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Original Research Report

Derivation of a New Human Embryonic Stem Cell Line, Endeavour-1, and Its Clonal Propagation

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ABSTRACT

Here we describe the derivation of a novel human embryonic stem (hES) cell line, Endeavour-1 (E1), its four new clonal lines (E1C1, E1C2, E1C3, E1C4), and their characterization. E1 and its clonal lines are propagated on human fetal fibroblasts (HFFs) derived and grown in a largely serum-free medium. Seven inner cell masses were isolated from 34 donated human embryos (27 survived), and one new hES cell line was obtained. E1 has been in culture for over 1 year and possesses all the typical features of stem cells, i.e., expression of stem cell surface markers (stage-specific embryonic antigens SSEA-3 and SSEA-4, and tumor recognition antigens TRA-1-60 and TRA-1-81), staining for alkaline phosphatase, and the presence of the pluripotent gene marker (nanog). This line shows pluripotency both under in vitro and in vivo conditions. E1 has a normal karyotype (46XX). Using our optimized procedure for cloning, four new clonal lines were derived from E1: E1C1, E1C2, E1C3, and E1C4. These clonal lines show normal characteristics: karyotype of that of the parent line (46XX) except for E1C3, which showed reciprocal translocation involving chromosomes 15 and 17; stem cell surface markers SSEA-4, TRA-1-60, and TRA-1-81; and gene expression for pluripotency (Nanog). All of these clonal lines formed embryoid bodies (EBs) in suspension cultures. After seeding, the EBs differentiated, forming cell lineages derived from all three germ layers as indicated by immunolocalization for the ectodermal marker β-III tubulin, the mesodermal marker CD34, and the endodermal marker α-fetoprotein (AFP). There were subtle differences in the expression of these markers between clones. These clonal lines showed pluripotency in vivo. E1 and its clonal lines can differentiate to definitive endoderm after treatment with activin A, and, as indicated by expression of SOX17, FOXa2, and GATA-4 by RT-PCR, there are some subtle differences between these clonal lines. This may help in selecting clonal lines for specific lineage specification and for developing future cell therapy for various diseases.

INTRODUCTION

Following the first report of successful derivation of five human embryonic stem (hES) cell lines by the Thomson group in 1998 [1], more new hES cell lines have been created [2,3], including one attempt to produce nuclear transferred (nt) blastocysts [4]. To date it is estimated than more than 414 new hES cell lines have been produced worldwide, out of which about 78 are listed in National Institutes of Health (NIH) Registry according to Guhr et al. [5]. Only about 179 these lines are characterized to some extent and available for research. Many of these hES cell lines are not clonal and have been derived under different culture conditions and propagated on different feeder layers [mouse embryonic fibroblasts (MEF), STO, fetal muscle, skin, and foreskin, adult fallopian tube epithelial cells, and some feeder free/serum free systems], hence comparison of these lines is very

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difficult [6–12]. In addition, differences in gene expression have been reported in some of these lines [13,14].

Recently some attempts have been made to derive new hES cell lines under more defined conditions such as serum-free or feeder-free [15–18]. However, almost all of these studies employed immunosurgery for dissection of the inner cell mass (ICM) and fetal calf serum (FCS) to grow feeder layers. Two of these xeno-free and feeder-free hES cell lines derived recently by Ludwig et al. [18] show chromosomal abnormalities. Ellerstrom and coworkers [19] also tried defining an in vitro culture system for the derivation of a new hES cell line under xeno-free conditions using human serum. The latter, however, was found to cause the differentiation of hES cells in long-term cultures [14,20].

Here we describe for the first time the derivation of a novel hES cell line called Endeavour-1 (E1) in culture conditions that are largely free of serum and animal products, and that is propagated on a human feeder layer. Clonal propagation of E1 by our optimized procedure [21] yielded four new clonal lines. These clonal lines show subtle differences in their ability to differentiate to various lineages and can be coaxed to differentiate to definitive endoderm by the nodal protein activin A.

MATERIALS AND METHODS

All reagents including culture media and sera were obtained from Gibco/Invitrogen (Carlsbad, CA; http://www.invitrogen.com). This study had institutional ethics approval (HREC 01270 and HREC 02247, respectively) to work on hES cell and human fetal fibroblast lines.

hES cell culture

Derivation of serum-free feeder layer. Serum-free HFFs were derived from human fetal skin after therapeutic termination of pregnancies and after obtaining maternal consent. Briefly, 2- × 3-mm^2 pieces of skin were washed twice with phosphate-buffered saline (PBS) containing 25 U/ml penicillin and 25 μg/ml streptomycin and finally chopped into small pieces with a pair of fine scissors. Single-cell suspensions were prepared by treating for 15 min at 37°C with an xeno-free protease, TripLE Select (Gibco/Invitrogen (Carlsbad, CA; http://www.invitrogen.com). After washing, the HFFs were cultured in serum preplacer (SR) medium (see the composition below) in T75 tissue culture flasks coated with human collagen IV (Sigma, MO). The primary cultures of HFFs were cryopreserved by a standard slow freezing procedure in 10% dimethylsulfoxide (DMSO). These newly derived serum-free HFFs were validated for supporting the undifferentiated growth of an existing line, hES 3, obtained from ESI Singapore for many passages.

hES cell culture. The hES cell colonies were maintained in six-well culture plates (Becton Dickinson, NJ; http://www.bdbiosciences.com) coated with human collagen Type IV and gamma-irradiated (45 Gy) in house-derived serum-free primary HFFs (passage 6) feeder layers (1.5×10^6 cells/ml). The incubations were carried out at 37°C, 5% CO_2 in SR medium consisting of high-glucose Dulbecco's knockout (KO-DMEM), supplemented with 20% KO-SR (Gibco, Carlsbad, CA), 2 mM L-glutamine, 0.1 mM nonessential amino acids, 0.01 mM 2-mercaptoethanol, 1× insulin-transferrin-selenium, 4 ng/ml, basic fibroblast growth factor (bFGF), 25 U/ml penicillin, and 25 μg/ml streptomycin.

Derivation of new hES cell line, E1

A total of 34 day-2 and day-5 embryos were thawed over a 12-month period at IVF Australia in Sydney. Twenty seven of the embryos survived and were cultured in Quinns Advantage Cleavage and Blastocyst medium supplemented with 5% human serum albumin (HSA; SAGE Biopharma, USA). Seven of the embryos hatched following laser breaching of the zonae pellucidae and were transferred to HFF feeder layers in six-well plates, where they were cultured in SR medium for a further 2–3 days. The attached inner cell masses (ICMs) were dissected manually and transferred into fresh feeder plates and transferred to the Diabetes Transplant Unit, Prince of Wales Hospital, Sydney, in a portable 5% CO_2 incubator. The appearance of a single hES cell colony was observed after 2 weeks in culture. The newly emerged hES cell colony was initially passaged two to three times by physical dissection before using enzymatic dissection with TripLE Select. E1 is amenable to both slow and fast freezing by using standard procedures [22].

Clonal propagation of E1

Preparation of hES single cells. Approximately 300–400 hES cell colonies were dissected with collagenase type IV (1 mg/ml in PBS without Ca^{2+};1 ml/well) for 7 min at 37°C from six-well plates after gently washing twice with PBS. The hES cell colonies were allowed to settle at the bottom of a 15-ml tube for 5 min, and the supernatant was aspirated. The hES cell colonies were dissociated into single cells by using 0.05% trypsin/0.25% EDTA at 37°C for 7 min and carefully triturated twice with a pipette. Finally, the single-cell preparations were resuspended at 1×10^6 cells/ml in conditioned medium collected from HFFs cultured in SR medium for 24 h.

Fluorescence-activated cell sorting of hES single-cell preparations. A FACS Calibur (Becton Dickinson) was used to select hES cells exclusively as described previously [21]. Each cell sorted by fluorescence-activated cell sorting (FACS) was dispersed into a well of a 96-well plate containing HFFs as a feeder layer and cultured in SR medium 5% CO_2 and 37°C for 2 weeks. The viability of single hES cells after FACS sorting was >98%, as assessed by fluorescent staining with carboxyfluorescein diacetate (CFDA) and propidium iodide (PI). Briefly, hES cells were washed with 500 μl PBS at 800 rpm for 3 min and resuspended in 250 μl of CFDA (0.1 mM in DMSO) and incubated for 30 min at 37°C. Cells were then washed in PBS and resuspended in 200 μl of PBS, before 10 μl of PI (100 μg/ml PBS) was added. The cells were incubated on ice for 5 min before counting viable (green fluorescence) and nonviable (red fluorescence) cells under the fluorescent microscope. Clones obtained were initially passaged by physical

dissection in 24-well plates and subsequently into six-well plates by trypsin.

Characterization of E1 and clonal lines

Stem cells surface and intracellular markers. Immunohistochemical localization of various stem cell surface markers, i.e., stage-specific embryonic antigens SSEA-1, SSEA-3, and SSEA-4 and tumor recognition antigens TRA-1-60 and TRA-1-81, were carried out using primary antibodies (1:40) against these surface markers and detected by using fluorescein isothiocyanate (FITC)-conjugated appropriate secondary antibodies as per the supplier's recommendation (Chemicon, VIC, Australia; http://www.chemicon.com.au) and as described previously [21]. The pluripotent intracellular marker alkaline phosphatase was assessed immunohistochemically by using a commercially available kit (Sigma-Aldrich) following the manufacturer's instructions.

Freezing and thawing. E1 and clonal lines were cryopreserved by slow and fast freezing procedures (vitrification) and thawed several times according to our modified procedure from [22]. This modification included replacing 20% fetal bovine serum (FBS) by 30% KO-SR for both slow- and fast-freezing procedures.

RT-PCR. Total RNA from hES cells was extracted using an RNeasy mini kit (Qiagen) with DNase treatment. The first strand of cDNA was synthesized using 5 μg of total RNA with Moloney murine leukemia virus reverse transcriptase (MMLV-RT; Gibco) and an oligo(dT) primer (Roche). The expression of pluripotent markers Nanog and differentiation markers for ectoderm (Nestin), mesoderm (Renin), endoderm (α-fetoprotein, AFP) and the definitive endoderm markers SOX-17, GATA-4, FOXa2 was assessed by semiquantitative PCR using the Gel Doc System (BIO RAD) as described previously [21].

Immunomicroarray phenotypic assessment. Expression for various hematopoietic markers in E1 and all of its clonal lines was analyzed using an immunomicroarray chip according to manufacturer's instructions (Medsaic Pty Ltd, Sydney, Australia)

Karyotyping. A standard G banding and multicolor spectra karyotyping (SKY) was used for analysis by using the SKY H-10 kit as per the manufacturer's instructions (Applied Spectra Imaging, Inc, Carlsbad, CA). For each sample, 20 metaphases were captured for modal determination.

Analysis of pluripotency in vivo. To assess in vivo pluripotency, approximately 1–2 $\times$ 10^6 cells were injected under the kidney capsule of nonobese diabetic severe combined immunodeficient (NOD-SCID) mice. The animals were euthanized 6–8 weeks later, and the grafts were examined histologically after Hematoxylin & Eosin staining of paraffin sections.

Analysis of pluripotency in vitro. hES cell colonies from each clone were dissected out from wells with collagenase and cultured in nontissue culture plates (suspension culture) in SR medium for 3–4 days to produce EBs. The EBs were seeded in gelatin-coated tissue culture dishes and cultured in SR medium without bFGF for 2 weeks to induce spontaneous differentiation. The expression of lineage markers in hES cell cultures after RNA extraction for ectoderm, mesoderm, and endoderm were evaluated by immunocytochemistry, as described previously [21].

Directed differentiation to definitive endoderm. We used a 5-day treatment with activin A protocol of D'Armour et al. [23] for directed differentiation of hES cells to definitive endoderm. The gene expressions for definitive endoderm, i.e., SOX-17, FOXa2 and GATA-4, and their protein expression was studied by RT-PCR and immunocytochemistry as described above.

Statistical analyses. Data in Figs. 7 and 8 (see below) were analyzed by using Student's *t*-test.

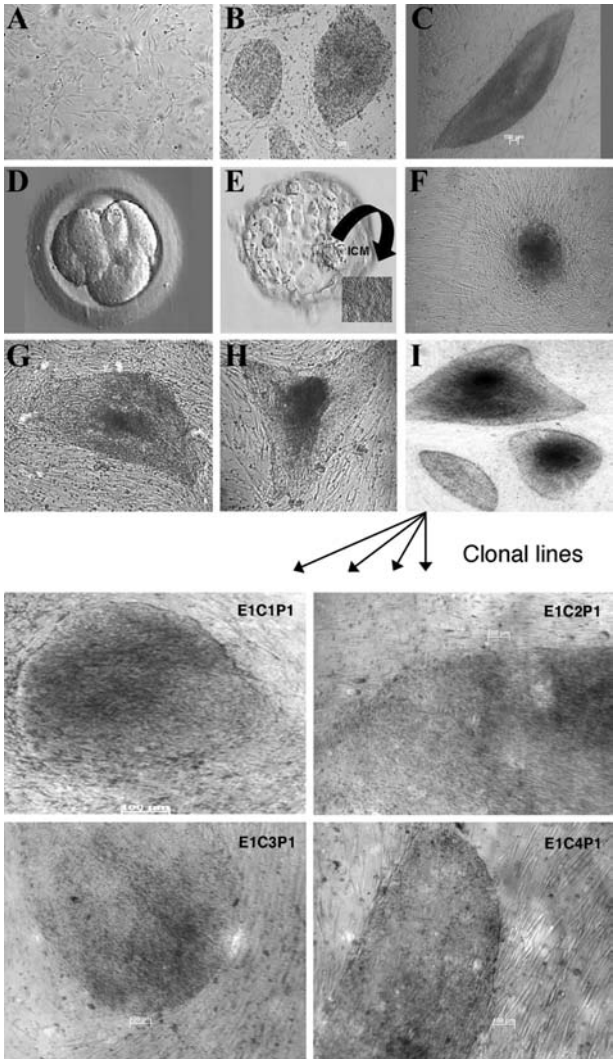

FIG. 1. (*Upper panel*) Derivation of E1 on serum-free HFFs used as feeder layer. (**A**) Serum-free HFFs as monolayer. (**B**) Normal growth of hES 3 colonies on serum-free HFFs. (**C**) Localization of alkaline phosphatase (marker of pluripotency) in a colony of E1. (**D**) A normal-looking human embryo after thawing. (**E**) Hatched blastocyst with visible ICM (higher magnification in inset). (**F**) A nascent colony of E1. (**G**) A fully grown colony of E1. (**H**) A normal-looking colony of E1 after first passage. (**I**) Normal-looking colonies of E1 at passage 9. (*Lower panel*) Derivation of four clonal lines, E1C1, E1C2, E1C3, and E1C4, from E1 with normal undifferentiated morphology and colonies with distinct boundaries.

RESULTS

We have demonstrated that largely serum-free HFFs produced in-house showed similar morphology to typical HFFs used previously and could maintain the undifferentiated growth of an existing hES cell line, hES 3, for several passages (Fig.1A,B). hES cell colonies grown on these HFFs show expression of the stem cell-specific surface markers SSEA-3, SSEA-4, TRA1-60, and TRA1-81, including alkaline phosphatase (pluripotent marker), and gene expression of a pluripotent marker, Nanog (data not shown), shown by RT-PCR. These HFFs at passage 6 were used for subsequent derivation of new hES cell lines.

Out of the seven ICMs dissected out from 27 surviving human embryos, one started growing and produced the hES cell line E1. The efficiency of derivation of a hES cell line depends largely on the initial quality and stage of development of donated embryos. E1 was derived from a blastocyst. The ICM from this blastocyst grew as a typical hES cell colony within 14 days (Fig. 1F). The first passage was carried out by physical dissection (Fig. 1G,H) to five to six pieces followed by enzymatic dissection (Fig. 1I) using TripLE Select at 5- to 6-day intervals. E1 has been in culture for over 1 year with 10–20% spontaneous differentiation occurring after each passage. This line has been cryopreserved many times by the slow- as well by fast-freezing methods and recovered after each thawing. Currently E1 is at passage 24 and growing well in culture. Our approach of using the in-house-produced serum-free HFFs as the feeder layer and the laser dissection of ICMs ensured largely serum-free and xeno-free conditions for derivation and propagation of hES cells.

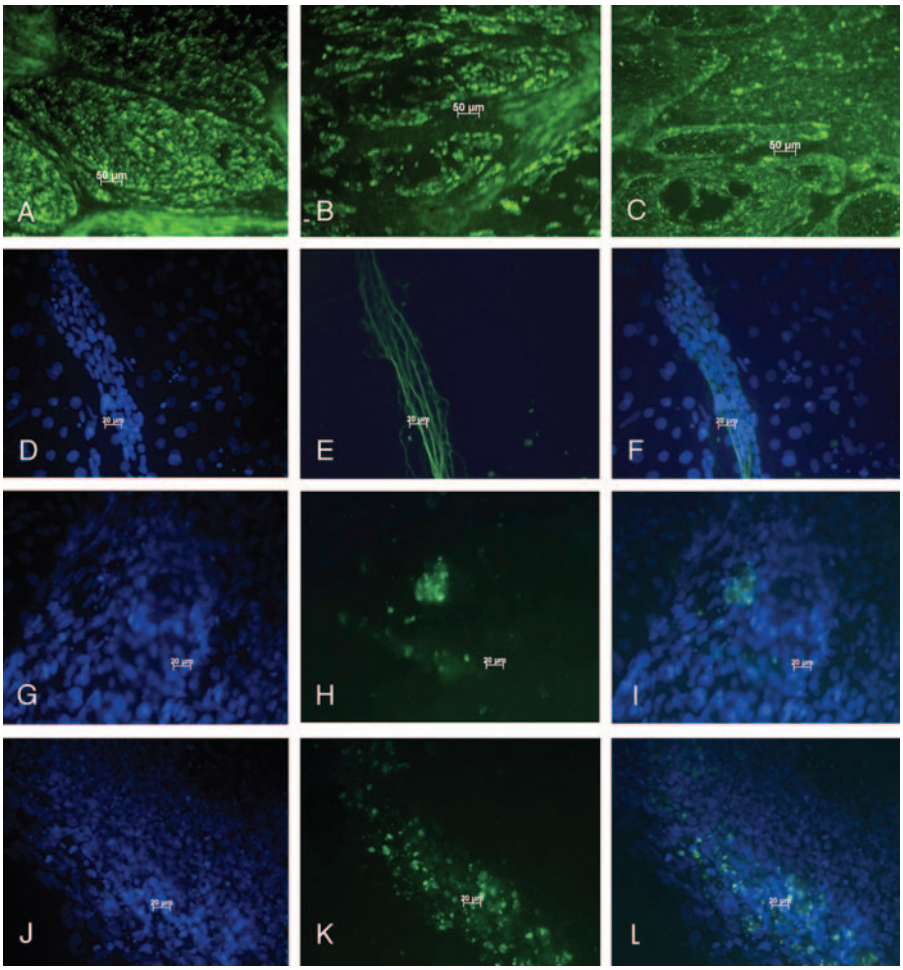

FIG. 2. Immunolacalization of stem cells surface markers and the markers of various lineages after spontaneous differentiation in E1. Lane 1: **A**, SSEA-4; **B**, TRA-1-60; **C**, TRA-1-81. Lane 2, β-tubulin III expression (ectoderm): **D**, DAPI staining for nuclei; **E**, β-tubulin filaments; **F**, overlap. Lane 3, AFP expression (endoderm): **G**, DAPI staining for nuclei; **H**, AFP expression; **I**, overlap. Lane 4, CD 34 expression (mesoderm): **J**, DAPI staining for nuclei; **K**, CD34 expression; **L**, overlap.

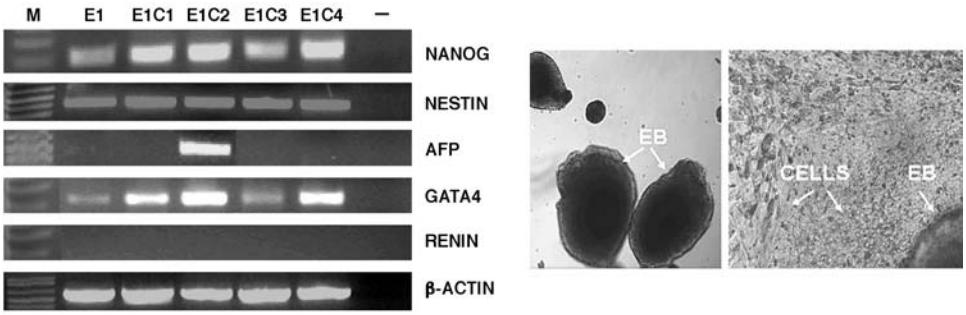

FIG. 3. Characterization of E1 and its clonal lines, E1C1, E1C2, E1C3, and E1C4. (**A**) RT-PCR expression of genes for pluripotency (Nanog) and markers of differentiation—AFP (endoderm), nestin (ectoderm), and rennin (mesoderm). (**B**) EBs (arrows) formed from E1 in suspension culture and their differentiation to different cell lineages (arrows). Similar cell types were also obtained after differentiation of clonal lines.

Characterization of E1and its clonal lines

Morphologically, E1 and its clonal lines E1C1, E1C2, E1C3, and E1C4 grew like normal hES cell colonies (Fig. 1I, lower panel) with clear boundaries and as a compact mass of cells that have a high nuclear-to-cytoplasmic ratio. The colonies (E1) showed uniform localization of alkaline phosphatase activity (Fig. 1C), a marker for pluripotency and a normal 46XX karyotype (see Fig. 4D). In situ staining of E1 colonies exhibited a strong expression of stem cell surface markers, including SSEA-4, TRA-1-160, and TRA-1-181 (Fig. 2A–C) as well as gene expression for a pluripotent marker, Nanog, shown by RT-PCR (Fig. 3A). Some expression of nestin and AFP in otherwise apparent undifferentiated colonies of E1 was also observed. Similar gene expression was observed for new clonal lines. These clonal lines show normal characteristics: karyotype of that of the parent line (46XX) (Fig. 4), except for E1C3, which showed reciprocal translocation involving chromosome 15 and 17 (Fig. 5); stem cell surface markers SSEA-4, TRA-1-60, and TRA-1-81 (Fig. 6); and gene expression for pluripotency (see Fig. 3). All of these clones formed EBs in suspension cultures, and after seeding the EBs differentiated, forming cell lineages derived from all three germ layers, as indicated by immunolocalization of the ectodermal

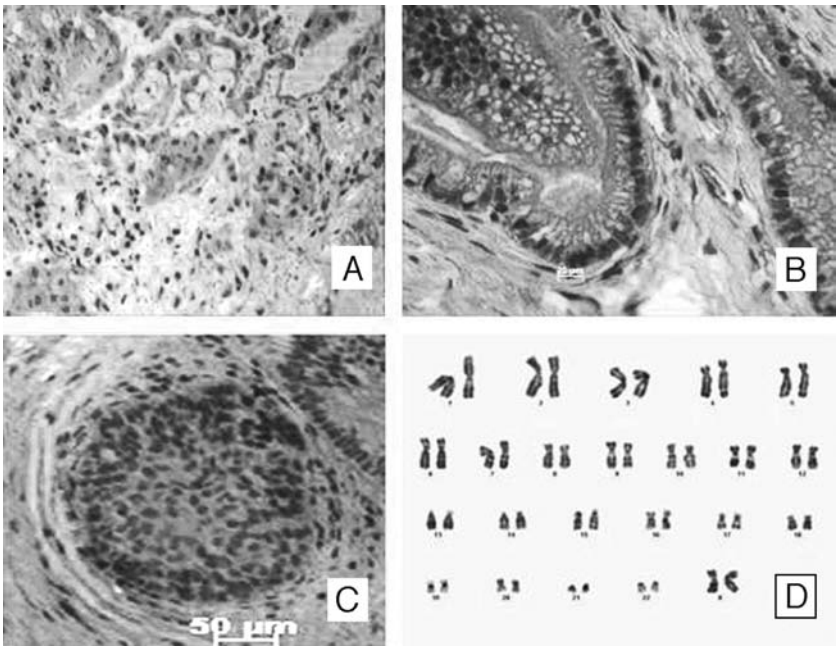

FIG. 4. Histological demonstrations of various tissues formed in the teratomas by E1 after injecting under the kidney capsule of NOD-SCID mice and its karyotyping analysis. (**A**) Neuroectoderm (arrow, ectoderm). (**B**) Gut-like structures (endoderm). (**C**) cartilage-like structure (mesoderm). (**D**) Karyotype, 46XX.

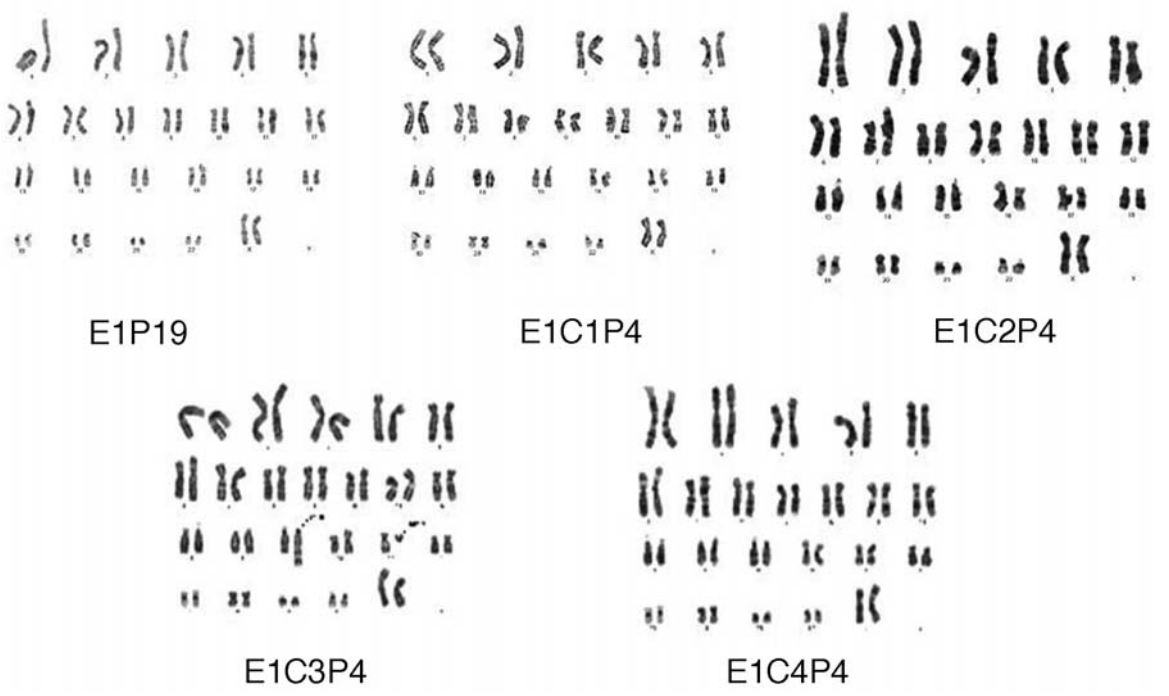

FIG. 5. Karyotype of the parent line, Endeavour (E1P19), and clonal lines, E1C1P4, E1C2P4, E1C3P4, and E1C4P4. The karyotype of E1C3P4 shows translocation.

marker β-III tubulin, the mesodermal marker CD34, and the endodermal marker AFP (Fig. 7). Subtle differences in these markers' expression were observed amongst different clonal lines (see bar diagrams in Fig. 7).

Pluripotency of E1 was also determined under both in vitro and in vivo conditions. In suspension culture, E1 formed EBs that on seeding in gelatin-coated plates differentiated into different cell types (Fig. 3B). Spontaneous differentiation of these EBs for 14 days produced various cell types derived from all three germ layers—ectoderm, endoderm, and mesoderm (Fig. 2 D–L). The ectoderm lineage (β-III tubulin$^+$) was obtained predominately, followed by mesoderm (CD34$^+$) and endoderm (AFP) lineages. Preliminary studies on directed differentiation of E1 and its clonal lines by activin A treatment for 5 days produced endoderm, as revealed by expression of GATA-4, SOX-17, and FOXa2 genes by RT-PCR and immunolocalization (Fig. 8A–C). Some subtle nonsignificant differences in gene expression for FOXa2, SOX-17, and GATA-4 after activin A treatment were observed (Fig. 8B). When E1 and its clonal lines were injected under the kidney capsule of SCID mice, they produced teratomas (both solid and cystic). Histological studies after Hematoxylin & Eosin staining of paraffin sections of these teratomas revealed the presence of tissues derived from three germ layers, including a gut-like structure (endoderm), cartilage-like structure (mesoderm), and neural ectoderm-like structures (ectoderm) (Fig 4A–C; Fig. 9). A comparative analysis of various characteristic features amongst E1 and its clonal lines is shown in Table 1, indicating subtle differences. E1C2 is more inclined toward endoderm differentiation.

In summary, E1 is a stable hES cell line under largely serum-free and xeno-free conditions used and has demonstrated a stable phenotype of undifferentiated status in vitro culture for over 1 year. With its ability to form all cell types derived from three germ layers under both in vitro and in vivo conditions, it shows pluripotency. Its ability to form clonal lines demonstrated self-renewal ability. E1 and its clonal lines can be made to differen-

FIG. 6. Immunolocalization of stem cell surface markers. From left, SSEA-4, TRA-1-60, and TRA-1-81 in four clones, E1C1, E1C2, E1C3, and E1C4.

FIG. 7. (*Upper panels*) Semiquantitative data (bar diagrams) from immunolocalization study for β-III tubulin, AFP, and CD34 as markers for ectoderm, endoderm, and mesoderm, respectively. (*Lower panel*) Immunolocalization of these markers in each block from top to bottom in E1, E1C1, E1C2, E1C3, and E1C4 and from left to right in each block: (*left*) nuclear staining with DAPI; (*middle*) specific localization of marker; and (*right*) merger. Data are means ± SEM, $n = 3$. Values compared using Student's *t*-test, i.e., AFP (E1 VS E1C2, $p < 0.05$; E1 vs. E1C4, $p < 0.05$; E1C1 vs. E1C3, $p < 0.01$; E1C1 vs. E1C4, $p < 0.050$, CD34 (E1 vs. E1C1 $p < 0.01$).

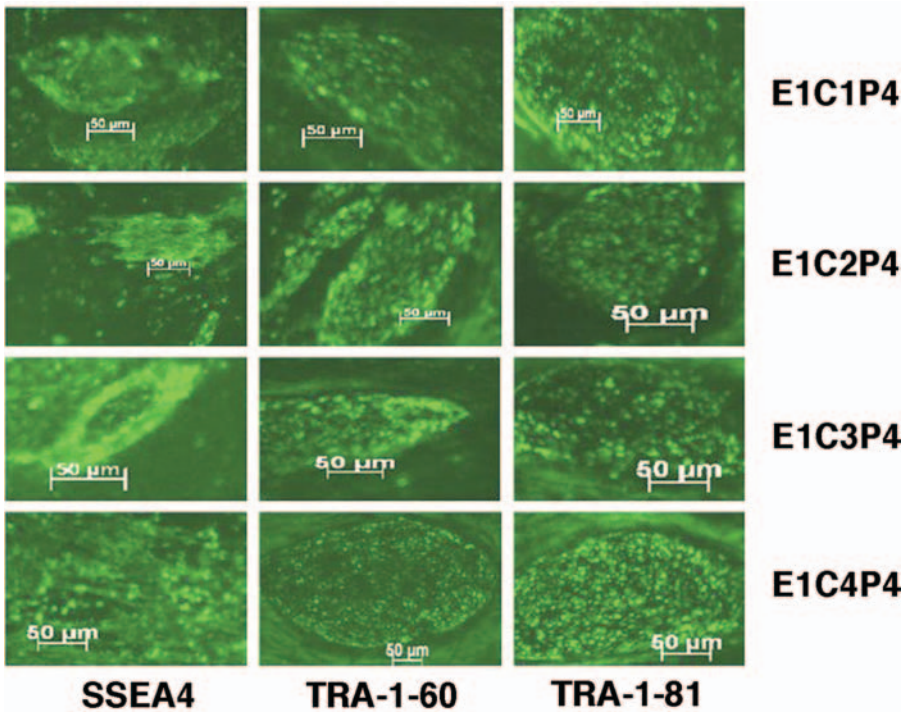

FIG. 6.

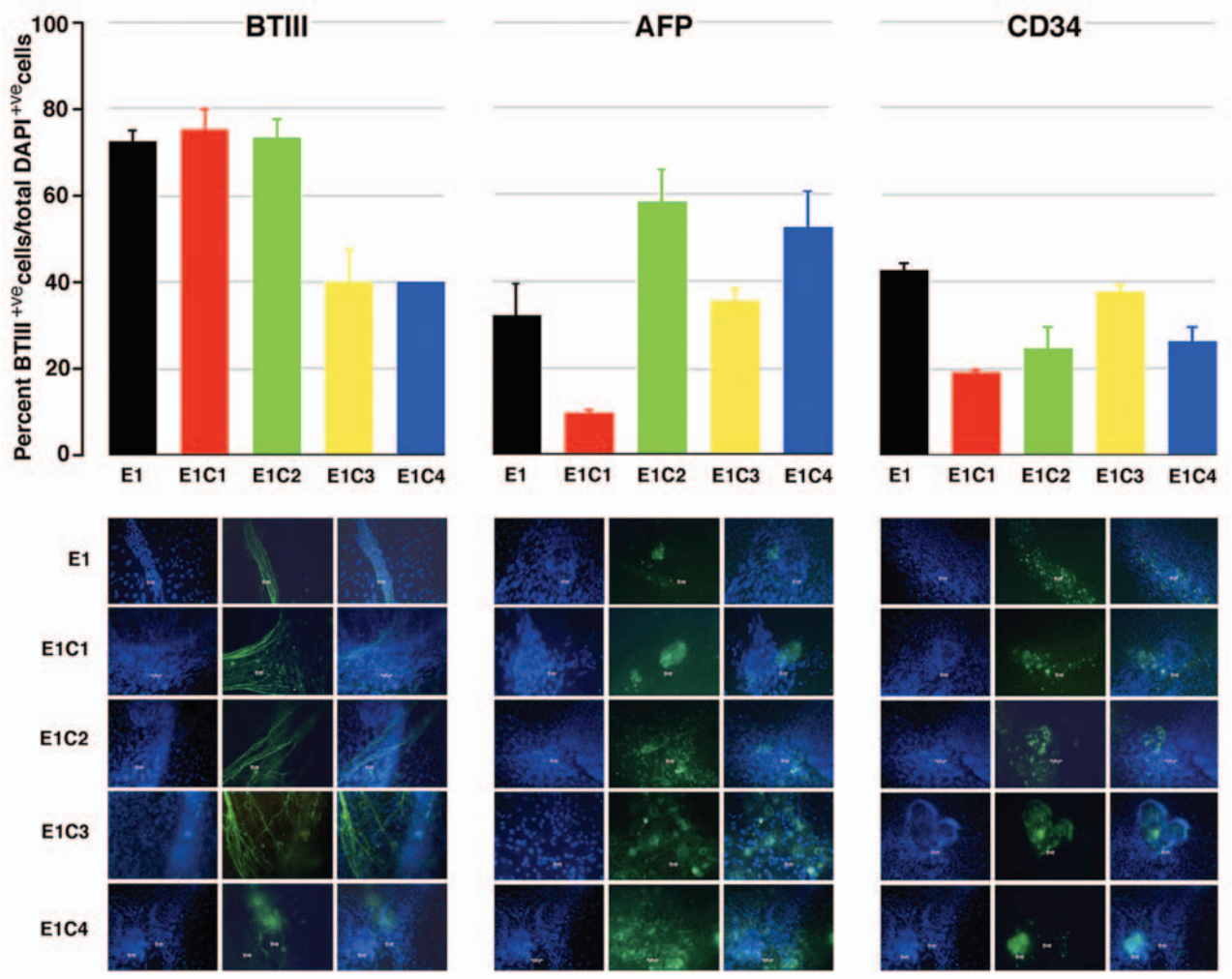

FIG. 7.

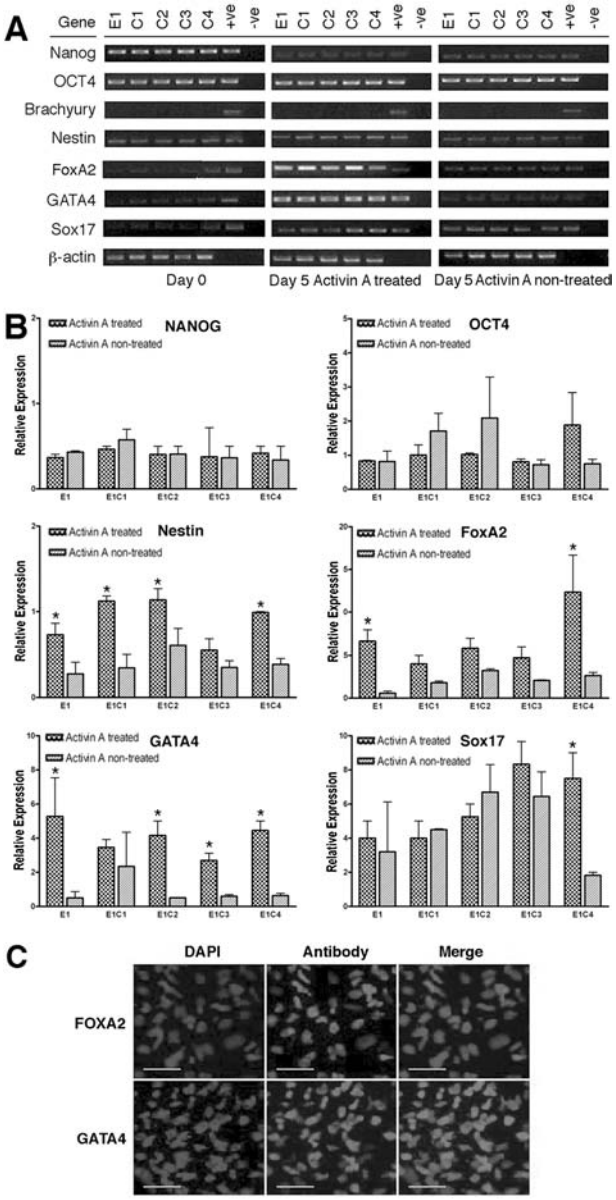

FIG. 8. Effect of activin A treatment on RT-PCR expression of definite endoderm marker genes FOXa2, GATA-4, and Sox-17; pluripotent marker genes Nanog, OCT-4; mesoderm marker gene brachyury; ectoderm marker gene nestin in E1 and its clonal lines. (**A**) RT-PCR. (**B**) Semiquantitative analysis of RT-PCR. Data are expressed as mean ± standard error mean (SEM). For each group $n = 3$, Student's t-test were analyzed on clones individually comparing treated and nontreated. $*p < 0.05$, represents a significant difference in each individual clone between treatment. (**C**) Immunolocalization of endoderm markers, FOXA2 and GATA-4 after activin A treatment in E1 (data from H Chung, KS Sidhu, and BE Tuch, unpublished).

tiate to definitive endoderm by Nodal protein. The subtle differences amongst clonal lines can be exploited for derivation of specified lineages for the subsequent development of cell therapies for various debilitating diseases such as type 1 diabetes, Parkinson, Alzheimer, and spinal cord injury.

DISCUSSION

Earlier hES cell lines were derived and propagated using MFFs as a feeder layer, and that approach was based on experience with derivation of murine (m) ES cell lines using fetal bovine serum (FBS) [1,24]. These animal-derived products run the risk of contaminating these hES cell lines with animal retroviruses and other nonhuman pathogens that may be transmitted to donors if used for transplantation purposes. Recently, it has been demonstrated that such culture procedures are liable to introduce nonhuman sialoproteins, which could be immunogenic in humans [25]. Thus, eliminating nonhuman pathogens that might be found in animal sera and feeder layers during the derivation of new hES cell lines has been the hallmark of some recent investigations (for review, see refs. 26, 27), so that these lines might be used for potential therapeutic purposes.

In this study, we have addressed all of these issues and defined all of the conditions that are largely serum-free and xeno-free and derived a new hES cell line, E1. The ICM was removed from blastocysts by laser dissection and grown on in-house-produced HFFs in a largely serum-free medium and grown on human-derived collagen IV as a substrate. E1 has been in culture for more than a year and is growing normally. It has all of the characteristic features of a pluripotent hES cell line that renews itself in culture, as revealed by gene expression and immnuocytochemistry, and produces teratomas after transplantation under the kidney capsule of SCID mice, indicating pluripotency. E1 is amenable to clonal propagation, further supporting its self-renewal ability in culture. Some expression of nestin and AFP observed in apparently undifferentiated colonies of E1 was considered to be normal, as in other studies [16,33].

Earlier attempts to replace MFFs with human source-derived fibroblasts as a feeder layer for hES cells [4,14,28–32] still used heterologous sera (FBS, KSR) and used immunosurgery to remove the ICM; hence, these were not xeno-free systems. Similarly feeder-free culture systems employed for similar purposes used complex extracellular matrices such as Matrigel, fibronectin, and laminin mostly derived from animal sources and were not xeno-free [7,33–35]. In these studies, either conditioned medium from mouse fibroblasts or growth factors [bFGF, transforming growth factor-β (TGF-β, activin A, Nodal, Noggin, leukemia inhibitory factor (LIF), platelet-derived growth factor (PDGF)] were used, and some degree of differentiation was observed in hES cell colonies in culture, including chromosomal abnormalities [13, 36–37]. Recently, Ludwig et al. [18] used defined feeder-

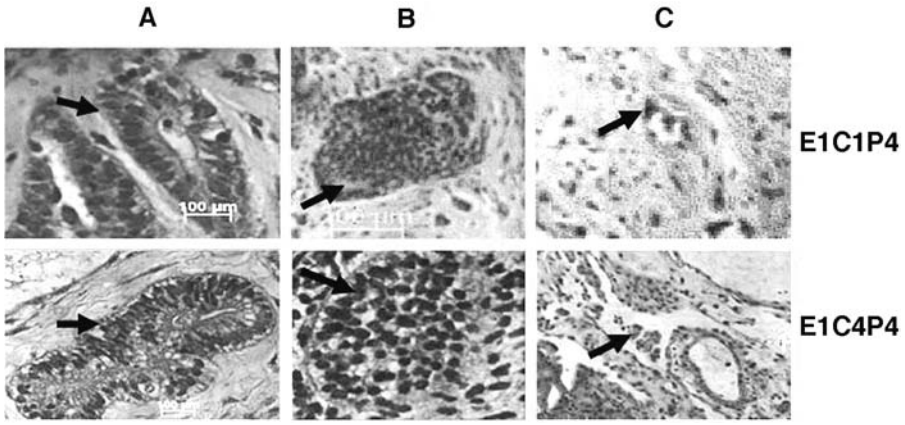

FIG. 9. Teratomas formation by clonal lines (E1C1P4 and E1C4P4) after injecting under the kidney capsule of NOD-SCID mice. E1C2P4 and E1C3P4also produced similar teratomas (histology not shown). (**A**) Intestine-like structures (arrow). (**B**) Cartilage-like structures (arrow). (**C**) Neuroectoderm (arrow).

free conditions for derivation of two new hES cell lines, but they also used immunosurgery for isolation of ICMs from blastocysts using antibodies derived from animal sources; karyotypically both of these new hES cell lines became unstable under feeder-free conditions. Thus, this may not be a suitable procedure for deriving transplantable hES cell lines until the molecular mechanism of self-renewal is fully understood. Ellerstrom et al. [19] defined a culture system for the derivation of a new hES cell line under xeno-free conditions using human serum. This method, however, has been found to cause the differentiation of hES cells in long-term cultures [14,20].

We have also described for the first time here the derivation and characterization of four clones, E1C1, E1C2, E1C3, and E1C4, from the parent line E1 by FACS sorting of single-cell preparations using our innovative procedure [21]. The efficiency of cloning was low (0.5–2%), but it is comparable to that reported for formation of clones from other hES cell lines [11,26–27]. These studies used manual picking of the cells under the microscope for cloning, which is a very subjective method and may not be very efficient given that the cloning efficiency is very low. The FACS sorting of single cells described by us is very objective and is an easy way of producing clones from hES cells despite the very low cloning efficiency reported in hES cells. We have also demonstrated that resuspending hES single cells in SR conditioned medium from HFFs maintained >98% cells viable after FACS sorting. Similarly concentrated conditioned medium from hES cells grown in the presence of FCS has been shown to promote cell survival and maintenance of an undifferentiated fate in newly created hES cell lines [11].

TABLE 1. SUMMARY OF THE CHARACTERISTIC FEATURES OF hES CELL CLONES

	Markers						
hES cell/clones	*SSEA-1*	*SSEA-3*	*SSEA-4*	*TRA-1-60*	*TRA-1-81*	*ALP*	*OCT-4*
E1 (Parent)	−	+	++	++	+++	++	+
E1C1	−	+	++	++	+++	++	+
E1C2	−	+	++	++	+++	++	+
E1C3	−	+	++	++	+++	+++	+
E1C4	−	+	++	+++	+++	++	+

	Gene expression/karyotype/teratoma					
	Nanog	*Nestin*	*Renin*	*AFP*	*Karyotype*	*Teratomas*
E1	−	++	−	−	46XX	3 Germ layers
E1C1	+	++	−	−	46XX	3 Germ layers
E1C2	+	++	−	++	46XX	3 Germ layers
E1C3	+	++	−	−	46XX	3 Germ layers
E1C4	+	++	−	−	46XX	3 Germ layers

++, strong; +, weak; −, absent; Nanog, Nestin, Ren in AFP gene expression by RT-PCR.

Previously described hES cell lines are not clonally derived, although phenotypically the cells appear similar [2–4]. Here we have demonstrated that individual hES cells in each of our newly created clones are pluripotent because they have been in continuous culture for more than 6 months and they continue to demonstrate developmental potential in vivo.

Although four of our clones are phenotypically very similar to the parent line E1, they do show some subtle differences in gene and protein expression. The expression of some transcriptional factors, such as Nanog, and lineage-specific genes like nestin were also comparable; the clone E1C2 also expressed endoderm markers, AFP, and more GATA-4, indicating that the latter is more inclined toward an endodermal lineage compared to other clones. The phenotypic expression shown by using an immunoarray chip also demonstrated subtle differences amongst clonal lines (data not shown).

The stable maintenance of the diploid chromosome number in all four clones (except E1C3) indicates that these clones have a stable karyotype, even after prolonged culture and after repeated freezing/thawing cycles. These clones also formed teratomas when injected under kidney capsule of NOD-SCID mice, and they showed different tissues derived from all three germ layers indicating pluripotency in vivo. These clones also formed EBs that after seeding in culture plates formed cells of different lineages, demonstrating pluripotency in vitro. Some differences in expression for various lineage-specific genes indicated differences between clones in their ability to differentiate. Taken together, these results suggest that the newly established four clones from the parent line E1 have similar properties, as also reported for other hES lines; they have stable karyotypes and show pluripotency both in vitro and in vivo. These clonal lines, including the parent E1, are amenable to directed differentiation to definitive endoderm after treatment with the nodal protein activin A, as revealed by gene and protein expression data. Using the protocols described here, we also produced four more clonal lines from a Boston hES cell line, Hues-3 [2], that also expressed subtle differences (data not shown). Selection of clonal lines that are inclined to a particular lineage will help to achieve unified lineage specification that can be used for future cell therapy for such debilitating diseases as type 1 diabetes, Parkinson, Alzheimer, and spinal cord injury.

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