HLA Homozygous Stem Cell Lines Derived from Human Parthenogenetic Blastocysts

E.S. REVAZOVA,[1] N.A. TUROVETS,[1,2] O.D. KOCHETKOVA,[1] L.S. AGAPOVA,[1]
J.L. SEBASTIAN,[1] M.V. PRYZHKOVA,[1] V.IU. SMOLNIKOVA,[2]
L.N. KUZMICHEV,[2] and J.D. JANUS[1]

ABSTRACT

Individual HLA homozygous parthenogenetic human stem cell (hpSC-Hhom) lines have the potential for cell-based therapy in a significant number of individuals, provided the HLA haplotype is prevalent. We report the successful derivation of four stable hpSC-Hhom lines from both HLA homozygous and HLA heterozygous donors. Of these, the hpSC-Hhom-4 line carries the HLA haplotype found most commonly within the U.S. population, and is shared by different racial groups. These hpSC-Hhom lines demonstrate typical human embryonic stem cell morphology, expressing appropriate stem cell markers and possessing high levels of alkaline phosphatase and telomerase activity. Additionally, injection of these cell lines into immunodeficient animals leads to teratoma formation. G-banded karyotyping demonstrates a normal 46,XX karyotype in lines hpSC-Hhom-1 and hpSC-Hhom-4, and chromosomal anomalies in lines hpSC-Hhom-2 and hpSC-Hhom-3, both derived from the same donor. HLA genotyping of all four hpSC-Hhom lines demonstrates that they are HLA homozygous. Furthermore, in the case of HLA heterozygous donors, the hpSC-Hhom lines inherit the haplotype from only one of the donor's parents. Single-nucleotide polymorphism (SNP) data analysis suggests that hpSC-Hhom lines derived from HLA heterozygous oocyte donors are homozygous throughout the genome as assessed by SNP analysis. The protocol used for deriving these HLA homozygous stem cell lines minimizes the use of animal-derived components, which makes them more appealing for potential clinical application.

INTRODUCTION

THE FIRST HUMAN EMBRYONIC STEM CELLS (ESC) were derived from a blastocyst inner cell mass (ICM) obtained from a fertilized oocyte (Thomson et al., 1998), capable of infinite division and differentiation into cells of all tissues types (Reubinoff et al., 2000). The ESC is therefore a potentially limitless source of totipotent cells for transplant-based cell therapies.

The greatest risk posed with allogeneic tissue and organ transplantation is that of immune rejection. The degree of risk is proportional to the degree of disparity between donor and recipient cell-surface antigen-presenting proteins. In the ideal transplant, donor tissue is histocompatible with the recipient at the major histocompatibility complex (MHC). The human leukocyte antigen (HLA) system is the nomenclature designating the human MHC, and represents antigens important for transplantation. Matching donor and recipient tissue for HLA antigens reduces the chance of a cytotoxic T-cell response in the recipient, and thus greatly increases the likelihood of transplant survival.

[1]International Stem Cell Corporation, Oceanside, California.
[2]Scientific Center for Obstetrics, Gynecology, and Perinatology RAMS, Moscow, Russia.

MHC class I and II HLA haplotypes are specific sets of HLA-A, -B, and -DR locus alleles inherited together from a parent (Ceppellini et al., 1967). Despite a high degree of HLA polymorphism, there are only 200 common HLA haplotypes in existence within the U.S. Caucasian population (Mori et al., 1997). This HLA diversity, in combination with a heterozygous selection coefficient, means that the chance of finding a donor–recipient match ranges from 1 in 1000 to one in several million due to the unique tissue type provided by the combination of these allelic variants in the heterozygous individual (Hansen et al., 1994).

Transplant-based stem cell therapies face the same HLA matching issues that limit solid organ allogeneic transplants due to immune rejection. HLA-matched stem cell lines may overcome the risk of immune rejection, as described in a recent protocol for derivation of patient-specific parthenogenetic human stem cell lines (Revazova et al., 2007). Here, HLA heterozygous cell lines are derived from HLA heterozygous donors by activating oocytes using a combination of A23187 and 6-DMAP. Since these cells are HLA-matched with the oocyte donor, their ability to provide tissue-matched derivatives is limited.

MHC compatibility between a donor and recipient increases significantly if the donor cells are HLA homozygous; that is, contain identical alleles for each antigen-presenting protein. Furthermore, if homozygous donor cells have a haplotype found with high frequency in a population, these cells may have application in transplantation-based stem cell therapies for a large number of individuals.

We believe that there are at least two potential approaches for deriving human HLA homozygous parthenogenetic stem cell lines. The first of these, which derives human HLA homozygous stem cell lines from HLA homozygous oocyte donors, is limited by the rarity of these donors within the population. We hypothesize that a second approach involves isolating HLA homozygous parthenogenetic ESC (hpSC-Hhom) from a heterozygous oocyte donor. This is based on earlier studies showing that oocyte activation by combining A23187 and puromycin results in the production of mouse haploid (Nakasaka et al., 2000) and human haploid (Nakagawa et al., 2001; Yamano et al, 2000) parthenotes, as well as the derivation of a homozygous diploid stem cell line from a mouse parthenogenetic haploid embryo (Kaufman et al., 1983). Isolating the hpSC-Hhom line provides a method for generating human HLA-matched cells and may potentially be a solution for creating histocompatible tissues for clinical use.

METHODS

Donor selection and informed consent process

Donors were recruited from a pool of women who first presented to the center for IVF and were found to be eligible for an IVF procedure according to clinical guidelines.

Each potential donor was approached by her doctor and informed and counseled about the study. If the donor chose to participate, the donor was presented with a comprehensive informed consent document (reviewed and approved by an independent U.S.-based ESCRO Committee), written in the Russian language, which outlined the purpose of the study and the procedures. If the potential donor had questions, a medical doctor was made available. Only potential donors who signed the informed consent participated in the study.

Donors voluntarily donated with no financial compensation for oocytes. The signed informed consent stated that all donated material was to be used for research and not for reproductive purposes, namely, the development of methods to derive human ES cells and their differentiated progeny.

Research eligibility was determined according to the FDA's Eligibility Determination for Donors of Human Cells, Tissues, and Cellular and Tissue-Based Products (FDA HCT/Ps, 2004), as well as Order No. 67 (02.26.2003) of the Russian Public Health Ministry. This included a thorough medical examination with chest X-ray, blood (including liver function tests), and urine analysis. Screening was also performed for *Chlamydia trachomatis*, *Neisseria gonorrhoeae*, syphilis, HIV, HBV, and HCV.

Potential donors and parents were screened further for research participation according to HLA type.

In this protocol, the priority for oocyte harvest was a successful IVF procedure. The best fully developed mature cumulus oocyte complexes were selected for IVF. If the total number of oocytes harvested was less than 11, the woman was au-

tomatically excluded from donating for research purposes.

Donor superovulation

Each donor underwent ovarian stimulation utilizing FSH (Gonal-F, Lab. Serono, Switzerland) from the 3rd to the 13th day of the menstrual cycle. A total of 1500 IU was 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 follicles 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. Ultrasound-guided transvaginal aspiration of follicular fluid from antral follicles was performed 35 h after hCG injection.

Oocyte activation and culture of parthenogenetic embryos

Cumulus oocyte complexes (COCs) were picked from the follicular fluid, washed in Flushing Medium (MediCult, Jyllinge, Denmark) and then incubated in Universal IVF medium (MediCult) with Liquid Paraffin (MediCult) 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 A23187 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 A23187 (Sigma, St. Louis, MO) for 5 min and 10 μg/mL puromycin (Sigma) or 1 mM 6-DMAP (Sigma) for 4 h, followed by careful washing of oocytes in Universal IVF medium. Oocytes were then placed in fresh IVF medium with paraffin overlay following culture. The next day (day 1), parthenogenetically activated oocytes were cultivated to the blastocyst stage using sequential BlastAssist System media (MediCult) according to the manufacturer's recommendations. From the derived blastocysts, the ICM was isolated on days 5 through 6 of culture.

Isolation of blastocyst inner cell mass and culture of hpSC-Hhom

The zona pellucida was removed by 0.5% pronase (Sigma) treatment. Whole blastocysts were placed on a feeder layer of mitomycin C mitotically inactivated human neonatal skin fibroblasts (NSF) (Revazova et al., 2007), in medium designed for the culture of hpSC-Hhom. When trophoblast cells spread following blastocyst attachment, the ICM became visible. After 3 to 4 days of additional culture, the ICM was isolated by mechanical slicing of the ICM from the trophectoderm outgrowth using a finely drawn glass pipette. The isolated ICM was plated on a fresh feeder layer and cultured for an additional 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 of culture. Early passage colonies were mechanically divided into clumps and replated. Further passing of hpSC-Hhom was performed with collagenase IV treatment and mechanical dissociation. The propagation of hpSC-Hhom was performed at 37°C, 5% CO_2 in a humidified atmosphere.

For the culture of ICM and hpSC-Hhom we used VitroHES (Vitrolife) supplemented with 4 ng/mL hrbFGF (Chemicon, Temecula, CA), 5 ng/mL hrLIF (Chemicon) and 10% human umbilical cord blood serum. The medium for the culture of NSF consisted of 90% DMEM (high glucose, with L-glutamine) (Invitrogen, Carlsbad, CA), 10% human umbilical cord blood serum, and penicillin–streptomycin (100 U/100 μg) (Invitrogen). Before medium preparation, human umbilical cord blood serum was screened for syphilis, HIV, HBV, and HCV.

Characterization of hpSC-Hhom

For immunostaining of embryonic stem cell markers, hpSC-Hhom colonies were fixed at room temperature for 20 min with 4% paraformaldehyde to identify SSEA-1, SSEA-3, and SSEA-4; 100% methanol was used for 5 min at -20°C to identify the remaining markers. Monoclonal antibodies used included: SSEA-1 (MAB4301), SSEA-3 (MAB4303), SSEA-4 (MAB4304), TRA-1-60 (MAB4360), and TRA-1-81 (MAB4381) from

Chemicon, as well as OCT-4 (sc-9081) from Santa Cruz Biotechnology (Santa Cruz, CA). Secondary antibodies included Alexa Fluor 546 (orange-fluorescent) and 488 (green-fluorescent) from Molecular Probes (Invitrogen). Nuclei were stained with DAPI (Sigma). Alkaline phosphatase and telomerase activity were detected with AP kit and TRAPEZE Kit (Chemicon). Chromosomal slides were prepared by the routing method. G-banding was performed according to trypsin-Giemsa technique, and 30–100 metaphases were karyotyped in each instance.

Embryoid body formation and neural differentiation

The hpSC-Hhom colonies were mechanically divided into clumps and placed in 24-well cluster plates precoated with 2% agarose (Sigma) in medium containing 85% Knockout DMEM, 15% human umbilical cord blood serum, $1 \times$ MEM NEAA, 1 mM Glutamax, 0.055 mM β-mercaptoethanol, penicillin–streptomycin (50 U/50 μg) (all from Invitrogen, except the serum). Embryoid bodies were cultured in suspension for 14 days, followed by either plating for outgrowth development or additional culturing in suspension for 1 week.

Neural differentiation was induced by the cultivation of 2-week-old embryoid bodies attached to a culture dish surface for 1 week in differentiation medium containing DMEM/F12, B27, 2 mM Glutamax, penicillin–streptomycin (100 U/100 μg) and 20 ng/mL hrbFGF (Invitrogen).

Immunocytochemistry of hpSC-Hhom differentiated derivatives

Embryoid bodies, placed on poly-D-lysine (Sigma)-treated microcover glasses (VWR Scientific Inc., West Chester, PA), were cultured for approximately 1 week in the appropriate differentiation medium. For immunostaining, differentiated cells were fixed with 100% methanol for 5 min at −20°C.

For detecting ectodermal markers, we used monoclonal mouse antineurofilament 68 antibodies (Sigma) and antihuman CD56 (NCAM) to highlight neuronal markers. For detecting mesodermal markers in 3-week-old embryoid bodies, we used monoclonal mouse antidesmin antibody (Chemicon) and antihuman alpha actinin antibody (Chemicon) as muscle specific markers. For detecting endodermal markers, we used monoclonal mouse antihuman alpha-fetoprotein anti-

body (R&D Systems, Minneapolis, MN). Secondary antibodies Alexa Fluor 546 (orange-fluorescent) and 488 (green-fluorescent) were obtained from Molecular Probes (Invitrogen). Nuclei were stained with DAPI (Sigma).

HLA genotyping

Investigation of HLA haplotypes and HLA genotyping was performed for both donors and their parents. Genomic DNA was extracted from blood, cumulus cells, hpSC-Hhom, and NSF with Dynabeads DNA Direct Blood from Dynal (Invitrogen). HLA genotyping was performed by PCR with allele-specific sequencing primers (PCR-SSP, Protrans, Indianapolis, IN). All tests were performed according to manufacturer's recommendations.

Affymetrix SNP microarray analysis

Genomic DNA was isolated from blood, cumulus cells, hpSC-Hhom, and NSF by the phenol/chloroform extraction method. DNA samples obtained from three donors, four hpSC-Hhom lines, and NSF were all genotyped with Affymetrix Mapping 250K Nsp Arrays. Since the initial data set containing 252,973 binary SNP markers exceeded the number necessary to determine equivalency of genomic samples, it was reduced to simplify computation.

The following criteria were used to select markers based on genetic considerations: (1) the greater the degree of heterozygosity in markers, the more information they provide in identifying the origins of the SNP samples. The heterozygosity of binary SNP markers is capped at a maximum of 0.5. We chose only SNP markers with heterozygosity greater than 0.375 (i.e., no allele has a frequency less than 0.25 or greater than 0.75 in the Caucasian population.); (2) all 22 autosomal chromosomes were used; (3) markers with low reliability were removed, as the identification of samples for common origins is highly sensitive to genotyping errors. In the Affymetrix dataset, high confidence scores correspond to low reliability, so those markers with high confidence scores were removed. At the default setting, no call is made for a marker if its confidence score exceeds 0.25. We chose to apply even more stringent requirements for reliability by choosing only those markers that have confidence scores less than or equal to 0.02 for all 12 samples.

Applying these criteria, the number of SNP markers was reduced from 252,973 to 4,444. One

final step was taken to reduce the number of markers (no random sampling was performed), by selecting only those where the intermarker distances were at least 0.1 cM (1 Mbp = 1 cM), since markers very close to each other provide less information due to the presence of tight linkage between them. These steps led to the final selection of 3993 markers.

The 3993 markers thus chosen 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). Relcheck provides a method for determining the relationship between a pair of SNP samples. This is based on calculating a likelihood ratio for observing a given configuration of markers according to the genetic relationship between samples. Our use of Relcheck is based on the knowledge that since monozygotic twins share the same DNA, the test of equivalency between two samples can be made by checking if those samples may have come from monozygotic twins. Relcheck was run with the assumption that genotyping error rates are approximately 0.4%. The Relcheck program identifies five types of relationships: monozygotic twins, parent/offspring pair, full siblings (full sibs), half siblings (half sibs), and unrelated (Boehnke and Cox, 1997; Broman and Webster, 1998).

Internal controls correctly identified the paired genotype relationship between split cultures derived from the same hpSC-Hhom line as "monozygous twins."

Teratoma formation and evaluation

All animal procedures were carried out by the Biological Testing Laboratory—Branch of Shemyakin & Ovchinnikov Institute of Bioorganic Chemistry of the Russian Academy of Sciences (Pushchino, Moscow Region, Russia) accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care International (AAALAC).

Immunodeficient severe combined immunodeficient (SCID) beige mice that were bred from CB17/ICR-PRKDC-SCID CRL mice received from Charles River laboratories Research Model and Services (Germany) were used for teratoma formation. For injection into mice, the hpSC-Hhom were enzymatically separated from culture dishes using collagenase type IV and then resuspended into clumps. Approximately 2 to 5 million hpSC-Hhom cells were injected into the upper hind limb subcutaneous space. After approximately 2 months, established 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 then sectioned in 60-μm slices using a vibratome. Sections were mounted on glass slides and stained with hematoxylin/eosin, Kraberg, Van Gieson, and picrofucsin.

RESULTS

Derivation of a hpSC-Hhom line from an HLA homozygous donor

With an initial goal of isolating an HLA homozygous parthenogenetic human stem cell line, we used oocytes from an HLA homozygous donor. HLA genotyping of both the donor (donor 1) and her parents demonstrated that both parents were heterozygous. The same haplotype A*25, B* 18, DRB1*15 was inherited from each parent, with the donor having an HLA homozygous genotype A*25, A*25, B*18, B* 18, DRB1*15, DRB1*15 (Table 2, Case 1).

Nineteen cumulus–oocyte complexes (COCs) were taken from donor 1, of which seven were used for research (Table 1). Parthenogenetic activation was performed using a previously described protocol with A23187 and 6-DMAP treatment (Revazova et al., 2007). Four parthenogenetic embryos achieved the blastocyst stage, from which one allowed isolation of the hpSC-Hhom-1 line.

TABLE 1. ORIGIN OF PARTHENOTES AND HLA HOMOZYGOUS PARTHENOGENETIC HUMAN STEM CELL LINES

Donor number	Oocytes harvested	Oocytes donated	Blastocysts	Cell lines	IVF Result
1	19	7	4	hpSC-Hhom-1	Successful
2	18	7	3	hpSC-Hhom-2	Successful
				hpSC-Hhom-3	(twin pregnancy)
3	20	10	0	0	Unsuccessful
4	27	14	2	hpSC-Hhom-4	Successful
Total	84	38	9	4	

Genotype relationship between cells of the hpSC-Hhom-1 line and donor 1 somatic cells were identified as "full siblings" (genetically matched) by SNP analysis. SNP marker comparison revealed that donor 1 cells appear to exhibit a pattern of heterozygosity, whereas hpSC-Hhom-1 cells display a lower proportion of heterozygosity (Supplemental material).

HLA genotyping demonstrated that the hpSC-Hhom-1 line was HLA homozygous: A*25, A*25,

TABLE 2. HLA GENOTYPING

Case		HLA-A	HLA-B	DRB1
1	Donor's mother	A*25	B*18	DRB1*15
		A*26	B*44	DRB1*11
	Donor's father	A*25	B*18	DRB1*15
		A*03	B*07	DRB1*15
	Donor	A*25	B*18	DRB1*15
		A*25	B*18	DRB1*15
	hpSC-Hhom-1	A*25	B*18	DRB1*15
		A*25	B*18	DRB1*15
2	Donor's mother	A*02	B*13	DRB1*07
		A*33	B*14	DRB1*01
	Donor's father	A*68	B*18	DRB1*13
		A*03	B*40	DRB1*03
	Donor	A*02	B*13	DRB1*07
		A*68	B*18	DRB1*13
	hpSC-Hhom-2	A*68	B*18	DRB1*13
		A*68	B*18	DRB1*13
	hpSC-Hhom-3	A*02	B*13	DRB1*07
		A*02	B*13	DRB1*07
3	Donor's mother	A*02	B*08	DRB1*03
		A*01	B*15	DRB1*01
	Donor's father	A*01	B*51	DRB1*11
		A*33	B*15	DRB1*04
	Donor	A*02	B*08	DRB1*03
		A*01	B*51	DRB1*11
	NO LINE			
		HLA haplotype N10: A*02 B*08 DRB1*03		
4	Donor's mother	A*02	B*07	DRB1*13
		A*02	B*40	DRB1*16
	Donor's father	A*01	B*08	DRB1*03
		A*24	B*44	DRB1*01
	Donor	A*02	B*40	DRB1*13
		A*01	B*08	DRB1*03
	hpSC-Hhom-4	A*01	B*08	DRB1*03
		A*01	B*08	DRB1*03
		HLA haplotype N1		
Feeder	NSF	A*25	B*15(62)	DRB1*04
		A*32	B*18	DRB1*15

Yellow signifies donor's mother's HLA haplotype; green signifies donor's father's HLA haplotype, each inherited by the donor and then subsequently by the hpSC-Hhom line.

B*18, B* 18, DRB1*15, DRB1*15 and completely HLA matched with the donor (Table 2, Case 1).

Derivation of hpSC-Hhom lines from an HLA heterozygous donor

Since HLA homozygous oocyte donors are a rare occurrence, we sought to isolate HLA homozygous cell lines from oocytes obtained from HLA heterozygous donors. In all, 18 COCs were obtained from donor 2, of which seven were donated for research (Table 1). These oocytes were parthenogenetically activated using a different protocol with A23187 and puromycin treatment. Following 18 h, we observed second polar body extrusion and formation of one pronucleus in the activated oocytes. Three blastocysts developed from these zygotes, allowing isolation of two hpSC-Hhom lines: hpSC-Hhom-2 and hpSC-Hhom-3.

SNP analysis performed between oocyte donor 2's somatic cells and the hpSC-Hhom-2 and hpSC-Hhom-3 lines showed the relationship as "parent/offspring pair." Moreover, both of these cell lines appeared to be homozygous throughout the genome (at the SNP markers evaluated) in contrast to the donor's heterozygous somatic cells (1578 heterozygous SNP markers; Supplemental material).

Based on the HLA-genotyping results, cells from both of these lines appeared HLA homozygous: hpSC-Hhom-2 line exhibited HLA genotype A*68, A*68, B*18, B*18, DRB1*13, DRB1*13, and hpSC-Hhom-3 line exhibited HLA genotype A*02, A*02, B*13, B*13, DRB1*07, DRB1*07 (at the loci investigated). We also found by HLA genotyping that each hpSC-Hhom line inherited a different HLA haplotype, one from the donor's father, and the other from the donor's mother. The donor's father's HLA haplotype (A*68, B*18, DRB1*13) was found in the homozygous state in the hpSC-Hhom-2 line and the donor's mother's HLA haplotype (A*02, B*13, DRB1*07) was found in the homozygous state in the hpSC-Hhom-3 line (Table 2, Case 2).

Isolation of hpSC-Hhom lines from oocytes of HLA heterozygous donors, selected according to HLA haplotype

As a final step, we sought to isolate hpSC-Hhom lines with an HLA haplotype known to have a high frequency among the population. HLA haplotype screening of IVF candidates produced two HLA heterozygous oocyte donors carrying a common haplotype. According to a published list of HLA haplotype frequencies (Mori et al., 1997), donor 3 (HLA haplotype A*02, B*08, DRB1*03) and donor 4 (HLA haplotype A*01, B*08, DRB1*03) (Table 2, Case 3 and Case 4) carried common haplotypes found within the U.S. population. However, with a heterozygous HLA genotype, each donor carried not only the frequent haplotype, but also a less common one as well. It was therefore not possible to predict with full accuracy which haplotype would be present in an isolated hpSC-Hhom line.

We used A23187 and puromycin for parthenogenetic activation of the donors' oocytes. We were not successful in isolating an hpSC-Hhom line from the oocytes of donor 3, from which 20 COCs were obtained, with 10 oocytes donated for research. None of these reached the blastocyst stage. Furthermore, the IVF procedure for this donor was unsuccessful in achieving pregnancy. Together, these findings may reflect the poor quality of oocytes from this particular donor (Table 1).

From donor 4, 27 COCs were obtained, with 14 oocytes donated for research. Following parthenogenetic activation of the oocytes, two blastocysts were obtained, from which the hpSC-Hhom-4 line was isolated (Table 1).

The genotype relationship between hpSC-Hhom-4 and donor 4 somatic cells was identified as "parent/offspring pair" by SNP analysis, similar to Case 2 (Supplemental material). The hpSC-Hhom-4 line appeared homozygous throughout the genome (at the SNP markers evaluated) in comparison to the donor's heterozygous somatic cells (1174 heterozygous SNP markers; Supplemental material).

According to HLA genotyping, the hpSC-Hhom-4 line was HLA homozygous (at the loci

TABLE 3. FREQUENCY AND RANKING ACCORDING TO RACIAL GROUP FOR HLA HAPLOTYPE A*01, B*08, DRB1*03 IN THE U.S. POPULATION

Racial group[a]	Frequency (%)[b]	Ranking[c]
CAU	5.1812	1
NAT	4.7439	1
AFR	1.2491	2
LAT	1.6733	3
ASI	0.3195	54

Adapted from Mori et al., 1997.
[a]Caucasian American (CAU), Native American (NAT), African American (AFR), Latin American (LAT) and Asian American (ASI).
[b]HLA-A, -B, -DR haplotype frequencies.
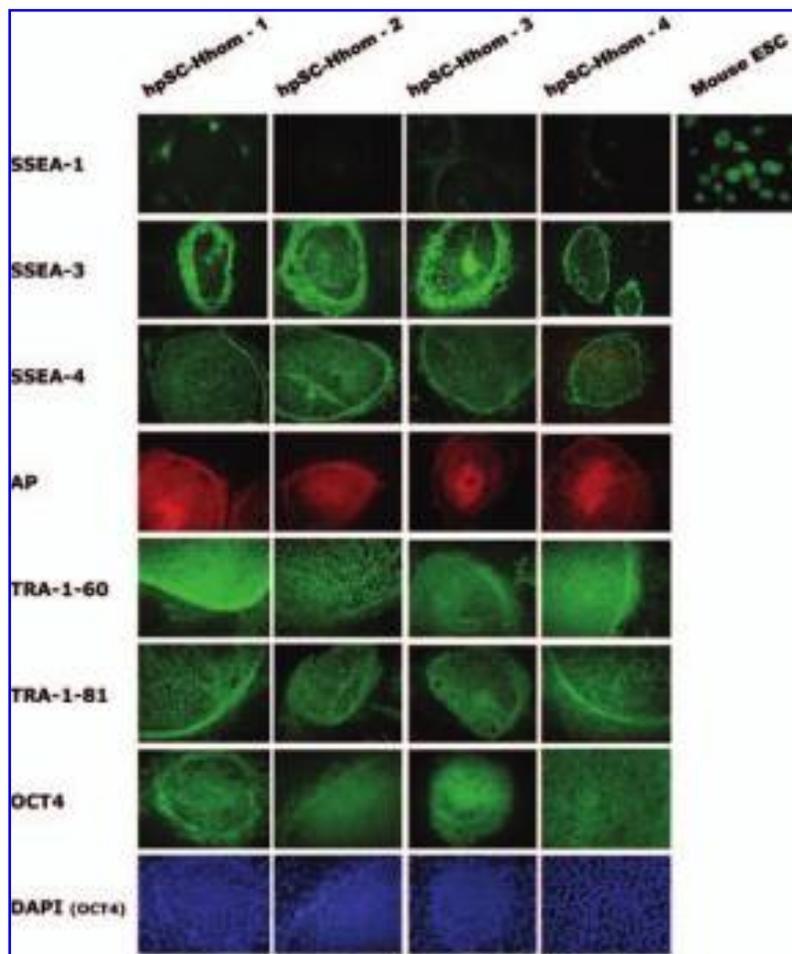[c]Respective ranking within each racial group.

FIG. 1. Characterization of hpSC-Hhom lines for human ESC-specific markers. Cells from all four hpSC-Hhom lines demonstrate positive staining for SSEA-3, SSEA-4, TRA-1–60, TRA-1–81, OCT-4 (nuclei stained by DAPI), alkaline phosphatase, and negative staining for SSEA-1. Mouse embryonic stem cells (ESC) were used as a positive control for SSAE-1 staining.

evaluated) and had the most common HLA haplotype in the U.S. population, shared by a number of racial groups (Table 3): A*01, B*08, DRB1*03, (Table 2, Case 4).

IVF procedures resulted in pregnancies in three of the four donors (donors 1, 2, and 4). The high IVF success rate was largely due to the selection of donors with a good prognosis for an IVF pregnancy. Interestingly, from donor 2 we isolated two hpSC-Hhom lines, with the donor having a twin pregnancy from her IVF procedure.

Characterization of the hpSC-Hhom lines

Cells from all of the hpSC-Hhom lines displayed morphology expected from human ESC, formed densely packed colonies, and displayed prominent nucleoli with a small cytoplasm to nucleus ratio. All cells expressed common human ESC markers SSEA-3, SSEA-4, TRA-1–60, TRA-1–81, and OCT-4, while not expressing SSEA-1, a positive marker for undifferentiated mouse ESC (Fig. 1). All lines exhibited high levels of alkaline phosphatase (Fig. 1) and telomerase activity (Fig. 2).

G-banded karyotyping demonstrated that hpSC-Hhom-1 and hpSC-Hhom-4 lines had a normal human 46,XX karyotype (Fig. 3A and B). The hpSC-Hhom-2 and hpSC-Hhom-3 lines, both derived from a single donor, displayed karyotype anomalies. Approximately 15% of cells from the hpSC-Hhom-2 line exhibited aneuploidy of chromosome 8: 47,XX, +8 karyotype (Fig. 3C) and 4.2% of cells from hpSC-Hhom-3 line exhibited aneuploidy of chromosome 1: 47,XX, +1 karyotype (Fig. 3D). We did not observe X chromosome heteromorphism for any cell line in the analysis of 100 metaphases (Fig. 3).

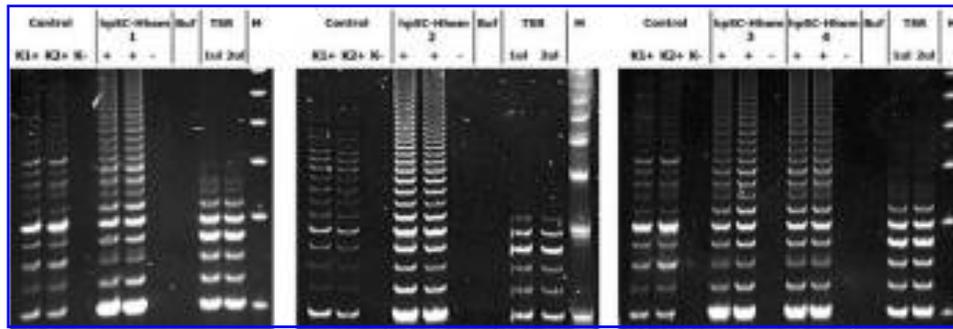The hpSC-Hhom-4 line remained undifferenti-

FIG. 2. The hpSC-Hhom lines demonstrate high levels of telomerase activity in comparison with positive control cells: "K1+"-telomerase positive cell extract (applied with TRAPEZE Kit); "K2+"—extract from 500 cells; "K−" or "−"—heat-treated cell extract with inactivated telomerase; Buf—CHAPS lysis buffer, primer-dimer/PCR contamination control; TSR—telomerase quantitative control template (0.1 and 0.2 amol/μL); "M"—marker, DNA ladder.

ated over 27 passages. The other cell lines were successfully cultured over at least 21 passages. The cells from hpSC-Hhom-4 line formed cystic embryoid bodies in suspension culture and gave rise to derivatives from all three germ layers—ectoderm, mesoderm, and endoderm—following differentiation *in vitro* (Fig. 4). Ectoderm differentiation was confirmed by positive immunocytochemical staining for neuron specific markers neurofilament 68 (Fig. 4A) and NCAM (Fig. 4B). Differentiated cells were also positive for mesoderm muscle specific markers desmin (Fig. 4C) and alpha-actinin (Fig. 4D). Endoderm differentiation was confirmed by positive staining for alpha-fetoprotein (Fig. 4E).

The ability of all hpSC-Hhom lines to form derivatives from all three germ layers was furthermore investigated *in vivo* by subcutaneous injection of hpSC-Hhom cells into immunodeficient mice (Fig. 5). All hpSC-Hhom lines were capable of forming teratomas approximately two months following injection. Teratocarcinoma formation was not observed. Approximately four million mitomycin-C-treated human fibroblasts used as feeder layers for the hpSC-Hhom cells were also injected as controls and did not exhibit teratoma growth.

Histological examination of cell transplants demonstrated the presence of organized structures including: various gland types (some producing a brown pigment, possibly biliary pigment), chondro-differentiation, well-formed bones, mesenchymal cells, a high production of collagen fibers, fat tissue, neural tubes, and stratified pavement epithelium with parakeratosis-pearls (Fig. 5). These findings suggest that hpSC-Hhom will differentiate *in vivo* into tissues derived from all three germ layers.

Future projects

With HLA haplotype screening, we are continuing our efforts to isolate hpSC-Hhom lines containing high-frequency haplotypes in the population. Our goal is to establish an hpSC-Hhom bank with haplotypes covering the world population. In parallel, we have begun differentiation studies of these cell lines aimed at establishing a tissue bank according to HLA type. Furthermore, our efforts are also directed at minimizing and eventually completely eliminating the presence of animal-derived components during the production of parthenotes and the isolation of stem cell lines. Current culture conditions include: SynVitro Hyadase (MediCult), containing synthetic components only for oocyte denudation; Universal IVF Medium (MediCult) and the BlastAssist System (MediCult) for embryo culture, both of which contain only human-derived or synthetic components; use of mechanical ICM separation; use of human umbilical cord blood serum during ICM and stem cell cultivation; and last, human NSF as feeder layers, propagated only with human umbilical cord blood serum.

DISCUSSION

We have used two different approaches for deriving human HLA homozygous parthenogenetic stem cell lines that may prove to be useful for transplantation-based stem cell therapies.

In our initial approach, one cell line was derived from an HLA homozygous donor. This was accomplished by using A23187 and 6-DMAP during oocyte activation, blocking extrusion of the
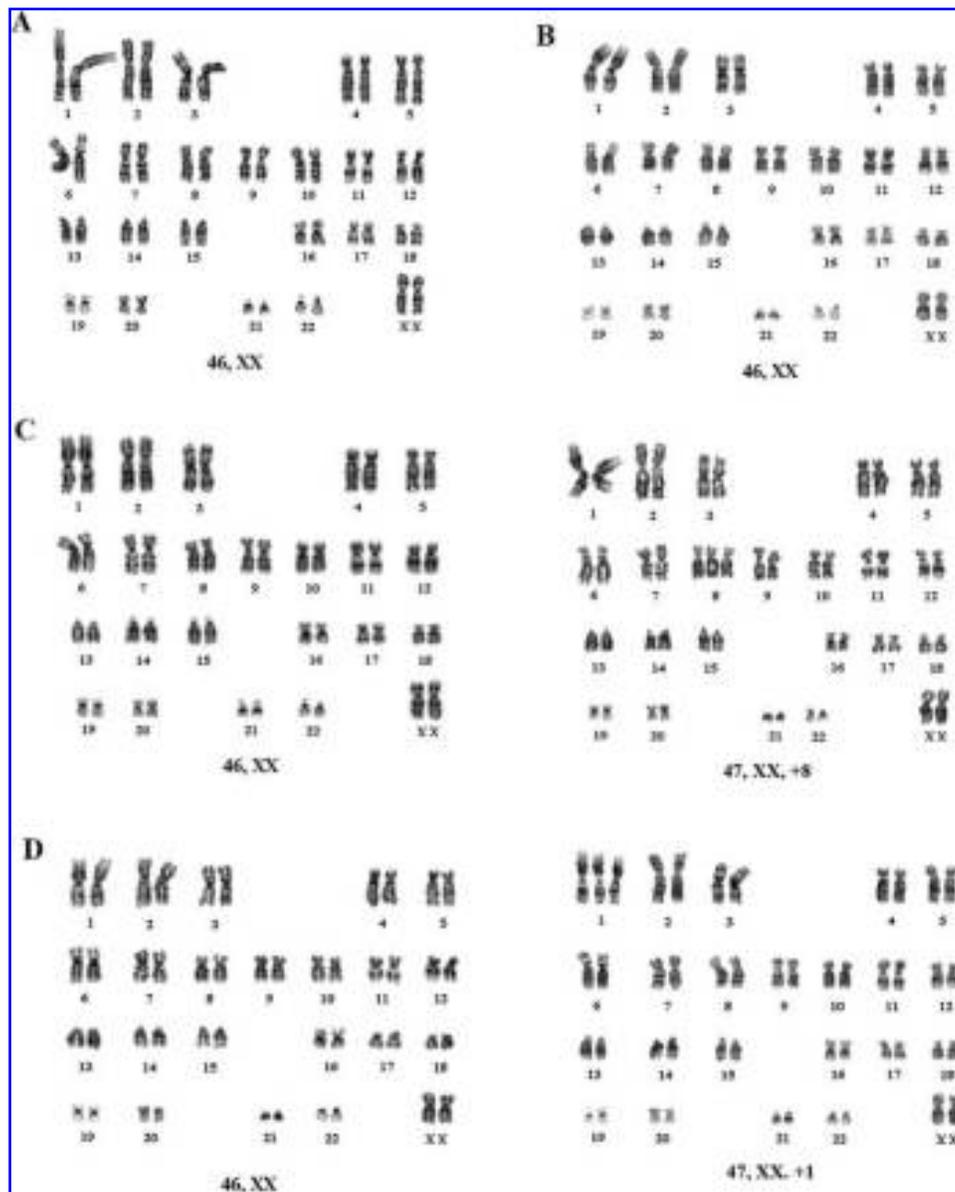
FIG. 3. G-banded karyotyping of the human HLA homozygous parthenogenetic stem cell lines: hpSC-Hhom-1 (**A**) and hpSC-Hhom-4 (**B**) lines have a normal 46,XX karyotype; the hpSC-Hhom-2 (**C**) line has 15% cells with 47,XX,+8 karyotype-aneuploidy of chromosome 8; the hpSC-Hhom-3 (**D**) line has 4.2% cells with 47,XX, +1 karyotype-aneuploidy of chromosome 1.

FIG. 5. *In vivo* differentiation of hpSC-Hhom-4. Teratoma formation in SCID mice. Derivatives from all three embryonic germ layers (ectoderm, endoderm, and mesoderm): well-formed respiratory-type glands surrounded by mesenchymal cells, hematoxylin/eosin (h/e) staining; original magnification ×140 (**A**). Likely neural tube with a single-layer of cells; to the right an endodermal gland and at the bottom chondro-differentiation, h/e staining; original magnification ×70 (**B**). Bone surrounded by mesenchymal cells, in the center is a tubular gland with cuboid epithelium, picrofucsin staining; original magnification ×140 (**C**). Well-formed bone surrounded by mesenchymal cells and fat tissue, h/e staining; original magnification ×140 (**D**). On the left are endodermal glands; mesodermal and fat tissue, collagen; bone is seen on the right and at the bottom, Kraberg staining; original magnification ×70 (**E**). An endoderm glandular structure surrounded by mesenchymal cells, a high production of collagen fibers, Van Gieson staining; original magnification ×280 (**F**). Stratified epithelium, in the center a hyperkeratotic pearl, on the left another gland, h/e staining; original magnification ×140 (**G**). A colony of glands, h/e staining; original magnification ×70 (**H**). Glands containing cells producing a brown pigment, possibly biliary pigment, surrounded by fat tissue and mesenchymal cells, h/e staining; original magnification ×140 (**I**).
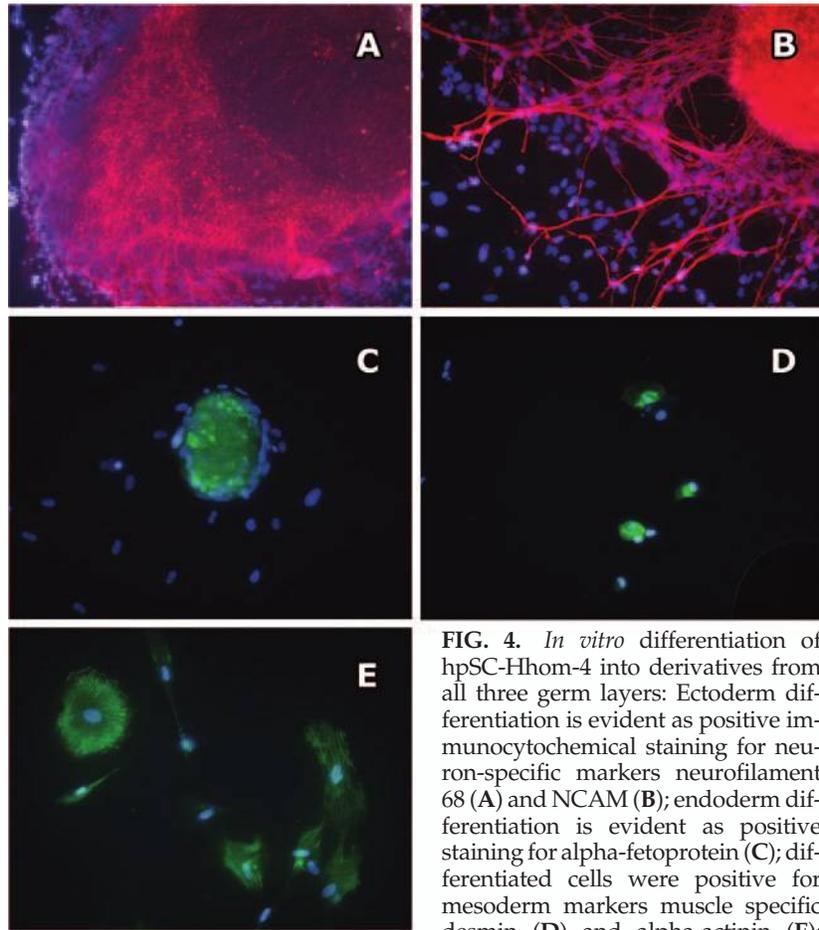
FIG. 4. *In vitro* differentiation of hpSC-Hhom-4 into derivatives from all three germ layers: Ectoderm differentiation is evident as positive immunocytochemical staining for neuron-specific markers neurofilament 68 (**A**) and NCAM (**B**); endoderm differentiation is evident as positive staining for alpha-fetoprotein (**C**); differentiated cells were positive for mesoderm markers muscle specific desmin (**D**) and alpha-actinin (**E**); original magnification ×200 (**A–E**).
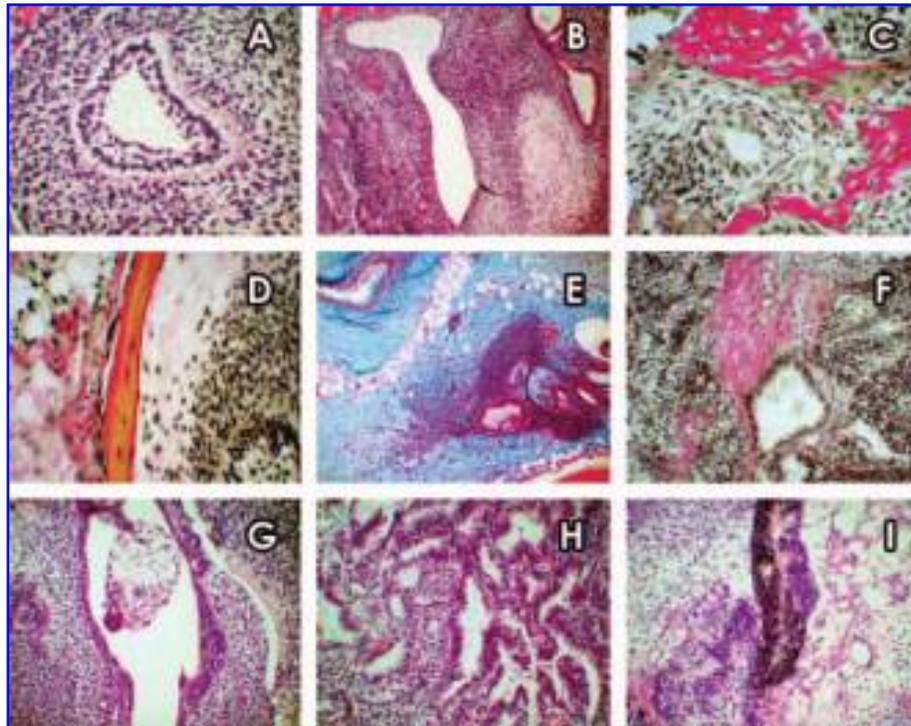

FIG. 5.

second polar body, thereby retaining all of the genetic material of the MII oocyte. The HLA genotype of stem cells derived from these oocytes matched that of the donor (Revazova et al., 2007). In a similar fashion, we derived the diploid hpSC-Hhom-1 line from an HLA homozygous donor.

A recent publication (Kim et al., 2007) suggests the prior unintentional creation of a human parthenogenetic ESC line (SCNT-hES-1) originally purported to be of somatic cell nuclear transfer (SCNT) origin.

In a subsequent approach, we successfully derived HLA homozygous ESCs from HLA heterozygous oocyte donors. Parthenogenetic activation of oocytes with a combination of A23187 and puromycin allowed extrusion of the second polar body. The activated oocyte therefore contained only half of a set of metaphase II chromosomes, which allowed formation of a homozygous genotype. It has been shown that 80% of human oocytes activated by a combination of calcium ionophore and puromycin display a pronucleus with the extrusion of the second polar body. Moreover, cytogenetic analysis demonstrates that 78% contain a normal haploid set of chromosomes (Yamano et al, 2000). In subsequent work, human oocytes activated with A23187 and puromycin displayed one pronucleus and two polar bodies with a haploid set of chromosomes (Nakagawa et al., 2001). It is interesting to note that in the mouse model similar oocyte activation produces a pronuclear parthenote with a normal set of haploid chromosomes. (Nakasaka et al., 2000).

Using this protocol, we derived three diploid HLA homozygous stem cell lines (hpSC-Hhom-2, hpSC-Hhom-3, hpSC-Hhom-4) from oocytes isolated from HLA heterozygous donors.

HLA genotyping data suggests that the HLA haplotype is inherited exclusively from one of the donor's parents. SNP analysis data suggests that these three cell lines are homozygous throughout the genome as assessed by SNP analysis. All three cell lines have a diploid karyotype, which corresponds to earlier work in which diploid stem cell lines were derived from haploid mouse embryos (Kaufman et al., 1983).

The exact mechanism and timing of duplication of haploid genetic material following oocyte activation is unclear. More than likely, DNA replication occurs in the absence of cell cleavage or division. Prior studies suggest that 80% of parthenogenetically activated mouse oocytes pre-serve their haploid state until the morula stage, with subsequent stem cell lines derived from these embryos becoming diploid (Kaufman et al., 1983).

Two of the hpSC-Hhom lines have karyotype anomalies: 15% of cells in the hpSC-Hhom-2 line exhibit trisomy of chromosome 8; and 4.2% of cells in the hpSC-Hhom-3 line exhibit trisomy of chromosome 1. We propose three possible explanations for these findings. First, this may be a natural result of embryo cleavage mosaicism (Bielanska et al., 2002). Second, as a protein synthesis and phosphorylation inhibitor, puromycin, may play a direct role. A third explanation may relate to an unrecognized anomaly with the donor, because both of these cells lines were derived from oocytes obtained from the same donor. Certainly any cell lines shown to have these abnormalities would have very limited clinical application. Therapeutic utility may also potentially be limited by the expression of recessive lethalities in fully homozygous cell lines, and this must be more carefully investigated for each hpSC-Hhom line prior to clinical use.

Cells from all four hpSC-Hhom lines demonstrate typical human ESC morphology, express appropriate stem cell markers and possess high levels of alkaline phosphatase and telomerase activity. hpSC-Hhom-1 and hpSC-Hhom-4 lines both have a normal 46,XX karyotype. Although the potential for parthenogenetic stem cells to differentiate into functional derivatives is debatable, prior studies of mouse and monkey parthenogenetic stem cells have shown formation of teratomas with derivatives from all three embryonic germ layers (Lin et al., 2003; Vrana et al., 2003). Furthermore, monkey parthenogenetic embryonic stem cells placed under selective culture conditions have differentiated into neural cells, with functional dopaminergic and serotonergic neurons (Vrana et al., 2003). In our previous work with heterozygous parthenogenetic human stem cell lines we showed differentiation into derivatives from all three germ layers both *in vitro* and *in vivo*. In the present study with homozygous lines (hpSC-Hhom), we have obtained similar results.

Given these findings, the hpSC-Hhom-1 and hpSC-Hhom-4 lines have potential to be useful in clinical applications following differentiation into multipotent progenitor cells, somatic cell precursors, and normal functional cells.

Uniquely suitable for therapeutic applications

may be the hpSC-Hhom line's MHC homozygosity. With proper selection of oocyte donors according to HLA haplotype, and FDA-approved manufacturing protocols, it is possible to generate a bank of cell lines, whose tissue derivatives collectively could be MHC-matched with a significant number of individuals. It has been suggested that a panel of only ten HLA homozygous human ESC lines selected for common types can provide a complete HLA-A, HLA-B, and HLA-DR match for 37.7% of United Kingdom recipients, and a beneficial match for 67.4%. (Taylor et al., 2005). Using the U.S. population calculations suggest that there are close to 200 common haplotypes per racial group (Mori et al., 1997). The hpSC-Hhom-4 line carries the most common haplotype, potentially providing an MHC match for nearly 5% of individuals within this population. We believe that the hpSC-Hhom lines are therefore ideally suited for establishing a repository of differentiated cells and tissues HLA-matched to the population, which could be available for immediate clinical application. A possible concern are hematopoietic derivatives that may potentially invoke graft-versus-host disease (Billingham, 1966) in a heterozygous recipient, in which case patient-specific parthenogenetic stem cells (Revazova et al., 2007) may provide a solution.

Aside from replacement therapy, a repository of cells and tissues derived from hpSC-Hhom lines may be invaluable in the treatment of genetic disorders. According to the Online Mendelian Inheritance in Man (OMIM), John Hopkins University and the National Center for Biotechnology Information (NCBI), there are more than 1000 genetic disorders, with the list continuing to expand. Examples include Alzheimer's disease, diabetes, Graves disease, hemophilia, Huntington's disease, muscular dystrophy, Parkinson's disease, sickle cell anemia, Phenylketonuria-PKU, and severe combined immune deficiency (SCID). In these situations, it would be important to use cell lines obtained from donors not carrying the same genetic defect.

In conclusion, we report the successful derivation of HLA homozygous parthenogenetic human stem cell lines, which can be HLA matched with large segments of the population. Establishing an HLA-matched tissue bank may potentially eliminate the concern for immune rejection. hpSC-Hhom lines may therefore have important applications in transplantation-based stem cell therapies.

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Address reprint requests to:
J.D. Janus
International Stem Cell Corporation
2595 Jason Court
Oceanside, CA 92056

E-mail: Janusj@lifelinecelltech.com

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