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Generation and Maintenance of Neural Progenitor Cell Lines Derived from Human Embryonic Stem Cells by Small Molecules

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Abstract

Background: A new research perspective for human neurodevelopment and neurological disease modeling involves the use of human embryonic stem cells (hESC). The development of robust protocols that yield adequate neural cell populations with definite regional identities were the prerequisites for these comparative studies. **Materials and Methods:** We used small molecules cocktail to generate two neural progenitor cell (NPC) lines from hESCs as following: the first experimental group included Noggin, Dorsomorphin, CHIR99021 and A83-01(NDCA) and the second group composed of Noggin, Dorsomorphin, CHIR99021 and SIS3 (NDCS). To validate our findings, we expanded both cell lines for over 20 passages *in vitro* and checked for chromosomal stability, as well as expressions of neural and regional identity markers by immunofluorescence staining. Gene expression analysis was quantified by RT-PCR at different passages up to passage 20. **Results:** Both cell lines proliferated in an adherent culture system in the presence of FGF2. They retained progenitor characteristics of NESTIN, SOX1, and PAX6 protein expression, formed rosette-like structures, and had the high neurogenic capacity. Importantly, the NPC populations in their first 10 passages expressed rostral markers (OTX2 and TH), and the next 10 passages (10-20) changed their specification toward the hindbrain where they expressed HOXA3and HOXB2, which correlated with a normal central nervous system development pattern. **Conclusion:** These NPCs offer a new system to study human central nervous system development and disease modeling of specific neurodegenerative diseases. **[GMJ.2017;6(2):145-56] DOI: 10.22086/GMJ.V6I2.764**

Keywords: Neural Progenitor Cells; Small Molecules; Regional Identity

Introduction

Neural progenitor cells (NPCs) are self-renewing, multipotent cells that produce neuronal subtypes, oligodendrocytes, and astrocytes [1]. Biomedical application of NPCs and determining the appropriate regional diversity that dictates or constrains their potency to differentiate into a specific neuronal subtype is a matter of debate [2]. During the early developmental stages of an embryo, the forebrain, midbrain, hindbrain, and spinal cord delineate themselves from each other and become more specialized along the ante-

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rior-posterior and dorso-ventral axes.
This regional identity of NPCs occurs on many levels, e.g., proliferation [3], gene expression [3-5], commitment to specific cell types, and migratory patterns [6].
There is increasing evidence from animal studies where the formation of the nervous system can be induced by signals released from a region of the embryo known as the "organizer" which secretes several molecules that contain direct neural activity, including Noggin, Chordin, and Follistatin. These molecules act as inhibitors of a highly conserved mechanism in neurogenesis, the bone morphogenetic protein (BMP) signaling pathway. The BMP antagonism is a key for initiating the process of neural induction, and neuroepithelial specification believed to occur as a default pathway [7].
Although the BMP signaling pathway plays an essential role in the molecular determination of neural induction, other pathways such as fibroblast growth factor (FGF) [8, 9], Notch [7], Wnt and Shh [10] signaling cooperate to establish competence, regulate inducing signals, and maintain the induced state. According to research, activation of Notch signaling in cooperation with FGF signaling accelerates neural differentiation upon withdrawal of self-renewal stimuli. In contrast, inhibition of Notch signaling suppresses commitment to a neural fate [11]. The neural induction process is a balanced view that incorporates both instructive and inhibitory signals.
Immediately after neuroectoderm specification, it is regionally identified along the rostro-caudal axis of the embryonic body. The exact time points and mechanisms of neuroaxial regionalization are in doubt. As the default model during neuralization, initially, rostral precursors are specified followed by caudalizing signals that re-specify their subdivisions toward a posterior neuroaxis identity [8].
The forebrain arises from the anterior neuroectoderm, whereas the midbrain, hindbrain, and spinal cord arise from the posterior neuroectoderm. Other local signals secreted by neighboring developing neurons appear to cooperate with the main, previously mentioned neural signaling pathways to fully determine the neuronal fate [12, 13].
Ethical issues, as well as similarity challenges between animal models and human phenotypes, complicate the study of neurological disorders. Hence, human embryonic stem cells (hESCs) are an alternative approach to both basic science and regenerative medicine applications. The emphasis by scientists on neural differentiation of hESCs has led to the use of several protocols for their *in vitro* differentiation in an attempt to generate NPCs that have definite regional identity [14, 15].
Aggregate and adherent culture systems are two different systems used to explore human neurodevelopment from hESCs [14]. The adherent culture system for hESCs differentiation shows significant similarity and high efficiency between *in vivo* neuroectodermal development and *in vitro* differentiation [7]. However, generation of a regional specific NPC population from hESCs remains elusive. Another challenge is the lack of an optimal protocol to generate a homogenous, renewable, easy to the culture of regional specific NPC population which suitable for specific neurodegenerative diseases.
The conventional method for differentiation induction in stem cell biology is based on the use of recombinant growth factors that are the key elements of developmental signaling pathways. Despite the high cost, the precise effect of these elements during the induction process remains to be determined [16, 17]. Small molecules, which are chemically based modulating signaling pathway targets, have recently been introduced as suitable tools for stem cell manipulation [18].
Here, we have focused on the establishment of two neural progenitor populations from hESCs by small molecules which are the inhibitors of neural induction signaling pathways. Then, we defined both the regional identity and differentiation potentials of these cell lines over 20 passages *in vitro*.

Materials and Methods

Materials and Reagans
Dorsomorphin, CHIR99021, and A83-01 were obtained from Stemgent (USA). SIS3 and Noggin were purchase from cyman and R&D, respectively. KOSR, DMEM/F12,

L-glutamine, non-essential amino acid, B27 supplement, penicillin/streptomycin and N2 supplement were obtained from Gibco (USA). Trypsin-EDTA, Poly-L-ornithine, laminin, 4% paraformaldehyde (PFA), Triton X-100, goat serum, rabbit serum, bovine serum albumin, fetal bovine serum (FBS), thymidine, phosphate buffered saline (PBS), ethidium bromide were obtained from Sigma-Aldrich (Germany). TRIzol reagent, colcemid, bFGF were purchase from Invitrogen (USA), Roche (Switzerland) and Royan Institute (Iran), respectively.

Culture of hESCs

Royan H6 (passage 42) hESCs were proliferated and passaged under feeder-free conditions in stem cell culture medium that consisted of 100 ng/ml of basic FGF, as previously described [19]. The culture medium was renewed every other day.

Generation of hESC-Derived NPCs

We evaluated the possibility of generating NPCs that had a rostro-caudal identity. Our experimental groups consist of different small molecule cocktail which described later. The first group cocktail included Noggin, Dorsomorphin, CHIR99021, A83-01 (NDCA) and the second group included Noggin, Dorsomorphin, CHIR99021, and SIS3 (NDCS).

In the proposed protocols for both experimental groups, we used dual SMAD inhibition that consisted of Dorsomorphin (3μM) and Noggin (250 ng/ml) accompanied by WNT signaling activation (CHIR99021, 3μM). The experimental groups compared two inhibitors of transforming growth factor beta (TGFβ) signaling, A83-01 (2 μM) and SIS3 (0.5 μM). The base neural induction medium for hESCs included DMEM/F12, 5% KOSR, 1mM non-essential amino acid, 1mM L-glutamine, penicillin/streptomycin 1mM, 1% N2 supplement, and 0.001% B27 supplement.

As soon as rosette structures, as the main indicator of neuroepithelium differentiation, appeared 9-12 days after induction. We manually selected these structures and re-plated them on 0.001% Poly-L-ornithine and 1 μg/ml laminin coated plates. A suitable medium for expansion of NPCs consisted of the above-mentioned base neural induction medium plus the addition of 40 ng/ml bFGF.

Afterwar, NPCs were expanded and passaged with trypsin/EDTA for 3-5 days. The media were renewed every other day [20]. The resultant cell populations were characterized by immunofluorescence staining and gene profile analysis.

Differentiation of NPCs in Vitro

We induced both spontaneous to validate the differentiation potential in both NPC lines. To induce neural differentiation, we placed 1×10^5 cells into Poly-L-ornithine/laminin coated six-well plates that contained neural expansion medium. After 24h, the medium was changed to differentiation medium. Spontaneous differentiation was induced in medium that consisted of neurobasal medium /DMEM-F12 (1:1), %, 1 mM non-essential amino acid, 1 mM L-glutamine, 1 mM penicillin/streptomycin, 1% B27, 5% KOSR, and 1% N2 supplement for 35 days. We renewed one-half of the medium every 5 days.

Immunofluorescence Staining

The cells were fixed in 4% PFA for 20 min, followed by permeabilization with 0.1% Triton X-100 for 10 min, and blocked in 10% goat or rabbit serum in PBS for 1h at room temperature (RT). Cells were subsequently incubated overnight with primary antibodies at 4°C, then washed and incubated with FITC- or Texas Red-conjugated secondary antibodies. Table-1 lists the primary and secondary antibodies for immunofluorescence and flow cytometry. The cells were analyzed with a fluorescent microscope IX71 (Olympus).

Flow Cytometry

Cells were singularized by 0.05% trypsin-EDTA and fixed in 4% PFA for 20 min at RT. Next, the cells were washed and permeabilized with 0.1% Triton X-100 for 10 min at RT, followed by the addition of 10% bovine serum albumin in PBS to block non-specific antibody binding. Then, the cell suspension was incubated overnight at 4°C with the appropriate primary antibody diluted in PBS that contained 0.1% FBS and 0.1% BSA. Subsequently, cells were washed three times and incubated for 60 min

at 4°C with secondary antibodies. Table-1 lists the primary and secondary antibodies. After washing with staining buffer, the cells were analyzed with a flow cytometer (FACS-Calibur; BD Biosciences). The experiments were performed in triplicate, and the acquired data were analyzed with Cell Quest software (BD Biosciences).

Karyotype Analysis

We treated the NDCA and NDCS groups with 0.66 mM thymidine for 16 h at 37°C in 5% CO2. Then, treated cells were washed with PBS; after 5h, they were treated for 30 min with 0.15 mg/ml colcemid. Both NPC groups were exposed to 0.075 M KCl at 37°C for 16 min and then fixed in three consecutive immersions in an ice-cold 3:1 ratio of methanol: glacial acetic acid, after which they were dropped onto pre-cleaned, chilled slides. Standard G-band staining was used to visualize the chromosomes. We screened more than 50 metaphase spreads after which 10 were evaluated for chromosomal re-arrangements.

RNA Isolation and Reverse Transcription for Gene Expression Analysis

The gene expression profile was studied at the rosette stage and in passages 5, 10, 15, and 20 compared to undifferentiated hESCs in both studied groups. For each sample, total RNA was prepared using TRIzol reagent according to the manufacturer's recommendations. Subsequently, 2µg of total RNA determined using a UV/visible spectrophotometer (WPA, Biowave II) were reverse transcribed to first-strand cDNA with a RevertAid First-strand cDNA Synthesis Kit and random hexamer primer (k1632, Fermentas) in a 20µl reaction according to the manufacturer's instructions. Standard polymerase chain reaction (PCR) was performed in a thermal cycler (Eppendorf). PCR products were run on a 2% agarose gel and visualized by ethidium bromide.

Table 1. Antibodies Used in This Study

Antibody name	Species	Clonality	Dilution	Source
Primary Ab				
NESTIN	Mouse	Monoclonal	1:100	Chemicon, MAB5326
PAX-6 (H-295)	Rabbit	Polyclonal	1:200	Santa Cruz, CA, USA, 11357
SOX1	Rabbit	Polyclonal	1:1000	Abcam, Cambridge, UK, 22572
β-TUBLIN III	Mouse	Monoclonal	1:250	Sigma-Aldrich, T8660
GLIAL FIBRILLARY ACIDIC PROTEIN (GFAP)	Mouse	Monoclonal	1:200	Sigma-Aldrich, G3893
NEUROFIBRILLARY FIBROBLAST (NF-H)	Mouse	Monoclonal	1:200	Sigma-Aldrich, N0142
Secondary Ab				
FITC anti-mouse IgG	Mouse	Monoclonal	1:200	Chemicon, AP308F
FITC anti-rabbit IgG	Rabbit	Monoclonal	1:200	Sigma-Aldrich, F1262
Texas red anti-mouse IgG	Mouse	Monoclonal	1:100	Jackson Immunoresearch Lab., 315-075-003, Hamburg, Germany
FITC anti-mouse IgM	Mouse	Monoclonal	1:100	Sigma-Aldrich, F9259

For quantitative real-time RT-PCR, the cDNA was diluted 1:7. A total of 2µl cDNA was used in a 20 µl PCR reaction that contained 10µl 2x Power SYBR Green Master Mix (Applied Biosystems) and 1µl of 5 pmole forward and reverses primers. Reactions were run on a Rotor-Gene 6000 (Corbett Life Science). All qRT-PCR experiments were performed with three technical and three independent biological replicates. The amount of mRNA was normalized to GAPDH mRNA and data analysis was performed using the ΔΔCt method. Primer sequences are listed in Table-2.

Cryopreservation of NPCs

Cryopreservation of both NDCA and NDCS groups were performed as previously described [20].

Statistical Analysis

We counted the numbers of cells from seven random fields per sample at different passages (5 and 20) and the total cell numbers for immunofluorescence staining. Flow cytometry data for both NPC groups was represented an average of three samples from each independent experiment. The whole statistical analysis was presented as mean±SEM.

Results

Generation of hESC-Derived NPCs

hESC-H6 cells were maintained and passaged weekly in feeder–free medium supplemented with 100 ng/ml bFGF (Figure-1) [19]. The hESCs were induced to generate NPCs by a combination of small molecules in the two different groups. Induction medium in the first group (NDCA) consisted of Noggin, Dorsomorphin, CHIR, and A83-01 (Figure-2), whereas the second group (NDCS) consisted of Noggin, Dorsomorphin, CHIR, and SIS3 (Figure-3). Neural rosette structures that appeared within 9 to 12 days after induction were manually selected after a mild treatment with 0.008% trypsin/ 2mM EDTA.

The isolated structures (rosettes) after enzymatic treatment were re-plated on Poly-L-ornithine and laminin coated plates in neural expansion medium supplemented with 40 ng/ml bFGF for 1 week. Both NPCs lines were passaged every 3-5 days.

Table 2. Primers Used in This Study

Genes	Primer sequences (5'-3')	Size (bp)	Annealing Temp. (°C)
GAPDH	F: 5' CTC ATT TCC TGG TAT GAC AAC GA 3' R: 5' CTT CCT CTT GTG CTC TTG CT 3'	121	60
NESTIN	F: 5' CTCCAGAAACTCAAGCACC 3' R: 5' TCCTGATTCTCCTCTTCCA 3'	144	60
SOX1	F: 5' GTG TAC CCT GGA GTT TCT G 3' R: 5' TAG TCT GTG CCT CTA AGG TG 3'	200	60
PAX6	F: 5' CGGTTTCCTCCTTCACAT 3' R: 5' ATCATAACTCCGCCCATT 3'	196	60
OTX2	F:5' CTC TGA ACC TGT CCA CCC 3' R:5' AGC AAG TCC ATA CCC GAA 3'	147	60
TH	F: 5' CTG TGG CCT TTG AGG AGA AG 3' R: 5' ATG GTG GAT TTT GGC TTC AA 3'	128	60
HOXA1	F: 5' AGT GCT GGA GCG AAG AAG AG 3' R: 5' TCT CGC CAG TTC ATC TTT CA 3'	148	60
HOXA3	F: 5' TATTCCTCTGCCCTGGACAC 3' R: 5' GGCAAAGTCTGGGAAACTCA 3'	79	60
HOXB2	F:5' GCC ACG TCT CCT TCT C 3' R:5' CTT CTC CAG TTC CAG CAG 3'	127	60
HOXC5	F:5' TCA AAG AGT CAC AAA TCA CC 3' R:5' TCC ATA GTT CCC ACA AGT T 3'	104	60

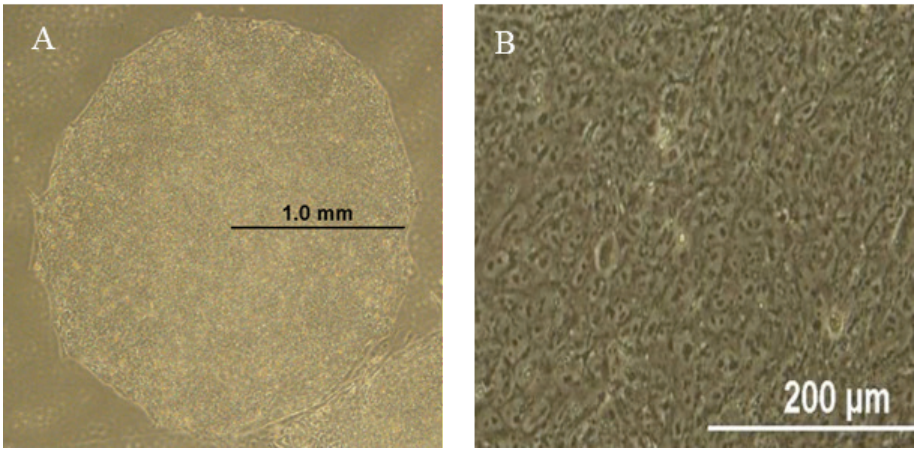

Figure 1. (A) hESC colony (scale bar 1µm, 4X microscope lens); **(B)** hESCs at higher magnification (scale bar 200µm, 20x microscope lens).

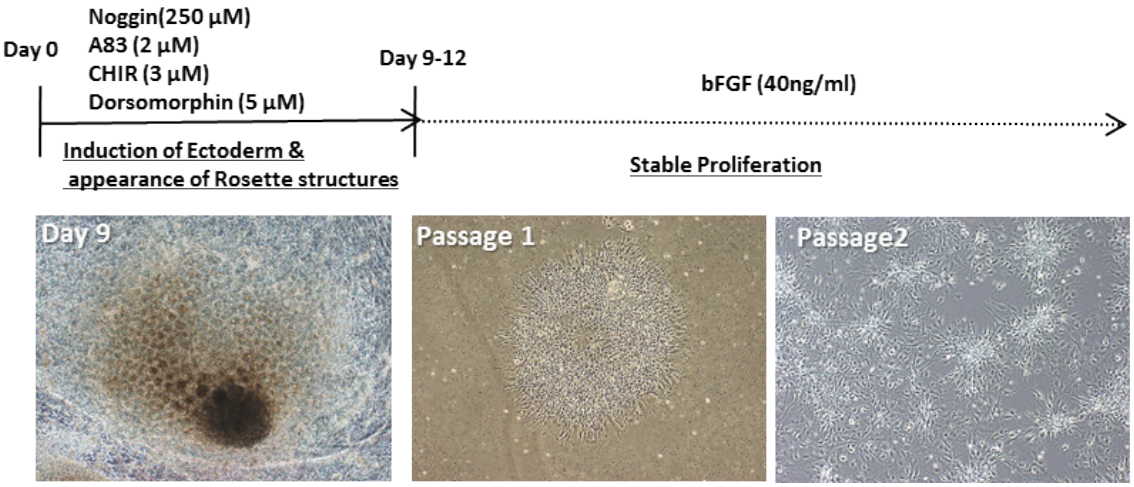

Figure2. Neural rosette structure formation after 9-12 days of induction with Noggin, Dorsomorphin, CHIR99021, and A83-01 (Day 9). Neural rosette structures after re-plating in the first passage (Passage1). The NPCs after 10 passages *in vitro* (Passage10).

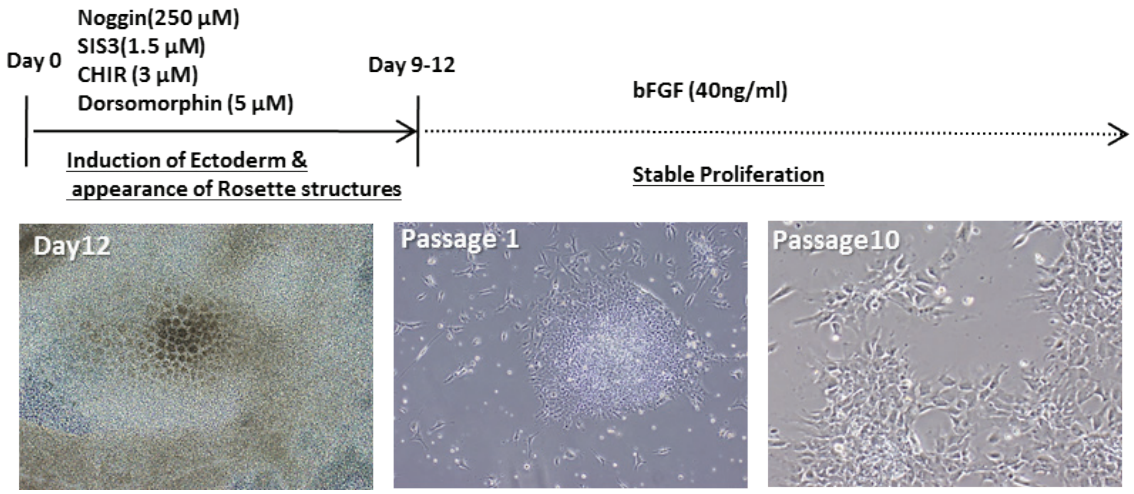

Figure 3. Neural rosette structure formation after 9-12 days of induction with Noggin, Dorsomorphin, CHIR99021, and SIS3 (Day 12). Neural rosette structures after re-plating in the first passage (Passage 1). The NPCs after 10 passages *in vitro* (Passage 10).

Characterization of NPCs

Both NPC lines (NDCA and NDCS) were evaluated for expression of neural progenitor markers by immunofluorescence staining, flow cytometry, karyotype, and gene expression analyses. These analyses were performed at different passages (rosette, 5, 10, 15, 20). The results of immunofluorescence staining and flow cytometry showed that the produced NPC cell lines uniformly expressed NESTIN, PAX6, and SOX1. A smaller number were positive for GFAP (Figure-4A and Figure-5A). The results confirmed their homogeneity and NPC identity. Under our culture conditions, karyotype analysis demonstrated a normal chromosomal stability after treatment with small molecules and passaging *in vitro* (Figure-4A and Figure-5A).

Real-time PCR analysis was used to evaluate potential regional specification of NPCs in both studied groups within different passage numbers (rosette, 5, 10, 15, 20). The result showed that both cell lines strongly expressed anterior markers OTX2 and TH during the first 10 passages *in vitro*. However, this marker significantly decreased at later passages. Interestingly, with continued passaging of both NPC lines the posterior markers (HOX genes) that included HOXA3, and HOXB2 up-regulated, whereas HOXC5 significantly unregulated during the second ten passages (Figure-6). However, the HOXC5 gene expression pattern differed from other HOX genes and expressed sharply in the middle passages (5-10 time) of both studied cell lines.

Moreover, our findings supported the results of previous research which demonstrated that long-term expanded human NPCs derived from hESCs underwent caudalization with progressive loss of rostral markers from early to later passages [21-23].

This specification property of hESC-derived NPCs remains to be elucidated. Different hypotheses are suggested to explain this distinct regional specification. Most likely, selection or re-patterning of NPCs occurs, which is induced by continuous exposure to bFGF.

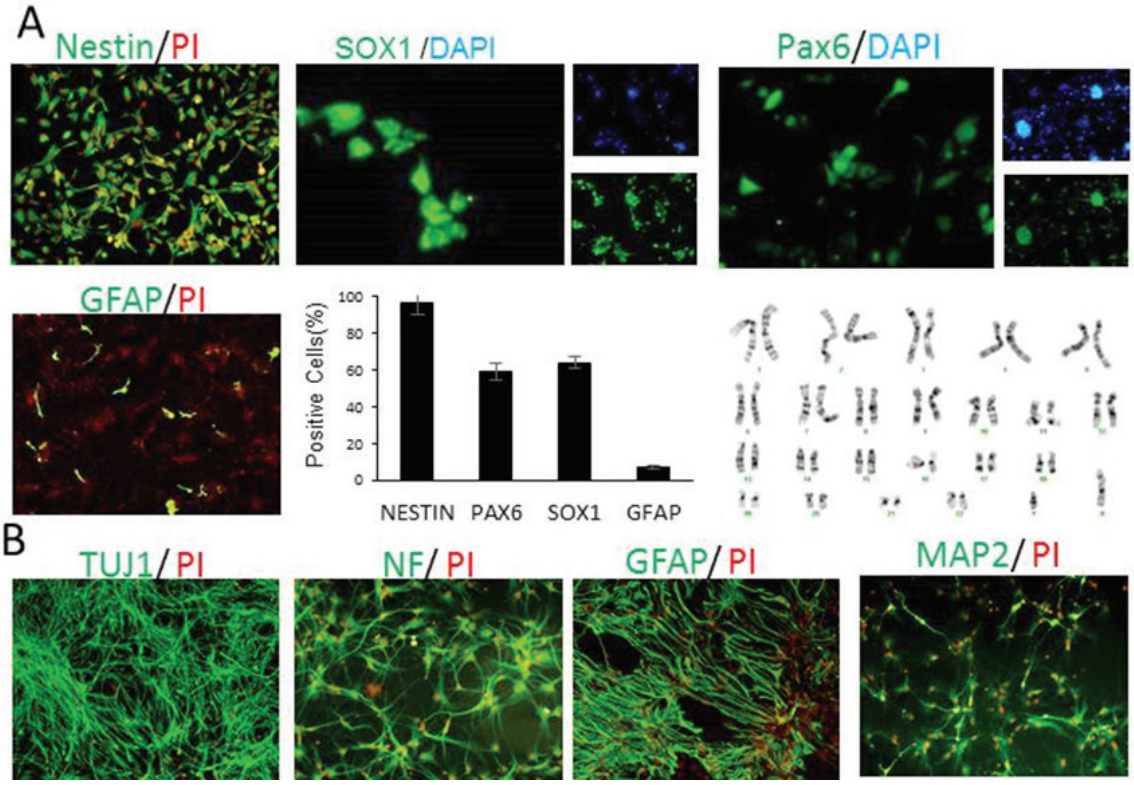

Figure 4. Characterization of the NDCA group of NPCs. **A:** Immunofluorescence staining for neural progenitor markers NESTIN, SOX1, PAX6, and GFAP. Flow cytometry analysis in the histogram for these markers. Karyotype analysis of chromosomal stability of NPCs after 10 passages of the NDCA group *in vitro*. **B:** The developmental potential of the NDCA group into neurons and astrocytes with spontaneous differentiation after 14 days of plating.

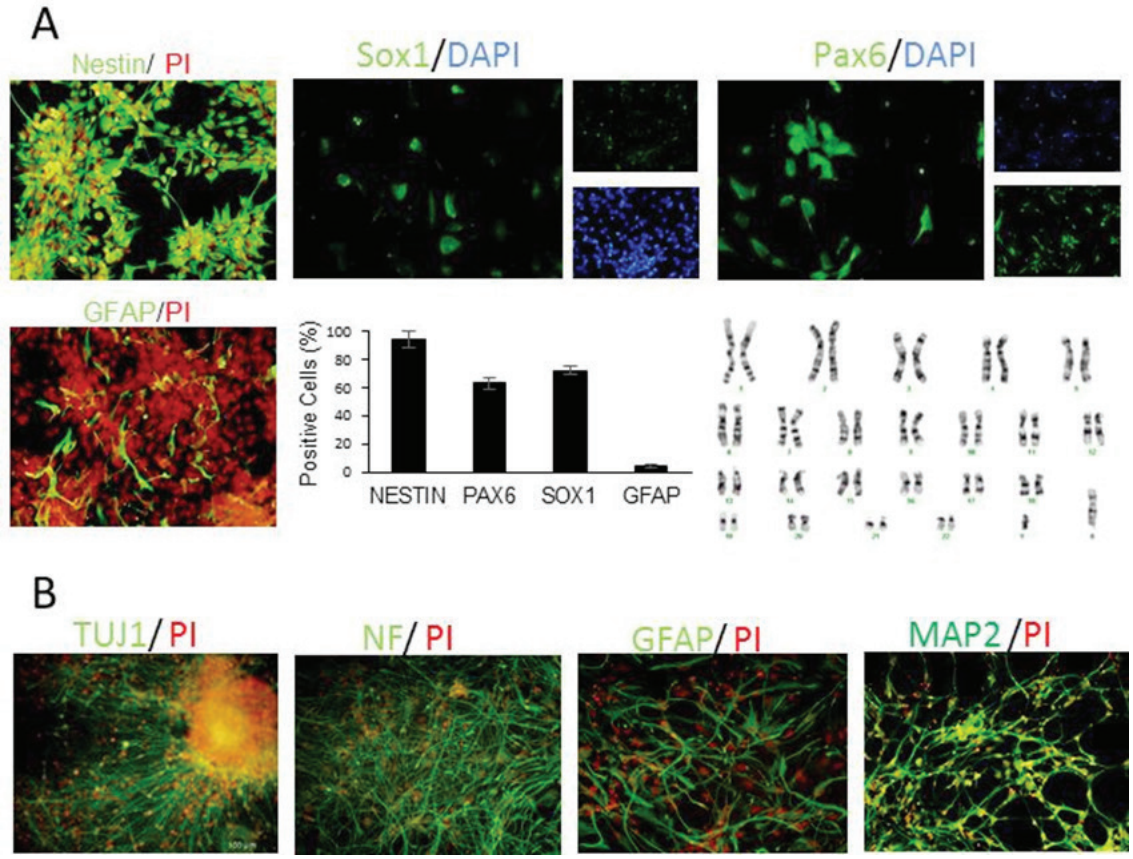

Figure 5. Characterization of the NDCS group of NPCs. **A:** Immunofluorescence staining for neural progenitor markers NESTIN, SOX1, PAX6, and GFAP. Flow cytometry analysis in the histogram for these markers. Karyotype analysis of chromosomal stability of NPCs after 10 passages of the NDCS group *in vitro*. **B:** The developmental potential of the NDCS group into neurons and astrocytes with spontaneous differentiation after 14 days of plating.

Finally, we assessed the differentiation capacity of both NDCA and NDCS to give rise to neurons and glia. Hence, we induced passage-15 cells to spontaneously differentiate in a medium based on expansion medium without growth factors over a period of 35 days.

The NDCA group differentiated into large numbers of beta III-tubulin (~ 90%), NF (~79%) and MAP2 (~ 63%) positive neurons and a smaller fraction of GFAP (~23%) positive astrocytes (around 35%; Figure-4B) within the first 14 days of re-plating.

However, the NDCS group differentiated into beta-III-tubulin (~ 86%), NF (~ 90%), MAP2 (~56%), and GFAP (~14%) positive neurons and astrocytes (Figure-5B). This differentiation pattern was observed during the maintenance period of the studied NPC populations in different passage numbers *in vitro* which demonstrated their potential to produce specific neurons in further direct differentiation studies.

Cryopreservation of NPCs

Post-thawing survival, the capability to retain proliferation ability, and stability in neural progenitor identity are the most important indications that cell lines can be cryopreserved. Cryopreservation of both groups has demonstrated that most cells in the NDCA (~ 85±5 percent) and NDCS (~ 93± 6.25 percent) groups survived after thawing. To improve the post-thawing viability rates, we used ROCK inhibitor Y-27632 overnight in the expansion culture medium, as previously described [20]. Both populations maintained their neural progenitor properties as evidenced by expression of neural markers (e.g., NESTIN) after plating on laminin/poly-l-ornithine coated dishes and preserved their chromosomal stability after thawing (Figure-7).

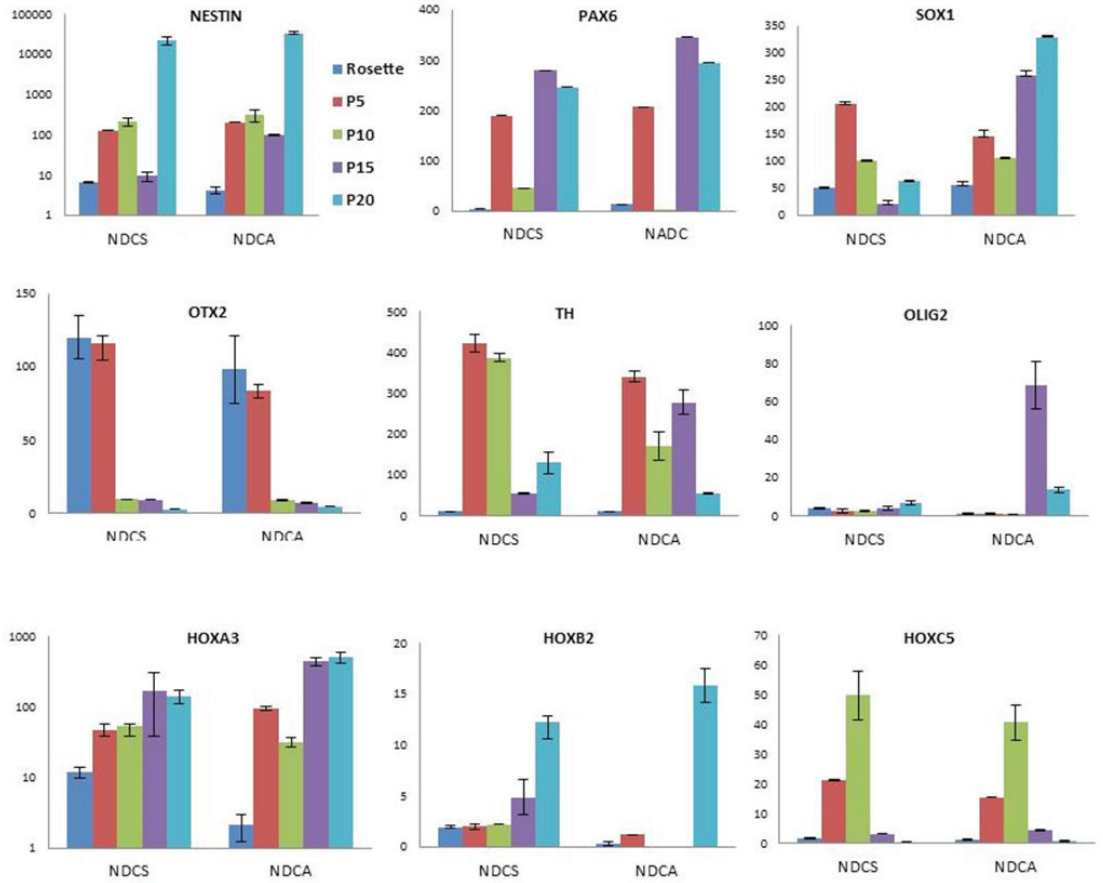

Figure 6. Gene expression profile of hESC-NPCs. **A:** Quantitative real-time RT-PCR of RNA samples collected at different passages to determine the regional specification of both NPC groups. Relative fold induction was calculated using the ΔΔCt method, relative to undifferentiated hESCs.

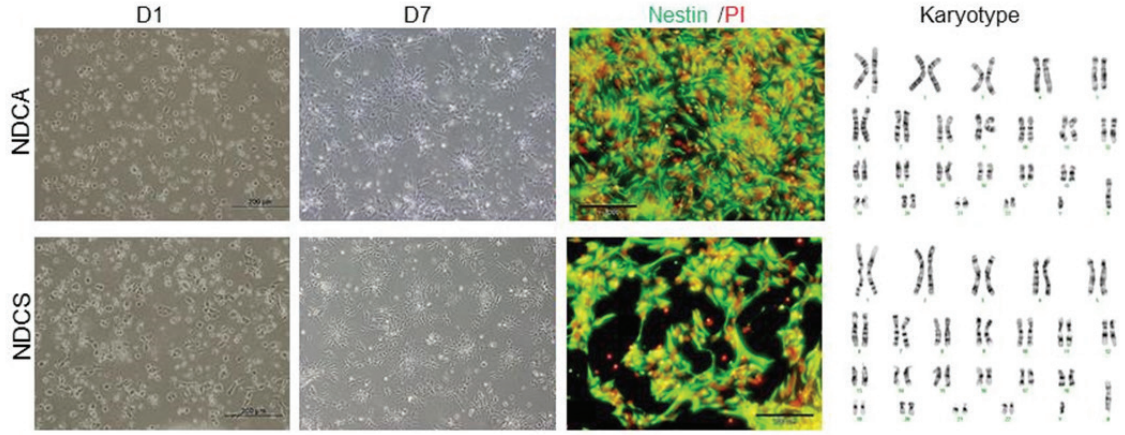

Figure 7. Cryopreservation and recovery of both NPCs groups (NDCA and NDCS). **D1:** Phase contrast image one day after thawing; **D7:** one week after defreeze; **Nestin/PI:** Immunofluorescence staining for NESTIN expression for both cell lines. Cytogenetic analysis after thawing.

Discussion

With the notion of stem cell differentiation studies information regarding developmental pathways have been raised. Regarding the landscape of analyzing the molecular pathogenesis of neurodegenerative diseases, hESC-derived NPCs have paved a new way for regenerative medicine. The introduction of defined, efficient protocols that are reproduc-

ible and quantitatively generate specific neuronal populations is inevitable. Finding the optimum protocols that generate regionally specific neural populations can accelerate the determination of disease-relevant mechanisms and increase the feasibility of their treatment.
Recently, significant progress exists with NPC therapy in regenerative medicine of neurodegenerative diseases such as spinal cord injury [24], Parkinson [25], multiple sclerosis (MS) [26], and stroke [27].
Koch *et al*. reported the successful generation of a long-term expandable NPC line from hESCs [21].
They used bFGF and hEGF growth factors to produce a pure population of *in vitro* NPCs which maintained clonogenicity and stable neurogenesis for over 150 passages.
Continuously, we produced long-term NPCs from iPSCs [20].
Our generated NPCs had the general capacity to differentiate into neural and glial cells and maintained in high concentrations of bFGF (100 ng/ml) and EGF (20 ng/ml).
Falk *et al*. described a protocol that generated neuroepithelial-like stem cells from three hESC and six iPSC lines.
All reported cell lines had continuous expandability in the presence of FGF2 and EGF, along with stable and hindbrain specific neural differentiation capacity [23].
More recently, the introduction of small molecules as chemically based developmental pathway inhibitors encouraged numerous scientists to use these molecules in research due to their cost effectiveness, ease of use, and non-immunogenic effects [28, 29].
In the current study, we used two combinations of small molecules involved in neural development pathways to reach expandable NPCs *in vitro*. These NPCs were evaluated during the first 20 passages by gene analysis.
In both studied groups, with dual SMAD inhibition (BMP signaling pathway) by Noggin and Dorsomorphin, neuroepithelium formation was induced.
The induction continued with WNT signaling activation using CHIR99021 (small molecule) to enhance cell proliferation and dorsalization of neural progenitor populations. In addition to above mentioned small molecules, TGFβ signaling inhibitors A83-01 (a specific inhibitor of ALK5, 4, 7 kinases) and SIS3 (a specific inhibitor of SMAD3 and receptor of ALK5) were compared in our neural induction.
Results showed that the large population of our produced cell lines in both groups had a stable expression of NPCs markers such as NESTIN, PAX6 and SOX1 during long-term expansion *in vitro*.
They had normal chromosomal stability after induction by small molecules and an *in vitro* thawing process. Interestingly, these two distinct NPC populations could be expanded long-term in the same culture conditions and cryopreserved successfully for long-term storage.
Following for clarifying of both NPCs regional identity, we used real-time PCR to evaluate the expressions of TH and OTX2 genes as anterior and HOXA3, HOXB2 and HOXC5 as posterior neural markers. Remarkably, the initial developmental stage of NDCA and NDCS cell lines seemed to show increased tendency to regional re-specification.
The current hypothesis came from the gene profile analysis of both groups which have shown up-regulation of anterior gene markers during the first 10 passages with the gradual dominance of posterior gene expression patterns at passages 10 to 20. This overall posteriorization supported the results of current and previous studies [20, 21, 23].
Results from spontaneous differentiation of the studied groups showed that general neuronal markers TUJ1, NF, MAP2 highly expressed in both NPC groups.

Conclusion

Taken together, by employing a chemical strategy, we could generate two NPC populations in adherent, defined and easy to culture system with repeated freeze-thaw capacity. These populations might supply a consistent source of human neurons for drug screening and biomedical applications. Also, based on their self-renewal potential, they were amenable to various genetic modifications with high efficiency.
Their regional specification makes them suitable tools for modeling a range of neurode-

generative diseases that affect the midbrain, hindbrain, and spinal cord.

Acknowledgments

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Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this article. This work was financially supported by Royan Institute for Stem Cell Biology and Technology.

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