발생과 생식 제 12권 제 1호, 87~95 (2008)
Dev. Reprod. Vol. 12, No. 1, 87~95 (2008)

Human Embryonic Stem Cell-derived Neuroectodermal Spheres Revealing Neural Precursor Cell Properties

Hyo-Won Han[1], Janghwan Kim[1], Man-Jong Kang[2], Seong-Ju Moon[2], Yong-Kook Kang[1] Deog-Bon Koo[1,†] and Yee Sook Cho[1,†]

[1]Center for Regenerative Medicine, KRIBB, 52 Eoeun-dong, Yuseong-gu, Daejeon 305-806, Korea
[2]Dept. of Animal Science, Chonnam National University, Gwangju 500-757, Korea

인간 배아줄기세포 유래 신경전구세포의 특성 분석

한효원[1] · 김장환[1] · 강만종[2] · 문성주[2] · 강용국[1] · 구덕본[1,†] · 조이숙[1,†]

[1]한국생명공학연구원 재생의학연구센터, [2]전남대학교 동물자원학부

ABSTRACT : Neural stem/precursor derived from pluripotent human embryonic stem cells (hESCs) has considerable therapeutic potential due to their ability to generate various neural cells which can be used in cell-replacement therapies for neurodegenerative diseases. However, production of neural cells from hESCs remains technically very difficult. Understanding neural-tube like rosette characteristic neural precursor cells from hESCs may provide useful information to increase the efficiency of hESC neural differentiation. Generally, neural rosettes were derived from differentiating hEBs in attached culture system, however this is time-consuming and complicated. Here, we examined if neural rosettes could be formed in suspension culture system by bypassing attachment requirement. First, we tested whether the size of hESC clumps affected the formation of human embryonic bodies (hEBs) and neural differentiation. We confirmed that hEBs derived from 500×500 μm square sized hESC clumps were effectively differentiated into neural lineage than those of the other sizes. To induce the rosette formation, regular size hEBs were derived by incubation of hESC clumps (500×500 μm) in EB medium for 1 wk in a suspended condition on low attachment culture dish and further incubated for additional 1~2 wks in neuroectodermal sphere (NES)-culture medium. We observed the neural tube-like rosette structure from hEBs after 7~10 days of differentiation. Their identity as a neural precursor cells was assessed by measuring their expressions of neural precursor markers (Vimentin, Nestin, MSI1, MSI2, Prominin-1, Pax6, Sox1, N-cadherin, Otx2, and Tuj1) by RT-PCR and immunofluorescence staining. We also confirmed that neural rosettes could be terminally differentiated into mature neural cell types by additional incubation for 2~6 wks with NES medium without growth factors. Neuronal (Tuj1, MAP2, GABA) and glial (S100β and GFAP) markers were highly expressed after 2~3 and 4 wks of incubation, respectively. Expression of oligodendrocyte markers O1 and CNPase was significantly increased after 5~6 wks of incubation. Our results demonstrate that rosette forming neural precursor cells could be successfully derived from suspension culture system and that will not only help us understand the neural differentiation process of hESCs but also simplify the derivation process of neural precursors from hESCs.

Key words : Human embryonic stem cells, Neural stem cells, Neural precursor cell, Neroectodermal sphere, Embryoid body.

요 약 : 만능성 인간 배아줄기세포로부터 확립된 신경줄기세포 또는 신경전구세포는 퇴행성 신경질환 세포치료제로 이용될 수 있는 다양한 종류의 신경세포로 분화 유도될 수 있다. 하지만, 인간 배아줄기세포로부터 신경세포를 생산하기 위한 기술은 아직 많은 장애를 가지고 있다. 인간 배아줄기세포 유래 신경전구세포에서 특징적으로 나타나는 신경관 유사로제트에 대한 이해는 인간 배아줄기세포 신경 분화의 효율을 높이는데 유용한 정보를 제공할 것으로 사료된다. 일반적으로 신경로제트(neural rosette)는 분화 중인 배아체를 부착 배양함으로써 유도하지만, 이 방법은 시

† Correspondence: Center for Regenerative Medicine, KRIBB, 52 Eoeun-dong, Yuseong-gu, Daejeon 305-806, Korea, Tel: +82-42-860-4479, +82-42-860-4418, Fax: +82-42-860-4608, E-mail: june@kribb.re.kr, dbkoo@kribb.re.kr

간이 걸리고 복잡하다는 단점이 있다. 본 연구에서는 신경로제트가 부착배양을 하지 않고 부유배양으로 형성될 수 있는지 조사하였다. 우선적으로, 배아체 형성 및 신경분화에 인간 배아줄기세포 클럼프(clump) 크기가 영향을 주는지를 조사하였고, 사방 500 μm 크기의 인간 배아줄기세포 클럼프가 신경 분화 유도에 가장 효과적임을 확인하였다. 로제트 형성을 유도하기 위해, 사방 500 μm 크기의 인간 배아줄기세포 클럼프를 1주일 동안 EB 배양배지에 부유 배양함으로써 균일한 크기의 배아체를 얻은 후, NES 배양 배지에서 부가적으로 1~2주 동안 계속 부유 배양한 결과, 7~10일 사이에 신경관 유사 로제트가 형성됨을 확인하였다. 로제트 형성 세포의 신경전구세포로서 특성은 RT-PCR과 면역형광염색법을 이용한 신경전구세포 특이적 마커(vimentivi, nestin, MSI1, MSI2, Sox1, Tuj1) 발현을 통해 확인하였다. 또한, 성장인자를 제외한 NES 배양 배지에서 신경로제트를 2~6주 동안 지속적으로 배양하면 성숙 신경세포로의 말단 분화가 유도됨을 확인하였다. 신경세포 특이적 마커(Tuj1, MAP2, GABA)와 신경아교 특이적 마커(S100β, GFAP)는 2~3주 또는 4주 후에 각각 발현이 유도됨을 확인하였고, 희소 돌기아교 특이적 마커(O1과 CNPase)는 5~6주 후에 발현이 증가함을 확인하였다. 본 연구결과는 신경로제트가 부유 배양시스템에서 성공적으로 형성됨을 보여주고 있으며, 이는 인간 배아줄기세포의 신경 분화를 이해하고, 신경전구세포 유도 과정을 단순화하는데 효과적으로 이용될 수 있을 것으로 사료된다.

INTRODUCTION

Human embryonic stem cells (hESCs), which are derived from inner cell mass of the human blastocysts, have a potential to differentiate into the three germ layers and possibly all tissues of the human body (Thomson et al., 1998; Assady et al., 2001; Odorico et al., 2001; Levenberg et al., 2002; Mummery et al., 2002). Thus, they are a promising source for the supply of tissue specific cell types and have been developed as a novel model system to explain the molecular basis for the development and differentiation of various lineages (Reubinoff et al., 2000).

Neural progenitor cells (NPCs) can be derived from hESCs and differentiate into mature neural cell types, neurons, astrocytes and oligodendrocytes (Reubinoff et al., 2000; Zhang et al., 2001). NPCs and NPCs-derived neural cells are valuable both in the study of early human neurogenesis and in the creation of an unlimited source of donor cells for neural transplantation therapy. EBs formed from hESCs in suspension culture has a potential to differentiate into all three germ layers (Itskovitz-Eldor et al., 2000). Generally, neural differentiation of hESCs is induced by initial use of EBs in the presence of serum/serum replacement or coculture of hESCs with stromal cell lines (Zhang et al., 2001; Carpenter et al., 2001; Perrier et al., 2004).

Neural rosette, radially organized columnar epithelial cells, was detected during hESC neural differentiation and expressed early neroectodermal markers such as Pax6 and Sox1 (Zhang et al., 2001; Perrier et al., 2004). Early rosette stage cells display broad differentiation potential into various region-specific neuronal and glial cell types. However, too many cells are lost in the process of differentiation of EBs from hESCs and NSCs from EBs. In addition, the samples of EBs are heterogeneously produced, and the results from EBs used in the differentiation of NSCs cannot be reproduced. Furthermore, the isolation point of rosettes in adherent cells is not clear. Therefore, many researches are focused on the identification of factors that direct the expansion and differentiation of NPCs. Recent studies demonstrated that neural rosette cells derived from hESCs are novel neural stem cells with broad differentiation potential toward CNS and PNS fates and they can be expanded in the presence of FGF2/EGF *in vitro* without losing rosette properties (Perrier et al., 2004; Elkabetz et al., 2008).

Here, we demonstrate that the successful derivation of NES forming multiple neural rosettes from CHA3 hESC-derived hEBs with NES culture medium in suspension culture. The proliferating NES could be maintained by passaging every week. Terminal differentiation of NES into mature neural cell types was induced by NES medium without growth factors such as bFGF, EGF, and LIF.

MATERIALS AND METHODS

1. Culture of STO Cells

The STO cell lines were obtained from American Type Culture Collection (ATCC) and grown in Dulbecco's medified Eagle's medium (DMEM; GIBCO-BRL) supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine (Sigma), 0.1 mM β-mercaptoethanol, and 1% nonessential amino acid (NEAA, GIBCO-BRL). Feeder STO Cells used for hES culture were prepared by exposure to 8 krad of γ-irradiation and plated on 0.1% gelatin-coated culture dishes 1 day before the hESCs were plated.

2. Culture of Undifferentiated hESCs

Human ES cell line hES CHA-3 (Pochon Cha University, Korea) was used in this study and maintained by coculture with γ-irradiated STO feeder cells in ES culture medium (DMEM/F12 (Invitrogen) supplemented with 20% knockout serum replacer (SR, Invitrogen), 100 U/mL penicillin-streptomycin (Invitrogen), 1% NEAA (Invitrogen), 0.1 mM β-mercaptoethanol and 4 ng/mL human bFGF (Invitrogen). The cells were found to maintain a normal karyotype (XX), as well being shown to be positive for Nanog, Oct-4, SSEA-3, SSEA-4, Tra1-60, Tra1-81, and alkaline phosphatase (AP), while being negative for SSEA-1.

3. Derivation of Neural Rosette-Forming NES from hESCs

Undifferentiated hESCs were disaggregated into regular-sized square using a tissue chopper (Joannides et al., 2006) or ESCD (Kim et al., 2007). To generate hEBs, small-sized hESC clumps were transfered into low-attachment dishes and cultured in EB culture medium (DMEM/F12 supplemented with 10% SR, 100 U/mL penicillin-streptomycin, 0.1 mM NEAA, and 0.1 mM β-mercaptoethanol) for 7 days. To derivate the neuroectodermal sphere (NES), hEBs were cultured in a NES culture medium (DMEM/F12 supplemented with N2 (Invitrogen), B27 (Invitrogen), 1 μg/mL Leukemia Inhibitory Factor (LIF, Sigma), 100 U/mL penicillin-streptomycin, 20 ng/mL epidermal growth factor (EGF, Invitrogen) and 20 ng/mL bFGF). Culture medium was refreshed in every 2 days. NES has sub-cultured in every week by tissue chopper (MCLLWAIN, USA) in the 100 μm. NES (Fig. 5A, Stage Ⅳ) was plated to ECM- coated culture dishes, incubated with NES culture medium without growth factors, bFGF, EGF, and LIF for 4 wks. Their neural differentiation was evaluated by immunohisochemistry and RT-PCR in every week.

4. RT-PCR and Quantitative Real-Time PCR

Total RNA was extracted from the cells prepared using the RNeasy mini kit (Qiagen). First-strand cDNA was synthesized with 2 μg total RNA using Superscript First Strand Synthesis System (Invitrogen). Diluted cDNAs were used for polymerase chain reaction (PCR). PCR was performed under the following conditions: 1 cycle of 5 min at 95℃, 25~30 cycles of 30 sec at 95℃, 30 sec at 53~60 ℃, and 30 sec at 72℃, and the final single cycle of 72℃ extension for 7 min. Gene transcripts were quantitatively analyzed by real-time RT-PCR using SYBR Green (QuantiTect SYBR Green PCR Master Mix, Qiagen), according to the manufacturer's instructions, on the 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA) under the following conditions: 1 cycle of 5 min at 95℃, 25~30 cycles of 30 sec at 95℃, 30 sec at 53~60℃, and 30 sec at 72℃, and the final single cycle of 72℃ extension for 7 min. product were analyzed on 1.5% agarose gel and visualized by ethidium bromide (EtBr) staining. PCR products were normalized based on the GAPDH amounts.

5. Immunocytochemistry

NPCs were fixed with 10% paraformaldehyde (PFA) for 30 minutes at room temperature. The cells were permeabilized with 0.1% Triton X-100 (Fisher scientific) for 30 minutes, washed in 0.05% PBS/tween20 (PBST, MP Biomedicals) followed by incubation in blocking buffer (2% BSA in PBS) for 1 hour at room temperature, and then incubated overnight with primary antibody in blocking buffer at 4℃. Secondary antibodies conjugated with Cy2

Table 1. Primers for RT-PCR and Real-time PCR

Gene		Primer sequence (5'-3')
Oct3/4	Sense	AGTGAGAGGCAACCTGGAGA
	Antisense	CAAAAACCCTGGCACAAACT
Nanog	Sense	CAAAGGCAAACAACCCACTT
	Antisense	ATTGTTCCAGGTCTGGTTGC
Brachyury	Sense	ACCCAGTTCATAGCGGTGAC
	Antisense	ATGAGGATTTGCAGGTGGAC
VEGFR2	Sense	AGCGATGGCCTCTTCTGTAA
	Antisense	ACACGACTCCATGTTGGTCA
α-FP	Sense	CCGAACTTTCCAAGCCATAA
	Antisense	TGGCATTCAAGAGGGTTTTC
GATA2	Sense	AAGGCTCGTTCCTGTTCAGA
	Antisense	TCTCCTGCATGCACTTTGAC
Tuj1	Sense	ACCTCAACCACCTGGTATCG
	Antisense	GGGATCCACTCCACGAAGTA
Vimentin	Sense	GAGAACTTTGCCGTTGAAGC
	Antisense	TCCAGCAGCTTCCTGTAGGT
Nestin	Sense	AACAGCGACGGAGGTCTCTA
	Antisense	TTCTCTTGTCCCGCAGACTT
MSI1	Sense	TTCGGGTTTGTCACGTTTGAG
	Antisense	GGCCTGTATAACTCCGGCTG
MSI2	Sense	TTTGTAGGCGGGTTATCTGC
	Antisense	GCCATAGCTTGGAGCAAATC
Sox1	Sense	GGGAAAACGGGCAAAATAAT
	Antisense	CCATCTGGGCTTCAAGTGTT
Otx2	Sense	CAACAGCAGAATGGAGGTCA
	Antisense	CTGGGTGGAAAGAGAAGCTG
Pax6	Sense	ATGAGGCTCAAATGCGACTT
	Antisense	CATTTGGCCCTTCGATTAGA
N-Cadherin	Sense	GCCACCTACAAAGGCAGAAG
	Antisense	CCGAGATGGGGTTGATAATG
Prominin-1	Sense	TCAGTGAGAAAGTGGCATCG
	Antisense	TGTTGTGATGGGCTTGTCAT
MAP2	Sense	GACATGCAAGGCACAGAAGA
	Antisense	TTTTCCCTCATGGGAGTCAG
GFAP	Sense	CCTCTCCCTGGCTCGAATG
	Antisense	GGAAGCGAACCTTCTCGATGTA
Hoxb4	Sense	CAAAGTTCACGTGAGCACGG
	Antisense	GTTGGGCAACTTGTGGTCTT

Table 1. Continued

Gene		Primer sequence (5'-3')
S100β	Sense	ACTACTGCCTGCCACGAGTT
	Antisense	CCGTTAAAACAGCCTTTGGA
CNPase	Sense	GCTGTGCAGCTGACGTAGAG
	Antisense	AGGTTTGCCTTTCCCGTAGT
GAPDH	Sense	GAAGGTGAAGGTCGGAGTC
	Antisense	GAAGATGGTGATGGGATTTC

(green, 1:300) and Cy3 (red, 1:300) were purchased from Jacson Laboratories for 1 hour at room temperature. The cells were then rinsed with PBST, counter-stained with the 1 μg/mL DNA-binding dye 4’-6’-diamidino-2-phenylindole (DAPI) in PBS for 5 minutes at room temperature. Coverslips were mounted with mounting medium (Dakocytomation fluorescent mounting medium) onto glass slides and examined under a fluorescence microscope (Olympus IX51 microscope). The primary antibodies used were PSA-NCAM (1:200, Chemicon), Nestin (1:200, Chemicon), Musashi (1:500, Chemicon), Tuj1 (1:500, Covance), MAP2 (1:1000, Chemicon), GFAP (1:200, Chemicon), O1 (1:300, R&D), GABA (1:100, Chemicon). For the staining of cell surface marker O1, the step was skipped of permeabilization and changed washing solution to PBS. hESCs were plated onto gelatin coated-plastic coverslips (SPL). The primary antibodies used were against stage specific embryonic antigen, SSEA1 (1:300, Chemicon), SSEA3 (1:300, Chemicon), SSEA4 (1:300, Chemicon), TRA-1-60 (1:300, Chemicon), TRA-1-81 (1:300, Chemicon), Oct4 (1:500 Chemicon) that were confirmed undifferentiated condition of hESCs.

RESULTS AND DISCUSSION

1. Size-Dependent Neural Differentiation of hEBs

In this study, we used human ES cell line CHA-3 (Pochon CHA University) to derive neuroectodermal sphere (NES), forming neural rosette. Undifferentiated CHA-3 hESCs were maintained in co-culture with feeder STO cells and passaged by cutting colonies into regular-sized square using

disposable needle (Fig. 2A, B, Stage I). The undifferentiated state of hESCs was confirmed by the expression of ESC-specific markers including Oct-4, alkaline phosphatase (AP), Tra1-60, Tra1-81, SSEA-3, SSEA-4 (Fig. 1A). Differentiation marker SSEA-1 was not detected in our culture condition (Fig. 1A). Conventionally, neural differentiation of hESCs is induced via hEB formation *in vitro*. Under suspension culture condition, hESC clumps spontaneously aggregate to form hEBs. To generate hEBs of uniform size, hESC colonies were gridded (300×300, 500×500, or 800×800 μm) by a tissue chopper (Joannides et al., 2006) or embryonic stem cell divider (ESCD) (Kim et al., 2007) and transferred onto low attachment dish. To choose the efficient hESC clump size for the hEB formation and neural differentiation, hEBs were formed in EB culture medium with 10% SR and without bFGF. By monitoring the expression of neural lineage markers, we assessed that hEBs generated from 300×300 or 500×500 μm hESC clumps are efficient for the EB formation and neural differentiation (Fig. 1B, C). Thus, we used hEBs formed from 500×500 μm hESC clumps for further studies.

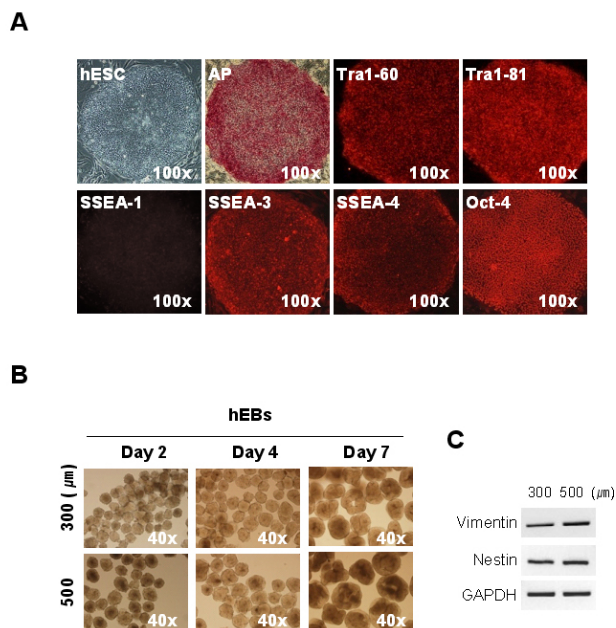

Fig. 1. hESC-derived EBs and their size dependency on neural differentiation. The fully grown hESCs cultured for 5~6 days were disaggregated into regular-sized hESC clumps by gridding using 21 gauge needle (Fig. 2B). For comparison, each group of hESC clumps in different size (300×300, 500×500, or 800×800 μm square) was separately cultured in suspension for 7 days using low attachment culture dishes. A. Phase contrast images of hESCs and immunostaining of hESC-specific markers. B. Phase-contrast images of suspended hEB. C. Expression of neural specific markers, vimentin and nestin, in hEBs.

2. Rosette Formation in Attached Culture

Recent studies showed that hESCs undergo neural differentiation in the presence of FGF-2 and form neural tube like structure called rosette (Zhang et al., 2001; Perrier et al., 2004). In general, hESC-derived neural precursor cells formed rosettes are generated from hEBs cultured in attached culture condition. We first examined if CHA-3 hESC-derived hEBs could undergo differentiation into neroectodermal linage and developed rosette structure, a characteristic neural stem/precursor cells in attachment condition. hESC-derived hEBs were formed in suspension culture for 4 days. hEBs were attached to matrigel or gelatin coated culture dishes and cultured in NES medium. Significantly, rosette structure resembling the early neural tube was observed from differentiating CHA-3 hEBs at 5~7 days after differentiation (Fig. 2A). With enzyme treatment, rosettes positive for Nestin and Sox1 can be isolated and expanded as free-floating cell aggregates, neural precursor cell aggregate, in a suspension culture (Zhang et al., 2001). They can be splitted into small-pieces every week and maintained up to about 8 passages. Further differentiation of neural precursor cell aggregates can be induced by plating onto ECM-coated culture dish and depletion of bFGF. All three major cell types of CNS (neuronal and glial cells, and oligodendrocytes) were differentiated from neural precursor cell aggregates formed form rosette (Zhang et al., 2001). As a result, we confirmed that CHA-3 hESCs could undergo neuroectodermal differentiation and form neural-tube-like rosette positive for neural precursor markers.

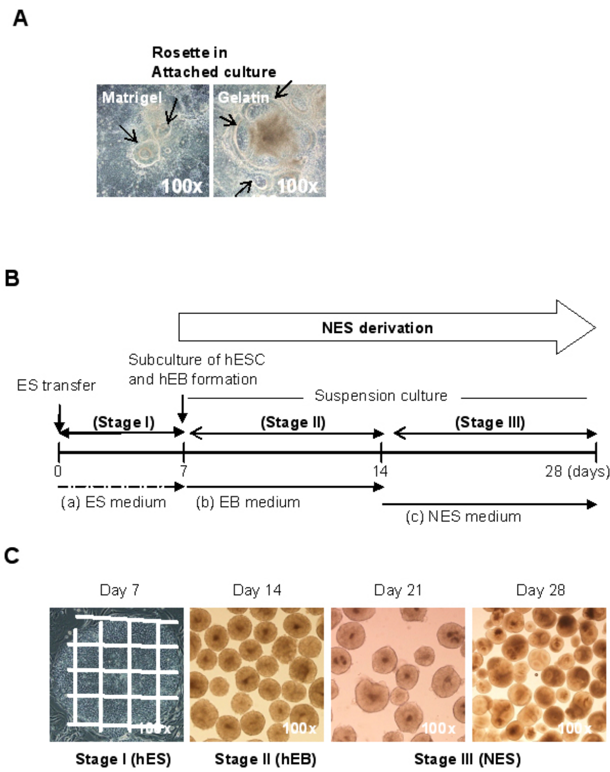

Fig. 2. Two distinct methods for "neural rosette formation" from hESCs. With our experimental conditions, size-specified hEBs, which were derived from hESCs clumps (500×500 mm square size), efficiently formed a neural rosette by culturing with NES culture medium either in attached (A) or suspension culture (B, C). A. Morphological observation of neural tube-like rosette structure formed from attached hEBs (indicated with arrow). B. Schematic diagram of neural rosette forming neuroectodermal sphere (NES) derivation from hESCs in suspension culture. C. Representative cell morphology (gridded hESCs, hEBs, and NES) at different stages of Fig. 2B.

3. Rosette Formation in Suspended Culture

In conventional method, hEBs should be attached into culture dish to generate rosette structure and detached from plate to maintain their self-renewal capacity retaining differentiation potential into neuron and glial lineage. Therefore, we speculated whether directed neuronal differentiation of hEB through bypassing conventional attachment step would generate rosette structure, neural precursor morphology. hEBs of uniform size were formed by incubation in EB culture medium for 7 days (Fig. 2B, C, Stage Ⅱ) and continuously cultivated after culture medium changed with NES culture medium supplemented with growth factors bFGF (20 ng/mL), EGF (20 ng/mL), and LIF (1 μg/mL) (Fig. 2B, C, Stage Ⅲ) in suspension. After 7～10 days of incubation, hEBs displayed morphological changes and rosette structure resembling neural tube or neurosphere (Zhang et al., 2000; Carpenter et al., 2001; Flax, et al., 1998) (Fig. 2C and 3A). In our experimental condition, over 90% of hEBs formed rosette structure (data not shown).

We further determined the neural identity of rosette forming hEBs in suspension culture by examining the expression level of neural precursor markers. The rosette forming hEBs showed positive expression of neural precursor markers such as Vimentin, Nestin, MSI1, MSI2, Prominin-1, Pax5, Sox1, N-cadherin, Otx2, and Tuj1, but negative expressions of the ESC markers Oct4 and Nanog, and other lineage markers specific endoderm (α-FP) or Mesoderm (VEGFR2, Brachyury, and GATA2) (Fig. 3B,

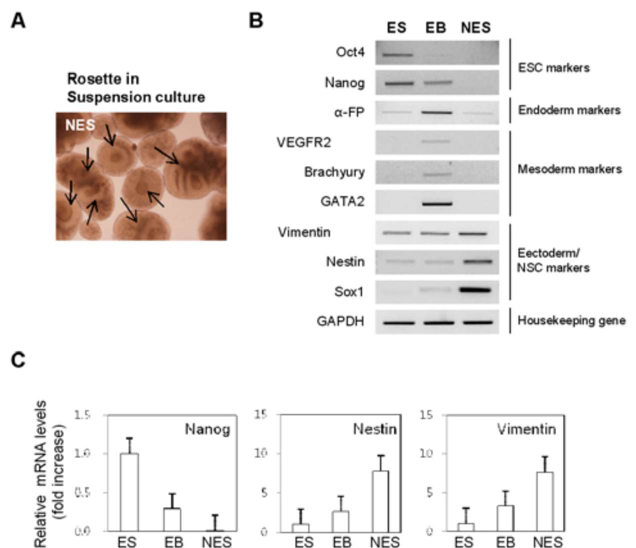

Fig. 3. Expression of NSC markers on rosette forming NES. NES was derived from hESCs in suspension culture as mentioned in Fig. 2B. Neural tube-like rosette structures were indicated with arrow (A). Expression of hESC-specific markers and lineage-specific markers were evaluated by RT-PCR (B), and by quantitative real-time PCR (C) in hESC, hEB, and NES.

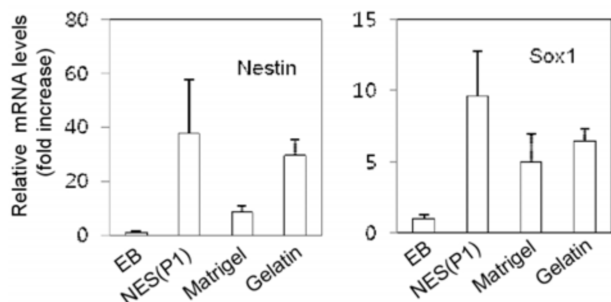

Fig. 4. Effect of extracellular matrix on neural differentiation of hEBs. Neural rosette was derived from hEBs in suspension or attached culture. For attached culture, extracellular matrixes, such as matrigel or gelatin, were coated on the plastic tissue culture dishes. Effect of extracellular matrix on neural differentiation was examined by determining the expression level of NSC markers Nestin (left panel) and Sox1 (right panel).

C and 5B). These results suggest that neuroectodermal spheres (NES) from hESCs were successfully derived *via* hEBs in suspension culture. Furthermore, the expression levels of NSC markers Nestin and Sox1 were higher at NES compared to neural precursor cells forming rosette in attached culture (Fig. 4). These results indicated that extracellular matrix does not appear to be required for the neural differentiation and rosette formation of hEBs. Proliferating NES was splitted into small-pieces (4-6 NES clumps) every week. Their expression level and self-renewal capacity of NSC markers was maintained during passaging process (data not shown).

4. Terminal Differentiation of NES

We next examined if self-renewing NES could undergo further differentiation into mature neural cell types. To induce terminal differentiation, NES of stage Ⅲ (Fig. 5A) was attached to matrigel-coated culture dish and incubated in NES culture medium without growth factors bFGF, EGF and LIF at stage Ⅳ (Fig. 5A). The expression level of neural lineage markers at various time points were analyzed during NES differentiation process by RT-PCR (Fig. 5B). Neuronal (Tuj1, MAP2, and GABA) and glial

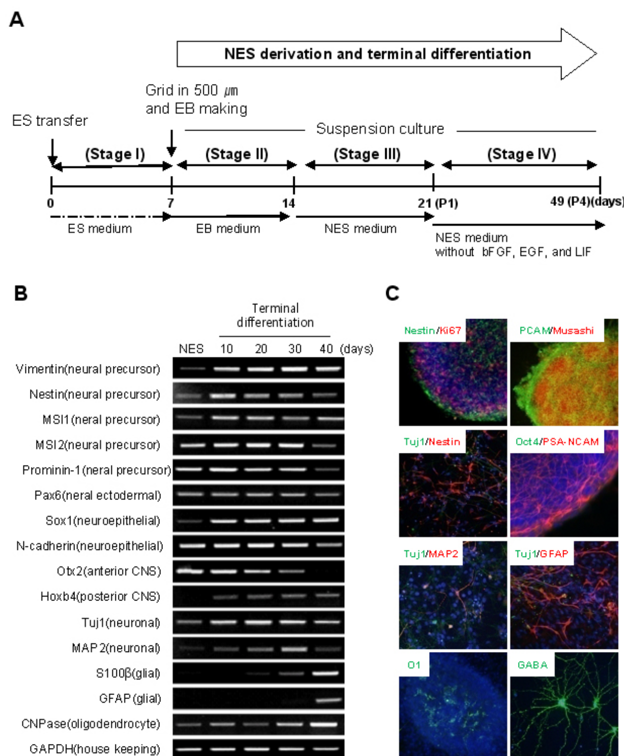

Fig. 5. Terminal differentiation of NES. Terminal differentiation of NES was induced by incubation with NES culture medium without growth factors such as bFGF, EGF, and LIF. A. Schematic diagram of terminal differentiation of NES. B. Expression of target genes during terminal differentiation process of NES. C. Immunostaining of neural specific markers during terminal differentiation process of NES.

(S100 β and GFAP) markers were highly expressed after 2～3 and 4～5 wks of differentiation, respectively. Expression of oligodendrocyte markers, O1 and CNPase, were significantly increased after 5～6 wks of differentiation. We also performed immunohistochemical analysis during NES differentiation process. Consistent with RT-PCR, the majority of differentiating NES positively expressed early neuronal markers such as Nestin, Tuj1, and Musashi, and proliferation marker Ki67 at the first week of differentiation (Fig. 5C). After 2～3 wks, more differentiated neuronal makers, MAP2 and GABA, were observed. The expression of glial cell markers, GFAP and S100 β, and

oligodendrocyte marker, O1, were detected after 4 and 5～6 wks of differentiation, respectively (Fig. 5C). These results demonstrated that NES could be a useful source of neural stem/precursor cells producing a variety cell types within the neural cell lineage. Recent report showed that functional dopamine neurons can be differentiated from hESC-derived spherical neural masses (SNMs) revealing neural precursor-like characteristics on a large scale (Cho et al., 2008), suggesting hESC-derived neural precursor cells are very useful for the production of mature neural cells.

In this study, we have developed the controlled method for the derivation of hESC-derived NES using hEBs of uniform size. Our results may help better understanding of neural differentiation of hESCs and simplification of generating neural cells.

ACKNOWLEDGEMENTS

We thank Professor Hyung-Min Chung for kindly providing CHA-3 human embryonic stem cells. This work was supported by KRIBB Research Initiative Program and KOSEF Stem Cell Research Program (M1064102000206N410200210).

REFERENCES

Assady S, Maor G, Amit M, Itskovitz-Eldor J, Skorecki KL, Tzukerman M (2001) Insulin production by human embryonic stem cells. Diabetes 50:1691-1697.

Carpenter MK, Inokuma MS, Denham J, Mujtaba T, Chiu CP, Rao MS (2001) Enrichment of neurons and neural precursors from human embryonic stem cells. Exp Neurol 172:383-397.

Cho MS, Lee YE, Kim JY, Chung S, Cho YH, Kim DS, Kang SM, Lee H, Kim MH, Kim JH Leem JW, Oh SK, Choi YM, Hwang DY, Chang JW (2008) Highly efficient and large-scale generation of functional dopamine neurons from human embryonic stem cells. Proc Natl Acad Sci USA 105:3392-3397.

Elkabetz Y, Panagiotakos G, Al Shamy G, Socci ND, Tabar V, Studer L (2008) Human ES cell-derived neural rosettes reveal a functionally distinct early neural stem cell stage. Genes Dev 22:152-165.

Flax JD, Aurora S, Yang C, Simonin C, Wills AM, Billinghurst LL, Jendoubi M, Sidman RL, Wolfe JH, Kim SU, Snyder EY (1998) Engraftable human neural stem cells respond to developmental cues, replace neurons, and express foreign genes. Nat Biotechnol 16:1033-1039.

Itskovitz-Eldor J, Schuldiner M, Karsenti D, Eden A, Yanuka O, Amit M, Soreq H, Benvenisty N (2000) Differentiation of human embryonic stem cells into embryoid bodies compromising the three embryonic germ layers. Mol Med 6:88-95.

Joannides A, Fiore-Heriche C, Westmore K, Caldwell M, Compston A, Allen N, Chandran S (2006) Automated mechanical passaging: a novel and efficient method for human embryonic stem cell expansion. Stem Cells 24: 230-235.

Kim MS, Kim J, Han HW, Cho YS, Han YM, Park JK (2007) Microfabricated embryonic stem cell divider for large-scale propagation of human embryonic stem cells. Lab Chip 7:513-515.

Levenberg S, Golub JS, Amit M, Itskovitz-Eldor J, Langer R (2002). Endothelial cells derived from human embryonic stem cells. Proc Natl Acad Sci USA 99:4391-4396.

Mummery C, Ward D, van den Brink CE, Bird SD, Doevendans PA, Opthof T, Brutel de la Riviere A, Tertoolen L, van der Heyden M, Pera M (2002) Cardiomyocyte differentiation of mouse and human embryonic stem cells. J Anat 200:233-242.

Odorico JS, Kaufman DS, Thomson JA (2001) Multilineage differentiation from human embryonic stem cell lines. Stem Cells 19:193-204.

Perrier AL, Tabar V, Barberi T, Rubio ME, Bruses J, Topf N, Harrison NL, Studer L (2004) Derivation of midbrain dopamine neurons from human embryonic stem cells.

Proc Natl Acad Sci USA 101:12543-12548.

Reubinoff BE, Pera MF, Fong CY, Trounson A, Bongso A (2000) Embryonic stem cell lines from human blastocysts: somatic differentiation *in vitro*. Nat Biotechnol 18: 399-404.

Thomson JA, Itskovitz-Eldor J, Shapiro SS, Waknitz MA, Swiergiel JJ, Marshall VS, Jones JM (1998) Embryonic stem cell lines derived from human blastocysts. Science 282:1145-1147.

Zhang SC, Wernig M, Duncan ID, Brustle O, Thomson JA (2001) *In vitro* differentiation of transplantable neural precursors from human embryonic stem cells. Nat Biotechnol 19:1129-1233.