STEM CELLS®

Experimental Protocols for Embryonic Stem Cell Research

Differentiation of Human Embryonic Stem Cells to Neural Lineages in Adherent Culture by Blocking Bone Morphogenetic Protein Signaling

LESLEY GERRARD, LEIGH RODGERS, WEI CUI

Department of Gene Function and Development, Roslin Institute, Roslin, Midlothian, United Kingdom

Key Words. Human embryonic stem cell • Neural differentiation • Noggin • Bone morphogenetic protein • Oct4

ABSTRACT

Human embryonic stem cells (hESCs) have extensive self-renewal capacity and are competent to differentiate into any cell type of the body. They are valuable not only for the study of early human development but also for regenerative medicine. However, how to direct differentiation of hESCs along a particular lineage pathway to a specific cell type remains a challenge. Although hESCs have been shown to differentiate in vitro into neural progenitors, the factors controlling their differentiation are poorly understood. In this study, we report the development of an in vitro adherent culture system to efficiently generate neural progenitors in which neither multicellular aggregates nor stromal cells are required. We show that inhibition of bone morphogenetic protein signaling by its antagonist noggin is sufficient to block extraembryonic cell fate, transiently sustain *Oct4* gene expression, and result in robust production of neural progenitors. Our findings will provide a platform for studying the molecular mechanism controlling neural differentiation. STEM CELLS *2005;23:1234–1241*

INTRODUCTION

The isolation and culture of human embryonic stem cells (hESCs) has opened up new opportunities for the study of basic stem cell biology, early human embryonic development, and regenerative medicine. hESCs are derived from the inner cell mass of preimplantation embryos and retain the developmental potency of embryonic founder cells, being able to differentiate into cells and tissues of all three germ layers in vitro and in vivo [1]. Therefore, they provide a novel model system to elucidate the molecular signals required for the development of various lineages.

Like mouse embryonic stem cells (ESCs), hESCs can be expanded to large numbers while retaining their differentiation potentials. This is particularly important for transplantation therapies, because hESCs promise an almost unlimited supply of specific cell types. However, directing hESC differentiation along specific lineage pathways remains a challenge. The clinical application of hESCs requires the generation of highly purified donor cells for specific tissues or organs. For neural differentiation of hESCs, most protocols available so far are based on the initial formation of embryoid bodies (EBs) in the presence of serum/serum replacement or coculture of hESCs with particular stromal cell lines [2–4]. In the first cases, hESCs were initially differentiated in suspension cultures to form EBs, which often contain cells of all three germ layers; clusters of neural cells are surrounded by flat non-neural cells, and even in the neural clusters there are mixed populations of neuronal (majority) and glial cells [2, 3]. Moreover, due to the complexity of multicellular aggregates, it is difficult to use this system for studying signaling pathways essential for the neural differentiation. In the latter case, the effect of stromal cells is attributed to an undefined neural-inducing activity [4].

In mouse ESCs, neuroectoderm differentiation has been achieved in an adherent monoculture system in a serum-free medium, without multicellular aggregation or coculture with other cell types [5]. This monolayer culture regimen enables us

Correspondence: Wei Cui, M.D., Ph.D., Department of Gene Function and Development, Roslin Institute, Roslin, Midlothian, EH25 9PS, U.K. Telephone: 44-0-131-527-4200; Fax: 44-0-131-440-0434; e-mail: wei.cui@bbsrc.ac.uk Received March 14, 2005; accepted for publication June 21, 2005; first published online in STEM CELLS *EXPRESS* July 7, 2005. 1066-5099/2005/$12.00/0 doi: 10.1634/stemcells.2005-0110

to directly visualize the process of neural conversion. In addition, because the process is in a chemically defined medium, it provides a good system in the future to investigate signaling pathways in controlling the ESC neural differentiation. Here we extend this study to hESCs, demonstrating that blocking bone morphogenetic protein (BMP) signaling with its antagonist noggin at the initial stage of the differentiation is sufficient for derivation of highly enriched and expandable populations of neural progenitors in adherent culture. Additionally, these progenitors can be further differentiated into various types of neurons in vitro but do not exhibit glial markers until long term in culture.

Materials and Methods

Derivation and Culture of Neural Progenitors

hESCs were cultured on matrigel (BD Biosciences, San Jose, CA, http://www.bdbiosciences.com) in mouse embryonic fibroblast–conditioned medium (MEF-CM) supplemented with 8 ng/ml basic fibroblast growth factor (bFGF) (Invitrogen, Carlsbad, CA, http://www.invitrogen.com) and propagated routinely with collagenase in 1:3 ratios [6]. For neural differentiation, nearly confluent hESCs were split with 0.5 mM EDTA/phosphate-buffered saline (PBS) in 1:5 ratios into culture dishes coated with matrigel or poly-L-lysine/laminin (Sigma-Aldrich, St. Louis, http://www.sigmaaldrich.com) and cultured in N2B27 medium (1:1 mix of D-MEM/F12 supplemented with N2 and Neurobasal medium supplemented with B27, all from Gibco [Grand Island, NY, http://www.invitrogen.com], supplemented with 100 ng/ml mouse recombinant noggin (R&D Systems Inc., Minneapolis, http://www.rndsystems.com). At this stage, cells were defined as passage 1 (P1). In the neural differentiation, the cells were consistently cultured in the same matrices-coated culture plastics. Cells of P1 and P2 were split by collagenase into small clumps, similar to hESC culture, and continuously cultured in N2B27 medium plus noggin. From P3, cells were disassociated into single cells by TrypLE express (Gibco), and noggin could be withdrawn from N2B27 medium. Some of the cells were cultured in N2B27 medium supplemented with 20 ng/ml bFGF from P4-5.

The growth curves were started at P5. The neural progenitors were plated at 1×10^5 per cm^2 in six-well culture plates and cultured in N2B27 medium with or without 20 ng/ml bFGF. The cells were counted when splitting, and population doublings were calculated.

For tyrosine hydroxylase (TH) neuron differentiation, neural progenitors at P4 or P5 were seeded in poly-L-lysine/laminin (PLL/Lam)-coated culture dishes and induced with sonic hedgehog (300–400 ng/ml), FGF8 (100 ng/ml) [7], and ascorbic acid (160 μM) for 1–2 weeks and then followed by brain-derived neurotrophic factor (BDNF) (20 ng/ml), glial cell line–derived neurotrophic factor (GDNF) (20 ng/ml), ascorbic acid (160 μM), and laminin (0.5 μg/ml) for another 2 weeks.

Immunocytochemistry

Cells were fixed at room temperature with 4% paraformaldehyde for 10 minutes, washed with PBS, and incubated with 10% goat serum and 0.02%–0.1% Triton X-100 for 1 hour. The cells were then incubated with primary antibodies at the appropriate dilution at 4°C overnight. Secondary antibody was applied for 30 minutes after washing with PBS. The cells were finally mounted with Mowiol (Calbiochem, San Diego, http://www.emdbiosciences.com) and then visualized and captured using Nikon TE2000-U (http://www.nikoninstruments.com) or Digital Pixel image analysis system (Brighton, U.K., http://www.digitalpixel.co.uk).

The following primary antibodies were used in this study: mouse monoclonal antibodies against nestin, polysialic acid–neural cell adhesion molecule (PSA-NCAM), and TH (1:200; all from Chemicon, Temecula, CA, http://www.chemicon.com), β-tubulin III (1:1,000; Sigma), and α-fetoprotein (1:500; Sigma); rabbit polyclonal antibodies against nestin and musashi (1:200), GAD65 (1:500), MAP2 and Pax6 (1:1,000; all from Chemicon), GABA and Sox1 (1:500; Sigma), GATA6 (1:50; Santa Cruz Biotechnology Inc., Santa Cruz, CA, http://www.scbt.com), and glial fibrilliary acidic protein (GFAP) (1:500; DakoCytomation, Glostrup, Denmark, http://www.dakocytomation.com). Secondary antibodies used were goat anti-rabbit fluorescein isothiocyanate (FITC), goat anti-mouse FITC, and goat anti-rabbit Texas red (all at 1:400; Jackson Laboratory, Bar Harbor, ME, http://www.jax.org), goat anti-mouse immunoglobulin G Alex Fluro 568, and goat anti-rabbit Alex Fluro 488 (all at 1:500–1:1,000; Molecular Probes Inc., Eugene, OR, http://probes.invitrogen.com).

RNA Isolation and Reverse Transcription–Polymerase Chain Reaction

Total RNA was extracted using QIAGEN RNeasy kit and DNase I treatment (Qiagen, Inc., Valencia, CA, http://www.qiagen.com). First-strand cDNA was synthesized with 2 μg total RNA in 20–25 μl as described previously [8]. The cDNAs were then diluted to 225 μl with distilled H_2O. Diluted cDNA 3–10 μl was used for polymerase chain reaction (PCR) in 50-μl reactions. PCR conditions were optimized and linear amplification range was determined for each pair of primers by varying annealing temperature and cycle numbers. Primer sequences and their PCR conditions are provided upon request. The expression levels were analyzed using multianalysis software and standardized by β-actin housekeeping gene expression.

Results

Noggin Blocked hESC Commitment to Extraembryonic Endoderm and Enhanced Neuroectoderm Formation

It has been shown previously that culturing mouse ESCs in modified N2B27 medium on gelatin-coated plastic can suffi-

ciently differentiate them into neural precursors [5]. A modified version of this procedure was applied to hESCs (H1, H7, and H9) in which hESCs were dissociated with EDTA rather than trypsin. EDTA-dissociated cells plated as small clumps (~4 to 10 cells/clump), but trypsin generated single cells, which exhibited low seeding efficiency and spontaneous differentiation. All three hESC lines showed very low attachment in gelatin-coated culture dishes and formed cell aggregates in suspension. Therefore, gelatin was replaced with matrigel or PLL/Lam because both matrices have been reported for hESC culture previously [6].

On both matrigel and PLL/Lam, hESC plating efficiency was improved. After 4 days of culture in N2B27 medium, flat cells started to emerge, which were very similar in appearance to BMP2-treated hESCs reported earlier by Pera et al. [9]. This flat cell population expanded rapidly (Figs. 1A, 1B, 1D, 1E), pushing other cell types into clumps, eventually forcing them to detach into the medium. This was most apparent in H9 cells and then H1 and its clonal derivatives and less evident in H7 cells. These flat cells showed positive staining with GATA6 antibody (Fig. 1G) and sometimes formed fluid-filled cysts (Fig. 1B, arrow) that expressed α-fetoprotein (AFP) (Fig. 1H). GATA6 has been implicated in mammalian yolk sac endoderm differentiation and function [10, 11]. Forced expression of GATA6 in mouse ESCs triggers differentiation to visceral endoderm [12]. Therefore, the results suggest that these cells are extraembryonic endoderm [9].

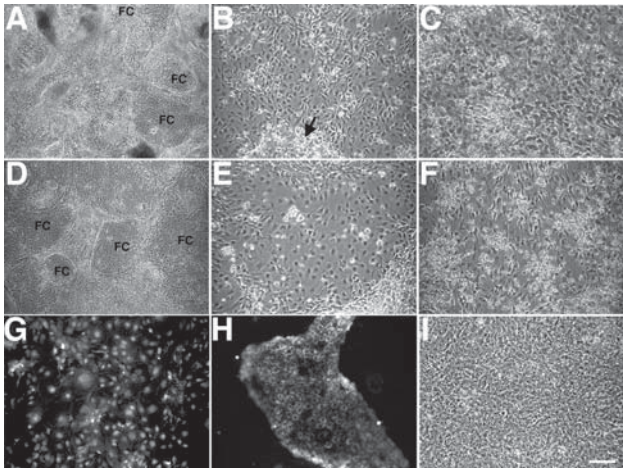


Figure 1. Differentiation of human embryonic stem cells in N2B27 medium with or without bone morphogenetic protein antagonists. H1 cells were cultured for 13 days in **(A, B, D, E, G, H)** N2B27 medium, **(C, F)** N2B27 medium supplemented with 100 ng/ml noggin, or **(I)** N2B27 medium supplemented with 50 ng/ml follistatin. **(A–C):** Cells cultured on matrigel. **(D–F):** Cells cultured on poly-L-lysine/laminin. **(A–F, I):** Phase-contrast images. **(G, H):** Immunostaining with GATA6 and α-fetoprotein antibodies, respectively. Arrow: Differentiated cells formed cyst. Scale bar = 250 μm in A and D, 75 μm in the others. Abbreviation: FC, flat cells.

It has been reported that BMP2 plays a critical role in ESCs differentiation to extraembryonic endoderm [9] and inhibits neuroectoderm differentiation [5, 13]. Therefore, we examined whether blocking BMP signaling with noggin and/or follistatin might restrain the primitive endoderm formation and promote neural differentiation. Both noggin and follistatin are well-characterized BMP antagonists and have been shown as neural inducers in *Xenopus* embryos [14–16]. However, noggin is known to bind BMP 2, 4, and 7 with higher affinity, consequently preventing ligation of their receptors, whereas follistatin has high binding affinity for activin but lower affinity for BMPs and its antagonizing effects of BMPs through different mechanism rather than interfering with the bindings of receptors. Our results showed that hESCs cultured in N2B27 medium supplemented with 100 ng/ml noggin significantly blocked extraembryonic endoderm formation in both matrigel and PLL/Lam-coated plastic and after 10 days neural progenitor cells are clearly visible (Figs. 1C, 1F), whereas follistatin did not show evident neuroectoderm formation, although it partially inhibited extraembryonic endoderm formation. The hESCs in follistatin-treated cultures exhibited mainly fibroblast-like morphology (Fig. 1I).

Analysis of gene expression in H1, T5 (a clonal Oct4-GFP cell line derived from H1 [17]), and H7 cell lines in the noggin-treated as well as nontreated culture conditions revealed that at day 6 of differentiation (Fig. 2A), noggin-treated cultures showed apparent downregulation of GATA6, Id1, and Id3 transcripts in all tested cell lines. Additionally, the downregulation of BMP4 was observed in most tested lines. Id proteins have been identified as targets of BMP signaling, and BMP2 induces expression of Id1 and Id3 [18]. Both Id1 and Id3 repress the promoter activation induced by neurogenic bHLH factors and inhibit neurogenesis [18]. These results indicate that noggin inhibited BMP signal pathways, and this may have been the mechanism that blocked ESC differentiation to extraembryonic endoderm. However, the levels of BMP2 expression exhibited upregulation in noggin-treated cultures. This could be due to the feedback regulation of downstream genes. There was no obvious expression of AFP at day 6 of differentiation in both conditions. By day 13, however, AFP expression was significantly upregulated in non–noggin-treated cultures (Fig. 2B), whereas in noggin-treated cultures, no or very low levels of AFP expression were detected.

Downregulation of *Oct4* Expression Is a Relatively Slower Process During Neural Differentiation

It has been reported that Oct4, the POU-domain transcription factor encoded by *Pou5f1*, may be required for neurogenesis [19]. We have previously generated *Oct4*-GFP reporter hESC lines, which retain hESC characteristics, and the expression of *Oct4*-GFP reporter transgene mimics the expression of endogenous *Oct4* gene [17]. The adherent differentiation culture protocol in addition to these reporter cell lines provides a noninvasive and

relatively direct model system to investigate *Oct4* expression during neural differentiation in vitro. Therefore, we took two *Oct4*-EGFP clonal hESC lines through the differentiation protocol (as described in the next section) and monitored the GFP expression regularly using a fluorescent microscope.

Oct4-GFP expression was readily visible in most cells throughout the first week of hESC differentiation (Fig. 3Aa). After approximately 10 days of differentiation, downregulation of GFP expression was initiated. The areas where the differentiated cells became smaller and the nuclei were less visible lost GFP expression, whereas the areas where the cells retained morphology of hESCs were still positive for GFP (Fig. 3Ab). After approximately 1 month of differentiation, cells exhibited typical neural progenitor morphology, and almost no GFP was visible (Fig. 3Ac). However, if the hESCs were cultured in N2B27 medium without noggin, approximately 50% of cells lost *Oct4*-GFP expression during the first week of differentiation and those cells exhibited extraembryonic endoderm-like morphology (Fig. 3B). The observations of GFP expression were compatible with the reverse transcription (RT)-PCR results (Fig. 4A) in which *Oct4* expression was retained longer during neural differentiation than other hESC markers, such as Nanog and human telomerase RT catalytic unit (hTERT). This is consistent with previous published reports [19, 4], suggesting that transiently sustained Oct4 levels may be required to bypass the initial differentiation to extraembryonic endoderm-like cells.

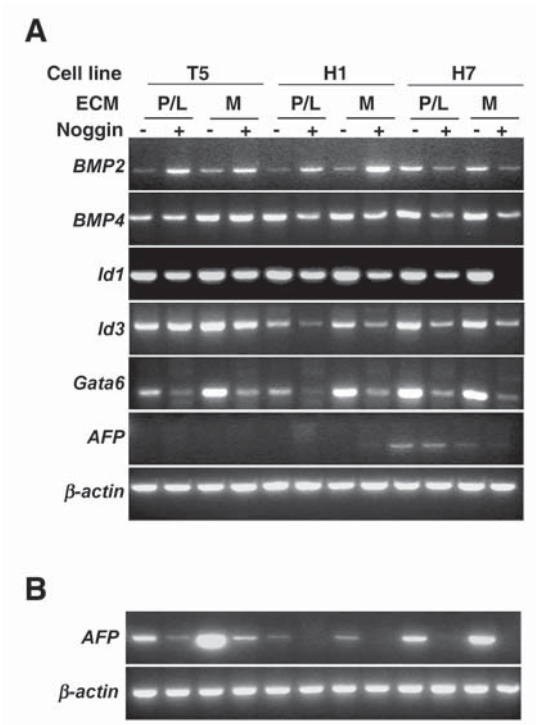


Figure 2. Comparison of gene-expression pattern during initial hESC differentiation in N2B27 with or without noggin treatment. hESCs were differentiated for **(A)** 6 days and **(B)** 13 days. Reverse transcription–polymerase chain reaction analyses show that BMP target genes *Id1* and *Id3* are downregulated upon noggin treatment and expression of extraembryonic endoderm markers GATA6 and AFP are inhibited. H1, H7, and T5 represent three hESC lines. Abbreviations: AFP, α-fetoprotein; BMP, bone morphogenetic protein; ECM, extracellular matrix; hESC, human embryonic stem cell; M, matrigel; P/L, poly-L-lysine/laminin.

Differentiation of Neural Progenitors and Neurons from hESCs

Our protocol of neural differentiation in adherent culture system comprises four stages (Fig. 5A). Stage 1 is hESC culture (Fig. 5Ba). hESCs were cultured in matrigel-coated six-well culture plates with MEF-CM supplemented with bFGF as previously described [6, 17]. When cells were almost confluent, they were disassociated with EDTA, seeded at a 1:5 ratio into new plates (~3 × 10^4 per cm^2), coated with either matrigel or PLL/lam, and cultured in N2B27 medium supplemented with mouse recombinant noggin at 100 ng/ml. These cells were defined as passage 1 (P1). During the initial week of induction stage, the cells formed more compacted colonies, devoid of surrounding spontaneously differentiated cells (Fig. 5Bb, stage 2, neural induction) compared with hESCs grown in MEF-CM culture. Gradually the cells in some areas started to look darker and smaller with less clearly visible nuclei, a sign of early neuroectoderm formation. These cells

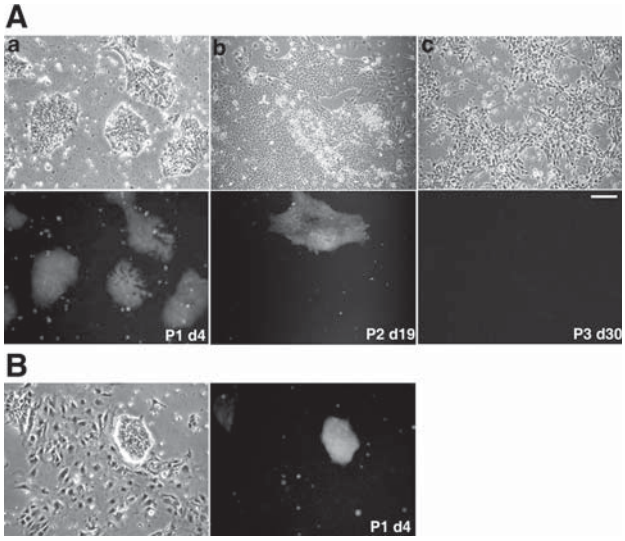
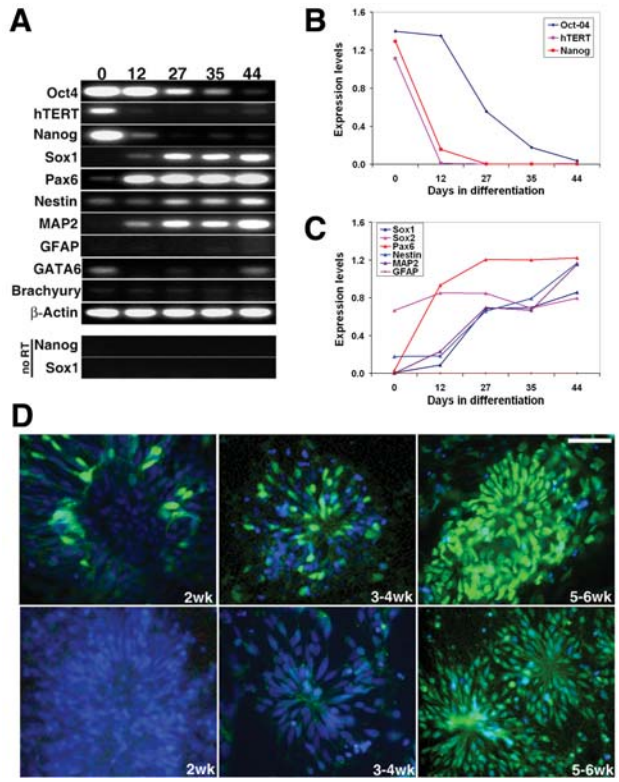


Figure 3. *Oct4*-GFP expression in *Oct4*-GFP clonal cell line, T5, during neural differentiation. **(A):** Phase-contrast and fluorescent images of T5 human embryonic stem cell (hESC) differentiation in N2B27 medium supplemented with noggin for (a) 4 days, (b) 19 days, and (c) 30 days. The expression of *Oct4*-GFP was sustained during the first week of differentiation and then downregulated. No GFP was visible after approximately 1 month of differentiation. **(B):** Phase-contrast and fluorescent images of T5 hESC differentiation in N2B27 medium for 4 days. The cells became flat, extraembryonic endoderm-like cells and the *Oct4*-GFP expression was dramatically downregulated in the flat cells. Scale bar = 100 μm in Ac and 140 μm in the others.



were split with collagenase at a 1:3 ratio (5 to 7 × 10^4 per cm^2) at days 8 through 14 and continuously cultured in the same medium. Collagenase was used rather than EDTA at this stage because the seeding efficiency with EDTA was much lower even though there were no significant morphological changes observed. The neural tube–like structure became clearly visible at P2 (approximately day 20), and some early rosette neural progenitors also started to appear (Figs. 5Bc, 5Bd, stage 3, formation of neuroectoderm). These early neural progenitors still prefer to be propagated in small clumps to give a high yield of neural lineages. We found that if we disassociated them into single cells at this stage, some of these cells became other cell types rather than neural progenitors. This could be due to three possibilities. One is that some cells

Figure 4. Alterations on expression levels of hESCs and neural markers during neural differentiation. **(A–C):** Semiquantities reverse transcription (RT)–polymerase chain reaction on hESCs and neural markers. **(D):** Immunostaining with anti-Pax6 and anti-Sox1 antibodies. Scale bar = 50 μm. Abbreviations: hESC, human embryonic stem cell; hTERT, human telomerase reverse transcription catalytic unit.

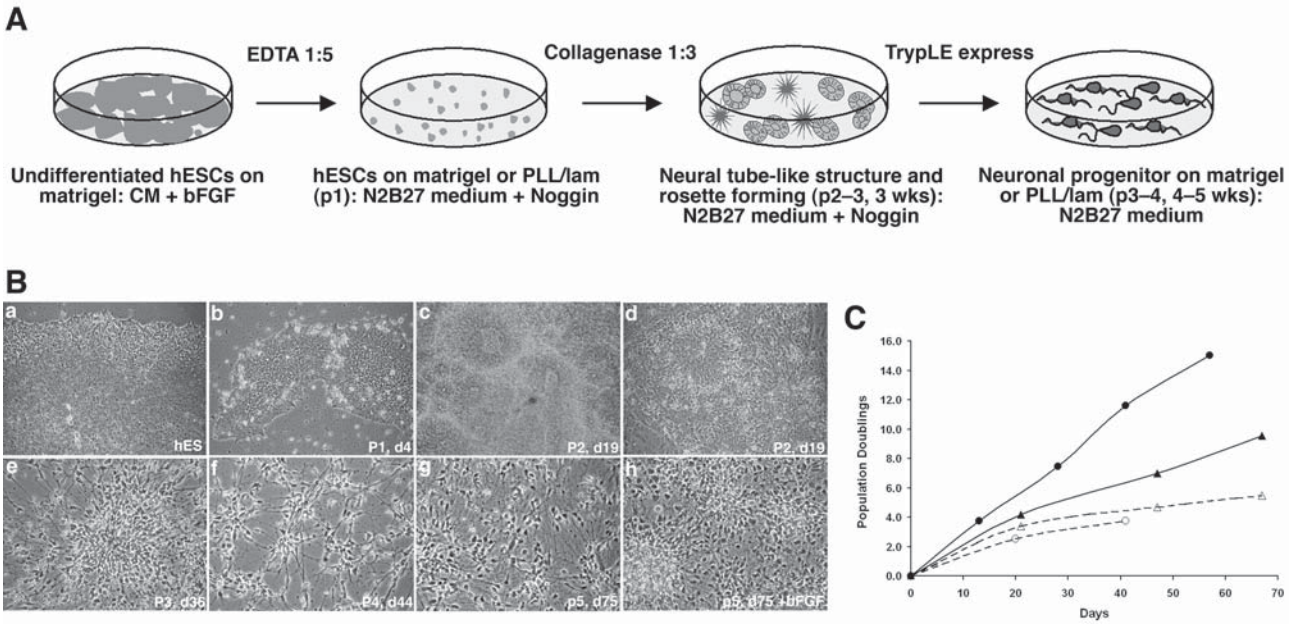


Figure 5. Differentiation of hESCs to neural progenitors in adherent culture system. **(A):** Schematic representation illustrating the sequential steps in the neural differentiation of hESCs. The timeline was counted from the start of differentiation (see text for details). **(B):** Sequential images during H1 cell differentiation in the matrigel-adherent culture as described in the text and Materials and Methods. **(a):** hESCs grown in CM with bFGF. **(b):** The first week of differentiation (P1), hESCs still exhibit ESC-like morphology. **(c, d):** Neural tube–like structures **(c)** and rosette **(d)** appeared at approximately 2–3 weeks of differentiation (P2). **(e, f):** Typical neural progenitors clearly emerged after 4 weeks of differentiation (P3-4). **(g-h):** Neural progenitors at P5 cultured in N2B27 without or with bFGF, respectively. Scale bar = 100 μm in a–d; 75 μm in e–h. **(C):** Growth curves of H1-T5 (triangle) and H7 (circle) neural progenitors (P5-P10) cultured in N2B27 with (solid symbol) or without (open symbol) bFGF supplement show that the cells proliferated faster in response to bFGF. Abbreviations: bFGF, basic fibroblast growth factor; CM, conditioned medium; hESC, human embryonic stem cell; PLL/Lam, poly-L-lysine/laminin.

were still undifferentiated at this stage so they could respond to the change in the microenvironment to become other cell types. Alternatively, the early neuroectoderm cells retained the capacity to generate other cell types in response to environment, or it could be a simple in vitro selection for non-neural cells.

At P3 (about 30 days of differentiation), the cells exhibited typical rosette neural progenitor cell morphology (Fig. 5Be) and showed robust staining with nestin antibody (Fig. 6A), indicating their neural progenitor properties. From P3, the hESC-derived neural progenitor cells could be disassociated and seeded efficiently with TrypLE express at approximately 1×10^5 per cm^2. Most cells exhibited a homogenous appearance of neural precursor/early neurons, as shown in Figure 5Bf, and showed positive staining for neural precursor markers, nestin, musashi, and PSA-NCAM (Figs. 6B–6D). By counting the cells positive for these markers over the number of cells positive for DAPI, 97.7% ± 0.55%, 96.3% ± 2.87%, and 92.3% ± 0.96% were positive for nestin, mushashi, and PSA-NCAM, respectively. We have repeated the experiments several times on H1, H7, and T5 cell lines, and the results are similar. The data were from three independent differentiation experiments of T5 cells. At P3, cells at the edge of the rosette also stained positive for MAP2 and β-tublin III, and by P4, even more cells were positive for MAP2 and β-tublin III (Figs. 6E, 6F). However, no GFAP-positive cells were observed (Fig. 6K), although they started to emerge after more than 80 days of differentiation in culture (Fig. 6L).

hESC-derived neural progenitors with this protocol were expandable by seeding them no less than 1×10^5 per cm^2 without addition of bFGF or epidermal growth factor, but the proliferation gradually slowed after six passages. However, the neural progenitors could be maintained at a relatively high dividing rate for much longer when bFGF was supplemented (Figs. 5Bg, 5Bh, 5C). At P3 without bFGF, cells in the center of the rosette retained strong proliferative potential, as shown by BrdU staining, and the surrounding cells positive for MAP2 antibody showed less BrdU incorporation (Fig. 6J). Some of the hESC-derived cells have been cultured for longer than 4 months and still proliferate vigorously with the addition of bFGF. We also found that although addition of bFGF from the beginning of differentiation enhanced the cell proliferation and slightly delayed the time of differentiation, it did not affect the overall neural differentiation outcome.

Characterization of hESC-Derived Neural Progenitors and Neurons

Expression analysis of the hESCs and their derived neural progenitors at various stages of differentiation confirmed morpho-

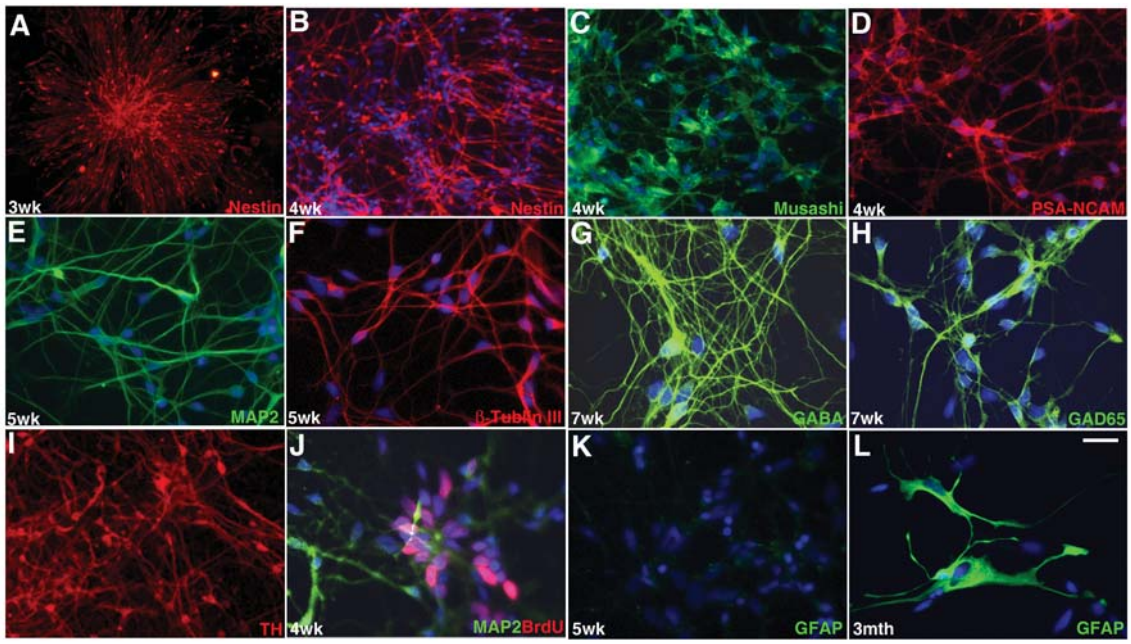


Figure 6. Characterization of hESC-derived neural progenitors and neurons by immunocytochemistry. **(A):** Typical rosette-neural progenitor appeared at approximately 3 weeks of differentiation and is positive with nestin antibody staining. After disassociation, the neural progenitors are also positive for other neural progenitor markers, such as **(C)** musashi and **(D)** PSA-NCAM, in addition to **(B)** nestin. These neural progenitors are expandable, and the cells in the center of the rosette are positive for BrdU labeling **(J)**. At P4, the cells are positive for early neuronal markers as well such as **(E)** MAP2 and **(F)** β-tubulin III. Most of the neurons differentiated from hESCs in the N2B27 medium are GABA neurons, indicated by their positive staining for **(G)** GABA and **(H)** GAD65 antibodies. TH neurons can be induced by exposure of neural progenitors at 4 weeks of differentiation to SHH and FGF8 **(I)**. No GFAP-positive cells were detected **(K)** until long term in culture **(L)**. Blue staining represents DAPI counterstain. Timelines are indicated at lower left corner. Scale bar = 75 μm in A, B, and I and 25 μm in the others. Abbreviations: DAPI, 4,6-diamidino-2-phenylindole; GABA, γ-aminobutyric acid; GFAP, glial fibrilliary acidic protein; hESC, human embryonic stem cell; TH, tyrosine hydroxylase.

logical observations and immunostaining results. The semi-quantitative RT-PCR showed a time-dependent reduction in expression of ESC markers, including Oct4, hTERT, and nanog (Figs. 4A, 4B), and the reduction of Oct4 was slower than hTERT and nanog. Furthermore, the reduction in expression of ESC markers was associated with increased expression of neural progenitor markers, such as Sox1, nestin, Pax6, and the neuronal marker MAP2 (Figs. 4A, 4C). The glial marker GFAP, however, was not detected, which corresponded to the immunostaining results. The mesodermal and endodermal markers AFP, GATA6, and brachyury showed almost undetectable expression without consistent increase or decrease during differentiation (Fig. 4A). Pax6 antibody staining was observed by the second week of differentiation and became more homogenous along the progress of differentiation (Fig. 4D, upper panel). No clear Sox1 antibody staining was observed until after 4 weeks of differentiation (Fig. 4D, lower panel). The sequence of Pax6 and Sox1 expression is similar to the neural differentiation with multicellular aggregates [20]. Musashi showed a similar timeline as Sox1 (Fig. 6C). Together, these results suggest that hESCs cultured with this serum-free adherent protocol mainly differentiated into the neural lineage, particularly neuronal cells.

Most neurons generated with this differentiation protocol by passage 5 (~60 days in differentiation) are immunopositive for γ-aminobutyric acid (GABA) (Fig. 6G) and GAD65, an enzyme required for GABA synthesis (Fig. 6H), with very few TH-positive neurons. No glutamatergic neurons were detected, as indicated by negative staining with GluRI and GLAST antibodies. However, exposing the cells to SHH, FGF8, and ascorbic acid for 1–2 weeks at passages 4 through 5 of differentiation (~40–50 days of differentiation) followed by treatment with BDNF, GDNF, ascorbic acid, and laminin for another 1–2 weeks resulted in a significant increase in the numbers of TH-positive neurons (Fig. 6I). These results indicate that the hESC-derived neural progenitors with this protocol have the potential for terminal differentiation into other neuronal types other than GABA neurons if optimal culture conditions for a particular neuronal type are provided. In the current protocol, neither GFAP-positive nor gal C–positive and O4-positive cells were detected by passage 5. However, GFAP-positive cells were detectable after long-term culturing (>80 days).

Discussion

The main finding of this study is the development of an adherent culture system using either matrigel or laminin as matrix. This culture system can efficiently differentiate hESCs to neural fate, particularly neuronal progenitors, without using multicellular aggregates or coculture. The progenitors generated by this method can be further differentiated to dopaminergic neurons, GABA neurons, and possibly other neural cell types if correct culture conditions are provided.

Mouse ESCs have been successfully converted to neuroectodermal precursors in adherent monoculture [5]. However, due to the differences between mouse ESCs and hESCs, we failed to differentiate hESCs efficiently into neural lineage using the same protocol. First, mouse ESCs can grow in culture after disassociation into single cells and seeding at low density, whereas hESCs are prone to spontaneous differentiation into flat or fibroblast-like cells if plated at low density [6, 9], indicating that hESCs require cell-cell contacts to control their fate. Second, there is no problem to culture mouse ESCs in gelatin-coated culture plastics, whereas hESCs are unable to efficiently attach in these dishes. This may reflect the differences of cell-surface molecules, including adhesion molecules, between these two ESCs. For example, mouse ESCs express SSEA1 cell-surface marker, but hESCs express SSEA3 and SSEA4. Therefore, instead of gelatin, matrigel and poly-lysine/laminin were used. Similar to mouse ESCs, maybe even more prevalent, hESCs produced large numbers of flattened cells when plated on matrigel or laminin. These flat cells were positive for GATA6 and AFP, indicating that they are extraembryonic endoderm [21, 22]. Addition of BMP antagonist noggin but not follistatin into the culture medium of hESCs effectively blocked the extraembryonic endoderm formation and promoted neural development. Such an effect of noggin has been observed in *Xenopus laevis* [23].

The neural progenitors generated in this study were mainly neuronal precursors. There were no detectable GFAP- and O4-positive cells until late passage in long-term cultures. These results differ from the neural progenitors produced by multicellular aggregates in the presence of serum. In the latter culture system, GFAP-positive glial cells are visible in less than 1 month of differentiation [2, 24]. This could be due to the application of BMP antagonist because BMP has been indicated as an important factor for glial cell formation [25]. The addition of noggin into the medium might have blocked the BMP signaling and inhibited the glial cell differentiation; withdrawing noggin at passage 3 or 4 may permit glial development in the long-term culture. It is also possible that cells in multicellular aggregates are at various developmental stages due to their complex microenvironments; some cells are more developmentally advanced than others, so they may develop into glial cells when most of the cells are still neuronal cells.

The adherent culture protocol facilitates visualization of the process of neural conversion. This process seemed to recapitulate early steps of the nervous system development in vivo in that undifferentiated ESCs become more compact and then neural tube–like structures are formed. This could provide an experimental tool to study factors affecting human neural tube formation under controlled conditions. We have used this system to investigate *Oct4* expression during neural differentiation and found that *Oct4* expression is temporally retained before down-regulation to become neural progenitor type of cells. The cells that

lost *Oct4* expression rapidly did not commit to neural fate, instead turning into the flattened extraembryonic cells. Our observations support previous findings that forced rapid downregulation of Oct4 in ESCs pushes them into extraembryonic cells [26, 27]. Our results and others [4, 19] suggest that transiently sustained levels of *Oct4* expression may be required for in vitro differentiation of human ESCs into neural lineages because rapid downregulation of *Oct4* expression in ESCs might have promoted the formation of primitive endoderm.

Understanding how to direct hESCs toward a specific lineage pathway and generate appropriate cell types robustly is very important not only for the study of developmental biology but also for potentially using these cells to treat human diseases. The results reported here provide a simple and relatively defined system for differentiation of hESCs to neural lineages, particularly neurons. This system will be useful to study factors for the determination of neural and other lineages.

Acknowledgments

We would like to dedicate this paper to Prof. John Clark in memory of his support to this work. We also thank Diana Wylie for her technical assistance and the other members of the laboratory. This work was supported by the Biotechnology and Biological Science Research Council and the Geron Corporation.

Disclosures

The authors indicate no potential conflicts of interest.

References

1 Thomson JA, Itskovitz-Eldor J, Shapiro SS et al. Embryonic stem cell lines derived from human blastocysts. Science 1998;282:1145–1147.

2 Zhang SC, Wernig M, Duncan ID et al. In vitro differentiation of transplantable neural precursors from human embryonic stem cells. Nat Biotechnol 2001;19:1129–1133.

3 Carpenter MK, Inokuma MS, Denham J et al. Enrichment of neurons and neural precursors from human embryonic stem cells. Exp Neurol 2001;172:383–397.

4 Perrier AL, Tabar V, Barberi T et al. Derivation of midbrain dopamine neurons from human embryonic stem cells. Proc Natl Acad Sci U S A 2004;101:12543–12548.

5 Ying QL, Stavridis M, Griffiths D et al. Conversion of embryonic stem cells into neuroectodermal precursors in adherent monoculture. Nat Biotechnol 2003;21:183–186.

6 Xu C, Inokuma MS, Denham J et al. Feeder-free growth of undifferentiated human embryonic stem cells. Nat Biotechnol 2001;19:971–974.

7 Lee SH, Lumelsky N, Studer L et al. Efficient generation of midbrain and hindbrain neurons from mouse embryonic stem cells. Nat Biotechnol 2000;18:675–679.

8 Cui W, Aslam S, Fletcher J et al. Stabilization of telomere length and karyotypic stability are directly correlated with the level of hTERT gene expression in primary fibroblasts. J Biol Chem 2002;277:38531–38539.

9 Pera MF, Andrade J, Houssami S et al. Regulation of human embryonic stem cell differentiation by BMP-2 and its antagonist noggin. J Cell Sci 2004;117:1269–1280.

10 Morrisey EE, Tang Z, Sigrist K et al. GATA6 regulates HNF4 and is required for differentiation of visceral endoderm in the mouse embryo. Genes Dev 1998;12:3579–3590.

11 Koutsourakis M, Langeveld A, Patient R et al. The transcription factor GATA6 is essential for early extraembryonic development. Development 1999;126:723–732.

12 Fujikura J, Yamato E, Yonemura S et al. Differentiation of embryonic stem cells is induced by GATA factors. Genes Dev 2002;16:784–789.

13 Tropepe V, Hitoshi S, Sirard C et al. Direct neural fate specification from embryonic stem cells: a primitive mammalian neural stem cell stage acquired through a default mechanism. Neuron 2001;30:65–78.

14 Balemans W, Van HW. Extracellular regulation of BMP signaling in vertebrates: a cocktail of modulators. Dev Biol 2002;250:231–250.

15 Bachiller D, Klingensmith J, Kemp C et al. The organizer factors Chordin and Noggin are required for mouse forebrain development. Nature 2000;403:658–661.

16 Wang XP, Suomalainen M, Jorgez CJ et al. Follistatin regulates enamel patterning in mouse incisors by asymmetrically inhibiting BMP signaling and ameloblast differentiation. Dev Cell 2004;7:719–730.

17 Gerrard G, Zhao D, Clark AJ et al. Stably transfected human embryonic stem cell clones express OCT4-specific green fluorescent protein and maintain self-renewal and pluripotency. STEM CELLS 2005;23:124–133.

18 Miyazono K, Miyazawa K. Id: a target of BMP signaling. Sci STKE 2002;E40.

19 Shimozaki K, Nakashima K, Niwa H et al. Involvement of Oct3/4 in the enhancement of neuronal differentiation of ES cells in neurogenesis-inducing cultures. Development 2003;130:2505–2512.

20 Li XJ, Du ZW, Zarnowska ED et al. Specification of motoneurons from human embryonic stem cells. Nat Biotechnol 2005;23:215–221.

21 Morrisey EE, Ip HS, Lu MM et al. GATA-6: a zinc finger transcription factor that is expressed in multiple cell lineages derived from lateral mesoderm. Dev Biol 1996;177:309–322.

22 Krumlauf R, Hammer RE, Tilghman SM et al. Developmental regulation of alpha-fetoprotein genes in transgenic mice. Mol Cell Biol 1985;5:1639–1648.

23 Munoz-Sanjuan I, Brivanlou AH. Neural induction, the default model and embryonic stem cells. Nat Rev Neurosci 2002;3:271–280.

24 Reubinoff BE, Itsykson P, Turetsky T et al. Neural progenitors from human embryonic stem cells. Nat Biotechnol 2001;19:1134–1140.

25 Rajan P, Panchision DM, Newell LF et al. BMPs signal alternately through a SMAD or FRAP-STAT pathway to regulate fate choice in CNS stem cells. J Cell Biol 2003;161:911–921.

26 Niwa H, Miyazaki J, Smith AG. Quantitative expression of Oct-3/4 defines differentiation, dedifferentiation or self-renewal of ES cells. Nat Genet 2000;24:372–376.

27 Hay DC, Sutherland L, Clark J et al. Oct-4 knockdown induces similar patterns of endoderm and trophoblast differentiation markers in human and mouse embryonic stem cells. STEM CELLS 2004;22:225–235.