Differentiation of GABAergic Neurons from Human Embryonic Stem Cells

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Abstract: Human embryonic stem cells (hESC) have the potential to proliferate indefinitely and to differentiate into all cell types comprising the body. For this reason, they may serve as an ideal source for cell replacement therapies to treat many forms of neurological and neurodegenerative diseases, such as Parkinson's disease and stroke. In order to be used for transplantation into particular disease types, it is essential to develop strategies that will enable hESC to differentiate into relevant cell types with sufficient amounts and the highest purity. In this study, we explored the optimal conditions for differentiating hESC into neural precursor (NP) cells using embryoid body (EB)-based suspension culture method, in which NP cells were stably generated from the neurospheres that were induced from the isolated neural rosette-like structures within EB. We further differentiated neurospheres into mature neurons, and found that they mainly consisted of forebrain-type or GABAergic neurons. Considering the differentiation potential of hESC-derived NP cells into forebrain-type or GABAergic neurons, we speculate that they can serve as useful cell sources for treating various neurological diseases, such as Huntington's disease.

Key words: human embryonic stem cells, neural differentiation, neural precursor cells, neurosphere, GABAergic neurons

1. Introduction

Human embryonic stem cells (hESC) are derived from the inner cell mass of the preimplantation-stage embryo, and have the potential to proliferate indefinitely and to differentiate into all cell types that constitute the body. For this reason, hESC have been widely regarded as ideal sources for cell replacement therapies to treat various forms of degenerative, metabolic and genetic diseases, including neurological disorders. For clinical application of hESC, it is essential to use pure population of relevant cell types in each corresponding disease. However, in the case of neural diseases and many other diseases, it has been shown that the fully differentiated specific cell types do not robustly survive following transplantation, making their effects minimal. To overcome these transplantation-associated survival problems, it is important to consider alternative cell types, in which they are committed to particular cell lineages and are less sensitive to cellular damages caused by transplantation procedures.

To date, many elegant protocols have been developed for inducing neural differentiation in ESC. These include embryoid body (EB)-based differentiation, in combination of treatments with retinoic acid, conditioned medium, or neural survival factors; the co-culture of ESC with stromal cells that possess neural-inducing activities; and the development of adherent feeder-free approaches. It has been shown that all three basic strategies can yield neural precursor (NP) cells, which can readily respond to appropriate developmental cues that direct fate toward midbrain dopamine neurons, somatic motor neurons, or forebrain neuron types (reviewed in Ref. 20). Therefore, it will be important to establish optimal conditions to isolate and maintain the early-stage ESC-derived NP cells for extended culture period, without losing their broad differentiation potential.

In this study, we explored the optimal conditions for differentiating hESC into NP cells using a step-wise differentiation protocol, in which neural induction was initiated using EB-based suspension culture method. In this protocol, NP cells were stably generated from the neurospheres that were induced from the isolated neural rosette-like structures within EB. We further differentiated neurospheres into mature neurons...
using specific conditions. Marker expression strongly suggests that the differentiated cells mainly consisted of forebrain-type or GABAergic neurons.

2. Materials and Methods

2.1 Culture of Human ES Cells

Human embryonic stem cells (hESC) were maintained according to the method described previously.\(^1,4,5\) Briefly, H9 hESC (obtained from WiCell, USA) at passage 50–55 were cultured on mitomycin C-treated mouse (CF-1) embryonic fibroblasts in hESC medium, which consists of DMEM/F-12 (Invitrogen) supplemented with 20% (v/v) knockout serum replacements (KSR, Invitrogen), penicillin (100 IU/ml, Welgene, Korea) and streptomycin (100 ug/ml, Welgene, Korea), 0.1 mM nonessential amino acids (Invitrogen), 0.1 mM \(\beta\)-mercaptoethanol (Invitrogen) and 4 ng/ml basic fibroblast growth factor (bFGF; R & D Systems, USA). Medium was changed daily. Passage of hESC was made about every 4 days by mechanical slicing of the undifferentiated region of the colony using a pulled Pasteur pipette following treatment of the culture with collagenase type IV (1 mg/ml, Invitrogen). Isolated hESC colonies were subsequently transferred onto freshly prepared mouse embryonic fibroblasts. All cultures were maintained at 37°C with 5% CO\(_2\) in atmosphere.

2.2 Neuronal Differentiation of Human ES Cells using the Suspension Culture Method

For EB formation, undifferentiated hESC were isolated using a pulled Pasteur following treatment with collagenase type IV, after which they were placed in non-adherent Petri dish for 3 days at 37°C for suspension culture. In this experiment, the EB medium consisted of DMEM/F-12 supplemented with 10% KSR, 1% penicillin, 1% streptomycin, 1% nonessential amino acids and 0.1% \(\beta\)-mercaptoethanol. After 3 days, medium was changed with the differentiation medium (DM), in which KSR of the EB medium was replaced by N2 supplements (Invitrogen) and 0.2 mM ascorbic acid (Sigma). Under this condition, EBs were maintained for additional 4 days.

2.3 Formation of Neural Rosette-Like Structures

At 7 days following the initiation of EB formation, EBs were transferred onto poly-L-ornithine (PLO) (15 ug/ml, Sigma) and fibroconnectin (FN) (1 ug/ml, Sigma)-coated tissue culture dishes in DM supplemented with 10 ng/ml bFGF. Under this condition, about 95% of EBs were attached on the PLO/FN-coated dishes. Within 2–3 days after attachment, neural rosette-like structures appeared. Medium was changed every other day.

2.4 Stable Generation of Neural Precursor (NP) Cells

To make single cells at NP stage, neurospheres were dissociated by treatment with Accutase\(^\text{TM}\) (Chemicon) for 10–15 min at 37°C. Following dissociation, NP cells were plated at a density of 5×10\(^4\) cells/cm\(^2\) onto PLO/FN-coated 60 mm\(^2\) tissue culture dishes for further expansion or reverse transcription polymerase chain reaction (RT-PCR) analysis, or PLO/FN-coated 4-well dishes containing 12 mm\(^2\) cover glasses for immunocytochemical analysis. At this stage, cells were maintained in the expansion medium, consisting of DMEM/F-12 supplemented with 1% penicillin, 1% streptomycin, 1% nonessential amino acids, 0.1% \(\beta\)-mercaptoethanol, N2 supplements and 20 ng/ml bFGF. Medium was changed every other day, and NP cells were sub-cultured every week. Cell analysis was performed according to immunocytochemical and RT-PCR methods.

2.5 Differentiation into GABAergic Neurons

To differentiate into mature neurons, neurospheres were directly attached onto PLO/FN-coated 60 mm\(^2\) tissue-culture dishes for RT-PCR analysis, or PLO/FN-coated 4-well dishes containing 12 mm\(^2\) cover glasses for immunocytochemical analysis. In this experiment, cells were maintained for 5 days in the neuronal differentiation medium, which consisted of DM medium supplemented with 20 ng/ml brain-derived neurotrophic factor (BDNF, R&D Systems) in the absence of bFGF. Cell phenotypes were analyzed using immunocytochemical and RT-PCR methods.

2.6 Immunocytochemistry

To analyze the marker expression of NP-stage and differentiated neuronal cells, cultures were maintained on 12 mm\(^2\) cover glasses. At each stage of differentiation, they were fixed in 4% paraformaldehyde for 15 min and were washed three times in Ca\(^++\), Mg\(^++\)-containing phosphate-buffered saline (PBS\(^+\)). Following primary antibodies were used in this study: Oct3/4 (1:500, Santa Cruz), stage-specific embryonic antigen 4 (SSEA4) (1:500, Hybridoma Bank), nestin (1:200, Chemicon), Sox2 (1:200, Chemicon), A2B5 (1:200, Chemicon), type III \(\beta\)-tubulin (Tuji) (1:500, Chemicon), Otx2 (1:500, Chemicon), Map2 (1:200, Chemicon), gamma-aminobutyric acid (GABA) (1:500, Sigma), glutamic acid decarboxylase 6 (GAD6) (1:100,