

Generation of cardiomyocytes from new human embryonic stem cell lines derived from poor-quality blastocysts.

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Abstract

Human embryonic stem (hES) cells represent a potential source for cell replacement therapy of many degenerative diseases. Most frequently, hES cell lines are derived from surplus embryos from assisted reproduction cycles, independent of their quality or morphology. Here, we show that hES cell lines can be obtained from poor-quality blastocysts with the same efficiency than from good- or intermediate-quality blastocysts. Furthermore, we show that the self-renewal, pluripotency, and differentiation ability of hES cell lines derived from either source are comparable. Finally, we present a simple and reproducible embryoid body-based protocol for the differentiation of hES cells into functional cardiomyocytes. The five new hES cell lines derived here should widen the spectrum of available resources for investigating the biology of hES cells and advancing toward efficient strategies of regenerative medicine.

Running title: Derivation of new hES cell lines

Human embryonic stem (hES) cells are permanent cell lines derived from pre-implantation human embryos, most frequently from the inner cell mass (ICM) of human blastocysts. Since 1998, when the first lines of hES cells were derived (Thomson et al. 1998), numerous hES cell lines have been produced by different laboratories (Allegrucci and Young 2007; Veiga et al. 2007). The two main defining features of these cells are their self-renewal capacity and pluripotency (i.e. their ability to differentiate into cell types of the three embryonic germ layers). Because of these properties, hES cells are thought to hold great potential as a source of cells for therapeutic use. Parkinson's disease, spinal cord injury, diabetes, heart failure, and bone marrow failure are examples of pathological conditions amenable to being treated by means of stem cell transplantation (Gerecht-Nir and Itskovitz-Eldor 2004; Liew et al. 2005; Menasche 2005; Semb 2005). In addition to their potential value as therapeutic agents, hES cells also appear as a powerful experimental model for studying early human development, provide a platform to develop and test new drugs and treatment protocols (Pera and Trounson 2004), and aid in research on human monogenic diseases (Mateizel et al. 2006; Pickering et al. 2003; Verlinsky et al. 2005).

Typically, hES cell lines originate from surplus embryos created during in vitro fertilization (IVF) treatments. Couples reaching an end to their IVF treatments with surplus embryos have a decision to make. These frozen surplus embryos can remain in cryostorage, they can be made available for adoption, they can be thawed and discarded, or they can be donated to research (Lyerly and Faden 2007). It is from this latter option that most hES cell lines to date have been derived. IVF programs often employ in vitro culture of pre-implantation

embryos to the blastocyst stage of development to achieve better implantation rates in specific groups of patients (Menezo et al. 1992; Veiga et al. 1995). Approximately 35% of cultured human embryos develop successfully to the blastocyst stage and not all of these show good quality morphology. The remainder show retarded or arrested development as well as abnormal morphology due to unequal cell division or cellular fragmentation (Gardner et al. 2000; Menezo et al. 1998).

Today, the methodology of hES cell derivation is still highly empirical, and various protocols are used in the different steps of the process, including feeder cell preparation (if feeder cells are used), embryo culture, inner cell mass (ICM) isolation, and initial steps of derivation. It is worth mentioning that an important goal in derivation attempts is to achieve derivation and culture of hES cells under feeder-free (Amit et al. 2004; Klimanskaya et al. 2005) and animal-free (Ellerstrom et al. 2006; Genbacev et al. 2005; Li et al. 2005; Rajala et al. 2007) conditions, and in chemically defined culture conditions (Li et al. 2005; Lu et al. 2006; Ludwig et al. 2006). Moreover, that the entire protocol is carried out following Good Manufacturing Practice (GMP) conditions will be necessary for the safe clinical use of hES cells in human therapy. Recent publications have revealed differences between hES cell lines related to the environment to which the cells have been exposed since the time of embryo culture and derivation (Adewumi et al. 2007; Allegrucci and Young 2007).

Here, we present our recent experience at the Center for Regenerative Medicine in Barcelona (CMRB) and describe the successful derivation of 5 new hES cell lines.

Interestingly, 3 out of the 5 new hES cell lines were derived from poor-quality blastocysts, underscoring the fact that not only embryos of good quality can be used for hES cell derivation. Moreover, one of these lines has been adapted to enzymatic passaging and displays extremely high plating efficiency from single cells, and can be routinely maintained on matrigel- or gelatin-coated plates for extended periods of time (over 30 passages) using fibroblast-conditioned media or serum-free chemically-defined media without loss of pluripotency or accumulation of karyotypic abnormalities. Finally, we show that functional cardiomyocytes can be reproducibly differentiated from hES cell lines using a modified embryoid body-based protocol, irrespective of the quality of the blastocyst used for their derivation.

Derivation of five new hES cell lines

A total of 61 human pre-embryos, which had been frozen at different stages of development (pronuclear-20, cleavage-35 and blastocyst-6) were used in this study (Table 1). The survival rate after thawing ranged from 80% for cleavage stage to 50% for pronuclear stage and averaged 69% overall. In vitro culture of pronuclear and cleavage stage embryos resulted in 13 reaching blastocyst stage from the 38 that survived thawing (34%). Four of the 6 embryos from blastocyst stage thaws survived yielding 17 blastocyst stage cultures overall. These relatively low rates of survival and blastocyst formation are likely attributed to the substandard freezing protocols that were used at the time these pre-embryos were placed into cryostorage. In addition, most supernumerary embryos were frozen irrespective of their quality. If an adequate selection had been performed prior to freezing, it is probable that derivation efficiency would have achieved higher rates of success (Sjogren et al. 2004).

In total, we obtained 17 blastocysts that were classified according to the criteria put forth by Stephenson and colleagues (Stephenson et al. 2006) into three groups: good, intermediate, or poor quality (Table 2). Four of these were graded as good-quality blastocysts, showing expanded morphology, an ICM with compacted cells and a trophectoderm forming a continuous or almost continuous layer. Four blastocysts were classified as intermediate because they had an ICM with few cells visible and not compacted. Nine embryos did not have a distinguishable ICM or it appeared degenerated and were classified as poor-quality blastocysts. Five of these poor quality pre-embryos did not show distinct blastocyst

morphology, the ICM being indistinguishable from the trophectoderm, and consisting of only a few surviving cells.

During the derivation process, the ICM could not be distinguished in some of the blastocysts and thus it was not possible to isolate it. As a consequence, in a first series of experiments, we performed immunosurgery to isolate the ICM only in good-quality blastocysts, while those scored as intermediate- or poor-quality blastocysts were seeded whole. In a second series of derivations, we avoided ICM isolation altogether for all blastocysts in order to eliminate the use of antibodies and complement from animal origin (Heins et al. 2004; Suss-Toby et al. 2004).

After 4-5 days in culture, a small clump of cells with hES cell morphology (a compact colony structure, a high nuclear to cytoplasmic ratio, and prominent nucleoli) (Reubinoff et al. 2000; Thomson et al. 1998) appeared among other cell types of unspecific morphology (Fig. 1A). These compact cell clumps were mechanically dissociated and replated onto fresh feeders after 10-12 days. During that time the outgrowth of such structures was carefully monitored and the culture media changed on a daily basis. Overall, from the 17 blastocysts seeded we obtained 5 hES cell lines (ES[2]-ES[6], Fig. 1B-C). One line (ES[5]) was derived from 1 of the 4 good-quality blastocysts (25%); one hES cell line (ES[3]) was derived out of the 4 intermediate-quality blastocysts (25%), and the remaining 3 lines (ES[2], ES[4], and ES[6]) were derived from the 9 poor-quality blastocysts (33%). The overall derivation efficiency was 29% and, surprisingly, did not depend on the quality of

blastocysts used. This contrasts with the widely held notion that embryo quality is correlated with developmental competence and embryo viability (Alikani et al. 1999), and with previous evidence suggesting that hES cells are more efficiently derived from good-quality embryos (Oh et al. 2005; Simon et al. 2005; Stojkovic et al. 2004; Zhang et al. 2006). Nevertheless, our efficiency in the derivation of hES cell lines from poor-quality blastocysts is consistent with previous studies reporting the derivation of 4 lines from 19 discarded embryos (21% derivation efficiency) (Mitalipova et al. 2003) and 2 lines from 19 blastocysts derived from day-3 embryos with low morphological scores (10% derivation efficiency) (Chen et al. 2005).

Characterization of the new hES cell lines

Karyotype analysis, human leukocyte antigen (HLA) typing, expression of pluripotency-associated markers, and evaluation of pluripotency in vitro and in vivo were used to characterize all 5 hES cell lines. A summary of the results obtained from these analyses is presented in Table 3.

The 5 hES cell lines were positive for all pluripotency markers tested, displaying high levels of alkaline phosphatase (Fig. 1D) and telomerase activity (data not shown), and high levels of Oct4, Nanog, Sox2, SSEA-3, SSEA-4, TRA-1-60, and TRA-1-81 immunoreactivity (Fig. 2 and data not shown). All 5 lines displayed a normal karyotype (46, XY; data not shown). We are currently investigating the possible significance of a male

karyotype for all 5 lines derived in this study. Four hES cell lines (ES[2]-[4] and ES[6]) have been continuously kept in culture for more than 50 passages (over 150 population-doublings, Table 3) without evident changes in their growth characteristics, expression of pluripotency-associated markers, or karyotype (data not shown).

All 5 hES cell lines formed teratomas in severe combined immunodeficient (SCID) beige mice. The tumors contained derivatives from the three embryonic germ layers, such as respiratory epithelium (Fig. 3A-B and data not shown), cartilage (Fig. 3 C-D and data not shown), or organized structures that stained positive for neural β -tubulin III, α -fetoprotein, or α -actinin (Fig. 3E-H and data not shown).

To ascertain the ability of hES cell lines to differentiate in vitro, we initially induced the formation of embryoid bodies from colony fragments maintained in suspension for 3-4 days in hES cell media. Under these conditions, embryoid bodies were formed in a reproducible manner and we could overcome the characteristic difficulty of hES cells to grow as aggregates (Reubinoff et al. 2000). Withdrawal of bFGF during this phase resulted in extensive cell death and failure to maintain initial cell aggregates, as has been previously reported (Reubinoff et al. 2000). After 3-4 days of growth in hES cell media, embryoid bodies generated in this way were plated onto gelatin-coated plates and allowed to undergo further differentiation by removal of bFGF and the addition of serum. Endoderm derivatives displaying strong α -fetoprotein immunoreactivity were readily observed after 2-3 weeks in all 5 hES cell lines (Fig. 4A and data not shown). However, neuroectoderm

derivatives were obtained at a very low frequency under these conditions. To promote differentiation of hES cells toward neuronal fates, we adapted a protocol of co-culture with the stromal cell line PA6 that has been described to enhance neural differentiation of mouse ES cells (Kitajima et al. 2005). All 5 hES cell lines co-cultured with PA6 cells gave rise to differentiated cells with the morphology of mature neurons that expressed high levels of β -tubulin III (Fig. 4B and data not shown).

Embryoid body-based cardiac differentiation of hES cells

In contrast to mouse ES cells, the differentiation of hES cells toward cardiomyocytes is notoriously inefficient (Reubinoff et al. 2000), particularly more so when using embryoid body-based differentiation protocols (Kehat et al. 2001; Laflamme et al. 2007; Laflamme et al. 2005). Alternatively, co-culture of hES cells with mouse visceral endoderm-like END-2 cells has been shown to result in robust induction of cardiomyocyte differentiation (Mummery et al. 2003). To characterize the ability of the new hES cell lines to differentiate into cardiomyocytes in vitro, and in an attempt to establish differentiation protocols that do not rely on co-culture systems, we initially set out to analyze the presence of heart muscle derivatives in embryoid bodies allowed to differentiate in serum-containing media for 2-4 weeks. In contrast to previous studies (Xu et al. 2002), but in agreement with observations from different laboratories using a variety of hES cell lines (Kehat et al. 2001; Laflamme et al. 2007; Laflamme et al. 2005), we did not observe rhythmically-beating cells under these conditions in any of the 5 new hES cell lines (Fig. 5A and data not shown). We next

analyzed the effect of ascorbic acid, since it has been shown that this compound enhances the cardiac differentiation of mouse ES cells (Takahashi et al. 2003) and potentiates the cardiogenic effect of END-2-hES cell co-cultures (Passier et al. 2005). The supplementation of the differentiation medium with 100 μ M ascorbic acid resulted in approximately 20% of the embryoid bodies generated from any of the 5 hES cell lines displaying rhythmically-beating areas (Fig. 5B and data not shown) that stained strongly positive for cardiac α -actinin (Fig. 5C and data not shown). Our results show that reproducible cardiac differentiation of hES cells can be obtained using relatively simple in vitro protocols and provide a valuable experimental platform to further optimize such protocols for the directed generation of large quantities of human cardiomyocytes, as well as for exploring the mechanisms that control cardiomyocyte differentiation.

Establishment of enzymatically-dispersable hES cell lines

Human ES cells typically display very low plating efficiency when passaged as single cells (Amit et al. 2000), and therefore require mechanical or mild enzymatic dissociation of cell clumps. These procedures are time-consuming, result in variable degrees of cell differentiation during hES cell culture maintenance, and make the establishment of genetically-modified hES cell clones difficult. The derivation of hES cell lines that can be routinely passaged as single cells (Cowan et al. 2004) and the adaptation to single-cell dissociation of pre-existing hES cell lines (Ellerstrom et al. 2007) are thus likely to facilitate the maintenance of undifferentiated hES cells and their experimental manipulation.

For these reasons, we attempted to establish sub-clones of hES cells that displayed high plating efficiency after single-cell trypsinization. Sub-clones were particularly easily adapted from early-passage (passage 3 or 4) ES[4] cells and have been maintained for over 40 passages on feeders after single-cell dissociation (scES[4]). Furthermore, scES[4] cells can also be propagated for extended periods of time in fibroblast-free cultures using feeder-conditioned hES cell media and matrigel- or gelatin-coated plates. It should be noted that, even though it has been suggested that such culture adaptations may increase the propensity of hES cells to accumulate karyotypic abnormalities (Baker et al. 2007; Enver et al. 2005), scES[4] cells continuously maintained in our laboratory for over 30 passages under feeder-free conditions using matrigel retain a normal karyotype and pluripotency in vivo and in vitro (data not shown). Finally, scES[4] cells have also been easily adapted to commercially-available serum-free, chemically-defined media (mTeSR™1 or STEMPRO® hESC SFM) and have been maintained undifferentiated for over 10 passages on matrigel- or gelatin-coated plates.

Conclusions

The five new hES cell lines derived in this study provide valuable tools for investigating the mechanisms of ES cell self-renewal, pluripotency, and differentiation toward specific cell-types. ES[2] and ES[3] are currently available to interested researchers through the Spanish Stem Cell Bank (http://www.isciii.es/htdocs/terapia/terapia_lineas.jsp). The

remaining 3 lines (ES[4], ES[5], and ES[6]) are in the process of being registered and deposited and will soon be similarly available.

Methods

Source of human pre-embryos. Human pre-implantation embryos were specifically donated for this research project by couples undergoing IVF cycles at Institut Universitari Dexeus (Barcelona). Embryos were used for the present study after the informed consent of the couples, following the protocol previously approved by the Institutional Ethics Committee on Clinic Investigation and the Spanish competent authorities (Comisión de Seguimiento y Control de la Donación de Células y Tejidos Humanos del Instituto de Salud Carlos III). Derivations were performed at the Stem Cell Bank of the Center of Regenerative Medicine in Barcelona.

Embryos were thawed using Vitrolife thawing media, Thaw-kit 1 or Thaw-kit Blast (Vitrolife, Göteborg, Sweden) depending on the embryo stage and according to the manufacturer's instructions.

Derivation of hES cell lines. Early-cleavage embryos were cultured in G1.2 medium (Vitrolife, Göteborg, Sweden) until day 3 and then in G2.2 medium (Vitrolife) to the blastocyst stage. Embryos thawed at the blastocyst stage were cultured in G2.2 medium

overnight to allow for re-expansion. Blastocysts were classified according to criteria reported elsewhere (Stephenson et al. 2006).

The zona pellucida was removed with 5mg/ml pronase (Roche) or with Tyrode's acid (Medicult). Fragments and degenerated cells were gently removed with a pulled Pasteur pipette (Humagen Fertility Diagnostics). When performed, immunosurgery for ICM isolation was performed according to published protocols (Solter and Knowles 1975).

Whole blastocysts or isolated ICMs were plated on top of irradiated (55Gy) human foreskin fibroblasts (CCD112Sk ATCC), seeded at a density of 7×10^4 cells/cm² on IVF dishes (Falcon, Becton Dickinson) coated with 0.1% gelatin (Chemicon), and cultured further at 37°C and 5% CO₂ in hES cell media consisting on KO-Dulbecco's modified Eagle's medium supplemented with 20% KO-Serum Replacement (Invitrogen), 2 mM GlutaMAX (Invitrogen), 50 µM 2-mercaptoethanol (Invitrogen), non-essential amino acids (Cambrex), 8 ng/ml basic fibroblast growth factor (bFGF) (Invitrogen), and penicillin-streptomycin (Invitrogen). Outgrowths were mechanically dissociated into small clumps after 10-12 days using a 150-µm diameter plastic pipette (The Stripper, Midatlantic Diagnostics) and replated onto a fresh feeder layer.

Propagation of hES cell lines. Individual undifferentiated colonies were mechanically dissociated into small clumps using a 150-µm diameter plastic pipette and replated every 5-7 days. In some cases, single-cell suspensions were prepared after enzymatic dispersion (0.05% Trypsin/EDTA, Invitrogen) and plated at 1:5-1:10 dilutions onto feeder layers, or

on plates coated with Matrigel (Becton Dickinson) diluted 1:15 with KO-DMEM, or 0.1% gelatin. In feeder-free cultures, we used hES cell media pre-conditioned by feeders. The following commercially-available serum-free, chemically-defined media were also assayed following the manufacturers' directions: mTeSR™1 (StemCell Technologies) and STEMPRO® hESC SFM (Invitrogen).

Karyotyping was performed after incubating colonies for 30 min in a 1:500 dilution of Colcemid (Invitrogen) by the G-banding method and imaged and processed using the software CytoVision (Applied Imaging, Olympus). A minimum of 15 metaphases were analyzed for each line.

Colonies were also periodically selected and cryopreserved in cryotube vials (Nunc, Roskilde) in 90% FBS and 10% DMSO (Sigma) at a cooling rate of 1°C/min and stored in liquid nitrogen. After thawing at 37°C, some colonies were plated on new feeder layers in hES medium to assess freezing/thawing survival.

In vitro differentiation. Embryoid bodies (EBs) were generated from large colony fragments obtained by mechanical splitting with a finely drawn Pasteur pipette or by treatment of confluent hES cell cultures with 1 mg/ml collagenase IV (Invitrogen) for 10 min at 37°C. Colony fragments were maintained in non-adherent dishes for 3-4 days in hES medium. Embryoid bodies were then plated in 0.1% gelatin-coated glass chamber slides and cultured in differentiation medium (DMEM supplemented with 20% fetal bovine serum,

2 mM GlutaMAX, 100 μ M 2-mercaptoethanol, non-essential amino acids, and penicillin-streptomycin) for 2-3 weeks. Neuronal differentiation of hES cells was induced by co-culture with the stromal cell line PA6 for 3–5 weeks (Kitajima et al. 2005). Differentiation into mesoderm derivatives was enhanced by supplementing the differentiation medium with 100 μ M ascorbic acid (Sigma).

Teratoma formation. Approximately 10^6 hES cells were resuspended in 20-40 μ l of hES cell media and injected intramuscularly into the gastrocnemius of severe combined immunodeficient (SCID) beige mice (Charles River Laboratories). Eight weeks after cell injection, mice were sacrificed and tumors were processed and analyzed following conventional immunohistochemistry protocols. All animal experiments were conducted following experimental protocols previously approved by the Institutional Ethics Committee on Experimental Animals, in full compliance with Spanish and European laws and regulations.

Immunofluorescence. Cells grown on chamber slides (LabTek, Nunc) were washed with PBS and fixed for 15 min with 4% paraformaldehyde at 4°C. We used the following primary antibodies for immunofluorescence: mouse anti-Oct4 (1:500, Santa Cruz Biotechnology), rabbit anti-Sox2 (1:100, Chemicon), rabbit anti-Nanog (1:500, Abcam), rat anti-SSEA-3, mouse anti-SSEA-4, mouse anti-TRA-1-60, mouse anti-TRA-1-81 (1:10, Chemicon), mouse anti- α -actinin (1:100, Sigma), mouse anti- β -tubulin III (1:1000, Covance), and rabbit anti- α -fetoprotein (1:400, Dako). Incubation with primary antibody

was for 24 h at 4°C. Incubation with FITC-, Cy2-, or Cy3-conjugated secondary antibodies (1:200, Jackson ImmunoResearch) was for 2h at RT followed by counterstaining with DAPI (10mg/ml, Sigma, St. Louis, MO).

Alkaline phosphatase activity was measured by using the Alkaline Phosphatase Red Membrane Substrate (Sigma) after fixation for 2 min with 4% paraformaldehyde.

Telomerase activity was detected using TRAPeze Telomerase Detection Kit (Chemicon International) following the manufacturer's directions.

Molecular typing. Human leukocyte antigen (HLA) typing of hES cell lines was performed by sequence based typification (SBT) with the AlleleSEQR HLA Sequencing Kit (Atria Genetics). Microsatellite DNA fingerprinting was performed by multiplex PCR of 9 microsatellites or short tandem repeats (STRs) plus amelogenine gene using AmpliSTR Profiler Plus Kit (Applied Biosystems).

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References

Figure Legends

Fig. 1. Derivation of five new human embryonic stem cell lines. (A) Initial cellular outgrowth of the successful hES cell derivations, surrounded by trophectoderm derivatives. Notice the atypical initial flat growth of the ES[4] line. (B) Morphology of hES cell colonies at early (2-4) passages. (C) Morphology of hES cell colonies after 15-20 passages. (D) Analysis of alkaline phosphatase activity on the same colonies shown in (C).

Fig. 2. Expression of pluripotency-associated markers. Morphology (as shown by differential interference contrast, DIC) and immunofluorescence localization of SSEA3, Oct4, TRA-1-60, Nanog, SSEA4 and SOX2 in colonies of ES[2] and ES[3] lines. Cell nuclei are counterstained with DAPI.

Fig. 3. Pluripotency of hES cells in vivo. Hematoxylin-eosin staining (A-D) and immunofluorescence localization (E-H) in paraffin sections of teratomas induced by injecting ES[2] (A, C, E, G) or ES[3] (B, D, F, H) cells into severe combined immunodeficient (SCID) beige mice. Histologically-recognizable structures include respiratory epithelia (A, B) and cartilage formations (C, D). (E, F) Neuroectoderm and endoderm derivatives are detected by strong immunoreactivity for β -tubulin III (green channel) and α -fetoprotein (red channel), respectively. (G-H) Mesoderm derivatives are detected by strong immunoreactivity for α -actinin (green channel). Cell nuclei are counterstained with DAPI in (E-H).

Fig. 4. In vitro differentiation of endoderm and neuroectoderm derivatives. ES[2] (A, C) and ES[3] (B, D) cells readily generate endoderm derivatives after embryoid body-based differentiation protocols (A, B), as evidenced by glandular structures immunoreactive for α -fetoprotein (green channel), and differentiated cells with morphology of mature neurons that stain positive for β -tubulin III (red channel) after co-culture with PA6 cells (C, D). Cell nuclei are counterstained with DAPI.

Fig. 5. Embryoid body-based in vitro differentiation of hES cells into cardiomyocytes. (A) Embryoid bodies of ES[3] cells after 3 weeks of differentiation in serum-containing media fail to differentiate into cardiomyocytes, as evidenced by the absence of α -actinin immunoreactivity (red channel). Cell nuclei are counterstained with DAPI in the lower panel. (B-C) Addition of ascorbic acid to the differentiation medium results in cardiomyocyte differentiation of ES[3]-derived embryoid bodies, as shown by the appearance of rhythmically-beating areas, encircled in (B), and strong immunoreactivity for α -actinin (red channel in C). A higher magnification of the area marked in (C) is shown in the right panels to better appreciate the sarcomeric striations of the α -actinin staining. Cell nuclei are counterstained with DAPI in (C).

Table 1. Summary of results of embryo thawing and derivation

	Pronuclear	Cleavage	Blastocyst	Total
Thawed	20	35	6	61
Embryo survival	10 (50%)	28 (80%)	4 (67%)	42 (69%)
Blastocyst rate	3 (30%)	10 (36%)		
Blastocyst/ICM seeded	3	10	4	17
Outgrowths	-	3	2	5
hES cell lines	-	3	2	5

Table 2. Scoring of blastocyst quality

Embryo #	Expansion	ICM	Trophectoderm	Quality score	Lines
1	1	D	C	poor	
2	2	D	C	poor	ES[2]
3	2	D	C	poor	
4	3	C	C	intermediate	ES[3]
5	2	D	C	poor	
6	3	B	B	intermediate	
7	2	B	A	good	
8	3	B	A	good	
9	3	C	A	intermediate	
10	1	D	C	poor	
11	2	D	C	poor	
12	2	A	B	good	
13	2	C	B	intermediate	
14	2	B	A	good	ES[5]
15	2	D	B	poor	ES[6]
16	1	D	C	poor	ES[4]
17	1	D	C	poor	

Blastocyst quality was scored following the criteria of Stephenson and colleagues (Stephenson et al. 2006), with respect to blastocyst expansion (1, no expansion in overall size, zona pellucida still thick; 2, some expansion in overall size, zona pellucida beginning to thin; 3, full expansion, zona pellucida very thin), ICM appearance (A, cells compacted, tightly adherent together and indistinguishable as individual cells; B, cells less compacted so larger in size, loosely adhered together, some visible as individual cells; C, very few cells visible, either compacted or loose, may be difficult to completely distinguish from trophoctoderm; D, cells of ICM appear degenerate), and trophoctoderm appearance (A, many small identical cells forming a continuous trophoctoderm layer; B, fewer, larger cells, may not form continuous trophoctoderm layer; C, sparse cells, may be very large, very flat or appear degenerate).

Table 3. Summary of characterization analyses of the 5 new hES cell lines

	ES[2]	ES[3]	ES[4]	ES[5]	ES[6]
Karyotype	46, XY				
Alkaline phosphatase	+	+	+	+	+
Telomerase	+	+	+	+	+
HLA typing	done *				
MDF	done *				
Number of passages	50	65	51	50	78
Pluripotency markers:					
SSEA-3	+	+	+	+	+
SSEA-4	+	+	+	+	+
TRA-1-60	+	+	+	+	+
TRA-1-81	+	+	+	+	+
Oct-4	+	+	+	+	+
NANOG	+	+	+	+	+
Sox-2	+	+	+	+	+
SSEA-1	-	-	-	-	-
Freezing/thawing	+	+	+	+	+
Pluripotency					
In vitro					
endoderm	+	+	+	+	+
ectoderm	+	+	+	+	+
mesoderm	+	+	+	+	+
In vivo					
endoderm	+	+	+	+	+
ectoderm	+	+	+	+	+
mesoderm	+	+	+	+	+

HLA, human leukocyte antigen; MDF, microsatellite DNA fingerprinting; +, positive/present; -, negative

* Full details are available upon request

References

- Adewumi O., Aflatoonian B., Ahrlund-Richter L., Amit M., Andrews P.W., Beighton G., Bello P.A., Benvenisty N., Berry L.S., Bevan S., Blum B., Brooking J., Chen K.G., Choo A.B., Churchill G.A., Corbel M., Damjanov I., Draper J.S., Dvorak P., Emanuelsson K., Fleck R.A., Ford A., Gertow K., Gertsenstein M., Gokhale P.J., Hamilton R.S., Hampl A., Healy L.E., Hovatta O., Hyllner J., Imreh M.P., Itskovitz-Eldor J., Jackson J., Johnson J.L., Jones M., Kee K., King B.L., Knowles B.B., Lako M., Lebrin F., Mallon B.S., Manning D., Mayshar Y., McKay R.D., Michalska A.E., Mikkola M., Mileikovsky M., Minger S.L., Moore H.D., Mummery C.L., Nagy A., Nakatsuji N., O'Brien C.M., Oh S.K., Olsson C., Otonkoski T., Park K.Y., Passier R., Patel H., Patel M., Pedersen R., Pera M.F., Piekarczyk M.S., Pera R.A., Reubinoff B.E., Robins A.J., Rossant J., Rugg-Gunn P., Schulz T.C., Semb H., Sherrer E.S., Siemen H., Stacey G.N., Stojkovic M., Suemori H., Szatkiewicz J., Turetsky T., Tuuri T., van den Brink S., Vintersten K., Vuoristo S., Ward D., Weaver T.A., Young L.A., and Zhang W. 2007. Characterization of human embryonic stem cell lines by the International Stem Cell Initiative. *Nat Biotechnol* **25**: 803.
- Alikani M., Cohen J., Tomkin G., Garrisi G.J., Mack C., and Scott R.T. 1999. Human embryo fragmentation in vitro and its implications for pregnancy and implantation. *Fertil Steril* **71**: 836.
- Allegrucci C., and Young L.E. 2007. Differences between human embryonic stem cell lines. *Hum Reprod Update* **13**: 103.
- Amit M., Carpenter M.K., Inokuma M.S., Chiu C.P., Harris C.P., Waknitz M.A., Itskovitz-Eldor J., and Thomson J.A. 2000. Clonally derived human embryonic stem cell lines maintain pluripotency and proliferative potential for prolonged periods of culture. *Dev Biol* **227**: 271.
- Amit M., Shariki C., Margulets V., and Itskovitz-Eldor J. 2004. Feeder layer- and serum-free culture of human embryonic stem cells. *Biol Reprod* **70**: 837.
- Baker D.E., Harrison N.J., Maltby E., Smith K., Moore H.D., Shaw P.J., Heath P.R., Holden H., and Andrews P.W. 2007. Adaptation to culture of human embryonic stem cells and oncogenesis in vivo. *Nat Biotechnol* **25**: 207.
- Chen H., Qian K., Hu J., Liu D., Lu W., Yang Y., Wang D., Yan H., Zhang S., and Zhu G. 2005. The derivation of two additional human embryonic stem cell lines from day 3 embryos with low morphological scores. *Hum Reprod* **20**: 2201.
- Cowan C.A., Klimanskaya I., McMahan J., Atienza J., Witmyer J., Zucker J.P., Wang S., Morton C.C., McMahan A.P., Powers D., and Melton D.A. 2004. Derivation of embryonic stem-cell lines from human blastocysts. *N Engl J Med* **350**: 1353.
- Ellerstrom C., Strehl R., Moya K., Andersson K., Bergh C., Lundin K., Hyllner J., and Semb H. 2006. Derivation of a xeno-free human embryonic stem cell line. *Stem Cells* **24**: 2170.
- Ellerstrom C., Strehl R., Noaksson K., Hyllner J., and Semb H. 2007. Facilitated expansion of human embryonic stem cells by single-cell enzymatic dissociation. *Stem Cells* **25**: 1690.
- Enver T., Soneji S., Joshi C., Brown J., Iborra F., Orntoft T., Thykjaer T., Maltby E., Smith

- K., Dawud R.A., Jones M., Matin M., Gokhale P., Draper J., and Andrews P.W. 2005. Cellular differentiation hierarchies in normal and culture-adapted human embryonic stem cells. *Hum Mol Genet* **14**: 3129.
- Gardner D.K., Lane M., Stevens J., Schlenker T., and Schoolcraft W.B. 2000. Blastocyst score affects implantation and pregnancy outcome: towards a single blastocyst transfer. *Fertil Steril* **73**: 1155.
- Genbacev O., Krtolica A., Zdravkovic T., Brunette E., Powell S., Nath A., Caceres E., McMaster M., McDonagh S., Li Y., Mandalam R., Lebkowski J., and Fisher S.J. 2005. Serum-free derivation of human embryonic stem cell lines on human placental fibroblast feeders. *Fertil Steril* **83**: 1517.
- Gerecht-Nir S., and Itskovitz-Eldor J. 2004. Human embryonic stem cells: a potential source for cellular therapy. *Am J Transplant* **4 Suppl 6**: 51.
- Heins N., Englund M.C., Sjoblom C., Dahl U., Tonning A., Bergh C., Lindahl A., Hanson C., and Semb H. 2004. Derivation, characterization, and differentiation of human embryonic stem cells. *Stem Cells* **22**: 367.
- Kehat I., Kenyagin-Karsenti D., Snir M., Segev H., Amit M., Gepstein A., Livne E., Binah O., Itskovitz-Eldor J., and Gepstein L. 2001. Human embryonic stem cells can differentiate into myocytes with structural and functional properties of cardiomyocytes. *J Clin Invest* **108**: 407.
- Kitajima H., Yoshimura S., Kokuzawa J., Kato M., Iwama T., Motohashi T., Kunisada T., and Sakai N. 2005. Culture method for the induction of neurospheres from mouse embryonic stem cells by coculture with PA6 stromal cells. *J Neurosci Res* **80**: 467.
- Klimanskaya I., Chung Y., Meisner L., Johnson J., West M.D., and Lanza R. 2005. Human embryonic stem cells derived without feeder cells. *Lancet* **365**: 1636.
- Laflamme M.A., Chen K.Y., Naumova A.V., Muskheli V., Fugate J.A., Dupras S.K., Reinecke H., Xu C., Hassanipour M., Police S., O'Sullivan C., Collins L., Chen Y., Minami E., Gill E.A., Ueno S., Yuan C., Gold J., and Murry C.E. 2007. Cardiomyocytes derived from human embryonic stem cells in pro-survival factors enhance function of infarcted rat hearts. *Nat Biotechnol* **25**: 1015.
- Laflamme M.A., Gold J., Xu C., Hassanipour M., Rosler E., Police S., Muskheli V., and Murry C.E. 2005. Formation of human myocardium in the rat heart from human embryonic stem cells. *Am J Pathol* **167**: 663.
- Li T., Zhou C.Q., Mai Q.Y., and Zhuang G.L. 2005. Establishment of human embryonic stem cell line from gamete donors. *Chin Med J (Engl)* **118**: 116.
- Liew C.G., Moore H., Ruban L., Shah N., Cosgrove K., Dunne M., and Andrews P. 2005. Human embryonic stem cells: possibilities for human cell transplantation. *Ann Med* **37**: 521.
- Lu J., Hou R., Booth C.J., Yang S.H., and Snyder M. 2006. Defined culture conditions of human embryonic stem cells. *Proc Natl Acad Sci U S A* **103**: 5688.
- Ludwig T.E., Levenstein M.E., Jones J.M., Berggren W.T., Mitchen E.R., Frane J.L., Crandall L.J., Daigh C.A., Conard K.R., Piekarczyk M.S., Llanas R.A., and Thomson J.A. 2006. Derivation of human embryonic stem cells in defined conditions. *Nat Biotechnol* **24**: 185.
- Lyerly A.D., and Faden R.R. 2007. Embryonic stem cells. Willingness to donate frozen

- embryos for stem cell research. *Science* **317**: 46.
- Mateizel I., De Temmerman N., Ullmann U., Cauffman G., Sermon K., Van de Velde H., De Rycke M., Degreef E., Devroey P., Liebaers I., and Van Steirteghem A. 2006. Derivation of human embryonic stem cell lines from embryos obtained after IVF and after PGD for monogenic disorders. *Hum Reprod* **21**: 503.
- Menasche P. 2005. The potential of embryonic stem cells to treat heart disease. *Curr Opin Mol Ther* **7**: 293.
- Menezo Y., Hazout A., Dumont M., Herbaut N., and Nicollet B. 1992. Coculture of embryos on Vero cells and transfer of blastocysts in humans. *Hum Reprod* **7 Suppl 1**: 101.
- Menezo Y., Veiga A., and Benkhalifa M. 1998. Improved methods for blastocyst formation and culture. *Hum Reprod* **13 Suppl 4**: 256.
- Mitalipova M., Calhoun J., Shin S., Wininger D., Schulz T., Noggle S., Venable A., Lyons I., Robins A., and Stice S. 2003. Human embryonic stem cell lines derived from discarded embryos. *Stem Cells* **21**: 521.
- Mummery C., Ward-van Oostwaard D., Doevendans P., Spijker R., van den Brink S., Hassink R., van der Heyden M., Opthof T., Pera M., de la Riviere A.B., Passier R., and Tertoolen L. 2003. Differentiation of human embryonic stem cells to cardiomyocytes: role of coculture with visceral endoderm-like cells. *Circulation* **107**: 2733.
- Oh S.K., Kim H.S., Ahn H.J., Seol H.W., Kim Y.Y., Park Y.B., Yoon C.J., Kim D.W., Kim S.H., and Moon S.Y. 2005. Derivation and characterization of new human embryonic stem cell lines: SNUhES1, SNUhES2, and SNUhES3. *Stem Cells* **23**: 211.
- Passier R., Oostwaard D.W., Snapper J., Kloots J., Hassink R.J., Kuijk E., Roelen B., de la Riviere A.B., and Mummery C. 2005. Increased cardiomyocyte differentiation from human embryonic stem cells in serum-free cultures. *Stem Cells* **23**: 772.
- Pera M.F., and Trounson A.O. 2004. Human embryonic stem cells: prospects for development. *Development* **131**: 5515.
- Pickering S.J., Braude P.R., Patel M., Burns C.J., Trussler J., Bolton V., and Minger S. 2003. Preimplantation genetic diagnosis as a novel source of embryos for stem cell research. *Reprod Biomed Online* **7**: 353.
- Rajala K., Hakala H., Panula S., Aivio S., Pihlajamaki H., Suuronen R., Hovatta O., and Skottman H. 2007. Testing of nine different xeno-free culture media for human embryonic stem cell cultures. *Hum Reprod* **22**: 1231.
- Reubinoff B.E., Pera M.F., Fong C.Y., Trounson A., and Bongso A. 2000. Embryonic stem cell lines from human blastocysts: somatic differentiation in vitro. *Nat Biotechnol* **18**: 399.
- Semb H. 2005. Human embryonic stem cells: origin, properties and applications. *Apmis* **113**: 743.
- Simon C., Escobedo C., Valbuena D., Genbacev O., Galan A., Krtolica A., Asensi A., Sanchez E., Esplugues J., Fisher S., and Pellicer A. 2005. First derivation in Spain of human embryonic stem cell lines: use of long-term cryopreserved embryos and animal-free conditions. *Fertil Steril* **83**: 246.
- Sjogren A., Hardarson T., Andersson K., Caisander G., Lundquist M., Wikland M., Semb H., and Hamberger L. 2004. Human blastocysts for the development of embryonic stem

- cells. *Reprod Biomed Online* **9**: 326.
- Solter D., and Knowles B.B. 1975. Immunosurgery of mouse blastocyst. *Proc Natl Acad Sci U S A* **72**: 5099.
- Stephenson E.L., Braude P.R., and Mason C. 2006. Proposal for a universal minimum information convention for the reporting on the derivation of human embryonic stem cell lines. *Regen Med* **1**: 739.
- Stojkovic M., Lako M., Stojkovic P., Stewart R., Przyborski S., Armstrong L., Evans J., Herbert M., Hyslop L., Ahmad S., Murdoch A., and Strachan T. 2004. Derivation of human embryonic stem cells from day-8 blastocysts recovered after three-step in vitro culture. *Stem Cells* **22**: 790.
- Suss-Toby E., Gerecht-Nir S., Amit M., Manor D., and Itskovitz-Eldor J. 2004. Derivation of a diploid human embryonic stem cell line from a mononuclear zygote. *Hum Reprod* **19**: 670.
- Takahashi T., Lord B., Schulze P.C., Fryer R.M., Sarang S.S., Gullans S.R., and Lee R.T. 2003. Ascorbic acid enhances differentiation of embryonic stem cells into cardiac myocytes. *Circulation* **107**: 1912.
- Thomson J.A., Itskovitz-Eldor J., Shapiro S.S., Waknitz M.A., Swiergiel J.J., Marshall V.S., and Jones J.M. 1998. Embryonic stem cell lines derived from human blastocysts. *Science* **282**: 1145.
- Veiga A., Camarasa M.V., Aran B., Raya A., and Izpisua Belmonte J.C. 2007. Selection of embryos for stem cell derivation: can we optimize the process? In *Stem Cells in Reproductive Medicine: Basic Science and Therapeutic Potential*. (ed. A.S. Pellicer, C.), p. 133. Informa Healthcare, London.
- Veiga A., Torello M.J., Boiso I., Sandalinas M., Busquets A., Calderon G., and Barri P.N. 1995. Optimization of implantation in the in-vitro fertilization laboratory. *Hum Reprod* **10 Suppl 2**: 98.
- Verlinsky Y., Strelchenko N., Kukhareno V., Rechitsky S., Verlinsky O., Galat V., and Kuliev A. 2005. Human embryonic stem cell lines with genetic disorders. *Reprod Biomed Online* **10**: 105.
- Xu C., Police S., Rao N., and Carpenter M.K. 2002. Characterization and enrichment of cardiomyocytes derived from human embryonic stem cells. *Circ Res* **91**: 501.
- Zhang X., Stojkovic P., Przyborski S., Cooke M., Armstrong L., Lako M., and Stojkovic M. 2006. Derivation of human embryonic stem cells from developing and arrested embryos. *Stem Cells* **24**: 2669.