

Neural differentiation of embryonic stem cells induced by conditioned medium from neural stem cell

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Embryonic stem cells can proliferate indefinitely and are capable of differentiating into derivatives of all three embryonic germ layers *in vitro*, including the neural lineage. The main objective of this study is to test the effects of neural stem cell conditioned medium on the neural differentiation of mouse embryonic stem cells. When cultured in neural stem cell conditioned medium, mouse embryonic stem cells can form floating cell spheres composed of many nestin-positive cells. After trypsinization and growth on gelatin,

these embryonic stem cell-derived neural progenitor cells can be expanded for more than 3 months without loss of neural progenitor characteristics. Both neuronal and glial cells can be readily generated from these cells under differentiation conditions. Thus, neural stem cell conditioned medium is a highly potent reagent for inducing the development of mouse embryonic stem cells into the neural lineage, especially neural progenitor cells. *NeuroReport* 17:981–986 © 2006 Lippincott Williams & Wilkins.

Keywords: conditioned medium, differentiation, embryonic stem cell, neural stem cell

Introduction

Mouse embryonic stem (ES) cells are pluripotent cells derived from the inner cell mass of blastocysts and have the ability to differentiate into various cell lineages *in vitro* and *in vivo* [1,2]. Inducing the ES cells to differentiate into neural lineage not only provides an ideal model for studying the molecular mechanisms of neural development, but also is a promising source of cell replacement therapies [3–5].

A variety of methods have been used to derive neural progenitor cells (NPCs) from ES cells [6–15]. Within these protocols, the common mode of ES cell differentiation is a multi-step method of the formation in suspension culture of embryoid bodies followed by retinoic acid or growth factor treatment and selection [7–9]. Although it is possible to give rise to a large variety of neural cells, there are still some problems. First, production of neural progenitors may be accompanied by other cell lineages because embryoid bodies contain many different types of cells, including mesodermal and endodermal cells. Second, neural precursors produced in response to retinoic acid induction appear to be developmentally restricted [8,9]. Furthermore, the teratogenicity of retinoic acid makes it unsuitable for therapeutic applications [7,16].

In an attempt to overcome these problems inherent in ES cell differentiation as embryoid bodies or with retinoic acid, several different systems [10–12,17,18] that enable enrichment for NPCs have been developed. Ideal protocols for generation of NPCs for the treatment of neurodegenerative diseases and cell loss within the nervous system, however, have not been achieved.

Here, we develop a simple and effective method for directed differentiation of mouse ES cells into NPCs *in vitro*. Neural lineage-specific induction by neural stem cell conditioned medium (NSC-CM), which requires neither supplement of neural inducer molecules nor coculture condition, provides a new powerful tool for therapeutic application. Moreover, this offers an opportunity for studying the mechanisms by which ES cells transition into the neural lineage.

Materials and methods

Generation of embryonic stem cell line

The mouse ES cell line SC1003 (free of mycoplasma contamination) was generated from blastocysts of C57BL/6J mice as described [19] and maintained undifferentiated on gelatin-coated dishes in ES cell medium. This medium was

composed of high-glucose Dulbecco's modified Eagle's medium (DMEM, Gibco Grand Island, New York, USA), supplemented with 10% fetal calf serum (HyClone, Logan, Utah, USA), 0.1 mM β -mercaptoethanol (Sigma, St Louis, Missouri, USA), 1% non-essential amino acids (Sigma), 2 mM glutamine (Sigma), 1000 U/ml leukemia inhibitory factor (Chemicon, Temecula, California, USA), 100 U/ml penicillin and 0.1 mg/ml streptomycin (Sigma). All cultures were fed by replacing medium every 2–3 days.

Neural stem cells isolation and in-vitro cultures

Neural stem cells (NSCs) were obtained as described previously [20]. Briefly, C57BL/6J mouse fetuses on embryonic day 13.5 (midnight of overnight mating is designated as embryonic day 0) were isolated from their mother under deep anesthesia and placed into ice-cold 1:1 DMEM and F-12 nutrient (DMEM/F-12, Gibco), cortical tissues were collected and triturated in same solution with a fine drawn Pasteur pipette to obtain single cell suspension. The dissociated cells were seeded in NSC medium that was DMEM/F-12 supplemented with epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) (both at 20 ng/ml, Peprotech, Rocky Hill, New Jersey, USA), B27 (1:50, Gibco) and heparin (5 μ g/ml, Sigma) to form primary neurospheres. To expand, the primary neurospheres were passaged every 3–4 days using 0.05% trypsin/0.008% ethylenediamine tetraacetic acid and cultured in NSC medium.

Conditioned medium preparation

NSCs at passage 5–20 were trypsinized to a single cell or near single cell suspension and seeded at 5×10^4 cells/cm² in NSC medium to give a ratio of 1.75×10^5 cells/ml medium. NSC-CM was collected after 4 days of cultures by centrifugation, filtered through a 0.22- μ m membrane to remove the cell debris and stored at -20°C .

Derivation of self-renewing neural progenitor cells from embryonic stem cells

Mouse ES cells were dissociated into single cells and transferred to 0.2% gelatin-coated 6-well plates in NSC-CM. Medium was renewed every 2 days. Stem cells gave rise to floating cell clusters called cell spheres within 24 h in NSC-CM. After 5 days, the cell spheres were trypsinized and replated in NSC-CM to differentiate for another 5 days. The resulting cells were then transferred to 0.2% gelatin-coated wells in fresh NSC medium to propagate. These ES cell-derived NPCs were passaged every 4–5 days. For storage, they were harvested and cryopreserved at -80°C for 2 months. The freezing medium was NSC medium plus 10% dimethylsulfoxide. The role of NSC medium was also

studied in this induction by culturing ES cells on 0.2% gelatin-coated cell culture dishes in NSC medium.

Neuronal and glial differentiation of the embryonic stem cell-derived neural progenitor cells

For neuronal and glial differentiation, the ES cell-derived NPCs were plated into gelatin-coated wells in NSC medium without EGF for 4–6 days and then FGF-2 was withdrawn for a further 6 days.

Immunocytochemistry

Cells were fixed with 4% paraformaldehyde for 10 min, washed with PBS and permeabilized with 0.2% Triton X-100 for 30 min. Primary antibodies were used at the following dilutions: mouse anti-nestin (1:200; Chemicon), rabbit anti-glial fibrillary acidic protein (GFAP) (1:200; DAKO, Carpinteria, California, USA), mouse anti-microtubule associated protein-2 (MAP2) (1:500; Sigma), mouse anti-RC2 (1:50; Development Studies Hybridoma Bank, Iowa City, Iowa, USA), rabbit anti- β -tubulin- β (TuJ1) (1:2000; R&D Systems, Minneapolis, Minnesota, USA), rabbit anti-tyrosine hydroxylase (TH) (1:100; Sigma), rabbit anti- γ -aminobutyric acid (GABA) (1:100; Sigma), goat anti-choline acetyltransferase (ChAT) (1:100; R&D Systems). Secondary antibodies were cy3-conjugated or fluorescein isothiocyanate-conjugated anti-mouse IgG (both 1:200; Sigma), or R-PE-conjugated anti-rabbit IgG (1:200; Southern Biotech, Birmingham, Alabama, USA) and proper controls, which included both negative controls that replace the primary antibody with serum, and positive controls that use the antibody with cells known to contain the protein, revealed non-specific staining. Cells were counter-stained with 300 nM Hoechst 33342 (Sigma) for 5 min before visualization.

Reverse-transcription polymerase chain reaction analysis

Total RNA was extracted using Trizol (Bio Basic Inc., Toronto, Canada). Single-stranded cDNA was generated by using a first-strand cDNA synthesis kit (Fermentas, Hanover, Maryland, USA). Polymerase chain reaction (PCR) was performed for 30 cycles for all markers. Details of primers [18] and amplicon size are provided in Table 1.

Cell counts and statistical analysis

Fluorescence was photographed under a fluorescence microscope with a high-resolution digital photograph system (Roper Scientific Photometrics, Tucson, Arizona, USA). The number of immunoreactive cells in 10 microscopic fields of three independent experiments was counted in a randomized fashion. All results are expressed as mean \pm SEM.

Table 1 Primer information

Gene	Sense primer	Antisense primer	Tann ($^\circ\text{C}$)	Product size (bp)
Oct-4	GGCGTTCTCTTTGGAAAGGTGTTC	CTCGAACACATCCTTCTCT	60	312
sox2	GGCGGCAACCAAGAAGAACAG	GCTTGGCCTGCGTCGATGAAC	62	196
sox3	AGACGCTGCTCAAGAAGGAC	CGTAGCCCAGCTGCTCCT	60	191
Nestin	GGAGTGTCGCTTAGAGGTGC	TCCAGAAAGCCAAGAGAAGC	61	327
<i>fabp7</i> (BLBP)	GGTAAGACCCGAGTTCCCTC	ATCACCACTTTGCCACCTTC	59	213
Olig2	GGCGTGGCTTCAAGTCATC	TAGTTTCGCGCCAGCAGCAG	61	250
Mash1	CTCGTCTCTCCGGAAGTATG	CGACAGGACGCCGCTGAAAG	57	301
β -Actin	AGAAGATCTGGCACCAACC	CAGCTGGTCTCCGTATGTCC	60	193

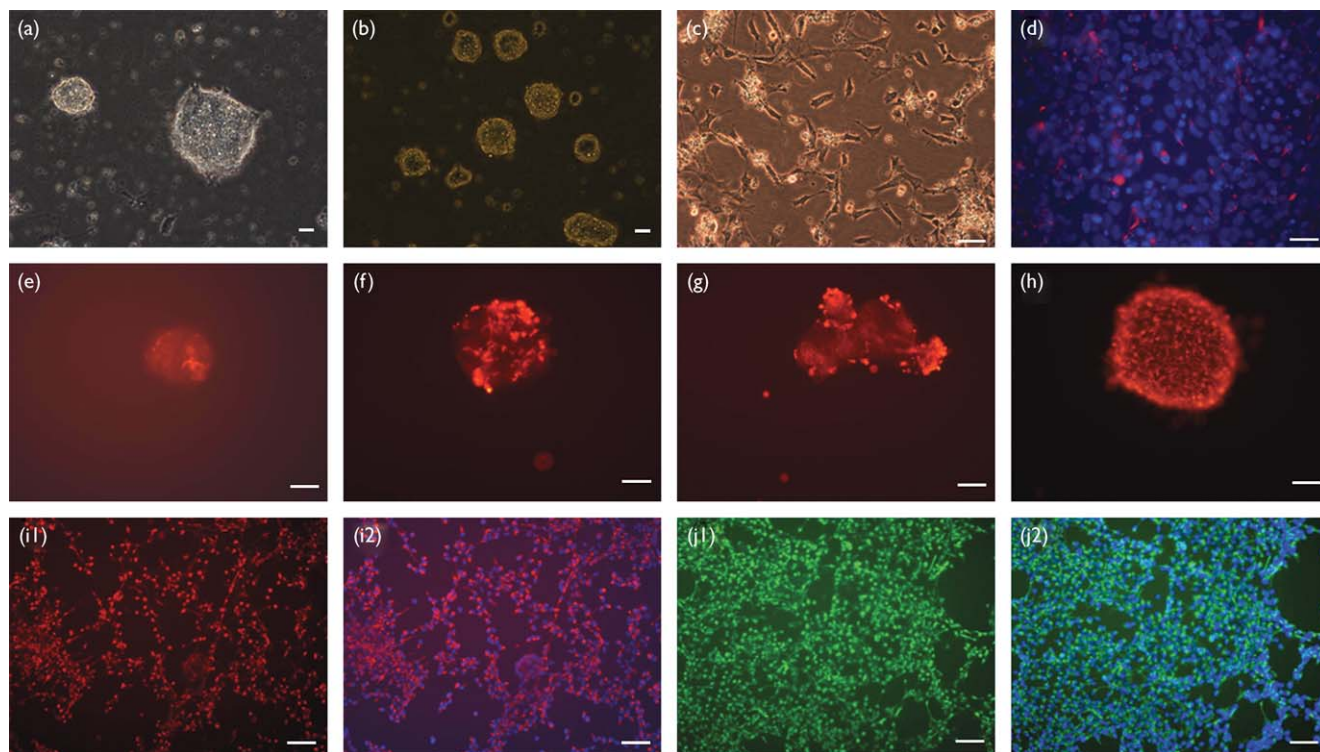


Fig. 1 Culture of ES cells and their neural specific differentiation. (a) Phase contrast image of an undifferentiated ES cell clone. (b) Phase contrast images of ES cells cultured in NSC-CM. (c–d) Phase contrast (c) and nestin immunostained (d) images of ES cells cultured in NSC medium for 10 days. (e–h) Nestin immunostained images of cell spheres cultured in NSC-CM for 24 h (e), 48 h (f), 72 h (g) and 96 h (h). (i–j) Immunostained images of the ES cell-derived NPCs. Expression of neural progenitor markers nestin (i) and RC2 (j) in ES cell-derived NPCs; (i1, j1) immunostained images; (i2, j2) merged with cellular nuclei stained with Hoechst 33342. Bars: (a–h) 20 μ m, (i–j) 40 μ m. ES, embryonic stem cell; NSC-CM, neural stem cell conditioned medium; NPC, neural progenitor cell.

In addition to SC1003 ES cell line that we isolated from blastocysts of C57BL/6J mice, we used another ES cell line, E14, and obtained similar results with this protocol.

Results

Neural differentiation of embryonic stem cells in the neural stem cell conditioned medium

When cultured in the NSC-CM, single ES cells developed into a number of floating clusters called cell spheres (Fig. 1). Parts of the cells in the cell spheres expressed the intermediate filament protein nestin, a marker of NSCs, 24 h after plating, and the nestin-positive cells increased gradually in the subsequent culture period (Fig. 1). At day 4, cells at the periphery of the cell spheres were nearly completely nestin-positive cells; however, the nestin-positive cells also existed at the core of the cell spheres. When the resulting cell spheres were dissociated and cultured in fresh NSC-CM for another 5 days, some of the cells attached and exhibited neural morphology. Bipolar cells of neural precursor morphology or rosette conformations appeared and increased in number over the following days. The other cells still formed floating cell spheres. After 5-day cultures in NSC-CM, both adherent cells and floating cell spheres were trypsinized and transferred to NSC medium, in which the majority of cells attached to the 0.2% gelatin-coated dishes. Many of the cells gave rise to cell clusters. After culture for 7 days in NSC medium, cells exhibited typical NSC morphology (Fig. 2). They generally had an elongated bipolar shape, lamellate extensions and

end-feet, and were identified as neural precursors by staining with nestin and RC2 (Fig. 1). The percentage of nestin-positive cells was $95 \pm 12.6\%$. Reverse-transcription (RT)-PCR analysis showed that Oct-4, a marker of pluripotent ES cells, is constitutively expressed in undifferentiated ES cells but not in ES cell-derived NPCs. All ES cell-derived NPCs examined expressed the neural stem/precursor marker genes *sox2*, *sox3*, *nestin*, *fabp7* (*brain lipid binding protein, BLBP*) and the bHLH (basic helix–loop helix) transcription factors *Olig2* and *Mash1* in accordance with the immunocytochemical detection of nestin and RC2 expression (Fig. 2). Beta-actin served as a control here. *Olig2* and *Mash1*, as bHLH transcription factors, play an important role in neuronal determination and differentiation. As a control, NSCs generated from fetal mouse brain similarly expressed the above marker genes.

On the other hand, when single ES cells were cultured in NSC medium, there was no cell sphere formation (Fig. 1). About 10% of cells survived and attached. After 10-day cultures in NSC medium, a minority of the survived cells expressed nestin (Fig. 1), and Oct-4 and nestin mRNA were detected using RT-PCR (Fig. 2). The results demonstrated that soluble factors from NSCs may instruct ES cells to develop into cell spheres and induce differentiation into NPCs.

Neurogenic and gliogenic differentiation of the embryonic stem cell-derived neural progenitor cells

After culture in differentiation medium lacking growth factor for 10–12 days, cells differentiated into neuronal and

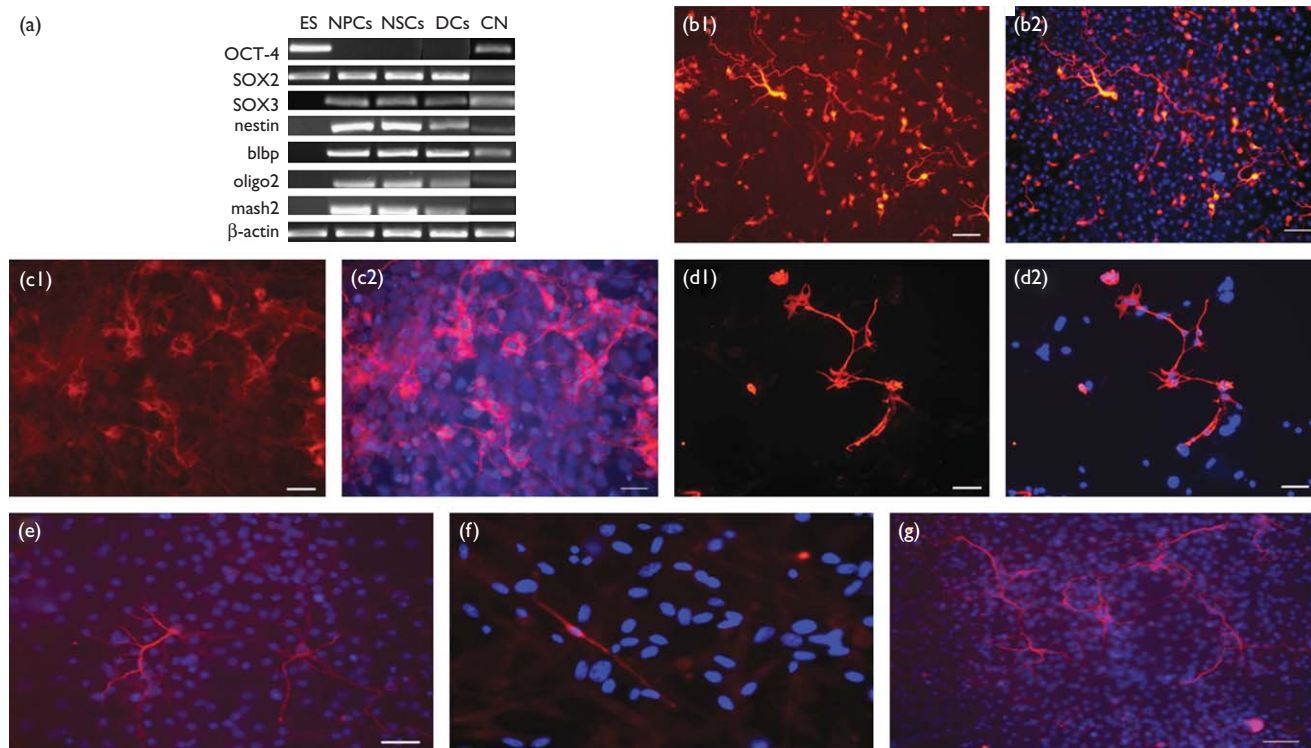


Fig. 2 (a) Reverse-transcription polymerase chain reaction analysis of the undifferentiated ES cells, ES cell-derived NPCs, NSCs derived from fetal mouse brain tissue, differentiated ES cell-derived NPCs. ES, undifferentiated ES cells; NPCs, ES cell-derived NPCs; NSCs, neural stem cells derived from fetal mouse brain tissue; DCs, differentiated ES cell-derived NPCs; CN, ES cells cultured in NSC medium. (b–h) Neuronal and glial differentiation of the ES cell-derived NPCs. (b1–d1) Immunostained images; (b2–d2) merged with cellular nuclei stained with Hoechst 33342. (b) β -Tubulin- β (TuJ1), (c) microtubule associated protein-2 (MAP2), (d) glial fibrillary acidic protein (GFAP), (e) γ -aminobutyric acid (GABA), (f) tyrosine hydroxylase (TH) and (g) choline acetyltransferase (ChAT). Bars: (b, g) 40 μ m, (c–f) 20 μ m. ES, embryonic stem cell; NSC-CM, neural stem cell conditioned medium; NPC, neural progenitor cell.

glial morphology. To examine the phenotypes of the differentiated cells, immunostaining was carried out with TuJ1, MAP2, GFAP, markers of early immature neurons, mature neurons and astrocytes, respectively. After 10 days of differentiation, $23 \pm 3.3\%$ of the total cells were TuJ1-positive (Fig. 2); 27.6 ± 9.2 and $29.7 \pm 11.6\%$ were MAP2 and GFAP-positive cells, respectively (Fig. 2). These data indicate that ES cell-derived NPCs are competent for neuronal and glial differentiation. We also tested whether specific types of neurons can be generated from the ES cell-derived NPCs. TH-positive and GABA-positive cells were present at 3.6 ± 1.4 and $1.1 \pm 0.7\%$, respectively. In addition to TH and GABA, some cells expressed ChAT ($5.8 \pm 1.6\%$), a marker of cholinergic neurons (Fig. 2). These results suggest that neural precursors generated in this protocol could be directed to differentiate into specific neuronal fates.

Subculture of the embryonic stem cell-derived neural progenitor cells and their differentiation

When the ES cell-derived NPCs were cultivated for more than 3 months on gelatin-coated dishes in NSC medium, they could be continuously and rapidly propagated and almost all the cells remained positive for nestin and RC2 (Fig. 3). The nestin-positive cells constituted $96.3 \pm 21.2\%$. These results were similar to the report by Conti *et al.* [18]. Furthermore, these cells had been cryopreserved at -80°C . The cryopreserved cells could be thawed and re-expanded on gelatin-coated dishes in NSC medium without losing

their characteristics. When terminally differentiated in medium lacking bFGF and EGF, the resulting cells expressing markers of neurons or glia were generated (Fig. 3). The percentages of positive cells for each marker were TuJ1 $20.9 \pm 3.7\%$ and GFAP $33.6 \pm 5.1\%$. The percentages of positive cells for nestin, TuJ1 and GFAP did not change significantly compared with the earlier culture.

Discussion

Here we have described the culture method by which cell spheres containing NPCs were generated from mouse ES cells instead of through the formation of embryoid bodies or retinoic acid treatment. Different from previous reports, this method produces neural progenitors simply by switching ES cells into NSC-CM. We speculate that soluble factors in NSC-CM instruct the formation of cell spheres and neural differentiation as ES cells grown in NSC medium fail to give rise to cell spheres and only differentiate into neural-specific lineages at a low frequency accompanied by dramatic decrease in cell number. As NSC medium contains FGF-2 and EGF, which are considered as capable of enhancing the survival and proliferation but not the induction of NPCs [21], the minority of ES cells that spontaneously differentiate into neural-specific lineage sustain propagation. The NSC medium here is much different from the medium described in previous studies [12,18]

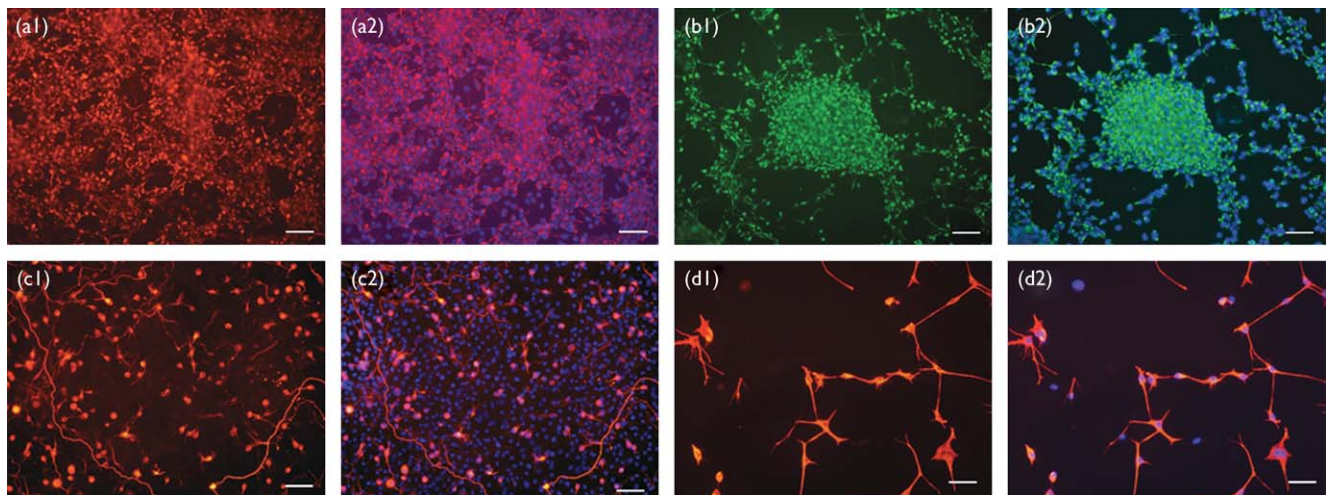


Fig. 3 Differentiation and expansion of ES cell-derived NPCs after passage 8. (a, b) Expression of neural progenitor markers nestin (a) and RC2 (b) in ES cell-derived NPCs. (c, d) Expression of β -tubulin- β (TuJ1) (c) and glial fibrillary acidic protein (GFAP) (d) in differentiated ES cell-derived NPCs. (a1–d1) immunostained images; (a2–d2) merged with cellular nuclei stained with Hoechst 33342. Bars: (a–c) 40 μ m, (d) 20 μ m. ES, embryonic stem cell; NPC, neural progenitor cell.

Nakayama *et al.* [15,22] reported a method to produce a large number of NSCs and neurons from mouse ES cells by coculture with astrocytes or using astrocyte-conditioned medium. The authors think the apparent molecular weights of effective fractions are from 3000 to 10000 as determined by ultrafiltration analysis of astrocyte-conditioned medium. These studies and our findings suggest that the same or equivalent factors to those in both NSC-CM and astrocyte-conditioned medium are involved in neurogenesis in brain development. We note that coculturing ES cells with stromal cells such as PA6 resulted in the formation of neural precursors [10,23]. These authors identified a stromal cell-derived inducing activity that induces differentiation of neural cells.

Given these reports and our findings, we expected that NSC-CM might provide an applicable method for the generation of neural lineage cells for cell replacement therapies. This method has some advantages. First, we can easily generate a vast number of highly homogeneous cells that nearly 100% express markers of NPCs by the present method. More importantly, the resulting cells can be cryopreserved and thawed to re-expand without loss of NPC characteristics. Second, NSC-CM is readily available, easy freezing for storage and ready to use. Third, as this method does not require any added factors such as retinoic acid, it is more secure for cell replacement therapies. Thus, this simple method may be applicable to the differentiation of human ES cells into neural progenitors for the treatment of neurodegenerative diseases such as Alzheimer's disease or Parkinson's disease, neural injury, or neural damage following stroke. For therapies of these diseases, it is important to yield a highly purified population of human neural precursors that could expand easily and retain the ability to form neurons and glia. To approach this objective, we have successfully differentiated human ES cells into neural lineage cells by this method (unpublished observations).

Conclusions

In summary, the present study provides a simple, efficient method for differentiating mouse ES cells into neural

progenitors and then into neurons and glia. Enough neural progenitors and neurons for cell replacement therapy can be expected to be generated conveniently by this method because the ES cell-derived neural progenitors could be cryopreserved and thawed to re-expand. Our results may also provide a tool to better understand the mechanisms for neural differentiation of ES cells.

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