2 kV/cm for 80 μ sec $\times$ two pulses, 0.1 sec apart; and C—1.3 kV/cm for 80 μ sec $\times$ two pulses, 0.1 sec apart, respectively. After electroactivation, oocytes were activated chemically with 2 mM 6-dimethylaminopurine added to G-1 medium with a paraffin overlay for 4 h after careful washing of oocytes in G-MOPS. A cluster of five oocytes was placed in balanced 50 μ L G-1 medium with a paraffin overlay following culture. Two days later, further cultivation of the parthenogenetic embryos was performed using G-2 medium and cocultured with 2×10^5 /mL human uterine endometrial epithelium cells (three to five embryos/0.8 mL G-2 medium). The total culture period in G-series medium was about 6–7 days, and the oocytes were observed every day and daily evaluation of development was performed. We applied proper egg parthenogenic activation indexes to activation of nuclear transfer embryos.

Nuclear transfer, cell fusion, activation, and culture of reconstructed embryos. A total of 58 human oocytes were incubated in G-1 medium supplemented with 7.5 μ g cytochalasin B/mL for 10 min before enucleation. To guarantee accurate enucleation, we employed a two-step approach, using the Oosight™ imaging system (SpindleView, CRI, MA) and Zona Infrared Laser Optical System (ZILOS-tk, Hamilton Thorne, Beverly, MA). SpindleView allowed noninvasive, polarized light imaging and detection of the spindle based on birefringence (Silva et al., 1999). An earlier version of this imaging system was used successfully for enucleation of monkey oocytes (Ng et al., 2004). Each oocyte was immobilized by the holding pipette and rotated with the use of the injection pipette until both the meiotic spindle and the polar body were clearly in focus in the oocyte equatorial plane. Oocyte rotation around the axis connecting the center of the oocyte with the first polar body allowed orientation of the meiotic spindle microtubules, which often becomes more favorable for visualization of the structure's birefringence (Cooke et al., 2003; Rienzi et al., 2003). An individual oocyte was positioned using the holding pipette with the first polar body at ~2 o'clock, and the metaphase

spindle was visualized as a small string of bead shaped structures (chromosomal complexes) usually adjacent to the polar body. Using this innovative approach, we were able to locate the spindle in real time. In the second step, the enucleation procedure was performed using the Leica inverted microscope equipped with the ZILOS-tk that typically is used in assisted reproduction technologies by application of a noncontact 1.48- μ m wavelength infrared diode laser beam. The oocyte, which was held by the holding pipette in the first step, was positioned so that a portion of the zone pellucida (ZP) was in the path of the laser beam. The laser beam then was activated and fired several times, at a duration of 1.5 msec, to open a single hole in the ZP at ~2 o'clock. The spindle and the polar body were removed with an injection pipette through the same hole that was opened in the second step, and a single donor cell was inserted under the ZP and placed in close contact with the cytoplasm. Forty-eight out of 58 enucleated oocytes were introduced with human fibroblast material and the remainder (10) were introduced with human lymphocyte material. We also enucleated 10 additional human oocytes using the two-step method described but without the insertion of human donor cells to observe the extent of enucleation.

Two 80- μ sec DC pulses of 1.2 kV/cm were used to induce embryo fusion and activation simultaneously. Successful fusion was confirmed by the disappearance of the donor cell in the perivitelline space. Fused embryos were activated by exposure to G-1 medium containing 2 mM 6-dimethylaminopurine for 4 h at 37°C in 6% CO₂. After careful washing of embryos in G-MOPS, a cluster of five embryos then was placed in balanced 50 μ L G-1 medium with a paraffin overlay following culture. On the third day, cleaved embryos were washed in G-MOPS and every five embryos were cultured in balanced 0.8 mL G-2 medium and cocultured with 2×10^5 /mL human uterine endometrial epithelium cells. The total culture period in G-series medium was about 6–7 days. Medium was replaced every 48 h, and developmental efficiency was calculated and the embryo quantities were recorded. The additional 10 enucleated human oocytes were subjected to the same protocol.

Short tandem repeat (STR) loci analysis. Oocyte donors' somatic cells, donor somatic cells, and SCNT blastocysts were used for independent microsatellite analyses. DNA was extracted with the standard Chelex® 100 (Bio-Rad, Hercules, CA) extraction procedure (Barber and Foran, 2006). The total volume of DNA extracted from blastocysts was about 15 μ L. Samples were amplified in DNAmplify PE9700 following the technical manual of Identifier (Applied Biosystems, Foster City, CA). Genescan analysis on the amplified fragments was performed for the following microsatellite markers: D8S1179, D21S11, D7S820, CSF1PO, D3S1358, TH01, D13S317, D16S539, D2S1338, D19S433, Vwa, TOPX, D18S51, AMELXY, D5S818, and FGA. Each amplification reaction contained 1 μ L DNA, 2 μ L primer mix, 4 μ L PCR premix, 0.25 μ L of Amplitaq Gold DNA polymerase, and 2.75 μ L sterile H₂O. Final reaction volumes were 10 μ L each. The cycling program started with an initial 11-min denaturation step at 95°C, followed by 28 cycles of 1 min at 94°C, 1 min at 59°C, 1 min at 72°C, and the final extension step of 60 min at 60°C (Suadi et al., 2007). SCNT blastocysts were identified by LCN-STR profiling (Lucy et al., 2007).

Single nucleotide polymorphism (SNP) identification of mitochondrial DNA (mtDNA). DNA from peripheral blood lymphocytes of a Parkinson's disease patient, and somatic cells of the oocyte donor and SCNT blastocysts derived from the lymphocyte were extracted as described in Analysis of STR loci section. Extracted DNA was used for SNP identification.

The 11 PCR primer pairs (3010, 4793, 10211, 5004, 7028, 7202, 16519, 12858, 4580, 477, and 14470, which represent the loci of SNPs) were selected using the publicly available Web-based primer selection software Primer3 (Rozen and Skaletsky, 2000). The concentration of each primer pair was determined according to Vallone's report (Vallone et al., 2004) and then diluted to 1/100 for PCR amplification. All primer pairs were synthesized by the Shanghai Shengong Biology Technology Company.

Multiplex amplification of the 11 unique amplicons was carried out using the PE9700 in a total volume of 15 μ L with 1- μ L mtDNA sample. The final concentration of each primer pair (22 total primers) was 0.045 μ M, and the other PCR reagent concentrations were determined according to Vallone's report (Vallone et al., 2004).

The conditions for multiplex PCR were as follows: 95°C for 11 min, 35 cycles of 94°C for 30 sec, 50°C for 30 sec, 72°C for 30 sec, 72°C for 7 min, and storage at 4°C.

The ABI3100-A Genetic Analyzer was used to process the data with the mixture of 0.3 μ L LIZ-120 internal sizing standard (Applied Biosystems, Bedford, MA), 7 μ L amplification product, and 10 μ L Hi-Di formamide (Applied Biosystems) after an appropriate spectral matrix had been created using materials from the matrix standard set DS-02 (Applied Biosystems). Data analysis was performed using Genemapper ID 3.2 software.

Quantitative analysis of mtDNA. Human oocytes, donor cells, IVF embryos at different stages, and SCNT embryos at different stages were used for quantitative mtDNA analysis. Samples were washed three times by PBS and put into 20 μ L embryo lysate (Chelex reagent containing 100 mg/mL proteinase K) at 56°C for 1 h, followed by 98°C for 10 min. Extracted DNA was kept at -20°C for further use.

DNA isolated from the samples was used to amplify the mtDNA ND1 gene with the following primers: forward, 5'-ATACCATGACCAACCTCCTACTCCTCATT-3' and reverse, 5'-CTGCTCGCAGTGCGCCGATCAGGGCGTAGT-3'.

PCR reactions were prepared as follows: 8.5 μ L distilled water, 1 μ L forward primer, 1 μ L reverse primer, 2 μ L DNA template, and 12.5 μ L power SYBR® green PCR master mix (including PCR buffer, dNTPs, Taq polymerase, and SYBR Green dye). Real-time PCR assays were performed using an iCycler (BIO-RAD, USA). Human mtDNA was amplified using the following parameters: 95°C for 5 min, followed by 38 cycles at 95°C for 30 sec, 55°C for 45 sec, 72°C for 45 sec, and 72°C for 7 min. The melting curve was constructed according to the fluorescence signal, which was detected continuously as the temperature increased to 95°C at 0.3°C/5 sec after 65°C for 10 sec. Standard curves were constructed using the CT value (threshold cycle) as ordinate, and log starting quantity of copy number as abscissa.

Statistical analysis

Quantitative values of two-cell, four-cell, eight-cell, 16-cell, morula, and blastocysts from human IVF and SCNT embryos

(Table 3) were analyzed using a *t*-test. Statistical significance was determined when $p < 0.05$.

Human ethics

With written informed consent and approval by Yuhuangding Hospital research and ethics committee obtained, a total of 135 oocytes were obtained from 12 healthy donors. Oocytes donors and somatic cell donors or their families were fully aware of the scope of our study, and each signed an informed consent form. All donated oocytes were obtained from egg donation cycles performed at the Reproductive Sciences Center of Yan-tai Yuhuangding Hospital, a fertility center located in Yan-tai, China. Its laboratory is licensed by the relevant hygiene authorities and Industrial and Commercial Administration, accredited by the Ministry of Health in 2005. All donations were performed using the same standard procedure as for clinical IVF procedures. All original data were kept at the Reproductive Sciences Center and were subject to inspection. The oocytes and embryos derived from SCNT were not available to other laboratories or for other uses. The second day after the formation of SCNT-derived blastocysts, DNA was isolated from the blastocysts for identification. Remaining isolated DNA was kept by for reexamination.

Results

Donor somatic cells

Human fibroblasts were used as donor cells after three passages. The shape of the primary passage cells was either spindle-like or triangle-like, with one to three nucleoli apparent in the nucleus, located at the middle of each cell. Fibroblasts had long pseudopodia (Fig. 1A1, B1). After passage, fibroblasts replicated rapidly and became clustered. Cytogenetic analysis showed 100% F1 ($n = 20$) maintained a diploid 22 chromosomes + XX karyotype (Fig. 1A2), and 100% AF 2 ($n = 20$) maintained a diploid 22 chromosomes + XY karyotype (Fig. 1B2). More than 98% of isolated human peripheral blood mononuclear cells were lymphocytes and positive for CD45+ by immunostaining (Fig. 1C1), and the lymphocyte karyotype was 44 chromosomes + XY karyotype (Fig. 1C2).

Feeder cells

Primary passage human uterine endometrial epithelium cells were used as feeder cells. After culture for 24 h, most cells were attached to the well bottom and were polygonal or tadpole in shape. Cells spread via filamentous structures, and had large, round nuclei with obvious nucleoli.

Culture of human IVF-derived embryos

We obtained 135 oocytes from 12 donors, with two oocytes classified as M I stage, and the remainder classified as M II stage.

Approximately 45% blastocysts were obtained from 20 IVF-derived human embryos (9 of 20). Six were classified as grade A and the other three were classified as grade B (Gardner et al., 2000; Puissant et al., 1987). The data were used as reference for the parthenogenesis embryo culture system.

Optimized protocols for parthenogenetic embryos

In Experiment 1, embryo development was significantly different under different culture conditions between groups

FIG. 1. Human somatic cells for SCNT. (A1) An image of human fibroblast cells 1 (F1) (magnification presented at $\times 200$). (A2) Chromosome spread of F1 (original magnification at $\times 1000$), 46,XX. (B1) An image of human fibroblast cells (AF2) (original magnification presented at $\times 200$). (B2) Chromosome spread of AF2 (original magnification at $\times 1000$), 46,XY. (C1) Identification of human peripheral blood mononuclear (CD45+) cells (scale bars equal $20\ \mu\text{m}$). (C2) Chromosome spread of CD45+ blood cells (original magnification at $\times 1000$), 46,XY.

FIG. 2. Development of a human embryo derived from SCNT. Visualization of the meiotic spindle in a human M II oocyte using the Oosight™ spindle imaging system during enucleation. (A) The spindle position of a cumulus matrix-free human metaphase II oocyte. The white arrow highlights the position of the spindle. (B) Enucleation pipette for the aspiration of the metaphase plate from human oocytes. The white arrow highlights the aspirated spindle. (C) Four-cell stage. (D) Eight-cell stage. (E) Morula. (F) Blastocyst. Original magnification at $\times 200$. Scale bars equal $50\ \mu\text{m}$.

TABLE 1. HUMAN SCNT EMBRYO DEVELOPMENT

Donated oocytes ^a	Fused oocytes ^b	Activated fused oocytes develop to: (%)					
		Two cell	Four cell	Eight cell	16 cell	Morula	Blastocysts
58	26	19–22 h ^c 25 (96.2)	36–38 h ^c 24 (92.3)	60–62 h ^c 16 (61.5)	85–87 h ^c 9 (34.6)	98–100 h ^c 7 (26.9)	110–132 h ^c 5 (19.2)

^aOut of 58 oocytes 2 were classified as M I, and the rest as M II.

^bFused oocytes were evaluated based on the disappearance of the donor cell.

^cTime lapse from the time of fusion to corresponding embryo stages.

Developmental efficiency is defined as a percentage based on number of fused oocytes as the rest of the stages.

TABLE 2. SCORING OF HUMAN EMBRYOS DERIVED BY SCNT

	Two cell			Four cell			Eight cell			16 cell			Morula			Blastocysts				
Score	2	3	4	2	3	4	2	3	4	2	3	4	2	3	4	Grade	A	A/B	B	C
Number per score	7	8	10	7	7	10	3	6	7	2	3	4	2	1	4		1	1	2	1

TABLE 3. STR-DNA ANALYSES OF THREE SCNT BLASTOCYSTS FOLLOWING NUCLEAR TRANSFER OF HUMAN FIBROBLAST DONOR CELLS (F1) USING OOCYTES OBTAINED FROM EGG DONOR 1

STR1 loci	Egg donor 1		Fibro donor 1		Three blastocyst from SCNT	
D8S1179	13		13	15	13/13/13	15/15/15
D21S11	31.2	32	30	33.2	30/30/30	33.2/33.2/33.2
D7S820	12		12		12/12/12	
CSF1PO	10	12	10		10/10/10	
D3S1358	15	18	16	17	16/16/16	17/17/17
TH01	9		6	9	6/6/6	9/9/9
D13S317	8	9	8	12	8/8/8	12/12/12
D16S539	9	12	9	13	9/9/9	13/13/13
D2S1338	19	24	23	24	23/23/23	24/24/24
D19S433	14		13		13/13/13	
Vwa	18		17	18	17/17/17	18/18/18
TPOX	8	11	8		8/8/8	
D18S51	16	22	16		16/16/16	
AMELXY	X		X		X/X/X	
D5S818	11		12	13	12/12/12	13/13/13
FGA	24	25	23	25	23/23/23	25/25/25

Egg donor 1 was the heterozygous at this locus.

Egg donor 1 was the homozygous at this locus.

Fibro donor 1 was the heterozygous at this locus.

Fibro donor 1 was the homozygous at this locus.

Three blastocysts derived from SCNT were heterozygous at this locus, consistent with Fibro donor 1.

Three blastocysts derived from SCNT were homozygous at this locus, consistent with Fibro donor 1.

TABLE 4. STR-DNA ANALYSES OF SCNT BLASTOCYSTS FOLLOWING NUCLEAR TRANSFER OF HUMAN FIBROBLAST DONOR CELLS (AF2) USING OOCYTES OBTAINED FROM EGG DONOR 2

<i>STR loci</i>	<i>Egg donor 2</i>		<i>Fibro donor 2</i>		<i>SCNT blastocyst</i>	
D8S1179	12	13	11	13	11	13
D21S11	30	32.2	30	32	30	32
D7S820	11		11		11	
CSF1PO	11	12	10		10	
D3S1358	15		16		16	
TH01	9		9		9	
D13S317	8	11	8	9	8	9
D16S539	9		9	12	9	12
D2S1338	19	20	18	20	18	20
D19S433	14	15	13	14	13	14
Vwa	18		17	19	17	19
TPOX	9		8	11	8	11
D18S51	13		13	21	13	21
AMELXY	X		X	Y	X	Y
D5S818			11	13	11	13
FGA			24	25	24	25

Egg donor 2 was the heterozygous at this locus.

Egg donor 2 was the homozygous at this locus.

Fibro donor 2 was the heterozygous at this locus.

Fibro donor 2 was the homozygous at this locus.

SCNT blastocyst was heterozygous at this locus, consistent with Fibro donor 2.

SCNT blastocyst was homozygous at this locus, consistent with Fibro donor 2.

A and B. In group A, embryo development was retarded and no blastocysts formed. In group B, one grade B blastocyst and one grade C blastocyst formed (Supplementary Tables 1 and 2) (see supplementary material at: www.liebertonline.com) (Gardner et al., 2000; Puissant et al., 1987). Embryo development in each group was recorded (Supplementary Fig. 1) and used as an optimization indicator for the culture of SCNT embryos.

In Experiment 2, a total of 27 oocytes were divided into three groups randomly and were induced to undergo parthenogenic activation under different activation conditions. Zero embryos developed to blastocysts in group A, while two and one embryos developed to blastocysts in groups B and C, respectively (Supplementary Tables 3 and 4) (Gardner et al., 2000; Puissant et al., 1987), and embryo development was recorded (Supplementary Fig. 2).

SCNT cloned embryos

A total of 26 fused units was obtained out of 58 oocyte-somatic cell units after electrical and chemical activation, of which 25 units (96.2%) cleaved, and five blastocysts (19.2%) formed after *in vitro* culture on days 5 to 6 (Table 1) (Fig. 2, and Supplementary Fig. 3). Each embryo was scored and

recorded (Table 2) (Gardner et al., 2000; Puissant et al., 1987). An additional 10 enucleated oocytes that were used as controls did not show embryo cleavage.

STR-DNA Identification

Five blastocysts (numbered #1, #2, #3, #4, and #5) were derived from three oocyte donors and two types of donor somatic cells (human fibroblasts and lymphocytes). Somatic cells from oocyte donors and SCNT blastocysts were used for STR-DNA assay. Of these, four blastocysts were derived from fibroblasts, and the rest from Parkinson's patient lymphocytes. Results from the analysis demonstrated that DNA from each SCNT blastocyst matched its corresponding donor somatic cell DNA at all loci (Tables 3, 4, 5, and Supplementary Figs. 4, 5, 6, 7, 8).

SNP identification of the SCNT blastocyst mtDNAs

Blastocyst #5, its corresponding donor somatic cells (numbered 5A), and oocyte donor's somatic cells (numbered 5B) were used for mtDNA SNP identification. The assay was designed to detect 11 SNP sites located in the mitochondrial genome. Blastocyst #5 showed a mixture of A and G at po-

TABLE 5. STR-DNA ANALYSES OF SCNT BLASTOCYST FOLLOWING NUCLEAR TRANSFER OF HUMAN LYMPHOCYTE DONOR CELLS (LYMPH DONOR) USING OOCYTES OBTAINED FROM EGG DONOR 3

<i>STR-loci</i>	<i>Egg donor 3</i>		<i>Lymph donor</i>		<i>SCNT blastocyst</i>	
D8S1179	13	15	10	14	10	14
D21S11	29	30	31	32.2	31	32.2
D7S820	10	11	12	13	12	13
CSF1PO	10	12	10	13	10	13
D3S1358	15	17	16		16	
TH01	7	9	9		9	
D13S317	8	11	9	11	9	11
D16S539	12	13	9		9	
D2S1338	21	25	22	23	22	23
D19S433	14.2	15.2	13	14	13	14
Vwa	17	18	17	18	17	18
TPOX	8	11	8		8	
D18S51	13	16	13	18	13	18
AMELXY	X		X	Y	X	Y
D5S818	11		9	10	9	10
FGA	22	23	19	21	19	21

Egg donor 3 was the heterozygous at this locus.

Egg donor 3 was the homozygous at this locus.

Lymph donor was the heterozygous at this locus.

Lymph donor was the homozygous at this locus.

SCNT blastocyst was heterozygous at this locus, consistent with the Lymph donor.

SCNT blastocyst was homozygous at this locus, consistent with the Lymph donor.

sition 16519, and matched that of the 5A (A at position 16519) and 5B (G at position 16519). These results showed that mtDNA in blastocyst #5 contained the SNP site of donor cells and oocyte donor somatic cells (Fig. 3), and we could conclude that mtDNA in blastocyst 5 was derived from its corresponding donor cell and recipient oocyte.

Quantitative analysis of derived embryo mtDNAs

Quantitative analysis of mtDNAs revealed no significant difference between IVF-derived and SCNT-derived embryos (Table 6).

Discussion

We applied the Oosight™ spindle imaging system with the Zona Infrared Laser Optical System for enucleation in the human SCNT process for the first time. In this report, we obtained five SCNT blastocysts, four from human fibroblasts and one from human lymphocytes. Evidence of successful SCNT was shown with STR-DNA analyses of five SCNT cloned blastocysts where embryos genomic DNA was that of the donor cells and was not parthenogenetic in origin. SNP examination of mtDNA showed one SCNT blastocyst (#5) was identical to its corresponding donor somatic cells and egg donor.

STR-DNA analysis of the donor somatic cells, oocyte donor somatic cells, and all cloned blastocysts was conducted to determine if the embryonic pathways in the donor nucleus had been reinitiated, and the results proved genomic DNA of five blastocysts was identical to their corresponding donor cells. Since the DNA of the donor cells used was different from that of recipient oocytes, parthenogenesis could be avoided. Modern forensic techniques allowed DNA to be extracted from very small amounts of cellular material. Low copy number (LCN) profiling enabled the production of STR profiles from small amounts of cells. We applied LCN on SCNT blastocysts and avoided negative results that might have occurred due to the loss of DNA.

Mature oocytes in different mammalian species contain on average 1.74 to 9.5×10^5 mtDNA copies (Cao et al., 2007; May-Panloup et al., 2005; Smith et al., 2005). In contrast, mtDNA copy numbers in somatic cells varied substantially, ranging from several hundred to several thousand (Cao et al., 2007; Frahm et al., 2005; King and Attardi, 1989; Shmookler and Goldstein, 1983). Recipient oocytes have approximately 100 to 250 times more mtDNA copies than donor cells based on copy number ratios of 0.4% to 0.9% (Inoue et al., 2004; Steinborn et al., 2000, 2002). Therefore, the resultant SCNT clones were assumed to have nuclear and mitochondrial genomes of different origins and slightly heteroplasmic

FIG. 3. mtDNA identification of blastocyst #5, its corresponding donor somatic cells, and oocyte donor somatic cells. (A) mtSNP of donor somatic cells at position 16519 was A; (B) mtSNP of oocyte donor somatic cells at position 16519 was G; (C) mtSNP of blastocyst #5 at position 16519 was mixture of A and G.

based on the quantitative participation of mtDNA from the two partners and on equal replication of their mitochondrial genomes.

The role and effect of heteroplasmy on the success of cloned embryo production has not been well documented. It has been reported that mtDNA recombination is likely to affect SCNT (Hiendleder *et al.*, 2005). SNPs analysis of blastocyst #5 indicated the coexistence of mtDNA from both lymphocyte and the recipient oocyte. Incompatibility between the cytoplasm of donor cells and the recipient cytoplasm may

influence the development of cloned embryos, and this may be the cause of different blastocyst formation rates between SCNT and IVF.

The key to producing human SCNT embryos is to enucleate the oocyte completely, with removal of only a small amount of cytoplasm in the process. In our early experiments, we tried several enucleation methods (Liu *et al.*, 2002, 2004; Mitalipov *et al.*, 1999; Westhusin *et al.*, 1992) using rabbit oocytes as the recipient cytoplasm, and found the enucleation method described in our report possessed several ad-

TABLE 6. QUANTITATIVE ANALYSIS OF MTDNAS

Groups	One cell	Two cell ^a	Four cell ^b	Eight cell ^c	16 cell ^d	Morula ^e	Blastocyst ^f
		(mean ⁶ ± SD 10 ⁵)	(mean ⁶ ± SD 10 ⁵)	(mean ⁶ ± SD 10 ⁵)	(mean ⁶ ± SD 10 ⁵)	(mean ⁶ ± SD 10 ⁵)	(mean ⁶ ± SD 10 ⁶)
Human oocyte	3.31 × 10 ⁶						
Human donor cell	9.79 × 10 ³						
Human IVF		1.62 ± 7.39	1.65 ± 7.0	1.65 ± 7.19	1.75 ± 7.04	1.95 ± 7.28	3.04 ± 1.24
SCNT embryos		1.61 ± 7.3	1.64 ± 7.2	1.70 ± 7.35	1.81 ± 6.68	2.14 ± 7.39	3.69 ± 1.37

There were no significant differences between ^{a,b,c,d,e,f} values at the same column that derived from Human IVF and SCNT embryos respectively ($p > 0.05$).

vantages. First, the Oosight Spindle Imaging System introduces a novel orientation-independent polarized microscopy system (polscope) coupled with image processing software that allows the visualization of meiotic spindle in a noninvasive way (Liu et al., 2000; Oldenbourg, 1999; Oldenbourg and Mei., 1995). Since the meiotic spindle is not visible with conventional light microscopy (due to the lack of contrast), use of the polscope system generates contrast to image the meiotic spindle on the basis of its birefringence. Parallel-aligned meiotic spindle microtubules shift the plane of the polarized light, inducing retardation of the light. To enhance sensitivity, the system uses digital image processing such that the meiotic spindle can be visualized irrespective of its orientation within the cell (Oldenbourg, 1999). Second, the microtubules of the meiotic spindle are highly sensitive to chemical and physical changes that may occur during oocyte handling, and the Oosight Spindle Imaging System can provide rapid and real-time observation of meiotic spindle without impairing oocyte viability. Third, a 1.48- μm infrared diode laser beam does not produce any thermal or mutagenic side effects (Germond et al., 1995), and is a rapid method that allows for the drilling of openings of a controlled-size micron range that can help injection by penetrating the ZP easily. Data generated by Simerly et al. (2003, 2004) showed that the use of a less-invasive enucleation method was a key factor for successful SCNT. Using this method, we could locate the spindle exactly, enucleate completely, and remove only a small amount of cytoplasm. We activated 10 additional enucleated oocytes using this method and did not observe embryo cleavage.

Exit of the ovulated oocytes from M II arrest normally is accomplished by fusion with spermatozoa, which commonly is referred to as "oocyte activation." The activation is triggered by intracellular calcium oscillations after sperm-oocyte membrane fusion (Whitaker and Patel, 1990). Since spermatozoa are not involved in SCNT, the oocyte must be stimulated artificially to fuse donor cells and oocytes, to activate the reconstructed oocytes and to initiate embryo development. Based on previous results with animal SCNT oocytes (Betthausen et al., 2000; Kasinathan et al., 2001; Kubota et al., 2000; Lai et al., 2002), we employed an activation protocol (simultaneous fusion and activation using electrical pulse followed by incubation with 2.0 mM 6-DMAP for efficient chemical activation) for human oocytes. In our earlier research, we tested several combinations of simultaneous fusion and activation with chemical activation for rabbit oocytes and observed that even a small voltage difference (we employed different voltage with only 0.1 kV/cm apart, such as 1.1, 1.2, 1.3, and 1.4 kV/cm) could result in a significant difference in fusion rate and blastocyst development rate (data not shown). In order to obtain the proper activation protocols for human SCNT embryos, we investigated the activation conditions for human parthenogenesis. When we induced parthenogenetic activation of human oocytes using electrical and chemical activation, we obtained an 88.9% activation rate with 25% blastocyst formation using two 80 μsec DC pulses of 1.2 kV/cm followed by incubation with 2.0 mM 6-DMAP for 4h. Cytoplasts exposed to voltage of 1.3 kV/cm resulted in poor blastocyst quality and less blastocyst formation. In our first human SCNT procedure, we adopted the same protocol for human oocyte SCNT and found that this protocol could provide efficient activa-

tion for human SCNT oocytes (which was represented by the formation of blastocyst); thus, it would appear that we have found the optimal voltage for producing the maximal rate of couple formation. Compared with Revazova et al. (2007) and French et al. (2008), the blastocyst formation rate in our report was lower. In the IVF process, the sperm activates development of the fertilized egg by inducing a prolonged series of oscillations in cytosolic free Ca^{2+} concentration (Roger et al., 2004). Oocytes can be activated after SCNT by exposure to electrical pulse and chemical agents, which act by stimulating calcium oscillations (Dale et al., 1999), and different activation rates and subsequent development may result from the different Ca^{2+} concentration oscillations when various activation protocols were employed.

In general, *in vitro*-cultured embryos compared with *in vivo*-cultured embryos appeared to have a low development rate in many animals (Booth et al., 2005; Gjorret et al., 2005; Pomar et al., 2005). One reason for this disparity could be an incomplete *in vitro* culture system. We first applied G-series medium for human parthenogenetic embryos and observed no blastocyst formation although we could obtain a 45% blastocyst development rate in human IVF using the same culture system. The different culture results of the same G-series medium between Lin et al. (2007) and the current experiment might be due to the oocyte treatment and different activation method used. Many studies have reported that coculture of embryos with uterine endometrial epithelium as feeder cells may help embryo development due to the secretion of growth factors that promote embryo development (Caballero-Campoo et al., 2002; Spandorfer et al., 2001). In order to obtain the proper culture conditions for human SCNT embryos, we investigated the culture conditions for human parthenogenesis. We used coculture of human uterine endometrial epithelium with human parthenogenetic embryos in G-2 medium and obtained about a 20% blastocyst development rate. Based on this result, we obtained a 19% blastocyst development rate for SCNT-derived embryos.

In most SCNT reports, either in primate or human (Beaujean et al., 2004; French et al., 2008; Hyan et al., 2003; Miyamoto et al., 2007; Zhou et al., 2006; Wakayama and Yanaqimachi, 1999), fibroblasts are the most common donor cells. In our report, a human lymphocyte was used as a donor cell and 2 mL of peripheral blood will provide enough lymphocytes for SCNT. This type of donor cell may be more acceptable by somatic cell donors in SCNT.

In conclusion, the use of an alternative enucleation method in SCNT allowed the successful generation of heterologous SCNT blastocysts from human fibroblasts and lymphocytes. STR-DNA identification analysis showed that all five SCNT blastocysts were derived from donor somatic cells, and one cloned blastocyst also was confirmed by mtDNA genetic polymorphism analysis. Quantitative analysis of embryo mtDNAs also showed the similarly changes between IVF embryos and embryos derived from SCNT. The method reported here will provide new information that may help to establish a human ES cell line from SCNT blastocysts.

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Author Disclosure Statement

The authors declare that no competing financial interests exist.

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