

ORIGINAL ARTICLE

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Human neural progenitor cells derived from embryonic stem cells in feeder-free cultures

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Abstract Derivation of human neural progenitors (hNP) from human embryonic stem (hES) cells in culture has been reported with the use of feeder cells or conditioned media. This introduces undefined components into the system, limiting the ability to precisely investigate the requirement for factors that control the process. Also, the use of feeder cells of non-human origin introduces the potential for zoonotic transmission, limiting its clinical usefulness. Here we report a feeder-free system to produce hNP from hES cells and test the effects of various media components involved in the process. Five protocols using defined media components were compared for efficiency of hNP generation. Based on this analysis, we discuss the role of basic fibroblast growth factor (FGF2), N2 supplement, non-essential amino acids (NEAA), and knock-out serum replacement (KSR) on the process of hNP generation. All protocols led to down-regulation of Oct4/POU5F1 expression (from 90.5% to <3%), and up-regulation of neural progenitor markers to varying degrees. Media with N2 but not KSR and NEAA produced cultures with significantly higher ($p < 0.05$) expression of the neural progenitor marker Musashi 1 (MSI1). Approximately 89% of these cells were Nestin (NES)+ after 3 weeks, but they did not proliferate. In contrast, differentiation media supplemented with KSR and NEAA

produced fewer NES+ (75%) cells, but these cells were proliferative, and by five passages the culture consisted of >97% NES+ cells. This suggests that KSR and NEAA supplements did not enhance early differentiation but did promote proliferating of hNP cell cultures. This resulted in an efficient, robust, repeatable differentiation system suitable for generating large populations of hNP cells. This will facilitate further study of molecular and biochemical mechanisms in early human neural differentiation and potentially produce uniform neuronal cells for therapeutic uses without concern of zoonotic transmission from feeder layers.

Key words human embryonic stem cells · neural differentiation · feeder-free culture

Introduction

Human neural stem or progenitor (hNP) cells have been derived from human embryonic stem (hES) cells using both spontaneous and directed differentiation protocols (Schulz et al., 2003; Gerrard et al., 2005; Shin et al., 2006; Nat et al., 2007). For spontaneous differentiation, ES cells were allowed to form an aggregated mass of differentiated cells known as an embryoid body (EB). These EBs contained cells of all three germ layers, including neuronal cells of ectodermal lineage. However, in this spontaneous process only about 0.2% of the differentiating ES cells formed hNP cells (Tropepe et al., 2001). Therefore, spontaneous differentiation is inefficient and leads to heterogeneous populations of cells, many of which are non-neural.

Higher efficiencies in neural/neuronal differentiation were achieved when hES cells were exposed to morphogens like retinoic acid (Schuldiner et al., 2001), conditioned medium (Schulz et al., 2003; Shin et al., 2006), stromal

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cells as supporting feeder (Perrier et al., 2004), and bone morphogenetic protein (BMP) inhibitors (Gerrard et al., 2005; Itsykson et al., 2005). Shin et al. (2006) derived hNP cells using an initial mouse embryonic fibroblasts (MEF) feeder step while Gerrard et al. (2005) generated hNP cells by blocking BMP signalling in a feeder-less culture system. Previous reports also suggested that serum (Krathwohl and Kaiser, 2004), N2 (Bottenstein and Sato, 1979; Garcia-Castro et al., 2002), basic fibroblast growth factor (FGF2) (Kitchens et al., 1994; Kuhn et al., 1997; Mayor et al., 1997; Xu et al., 1997; Lillien and Raphael, 2000; Tureyen et al., 2005), and non-essential amino acid (NEAA) (Miranda-Contreras et al., 2002; Hamdane et al., 2003; Aharoni et al., 2005) play significant roles during neural differentiation and maintenance.

Neural progenitors are often characterized *in vitro* by either gene expression, or immunocytochemistry for various markers such as Nestin (NES), Musashi 1 (MSI1), SOX1, SOX2, SOX3, and polysialylated neural cell adhesion molecule (Schulz et al., 2003; Ellis et al., 2004; Gerrard et al., 2005; Shin et al., 2006). We studied the effect of various media components on hNP cell derivation with the goal to identify an inhibitor- and feeder-free system that could generate uniform populations of hNP cells capable of further differentiation to neuronal phenotypes. This defined system could then be used to investigate the differentiation pathways to uniform neural stem cell formation using biochemical and molecular approaches.

Methods

ES cell culture

The work was performed with NIH approved karyotypically normal WA09 (WiCell Research Institute) cells and further verified in the BG02 (Bresagen, Athens, GA) cell line. All tissue culture ingredients were purchased from Gibco (Grand Island, NY), unless mentioned otherwise. BG02 ES cells were maintained and manually subcultured on MEF as described previously (Mitalipova et al., 2003). WA09 ES cells were cultured in a similar fashion using a different medium, called 20% knock-out serum replacement (KSR) ES medium (DMEM/F12 supplemented with 20% KSR, 1% NEAA, 1 mM L-glutamine, 4 ng/ml FGF2, 1 × penicillin-streptomycin (Pen-Strep), and 0.1 M β-mercaptoethanol). Before starting the neural progenitor derivation, to remove MEF from the system, we propagated ES cells manually for four to five passages on laminin (1 μg/cm², Sigma, St. Louis, MO) coated 35 mm tissue culture dishes (Falcon BD Biosciences, San Jose, CA). The culture was supported by conditioned ES medium prepared by exposing ES medium to MEF cells for 24 hr.

Neural progenitor derivation and propagation

Derivation and propagation of hNP cells were done on laminin (1 μg/cm²) coated 35 mm tissue culture dishes, if not described otherwise. We tested five different derivation protocols (each 21 days in duration) for their ability to generate feeder-free hNP cells. ES cells were trypsinized (0.25% trypsin in EDTA) and about

Table 1 Definition of differentiation protocols for derivation of hNP cells from human ES cells

Protocol	Days 1–7 (Stage I)	Days 8–14 (Stage II)	Days 15–21 (Stage III)
Protocol 1	Medium 1	Medium 2	Proliferation medium
Protocol 2	Medium 1	Medium 1	Proliferation medium
Protocol 3	Medium 2	Medium 2	Proliferation medium
Protocol 4	Medium 1+N2(1 ×)	Medium 2	Proliferation medium
Protocol 5	Medium 1+N2(1 ×)+ FGF2 (4 ng/ml)	Medium 2	Proliferation medium

hNP, human neural progenitors; FGF2, fibroblast growth factor 2.

3×10^5 ES cells were plated without feeder cells in ES medium. After 24 hr (Day 1), the ES medium was changed to the appropriate medium defined for each protocol (Table 1). During the initial 2 weeks of differentiation, each protocol used either Medium 1 (DMEM/F12 supplemented with 20% KSR, 1% NEAAs, 1 mM L-glutamine, and 1 × Pen-Strep but no FGF2) or Medium 2 (DMEM/F12 with 1 × N2, 4 ng/ml FGF2, 2 mM L-glutamine, and 1 × Pen-Strep) or variation of these media containing different amounts of FGF2 and N2 supplement. In each protocol, cells were expanded in neural proliferation medium (neurobasal medium supplemented with 1 × Pen-Strep and 1 × B27, 2 mM L-glutamine, 20 ng/ml FGF2, and 10 ng/ml LIF) for the last 7 days. Medium was changed daily during the entire 3 weeks. After 3 weeks of differentiation, hNP cells were sub-cultured with neural proliferation medium (Fig. 1, Table 1).

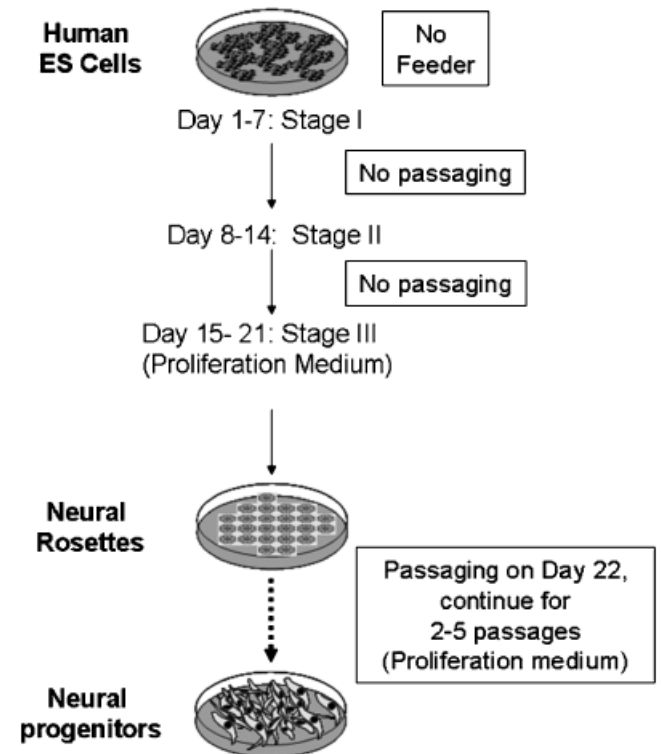


Fig. 1 Schematic diagram of neural progenitor derivation from human embryonic stem cells. Five protocols were used with each one consisting of three stages (I, II, and III), namely, days 1–7, days 8–14, and days 15–21. For each protocol we used partially defined media described in Table 1. Cells were re-plated only after 21 days of differentiation for another two to five passages.

Differentiation of neural progenitors

To examine the differentiation potential, hNP cells were plated on laminin coated eight-chamber Permax slides (Nalgene Nunc International, Rochester, NY) as described above. They were grown for 14–21 days in proliferation medium lacking FGF2 and LIF to promote random differentiation. For differentiation to astroglial cells (astrocytes and oligodendrocytes), hNP cells were grown for 2 weeks in proliferation medium supplemented with ciliary neurotrophic factor (CNTF) (10 ng/ml, Sigma).

Immuno-fluorescence staining

Cells were grown on slides, fixed in 4% paraformaldehyde (Sigma-Aldrich) and stained with NES (1:100, NeuroMics, Edina, MN), Oct4 (1:200, Santa Cruz Biotechnology, Santa Cruz, CA), SOX2 (1:200, R&D Systems, Minneapolis, MN), MS11 (1:100, NeuroMics), Tuj1 (1:500, NeuroMics), GFAP (1:500, Sigma), and myelin basic protein (MBP) (1:200, Chemicon, Billerica, MA) antibodies with appropriate fluorescent secondary antibodies (1:1,000 Molecular Probes-Invitrogen, Carlsbad, CA). Images were acquired using a Nikon inverted fluorescence microscope (Nikon, Melville, NY) and Image Pro Plus software (Media Cybernetics, Bethesda, MD).

Flow cytometry analysis

After 21 days of hES differentiation (Table 1), cells were used for flow cytometry or expanded two to five more passages before additional flow analysis. For preparing single cell suspensions from adherent culture, cells were minimally trypsinized (0.25% Trypsin in EDTA, neutralized by 3% BSA in PBS), followed by vigorous pipetting, then fixed, and stained as described (Shin et al., 2006). Flow cytometry was performed using FACS Calibur system (Becton Dickinson-BD Biosciences, San Jose, CA) and the data analyzed using FlowJo software (Tree Star, Ashland, OR). Forward- and side-scatter plots were used to exclude dead cells and debris from the histogram analysis. For this experiment, we used the following antibodies: NES (conjugated with PE, R&D 1:200); CD146 (conjugated with PE, Pharmingen-BD Biosciences, San Jose, CA, 1:50); α -fetoprotein (AFP, Santa Cruz Biotechnology, 1:100), Oct4 (Santa Cruz, 1:200), and Tra-1-60 (Chemicon, 1:200) detected by Alexafluor 488 fluorescent dye tagged secondary antibodies (Molecular Probes).

RNA isolation and real-time reverse-transcription polymerase chain reaction (RT-PCR) for gene expression analysis

RNA was isolated from cells after 21 days of differentiation by each of five protocols using RNeasy kit (Qiagen, Valencia, CA) and quantified using an RNA 600 Nano Assay by the Agilent 100 Bio-analyzer (Agilent Technologies, Santa Clara, CA). cDNA was synthesized from 1 μ g of RNA using Archive Kit (Applied Biosystems, Foster City, CA). cDNAs were subjected to gene expression analysis using quantitative real-time RT-PCR. In order to analyze expression of several genes at a time, we used a customized Taqman™ pre-designed gene array (Applied Biosystems) containing 47 target genes and 18S ribosomal RNA internal control. PCR was performed in ABI HT7900 system and data was acquired using SDS 2.2.1 software (Applied Biosystems). Those target genes covered markers of pluripotency and of all three germ lines, namely, ectoderm, endoderm, and mesoderm. Analysis was performed as previously described (Tibbitts et al., 2006) and briefly discussed under data procurement and statistical analysis below. We compared gene expression in cells generated by the different protocols and normalized this to gene expression levels in undifferentiated hES cells.

Procurement of gene expression data and statistical analysis

Gene expression data (three replications) were acquired and SDS software was used to estimate relative fold change values using $\Delta\Delta C_t$ quantitation method. We used endogenous 18S ribosomal RNA for normalization and calibrated gene expression for each sample against undifferentiated hES cells. Individual expression data was subjected to statistical analysis using a suitable linear model considering treatments or factors in medium as independent variables. Analysis of variance (ANOVA) was used to identify the significant effect from the model and then means were compared and grouped using Tukey's test at 5% level of significance. All analyses were performed using GLM procedure by SAS 8.01 (SAS Institute, Cary, NC).

Results

To find the optimal method of hNP derivation from hES cells in an adherent culture system, we compared five different protocols which helped to identify combinations of factors leading to high percentages of hNPs. We evaluated the efficiency of our protocols using flow cytometry, real-time RT-PCR, and immunostaining.

Flow cytometry quantification of cultures for pluripotent, neural and non-neural phenotypes

To identify the lineage of differentiated cells by flow cytometry at day 21, we tested for expression of the following proteins: NES (a neural progenitor marker) and other non-neural lineage markers CD146 (pan-endothelial marker, Bardin et al., 2001), AFP (extra-embryonic endoderm marker, Dziadek and Adamson, 1978; Thomson et al., 1998), and two common ES cell markers, Oct4 (nuclear), and Tra-1-60 (cell surface) (Thomson et al., 1998).

The results of flow cytometry experiments are presented in Table 2. In comparison with the hES cells and regardless of protocol, the percentage of NES expressing cells increased while there was a decrease in the cells expressing pluripotent markers Oct4 and Tra-1-60. The number of CD146 and AFP expressing cells did not change. Protocol-specific changes were as

Table 2 Flow cytometry of differentiated putative hNP cells examined for expression of Nestin, CD146, α -Fetoprotein (AFP), Oct4, and Tra-1-60

Protocol/gene ¹	Nestin	CD146	AFP	Oct4	Tra-1-60
Protocol 1	74.50	1.65	1.50	8.77	5.81
Protocol 2	69.50	1.93	1.80	11.20	5.34
Protocol 3	88.70	0.44	1.07	8.40	3.43
Protocol 4	72.60	1.94	2.39	11.50	7.04
Protocol 5	67.70	5.46	2.90	13.90	5.70

¹Percent of cells expressing the gene for each derivation protocol (WA09 ES, HUVEC, and HepG2 cells used as control). hNP, human neural progenitors.

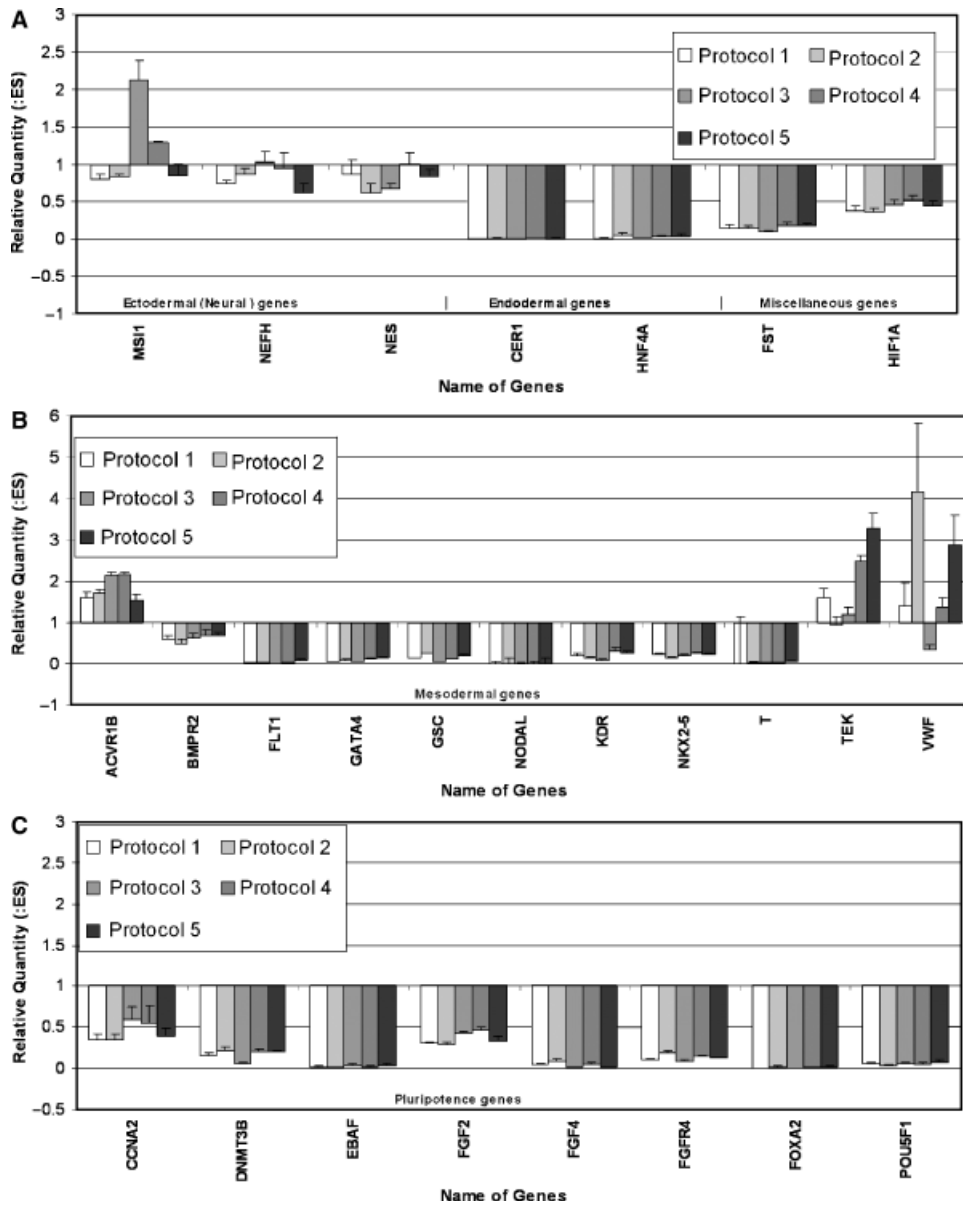


Fig.2 Gene expression profile of differentiation by five protocols. Human embryonic stem (hES) cells were differentiated to produce neural progenitors by five different protocols. Quantitative real-time reverse-transcription polymerase chain reaction was performed on RNA samples collected at day 21 of differentiation (three replications). Twenty six genes were identified to have significantly different levels of expression in comparison to undifferentiated ES cells. Bar diagrams with standard errors represent means of gene expression for each protocol measured as fold change relative to undifferentiated hES cells. Diagrams are grouped according to lineage: (A) neural (ectodermal), endodermal, and miscellaneous genes; (B) mesodermal genes; and (C) pluripotency genes.

follows: percentage of cells expressing Oct4 ranged from 8.4% (in Protocol 3) to 13.9% (in Protocol 5), whereas the other pluripotency marker Tra-1-60 ranged between 3.43% (Protocol 3) and 7.04% (Protocol 4). In comparison, undifferentiated hES cell cultures were highly positive for pluripotent markers 90.5% (Oct4) and 77.8% (Tra-1-60). However, about 5% or less of cells expressed AFP or CD146 markers at day 21. For each of the protocols, a majority of the cells expressed NES, ranging from 68% (Protocol 5) to 89% (Protocol 3) after 21 days of differentiation. This suggests that with our protocols, 21 days treatment was sufficient to generate predominantly differentiated cell populations and depending upon the protocol, we obtained varying efficiency for generating cells of a potentially neural lineage.

Real-time RT-PCR quantification of cultures for pluripotent, neural and non-neural genes

At the end of 21 days we also studied the relative abundance of RNA transcripts (three embryonic germ layers and of pluripotency) in cells differentiated by each of these five protocols. Running real-time RT-PCR we calculated the relative quantity of expression (with respect to undifferentiated WA09 hES cells) of each of the 47 target genes. The expression of 26 genes was significantly (minimum at $p \leq 0.05$ level) different than undifferentiated hES cells (Fig. 2 and Supplementary Table). For 14 genes, there was no change in expression level whereas for the remaining seven genes, expression level was highly variable from

experiment to experiment and hence data was not further analyzed.

Six genes were up-regulated in at least one of the five protocols (Supplementary Table and Fig. 2), MS11 ($p < 0.0003$), NEFH ($p < 0.011$), NES ($p < 0.011$) (all three neuronal) (Fig. 2A), ACVR1B ($p < 0.0009$), TEK ($p < 0.0003$), and VWF ($p < 0.005$) (all mesodermal) (Fig. 2B). The only gene up-regulated in all protocols was ACVR1B. Out of three neural markers, MS11 was up-regulated more than twofold only in Protocol 3. Although overall effect was significant for both NES (hNP marker) and NEFH (late neuronal marker), RT-PCR did not detect significant up-regulation compared with expression-levels in undifferentiated hES cells. Endothelial markers (mesodermal lineage), VWF and TEK were expressed in relatively higher quantity in all protocols except for Protocol 3 (Fig. 2B). In this protocol VWF was down-regulated and TEK expression did not change in comparison with hES cell expression level.

The genes that were significantly ($p < 0.0001$) down-regulated in all five protocols include DNMT3B, EBAF, FGF2, FGF4, FGFR4, FOXA2, and POU5F1 (all pluripotent markers; Fig. 2C); CER1 and HNF4A (both endodermal markers; Fig. 2A); FLT1, GATA4, GSC, KDR, NKX2.5, NODAL, T (all mesodermal markers; Fig. 2B); and FST and HIF1A (both classified under miscellaneous genes; Fig. 2A; Supplementary Table). The mesodermal gene BMPR2 ($p < 0.05$ in Protocol 2) and pluripotency gene CCNA2 ($p < 0.01$ in Protocols 1, 2, and 5) were also down-regulated.

As mentioned before, expression of fourteen genes did not change amongst the hNP derivation protocols. The list includes two pluripotency genes SALL2 (EGFR), SOX2 (also marker of hNP cells), AFP (extra-embryonic endoderm gene), FGF5, FN1 (both ectodermal genes), NOS3, ACVR1C, PECAM1 (CD31), CDH5, CD34, RUNX1, VEGF (mesodermal genes); EPO and TGFB1 (miscellaneous group). Because of high variability, seven genes (CD45, BMP4, GATA3, HEY1, SMAD1, SMAD2, and SMAD3) were excluded from the final analysis.

In the five different protocols, we evaluated the effects of media components on hES cells for deriving neural progenitors by comparing the expression of three neural genes, MS11, NES and, NEFH. KSR, NEAA, and N2 did not affect expression of NES and NEFH genes (data not shown) measured by real-time RT-PCR; however, MS11 expression was significantly affected by these medium components (Fig. 3). Absence of KSR and NEAA from the media independently showed a more than twofold increase in MS11 expression with respect to hES cells ($p < 0.0001$). N2 in culture medium for the first 14 days also significantly ($p < 0.03$) increased the MS11 expression, though not as dramatically as the lack of KSR and NEAA. Surprisingly, FGF2, independent of the other components, did not significantly

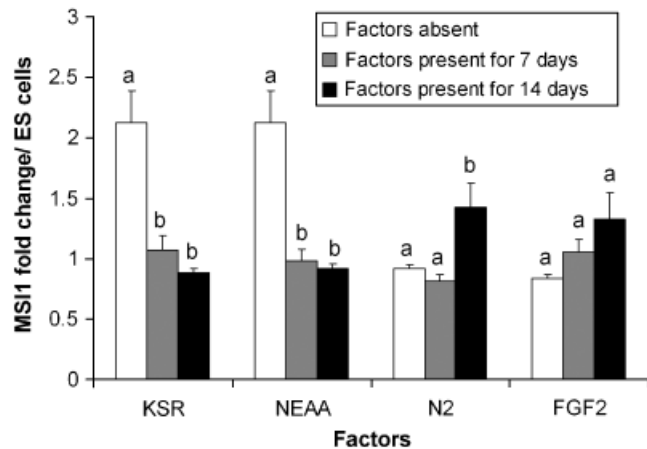


Fig. 3 Effects of media components (knock-out serum replacement, non-essential amino acids, N2, and fibroblast growth factor 2) on Musashi 1 (MS11) expression. MS11 level was quantified by real-time reverse-transcription polymerase chain reaction in hES cells differentiated (for 21 days) by medium with each of these factors present for varying periods (0, 7, and 14 days). Presented in bar diagrams are the means of fold change relative to hES cell expression of MS11. Significant factors ($p < 0.05$) are indicated by different letters above each bar.

affect NES, NEFH (data not shown), or MS11 expression during this derivation process (Fig. 3).

Temporal changes in phenotype during differentiation

As the cultures became more dense, neural-rosettes (neuroepithelial cells arranged radially about a center, resembling the cross section of a neural tube in a developing embryo) were formed in all five protocols (Figs. 4A,4B). Rosettes appeared earliest in Protocol 3, on Day 7. In this protocol, hES cells were exposed to both N2 and FGF2 7 days earlier than the other protocols. Protocol 1 also produced a large number of rosettes but they appeared later, around 10–12 days of differentiation (that is, 2–5 days after exposure to FGF2 and N2). In all protocols, cells within the rosettes were positive for NES (Fig. 4B). Sub-cultured rosette cells were positive for both NES (Fig. 4C) and MS11 (Fig. 4D) suggesting the cells were neural progenitors. These results held true not only for WA09 cells, but also for another hES line, BG02. We found that BG02 responded to all differentiation protocols as did WA09 and produced hNP cells in the form of rosettes expressing NES (Fig. 4B).

Propagation increases in NES+ cells

Based on the flow cytometry results, Protocols 1 and 3 were the most efficient in producing NES+ hNP cells. These hNP cells derived by both protocols survived (without passage) in culture for more than 60 days. The cells derived by Protocol 1 were propagated for an

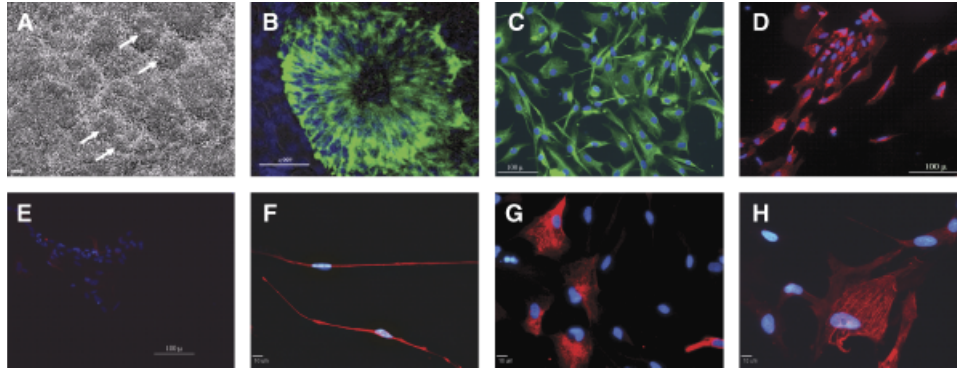


Fig. 4 Immunostaining of human neural progenitors before and after differentiation. The culture was highly homogenous with neural rosettes. (A) Neural rosettes (white arrows, bright field) from WA09 cells. A similar result also obtained with BG02 cell line. (B) Shown here, a neural rosette stained with Nestin (NES) (green) antibody. Propagated Neural progenitors showed

additional 30–40 days (~ 10 – 12 passages) whereas for Protocol 3, very few cells were cultured beyond 10–15 days (~ 3 – 4 passages) under these culture conditions. For this reason, we limited further characterization of the hNP to Protocol 1-derived cells. The percentages of NES+ cells in three independently derived hNP cell lines using Protocol 1 were determined (Fig. 5). After five passages, two lines were 99.6% and 97.4% NES+ while at passage 2, the third line was 98.1%. This was also accompanied by further suppression of Oct4 from an initial $\sim 9\%$ (Table 2: Protocol 1) to $<1.6\%$ of cells after five passages (Fig. 5A). Within the cell population that underwent fewer passages, there existed a relatively higher percentage of

expression of marker genes, NES (C) and Musashi 1 (D) but not SOX2 (E). Further differentiation produced neurons (Tuj1) (F), astrocytes (GFAP) (G) and oligodendrocytes (myelin basic protein) (H) (lower panel). DAPI (blue) was used for staining the nuclei (scale bar for (A) through (E) is $100\ \mu\text{m}$ and for the remaining figures $10\ \mu\text{m}$).

Oct4+ cells (e.g., line 5, p2, Fig. 5A). The histogram generated by this data also showed the overall intensity of NES expression increased in the hNP lines with respect to renal cells, and as expected the Oct4 intensity decreased compared with hES cells (Fig. 5B). All hNP cells derived by Protocol 1 expressed NES (Fig. 4C) and MSI1 (Fig. 4D) but did not express transcription factor SOX2 (Fig. 4E) or POU5F1 (Fig. 2C).

Properties of hNP cells

(a) *Differentiation of hNP cells produced neurons, astrocytes and oligodendrocytes:* In order to test the

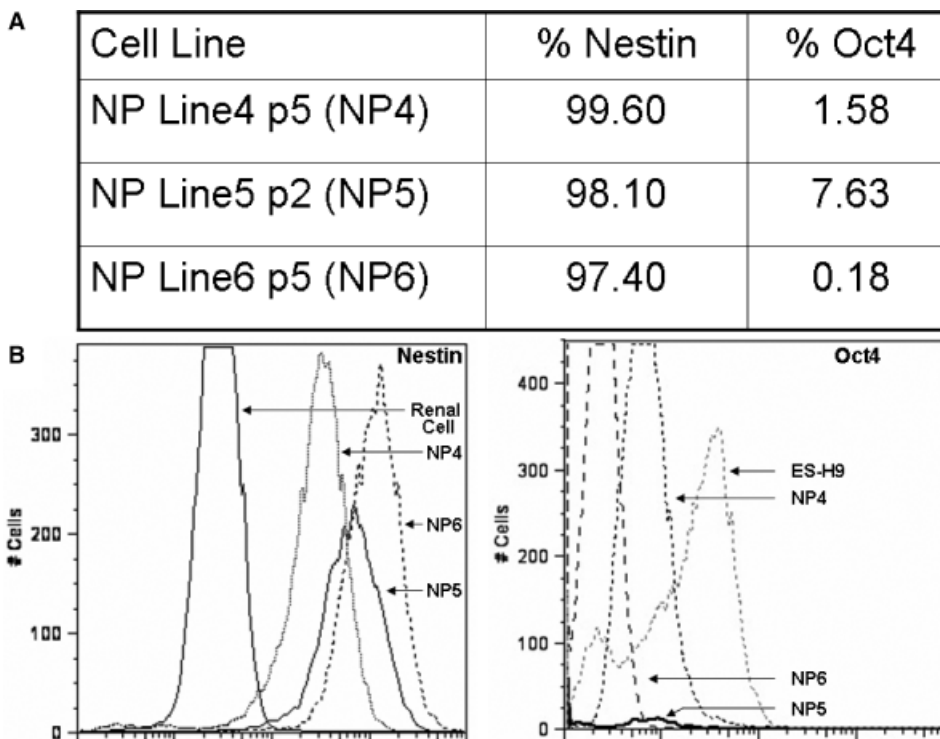


Fig. 5 Flow cytometry quantifying Nestin (NES) and Oct4 expression in human neural progenitors (hNP). (A) NES and Oct4 expressing cells (in percent). Three cell lines (NP Line 4, 5, and 6) derived independently by Protocol 1 expressed $>97\%$ NES and had reduced Oct4 expression (less than 8%) after two to five passages. (B) The same data shown in histogram; human embryonic stem cells (WA09) and renal cells used as control. The x -axis represents the intensity of marker expression and y -axis shows the number of cells at a specific intensity.

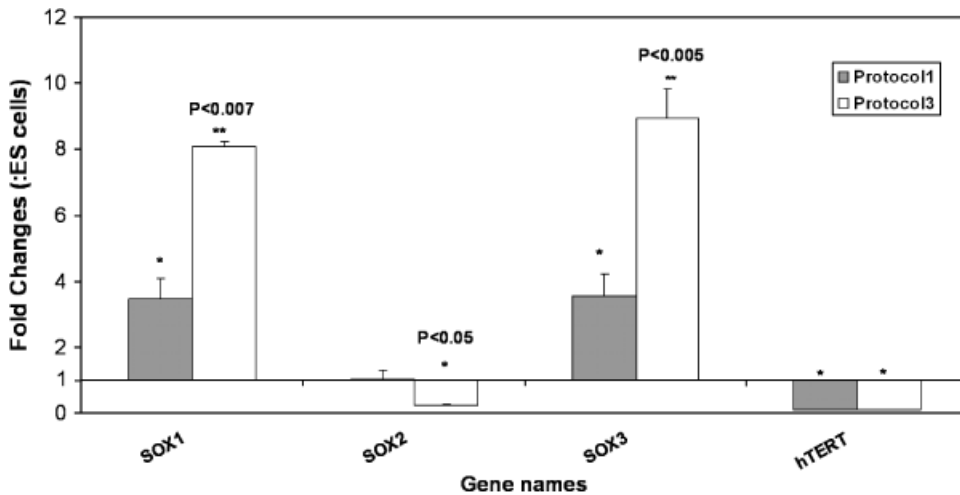


Fig. 6 Expression of SOX1, SOX2, SOX3, and human telomerase reverse transcription (hTERT) in derivation culture. Expression of SOX genes and hTERT in Protocols 1 and 3 derivation cultures (after 21 days of differentiation, $n = 3$) was quantified by real-time polymerase chain reaction and fold changes were normalized to WA09 human embryonic stem (hES) cells baseline gene expression. Protocol 3-derived cells show higher levels of SOX1 and SOX3 (but lower SOX2) expression than cells derived by Protocol 1 (*or** indicate level of significance compared to undifferentiated WA09 ES cells at $p < 0.05$ and < 0.01 , respectively). Numerical probability values indicate significance level between Protocols 1 and 3).

differentiation potential of hNP cells derived by Protocol 1, we sub-cultured them in proliferation medium lacking FGF2 and LIF. After 2 weeks of differentiation, hNP cells exhibited neuronal morphology and positively stained with Tuj1 (β III tubulin, neuronal marker) (Fig. 4F). In the same culture, we failed to detect any cell expressing O1 (late oligodendrocyte progenitor marker), O4 (marker of both types I and II pro-oligodendrocytes but not of O-2A-progenitor cells), or MBP (marker of oligodendrocytes) (data not shown). However, when hNP cells were differentiated in the presence of CNTF for 2 weeks, in addition to Tuj1, we detected glial fibrillary acidic protein (GFAP, marker of astrocytes) and MBP (Figs. 4G,4H).

(b) *SOX2 is down-regulated in cells by Protocol 3 only, whereas human telomerase reverse transcription (hTERT) is down-regulated in both Protocols 1 and 3:* Because SOX1B members are known to express in NP cells, using quantitative real-time RT-PCR we determined the relative expression of SOX1, SOX2, and SOX3. In addition, we also quantified hTERT (catalytic unit, marker for proliferative cells) expression in hNP cells derived by both Protocols 1 and 3. When compared with expression levels in hES cells, both SOX1 and SOX3 transcriptions were up-regulated in a similar way in Protocol 1 (both genes by ~ 3.5 -folds; $p < 0.05$) and Protocol 3 (eightfold in SOX1 and ninefold in SOX3; $p < 0.01$). Expression levels of both genes were significantly higher in Protocol 3 ($p < 0.007$ for SOX1 and $p < 0.005$ for SOX3) than in Protocol 1. Although protein was not detected by immunostaining, we found a similar level of SOX2 transcripts in both Protocol 1-derived hNP and undifferentiated hES cells. However, in cells derived by Protocol 3, SOX2 transcripts were significantly down-regulated ($p < 0.05$; reduced to $\sim 25\%$) compared with undifferentiated hES cells. For hTERT, there was a significant ($p < 0.05$) down-regulation in both protocols (reduced to 10% and

8% of hES expression level, respectively, in cells derived by Protocols 1 and 3) (Fig. 6) suggesting hNP generated here are not as proliferative as hES cells.

Discussion

In vitro neural differentiation of hES cells serves as a model to study early neurogenesis in humans and it also potentially offers an unlimited source of cells for both replacement therapy as well as for drug screening in tissue culture. We derived large amounts of hNP cells (about 50 million from each million of hES cells) in a feeder-free culture system without the addition of retinoic acid or BMP-inhibitors (i.e., noggin, chordin). We also have identified media components that either had a negative (KSR and NEAA) or positive (N2) effect on hES cell differentiation to neural lineage. In our system, high density hES cultures generated hNP without requiring any genetic manipulation or intermediate passaging. The hNP cells express neural progenitor markers, and can produce all three later cell types, namely, neurons, oligodendrocytes, and astrocytes.

Here, we examined several marker genes expression at day 21 of differentiation using flow cytometry and real-time RT-PCR for differentiation of ES cells to the neural lineage (Shin et al., 2006; Tibbitts et al., 2006; Nat et al., 2007). In flow cytometry experiments, we saw a loss of pluripotency, confirmed by a marked reduction of both Tra-1-60+ (about 5%) and Oct4+ ($\sim 9\%$) cells after 21 days of differentiation. In contrast, 14 days of MEF co-culture followed by 3 days of feeder-less culture produced 17% Oct4+ cells (Shin et al., 2006). Thus, absence of feeder cells may have accelerated differentiation in the protocols reported here. The Oct4+ cells may represent residual undifferentiated hES cells and as previous reports have suggested

(Shimozaki et al., 2003; Perrier et al., 2004; Gerrard et al., 2005), they may also play some role in directing differentiation toward the neural fate bypassing extra-embryonic endoderm formation. Reduction of pluripotent cells in differentiated cultures is also supported by real-time PCR data wherein we found many ES cell markers were down-regulated (Fig. 2C).

The hNP cell were characterized by detecting markers and also by testing what cell types they can produce upon further differentiation. Although not an exclusive and unique marker, NES is widely used in neural progenitor studies (Cattaneo and McKay, 1990; Lendahl et al., 1990; Lardon et al., 2002), and it was used here (Shin et al., 2006; Nat et al., 2007) to identify the two most efficient protocols (Protocols 1 and 3) for hNP derivation (Table 2). We further confirmed that hNP have neural identity as they express a progenitor marker MS11 (Sakakibara et al., 1996) and can differentiate into neurons, oligodendrocytes, and astrocytes (Fig. 4). *In vitro* differentiation of mouse ES cells has been shown to generate lineage-restricted neural precursors (Mujtaba et al., 1999), which share characteristics associated with *in vivo* neural precursor, such as expression of markers (NES, Sox1, Sox2, MS11) (Lang et al., 2004) or these NP-like cells are capable of differentiating into all three cell types (Okabe et al., 1996; Li et al., 1998). Thus, hNP generation occurs in a similar manner to mouse ES cells and further differentiation of hNP to astroglial lineage in our culture is consistent with studies in rodent and human stem/precursor cells (Hughes et al., 1988; Mi and Barres, 1999; Galli et al., 2000; Shimazaki et al., 2001). In terms of marker expression, our results are comparable with several previously published hES cell reports (Schulz et al., 2003; Gerrard et al., 2005; Shin et al., 2006). Also, Tibbitts et al. (2006) derived feeder-free rhesus monkey neural progenitors (rhNP) and evaluated the efficiency using large numbers of genes. Despite some technical differences during differentiation (different extracellular matrices, higher FGF2 concentration from day 10, and no B27 for propagation), the expression profile of many genes covering all lineages (e.g., pluripotent genes: POU5F1, EBAF, DMNT3B, mesodermal markers: GSC, FLT1, KDR, endodermal marker: CER1, and NP markers: NES and MS11) bear striking similarities in NP cells of these two species.

Among the protocols described here, Protocol 3 generated the highest proportion of NES+ cells (89%) at 21 days, but these cells did not proliferate beyond three to four passages. In contrast, the percentage of NES+ cells in Protocol 1 at 21 days was lower (75%), but increased with passaging to yield highly enriched hNP cell (97%–100%) culture. These cells also survived 10–12 passages, making this protocol the best suited for generating large numbers of hNP cells.

Although they expressed similar neural markers, hNP cells derived by each protocol (1 and 3) had

different proliferative potential. The difference in proliferation was evident in SOX and hTERT gene expression level. SOX2, a SOXB1 family transcription factor, is expressed early in the inner cell mass (source of ES cells) and expression continues throughout neural tube development (Wood and Episkopou, 1999). It functions synergistically with Oct4 to maintain the proliferative state of both pluripotent ES (Ambrosetti et al., 1997; Avilion et al., 2003) and potential hNP cells. In addition to SOX2, SOX1 and SOX3 are also expressed in vertebrate NP cells (Uwanogho et al., 1995; Bylund et al., 2003; Shin et al., 2006). In our experiments, we note up-regulation of SOX1 and SOX3 in both Protocols 1 and 3. However, this is higher in Protocol 3 than in 1 (Fig. 6). In contrast, expression of SOX2 was down-regulated in only Protocol 3 which may account for their diminished self-renewal compared with Protocol 1 (Bylund et al., 2003). In both protocols, there was down-regulation of hTERT (another gene associated with pluripotency and proliferation) which is consistent with previous reports (Gerrard et al., 2005). The developmental consequences of the relative changes in the SOXB1 gene expression profiles deserve further investigation.

Our culture technique uses partially defined media without feeder cells, giving us the opportunity to look at the role of different factors in differentiation. We tested four factors (FGF2, N2, KSR, and NEAA) in the media for their effect on expression of MS11 levels in the resulting hNP cell culture. It is generally believed that FGFs (including FGF2) are indirect neural inducers in vertebrates (Strong et al., 2000; Delaune et al., 2005; Stern, 2005). Here FGF2 treatments during differentiation of hES to hNP cells did not increase MS11 expression, suggesting that FGF2 may not act as a neural inducer in this culture. N2 preparation is a serum-free supplement (with transferrin, insulin, progesterone, putrescine, and selenite) frequently used as a media supplement in neuronal culture (Svendsen et al., 1995) and hNP derivation studies (Gerrard et al., 2005; Shin et al., 2006). Use of N2 for 14 days increased MS11 expression, suggesting it promotes hNP derivation (Svendsen et al., 1995). When NEAA supplement (frequently used in hES maintenance (Thomson et al., 1998)) was omitted, MS11 expression increased whereas no change in MS11 was observed with its inclusion confirming its role in hES cell self-renewal, not in hNP cells. The lack of KSR in the media produced similar results and is consistent with previous studies where serum-free protocols were used to produce hNP cells (Schulz et al., 2003; Shimozaki et al., 2003; Shin et al., 2006).

To further determine the media component effects on directed differentiation toward a neural lineage we quantified the expression of several non-neural genes and hNP markers using real time PCR (Fig. 2). This important added proof had not been attempted in previous hNP experiments. As, in the presence of N2-B27,

a majority of hES cells generate extra-embryonic endoderm-like cells (Gerrard et al., 2005), we quantified AFP+ cells in culture. In addition we quantified the pan-endothelial marker CD146 (Bardin et al., 2001) because of the close interaction between neural and vascular systems and the potential for conversion of neural stem cells to endothelial cells (Shen et al., 2004). In most protocols we did not detect AFP (<3%) or CD146 (~5% cells). However, in Protocol 5 we detected up-regulation of some endothelial genes (VWF and TEK, Fig. 2B) suggesting it may prove useful in generating endothelial lineages. In all protocols, we noted up-regulation of a mesodermal gene ACVR1B (a transducer of TGF- β superfamily ligands) involved in a variety of cellular functions including mesoderm formation and early patterning (Zhang et al., 2004). How this gene is connected to neural differentiation is not clear. Overall, quantitative PCR of several non-neural genes and markers indicate that differentiation was restricted to a neural lineage (Fig. 2). Thus, lack of both undifferentiated and differentiated phenotypes of non-neural lineages in culture suggests our protocol is as efficient at generating hNP cells as previous feeder-dependant techniques. Because we used FGF2 (that maintains progenitors with forebrain specification) and B27 (containing retinyl acetate that promotes caudal subtype cells) for proliferation of hNP cells (Zhang, 2006), it is possible they belong to both fore- and hind brain neural subtypes. However, this remains to be confirmed by further studies.

Previously hNPs have been derived from hES cells using various methodologies (Reubinoff et al., 2001; Zhang et al., 2001; Schulz et al., 2003; Gerrard et al., 2005; Itsykson et al., 2005; Pomp et al., 2005; Shin et al., 2006). Recently, Nordberg and colleagues derived neurogenic neuroepithelial and radial glial cells in both adherent and suspension culture without growth factors and morphogens (Nat et al., 2007). However, the yield of neural tissue and its purity varied greatly. Here we have developed a protocol capable of generating large quantities of enriched neural progenitors (>97%) with potential for multiple applications. Our methods do not use non-human support cells, reducing potential zoonotic transmission in future therapeutic applications of our hNP cells. Moreover, being a partially defined system without interference by undefined factors from MEF or stromal cells makes this a better system. This can serve to dissect genetic and molecular pathways involved in human neural development *in vitro* without the need for complex cocktails of growth factors, inhibitors or feeder cells.

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Supplementary material

The following material is available for this article online:

Table S1. Least squares means and standard errors of relative fold changes (with respect to undifferentiated WA09 ES cell) in gene expression during differentiation. Probability value indicates level of significance

from ANOVA; means with same superscript alphabets are not significantly different (based on Tukeys test). (Msc. = Miscellaneous).

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