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Derivation of Three Clones from Human Embryonic Stem Cell Lines by FACS Sorting and Their Characterization

KULDIP S. SIDHU and BERNARD E. TUCH

ABSTRACT

Here we describe the first report of three human embryonic stem cell (hESC) clones, hES 3.1, 3.2, and 3.3, derived from the parent line hES3 by sorting of single-cell preparations by flow cytometry. The viability of single-cell preparations before and after cell sorting remained >98%. The hESC were selected by size gating and forward-angle light scatter and were dispersed directly as single-cell/well into 96-well plates containing human fetal fibroblasts as feeder layers. Single stem cell dispersion into 96-well plates was confirmed by using cells from a hES3 line that constitutively expressed green fluorescence protein (eGFP) under similar conditions of flow cytometry. Three clones were obtained from the parent line hES3—hES3.1, 3.2, and 3.3—and they have been in continuous culture for more than 1 year. The cloning efficiency was less than <0.5%. These hESC clones show normal stem cell characteristics, such as undifferentiated growth, high nucleocytoplasmic ratio, the same karyotype as that of the parent line (46 XX), stem cell surface markers (i.e., SSEA3, SSEA4, OCT4, TRA-1-60, and TRA-1-81), and gene expression for pluripotency (Oct-4 and nanog). They all formed embryoid bodies in suspension cultures, and after seeding in culture plates they showed pluripotency in vitro by forming cell lineages derived from all three germ layers as indicated by expression of the ectodermal marker nestin, the mesodermal marker renin, and the endodermal markers α-fetoprotein and GATA6. All clones showed normal expression of alkaline phosphatase activity, a marker of in vitro pluripotency. When hESC clones ($1–2 \times 10^6$ total) were injected into nonobese diabetic-severe combined immunodeficiency (NOD-SCID) mice under the kidney capsule, all formed teratomas within 6–8 weeks. Analysis of the stem cell surface marker TRA-1-160 by flow cytometry showed nonsignificant ($p < 0.05$) differences between the clones and the parent line. The clones also differed in their expression of genes, with only one, hES 3.2, expressing the endodermal markers, i.e., α-fetoprotein and GATA6. The ability to produce clones from a parent hESC line rapidly by FACS sorting will help provide a homogeneous population of cells for achieving uniformed lineage specifications for future transplantation therapies and biomedical research.

INTRODUCTION

Following the first report of successful derivation of five human embryonic stem cell (hESC) lines (1), many more hESC lines have been created recently (2–4). To date it is estimated than more than 220 new hESC lines have been produced world-wide, and out of which 78 are listed in the National Institute Health (NIH) Registry. Only about 26 these lines are characterized to some extent and available for research. Many of these hESC lines are not clonal and are derived under different culture conditions and propagated on different feeder layers

Stem Cell Group, Diabetes Transplant Unit, Prince of Wales Hospital and University of New South Wales, Sydney, NSW 2031 Australia.

(mouse embryonic fibroblasts, immortal mouse fibroblasts, fetal muscle, skin, and foreskin, adult fallopian tube epithelial cells, including some feeder-free system); hence, comparison of these lines is difficult (5–7).

During the last 5 years or so, emphasis has been on improving culture conditions (5–6,8–10), genetic manipulation (11,12), and optimizing differentiation protocols to produce different cell lineages from hESC for transplantation and drug testing (10,13–16). However, research has been seriously limited by the lack of optimized protocols for obtaining pure populations of a specified lineage from hESC lines due to the lack of quality control measures, inherent variability, and lack of uniform procedures for propagating these hESC lines. Only a handful of studies have investigated these parameters potentially for achieving uniformity in lineage selections (5–7,10,17).

Currently the selection criteria used for quality control on hESC lines are: (1) a typical phenotype (high nucleocytoplasmic ratio), (2) surface markers (SSEA3, SSEA 4, TRA1-60, TRA1-81, GTCM2, TGT3430), (3) intracellular markers (Nanog, Oct-4, REX1), (4) high telomerase activity, (5) pluripotency in vitro and in vivo, and (6) ability to sustain cryopreservation and maintenance of these characteristics over an extended period of propagation. It has been demonstrated that even fully characterized hESC lines show variability of gene expression against the above criteria (7,18,19). The potential of these lines to differentiate into different lineages under in vitro conditions is highly variable (20). Although some hESC lines can be maintained for prolonged periods of time without losing stem cell characteristics, quantitative analysis of antigen expression by flow cytometry and gene expression by microarray suggests some subtle differences in the expression of small subsets of genes upon long-term culture (18,19,21–23) including a gain of chromosomes 17q and 12 (24).

The ability of a typical hESC colony to show clonal expansion despite the heterogeneous nature of cells present may be an important criterion to define pluripotency in these cells, although the clonal efficiency is very low (10). Currently the conditions and procedure used for deriving these clones from hESC lines are far from optimal. To date only a few single-cell clones from the parental hESC lines H1, H9, H13, H16, and J3 have been described; this was achieved by physically picking up of single cells under the microscope, with a maximum clonal efficiency of 0.83% (25,26). This procedure described for clonal derivation from hESC lines is very labor-intensive and highly subjective.

We describe here the derivation of three clones (hES 3.1, 3.2, 3.3) from the hES3 line by fluorescence-activated cell sorting (FACS) of single cells for the first time. The procedure described is efficient and objective, although the clonal efficiency continues to be low. Most of the hESC lines described are not clonally derived and hence pluripotency may be restricted to a small subpopulation. In addition, the possibility exists that within an apparent homogeneous population of a hESC colony there were multipotent precursor cells of different lineages forming three germ layers. Therefore, the derivation of hESC clones by this procedure, along with authentic profiling of all available hESC lines for genetic, epigenetic, chromosomal, molecular, and biological characteristics, may be very pertinent for achieving uniformed lineage specifications for future transplantation therapies.

MATERIALS AND METHODS

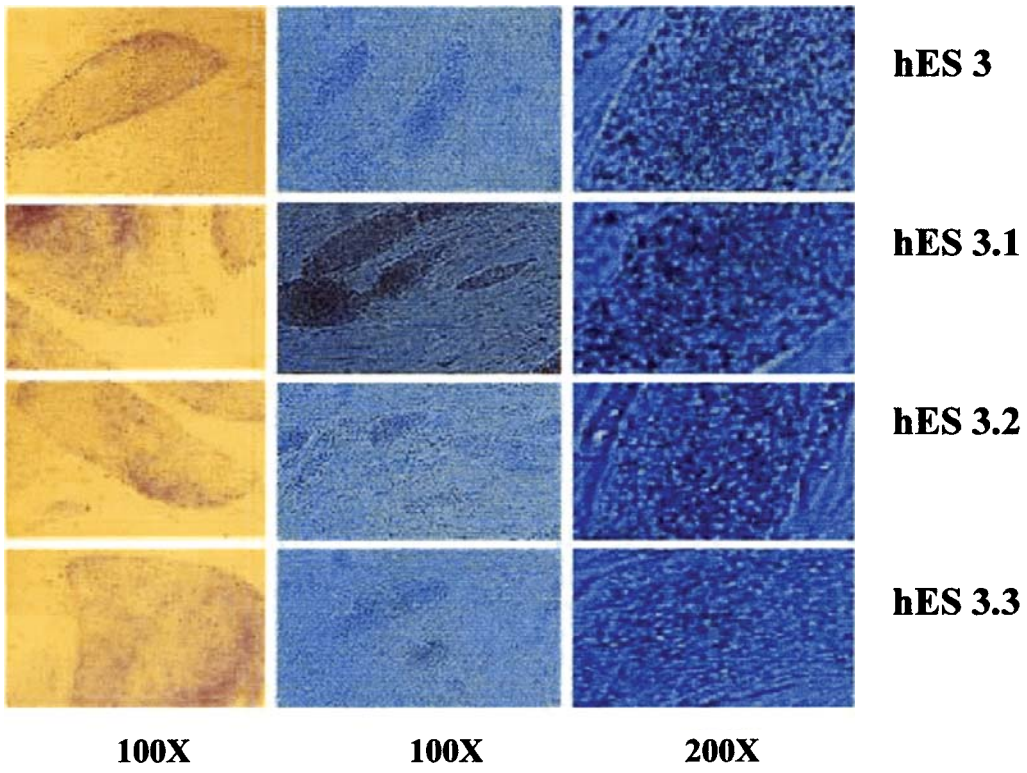
All reagents including culture media, sera were obtained from Gibco/Invitrogen (Carlsbad, CA, http://www.invitrogen.com).

hESC line

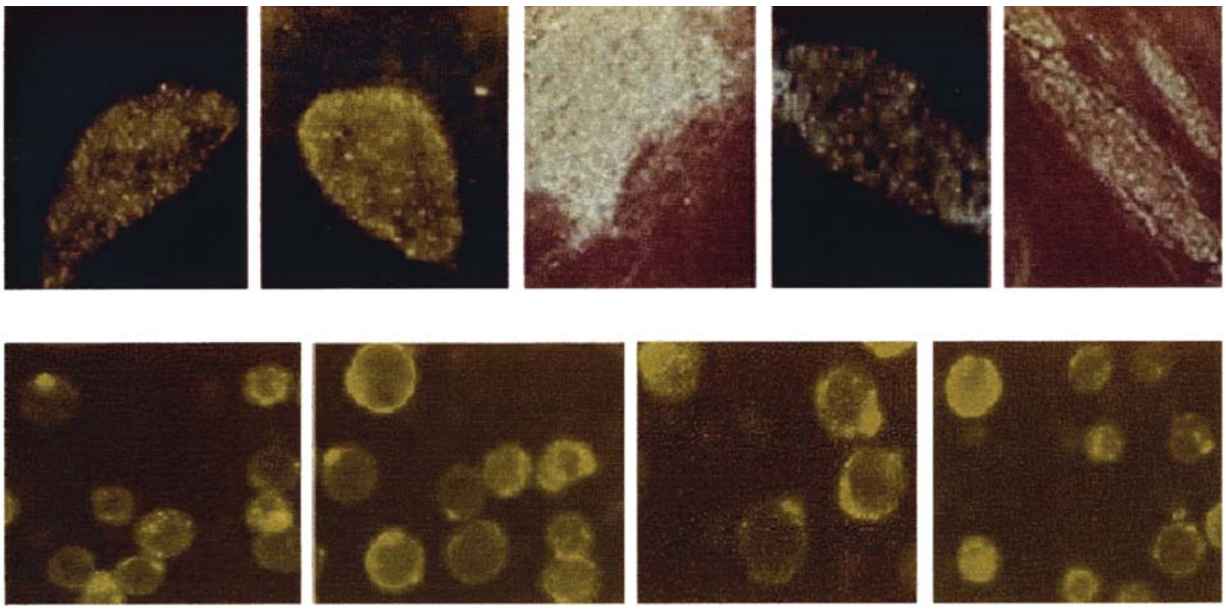
The hESC line ESI-hES3 was obtained from Embryonic Stem Cell International Pte, Ltd. (Singapore) The hES3 cell line that constitutively expresses green fluorescent protein (GFP) (Envy line) was obtained from the Monash Immunology and Stem Cell Laboratories, Melbourne (courtesy Dr. Andrew Elefanty). The hESC colonies were maintained in gelatin-coated six-well culture plates (Becton Dickinson, NJ, http://www.bdbiosciences.com) on gamma-irradiated (45 Gy) primary human fetal fibroblast (HFF; passage 6) feeder layers (1.5×10^5 cells/ml) and cultured at 37°C, 5% CO_2 in serum replacer (SR) medium consisting of Dulbecco knockout (KO-DMEM) high glucose, supplemented with 20% knockout serum replacer (Gibco, Carlsbad, CA), 2 mM L-glutamine, 0.1 mM nonessential amino acids, 0.01 mM 2-mercaptoethanol, 1× insulin-transferrin-selenium, basic fibroblast growth factor (bFGF), 4 ng/ml, 25 U/ml penicillin, and 25 μg/ml streptomycin. This study to work on hESC and HFF lines had Institutional ethics approval (HREC 01270 and HREC 02247, respectively). With this scale-up procedure, routinely >75 hESC colonies were grown per well of a six-well culture plate. The subculturing of hESC colonies, with a 1:6 split, was performed every 6–7 days by using 0.05% trypsin for 2 min. Cryopreservation of these clones were carried out by vitrification in open pulled straws as well as by slow freezing in cryovials (27).

Preparation of hES single cells

Aliquots of 300–400 hESC colonies, including that from the Envy line, were dissected from six-well plates by gently washing twice and with collagenase type IV [1 mg/ml in phosphate-buffered saline (PBS) without Ca^{2+}; 1 ml/



COLOR PLATE 1. Gross morphology of hES colonies (*two right-hand panels*) and alkaline phosphatase localization (*left panel*) in clones (hES 3.1, 3.2, and 3.3) and parent line (hES 3).



COLOR PLATE 2. (*Upper panel*) Immunolocalization of stem cell-surface markers. From left, OCT4, SSEA 3, SSEA4, TRA-1-81, and TRA-1-60 in clone hES 3.1. Similar expression of these surface markers was observed in other clones. (*Lower panel*) FACS-sorted TRA-1-60-positive bright hESCs. From left, hESC 3, clone 3.1, clone 3.2, and clone 3.3. (Magnification, 400×).

well] for 7 min at 37°C. The hESC colonies were allowed to settle at the bottom of a 15-ml tube for 5 min, and the supernatant was aspirated. The hESC colonies were dissociated into single cells by using 0.05% trypsin and 0.25% EDTA at 37°C for 7 min, triturated carefully twice with a pipette. Finally, the single-cell preparations were resuspended at 1×10^6 cells/ml in conditioned medium collected from HFF cultured in SR medium for 24 h.

FACS sorting of hES single-cell preparations

A FACS Calibur (Becton Dickinson) was used to select hESCs exclusively, and not feeder fibroblast, by size gating and forward-angle light scatter. The selection of stem cells alone by this procedure was confirmed by using FACS sorting of a single-cell preparation from an Envy hESC line that constitutively expresses eGFP. Each cell sorted by FACS was dispersed into a well of a 96-well plate containing HFF as a feeder layer and cultured in SR medium 5% CO2 and 37°C for 2 weeks. Each sorted cell in a well was observed to have green fluorescence when examined with a fluorescene microscope. The viability of single hESC after FACS sorting was >98% as assessed by fluorescent staining with carboxyfluorescein diacetate (CFDA) and propidium iodide (PI). Briefly, hESCs were washed with 500 μl of PBS at 800 rpm for 3 min and resuspended in 250 μl of 0.1 mM CFDA in dimethyl sulfoxide (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 and incubated on ice for 5 min. Viable (green fluorescence) and nonviable (red fluorescence) cells were counted under the fluorescent microscope.

Clones obtained were initially passaged by physical dissection into 24-well plates and subsequently into six-well plates by trypsin.

Procedures for characterization of clones

Stem cell-surface and intracellular markers: Immunohistochemical localization of various stem cell surface markers, i.e., stage-specific embryonic antigens, SSEA-1, SSEA-3, SSEA-4; tumor recognition antigens, TRA-1-60, TRA-1-81; a POU-domain transcription factor, OCT-4 were carried out using primary antibodies (1:250) against these surface markers and detected by using fluorescein isothiocyanate (FITC)-conjugated appropriate secondary antibodies (Chemicon, VIC, Australia; http://www.chemicon.com.au). The expression of the stem cell-surface marker TRA-1-60 on single-cell preparations was also examined by flow cytometric analysis according to the procedure described by Carpenter et al. (5) with some modifications. Briefly, a single-cell preparation (approximately 4–5 $\times 10^6$ cells) was suspended in 100 μl of staining buffer, consisting of 2% bovine serum albumin (BSA), 2 mM EDTA, and 0.1% sodium azide in PBS, for blocking nonspecific binding sites. The primary monoclonal antibodies (mAbs) were added at a final dilution of 1:100 for 1 h with FITC-conjugated anti-mouse immunoglobulin G (IgG) antibodies (1:50) added to detect the binding of TRA-1-60. The pluripotent intracellular marker alkaline phosphatase was assessed immunohistochemically by using a commercially available kit (Sigma-Aldrich) following the manufacturer's instructions.

RT-PCR: Total RNA from hESCs 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 MMLV-RT (Gibco) and an oligo(dT) primer (Roche). Expression of pluripotent markers Nanog and OCT-4 and differentiation markers for ectoderm (Nestin), mesoderm (Renin), and endoderm (α-fetoprotein + GATA6) were assessed by semiquantitative PCR using the Gel Doc System (BIO RAD) and Quantity One-4.5.2 software. The expression levels of the markers were compared to β-actin, a housekeeping gene, as described previously (28).

Karyotyping: A standard G banding and multicolor spectra karyotyping (SKY) was used for analysis by using 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.

Formation of teratomas in vivo: To assess in vivo pluripotency, approximately 2×10^6 cells from each clone were injected under the kidney capsule of nonobese diabetic-severe combined immunodeficiency (NOD-SCID) mice. The animals were euthanized 6–8 weeks later, and grafts were examined histologically.

Formation of embryoid bodies: hESC colonies from each clone were dissected from wells with collagenase and cultured in non-tissue culture plates (suspension culture) in SR medium for 1 week to produce embryoid bodies (EBs). The EBs were then seeded in tissue culture dishes and SR medium without bFGF for 2 weeks to induce differentiation. The expression of lineage markers in hESC cultures after RNA extraction for ectoderm, mesoderm, and endoderm was evaluated by RT-PCR as described previously.

Freezing and thawing: The clones were cryopreserved by slow and fast freezing procedures (vitrification) and thawed several times as described previously (27).

Statistical analysis: The FACS data (mean from four separate experiments) were compared using Student's *t*-test using the computer program Sigma Plot (San Rafael, CA).

RESULTS

Derivation of hESC clones

Subculturing (splitting at 1:6 ratio) of hESCs at a seeding density of >75 colonies per well by trypsin did not pro-

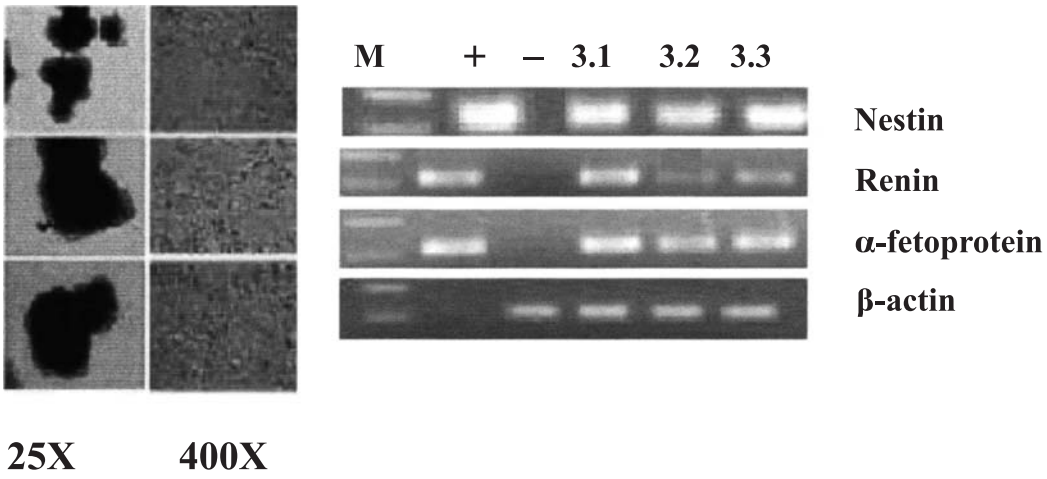


FIG. 1. EB formation by clones, from top to bottom, clone 3.1, 3.2, and 3.3 (*left panel*) their in vitro differentiation to different cell types ([*middle panel*) and gene expression for ectoderm (nestin), mesoderm (renin), and endoderm (α-fetoprotein) (*right panel*).

duce any significant induction of differentiation. For obtaining single-cell preparations, hESC colonies were collected by collagenase treatment followed by trypsin digestion to obtain single-cell preparations. This procedure also helped to selectively remove differentiated hESC colonies (pick to loose, PTL, or pick to keep PTK) the undifferentiated colonies by collagenase. Sedimentation of hESC colonies after collagenase treatment also helped to eliminate most of the fibroblasts. A relatively pure population of hESC with viability >98% was obtained that could be dispersed as single cells by flow cytometry. Three clones were obtained, hES 3.1, hES 3.2, and hES 3.3, after FACS sorting of single-cell preparations from the hES 3 line in 96-well plates with an overall efficiency of <0.5%.

Characterization of hESC clones

To evaluate whether the hESC clones were stem cells, they were characterized according to their morphology and EB formation, expression of pluripotent genes, stem cell surface markers, and ability to differentiate both in vitro and in vivo.

Morphology and EB formation

Under the culture conditions described for hESCs, all of the three derived clones, hES 3.1, 3.2, and 3.3, now at passage 23 including the parent line, hES 3 at passage 155, form large compact colonies with a distinct stem cell morphology (Color Plate 1).

The gross degree of spontaneous differentiation as evidenced by appearance of cobblestone morphology in colonies from all these clones was found to be 5–10%, which is comparable to the parent line. However, the hES 3.2 clone showed a higher degree of differentiation (>20%) if propagated after day 5. These clones were successfully cryopreserved by both vitrification and slow freezing methods several times and brought up with plating efficiencies of >90% and >75%, respectively (data not shown). These clones also formed EBs in suspension cultures for 7 days and after seeding in culture plates formed various types of cells. These cells showed marker expression for three germ layers and the disappearance of the pluripotent marker nanog during culture (Fig. 1).

RT-PCR analysis of gene expression

Figure 2 shows a semiquantitative RT-PCR analysis of cDNA carried out for expression of the pluripotent marker gene nanog and also the lineage marker genes nestin, renin, and α-fetoprotein + GATA 6 from different batches of clones and the parent line for comparison. Nanog was expressed strongly in all hES clones, 3.1, 3.2, 3.3, and the hES 3 parent line and remained so at least for 6 months in culture during different passages. α-Fetoprotein and GATA6 (data not shown) were present in clone 3.2 but not the other two clones or the parent cell

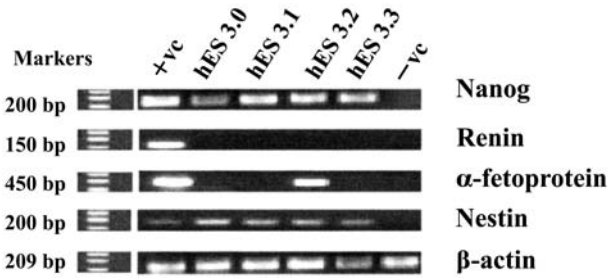


FIG. 2. RT-PCR expression of undifferentiating marker, nanog and differentiating markers, nestin (ectoderm), α-fetoprotein (endoderm), and renin (mesoderm) and corresponding molecular masses on left side as compared to housekeeping gene β-actin.

Table 1. Summary of the Characteristic Features of hESC Clones

	Markers						
hESC/clones	*SSEA-1*	*SSEA-3*	*SSEA-4*	*TRA-1-60*	*TRA-1-81*	*ALP*	*OCT-4*
hES 3 (parent)	−	+	++	++	++	++	+
hES 3.1 (clone)	−	+	++	++	++	++	+
hES 3.2 (clone)	−	+	++	++	++	++	+
hES 3.3 (clone)	−	+	++	++	++	++	+

	Gene expression/karyotype/teratoma					
	Nanog	*Nestin*	*Renin*	*α-Fetoprotein GATA6*	*Karyotype*	*Teratoma*
hES 3	++	+	−	−	46XX	Three germ layers
hES 3.1	++	+	−	−	46XX	Three germ layers
hES 3.2	++	+	−	+	46XX	Three germ layers
hES 3.3	++	+	−	−	46XX	Three germ layers

++, Strong; +, weak; −, absent; ALP, alkaline phosphatase.
Nanog, Nestin, Ren in α-Fetoprotein, GATA6 gene expression by RT-PCR.

line. Renin was not expressed in any of the clones or the parent line, whereas nestin was.

Stem cell markers

Table 1 summarizes the results of characterization of the three clones hES3.1, 3.2, and 3.3. All clones, including the parent hESC line, showed expression of the surface markers OCT4, SSEA-3, SSEA-4, TRA-1-60, and TRA-1-81 (Color Plate 2) and the intracellular marker alkaline phosphatase (Color Plate 1). A quantitative analysis of the stem cell-surface marker TRA-1-60 by FACS sorting indicated a nonsignificant difference ($p < 0.05$) in percentage of bright green fluorescent stem cells between the parent line hES 3 (67.52 ± 0.82) and the three clones, hES 3.1, 3.2, and 3.3 (75.17 ± 10.03, 59.95 ± 15.17, and 76.3 ± 32, respectively).

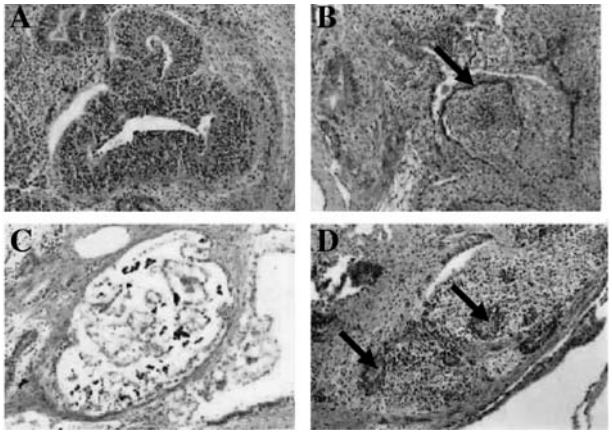


FIG. 3. Formation of teratomas after transplanted hES clone 3.1 into NOD-SCID mice. (**A**) Gut-like structure (endoderm); (**B**) cartilage-like structure (arrow, mesoderm); (**C**) blood vessel-like (endothelial); (**D**) neural rosettes-like structure (arrows, ectoderm).

Pluripotency in vivo

Clumps of hES 3.1, 3.2, and 3.3 at passage 10 and the parent line hES 3 at passage 150, when injected under the kidney capsule of SCID mice, formed teratomas after 4–6 weeks. The cysts containing teratomas derived from these cells consisted of highly differentiated cells and tissues derived from all three germ layers, including gut epithelium (endoderm), cartilage-like (mesoderm), and neural rosettes (ectoderm) (Fig. 3).

Karyotyping

Cytogenetic evaluation of clones at passage 10 and the parent line at passage 150 by standard G-banding (20 cells for each culture) showed a normal 46 XX karyotype (Fig. 4).

DISCUSSION

Here we describe for the first time the derivation and characterization of three clones, hES 3.1, 3.2, and 3.3 from the parent line hES 3 by FACS sorting of single-cell preparations. The efficiency of cloning was low (<0.5%) but comparable to that reported for formation of clones from other hESC lines (10,25,26). These later studies used manual picking of the cells under the microscope for

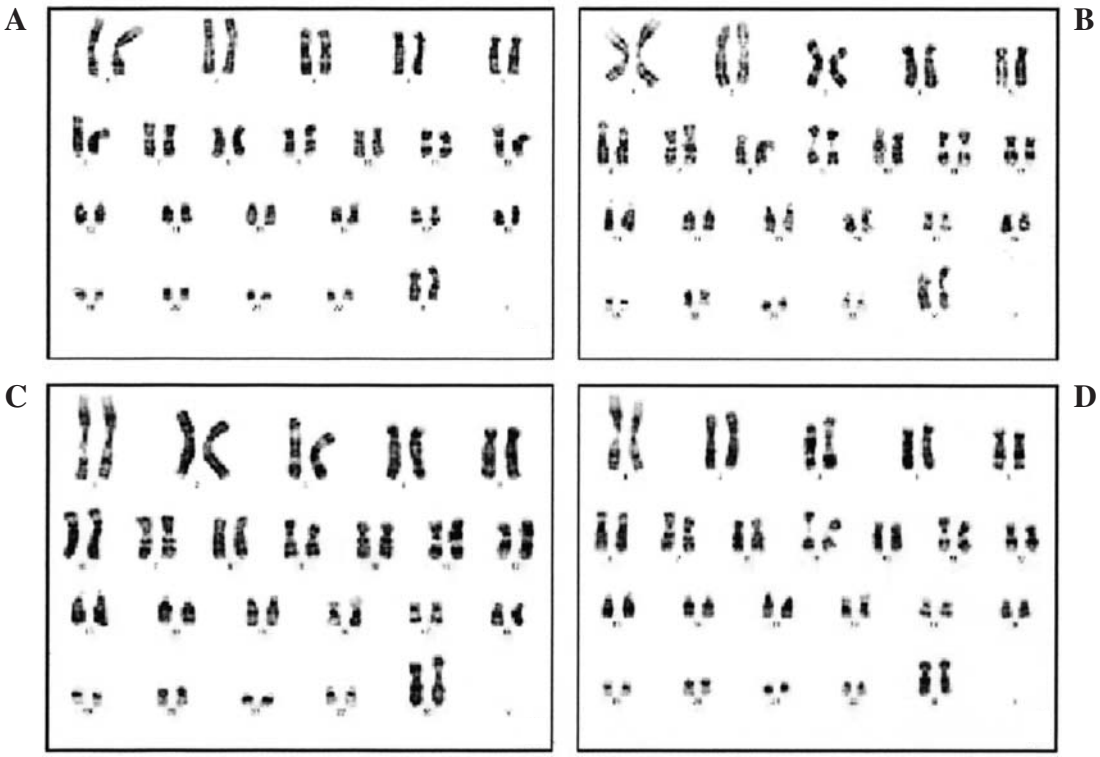


FIG. 4. Karyotypes of clones, hES 3.1 (**A**), hES 3.2 (**B**), hES 3.3 (**C**), and hES 3 (**D**).

cloning, which is very subjective method and may not be very efficient given that the cloning efficiency is very low. The FACS sorting of single cells described here is a very objective and easy way of producing clones from hESCs despite the very low cloning efficiency reported in hESCs. The validity of the procedure described for picking up only embryonic stem cells was also confirmed by using eGFP-labeled hESCs for quick detection. We have also demonstrated that resuspending hES single cells in SR conditioned medium from HFF maintained >98% of the cells as viable after FACS sorting. Similarly concentrated conditioned medium from hES cells grown in the presence of fetal calf serum (FCS) has been shown to promote cell survival and maintenance of an undifferentiated fate in newly created hESC lines (10).

The PTL and PTK procedures described for propagation of hESC gave a lot of flexibility in handling a variety of hESC cultures containing mixture of differentiated and undifferentiated hES colonies. Using these procedures, normally $3–5 \times 10^6$ hESCs per six-well plates could be produced easily. Subculturing of the hES 3 line by trypsin treatment so far at passage 155 has not produced any chromosomal abnormalities. This is a much more efficient procedure for subculturing compared to the labor-intensive physical dissection used by others (27).

Previously described hESC lines are not clonally derived, although phenotypically the cells appear similar (1–3). We have demonstrated here that individual hES cells in each of our newly crated clones, hES 3.1, 3.2, and 3.3, are pluripotent because they are in continuous culture for more than 1 year and they continued to demonstrate developmental potential in vivo.

Although three of our clones, hES 3.1, 3.2, and 3.3, are phenotypically very similar to the parent line hES 3, they do show some subtle differences. Morphologically, theses clones show more discrete boundaries of their colonies as compared to those in the parent line. All clones, including the parent line, do show comparable expression patterns of stem cell-surface marker and the intracellular marker ALP. However, nonsignificant differences were observed in bright cells after TRA-1-60 labeling in clones compared to that in parent line. Although the expression of some transcriptional factors, i.e., nanog, and lineage-specific genes like nestin were also comparable, the clone hES 3.2 also expressed the endodermal markers α-fetoprotein and GATA6, indicating that the latter is more inclined toward an endodermal lineage. All of our clones, including the parent line, showed comparable expression of the neuroectodemal marker nestin, which has also been reported in other hESC lines even in an apparent undifferentiated state (10).

The stable maintenance of diploid chromosome number in all of our clones indicates that these clones have a stable karyotype, even after prolonged culture and repeated freezing/thawing cycles. These clones also formed teratomas when injected under the kidney capsule of NOD-SCID mice, and these mice 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. Taken together, these results suggest that the newly established clones from parent line hES 3 have properties similar to those reported for other hES lines, they all have stable karyotypes, and they show pluripotency both in vitro and in vivo. They all show some subtle differences in hES colony morphology, stem cell-surface marker, and gene expression, i.e., hES 3.2 is more inclined toward endoderm differentiation compared to the parent line. Further studies will reveal more or bring out those differences that could be relevant for directed differentiation to desirable lineages.

The clonal expansion of hESCs may be taken as the strict criterion of pluripotency. Most of hESC lines reported in the literature are not clonally derived, and, although all show normal stem cell-surface markers, their pluripotency may be restricted to only a subpopulation. Currently, the culturing conditions for hESCs are suboptimal, and a selective pressure for enhanced survival in a subpopulation of hES cells does occur. Hence future improvement in procedure for deriving clones from hESC lines may mitigate or reduce this selection pressure. The selective and directed differentiation of newly derived clones described in this manuscript has yet to be attempted. The normal karyotype of all new hESC clones derived here may indicate a homogeneous euploid population that may be relevant to potential therapeutic applications.

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Address reprint requests to:
Dr. Kuldip S. Sidhu
Diabetes Transplant Unit
Prince of Wales Hospital
High Street Randwick
Sydney, NSW 2031 Australia

E-mail: k.sidhu@unsw.edu.au

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