Reprod Dev Biol 31(4) : 267-272 (2007)

In Vitro Expansion of Homogeneous Neural Precursor Cells Derived from Human Embryonic Stem Cells

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ABSTRACT

Human embryonic stem (ES) cells are derived from the inner cell mass of the preimplantation embryo and have the capacity to differentiate into various types of cells in the body. Hence, these cells may potentially be an indefinite source of cells for cell therapy in various degenerative diseases including neuronal disorders. For clinical applications of human ES cells, directed differentiation of these cells would be necessary. The objective of this study is to develop the culture condition for the expansion of neural precursor cells derived from human ES cells. Human ES cells were able to differentiate into neural precursor cells upon a stepwise culture condition. Neural precursor cells were propagated up to 5000-fold in cell numbers over 12-week period of culture and evaluated for their characteristics. Expressions of *sox1* and *pax6* transcripts were dramatically up-regulated along the differentiation stages by RT-PCR analysis. In contrast, expressions of *oct4* and nanog transcripts were completely disappeared in neural precursor cells. Expressions of nestin, *pax6* and *sox1* were also confirmed in neural precursor cells by immunocytochemical analysis. Upon differentiation, the expanded neural precursor cells differentiated into neurons, astrocytes, and oligodendrocytes. In immunocytochemical analysis, expressions of type III β-tubulin and MAP2ab were observed. Presence of astrocytes and oligodendrocytes were also confirmed by expressions of GFAP and O4, respectively. Results of this study demonstrate the feasibility of long-term expansion of human ES cell-derived neural precursor cells *in vitro*, which can be a potential source of the cells for the treatment of neurodegenerative disorders.

(Key words : Human embryonic stem cells, Differentiation, Neural precursor cells, *In vitro* expansion, Embryonic stem cells)

INTRODUCTION

Transplantation of human fetal neural progenitors has been employed as a successful treatment for patients with the progressive neurological diseases (Olanow, 1992; Dunnett *et al.*, 1999; Hagell *et al.*, 1999). However, availability of fetal tissues restricts the practical application of fetal tissue transplantation for the treatment of the degenerative neurological disorders (Hoffer *et al.*, 1992). The shortage of fetal tissues would be overcome by the use of stem cells, such as neural stem (NS) cells and embryonic stem (ES) cells. Neural stem cells are multipotent stem cells that can differentiate into neural lineages of cells, including neuronal and glial cells. Although researches have been carried out on expansion of NS cells *in vitro*, culture conditions for expansion of NS cells has not yet been successfully established. Embryonic stem cells are pluripotent cells that retain the capacity of self-renewal and give rise to the entire tissues of body, including ectoderm, mesoderm and endoderm. Recently, human ES cells were isolated from inner cell mass of blastocyst, demonstrating various characteristics of ES cells (Thomson *et al.*, 1998). Human ES cells have been suggested to be a valuable resource for the treatment of degenerative diseases. Directed differentiation of human ES cells into specific cell types *in vitro* has been a major goal to accomplish their usefulness in therapeutic applications. Neural precursor cells have been successfully derived from human ES cells and shown to retain their multipotency *in vivo* (Carpenter *et al.*, 2001; Reubinoff *et al.*, 2001; Zhang *et al.*, 2001; Kim *et al.*, 2004; Perrier *et al.*, 2004). Furthermore, neural pre-

* This resarch was supported by a grant (SC2190) from Stem Cell Research Center of the 21st Century Frontier Research Program funded by the Ministry of Science and Technology, Republic of Korea

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cursor cells have been shown to engraft into host tissues and differentiate into neural cells *in vivo* (Ko *et al.*, 2007). However, a reliable culture condition for expansion of neural precursor cells has been limited, though a large scale-up of neural precursor cells is a prerequisite for their clinical applications.

In this study, a culture condition was investigated for expansion of neural precursor cells differentiated from human ES cells *in vitro.*

MATERIALS AND METHODS

Culture of Human ES Cells

Human ES cells (CHAhES-4) were maintained according to the method previously described (Zhang *et al.*, 2001). Briefly, human ES cells were cultured on mitotically inactivated STO cells in ES cell medium that consisted of Dulbecco's modified Eagle's medium (DMEM)/F12 (1:1) supplemented with 20% Knockout Serum Replacement (KSR), 2 mM L-glutamine, 0.1 mM minimal essential medium nonessential amino acids, 100 U/ml penicillin, 100 μ g/ml streptomycin, 55 mM β -mercaptoethanol and 4 ng/ml recombinant basic fibroblast growth factor (bFGF; R&D Systems, MN, USA). STO cells were cultured in DMEM supplemented with 100 mM MEM nonessential amino acid, 100 U/ml penicillin, 100 μ g/ml streptomycin and 55 mM β -mercaptoethanol and 10% fetal bovine serum (FBS). Human ES cell were manually subcultured by fire-thrown pasteur pipettes on freshly prepared STO cells at 7-day interval. Media were changed daily. All cultures were maintained at 37℃, 5% CO_2 in atmosphere. All reagents were purchased from Gibco BRL (Carlsbad, California, USA), otherwise indicated.

Neural Differentiation of Human ES Cells

Neural differentiation of human ES cells was carried out by procedure modified from the previously described (Kim *et al.*, 2004) Briefly, undifferentiated human ES cells were enzymatically isolated from STO cells and cultured as aggregates (embryoid bodies, EB) in suspension for 7 days. Culture medium of differentiation consisted of DMEM/F12 (1:1), 100 mM nonessential amino acids and 2 mM L-glutamine supplemented with N2 supplement. Embryoid bodies were allowed to attach onto culture plates coated with matrigel (1:30 diluted in culture medium) in differentiation medium supplemented with 20 ng/ml bFGF for additional 7 days. Neuroepithelial (NE) cells with rosettes structures were mechanically isolated from the adherent cultures. For long-term expansion, NE cells were dissociated into small clumps (about 10～20 cells) by incubation with accutase (Sigma, St. Louis, MO, USA) for 10 min and transferred to culture plates coated with polyornithine (15 μ g/ml)/matrigel (1:30 diluted in culture medium) at $1\sim2\times10^5$ cells/cm^2 in differentiation medium supplemented with 20 ng/ml bFGF and 20 ng/ml recombinant human epidermal growth factor (EGF). The expanded neural precursor cells were subcultured every 14 days at 50,000 cells/ml. For the differentiation of the expanded neural precursor cells, cells were dissociated into single cells by incubation with trypsin 0.25% and plated onto culture plate coated with polyornithine (15 μ g/ml)/matrigel (1:30 diluted in culture medium) in neuronal differentiation medium for 7 to 10 days. Neuronal differentiation medium consisted of Neurobasal medium supplemented with N2 supplement and 200 μ M ascorbic acid (Sigma) in the absence of growth factors. Culture media were changed every other day.

RT-PCR Analysis

Total RNA preparation, cDNA synthesis and RT-PCR reactions were performed as described previously by Kim *et al.* (2004). Total RNA was extracted from undifferentiated human ES cells, NE cells and expanded neural precursor cells by using TRIzol Reagent (Invitrogen, Carlsbad, California, USA) followed by treatment with DNase I (Ambion, Austin, TX, USA). Total RNA (5 μ g each) was reverse transcribed using oligo-dT and Superscript II reverse transcriptase (Invitrogen). The PCR reaction was carried out with 1 μ l cDNA template, 1 μ l 10 mM dNTP mixtures and 10 pmol of each primers using standard conditions with Taq DNA polymerase (Invitrogen). Complementary DNAs were amplified as follows: 5 min for denaturation at 94℃, followed by 30 cycles at 94℃ for 30 sec, 55℃ for 30 sec and 72℃ for 1 min, and extension at 72℃ for 7 min in GeneAmp 9700 (Applied Biosystems, Foster City, CA, USA). As a control for mRNA quality, GAPDH transcripts were analyzed. Products were analyzed on 1.0% agarose gel and visualized by ethidium bromide staining. For reverse transcription-polymerase chain reaction (RT-PCR) analysis, 5 μ g of total RNA was transcribed into cDNA using the SuperScript Preamplification Kit (Life Technologies) and oligo (dT) primers. Followings are primer sequences for each gene:

oct4: 5'-CTGGAAGCTGGAGAAGGAGAATCTG-3',
5'-CAAGGGCCGCAGCTTACACATGTTC-3';
nanog: 5'-GCTTGCCTTGCTTTGAAGCA-3',
5'-TTCTTGACTGGGACCTTGTC-3';
pax6: 5'-GGCAACCTACGCAAGATGGC-3',
5'-TGAGGGCTGTGTCTGTTCGG-3';
sox1: 5'-CTCTGGACCAAACTGTGGCG-3',
5'-CAATGCGGGGAGGAGAAGTC-3';
gapdh: 5'-TGGTATCGTGGAAGGACTCA-3',
5'-CCTGCTTCACCACCTTCTTG-3'.

Cell Proliferation Analysis

Cell proliferation assay was performed by counting total cells at every passage. Cell counts were carried out at 3 independent experiments with 3 replicates. Data were expressed as mean±standard deviation.

Immunochemical Analysis

Cells were fixed with 4% paraformaldehyde (Sigma) for 15 min at 25℃ and incubated with primary antibodies for over night at 4℃ after treatment with 5% normal goat serum (Sigma) in PBS containing 0.1% Triton-X (Sigma). Cells were incubated with secondary antibodies in PBS (1:200) for 1 hr at room temperature containing 5% normal goat serum and 0.1% Triton X-100. Cells were analyzed for expressions of *OCT4* (1:100, Santa Cruz Biotechnology, Santa Cruz, California, USA), Nestin (mouse monoclonal, 1:200, Chemicon, Temecula, CA, USA), *SOX1* (rabbit polyclonal, 1:300, Chemicon), *PAX6* (rabbit polyclonal, 1:300, Chemicon), type III β -tubulin (mouse monoclonal IgG, 1:500, Chemicon), MAP2ab (rabbit polyclonal, 1:500, Chemicon), GFAP (mouse monoclonal IgG, 1:600, Chemicon) and O4 (mouse monoclonal IgM, 1:150, Chemicon). Secondary antibodies were goat anti-mouse IgM conjugated with Alexa-488 (1:300, Chemicon), goat anti-mouse IgG conjugated with Cy3 (1:300, Chemicon), goat anti-rabbit IgG (1:300, Chemicon). Cells were visualized under a laser-scanning confocal microscope imaging system (LSM510, Carl Zeiss, Thornwood, NY, USA). Negative controls without primary antibody were performed in all experiments to monitor nonspecific staining.

RESULTS

Differentiation of Human ES Cells into Neural Precursor Cells

Human ES cells were maintained in undifferentiated state under the standard culture condition (Fig. 1A). These cells were induced to differentiate into neural lineages in stepwise processes. Differentiation of ES cells was initiated by suspension culture of EB in serum-free condition in the absence of bFGF. After replating EB, NE cells with rosette morphology appeared from the center of the cell aggregates replated (Fig. 1B). As shown in Fig. 1C, the purified NE cells expressed nestin, a marker for neuroectodermal cells, but failed to express *oct4* protein which is a cellular marker for undifferentiated ES cells, suggesting that they were virtually homogeneous population of NE cells.

Expansion and Characterization of Neural Precursor Cells

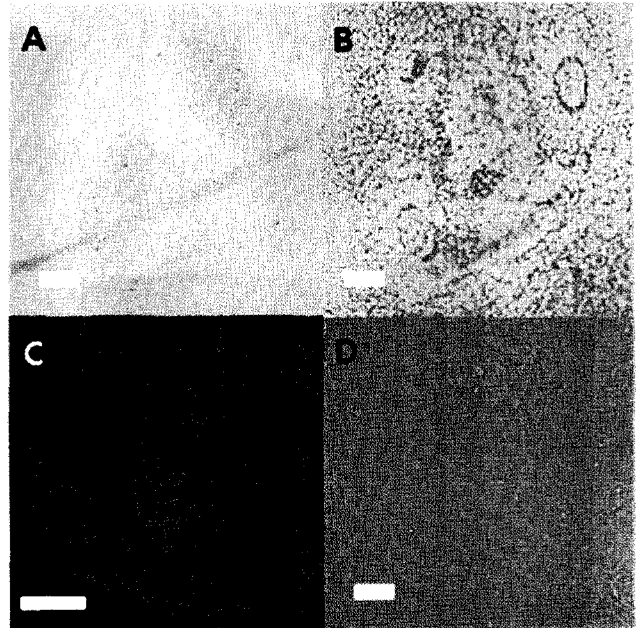

Fig. 1. Morphologies of human ES, NE and expanded neural precursor cells. (A) Undifferentiated human ES cells. (B) Neuroepithelial cells derived from human ES cells. (C) Expression of *oct4* and nestin. (D) Morphology of expanded neural precursor cells. Scale bar, 50 μm.

In vitro expansion of neural precursor cells were carried out by culture of NE cells on matrigel-coated culture plates. Neuroepithelial cells isolated based on their distinctive morphology were cultured on matrigel in the presence of bFGF and EGF. Neuroepithelial cells migrated radially away from the cell clumps, resulting in a cell monolayer around the clumps. After primary culture of NE cells, the proliferating cells were enzymatically dissociated and subcultured in the same culture conditions. The resulting cells were termed as neural precursor cells. Proliferation of neural precursor cells was measured quantitatively by counting total cell numbers over the 12-week culture period. As demonstrated in Fig. 2, neural precursor cells slowly proliferated, and the number of neural precursor cells increased by 5000-fold over 12 weeks.

In order to characterize the identity of cells expanded, RT-PCR and immunocytochemical analyses were carried out. The expanded cells were shown to retain the characteristics of neural precursor cells as demonstrated in Fig. 3A. Gene expressions of *pax6* and *sox1*, specific for neural precursor cells, were dramatically up-regulated along the stages of differentiation. Especially, their expressions were not different between NE and the expanded neural precursor cells. Immunocytochemical analysis also confirmed expressions of nestin, *pax6* and *sox1* on the expanded neural precursor cells (Fig. 3B~D). Multipotency of the expanded neural precursor cells were examined by neural induction of

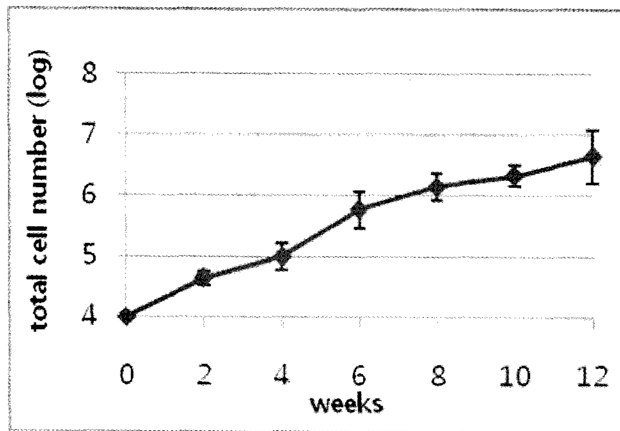

Fig. 2. Proliferation rate of neural precursor cells *in vitro*.

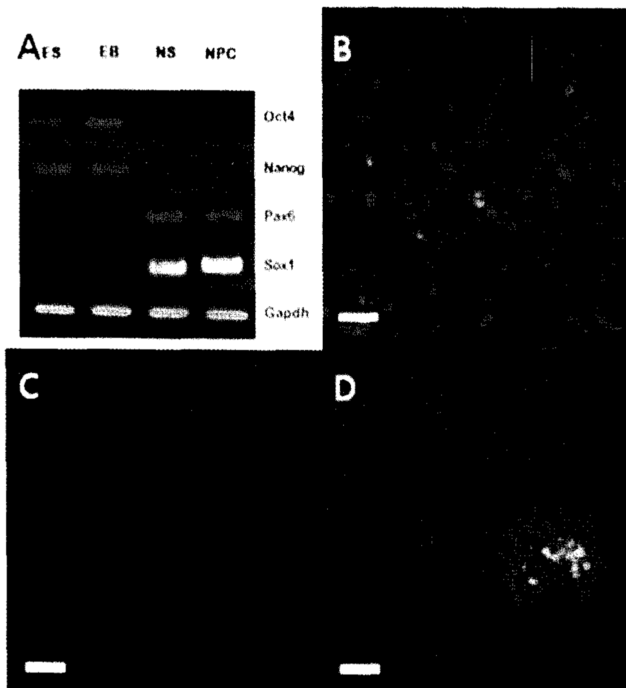

Fig. 3. RT-PCR and imunocytochemical analyses of neural precursor-specific markers in expanded neural precursor cells. (A) Expressions of neural precursor-specific genes. (B) Expression of nestin. (C) Expression of *pax6*. (D) Expression of *sox1*. Scale bar, 100 µm.

the cells. As shown in Fig. 4, the expanded cells were able to differentiate into neuron, astrocytes and oligodendrocytes upon differentiation. Expressions of type III β -tubulin and MAP2ab confirmed the neuronal differentiation (Fig. 4A and B). Astrocytes and oligodendrocytes were confirmed by expressions of GFAP and O4, respectively (Fig. 4C and D). Results from this study demonstrated the expansion of neural precursor cells derived from human ES cells *in vitro*, while maintaining their differentiation capacities.

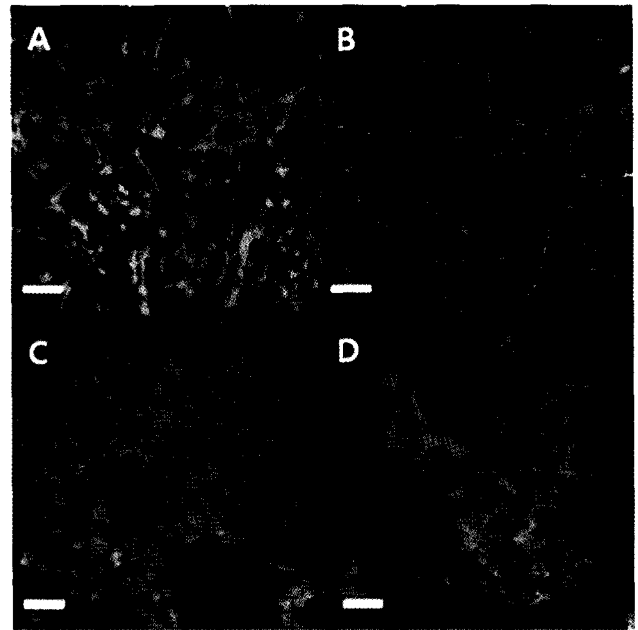

Fig. 4. Differentiation potentials of expanded neural precursor cells. (A) Expression of type III β-tubulin. (B) Expression of MAP2ab. (C) Expression of GFAP. (D) Expression of O4. Scale bar, 100 µm.

DISCUSSION

Desirable type of cells for CNS transplantation will be an expandable population of cells which can differentiate into the appropriate phenotypes. Transplantation of fetal CNS tissues has been successfully performed in human patients suffering from the degenerative neural diseases, resulting in some clinical improvement (Olanow, 1992; Dunnett *et al.*, 1999; Hagell *et al.*, 1999). However, a limited availability of fetal tissues has hampered the practical application of fetal cells for clinical trials. Recent progress from research on NS cells has made it possible to expand neural progenitor cells obtained from a small amount of fetal CNS tissues *in vitro* (Reynolds and Weiss, 1992; Chung *et al.*, 2006). However, expansion of NS cells *in vitro* has generated the conflicting data among researchers. Furthermore, expanded NS cells often showed that their differentiation capacities were limited in a specific neural lineage or lost (Yan *et al.*, 2001; Ko *et al.*, 2005; Chung *et al.*, 2006). Whereas fetal neural tissues are limited in their practical application due to their availability, ES cells are readily available for clinical applications in large scale.

The present study demonstrates that neural precursor cells could be differentiated from human ES cells and expanded without losing their multipotency in long-term culture. Neuroepithelial cells could easily be derived from human ES cells based on their distinctive

morphology. Culture medium for differentiation employed in this study was commonly used in culture of neural stem cells. Recent reports from others that NE cells were differentiated from human ES cells (Carpenter *et al.*, 2001; Reubinoff *et al.*, 2001; Zhang *et al.*, 2001, Perrier *et al.*, 2004). Previous report from our laboratory also showed that NE cells were generated from human ES cells (Lee *et al.*, 2003; Kim *et al.*, 2004). However, efficiency for differentiation of NE cells was low in the previous reports. Employment of medium for routine culture of neural stem cells improved the efficiency of neural differentiation from ES cells.

Expansion of ES-derived neural precursor cells was previously reported, in which neural precursor cells were maintained either in suspension culture (Reubinoff *et al.*, 2001; Zhang *et al.*, 2001) or adherent culture (Ying *et al.*, 2003). However, the culture conditions for expansion were laborious and not applicable for long-term culture of neural precursor cells (Joannides *et al.*, 2006; Shin *et al.*, 2006). In order to develop the culture condition for extensive expansion of neural precursor cells as a homogeneous population, adherent culture method was employed in this study. The purified NE cells were seeded and maintained on the substrates, polyornithine/matrigel, in the condition previously described for the culture of neurogenic cells (Nunes *et al.*, 2003). Especially, use of matrigel seemed to facilitate the adhesion and proliferation of NE cells. Furthermore, proliferation of neural precursor cells was dependent on the addition of growth factors, such as bFGF and EGF, throughout the culture period. Both EGF and bFGF have been added in culture of various progenitor and stem cells (Gritti *et al.*, 1995; Reynolds *et al.*, 1996; Palmer *et al.*, 1997). Results from studies on rodent cells demonstrated that proliferation of NS cells isolated in early neurogenesis depends on presence of bFGF and EGF in culture (Kalyani *et al.*, 1997; Tropepe *et al.*, 1999). Although addition of bGFG and EGF enhanced proliferation of neural precursor cells in this study, it is not clear that the condition is the optimal for growth of neural precursor cells. Effects of various mitogens need to be evaluated on the proliferation and differentiation of neural precursor cells *in vitro*. For example, ciliary neurotrophic factor was shown to be effective on the culture of the early neuronal precursors (Ip *et al.*, 1993) and also on the maintenance of the pluripotentiality of ES cells (Conover *et al*, 1993). Proliferation of neural precursor cells was quantitatively examined by counting total cell number over 3 months of culture period. Neural precursor cells were continuously expanded up to 5,000-fold under the same culture condition, although their proliferation rates were slow. Similar results were reported in other study (Ko *et al.*, 2007). Neural precursor cells isolated from the ventral mesencephalons were expanded only 1,000-fold (Chung *et al.*, 2006). The culture conditions in the present study were shown to be superior to those of other study in expansion of neural precursor cells. After expansion of neural precursor cells, they were examined for their characteristics. Molecular and immunocytochemical analyses were used to confirm the identity of neural precursor cells. Expressions of *pax6* and *sox1* known to be specific for neural precursor cells were dramatically up-regulated during the processes of differentiation. Immunocytochemical analysis also showed that expressions of nestin, *pax6* and *sox1* were observed in the most of the expanded cells. Results from both analyses confirmed that the expanded cells were homogeneous population of neural precursor cells. Because of neural precursor cells are multipotent cells, they were analyzed for their capacity for differentiation into mature neuronal and glial lineages. Assessment of mature neuronal cells was carried out by immunocytochemical analysis upon differentiation. Expressions of type III β -tubulin and MAP2ab were detected in the differentiated neural precursor cells, confirming that the expanded neural precursor cells differentiate into mature neurons. Furthermore, expressions of GFAP and O4 in the differentiated neural precursor cells also confirmed the presence of astrocytes and oligodendrocytes, respectively. Neural precursor cells derived from fetal neural tissues were shown to lose their differentiation capacities after expansion *in vitro* (Chung *et al.*, 2006). In contrast, neural precursor cells retained their multipotency even after expansion in long-term culture in this study. Results from both RT-PCR and immunocytochemical analyses strongly support that the expanded cells were cells with identity of neural precursor cells.

In conclusion, this study demonstrates that neural precursor cells can be generated from human ES cells and expanded without losing their differentiation capacities in serum-free culture condition in this study. Expansion and differentiation of neural precursor cells were confirmed by molecular and biochemical analyses. The protocols for expansion of neural precursor cells in the present study provide efficient systems for application of human ES cells in various areas. Neural precursor cells will be useful for the study of the underlying mechanisms on neural development in humans. These cells also provide the screening systems for the development of new drugs. Furthermore, neural precursor cells may also be used for the treatment of various neurodegenerative disorders. Considering ES-derived neural precursor cells as a potential source for cell replacement therapy, development of an optimal culture system for the expansion of neural precursor cells needs to be achieved.

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(Received: 5 November 2007/ Accepted: 10 December 2007)