Derivation of Neural Precursor Cells 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. Human ES cells have the capacity to differentiate into various types of cells in the body. Human ES cells are indefinite source of cells for cell therapy in various degenerative disorders including neuronal disorders. Directed differentiation of human ES cells is a prerequisite for their clinical application. The objective of this study is to develop the culture condition for the derivation of neural precursor cells from human ES cells. Neural precursor cells were derived from human ES cells in a stepwise culture condition. Neural precursor cells in the form of neural rosette structures developed into neurospheres when cultured in suspension. Suspension culture of neurospheres has been maintained over 4 months. Expressions of *nestin, sox1, sox2, pax3* and *pax6* transcripts were upregulated during differentiation into neural precursor cells by RT-PCR analysis. In contrast, expression of oct4 was dramatically downregulated in neural precursor cells. Immunocytochemical analyses of neural precursor cells demonstrated expression of nestin and SOX1. When induced to differentiate on an adhesive substrate, neurospheres were able to differentiate into three lineages of neural systems, including neurons, astrocytes and oligodendrocytes. Transcripts of *sox1* and *pax6* were downregulated during differentiation of neural precursor cells into neurons. In contrast, expression of *map2ab* was elevated in the differentiated cells, relative to those in neural precursor cells. Neurons derived from neurai precursor cells expressed NCAM, Tuj1, MAP2ab, NeuN and NF200 in immunocytochemical analyses. Presence of astrocytes was confirmed by expression of GFAP immunocytochemically. Oligodendrocytes were also observed by positive immuno-reactivities against oligodendrocyte marker O1. Results of this study demonstrate that a stepwise culture condition is developed for the derivation of neural precursor cells from human ES cells.

(Key words : Embryonic stem cell, Differentiation, Neural precursor cell, Human)

INTRODUCTION

Embryonic stem (ES) cells are pluripotent cells that 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 (Thomson et al., 1998). Undifferentiated human ES cells expressed oct4 and cell surface markers, including SSEA-3, 4, TRA-1-60, TRA-1-81, and alakaline phosphatase. Human ES cells were able to differentiate into various tissues *via* formation of embryoid body (EB) *in vitro*. Human ES cells have been suggested to be a valuable resource for the 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.

Neurogenesis has been considered as the most complex event of organogenesis during embryonic development, in which a precise signaling and cellular interaction cascade are involved in generation of a functional neural network. During neurulation, neuroectodermal cells develop into neural plate that eventually forms neural tube by its closure at dorsal areas of embryos along anterioposterior axis. Neuronal and glial lineages are differentiated from the neuroectodermal cells of neural tube, developing nevous systems in mammals. Differentiation of mouse ES cells into neural precursor cells has been reported using various culture conditions (Bain et al., 1995; Kawasaki et al., 2000; Lee et al., 2000; Kim et al., 2002; Rathjen et al., 2002; Wichterle et al., 2002; Ying et al., 2003). Their differentiation capacities were shown both *in vivo* and *in vitro*. Neural precursor cells have also been successfully isolated from human ES

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cells (Reubinoff et al., 2001; Zhang et al, 2001; Lee et al., 2004; Perrier et al., 2004). Similar results were obtained for their differentiation capacities *in vitro*, in which neural precursor cells differentiate into neurons, astrocyte, oligodenderocyte. Neural precursor cells were detected in subventricular region of the lateral ventricle, the hippocampus, the cerebral cortex and spinal cord (Reubinoff et al., 2001; Zhang et al, 2001), suggesting their potential applications for treatments of neuronal disorders.

In the present study, culture conditions at stepwise manners were examined for the generation of neural precursor cells from human ES cells *in vitro*.

METHODS AND MATERIALS

Culture of Human ES Cells

Two human ES cell lines (SNU-hES3 and Miz-hES1) were used in this study (Park et al., 2003). Human ES cells were cultured on mitotically inactivated STO cells (ATCC CRL-1503) in ES cell medium that consisted of DMEM/F12 (1:1) supplemented with 100 mM MEM nonessential amino acid, 100 U/ml penicillinm, 0.1 mg/ml streptomycin, 55 mM -mercaptoethanol, 20% Knockout Serum Replacement and 4 ng/ml recombinant human basic fibroblast growth factor (bFGF) (R&D systems). Feeder cells were cultured in DMEM supplemented with 100 mM MEM nonessential amino acid, 100 U/ml penicillinm, 100 ug/ml streptomycin and 55 mM -mercaptoethanol and 10% fetal bovine serum (FBS, Hyclone). Media were changed daily. Human ES cell were manually subcultured by fire-thrown pasteur pipettes on freshly prepared STO cells at 7-day interval. All cultures were maintained at 37C, 5% CO_2 in atmosphere. All reagents were purchased from Gibco BRL, otherwise indicated.

Differentiation of Human ES Cells into Neural Precursor Cells

Formation of EB were carried out by suspension culture of ES cell clumps in bacteriological plate coated with F-127 (Sigma) in N2B27 medium (Ying et al., 2003) in the presence of bFGF (10 ng/ml) that consisted of DMEM/F12 (1:1) and Neurobasal medium (Gibco BRL) supplemented with N2 supplement (Gibco BRL), B27 supplement (Gibco BRL), 2mM L-glutamine (Gibco BRL). Embryoid bodies were cultured for 14 days and medium was changed every other day. Fourteen-old EBs were replated onto culture plates coated with 0.2% gelatin and cultured for 14-21 days in N2B27 medium. Medium was changed every other day. Neuroepithelium cells with rosette structures appeared after 7 days in culture were manually isolated from the culture and transferred into bacteriological plate coated with F-127 in neurosphere (NS) medium (Zhang et al., 2001). Neurospheres were formed in suspension culture within 24 hrs and subcultured every 14 days. Neurosphere medium consisted of DMEM/F12 (1:1) supplemented with 2mM L- glutamine, 25 μg/mℓ human insulin (Sigma), 100 μg/mℓ human transferrin (Sigma), 20nM progesterone (Sigma), 60uM putrescine (Sigma), 30nM sodium selenite (Sigma), 2 μg/mℓ heparin (Sigma), recombinant human epidermal growth factor (EGF) (R&D, Systems), 20 ng/mℓ FGF-2. Medium was changed every other day. For the differentiation of neural precursor cells into neuronal lineages, neurospheres were cut into small pieces and plated onto culture plates coated with 10 μg/mℓ fibronectin (Sigma) in NS medium in the absence of growth factors and cultured for 7~14 days. For the glial differentiation of neural precursor cells, neurospheres were cultured in DMEM/F12 (1:1) supplemented with B27 supplements and 0.5% FBS for 21 days.

Immunocytochemical Analysis

Cells were fixed in 4% formaldehyde for over night at 4℃ and incubated with primary antibodies for over night at 4℃ after treatment with 5% normal goat serum (Sigma) in PBS containing 0.01% Triton-X (Sigma). Cells were incubated with secondary antibodies in PBS (1:200) for 1 hr at room temperature. Cells were analyzed for expressions of OCT4 (1:100, Santa Cruz), SSEA-4 (1:200, Chemicon), SOX1 (1:800, Chemicon), nestin (1:200, R&D Systems), NCAM (1:400, Chemicon), Tuji (1:800, Chemicon), MAP2ab (1:1000, Chemicon), NeuN (1:400, Chemicon), NF68 (1:400, Sigma), NF200 (1:200, Chemicon), A2B5 (1:500, Chemicon), GFAP (1:800, Dako), MBP (1:500, Chemicon) and 01 (1:500, Chemicon). Secondary antibodies were rabbit anti-mouse IgG (1:400, Chemicon), goat anti-mouse IgM (1:400, Chemicon), goat anti-rat IgG (1:400. Chemicon) and goat anti-rabbit IgG (1:1000, Dako) either conjugated with rhodamine or FITC. Cells were visualized under the fluorscent microscope.

RT-PCR Analysis

Total RNA was extracted from undifferentiated human ES cells, EB, rosette structures and neurospheres by using Trizol (Invitrogen) and DNase I treatment (Invitrogen). Total RNA (1ug each) was reverse transcribed using oligo-dT and Superscript II reverse transcriptase (Invitrogen). The PCR reaction was carried out with 1μℓ cDNA template, 1μℓ 10mM dNTP mixtures and 10pmol of each primers using standard conditions with Taq DNA polymerase (Invitrogen). Complementary DNAs were amplified as follows: 5 minutes for denaturation at 94℃, followed by 30 cycles at 94℃ for 30 seconds, 55℃ for 30 seconds and 72℃ for 1minute, and extension at 72℃ for 7 minutes in GeneAmp 9700 (Perkin-Elmer). As a control for mRNA quality, GAPDH transcripts were

analyzed. Products were analyzed on 1.0% agarose gel and visualized by ethidium bromide staining. Followings are primer sequences for each genes:

oct4: 5'-CTGGAAGCTGGAGAAGGAGAATCTG-3', 5'-CAAGGGCCGCAGCTTACACATGTTC-3';
nestin: 5'-GCCCTGACCACTCCAGTTTA-3', 5'-GGAGTCCTGGATTTCCTTCC-3';
sox1: 5'-AGAACCCCAAGATGCACAAC-3', 5'-GCCAGCGAGTACTTGTCCTT-3';
sox2: 5'-AGAACCCCAAGATGCACAAC-3', 5'-GGGCAGCGTGTACTTATCCT-3';
pax3: 5'-GACAGCAGCTCTGCCTACTG-3', 5'-GGTCCATACTGTAGCCTGTG-3';
pax6: 5'-CGAGACTGGCTCCATCAGAC-3', 5'-AGCCATCTTGCGTAGGTTGC-3';
hnf3β : 5'CACAAGTGAGAGAGCAAGTG-3', 5'-ACAGTAGTGGAAACCGGAG-3';
brachury: 5'-CTGGACCCCAACGCCATGTA-3', 5'-GGGTCTCAGGGAAGCAGTGG-3';
map2ab: 5'-GCATATGCGCTGATTCTTCA-3', 5'-CTTTCCGTTCATCTGCCATT-3';
gapdh: 5'-TGGTATCGTGGAAGGACTCA-3, 5'-CCTGCTTCACCACCTTCTTG-3'.

RESULTS

Differentiation of Human ES Cells into Neural Precursor Cells

Human ES cells were maintained in undifferentiated state under the standard serum-free condition (Fig. 1A), which were confirmed by expressions of oct4 (Rosner et al., 1990; Scholer et al., 1990) immunocytochemically. In addition, ES cells were assessed for their stemness by expressions of stem cell surface marker, SSEA-4 (Draper et al., 2002; Henderson et al., 2002). Human ES cells were induced to differentiate into neural fates in stepwise processes. Differentiation of ES cells was initiated by formation of embryoid body (EB) in suspension culture under the serum-free condition in the presence of bFGF (Fig. 1D).

At 7~10 days after replating EB, neuroepithelial cells that were arranged in rosettes emerged from the center of the replated EB (Fig. 1E). Neuroepithelial cells were radiated from the core of the rosette structures that were similar to the neuroectodermal cells of the developing embryo. Based on their distinctive morphology, the rosettes structures were physically purified by fire thrown pasteur pipettes and cultured in suspension toform neurospheres under the neurosphere (NS) medium supplemented with bFGF and EGF. Neural precursor cells were maintained as neurospheres for the limited time. The neurospheres were spontaneously

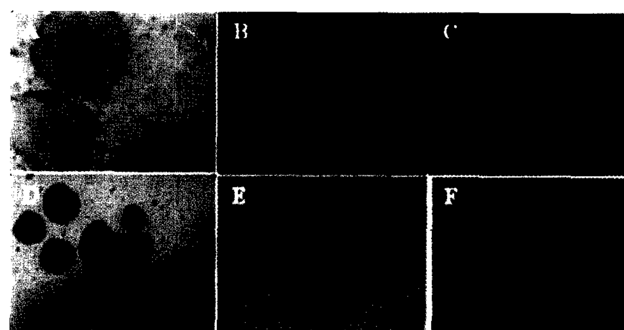

Fig. 1. Morphologies of human ES cells and ES-derived neural precursor cells. (A). Undifferentiated human ES cells (40×), (B). Immunocytochemical analysis of OCT4 in human ES cells (100×), (C). Immunocytochemical analysis of SSEA-4 in human ES cells (100×), (D). EB (40×), (E). Neural precursor cells (neural rosettes structure, 100×), (F). Neurons differentiated from neural precursor cells (200×).

able to diff- erentiate into neurons in NS medium when seeded onto the substrates (Fig. 1F). At 7~10 days after seeding, neurites were grown out of the cells within neurospheres and developed their bundles. Both human ES cell lines, SNU-hES3 and MizhES1, showed similar patterns in the generation of neural precursor cells.

Characterization of Neural Precursor Cells

Characterization of neural precursor cells was carried out by immunocytochemical and RT-PCR analyses. Expressions of genes specific for neural precursor cells were demonstrated in figure 2A. As expected, expression of *oct4* specific for the undifferentiatied ES cells was downregulated upon differentiation into neural lineages. Expression of *hnf3* specific for endodermal lineages was absent in both undifferentiated ES and neural precursor cells. Similar results were obtained for expression of *brachury* specific for nascent mesodermal cells. Expression of *sox1* was upregulated in freshly isolated neural precursor cells and maintained in neurospheres up to 2 weeks in cultures. Consistent results were obtained for expressions of other genes specific for neural precursor cells, including *sox2*, *nestin*, *pax3* and *pax6*. After 3 weeks in suspension culture, genes specific for neural precursor cells were downregulated unless neurospheres were subcultured. Neural precursor cells were also analyzed for the expression of cellular markers by immunocytochemical analyses. Figure 3 showed the expressions of cellular markers specific for neural precursor cells. Most of cells within neurospheres expressed SOX1 (Fig 2B). Similar results were obtained for expression of nestin (Fig. 2C). Especially, morphological appearance of neural rosettes was clearly observed by expression of nestin.

Genes specific for mature neurons were also analyzed for neural precursor cells and their differentiated neurons. Expression of *map2ab* was first detected in freshly

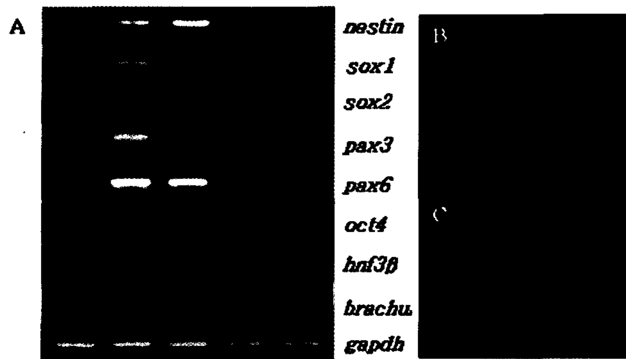

Fig. 2. RT-PCR and immunocytochemical analyses for neural precursor-specific markers in neural precursor cells. (A). lane1: undifferentiated ES cells, lane2: neural rosettes, lane3: 7-day neurospheres, lane4: 14-day neurospheres, lane5: 42-day neurospheres, (B). SOX1 (100×). (C). Nestin (100×).

Fig. 3. RT-PCR and immunocytochemical analyses of neuron-specific markers in neural precursor cells and their differentiated neurons. (A). lane1: undifferentiated ES cells, lane2: neural rosettes, lane3: 7-day neurospheres, lane4: 14-day neurospheres, lane5: differentiated neurons, (B). NCAM (200×), (C). Tuj1 (100×), (D). MAP 2ab (200×), (E). NeuN (400×), (F). NF68(100×), (G). NF200 (100×).

isolated neural precursor cells and neurospheres, and increased in their differentiated neurons. Similar results were obtained for expressions of *sox1* and *pax3* specific for neural precursor cells. Differentiation capacity of neural precursor cells into neurons was also analyzed by immunocytochemical analyses of various neuronal markers. Expression of NCAM was clearly detected in the surface of mature neurons and neuritis (Fig. 3B). Other neuronal markers, such as Tuj1and MAP2ab, were also detected in the neurons differentiated from neural pecursor cells (Fig 3C and D, respectively). Especially, expression of NeuN was localized in the neucli of neurons (Fig. 3E). Figure 3F and 3G demonstrated expression of NF68 and NF200 in the bundles of neuritis projected from neurons, respectively. To confirm differentiation capacity of neural precursor cells into glial lineages, cellular markers specific for astrocytes and oligodendrocytes were analyzed upon differentiation. Expression of A2B5 specific for glial progenitor cells was detected in the culture of glial differentiation (Fig. 4A). Marker for astrocytes, GFAP, was also expressed in thecells differentiated from neural precursor cells (Fig. 4B). Low frequency of MBP (+) cells was detected in differentiated cells (Fig. 4C). There were a few of O1 (+)

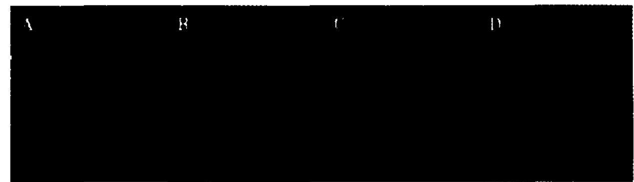

Fig. 4. Immunocytochemical analyses of glial markers in the cells differentiated from neural precursor cells. (A). A2B5 (100×), (B). GFAP (400×), (C). MBP (100×), (D). O1 (100×).

cells found in the culture. Results from this study demonstrate the *in vitro* differentiation of human ES cells into neural precursor cells.

DISCUSSION

This study demonstrates that neural precursor cells were differentiated and isolated from culture of human ES cells by formation of EB in serum-free conditions. Neural precursor cells were easily isolated from the differentiated culture of human ES cells based on their distinctive morphology. Culture medium employed in this study was first used for the differentiation of mouse ES cells into neural precursor cells (Ying et al., 2003). Mouse ES cells were able to differentiated into neural precursor cells at high efficiency when cultured as an adherent culture under the same culture medium as used in this study. However, an adherent culture failed to induce human ES cells to differentiate into neural fates under the same condition as in mice (data not shown), although spontaneous differentiation of human ES cells into neural precursor cells was reported under the prolonged culture of human ES cells (Reubinoff et al., 2001). Recent report from others showed that neural precursor cells were generated from human ES cells by the formation of EB (Zhang et al, 2001). Previous report from this laboratory also showed that neural precursor cells were isolated from human ES cells in conventional differentiation systems using formation of EB in serum-containing culture medium (Lee et al., 2004). However, serum-containing culture condition produced low frequency of neural precursor cells from human ES cells. The low frequency for the generation of neural precursor cells may be contributed to the differentiation of mesodermal lineages from ES cells under the serum-containing conditions in the deficit of neural lineages (Kim et al., 2002).

During development of neural tissues in mammals, neuroepithelial cells express various genes, including *sox1* (Pevny et al., 1998;Wood et al., 1999), *sox2* (Yuan et al., 1995; Avilion et al., 2003), *pax3* (Phelan et al. 1997; Goulding et al., 1991), *pax6* (Walther and Gruss, 1991; Stoykova et al., 1997; Pratt et al., 2000) and *nestin* (Lendahl et al.,1990). Consistent results were obtained for the

neural precursor cells differentiated from human ES cells in the present study, in which expressions of *sox1*, *sox2*, *pax3*, *pax6* and *nestin* were upregulated during neural differentiation. It was shown that expression of *sox2* is maintained in both undifferentiated ES and neuroectodermal cells (Yuan et al., 1995). Furthermore, SOX2 is required in the formation of neuroectoderm in *Xenopus* (Kishi et al., 2000). Consistently, expression of *sox2* was maintained in neural precursor cells as well as the undifferentiated human ES cells in this study. Results of this study showed that transcripts of *nestin* and *sox1* were expressed in undifferentiated ES cells. Similar results were reported in another human ES cell line, BG01 (BresaGen, Inc.), in which undifferentiated ES cells express *nestin* and *sox1* (Zeng et al., 2004). Interestingly, expression of nestin was not detected in undifferentiated ES cells by immunocytochemical analysis in this study, although expression of mRNA was present (data not shown). In contrast, low level of *oct4* still persisted in the isolated neural precursor cells, although its expression was dramatically downregulated during differentiation of human ES cells. These results can be explained by the report that OCT4 promoted neuroectoderm development and subsequent neuronal differentiation from mouse ES cells (Shimozaki et al., 2003). Results from immunocytochemical analyses confirmed those from RT-PCR analyses in protein level. Expressions of SOX1 and nestin in the differentiated cells confirmed the differentiation of neural precursor cells from human ES cells *in vitro*. Results from both RT-PCR and immunocytochemical analyses strongly support that human ES cells can be specifically induced to differentiate into neural precursor cells *in vitro*.

Because neural precursor cells are multipotent cells, they were analyzed for their capacity for differentiation into mature neuronal and glial lineages *in vitro*. Assessments of the mature neurons were carried out at 2 weeks after spontaneous differentiation on substrates in NS medium. Neurons were grown out of the neurospheres at 7~10 days after seeded on laminin. Neurites were projected from the cells in periphery of neurospheres. When analyzed by RT-PCR, cellular marker for mature neurons, *map2ab*, was expressed in both neural precursor cells and their differentiated cells, indicating that differentiation processes have already occurred even in the population of neural precursor cells freshly isolated. Since the neural precursor cells have the tendency to differentiate spontaneously under the culture conditions used in this study, optimization of culture condition needs to be developed for the maintenance of neural precursor cells *in vitro*. With another approach to improve their purity in culture, neural precursor cells need to be isolated before differentiation processes occurred. In contrast to expression of *map2ab*, those of *sox1* and *pax3* were maintained in the differentiated neurons, suggesting the presence of undifferentiated neural precursor cells even in the differentiated culture. The mixture of precursor cells and mature neurons in the culture potentially hampers the study for mechanisms underlying the early neural differentiation in humans. Results from immunochemical analyses of neuronal markers also confirmed the multipotency of neural precursor cells *in vitro*. With neuronal differentiation, neural precursor cells were able to differentiate into glial lineages, including astrocytes and oligodendrocytes *in vitro*. Extensive expression of GFAP in culture indicated the predominancy of astrocytes with minor population of oligodendrocytes under the condition used in this study. The optimized conditions needs to be developed for the directed differentiation of neural precursor cells into oligodendrocytes *in vitro*.

In conclusion, human ES cells can differentiate into neural precursor cells and mature neurons under serum-free conditions in this study. The formation and differentiation of neural precursor cells from human ES cells were confirmed by molecular and biochemical analyses. The protocols for generation of neural precursor cells in the present study provide efficient systems for applications of human ES cells in various areas. The neural precursor cells can be useful for the study of mechanisms on embryonic neural differentiation in humans. Neural precursor cells can also be used as a screening system for the development of new drugs. Recently, therapeutic applications of human ES cells have been suggested to cure various neurodegenerative diseases, including Parkinson's disease, Huntingtons disease, spinal cord injury and stroke. Based on their capacities for differentiation, neural precursor cells can be good source for the treatment of those disorders. Further studies on optimization of culture conditions for proliferation and differentiation need to be carried out.

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