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Patient-Specific Stem Cell Lines Derived from Human Parthenogenetic Blastocysts

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ABSTRACT

Parthenogenetic activation of human oocytes may be one way to produce histocompatible cells for cell-based therapy. We report the successful derivation of six pluripotent human embryonic stem cell (hESC) lines from blastocysts of parthenogenetic origin. The parthenogenetic human embryonic stem cells (phESC) demonstrate typical hESC morphology, express appropriate markers, and possess high levels of alkaline phosphatase and telomerase activity. The phESC lines have a normal 46, XX karyotype, except one cell line, and have been cultured from between 21 to 35 passages. The phESC lines form embryoid bodies in suspension culture and teratomas after injection to immunodeficient animals and give differentiated derivatives of all three embryonic germ layers. DNA profiling of all six phESC lines demonstrates that they are MHC matched with the oocyte donors. The study of imprinted genes demonstrated further evidence of the parthenogenetic origin of the phESC lines. Our research has resulted in a protocol for the production of human parthenogenetic embryos and the derivation of stem cell lines from them, which minimizes the presence of animal-derived components, making the derived phESC lines more suitable for potential clinical use.

INTRODUCTION

HUMAN EMBRYONIC STEM CELLS have the potential to give significant therapeutic benefit to patients, provided that the problem of immune rejection can be solved (Conley et al., 2004; Reubinoff et al., 2000; Thomson et al., 1998). Embryonic stem cells that are genetically related to the recipient may overcome such rejection problems. In this regard, parthenogenetic stem cells may be an alternative to embryonic stem cells derived by somatic cell nuclear transfer (SCNT) technology for women of reproductive age.

Parthenogenetic activation of oocytes is a relatively simple method to create histocompatible stem cells in comparison to SCNT, because it does not require the complex equipment necessary to micromanipulate an oocyte (Marshall et al., 1998). Parthenogenetic stem cells are produced from unfertilized oocytes and contain genetic material exclusively from the oocyte donor (the potential patient). It is proposed that following directed cell differentiation, autologous cells could be transplanted without the threat of immune rejection. Before now, all attempts to produce human parthenogenetic embryonic stem cell lines have failed (Cibelli et al., 2001; Lin et al., 2003). Parthenogenetic mouse major histocompatibility complex (MHC)-homozygous stem cell lines and one parthenogenetic primate heterozygous em-

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bryonic stem cell line (Cyno-1) have already been derived, and cell pluripotency has been demonstrated in these lines (Lin et al., 2003; Vrana et al., 2003).

Possible clinical use of hESC lines will require that their derivation and culture be performed using conditions that are free from components of animal origin. Attempts have been made in this direction (Kim et al., 2005); unfortunately, satisfactory methods for the derivation and culture of hESCs were not developed.

In the present report we describe the derivation of genetically MHC-matched pluripotent phESC lines from human parthenogenetic blastocysts using a protocol with minimal animal-derived components.

METHODS

Donor consent

Donors voluntarily donated oocytes, blood (for DNA analysis) with no financial payment. Donors signed comprehensive informed consent documents and were informed that all donated materials were to be used for research and not for reproductive purposes, namely the development of methods to derive human embryonic stem (ES) cells and their differentiated progeny.

In our research we used only shared oocyte donation. The quantity of oocytes used in our experiments did not affect donor pregnancy rates. Four donors out of five became pregnant. One donor was not successful in achieving embryo implantation, even though her *in vitro* fertilization (IVF) cycle utilized 11 mature oocytes.

Before ovarian stimulation, oocyte donors underwent medical examination for suitability according to Food and Drug Administration (FDA) eligibility determination guidelines for donors of human cells, tissues, and cellular and tissue-based products (FDA, 2004) and Order N 67 (02.26.2003) of the Russian Public Health Ministry. It included chest X-ray, blood and urine analysis, and liver function test. Donors were also screened for chlamydia trachomatis, neisseria gonorrhoeae, syphilis, HIV, hepatitis B virus (HBV), and hepatitis C virus (HCV).

Donor superovulation

Each donor underwent ovarian stimulation by FSH (Gonal-F, Lab. Serono, Switzerland) from the 3rd to the 13th day of their menstrual cycle. A total of 1500 IU were given. From the 10th to the 14th day of the donor's menstrual cycle gonadoliberin antagonist Orgalutran (Organon, Holland) was injected at 0.25 mg/day. From the 12th to the 14th day of the donor's menstrual cycle a daily injection of 75 IU FSH + 75 IU LH (Menopur, Ferring GmbH, Germany) was given. If an ultrasound examination displayed follicules between 18 and 20 mm in diameter, a single 8000 IU dose of hCG (Choragon, Ferring GmbH, Germany) was administered on the 14th day of the donor's menstrual cycle. Transvaginal punction was performed 35 h after hCG injection on approximately the 16th day. Follicular fluid was collected from the antral follicles of anesthetized donors by ultrasound-guided needle aspiration into sterile test tubes.

Oocyte activation and culture of parthenogenetic embryos

Cumulus oocyte complexes (COCs) were picked from the follicular fluid, washed in Flushing Medium (MediCult) and then incubated in Universal IVF medium (MediCult) with Liquid Paraffin (MediCult, Jyllinge, Denmark) overlay for 2 h in a 20% O_2, 5% CO_2, 37°C humidified atmosphere. Before activation, COCs were treated with SynVitro Hyadase (MediCult) to remove cumulus cells, followed by incubation in Universal IVF medium with Paraffin overlay for 30 min. Further culture of oocytes and embryos was performed in a humidified atmosphere at 37°C with an O_2 reduced gas mixture (90% N_2 + 5% O_2 + 5% CO_2) with the exception of the ionomycin treatment, which was performed at conditions described for culture of COCs. Activation was performed in Universal IVF medium with Paraffin overlay by consecutive exposure of oocytes to 5 μM calcium ionomycin (Sigma, St. Louis, MO) for 5 min and 1 mM 6-DMAP (Sigma) for 4 h with careful washing of oocytes from each reagent in Universal IVF medium. Oocytes were then placed in fresh IVF medium with Paraffin overlay following culture. The next day (day 1) further cultivation of the parthenogenetic embryos was performed using sequential BlastAssist System media (MediCult) according to the manufacturer's recommendations. On days 5 through 6 of culture, derived blastocysts were used for the isolation of inner cell mass (ICM).

Isolation of ICM of blastocysts and culture of parthenogenetic hESCs

The zona pellucida was removed by 0.5% pronase (Sigma) treatment. For immunosurgery, the blastocyst was incubated with horse antiserum to human spleen cells followed by exposure to guinea pig complement. Trophectoderm cells were removed from the ICM by gently pipetting the treated blastocyst. For the derivation of phESC from the whole blastocysts, the blastocysts were placed on a feeder layer of mitomycin C mitotically inactivated human neonatal skin fibroblasts (NSF) in medium designed for the culture of phESC. When a blastocyst attached and trophoblast cells spread, the ICM became visible. Through 3 to 4 days of additional culture, the ICM was isolated through mechanical slicing of the ICM from the trophectoderm outgrowth using a finely drawn glass pipette. Further culture of isolated ICMs was performed on mitomycin C mitotically inactivated human NSF derived under informed consent from a genetically unrelated donor. NSF were derived using medium containing human umbilical cord blood serum instead of animal serum. Before medium preparation, serum was screened for syphilis, HIV, HBV, and HCV.

The medium for the culture of NSF consisted of 90% DMEM (high glucose, with L-glutamine) (Invitrogen, San Diego, CA), 10% human umbilical cord blood serum, and penicillin–streptomycin (100 U/100 μg) (Invitrogen).

For the culture of ICM and phESC we used VitroHES (Vitrolife, Kangsbacka, Sweden) supplemented with 4 ng/ml hrbFGF (Chemicon, Temecula, CA), 5 ng/mL hrLIF (Chemicon), and 10% human umbilical cord blood serum. The ICM was mechanically plated on a fresh feeder layer and cultured for 3 to 4 days. The first colony was mechanically cut and replated after 5 days of culture. All subsequent passages were made after 5 to 6 days in culture. For early passages, colonies were mechanically divided into clumps and replated. Further passing of phESC was performed with collagenase IV treatment and mechanical dissociation. The propagation of phESC was performed at 37°C, 5% CO_2 in a humidified atmosphere.

The phESC characterization

For immunostaining, phESC colonies were fixed with 4% paraformaldehyde during 20 min at room temperature for staining of SSEA-1, 3, 4 markers, or with 100% methanol for 5 min at −20°C for other markers. Monoclonal antibodies used were: SSEA-1 (MAB4301), SSEA-3 (MAB4303), SSEA-4 (MAB4304), OCT-4 (MAB4305), TRA-1-60 (MAB4360), and TRA-1-81 (MAB4381) from Chemicon. Secondary antibodies Alexa Fluor 546 (orange-fluorescent) and 488 (green-fluorescent) were from Molecular Probes (Invitrogen). Nuclei were stained with DAPI (Sigma). Alkaline phosphatase and telomerase activity were detected with an AP kit and a TRAPEZE Kit (Chemicon). Karyotype was determined by a standard G-banding method.

Embryoid body formation and further differentiation

The phESC colonies were mechanically divided into clumps and placed in the wells of 24-well cluster plates precoated with 2% agarose (Sigma) in medium containing 85% Knockout DMEM, 15% human umbilical cord blood serum, 1×MEM NEAA, 1 mM Glutamax, 0.055 mM β-mercaptoethanol, penicillin–streptomycin (50 U/50 μg) (all from Invitrogen, except serum). Embryoid bodies were cultured for 14 days in suspension and then placed onto a dish to develop outgrowths, or cultivated in suspension for an additional week.

Neural differentiation was induced by the cultivation of 2-week-old embryoid bodies attached to a culture dish surface over the period of a week in differentiation medium: DMEM/F12, B27, 2 mM Glutamax, penicillin–streptomycin (100 U/100 μg) and 20 ng/mL hrbFGF (all from Invitrogen). Some embryoid bodies gave rise to differentiated cells with neural morphology; others were dissected and additionally cultured to produce neurospheres.

Beating embryoid bodies appeared spontaneously following 5 days of culture after plating on an adhesive surface in the same medium as was used for embryoid body generation.

Immunocytochemistry of differentiated derivatives of phESC

Embryoid bodies, neurospheres, or contractile embryoid bodies were placed on poly-D-lisyne (Sigma) treated microcover glasses (VWR Scientific Inc., West Chester, PA) and cultured for approximately 1 week in the appropriate differen-

tiation medium. For immunostaining, differentiated cells were fixed with 100% methanol for 5 min at −20°C.

For the detection of ectodermal markers, we used monoclonal mouse antineurofilament 68 antibody (Sigma), anti-human CD56 (NCAM) antibody (Chemicon), and anti-beta III tubulin antibody (Chemicon) to highlight neuronal markers. We used antiglial fibrillary acidic protein (GFAP) antibody (Chemicon) to detect the glial cell marker.

For the detection of the mesodermal markers in 3-week-old embryoid bodies or in contractile embryoid bodies, we used monoclonal mouse antidesmin antibody (Chemicon), antihuman alpha-actinin antibody (Chemicon) as the muscle specific markers, and antihuman CD31/PECAM-1 antibody (R&D Systems, Minneapolis, MN), antihuman VE-Cadherin (CD144) antibody (R&D Systems) as the endothelial markers.

For the detection of the endodermal markers in embryoid bodies, we used monoclonal mouse antihuman alpha-fetoprotein antibody (R&D Systems). Secondary antibodies Alexa Fluor 546 (orange-fluorescent) and 488 (green-fluorescent) were from Molecular Probes (Invitrogen). Nuclei were stained with DAPI (Sigma).

HLA typing

Genomic DNA was extracted from blood, cumulus cells, phESC, and NSF with Dynabeads DNA Direct Blood from Dynal (Invitrogen). HLA typing was performed by PCR with allele-specific sequencing primers (PCR-SSP, Protrans, Indianapolis, IN). All manipulations were performed accordingly manufacturer's recommendations.

Affymetrix SNP microarray analysis

Genomic DNA was isolated from blood, cumulus cells, phESC, and NSF by phenol/chlorophorm extraction method. The DNA samples obtained from four Caucasian subjects were genotyped with Affymetrix Mapping 50 K Hind 240 Array (part of Affymetrix GeneChip Mapping 100 K kit). Initially, the dataset contained 57,244 binary SNP markers. Since the number of markers is more than would be necessary to identify the equivalency of genomic samples and to study heterozygosity, 15 (chromosomes 1–15) out of 22 autosomal chromosomes were chosen. The shorter seven chromosomes were removed to reduce the chance that no marker, or only a single marker for a given chromosome, is selected during random sampling. The 1459 markers tested were analyzed with Relcheck (version 0.67, copyright (©) 2000 Karl W. Broman, Johns Hopkins University, Licensed under the GNU General Public License version 2, June, 1991) (Boehnke and Cox, 1997; Broman and Weber, 1998). The Relcheck program identifies five types of relationships; monozygous (MZ) twins, parent/offspring pair, full siblings (full sibs), half sibs, and unrelated. Full sibs share approximately 50% of their genome identical by descent. In the results from paired samples, the marker data were consistent with this proportion. An "unrelated" pair shares less of their genome than "half-sibs."

Analysis of imprinted genes

Total RNA was extracted as described (Chomczynski and Sacchi, 1987) and precipitated with isopropanol. Residual genomic DNA was removed using an RNAse free DNAse treatment (Promega, Madison, WI). cDNA was synthesized from 1 μg total RNA using RevertAid M-MuLV reverse transcriptase (Fermentas) in 20 μL of the reaction volume. The PCR reactions were performed with 1 μL cDNA, using Taq DNA polymerase (Fermentas, Hanover, MD). All reactions were performed according to the manufacturer's instructions.

The sequence of the primers and PCR conditions were as follows: TSSC5 (Lee et al., 1998) forward primer 5′-GCTCTTCATGGTCATGTTCTCCA-3′ and reverse primer 5′-GGAGCAGTGGTTGTACAGAGG-3′, at conditions of 94°C for 4 min for one cycle; 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min for 33 cycles. The product size was 364 bp. H19 (Hashimoto et al., 1995) forward primer 5′-TACAACCACTGCACTACCTG-3′ and reverse primer 5′-TGGCCATGAAGATGGAGTCG-3′, at conditions of 94°C for 4 min for one cycle; 94°C for 1 min, 52°C for 1 min, and 72°C for 1 min for 38 cycles. The product size was 148 bp. PEG1_1 (Li et al., 2002) forward primer 5′-GAG TCC TGT AGG CAA GGT CTT ACC T-3′ and reverse primer 5′-CTT GCC TGA AGA CTT CCA TGA GTG A-3′, at conditions of 94°C for 4 min for one cycle; 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min for 35 cycles. The product size was 155 bp. PEG1_2 (Li et al., 2002) forward primer 5′-GCT GCT GGC CAG CTC TGC ACG GCT G-3′ and reverse primer 5′-CTT GCC TGA AGA CTT CCA TGA GTG A-3′, at conditions of 94°C for 4 min for one cycle; 94°C for 1 min, 65°C for 1 min, and 72°C for 1 min for 39

cycles. The product size was 230 bp. SNRPN (Glenn et al., 1993) forward primer 5′-CTTAGCTGAGACACCAAGAGG-3′ and reverse primer 5′-GCAGCATCTTGCTACTCTTGC-3′, at conditions of 94°C for 4 min for 1 cycle; 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min for 33 cycles. The product size was 246 bp. GAPDH (Adjaye et al., 1999) forward primer 5′-ACCACAGTCCATGCCATCAC-3′ and reverse primer 5′-TCCACCACCCTGTTGCTGTA-3′, at conditions of 94°C for 4 min for 1 cycle; 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min for 21 cycles. The product size was 450 bp.

The PCR products were analyzed by 5% polyacrylamide gel electrophoresis (5 μL/line), stained with ethidium bromide, and documented using the BioImaging system (UVP, Upland, CA). RT-PCR experiments were performed repeatedly with reproducible results. GAPDH served as a ubiquitously expressed control. Genomic contamination was ruled out by including an RT-negative sample (without reverse transcriptase, at the reverse transcription step) in each PCR set as a control.

Teratoma formation and immunohistochemical analysis

All animal procedures were carried out by Biocon (Rockville, MD) in accordance with Animal Welfare Act; the Guide for the Care and Use of Laboratory Animals; and the Principles for the Use of Experimental Animals. Biocon is accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care International (AAALAC) since 1981, #000529, and has an approved Animal Welfare Assurance Statement on file with the Office of Laboratory Animal Welfare (OLAW), #A3267 01, and is registered with the U.S. Department of Agriculture (USDA), #51 R 0032.

For injection to immunodeficient severe combined immunodeficient (SCID) beige mice and HSDRHCOr rats (Harlan) the phESC were enzymatically removed from the culture dishes using collagenase type IV and resuspended into clumps. Approximately 2 to 5 million phESC cells were injected into the upper hind limb subcutaneous space. After approximately 2 months of growth, formed teratomas were removed and fixed with 4% paraformaldehyde. Half of the tissue was cryoprotected in sucrose and the other half was mounted in 5% agar–agar and sectioned in 60-μm slices using a vibratome. Sections were mounted on glass slides and stained with hematoxylin/eosin or used for immunochistochemistry. Hematoxylin was from Fisher and eosin-Y from Sigma. Antibodies used were mouse monoclonal antibody against neuronal class III beta-tubulin (TUJ1) (Covance, Richmond, CA) for ec-

Table 1. Generation of Parthenotes and Parthenogenetic Embryonic Stem Cell Lines

					Blastocysts derived[f]			
Donor	*Oocytes derived*	*Oocytes donated*	*Oocytes activated*	*Parthenotes created*[e]	*with ICM*	*without visible ICM*	*Lines generated*	*Donor destiny*
1	8	4	4	4	2	—	phESC-1 immunosurgery	pregnant
2	15	8	8	8	3	3	phESC-3 phESC-4 phESC-5 all from whole blastocysts	pregnant twins
3	27	14	14[a,b]	11	3	2	phESC-6 from whole blastocyst	pregnant
4	22	11	11[c]	10	2	3	phESC-7 from whole blastocyst	pregnant
5	20	9[d]	7	7	1	4	no cell line generated	not pregnant

[a]Two oocytes were not activated.
[b]One oocyte degenerated after activation.
[c]One oocyte was not activated.
[d]Two oocytes were at metaphase I stage and were discarded.
[e]Total parthenogenetically activated oocytes = 40.
[f]Total blastocytst derived = 23.

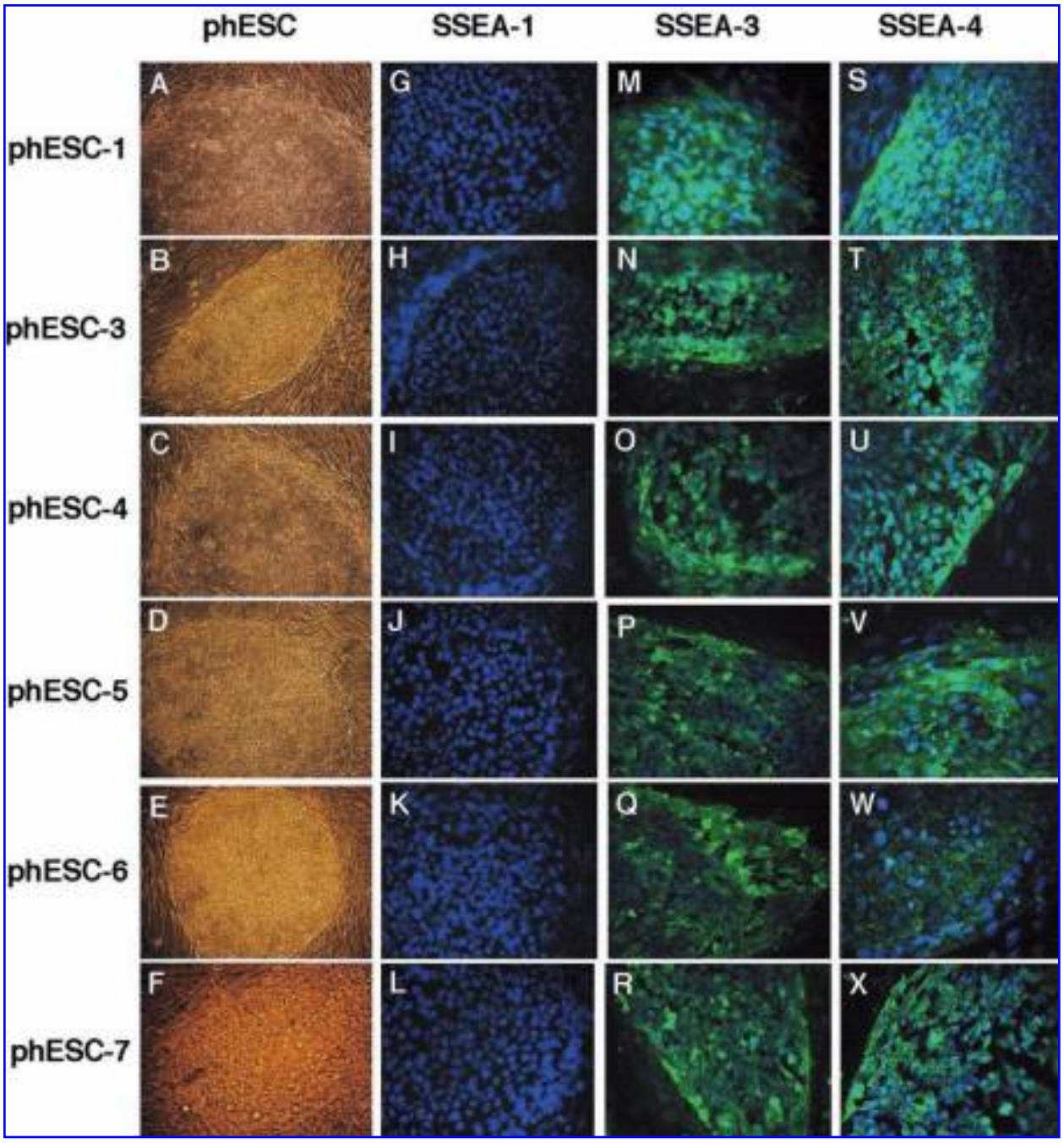


FIG. 1. Characterization phESC lines for specific markers. Undifferentiated colonies of phESC on human feeder layer cells (**A–F**), negative staining for SSEA-1 (**G–L**), expression of cell surface markers SSEA-3 (**M–R**), SSEA-4 (**S–X**). Original magnification (**A–E**) ×100; (**F**) ×200; (**G–X**) ×400. Alkaline phosphatase-positive staining of phESC colonies on feeder cells (**A–F**), OCT-4 (**G–L**), TRA-1-60 (**K–R**), and TRA-1-81 (**S–X**). Original magnification (**A, B, O, R**) ×100; (**C–F, M, S, X**) ×200; (**G–L, N, P, Q, T–W**) ×400.

toderm; mouse antihuman muscle actin monoclonal antibody (Dako, Carpinteria, CA) and polyclonal rabbit antihuman fibronectin antibody (Dako) for mesoderm; and mouse monoclonal anti-alpha-fetoprotein antibody (Sigma) for endoderm. Secondary goat antimouse Alexa Fluor 546 (orange-fluorescent) and goat antirabbit Alexa Fluor 488 (green-fluorescent) antibody were from Molecular Probes (Invitrogen). Nuclei were stained with DAPI (blue) (Sigma).

About 2 to 5 million mitomycin-C-treated human fibroblasts used as feeder layers for the phES

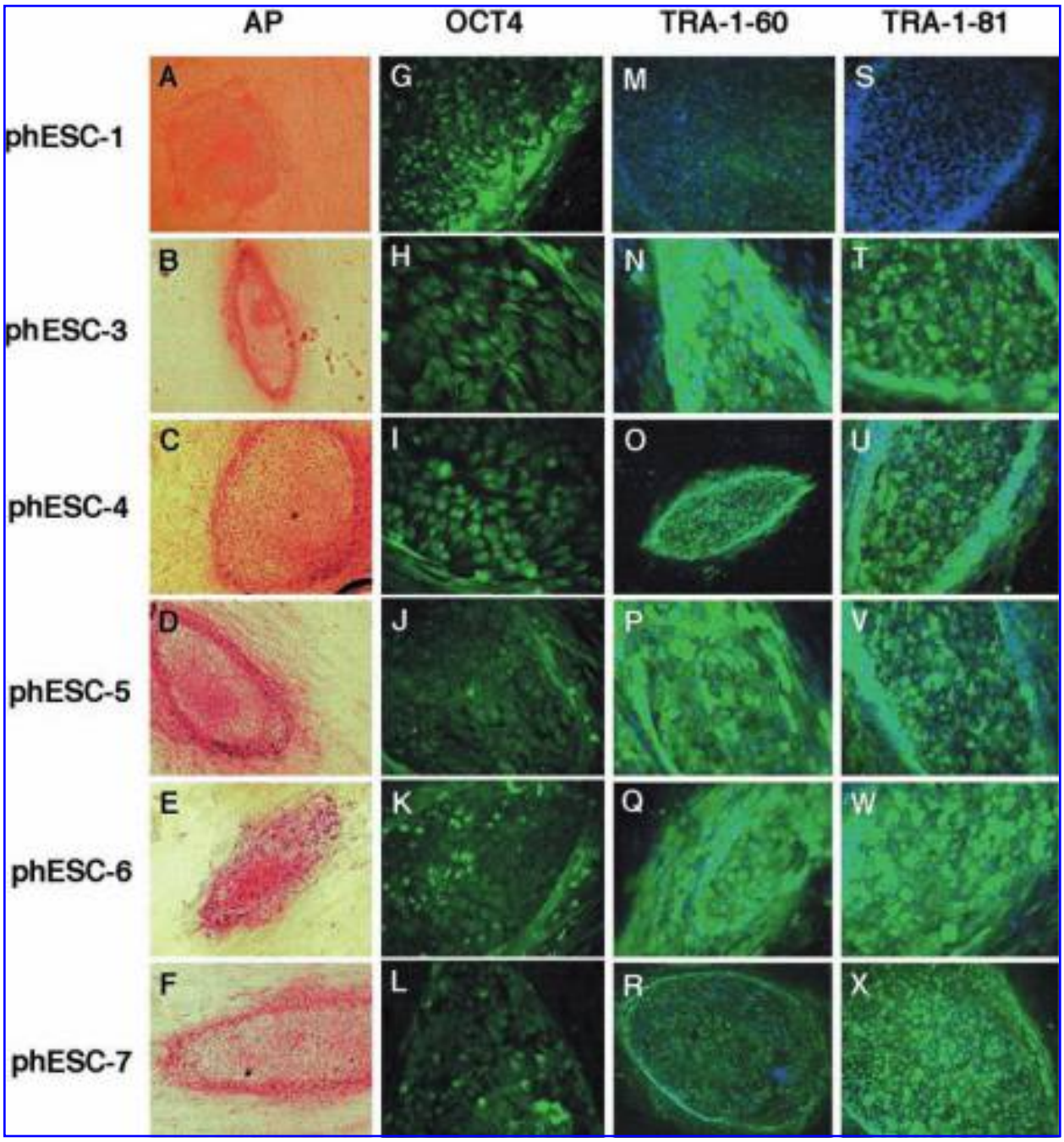


FIG. 1. (*Continued*).

cells were injected as controls. No teratoma growth was observed in the control animals.

RESULTS

Generation of parthenogenetic embryos

Five oocyte donors, all over 31 years of age, participated in this study. Oocytes were obtained using hormonal stimulation with the primary intent of IVF. A total of 46 COCs were taken from five donors and used for this study (Table 1). Before oocyte activation, COCs were held at atmospheric oxygen tension. After removal of cumulus cells only normal metaphase II oocytes with distinct first polar body were taken for activation procedure. We activated oocytes with 5 μM ionomycin for 5 min, followed by incubation with 1 mM 6-DMAP for 4 h to prevent the extrusion of the second polar body and produce diploid em-

bryos. Manipulation and culture of oocytes and embryos was performed in MediCult media in accordance with manufacturer's recommendations using standard IVF procedures and under reduced oxygen (90% N_2 + 5% O_2 + 5% CO_2). Only 40 oocytes were capable of cleavage after parthenogenetic activation. These procedures permitted us to produce 23 blastocysts on day 5 or 6 of embryo culture. Eleven of the blastocysts had visible ICMs (Table 1).

Derivation of parthenogenetic hESC lines

It is very important to minimize, if not eliminate, components of animal origin in the derivation and culture of hESCs destined for clinical use. To this end, some researchers did not use immunosurgery for ICM isolation, but rather used mechanical means to isolate the ICMs from whole blastocysts (Kim et al., 2005). Culture media and feeder cells may also contain animal pathogens. To eliminate possible contamination, researchers have used human cells as feeder instead of mouse fibroblasts (Cheng et al., 2003; Hovatta et al., 2003; Stojkovic et al., 2005) or have cultured hESCs in feeder-free and serum-free conditions (Amit et al., 2004; Klimanskaya et al., 2005). Taking into account these previous investigations, we modified the culture conditions under which we isolated the ICM and cultured the phESC.

For the derivation and culture of phESC we used mitomycin C mitotically inactivated human NSF as feeder cells. These cells originally were derived and propagated with medium containing human umbilical cord blood serum instead of animal serum. The phESC culture medium consisted of VitroHES medium (Vitrolife) supplemented with human serum derived from umbilical cord blood, hrbFGF, and hrLIF. The phESC were propagated in a 37°C, 5% CO_2, humidified atmosphere.

All derived parthenogenetic blastocysts were initially treated with 0.5% pronase to remove the zona pellucida. We isolated well-formed ICMs from two blastocysts obtained from the first oocyte donor using trypsin treatment (Li et al., 2003) and traditional immunosurgery (Solter and Knowles, 1975). The ICMs were further placed on human feeder cells at described conditions to produce phESC. The ICM from trypsin-treated blastocyst did not give live cells. The ICM derived after immunosurgery displayed cell outgrowth, resulting in the creation of the phESC-1 cell line. The other 21 whole blastocysts were initially placed on the feeder at described conditions. The ICMs were isolated by mechanical slicing from sprawled trophoblast cells and replaced onto fresh feeder cells. Five phESC lines (from phESC-3 to phESC-7) were generated in this manner (Table 1).

The characterization of phESC lines

The phESC lines display a morphology expected in hESCs and form colonies with tightly packed cells, prominent nucleoli, and a small cytoplasm to nucleus ratio (Fig. 1). These cells

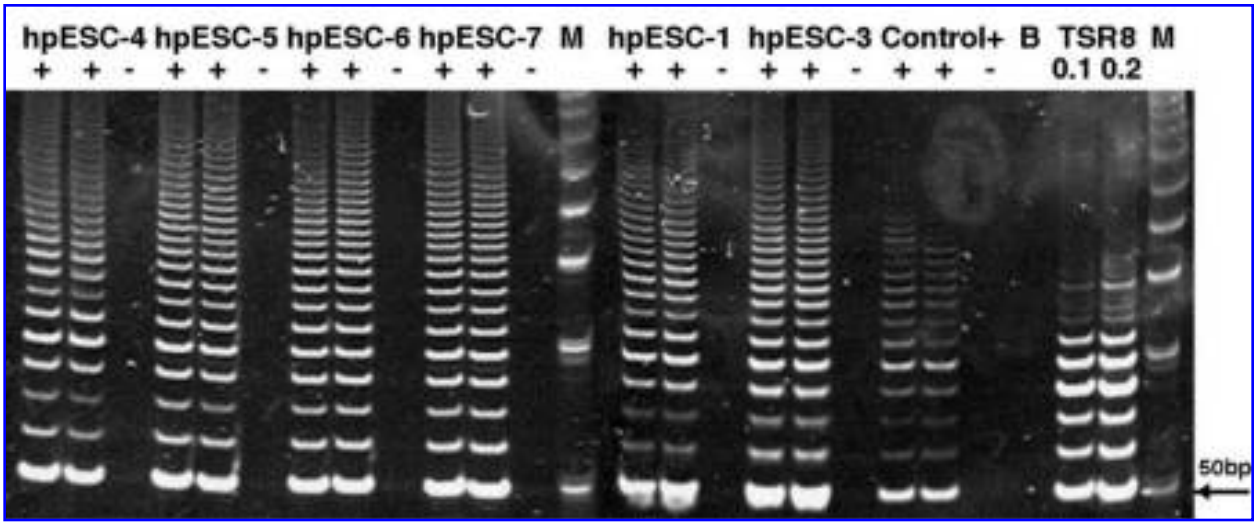


FIG. 2. The phESC demonstrate high level of telomerase activity by comparison with positive control cells: "+"—the extract from 500 cells; "−"—heat-treated cell extract with inactivated telomerase; "Control +"—telomerase-positive cell extract (applied with TRAPEZE Kit); "B"—CHAPS lysis buffer, primer-dimer/PCR contamination control; TSR8—telomerase quantitative control template (0.1 and 0.2 amol/μL); "M"—marker, DNA ladder.

express traditional hES cell markers SSEA-3, SSEA-4, (Fig. 1) TRA-1-60, TRA-1-81, and OCT-4, (Fig. 1) and do not express SSEA-1, a positive marker for undifferentiated mouse embryonic stem cells (Fig. 1). The cells derived from all lines demonstrate high levels of alkaline phosphatase (Fig. 1) and telomerase activity (Fig. 2).

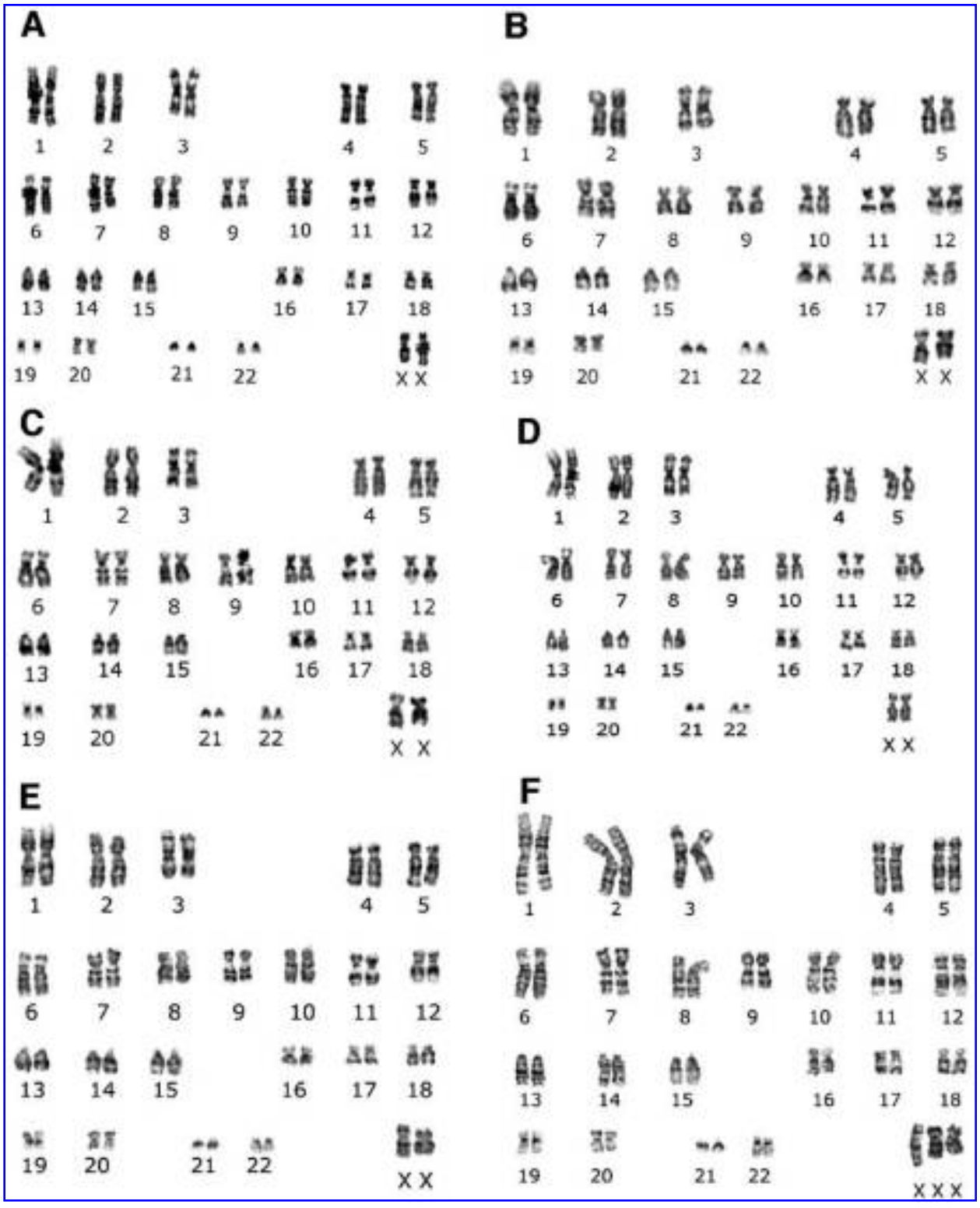
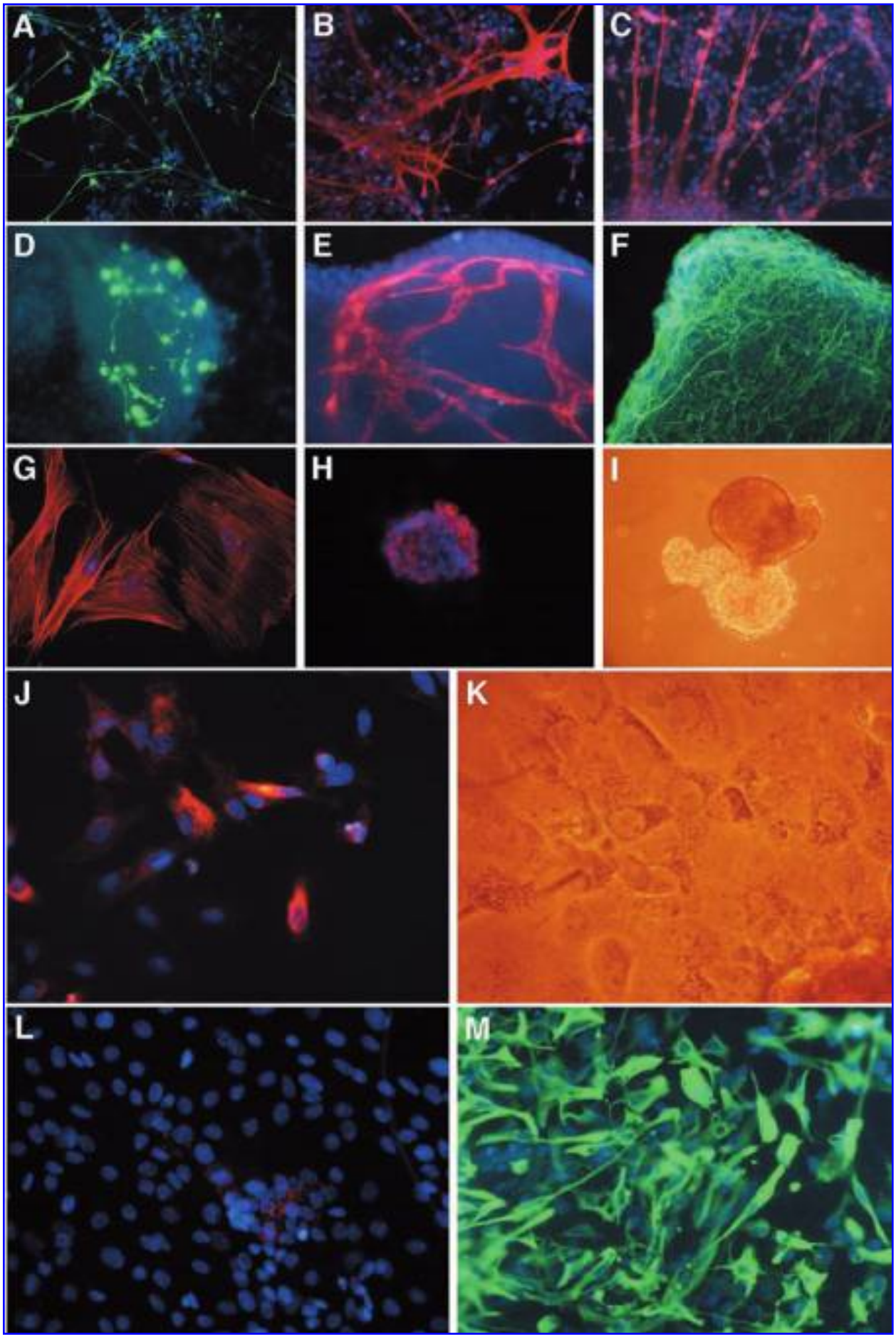


FIG. 3. The G-banded karyotyping of phESC lines. The phESC-1 (**A**), phESC-3 (**B**), phESC-4 (**C**), phESC-5 (**D**), and phESC-6 (**E**) lines have a normal 46, XX karyotype. The phESC-7 line has 47,XXX karyotype (**F**).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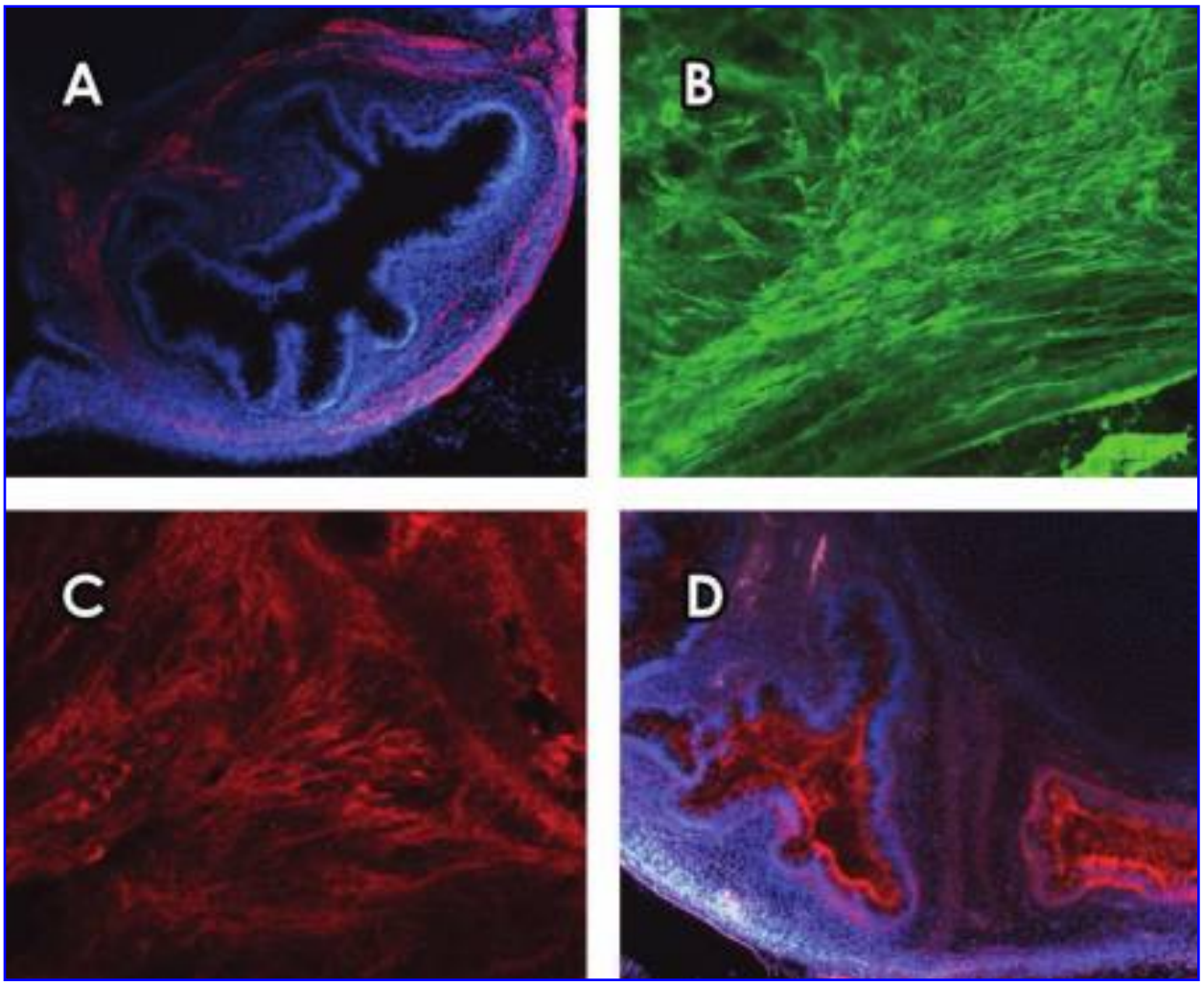

FIG. 5. The *in vivo* differentiation of phESC and teratoma formation in SCID mice. Immunofluorescence staining for the markers of three germ layers. The muscle actin, a mesodermal cell marker, is organized surrounding other components and is clearly identifiable (**A**). The presence of fibronectin in higher quantities is specific for connective tissue of mesodermal origin (**B**) The areas of neural differentiated cells (ectodermal origin) are extensive and labeled intensively with antybodies for beta tubulin (**C**). Alpha-fetoprotein, an immature endodermal cell marker, can be retrieved in areas of glandular appearance (**D**). The nuclei were staned with DAPI (blue)—(**A, D**). Original magnification (**A**), (**C**), (**D**) ×100; (**B**) ×200.

G-banded karyotyping showed that phESC lines have a normal human 46,XX karyotype, with the exception of the phESC-7 line (Fig. 3). Approximately 91% of cells from the phESC-7 line have a 47,XXX karyotype and 9% of the cells have a 48,XXX,+6 karyotype. We observed a different degree of X chromosome heteromorphism by analysis of 100 metaphases in our cell lines: approximately 12% of cells for the phESC-1 and phESC-6 lines showed X chromosome heteromorphism; 42% of cells for the phESC-5 line and in 70%, 80%, and 86% for the cell lines phESC-7, phESC-3, and phESC-4, respectively, showed X chromosome heteromorphism (Fig. 3).

FIG. 4. The *in vitro* differentiation of phESC into derivatives of all three germ layers. Ectoderm differentiation is presented by positive immunocytochemical staining for neuron-specific markers neurofilament 68 (**A**), NCAM (**B**), beta III-tubulin (**C**), and glial cell marker GFAP (**D, M**). Differentiated cells were positive for mesoderm markers: muscle specific alpha-actinin (**G**) and desmin (**J**), endothelial markers PECAM-1 (**E**), and VE-Cadherin (**F**). Endoderm differentiation is presented by positive staining for alpha-fetoprotein (**H, L**). The phESC produce pigmented epithelial-like cells (**I, K**). Original magnification (**I**) ×100; (**A–H, J–M**) ×400.

Table 2. Identifying DNA Samples from phESC and Related Donors

Genotype 1	Genotype 2	Putative relationship	Inferred relationship	IBS			n_typed	LOD				
				0	1	2		MZtwins	Par/off	Fullsibs	Halfsibs	Unrelated
1	2	unrelated	unrelated	166	662	631	1459	−1503.03	−300.45	−23.15	−8.41	0
1	3	unrelated	unrelated	241	616	602	1459	−1560.65	−434.85	−28.04	−12.22	0
1	4	unrelated	unrelated	225	623	611	1459	−1535.94	−400.61	−31.39	−14.39	0
1	5	unrelated	unrelated	225	623	611	1459	−1535.94	−400.61	−31.39	−14.39	0
1	6	unrelated	unrelated	243	644	572	1459	−1642.35	−445.78	−31.74	−14.54	0
1	7	unrelated	unrelated	252	638	569	1459	−1641.11	−453.5	−29.25	−12.86	0
1	8	unrelated	unrelated	250	643	566	1459	−1656.02	−460.02	−32.86	−15.32	0
1	9	unrelated	unrelated	219	657	583	1459	−1605.31	−382.39	−27.37	−11.58	0
1	10	unrelated	unrelated	158	707	594	1459	−1591.43	−279.21	−26.37	−10.89	0
1	11	unrelated	unrelated	193	668	598	1459	−1584.71	−354.76	−29.65	−13.31	0
1	12	unrelated	unrelated	166	671	622	1459	−1523.1	−300.5	−30.53	−13.92	0
2	3	unrelated	full sibs	0	282	1177	1459	−440.02	−146.3	0	−167.42	−363.63
2	4	unrelated	unrelated	233	627	599	1459	−1569.66	−423.24	−28.24	−12.91	0
2	5	unrelated	unrelated	233	627	599	1459	−1569.66	−423.24	−28.24	−12.91	0
2	6	unrelated	unrelated	217	650	592	1459	−1584.75	−388.44	−22.62	−8.53	0
2	7	unrelated	unrelated	243	650	566	1459	−1645.94	−437.91	−23.23	−8.72	0
2	8	unrelated	unrelated	225	649	585	1459	−1603.18	−404.41	−27.04	−11.97	0
2	9	unrelated	unrelated	210	639	610	1459	−1532.75	−360.46	−24.72	−9.89	0
2	10	unrelated	unrelated	144	683	632	1459	−1491.18	−243.56	−16.82	−4.51	0
2	11	unrelated	unrelated	172	680	607	1459	−1556.46	−310.03	−23.5	−9.7	0
2	12	unrelated	unrelated	176	667	616	1459	−1538.57	−327.95	−27.31	−12.06	0
3	4	unrelated	unrelated	336	457	665	1459	−1391.57	−599.92	−30.6	−14.62	0
3	5	unrelated	unrelated	336	457	665	1459	−1391.57	−599.92	−30.6	−14.62	0
3	6	unrelated	unrelated	322	482	655	1459	−1415.98	−571.23	−26.08	−11.86	0
3	7	unrelated	unrelated	369	442	648	1459	−1432.05	−664.95	−27.39	−11.93	0
3	8	unrelated	unrelated	334	483	642	1459	−1449.86	−597.75	−31.68	−15.14	0
3	9	unrelated	unrelated	307	493	659	1459	−1395.19	−530.45	−24.56	−10	0
3	10	unrelated	unrelated	215	623	621	1459	−1503.92	−364.97	−17.26	−4.43	0
3	11	unrelated	unrelated	264	582	613	1459	−1531.91	−473.48	−28.41	−12.81	0
3	12	unrelated	unrelated	254	595	610	1459	−1544.73	−460.57	−29.92	−13.88	0
4	5	unrelated	MZ twins	0	0	1459	1459	0	−379.58	−45.47	−401.67	−677.74
4	6	unrelated	unrelated	334	475	650	1459	−1436.59	−599.55	−342.73	−15.19	0
4	7	unrelated	unrelated	365	439	655	1459	−1418.34	−656.01	−31.6	−14.56	0
4	8	unrelated	unrelated	329	486	644	1459	−1450.75	−586.4	−32.06	−14.88	0
4	9	unrelated	unrelated	332	466	661	1459	−1395.18	−590.12	−28.69	−12.94	0
4	10	unrelated	unrelated	245	606	608	1459	−1542.32	−438.93	−28.75	−12.74	0
4	11	unrelated	unrelated	273	569	617	1459	−1530.97	−492.84	−29.03	−12.34	0
4	12	unrelated	full sibs	0	224	1235	1459	−326.17	−162.34	0	−183.44	−393.46
5	6	unrelated	unrelated	334	475	650	1459	−1436.59	−599.55	−32.73	−15.19	0
5	7	unrelated	unrelated	365	439	655	1459	−1418.34	−656.01	−31.6	−14.56	0
5	8	unrelated	unrelated	329	486	644	1459	−1450.75	−586.4	−32.06	−14.88	0

5	9	unrelated	unrelated	332	466	661	1459	−1395.18	−590.12	−28.69	−12.94	0
5	10	unrelated	unrelated	245	606	608	1459	−1542.32	−438.93	−28.75	−12.74	0
5	11	unrelated	unrelated	273	569	617	1459	−1530.97	−492.84	−29.03	−12.34	0
5	12	unrelated	full sibs	0	224	1235	1459	−326.17	−162.34	0	−183.44	−393.46
6	7	unrelated	full sibs	45	176	1238	1459	−277.78	−217.21	0	−165.72	−390.62
6	8	unrelated	full sibs	44	187	1228	1459	−289.8	−201.32	0	−153.75	−365.51
6	9	unrelated	unrelated	333	481	645	1459	−1436.5	−595.4	−30.3	−13.77	0
6	10	unrelated	unrelated	240	601	618	1459	−1518.17	−425.03	−27.11	−11.53	0
6	11	unrelated	full sibs	0	164	1295	1459	−209.27	−191.66	0	−213.25	−440.56
6	12	unrelated	unrelated	234	615	610	1459	−1547.15	−416.14	−30.21	−13.64	0
7	8	unrelated	full sibs	38	225	1196	1459	−326.62	−150.16	0	−121.55	−334.09
7	9	unrelated	unrelated	359	473	627	1459	−1479.28	−642.41	−30.61	−14.47	0
7	10	unrelated	unrelated	252	623	584	1459	−1598.35	−443.81	−28.88	−13.09	0
7	11	unrelated	full sibs	0	230	1229	1459	−318.49	−137.93	0	−159.55	−389.58
7	12	unrelated	unrelated	265	583	611	1459	−1539.33	−472.91	−30.55	−13.87	0
8	9	unrelated	unrelated	347	480	632	1459	−1472.41	−625.68	−30.93	−14.31	0
8	10	unrelated	unrelated	244	614	601	1459	−1561.3	−434	−28.07	−12.37	0
8	11	unrelated	full sibs	0	175	1284	1459	−223.73	−178.56	0	−200.12	−428.04
8	12	unrelated	unrelated	236	610	613	1459	−1539.08	−17.14	−29.32	−13.14	0
9	10	unrelated	full sibs	0	228	1231	1459	−315.15	−152.88	0	−174.27	−392.91
9	11	unrelated	unrelated	269	567	623	1459	−1502.69	−479.57	−28.47	−12.55	0
9	12	unrelated	unrelated	245	612	602	1459	−1557.25	−438.53	−26.07	−11.15	0
10	11	unrelated	unrelated	187	635	637	1459	−1478.7	−328.06	−25.52	−10.6	0
10	12	unrelated	unrelated	181	662	616	1459	−1534.36	−329	−25.2	−10.6	0
11	12	unrelated	unrelated	189	645	625	1459	−1520.01	−337.88	−27.33	−11.72	0

DNA samples were numbered as follows: 1—human neonatal skin fibroblasts, 2—phESC-7 line donor, 3—phESC-7 line, 4—phESC-1 line, 5—phESC-1 line, 6—phESC-3 line, 7—phESC-4 line, 8—phESC-5 line, 9—phESC-6 line, 10—phESC-6 line donor, 11—phESC-3 to phESC-5 lines donor, 12—phESC-1 line donor. The IBS columns in the output display the number of markers at which the pair are both typed and share 0, 1, or 2 alleles identical by state. (For MZ twins under the ideal condition of no genotyping errors, all markers must be placed under IBS = 2). The output does not display P (observed markers | given relationship) directly, but it displays LOD score = $\log_{10}$ [P (observed markers | putative relationship)/P(observed markers | relationship for which maximum likelihood was obtained and thus the call was made)] as a measure of similarity. The smaller this LOD score is, the less likely is the putative relationship between two samples.

Differentiation capacity of phESC

The phESC-1 line remained undifferentiated during 10 months of culture spanning 35 passages. The other cell lines were successfully cultivated over at least 21 passages. The cells from all phESC lines formed cystic embryoid bodies in suspension culture and gave rise to derivatives of all three germ layers, ectoderm, mesoderm, and endoderm, after differentiation *in vitro* (Fig. 4). Approximately 5% of embryoid bodies from the phESC-1 line gave rise to beating cells 5 days following plating (Movie 1, supplemental material; www.liebertonline.com/doi/suppl/10.1089/clo.2007.0033/suppl_file/MovieS1.avi). The phESC-6 line produced pigmented epithelial-like cells (Fig. 4I and K). Ectoderm differentiation is presented by positive immunocytochemical staining for neuron specific markers neurofilament 68 (Fig. 4A), NCAM (Fig. 4B), beta III-tubulin (Fig. 4C), and the glial cell marker GFAP (Fig. 4D and M). Differentiated cells were positive for mesoderm markers including alpha-actinin (Fig. 4G) and desmin (Fig. 4J), which are muscle-specific markers, and the endothelial markers PECAM-1 (Fig. 4E) and VE-Cadherin (Fig. 4F). Endoderm differentiation is presented by positive staining of differentiated derivatives for alpha-fetoprotein (Fig. 4H and L). The ability of phESC lines to form derivatives from all three germ layers was investigated *in vivo* by subcutaneous injection of phESC into immunodeficient mice and rats (Fig. 5). Cells from all phESC lines were capable of forming teratomas approximately two months after injection. Histological examination demonstrated the presence of organized structures, including epithelia, capsula, smooth muscle, adipose tissue, hematogenic tissue, neural tubes, and glandular epithelia (data not shown). Immunohistochemical analysis revealed positive staining for beta-tubulin (Fig. 5C)—ectoderm marker, fibronectin (Fig. 5B), and muscle actin (Fig. 5A)—mesoderm markers, and alpha-fetoprotein (Fig. 5D)—endoderm marker. These data demonstrate that phESC can be differentiated *in vivo* into the three germ layers that lead to all cell types found in a human body.

DNA profiling of phESC lines

We performed comparative DNA profiling of all the phESC lines, the donor somatic cells, and the feeder cells. These studies used Affymetrix single-nucleotide polymorphism (SNP) microarrays (Mapping 50 K Hind 240 Arrays) to confirm the genetic similarity of the phESC to the donor's somatic cells. A total of 1459 SNP markers across 15 autosomes (chromosomes 1–15) were chosen with median intermarker distance of 1.12 Mbp. All paired genotype relationships between phESC lines and their associated donor somatic cells were identified as "full siblings" (genetically matched), and all the other combinations of pairs were identified as "unrelated." Internal controls identified the paired genotype relationship between split cultures derived from the same phESC line as "monozygous twins" (Table 2).

Comparative analysis of SNP markers revealed, that on the whole, donor cells do not seem to exhibit a clear pattern of heterozygosity trend across distances from centromeres, whereas stem cells display somewhat lower proportions of heterozygosity near centromeres and telomeres in comparison to heterozygosity proportions in the middle as a result of probable chromosome recombination (Supplemental material; www.liebertonline.com/doi/suppl/10.1089/clo.2007.0033/suppl_file/SNP_Analysis.pdf).

Based on the HLA-typing results, stem cells derived of all phESC lines appeared MHC-matched with the oocyte donors, making this a possible method to create cells for therapeutic use (Table 3). HLA-analysis of the genetic material from the human fibroblasts used as feeder cells revealed no contamination of the phESC lines with material from the human fibroblasts (Table 3).

Analysis of imprinted genes

Alterations of genomic imprinting in human embryos can contribute to the development of disorders linked to maternally or paternally expressed genes (Gabriel et al., 1998). Studies of imprinting in phESC require a detailed investigation because of the possible influence upon phESC differentiation and functionality of their derivatives. As a preliminary study, we performed expression analysis of the human imprinted genes (Morison et al., 2005) TSSC5, H19, PEG1, and SNRPN in undifferentiated phESC (Fig. 6). Two hESC lines derived from discarded IVF embryos were used as controls. The transcripts of maternally expressed genes TSSC5 (Morison et al., 2005) and H19 (Morison et al., 2005) were observed in all phESC lines and also in control lines. The human PEG1 gene is transcribed from two alternative promoters (Li et al., 2002). The gene region from the first promoter is biallelically

Table 3. HLA Typing Results

	MHC I			*MHC II*		
	HLA-A	*HLA-B*	*HLA-C*	*DRB1*	*DQB1*	*DQA1*
phESC-1	A*01	B*15 (63)	Cw*04	DRB1*12	DQB1*06	DQA1*01
	A*02	B*35	Cw*0708	DRB1*13	DQB1*03	DQA1*0505
phESC-1	A*01	B*15 (63)	Cw*04	DRB1*12	DQB1*06	DQA1*01
donor	A*02	B*35	Cw*0708	DRB1*13	DQB1*03	DQA1*0505
phESC-3,	A*02	B*52	Cw*03	DRB1*01	DQB1*05	DQA1*0101
4, 5	A*03	B*22	Cw*04	DRB1*03	DQB1*02	DQA1*05
phESC-3,	A*02	B*52	Cw*03	DRB1*01	DQB1*05	DQA1*0101
4, 5 donor	A*03	B*22	Cw*04	DRB1*03	DQB1*02	DQA1*05
phESC-6	A*02	B*07	Cw*04	DRB1*04	DQB1*06	DQA1*01
	A*03	B*27	Cw*07	DRB1*15	DQB1*03	DQA1*03
phESC-6	A*02	B*07	Cw*04	DRB1*04	DQB1*06	DQA1*01
donor	A*03	B*27	Cw*07	DRB1*15	DQB1*03	DQA1*03
phESC-7	A*01	B*38	Cw*06	DRB1*13	DQB1*06	DQA1*0106
	A*02	B*57	Cw*12	DRB1*14	DQB1*06	DQA1*0103
phESC-7	A*01	B*38	Cw*06	DRB1*13	DQB1*06	DQA1*0106
donor	A*02	B*57	Cw*12	DRB1*14	DQB1*06	DQA1*0103
NSF	A*25	B*15(62)	Cw*12	DRB1*04	DQB1*06	DQA1*01
	A*32	B*18	Cw*12	DRB1*15	DQB1*03	DQA1*03

expressed, and the gene region from the second promoter (isoform 1) is paternally expressed (Li et al., 2002). Expression of the PEG1 gene from the first promoter was not affected in our phESC lines. Analysis of the paternally expressed region of the PEG1 gene and the paternally expressed SNRPN gene (Morison et al., 2005) demonstrated that expression of these genes was significantly downregulated in phESC lines in comparison with control hESC lines (Fig. 6). These results provide further evidence of the parthenogenetic origin of the described phESC lines.

DISCUSSION

The present paper describes the protocol for *in vitro* human parthenogenetic embryo development and subsequent embryonic stem cell derivation. For the generation of parthenogenetic human embryos, we used MediCult media developed for human embryo culture and procedures applied for standard IVF embryos, and cultured them at reduced oxygen. The use of low oxygen concentration in the gas mixture for the development of human parthenogenetic embryos to the blastocyst stage *in vitro* was critical. In general, the oxygen tension in a mammal oviduct and uterus is much less than half of that found in the normal atmosphere (Fischer and Bavister, 1993; Kaufman and Mitchell, 1994). For successful culture of human embryos after IVF, oxygen concentrations of 20% as well as 5% have been used. However, increased oxygen can generate reactive oxygen species that can induce apoptosis (Van Soom et al., 2002). It has been reported that low oxygen concentration increases the viability of preimplantation embryos, assists their normal development, and gives higher incidence of the formation of healthy blastocysts as indicated by greater cell number and a well formed ICM (Dumoulin et al., 1999). In previous investigations, human parthenogenetic embryos were developed *in vitro* using gas mixtures with high (20%) oxygen content (Cibelli et al., 2001; Lin et al., 2003). To produce human parthenogenetic embryos, we used a gas mixture containing 5% oxygen and achieved of 57.5% success rate in the formation of blastocysts.

The next step in the study was to select optimal methods for ICM isolation and phESC culture giving the best possible chance for clinical use of the phESC lines. To accomplish this goal, we derived human skin fibroblasts, propagated them with human umbilical cord blood serum (HUCBS) instead of animal serum and used them as feeder cells. Derivation and culture of phESC lines was performed in VitroHES medium (Vitrolife) designed for hESC culture with the addition of HUCBS. The use of HUCBS in production of hESCs has not been reported in earlier work, and we observed it had positive effects on ICM outgrowth and phESC propagation. The growth of phESC in VitroHES medium was better with

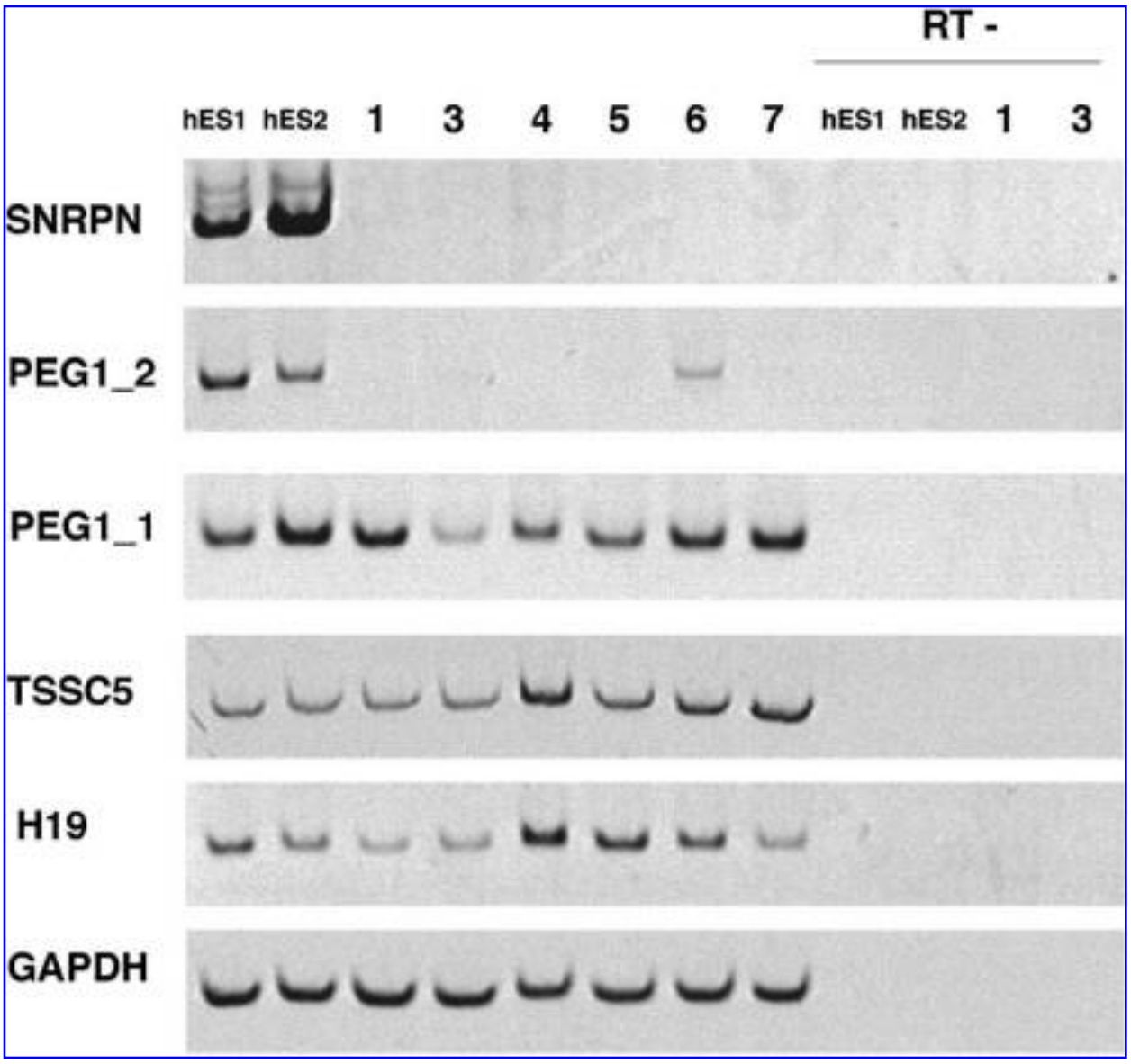

FIG. 6. RT-PCR analysis of imprinted gene expression. Two hESC lines hES1 and hES2 from discarded IVF embryos were used as positive controls for the expression analysis of the paternally expressed genes SNRPN and PEG1_2 and the maternally expressed genes TSSC5 and H19 in phESC lines: phESC-1, phESC-3, phESC-4, phESC-5, phESC-6, and phESC-7 (1, 3, 4, 5, 6, and 7, respectively.) The PEG1_1 gene is biallelically expressed and was used as an additional control. GAPDH was included as mRNA quantitative control. RT data demonstrate no genomic contamination of RT samples.

the addition of HUCBS than without it. The isolation ICMs from whole blastocysts by mechanical slicing from the trophectoderm outgrowth appeared to be a more gentle and preferable method versus immunosurgery and trypsin treatment. Moreover, this method permitted the exclusion of interaction with animal-derived reagents.

The six phESC lines were derived from oocytes given by four donors. IVF procedures resulted in pregnancies in all four donors. A contributing factor to this high success rate for donor IVF pregnancy was the selection of donors with a good prognosis for IVF pregnancy. The IVF procedure for donor 5 did not result in a pregnancy. In contrast to the other four, the quality of parthenogenetically activated oocytes from donor number 5 was poor, and we were unable to generate phESC lines. So the development of human parthenogenetic embryos may depend on the quality of donor

oocytes and may be conditioned by donor peculiarities.

In prior research, parthenogenetic activation of mouse oocytes resulted in MHC-homozygous embryonic stem cell lines (Lin et al., 2003). In our study the suppression of the second meiotic division after parthenogenetic activation of human metaphase II oocytes and the generation of diploid embryos led to the derivation of MHC-heterozygous phESC.

Although the phESC lines we generated present typical characteristics displayed by hESC lines, they show unique characteristics, including genotypes that are practically identical to those of the oocyte donors, as seen in the parthenogenetically derived monkey ES cell line Cyno-1 (Vrana et al., 2003). The phESC lines provide a unique model for clinical and scientific research, and the creation of hESC lines from parthenogenetic embryos may be a superior way to generate MHC-matched and possibly histocompatible ES cells in comparison to SCNT.

Further investigations of the characteristics of phESC lines and their immune matching are necessary to determine their suitability for use in cell therapy. As an example, the altered karyotype of phESC-7 may be a reason to exclude it from clinical use. Doubts have been raised regarding the capability of parthenogenetic stem cells to be differentiated into functional derivatives. In this regard, the additional analysis of the influence of imprinted genes on functionality of phESC differentiated derivatives is needed. However, as previous studies of mouse and monkey parthenogenetic stem cells have shown, these cells can form teratomas with derivatives from all three embryonic germ layers (Lin et al., 2003; Vrana et al., 2003). Monkey parthenogenetic ES cells under selective culture conditions have been differentiated into neural cells and functional dopaminergic and serotonergic neurons (Vrana et al., 2003). In our study we have shown that phESC can also be differentiated into derivatives of all three germ layers *in vitro* and *in vivo* and are pluripotent. Moreover, embryoid bodies from phESC were capable of giving rise to beating cardiomyocyte-like cells (Movie 1, supplement material).

We have demonstrated a method of creating parthenogenetic human embryonic stem cells and have generated experimental data showing that phESC can be differentiated into functional cells that may be of great value in future treatment of human degenerative diseases and for use in stem cell research.

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