Embryonic stem cell lines from human blastocysts: somatic differentiation in vitro

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We describe the derivation of pluripotent embryonic stem (ES) cells from human blastocysts. Two diploid ES cell lines have been cultivated in vitro for extended periods while maintaining expression of markers characteristic of pluripotent primate cells. Human ES cells express the transcription factor Oct-4, essential for development of pluripotential cells in the mouse. When grafted into SCID mice, both lines give rise to teratomas containing derivatives of all three embryonic germ layers. Both cell lines differentiate in vitro into extraembryonic and somatic cell lineages. Neural progenitor cells may be isolated from differentiating ES cell cultures and induced to form mature neurons. Embryonic stem cells provide a model to study early human embryology, an investigational tool for discovery of novel growth factors and medicines, and a potential source of cells for use in transplantation therapy.

Key words: embryonic stem cell, differentiation, Oct-4, neural progenitor, neuron

Pluripotential cells in the embryo have the capacity to give rise to differentiated progeny representative of all three embryonic germ layers, as well as the extraembryonic tissues that support development. In mammals the property of pluripotentiality is restricted to the oocyte, the zygote, early embryonic cells, primordial germ cells, and the stem cells of tumors derived from pluripotential cells (embryonal carcinomas). Under certain conditions, pluripotential stem cells can be propagated indefinitely in vitro and still maintain the capacity for differentiation into a wide variety of somatic and extraembryonic tissues. Pioneering work on mouse embryonal carcinoma (EC) cells led to the derivation of pluripotent diploid embryonic stem (ES) cells directly from the mouse blastocyst in 1981[1,2]. Since the first description of mouse ES cells, it has been recognized that the derivation of human ES cells could provide a unique resource for the functional analysis of early human development. For example, human ES cells could be used to identify polypeptide factors involved in differentiation and proliferation of committed embryonic progenitor cells. Furthermore, because ES cells can in principle serve as an unlimited source of any cell type in the body, human ES cells could yield highly effective in vitro models for use in drug discovery programs, and provide a renewable source of cells for use in transplantation therapy.

Previously, cultured cell lines derived from embryonal carcinomas have been used as models for cell differentiation in early human development (e.g., see refs 3–5). Pluripotential human EC cells, which are capable of spontaneous differentiation into all three germ layers, resemble mouse ES cells in their dependence on a feeder cell layer for continuous growth, and in their expression of the transcription factor Oct-4[4,6]. However, human EC cells differ from mouse ES cells in their morphology, their expression of cell surface markers, and their lack of response to leukemia inhibitory factor (LIF). More recently Thomson and colleagues reported the isolation and characterization of diploid rhesus and marmoset monkey ES cells with extensive capacity for differentiation[7,8]. These monkey ES cells resemble human EC stem cells in their morphology, marker expression, and lack of response to LIF.

Earlier we described the characteristics of primary cultures of undifferentiated cells from the human blastocyst that were able to undergo limited replication in vitro[9]. Since these early studies did not use embryonic feeder cell support (required for proliferation of pluripotent human EC and nonhuman primate ES cells) but relied instead on LIF supplementation of the culture medium, these cells eventually underwent differentiation or death. Therefore, we subsequently employed a culture system incorporating embryonic fibroblast feeder cell layers to derive human ES cells from blastocysts. While this work was in progress, Thomson and coworkers[10] reported the derivation of ES cell lines from the human blastocyst. We confirm these results and extend the characterization of the human ES cell phenotype. We further show that human ES cells express the transcription factor Oct-4 and undergo somatic differentiation in vitro, and we describe the isolation of neural progenitor cells from ES cultures.

Results

Derivation of cell lines HES-1 and HES-2. The outer trophectoderm layer was removed from four blastocysts by immunosurgery to isolate inner cell masses (ICM), which were then plated onto a feeder layer of mouse embryo fibroblasts (Fig. 1A). Within several days, groups of small, tightly packed cells had begun to proliferate from two of the four ICM. The small cells were mechanically dissociated from outgrowths of differentiated cells, and following replating they gave rise to flat colonies of cells with the morphological appearance of human EC or primate ES cells (Fig. 1B,C). These colonies were further propagated by mechanical disaggregation to clumps, which were replated onto fresh feeder cell layers. Growth from small clumps of cells (<10 cells) was not possible under the conditions of these cultures. Spontaneous differentiation, often yielding cells with the morphological appearance of early endoderm, was frequently observed during routine passage of the cells (Fig. 1D). Whereas LIF was used during the early phases of the establishment of the cell lines, it was subsequently found to have no effect on the growth or differentiation of established cultures (not shown). Differentiation occurred rapidly if the cells were deprived of a feeder layer, even in

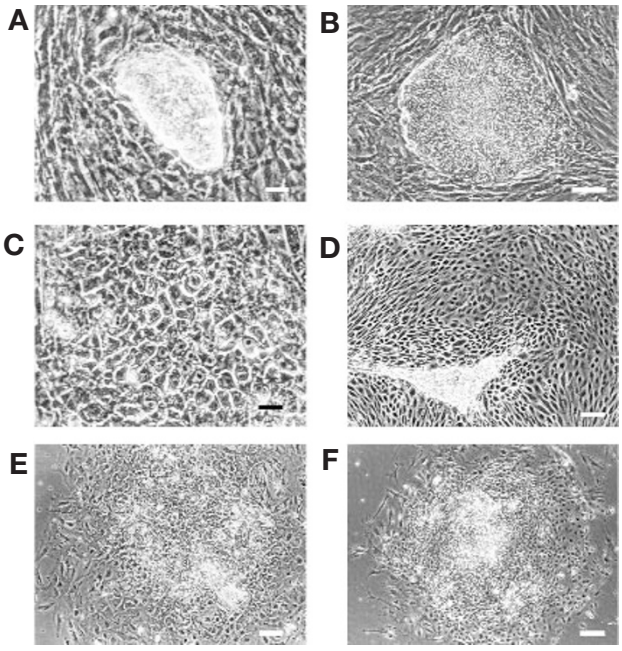

Figure 1. Phase contrast micrographs of ES cells and their differentiated progeny. (A) Inner cell mass three days after plating. (B) Colony of ES cells. (C) Higher magnification of an area of an ES cell colony. (D) An area of an ES cell colony undergoing spontaneous differentiation during routine passage. (E) A colony four days after plating in the absence of a feeder cell layer but in the presence of 2,000 units ml^{-1} hLIF undergoing differentiation in its periphery. (F) Neuronal cells in a high-density culture. Scale bars: (A, C) 25 μm; (B, E) 100 μm; (D, F) 50 μm.

the presence of LIF (Fig. 1E). Likewise, supplementation of the culture medium with cultivation in the presence of the soluble interleukin-6 (IL-6) receptor plus IL-6 had no effect on the growth or differentiation of the human ES cells under standard growth conditions, and and also failed to block differentiation of human ES cells in the absence of feeder layer (not shown). Cell line HES-1 has been grown for 64 passages in vitro and HES-2 for 44 passages, corresponding to a minimum of approximately 384 and 264 population doublings, respectively, based on the average increase in colony size during routine passage, and both cell lines still consist mainly of cells with the morphology of ES cells. Both cell lines have been successfully recovered from cryopreservation. (Reubinoff, Pera and Trounson, manuscript in preparation).

Marker expression and karyotype of the human ES cells. Marker and karyotype analysis were performed on HES-1 at passage levels 5–7, 14–18, and 24–26, and 44–46, and on HES-2 at passage levels 6–8 and 40–42. Embryonic stem cells contained alkaline phosphatase activity (Fig. 2A). Immunophenotyping of the ES cells was carried out using a series of antibodies that detect cell surface carbohydrates and associated proteins found on human EC cells[11]. The ES cells reacted positively in indirect immunofluorescence assays with antibodies against the SSEA-4 and TRA 1-60 carbohydrate epitopes, and the staining patterns were similar to those observed in human EC cells (Fig. 2 B,C). Embryonic stem cells also reacted with monoclonal antibody GCTM-2, which detects an epitope on the protein core of a keratan sulfate/chondroitin sulfate pericellular matrix proteoglycan[12–14] found in human EC cells (Fig. 2D). Like human EC cells, human ES cells did not express SSEA-1, a marker for mouse ES cells. Both cell lines stably retained a normal karyotype, and both were derived from female blastocysts.

Oct-4 is a POU domain transcription factor whose expression is limited in the mouse to pluripotent cells, and recent results show directly that zygotic expression of Oct-4 is essential for establishment

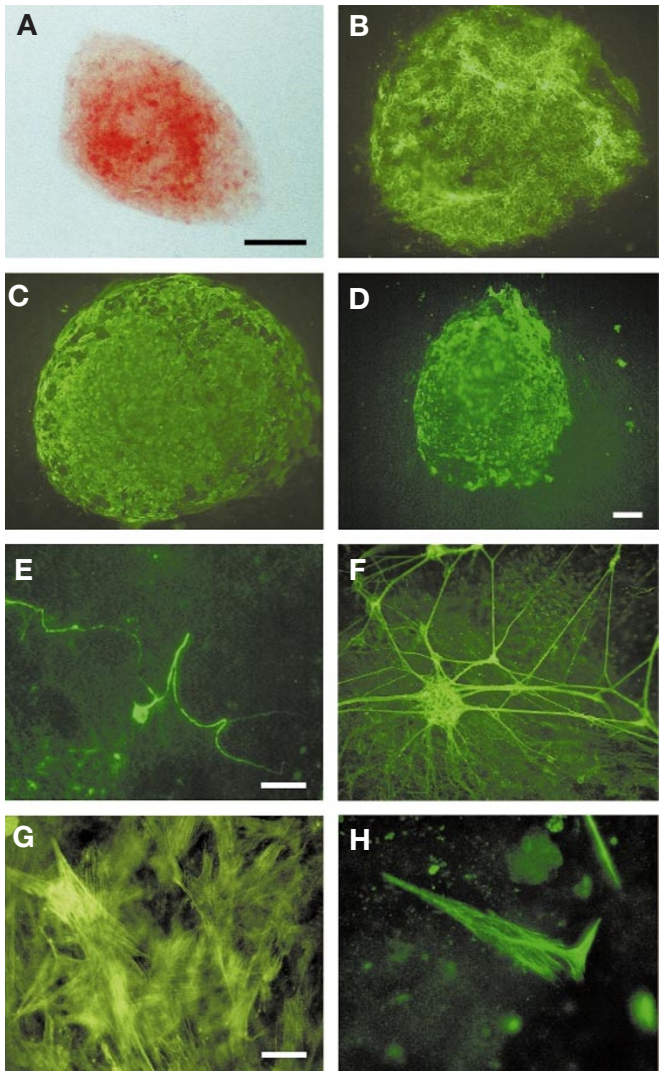

Figure 2. Marker expression in ES cells and their differentiated progeny. (A) ES cell colony showing histochemical staining for alkaline phosphatase. (B) ES cell colony stained with antibody MC-813-70 recognizing the SSEA-4 epitope. (C) ES cell colony stained with antibody TRA 1-60. (D) ES cell colony stained with antibody GCTM-2. (E) High-density culture, cell body and processes of a cell stained with anti-neurofilament 68 kDa protein. (F) High-density culture, cluster of cells and network of processes emanating from them stained with antibody against N-CAM. (G) High-density culture, cells showing cytoplasmic filaments stained with antibody to muscle actin. (H) High-density culture, cell showing cytoplasmic filaments stained with antibody to desmin. Scale bars: (A) 100 μm; (B-D, F) 200 μm; (E, G, H) 50 μm.

of the pluripotent stem cell population of the ICM[15]. Oct-4 is also expressed in human EC cells, and its expression is downregulated when these cells differentiate. Using reverse transcriptase polymerase chain reaction (RT-PCR) to carry out mRNA analysis on isolated colonies consisting mainly of stem cells, we showed that human ES cells also express Oct-4 (Fig. 3A, lanes 2–4). The PCR product was cloned and sequenced and shown to be identical to human Oct-4 (not shown).

Differentiation of human ES cells in xenografts. When HES-1 or HES-2 colonies of either early passage level (6; HES 1 and 2) or late passage level (HES-1, 14 and 27, HES-2, 27) were inoculated beneath the testis capsule of severe combined immunodeficient (SCID) mice, testicular lesions developed and were palpable from about five weeks after inoculation. All mice developed tumors, and in most cases both testis were affected. Upon autopsy we observed lesions consisting of cystic masses filled with pale fluid and areas of solid tissue. There was no gross evidence of metastatic spread to other sites within the peritoneal cavity.

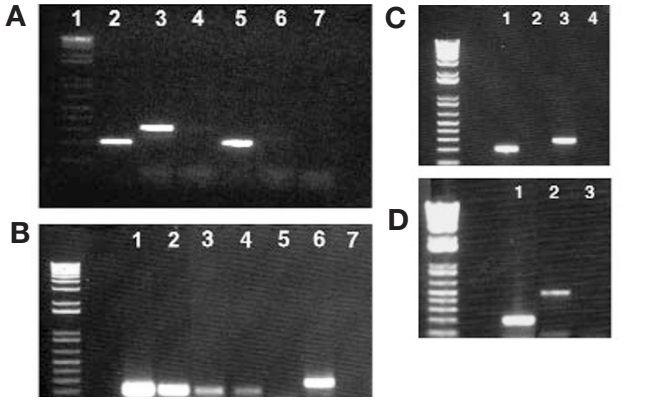

Figure 3. RT-PCR analysis of gene expression in ES cells and their differentiated derivatives. (A) Expression of Oct-4 and β-actin in ES stem cells and high-density cultures. Lane 1, 100 bp DNA ladder. Lane 2, stem cell culture, β-actin. Lane 3, stem cell culture, Oct-4. Lane 4, stem cell culture, PCR for Oct-4 carried out with omission of reverse transcriptase. Lane 5, high-density culture, β-actin. Lane 6, high-density culture, Oct-4. Lane 7, high-density culture, PCR for Oct-4 carried out with omission of reverse transcriptase. β- actin band is 200 bp and Oct-4 band is 320 bp. (B) Expression of nestin and Pax-6 in neural progenitor cells. Left lane, 100 bp DNA ladder; lane 1, β-actin in HX 142 neuroblastoma cell line (positive control for nestin PCR); lane 2, β-actin in neural progenitor cells; lane 3, nestin in HX 142 neuroblastoma cell line; lane 4, nestin in neural progenitor cells; lane 5, nestin PCR on same sample as lane 4 without addition of reverse transcriptase; lane 6, Pax-6; lane 7, Pax-6 PCR on same sample as line 6 without addition of reverse transcriptase. Nestin band is 208 bp, Pax-6 is 274 bp. (C) Expression of glutamic acid decarboxylase in cultures of neurons. Left lane, 100 bp DNA ladder; lane 1, β-actin; lane 2, β-actin PCR on same sample as lane 1 without addition of reverse transcriptase; lane 3, glutamic acid decarboxylase; lane 4 glutamic acid decarboxylase on same sample as lane 3 without addition of reverse transcriptase. Glutamic acid decarboxylase band is 284 bp. (D) Expression of $GABA_A$ α2-receptor. Left lane, 100 bp DNA ladder; lane 1, β-actin; lane 2, $GABA_A$ α2-receptor; lane 3, PCR without addition of reverse transcriptase. $GABA_A$ α2-receptor subunit band is 471 bp.

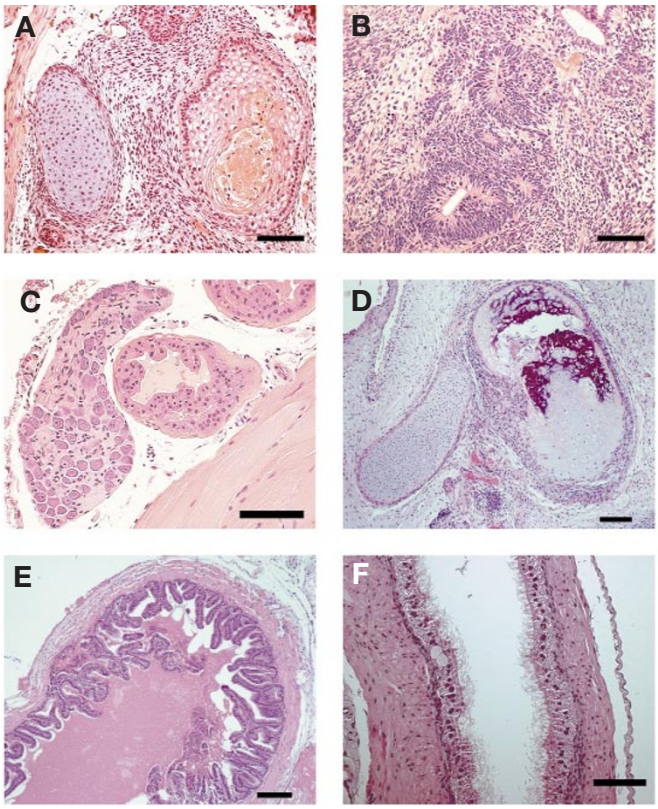

Figure 4. Histology of differentiated elements found in teratomas formed in the testis of SCID mice following inoculation of HES-1 or HES-2 colonies. (A) Cartilage and squamous epithelium, HES-2. (B) Neural rosettes, HES-2. (C) Ganglion, gland, and striated muscle, HES-1. (D) Bone and cartilage, HES-1. (E) Glandular epithelium, HES-1. (F) Ciliated columnar epithelium, HES-1. Scale bars: (A-E) 100 μm; (F) 50 μm.

Histological examination revealed that the lesion had displaced the normal testis and contained solid areas of teratoma. Embryonal carcinoma was not observed in any lesion. All teratomas contained tissue representative of all three germ layers. Differentiated tissues seen included cartilage, squamous epithelium, primitive neuroectoderm, ganglionic structures, muscle, bone, and glandular epithelium (Fig. 4). Embryoid bodies were not observed in the xenografts.

Differentiation of human ES cells in vitro. Both cell lines underwent spontaneous differentiation under standard culture conditions, but the process of spontaneous differentiation could be accelerated by suboptimal culture conditions. Thus, as with human EC cells, cultivation to high density for extended periods (four to seven weeks) without replacement of a feeder layer promoted differentiation of human ES cells. In high-density cultures, expression of the stem cell marker Oct-4 was either undetectable or strongly downregulated relative to the levels of the housekeeping gene β-actin (Fig. 3A, lanes 5–7). α-Fetoprotein and the β-subunit of human chorionic gonadotrophin were readily detected by immunoassay in the supernatants of cultures grown to high density. α-Fetoprotein is a characteristic product of endoderm cells and may reflect either extraembryonic or embryonic endodermal differentiation; the levels observed (1,210–5,806 ng ml^{-1}) are indicative of extensive endoderm present. Human chorionic gonadotrophin secretion is characteristic of trophoblastic differentiation; the levels observed (6.4–54.6 IU/L) are consistent with a modest amount of differentiation along this lineage.

In these high-density cultures, there was no consistent pattern of structural organization suggestive of the formation of embryoid bodies similar to those formed in mouse ES cell aggregates or arising sporadically in marmoset ES cell cultures[8]. Cultivation of clumps of ES cells in hanging-drop cultures, or as aggregates on bacteriological petri dishes, in standard medium without feeder cells resulted in considerable cell death, and only a minority of the clumps survived. There was no evidence of growth or formation of distinct tissue layers in the aggregates. When the surviving clumps were replated onto tissue culture plastic in standard culture medium, cell death was evident, and no extensive outgrowth occurred. Thus, manipulations used in our laboratory and elsewhere to facilitate embryoid body formation and multilineage differentiation of mouse ES cells induced death of human ES cells.

Nevertheless, somatic differentiation could be established under conditions such as prolonged cultivation, which allow the survival of human ES cells but are nonpermissive for stem cell growth and are unfavorable for extensive extraembryonic differentiation. After prolonged cultivation to high density (four to seven weeks), multicellular multicellular aggregates or vesicular structures formed above the plane of the monolayer, and among these structures we observed clusters of cells or single cells with elongated processes that extended out from their cell bodies, forming networks as they contacted other cells (Fig. 1F). The cells and the processes stained positively with antibodies against neurofilament proteins and the neural cell adhesion molecule (N-CAM, Fig. 2E, F). Contracting muscle was also seen infrequently in these cultures. Although contracting muscle was a rare finding, we often observed bundles of cells that were stained positively with antibodies directed against muscle-specific forms of actin, and less commonly cells containing desmin intermediate filaments (Fig. 2G, H).

Isolation and differentiation of neuronal progenitor cells from human ES cell cultures. Early stages of neuroectodermal differentia-

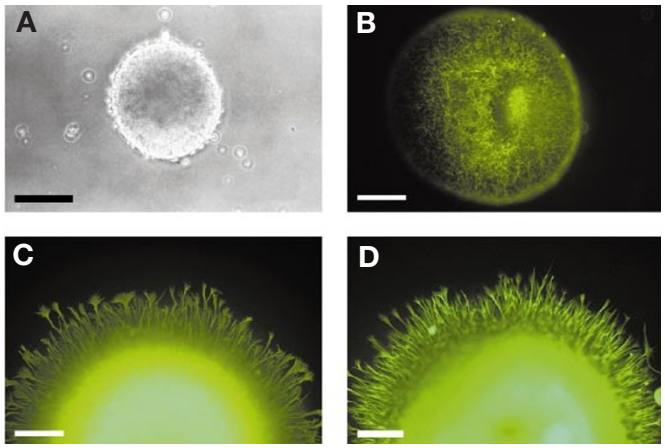

Figure 5. Phase contrast microscopy and immunochemical analysis of marker expression in neural progenitor cells isolated from differentiating ES cultures. (A) Phase contrast image of a sphere formed in serum-free medium. (B-D) Indirect immunofluorescence staining of spheres, 4 h after plating on adhesive substrate, for N-CAM, nestin, and vimentin, respectively. In C and D, cells at the base of the sphere were placed in plane of focus to illustrate filamentous staining; confocal examination revealed that cells throughout the sphere were decorated by both antibodies. Scale bar = 100 μm in all panels.

tion were first detected in cultures grown for approximately three weeks at high density by the appearance of areas containing cells with short processes that expressed polysialylated N-CAM. At this time, areas of colonies cells destined to give rise to these could be identified in high density cultures by characteristic morphological features visualized under phase contrast or stereo microscopy. These areas of cells were isolated and replated in serum-free medium, whereupon they formed spherical structures within 24 h (Fig. 5A). Cells in these spheres initially expressed markers of primitive neuroectoderm, such as polysialylated N-CAM (Fig. 5B), the intermediate filament proteins nestin (immunostaining, Fig. 5C; RT-PCR, Fig. 3B) and vimentin (Fig. 5D), and the transcription factor Pax-6 (Fig. 3B). When plated on an appropriate substrate, the spheres attached, and differentiated cells grew out onto the monolayer from them (Fig. 6A). These differentiated cells displayed morphology and expression of structural markers characteristic of mature neuronal differentiation, such as the 200 kDa neurofilament proteins and b-tubulin (Fig. 6 B,H). Moreover, the differentiated cells expressed specific markers of mature neurons such as the and 160 kDa neurofilament proteins (Fig. 6C), Map2a + b (Fig. 6D), and synaptophysin (Fig. 6F). and b tubulin (Figure 7h)Moreover,Finally, the cultures contained cells that synthesized glutamate (Fig. 6E), and expressed the rate-limiting enzyme in GABA biosynthesis (glutamic acid decarboxylase, Figs 3C and 6G), as well as receptor subunits for this latter neurotransmitter ($GABA_A$ α2-receptor subunit, Fig. 3D).

Discussion

The ES cell lines derived by us are similar in properties to those described by Thomson and coworkers[10] and share many features of pluripotent human EC cells and monkey ES cells. Common features of pluripotent, spontaneously differentiating human EC[4,16] and primate ES cells (refs 7,8,10, and this report) include similar morphology, expression of surface carbohydrate epitopes (SSEA-3 and -4 and TRA 1-60 and 1-81) and the pericellular matrix proteoglycan detected by GCTM-2 (which carries the TRA 1-60 epitope[14]), pluripotentiality, and a lack of response to LIF or related members of this cytokine family. Blastocyst-derived human ES cells differ somewhat from the stem cell lines derived from primordial germ cells by Shamblott and colleagues[17], which do not grow as flat monolayers, express the SSEA-1 epitope on their cell surface, and are at least partially dependent upon exogenous LIF and basic fibroblast growth factor.

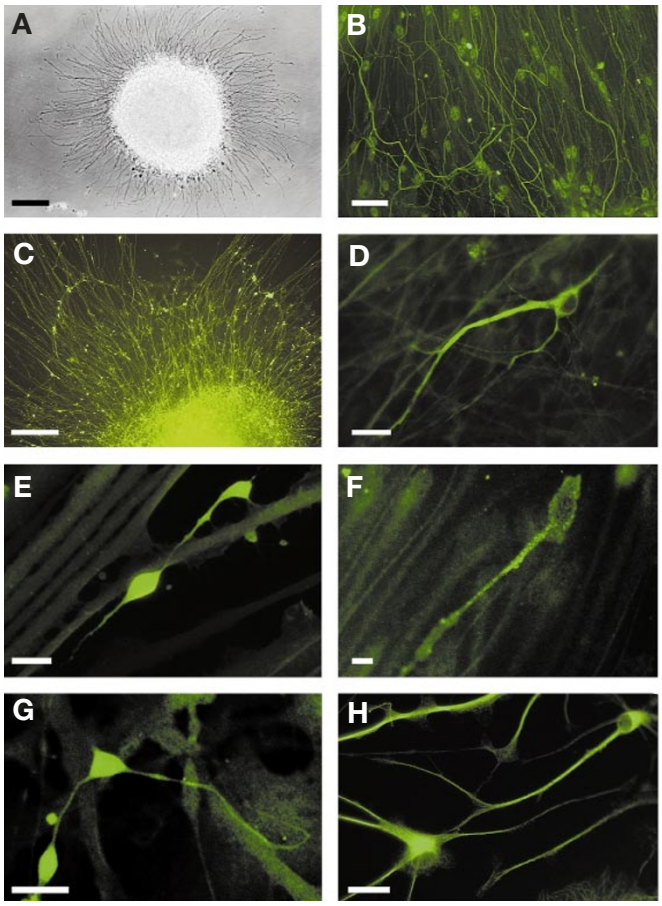

Figure 6. Phase contrast appearance and marker expression in cultures of neurons derived from progenitor cells shown in Fig. 5. (A) Phase contrast micrograph of differentiated cells emanating from a sphere plated onto adhesive surface. (B-H) Indirect immunofluorescence microscopy of differentiated cells decorated with antibodies against (B) 200 kDa neurofilament protein (C) 160 kDa neurofilament protein (D) MAP2Aba+b (E) glutamate (F) synaptophysin (G) glutamic acid decarboxylase, and (H) β-tubulin. Scale bars: (A, B) 100 μm; (C) 200 μm; (D) 20 μm; (E, F) 10 μm; (G) 20 μm; (H) 25 μm.

Human and monkey ES and EC cells differ substantially from mouse ES cells in terms of morphology, surface marker expression, and response to LIF. The differences between human and mouse ES cells may result from fundamental differences in embryonic development between the species, or they may reflect a difference in the embryonic stage of origin of ES cells in mouse and primate. Both ES cells and EC cells of murine and human origin express the transcription factor Oct-4 and downregulate expression of this factor during differentiation. In the mouse, Oct-4 expression is limited to pluripotential cell populations and has recently been shown to be essential for the establishment of pluripotential cell lineages during mouse embryonic development[15]. Oct-4 may be one of a small number of regulatory molecules characteristic of all mammalian pluripotential cells at all developmental stages.

It is clear from this study and that of Thomson and coworkers[10] that culture in the presence of embryonic fibroblasts can support the establishment and serial cultivation of human ES cells from blastocysts at a relatively high frequency. These encouraging findings provide a starting point for further refinement and eventual clinical applications of human ES cell technology. Present human ES culture systems do have limitations. Spontaneous differentiation occurs readily, and the culture system does not support clonal growth of human ES cells. Even human EC cells are difficult to passage as single cells and show relatively low cloning efficiency. It is possible that

autocrine growth factors produced by the primate stem cells themselves are required for renewal of the stem cell population. It should be noted that because no diploid human pluripotential stem cell has thus far been cloned, there is a remote possibility that more than one cell type is present in the cultures and may account for the variety of differentiated cells observed in vitro and in vivo.

We document somatic differentiation in vitro of blastocyst-derived human ES cells for the first time in this report. As shown previously for human EC cells[4], ES cells grown for extended periods at high density without renewal of a feeder layer form aggregates and vesicular structures within which are found various types of differentiated cells, including neurons and muscle. Somatic differentiation is obtained under conditions that limit stem cell renewal without inducing cell death and that block unidirectional differentiation into extraembryonic tissue. It seems likely that interactions between various cells at early stages of differentiation mimic in an unstructured fashion the inductive processes occurring during axis formation[18] in the embryo. Identification of the factors involved in these interactions will facilitate directed differentiation of the stem cells. There are clues from work on human EC cells indicating that, similar to embryonic cells of many species, human pluripotential stem cells can be induced to differentiate along specific lineages in response to members of the transforming growth factor-β superfamily[6,19,20].

It will be of interest to compare the properties of the neuronal progenitors described here with those of neural stem cells obtained from fetal[21] or adult[22] human brain. The isolation of these neuronal progenitors in a pure form from differentiating cultures of human ES cells shows that both committed somatic stem progenitor cell populations and their differentiated progeny may be obtained from human ES cultures undergoing multilineage differentiation. The findings also demonstrates the utility of human ES cells for basic studies of the early steps in the establishment of somatic stem cell lineages.

Much attention recently has been devoted to the potential applications of stem cells in biology and medicine. The properties of pluripotentiality and immortality (correlated with telomerase expression[10]) are unique to ES cells and enable investigators to approach many issues in human biology and medicine for the first time. Embryonic stem cells potentially can address the shortage of donor tissue for use in transplantation procedures, particularly where no alternative culture system can support growth of the required committed stem cell. However, it must be noted that almost all of the wide-ranging potential applications of ES cell technology in human medicine—basic embryological research, functional genomics, growth factor and drug discovery, toxicology, and cell transplantation—are based on the assumption that it will be possible to grow ES cells on a large scale, to introduce genetic modifications into them, and to direct their differentiation. Present systems fall short of these goals, but there are indications of progress to come. The identification of novel factors driving pluripotential stem cell growth[16,23,24], or stem cell selection protocols to eliminate the inhibitory influence of differentiated cells[25], both offer a way forward for expansion and cloning of human ES cells. Our finding that Oct-4 is expressed in stem cells and downregulated during differentiation strongly indicates that stem cell selection using drug resistance genes driven by the Oct-4 promoter will be a useful avenue for manipulating human ES cells. Directed differentiation using growth factors, discussed above, or the complementary strategy of lineage selection coupled with growth factor enhancement[26] (exemplified here by morphological identification and isolation of neuronal progenitors) could enable the selection of populations of pure committed progenitor cells from spontaneously differentiating cells, as described here. Given adequate public support and open collaboration between workers in this area, it is likely that the impact of human ES cells on biology and medicine will soon equal and surpass even that of the revolution brought about by their murine counterparts.

Experimental protocol

Derivation and propagation of ES cells. Human blastocysts were donated to the study following institutional review board approval and informed consent by couples undergoing in vitro fertilization treatment in Singapore, where the cell lines were initiated. Fertilized oocytes were cultured to the blastocyst stage (day 6 after insemination), in sequential media, according to our standard coculture free protocol[27]. After zona pellucida digestion by pronase (Sigma, St. Louis, MO)[28], ICM were isolated by immunosurgery[29] using anti-human serum antibody (Sigma) followed by exposure to guinea pig complement (Life Technologies, Gaithersburg, MD). Subsequently, ICM were cultured on mitomycin C mitotically inactivated mouse embryonic fibroblast feeder layer (isolated from day 13.5 post coitum fetuses of either the 129/Sv strain or F_1 crosses of this strain with C57/BL6; used at 75,000 cells cm^{-2}) in gelatin-coated tissue culture dishes. The culture medium consisted of Dulbecco's modified Eagle medium (DMEM, without sodium pyruvate, glucose 4500 mg L^{-1}; Life Technologies, Rockville, MD) supplemented with 20% fetal bovine serum (FBS; Hyclone, Logan, UT), 0.1 mM β-mercaptoethanol, 1% nonessential amino acids, 2 mM glutamine, 50 units ml^{-1} penicillin, and 50 μg ml^{-1} streptomycin (Life Technologies). During the isolation and early stages of ES cell cultivation, the medium was supplemented with human recombinant leukemia inhibitory factor (hLIF) at 2,000 units ml^{-1} (Amrad, Melbourne, Australia). Six to eight days after initial plating, ICM-like clumps were removed mechanically by a micropipette from differentiated cell outgrowths and replated on fresh feeder layer. The resulting colonies were further propagated in clumps of ~100 stem cell-like cells, on mouse feeder layer, about every seven days. The clumps were either dissociated mechanically, or with a combined approach of mechanical slicing followed by exposure to dispase (10 mg ml^{-1}; Life Technologies). In some experiments, clumps of cells were subcultured onto tissue culture dishes in standard medium supplemented with hLIF at 2,000 units ml^{-1} or a combination of IL-6 (2 ng ml^{-1}) and the soluble IL-6 receptor (33 ng ml^{-1}; R&D Systems, Minneapolis, MN) in the absence of a feeder cell layer. In other experiments, clumps of cells were cultivated in standard medium in 25 μl hanging drops, or on bacteriological dishes, before replating onto tissue culture plastic.

Stem cell characterization. Colonies were fixed in the culture dishes by 100% ethanol for immunofluorescence demonstration of the stem cell surface markers GCTM-2, TRA 1-60, and SSEA-1, whereas 90% acetone in H_2O fixation was used for SSEA-4. The sources of the monoclonal antibodies used for the detection of the markers were as follows: GCTM-2, this laboratory; TRA 1-60, a gift of Peter Andrews, University of Sheffield; SSEA-1 (MC-480) and SSEA-4 (MC-813-70), Developmental Studies Hybridoma Bank (Iowa City, IA). Antibody localization was performed by using rabbit anti-mouse immunoglobulins conjugated to fluorescein isothiocyanate (Dako, Carpinteria, CA).

Alkaline phosphatase activity was demonstrated as described[30].

Standard G-banding techniques were used for karyotyping.

Oct-4 expression studies. To monitor expression of Oct-4, RT-PCR was carried out on colonies consisting predominantly of stem cells, or colonies that had undergone spontaneous differentiation as described below. mRNA was isolated on magnetic beads (Dynal AS, Oslo) following cell lysis according to the manufacturer's instructions, and solid-phase first-strand cDNA synthesis was performed using Superscript II reverse transcriptase (Life Technologies). The PCR for Oct-4 was identical to that of van Eijk and colleagues[31] using the solid-phase cDNA as template and *Taq* polymerase (Pharmacia Biotech, Hong Kong). As a control for mRNA quality, we assayed β-actin transcripts using the same RT-PCR and the following primers: 5′-CGCACCACTGGCATTGTCAT-3′ (forward), 5′-TTCTCCTTGATGTCACGCAC-3′ (reverse). Products were analyzed on a 1.5% agarose gel and visualized by ethidium bromide staining.

Teratoma formation in SCID mice. At the time of routine passage, clumps of about 200 cells with an undifferentiated morphology were harvested as described above, and injected into the testis of four- to eight-week-old SCID mice (CB17 strain from the Walter and Eliza Hall Institute, Melbourne, Australia, 10–15 clumps per testis). Six to seven weeks later, the resulting tumors were fixed in neutral buffered formalin 10%, embedded in paraffin, and examined histologically after hematoxylin and eosin staining.

In vitro differentiation. Colonies were cultured on mitotically inactivated mouse embryonic fibroblasts to confluency (about two or three weeks) and further on up to seven weeks after passage. The medium was replaced every day. Levels of α-fetoprotein and the β-subunit of human chorionic gonadotropin were measured in medium conditioned by HES-1 and HES-2 at passage levels 17 and 6, respectively. After four to five weeks of culture,

conditioned medium was harvested 36 h after last medium change, and the protein levels were determined by a specific immunoenzymometric assays (Eurogenetics, Tessenderllo, Belgium) and a fluorometric enzyme immunoassay (Dade, Miami, FL), respectively. These proteins were not detected in control medium conditioned only by feeder layer.

Differentiated cultures were fixed three to seven weeks after passage (26 for HES-1 and 9 for HES-2 for immunofluorescence detection of lineage-specific markers. After fixation with 100% ethanol, specific monoclonal antibodies were used to detect the 68 kDa neurofilament protein (Amersham, Amersham, UK) and N-CAM (UJ13a, Dako). Muscle-specific actin and desmin were also detected by monoclonal antibodies (Dako; HHF35 and D33, respectively) after fixation with methanol/acetone (1:1). Antibody localization was performed as described above.

Isolation and characterization of neuronal progenitors and cells derived from them. Clusters of cells destined to give rise to neural precursors were identified by their characteristic morphological features in central areas ofdifferentiating ES cell colonies of both cell lines three weeks after plating. The clusters were dissected mechanically by a micropipette and replated in fresh serum-free medium. Within 24 h they formed spherical structures. The spheres were plated on coverslips coated with poly-D-lysine (30–70 kDa; Sigma) and laminin (Sigma), fixed after 4 h, and examined by indirect immunofluorescence analysis for expression of N-CAM (acetone fixation, antibody as described above), nestin (4% paraformaldehyde fixation, rabbit antiserum, a kind gift of Dr. Ron McKay), and vimentin (methanol fixation, mouse monoclonal antibody Vim3B4 from Roche Diagnostics Australia, Castle Hill, NSW). The expression of β-actin, nestin (primers[22]), and the transcription factor Pax-6 (primers[22]) in the spheres was demonstrated by RT-PCR as described above. In some experiments, the plated spheres were cultured in serum-free medium supplemented with all-*trans*-retinoic acid (1 μM) for 7–15 days, and cells growing out from them were analyzed by indirect immunofluorescence for expression of the following markers: 200 kDa neurofilament protein (4% paraformaldehyde fixation, mouse monoclonal antibody RT97 from Novocastra, Newcastle, UK), 160 kDa neurofilament protein (methanol fixation, mouse monoclonal NN18 from Roche), MAP2a+b (4% paraformaldehyde fixation, mouse monoclonal AP20 from Neomarkers, Union City CA), glutamate (1% paraformaldehyde and 1% glutaraldehyde, rabbit antiserum from Sigma), synaptophysin (4% paraformaldehyde, mouse monoclonal SY38 from Dako), glutamic acid decarboxylase (1% paraformaldehyde, 1 % glutaraldehyde, rabbit antiserum from Chemicon, Temecula, CA), and β-tubulin (4% paraformaldehyde, mouse monoclonal TUB 2.1 from Sigma). RT-PCR analysis for the expression of β-actin, glutamic acid decarboxylase (primers[32]), and $GABA_A$ receptor subunit α2 (primers[33]) was carried out as described above.

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1. Evans, M.J. & Kaufman, M. Establishment in culture of pluripotential stem cells from mouse embryos. *Nature* **292**, 151–156 (1981).
2. Martin, G.R. Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. *Proc. Natl. Acad. Sci USA* **78**, 7634–7638 (1981).
3. Andrews, P.W. et al. Pluripotent embryonal carcinoma clones derived from the human teratocarcinoma cell line Tera-2. *Lab. Invest.* **50**, 147–162 (1984).
4. Pera, M.F., Cooper, S., Mills, J. & Parrington, J.M. Isolation and characterization of a multipotent clone of human embryonal carcinoma-cells. *Differentiation* **42**, 10–23 (1989).
5. Thompson, S. et al. Cloned human teratoma cells differentiate into neuron-like cells and other cell types in retinoic acid. *J. Cell Sci.* **72**, 37–64 (1984).
6. Pera, M.F. & Herszfeld, D. Differentiation of pluripotent teratocarcinoma stem cells induced by bone morphogenetic protein-2. *Reprod. Fertil. Devel.* **10**, 551–556 (1999).
7. Thomson, J.A. et al. Isolation of a primate embryonic stem cell line. *Proc. Natl. Acad. Sci. USA* **92**, 7844–7844 (1995).
8. Thomson, J.A. et al. Pluripotent cell lines derived from common marmoset (*Callithrix jacchus*) blastocysts. *Biol. Reprod.* **55**, 254–259 (1996).
9. Bongso, A., Fong, C.Y., Ng, S.C. & Ratnam, S. Isolation and culture of inner cell mass cells from human blastocysts. *Hum. Reprod.* **9,** 2110–2117 (1994).
10. Thomson, J.A. et al. Embryonic stem cell lines derived from human blastocysts. *Science* **282**, 1145–1147 (1998).
11. Andrews, P.W. et al. Comparative-analysis of cell-surface antigens expressed by cell-lines derived from human germ-cell tumors. *Int. J. Cancer* **66**, 806–816 (1996).
12. Cooper, S., Pera, M.F., Bennett, W. & Finch, J.T. A novel keratan sulfate proteoglycan from a human embryonal carcinoma cell-line. *Biochem. J.* **286**, 959–966 (1992).
13. Pera, M.F. et al. Analysis of cell-differentiation lineage in human teratomas using new monoclonal-antibodies to cytostructural antigens of embryonal carcinoma-cells. *Differentiation* **39**, 139–149 (1988).
14. Badcock, G., Pigott, C., Goepel, J. & Andrews, P.W. The human embryonal carcinoma marker antigen TRA-1-60 is a sialylated keratan sulphate proteoglycan. *Cancer Res.* **59,** 4715–4719 (1999).
15. Nichols, J. et al. Formation of pluripotent stem cells in the mammalian embryo depends on the POU transcription factor Oct4. *Cell* **95**, 379–391 (1998).
16. Roach, S., Cooper, S., Bennett, W. & Pera, M.F. Cultured cell lines from human germ cell tumours: windows into tumour growth and differentiation and early human development. *Eur. Urol.* **23**, 82–88 (1993).
17. Shamblott, M.J. et al. Derivation of pluripotent stem cells from cultured human primordial germ cells. *Proc. Natl. Acad. Sci. USA* **95,** 13726–13731 (1998).
18. Beddington, R.S.P. & Robertson, E.J. Axis development and early asymmetry in mammals. *Cell* **96**, 195–209 (1999).
19. Andrews, P.W. et al. Inhibition of proliferation and induction of differentiation of pluripotent human embryonal carcinoma cells by osteogenic protein-1 (or bone morphogenetic protein 7). *Lab. Invest.* **71**, 243–251 (1994).
20. Caricasole, A.D. et al. In *Inhibin, activin and follistatin: regulatory functions in system and cell biology* (eds Aono, T., Sugino, H. & Vale, W.W.) 308–311 (Springer, New York, NY; 1997).
21. Flax, J.D. et al. Engraftable human neural stem cells respond to developmental cues, replace neurons, and express foreign genes. *Nat. Biotechnol.* **16**, 1033–1039 (1998).
22. Kukekov, V.G. et al. Multipotent stem/progenitor cells with similar properties arise from two neurogenic regions of adult human brain. *Exp. Neurol.* **156**, 333–344 (1999).
23. Dani, C. et al. Paracrine induction of stem cell renewal by LIF-deficient cells—a new ES cell regulatory pathway. *Dev. Biol.* **203**, 149–162 (1998).
24. Rathjen, J. et al. Formation of a primitive ectoderm like cell population, EPL cells, from ES cells in response to biologically derived factors. *J. Cell Sci.* **112**, 601–612 (1999).
25. McWhir, J. et al. Selective ablation of differentiated cells permits isolation of embryonic stem cell lines from murine embryos with a non-permissive genetic background. *Nat. Genet.* **14**, 223–226 (1996).
26. Li, M., Pevny, L., Lovell-Badge, R. & Smith, A. Generation of purified neural precursors from embryonic stem cells by lineage selection. *Curr. Biol.* **8**, 971–974 (1998).
27. Fong, C.Y. & Bongso, A. Comparison of human blastulation rates and total cell number in sequential culture media with and without co-culture. *Hum. Reprod.* **14**, 774–781 (1999).
28. Fong, C.Y. et al. Ongoing pregnancy after transfer of zona-free blastocysts: implications for embryo transfer in the human. *Hum. Reprod.* **12**, 557–560 (1997).
29. Solter D. & Knowles, B. Immunosurgery of mouse blastocyst. *Proc. Natl. Acad. Sci. USA* **72**, 5099–5102 (1975).
30. Buehr, M. & Mclaren, A. Isolation and culture of primordial germ cells. *Methods Enzymol.* **225**, 58–76 (1993)
31. van Eijk, MJ et al. Molecular cloning, genetic mapping, and developmental expression of bovine POU5F1. *Biol. Reprod.* **60**, 1093–1103 (1999).
32. Vescovi, A.L. et al. Isolation and cloning of multipotential stem cells from the embryonic human CNS and establishment of transplantable human neural stem cell lines by epigenetic stimulation. *Exp. Neurol.* **156**, 71–83 (1999).
33. Neelands, T.R. et al. $GABA_A$ receptor pharmacology and subtype expression in human neuronal NT2-N cells. *J. Neurosci.* **18**, 4993–5007 (1998).