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Neural precursors derived from human embryonic stem cells maintain long-term proliferation without losing the potential to differentiate into all three neural lineages, including dopaminergic neurons

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Abstract

Human embryonic stem (hES) cells have the ability to renew themselves and differentiate into multiple cell types upon exposure to appropriate signals. In particular, the ability of hES cells to differentiate into defined neural lineages, such as neurons, astrocytes, and oligodendrocytes, is fundamental to developing cell-based therapies for neurodegenerative disorders and studying developmental mechanisms. However, the utilization of hES cells for basic and applied research is hampered by the lack of well-defined methods to maintain their self-renewal and direct their differentiation. Recently we reported that neural precursor (NP) cells derived from mouse ES cells maintained their potential to differentiate into dopaminergic (DA) neurons after significant expansion *in vitro*. We hypothesized that NP cells derived from hES cells (hES-NP) could also undergo the same *in vitro* expansion and differentiation. To test this hypothesis, we passaged hES-NP cells and analyzed their proliferative and developmental properties. We found that hES-NP cells can proliferate approximately 380 000-fold after *in vitro* expansion for 12 weeks and maintain their potential to generate Tuj1$^+$ neurons, GFAP$^+$ astrocytes, and O4$^+$ oligodendrocytes as well as tyrosine hydroxylase-positive (TH$^+$) DA neurons. Furthermore, TH$^+$ neurons originating from hES-NP cells expressed other midbrain DA markers, including Nurr1, Pitx3, Engrail-1, and aromatic L-amino acid decarboxylase, and released significant amounts of DA. In addition, hES-NP cells maintained their developmental potential through long-term storage (over 2 years) in liquid nitrogen and multiple freeze–thaw cycles. These results demonstrate that hES-NP cells have the ability to provide an expandable and unlimited human cell source that can develop into specific neuronal and glial subtypes.

Keywords: astrocytes, dopaminergic neurons, human embryonic stem cells, neural precursors, neurons, oligodendrocytes.

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Human embryonic stem (hES) cells can provide a useful source of cells for basic developmental studies, cell-based therapies or drug screening for treatment of neurological disorders. Initial work with mouse embryonic stem (mES) cells provided the knowledge base for studies of hES cells. Several reports have demonstrated the capacity of mES cells to generate neural precursor (NP) cells that can differentiate into dopaminergic (DA) and motor neurons using optimal culture conditions and/or genetic manipulation (Kawasaki *et al.* 2000; Lee *et al.* 2000; Chung *et al.* 2002; Barberi *et al.* 2003; Kim *et al.* 2006). In particular, we recently showed that NP cells derived from mES cells (mES-NP) maintain the potential to differentiate into DA neurons after expansion *in vitro*. Therefore, it is reasonable to hypothesize that NP cells derived from hES cells would maintain their potential to differentiate into three cell types of neural lineage and DA neurons during *in vitro* differentiation after long-term mitogenic expansion. However, there are fundamental differences between mouse and human stem cell studies: dissociated single hES cells have poorer survival than mES

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Abbreviations used: AA, ascorbic acid; bFGF, basic fibroblast growth factor; DA, dopaminergic/dopamine; EGF, epidermal growth factor; FN, fibronectin; GFAP$^+$, glial fibrillary acidic protein-positive; hES, human embryonic stem; hES-NP, hES-derived NP; LIF, leukemia inhibitor factor; MEFs, mouse embryonic fibroblasts; mES, mouse embryonic stem; MS5-SHH, MS5 cells stably over-expressing sonic hedgehog; NDS, normal donkey serum; NP, neural precursor; O4$^+$, oligodendrocyte marker-positive; P, passage; PCA, perchloric acid; PLO, poly-L-ornithine; TH, tyrosine hydroxylase.

cells (Amit *et al.* 2000), human leukemia inhibitor factor (LIF) is unable to maintain the pluripotent state of hES cells (Daheron *et al.* 2004; Sato *et al.* 2004), and human development is considerably longer than that of mouse as is reflected in longer cell cycle times. In addition, the maintenance of hES cells' ability for self-renewal will be critical to their large-scale growth and differential potential. It is important to note that preparation of feeder layers, such as fibroblasts for self-renewal and stromal cells or immortalized astrocytes for differentiation of hES cells is labor intensive. Furthermore, variations between batches of feeder layers and different passages of hES cells can cause undesirable experimental complexities.

Recently, many laboratories have reported that hES cells can efficiently differentiate into DA neurons through an intermediate stage that has been known as hES-derived NP (hES-NP) cells (Reubinoff *et al.* 2001; Ben-Hur *et al.* 2004; Park *et al.* 2004; Perrier *et al.* 2004; Li *et al.* 2005; Park *et al.* 2005; Tabar *et al.* 2005; Yan *et al.* 2005; Sonntag *et al.* 2007). However, the proliferative and developmental potentials of hES-NP cells are still poorly understood although multipotent NP cells could be efficiently derived from ES cells. To address these, we expanded NP cells that were derived from H1 and HSF6 hES cell lines and investigated their developmental properties *in vitro*. Here, we report that hES-NP cells were able to proliferate approximately 380 000-fold after expansion for 12 weeks, and maintained the potential to generate $Tuj1^+$ neurons, glial fibrillary acidic protein-positive ($GFAP^+$) astrocytes and oligodendrocyte marker-positive ($O4^+$) oligodendrocytes as well as functional DA neurons. In addition, hES-NP cells maintained their differentiation potential through long-term storage of over 2 years and multiple freeze–thaw cycles. We report that hES-NP cells are fast, dependable, and unlimited source of neuronal cells for basic and applied research.

Materials and methods

hES cells culture

Human ES (hES) cell lines, H1 (provided by WiCell Research Institute) and HSF-6 (University of California, San Francisco, CA, USA), were cultured on mitotically inactivated mouse embryonic fibroblasts (MEFs) in DMEM/F12 medium with 20% knockout serum replacement, penicillin (100 IU/mL), streptomycin (100 μg/mL), 1 mmol/L L-glutamine, 1% non-essential amino acids, 0.1 mmol/L β-mercaptoethanol, and 4 ng/mL basic fibroblast growth factor (all from Invitrogen, Carlsbad, CA, USA). For the maintenance of undifferentiated hES cells, cultures were passaged about once every week by mechanic dissection and enzymatic treatments and then small clusters were transferred on freshly prepared MEF feeder.

In vitro differentiation of ES cells

Neural differentiation of hES cells was induced by co-culture on MS5 stromal cells or MS5 cells stably over-expressing sonic hedgehog (MS5-SHH). MS5 stromal feeder cells were maintained in α-minimum essential medium containing 10% fetal bovine serum and 2 mmol/L L-glutamine (Barberi *et al.* 2003). The MS5-SHH stable cell line has been established by viral transduction using retroviral construct expressing human SHH N-terminus followed by blasticidine (10 μg/mL, Invitrogen) selection (Park *et al*. 2005). Undifferentiated hES colonies were detached from MEF feeders by incubation with 200 U/mL collagenase IV (Invitrogen) for 15 min at 37°C, followed by gentle dissociation into small clusters with pipet and then cells were resuspended in serum-free N_2 medium with 0.2 mmol/L ascorbic acid (AA; Sigma-Aldrich, New London, NH, USA). The clusters on a layer of MS5 stromal cells were cultured for 7 days, and then passaged on freshly prepared feeder of MS5-SHH, and further cultured for 14 days. At the end of the co-culture, clusters of 20–300 cells in N_2 supplemented with 20 ng/mL basic fibroblast growth factor (bFGF), 10 ng/mL epidermal growth factor (EGF), and AA were replated on poly-L-ornithine/fibronectin (PLO/FN)-coated dishes. After 7 days of culture in N_2 + bFGF + EGF + AA, cells were transferred on PLO/FN-coated glass coverslips as clusters or a single cell. NP cells were frozen by suspension of small clusters in N_2 + bFGF + EGF + AA media containing 10% dimethyl sulfoxide and placed in a Styrofoam container at −80°C to ensure a gradual decrease in temperature. After 24 h, frozen cells were moved to a liquid nitrogen tank. Frozen NP cells were thawed in a 37°C water bath, and then plated on PLO/FN-coated plates in N_2 + bFGF + EGF + AA media. For neuronal differentiation, the NP cells were cultured by withdrawing bFGF and EGF from the media for 14 days or more.

Immmunocytochemistry

Cells were fixed in 4% paraformaldehyde in 1× phosphate buffered saline (PBS), rinsed with PBS, and then incubated with blocking buffer [PBS/10% normal donkey serum (NDS)/0.1% Triton X-100] for 30 min at 22°C. Fixed cells were incubated overnight at 4°C with primary antibodies diluted in PBS containing 2% NDS. The following primary antibodies were used: mouse anti-mouse Nestin (1 : 500; Chemicon, Temecula, CA, USA; http://www.chemicon.com), rabbit anti-β-tubulin (1 : 2000; Covance, Princeton, NJ, USA; http://www.covance.com), rabbit anti-GFAP (1 : 250; DakoCytomation, Glostrup, Denmark, http://www.dakocytomation.com), mouse anti-O4 (1 : 100; R&D Systems, Minneapolis, MN, USA; http://www.rndsystems.com), sheep anti-myelin basic protein (1 : 200; Chemicon), rabbit anti-tyrosine hydroxylase (TH, 1 : 250; Pel-Freez, Rogers, AK, USA; http://www.pelfreez-bio.com). After additional rinsing in PBS, the coverslips were incubated in the appropriate Alexa 488- and Alexa 594-labeled secondary antibodies (Invitrogen; http://www.invitrogen.com) in PBS with 2% NDS for 60 min at 22°C. After rinsing 3 × 10 min in PBS, cells were counter-stained using 1.5 μg/mL 4′,6-diamidino-2-phenylindole and then mounted onto slides in Gel/Mount (Biomeda corp., Foster City, CA, USA; http://www.biomeda.com). Stained cells were analyzed under Axioskope 2 *plus* fluorescence microscope (Carl Zeiss, Oberkochen, Germany).

Quantitative real-time RT-PCR analysis

Total RNAs were prepared from *in vitro* differentiated hES-NP cells using TriReagent (Sigma) followed by treatment with DNase I (Ambion, Austin, TX, USA). Two micrograms of total RNA were

reverse-transcribed into cDNA using oligo (dT) primers, according to the SuperScript Preamplification Kit (Invitrogen). The cDNA was then analyzed by PCR using the following primers:

Glyceraldehyde-3-phosphate dehydrogenase: 5′-TGACATCAAGAAGGTGGTGAAGC-3′, 5′-CCCTGTTGCTGTAGCCGTATTC-3′, Nestin: 5′-CAGCGTTGGAACAGAGGTTGG-3′, 5′-TGGCACAGGTGTCTCAAGGGTAC-3′, β-tubulin III: 5′-CAACAGCACGGCCATCCAGG-3′, 5′-CTTGGGGCCCTGGGCCTCCGA-3′, TH: 5′-GAGTACACCGCCGAGGAGATTG-3′, 5′-GCGGATATACTGGGTGCACTGG-3, Engrail-1: 5′-GCA ACCCGGCTATCCTACTTATG-3′, 5′-ATGTAGCGGTTTGCCTGGAAC-3′, Nurr1: 5′-TTCTCCTTTAAGCAATCGCCC-3′, 5′-AAGCCTTTGCAGCCCTCACAG-3′, Pitx3: 5′-GGAATGGTCACCCTGACATGAG-3′, 5′-TGAAGGCGAACGGGAAGGTCT-3′, aromatic L-amino acid decarboxylase: 5′-CTCGGACCAAAGTGATCCAT-3′, 5′-GTCTCTCTCCAGGGCTTCCT-3′, GFAP: 5′-GGCACGTGCGGGAGGCGGCC-3′, 5′-TCTCATCACATCCTTGTGC-5′, Myelin Basic Protein: 5′-AAGGACTCACACCACCCGGC-3′, 5′-TTTCAGCGTCTAGCCATGGG-3′, O4 : 5′-CTACTGCTCTGGGTCCCAGG-3′, 5′-CTGCCACTGAACCGAGATGG-3′.

PCR reactions were carried out in a PCR Reaction Buffer (Promega, Madison, WI, USA) containing 1.4 nmol/L of each primer and 1.25 U Taq I DNA polymerase (Promega). Samples were amplified in a Thermal Cycler (Eppendorf, Westbury, NY, USA) and a Continuous Fluorescence Detector (MJ Research, Waltham, MA, USA) using DNA Engine Opticon software under the following conditions: denaturing step at 94°C, 30 s; annealing step at 55°C, 30 s; extension step at 72°C, 30 s for 50 cycles and a final extension step at 72°C, 10 min. Relative expression of mRNAs was assessed by normalizing the levels of cDNA to the signal from glyceraldehyde-3-phosphate dehydrogenase mRNA.

Cell counting and statistical analysis

Cell density of three neural lineages (neurons, astrocytes, and oligodendrocytes) and DA neurons was determined by counting the numbers of β-tubulin$^+$, GFAP$^+$, O4$^+$, and TH$^+$ cells per field at ×100 magnification using an Axioskope 2 *plus* fluorescence microscope (Carl Zeiss). Seven visual fields were randomly selected and counted for each sample. Numbers presented in figures represent the average percentage and SEM of TH$^+$ cells over β-tubulin$^+$ from three samples per each hES-NP cell passage.

All statistical analyses were conducted using SAS v 9.1 (SAS Institute, Cary, NC, USA) for Windows 2000 Professional. The analysis was conducted on these means using a mixed models ANOVAprocedure (SAS) to determine possible statistical differences between group means.

Analysis of DA release

Differentiated hES-NP cells were treated with 200 μL of serum-free N_2 medium supplemented with 56 mmol/L KCl in six-well plates. Their media were collected after 30 min and concentrated solutions of perchloric acid (PCA) were added to a final concentration of 0.1 mol/L PCA/0.1 mmol/L EDTA. These deproteinated samples were centrifuged, and their supernatants were kept at −80°C until further analysis. Samples were further purified by using a 0.22 μm nylon filter (Osmonics, Inc., Trevose, PA, USA; http://www.osmonics.com). The DA content of the supernatants was measured by reverse-phase HPLC using a Velosep RP-18 column (100 × 3.2 mm; Brownlee Labs, Shelton, CT, USA; http://las.perkinelmer.com) and an ESA Coulochem II electrochemical detector (ESA, Inc., Chelmsford, MA, USA; http://www.esainc.com) equipped with model 5014 analytical cell as we previously described (Wachtel *et al.*, 1997). The mobile phase was composed of 0.1 mol/L sodium phosphate buffer (pH 2.65), 0.1 mmol/L EDTA, 0.4 mmol/L sodium octyl sulfate, and 9% (vol/vol) methanol and operated at a flow rate of 0.8 mL/min. The potential of the guard cell was set at 330 mV. The potential of the first electrode in the analytical cell was set at 0 mV and the second at 310 mV. L-DOPA, DA, dihydroxyphenyl acetic acid, and homovanillic acid were identified by retention time and quantified by their peak using an EZChrom Chromatography Data System (ESA, Inc.). The limit of detection for all compounds was <1 pg. DA content of each sample was normalized with the amount of total cellular proteins. For protein measurement, the cells were harvested in 0.1 mol/L PCA/0.1 mmol/L EDTA, precipitated, resuspended in 0.2% Triton X-100/10 mmol/L potassium phosphate buffer (pH 7) and sonicated. The protein content was measured by the Bradford method (Bio-Rad Laboratories, Hercules, CA, USA; http://www.bio-rad.com).

Results

In vitro differentiation of hES cells

Human embryonic stem colonies (HSF6 and H1) were detached from a feeder layer, dissociated into small clusters, and cultured first for 7 days on a layer of MS5 stromal feeder cells and then for 14 days on a MS5-SHH feeder in the serum-free N_2 medium supplemented with AA (Fig. 1a). During co-culture, the center cells developed a columnar morphology and formed neural tube-like rosettes around days 12–14 (Fig. 1b). Over the next 5–7 days (days 19–21), hES colonies containing neural lineages were disrupted into small clusters, and then replated on PLO/FN-coated dishes in serum-free N_2 medium with bFGF, EGF, and AA. After 7 days in culture, most of cells were positive for NP marker nestin (Lendahl *et al.*, 1990), and the cell number increased by 3–4-fold (Fig. S1a). NP cells were differentiated into DA neuronal cells by withdrawing bFGF and EGF from the media for 14 days (Fig. 1b). The morphology and phenotypes of NP cells were not altered during several passages for additional NP expansion. NP cells that had been frozen in liquid nitrogen for several months survived well after plating them on dishes (data not shown).

hES-NP cells can be expanded *in vitro* in the presence of bFGF and EGF

Human embryonic stem cells-derived NP cells were cultured up to 12 weeks by passaging once every 7 days in serum-free N_2 medium supplemented with bFGF, EGF, and AA. hES-NP cells were proliferated exponentially, reaching approximately 380 000-fold after expansion for 12 weeks (Fig. 2a). Each passage of hES-NP cells were about 95% positive for the NP marker nestin. A small number of NP cells spontaneously differentiated into β-tubulin III$^+$ neuron and GFAP$^+$ astrocytes (Fig. 2b–g). The

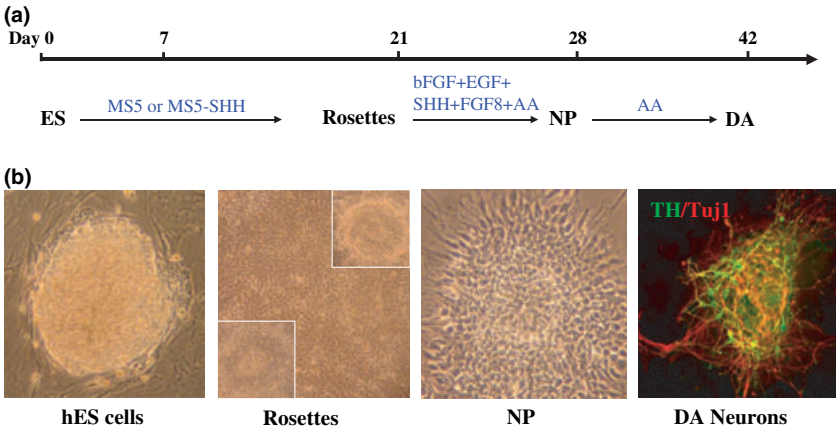

Fig. 1 Generation of *in vitro* dopaminergic neurons from hES cells on MS5 or MS5-SHH stromal feeder cells. (a) A scheme for induction and differentiation of dopaminergic neurons from hES cells. Undifferentiated hES cells are used for their neuralization, followed by dopaminergic induction. (b) Representative images during *in vitro* differentiation of dopaminergic neurons from hES cells. Rosettes were formed on the feeder layers at day 21. Rosettes were dissociated to small clumps and then transferred and expanded as neural precursor cells in N_2 medium with bFGF and EGF for 7 day. Neural precursor cells were developed to dopaminergic neurons as withdrawing bFGF and EGF for 14 days. TH^+ cells co-localized to β-tubulin$^+$ cells.

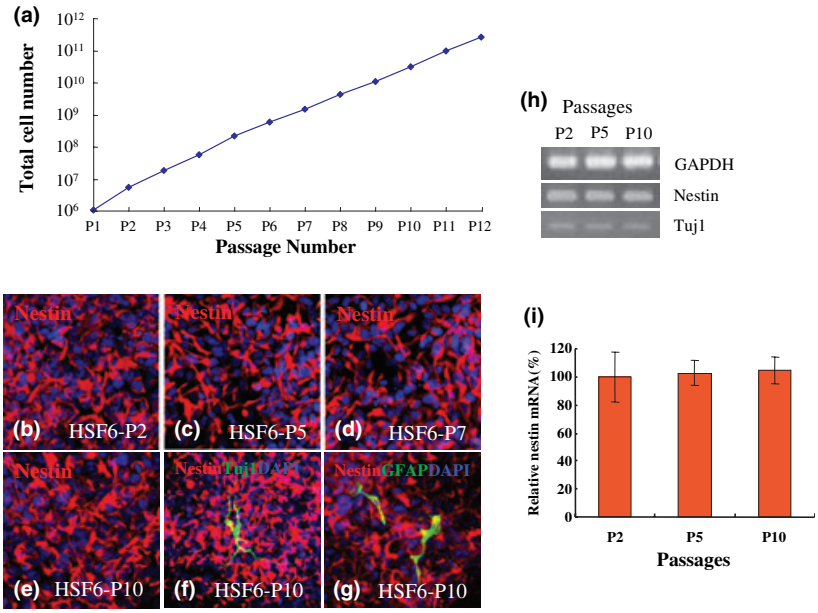

Fig. 2 Long-term proliferation of nestin$^+$-NP cells derived from hES cells (a) Expansion curve of HSF6 hES cells-derived NP cells from short-(P1) and long-term (P12) passages. (b–e) Detection of Nestin (NP cells marker in red) in different passages of NP cells: P2 (b), P5 (c), P7 (d), and P10 (e). (f–g) Show immunostaining with Nestin and Tuj1 (neuronal marker in green) or GFAP (astrocytes marker in green), and a small number of NP cells are Tuj1$^+$ (f) and GFAP$^+$ (g). DAPI counter-staining (in blue) shows nuclear morphology. (h) Semi-quantitative RT-PCR analysis using neural precursor cells marker, *nestin*. Total RNA samples were prepared from different passages (P2, P5, P7, and P10) of NP cells. cDNA prepared with *oligo dT* primers were amplified using primers specific for *GAPDH* and *nestin*. (i) Quantitative real-time RT-PCR analysis at different passages (P2, P5, and P10) of NP cells. The expression levels of *nestin* gene were normalized with those of *GAPDH* gene. Relative expression levels were determined by setting those in P2 NP cells at 100. ANOVA revealed that there is no significant difference between groups (n = 3 for each P2, P5, and P10 hES-NP cell).

behavior of hES-NP cells was comparable across H1 and HSF6 (Fig. S1b and c). Semi-quantitative RT-PCR analysis showed that expression of nestin was comparable in short- (P2) to long-term (P10) passaged hES-NP cells (Fig. 2h). Real-time RT-PCR analysis confirmed that consistent expression levels of nestin mRNAs were maintained in short- to long-term passaged hES-NP cells (Fig. 2i). These results show that long-term expanded hES-NP cells

maintain their NP state properties upon significant *in vitro* expansion.

In vitro expanded hES-NP cells do not lose their developmental potential and can differentiate into Tuj1$^+$ neurons, GFAP$^+$ astrocytes, and O4$^+$ oligodendrocytes

Neural precursor cells generate neuronal, astrocytic and oligodendrocytic progeny. We examined whether the proliferative hES-NP cells, such as passage 2 (P2), P5, P7, and P10, can differentiate into three cell types of the neural lineage by using different culture conditions. For both neurons and astrocytes, hES-NP cells were cultured by withdrawing bFGF and EGF from the culture media for 14 days. In order to generate oligodendrocytes, hES-NP cells were cultured in the presence of platelet-derived growth factor, bFGF, and EGF for 7 days, and then their differentiation was induced by withdrawing platelet-derived growth factor, bFGF, and EGF from the culture medium for 21 days or more. hES-NP cells were induced into cells positive for neuronal marker Tuj1, astrocyte marker GFAP, and oligodendrocyte marker O4 (Fig. 3a and b), suggesting that differentiation potential of hES-NP cells could be maintained for long-term periods. We also confirmed gene expression of three neural lineage markers by semi-quantitative RT-PCR. mRNA expressions of all three neural lineages such as Tuj1, GFAP, and O4 were all well preserved (Fig. 3b).

For quantitative analysis, we performed cell counting as described in Materials and methods. After *in vitro* differentiation, P2 and P10 hES-NP cells contained 37.2 ± 1.27% and 34.4 ± 1.04% β-tubulin$^+$ neurons/total cells, 7.42 ± 0.85% and 8.42 ± 0.48% GFAP$^+$ astrocytes/total cells, 0.06 ± 0.009% and 0.08 ± 0.007% O4$^+$ oligodendrocytes/total cells, respectively (Fig. 3c).

In vitro expanded hES-NP cells differentiate into functional midbrain DA neurons

We examined whether the NP cells derived from HSF6 and H1 hES cell lines retain their abilities to differentiate into DA neurons. The *in vitro* expanded hES-NP cells differentiated into DA neurons and expressed DA neuronal marker TH and neuronal marker β-tubulin III (Fig. 4a and b). We also checked the DA marker gene expression by semi-quantitative RT-PCR. mRNA expression of midbrain DA markers such as TH, Nurr1, Pitx3, En-1, and aromatic L-amino acid decarboxylase was evident even through the long-term (P10) expansion of hES-NP cells (Fig. 4c). Furthermore, we counted the number of TH$^+$ neurons from hES-NP cells that were passaged twice and 10-times (P2 and P10). Following *in vitro* differentiation, these cells contained 8.9 ± 1.11% and 10.4 ± 0.91% TH$^+$/total cells, respectively (Fig. 4d), and 32.35 ± 2.03% and 33.7 ± 1.65% TH$^+$/β-tubulin$^+$, respectively (Fig. 4e). Authentic DA neurons have the ability to synthesize and release DA in response to membrane depolarization. To test whether DA is released after membrane depolarization, we treated *in vitro* differentiated cells from short-term and long-term passaged hES-NP cells with 50 mmol/L KCl for 30 min and analyzed the released DA in the media by HPLC. In response to membrane depolarization, 3.18 ± 0.13 and 3.32 ± 0.12 pg DA/μg cellular proteins were released from passaged hES-NP cells (P2 and P10), respectively (Fig. 4f). In summary, neurons derived from short-term and long-term expanded hES-NP cells can maintain the ability to differentiate into physiologically functional midbrain-like DA neurons.

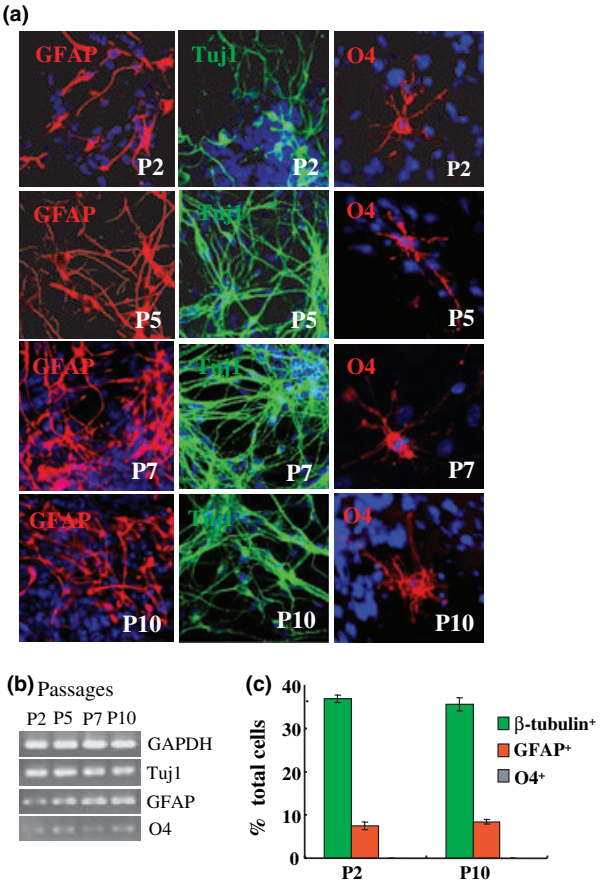

Fig. 3 Developmetal potential of different passages of hES cells-derived NP cells into neurons, astrocytes, and oligodendrocytes. (a) Detection of GFAP (astrocytes marker in red), Tuj1 (neuronal cells marker in green), and O4 (oligodendrocytes marker in red) in differentiated cells from different passages (P2, P5, P7, and P10). DAPI counter-staining (in blue) shows nuclear morphology. (b) Semi-quantitative RT-PCR analysis using neuronal cell marker *β-tubulin III,* astrocytes marker *GFAP,* and oligodendrocytes marker *O4.* Total RNA samples were prepared in cells differentiated from different passages (P2, P5, P7, and P10) of NP cells. cDNA prepared with *oligo dT* primers were amplified using primers specific for *GAPDH*, *β-tubulin III, GFAP, and O4.* (C) The cell number of β-tubulin$^+$, GFAP$^+$, and O4$^+$ cells from seven random fields per sample was counted at different passages (P2 and P10), and the total cell numbers of cells are shown. Each group represents an average of three samples from each independent experiment. ANOVA revealed that there is no significant difference between groups (n = 3 for each P2 and P10 hES-NP cell).

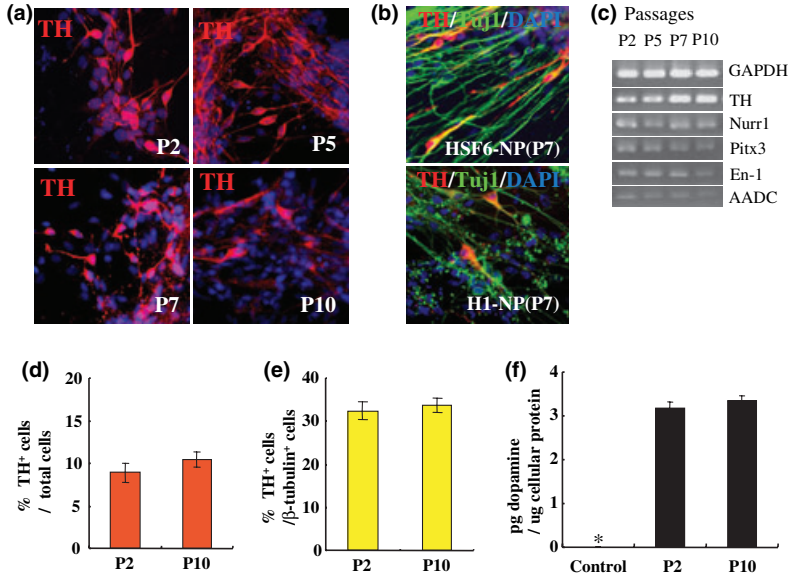

Fig. 4 Differentiation of hES-NP cells into DA neurons. (a) Detection of TH in differentiated cells from different passages (P2, P5, P7, and P10) of NP cells. DAPI counter-staining (in blue) shows nuclear morphology. (b) Representative images of TH/Tuj1-positive neurons differentiated from the hES-NP (P7) cells derived from the hES cell lines HSF6 and H1. (c) Semi-quantitative RT-PCR analysis using DA markers, including *TH, Nurr1, Pitx3, Engrain-1 (En-1),* and *AADC.* Total RNA samples were prepared in cells differentiated from different passages (P2, P5, P7, and P10) of NP cells. (d) The cell number of TH^+ cells from seven random fields per sample was counted at different passages (P2 and P10), and the total cell numbers of cells are shown. Each group represents an average of three samples from each independent experiment. ANOVA revealed that there is no significant difference between groups ($n = 3$ for each P2 and P10 hES-NP cell). (e) The proportion of TH+/β-tubulin$^+$ cells was calculated by dividing the number of TH+ cells by that of β-tubulin$^+$ cells ($n = 3$). ANOVA revealed that there is no significant difference between groups ($n = 3$ for each P2 and P10 hES-NP cell). (f) Analysis of dopamine release. *In vitro* differentiated NP cells were treated with 50 mmol/L KCl and the media were assayed for DA levels by reverse-phase HPLC. Each group represents an average of three samples from two independent experiments. ANOVA revealed $F = 492.36$, $p < 0.001$. The asterisk indicates significant difference from P2 and P10 cells.

Long-term storage and freeze–thaw cycles do not cause hES-NP cells to lose their developmental potential

To establish hES-NP cells as convenient and unlimited cell source for basic study and clinical therapeutic application, they should be stable in liquid nitrogen without losing their developmental and proliferative potential. We tested hES-NP cells for their ability to maintain their differential potential after long-term storage and freeze–thaw cycles. hES-NP cells were stored in liquid nitrogen, thawed by a simple freeze–thaw method and plated on PLO/FN-coated dishes in serum-free N_2 media supplemented with bFGF and EGF. As shown in Fig. 5, differentiated cells were not only positive for the neuronal marker Tuj1, the astrocyte marker GFAP, and the DA neuronal marker TH (Fig. 5b), but also were as efficient as those without the freeze–thaw cycles (Fig. 5a). We observed the same results with NP cells derived from the H1 and HSF6 lines (Fig. 5a and b). Furthermore, hES-NP cells preserved their developmental potential to differentiate into $GFAP^+$ astrocytes, $Tuj1^+$, and TH^+ neurons, even after being stored in liquid nitrogen up to 2 years (Fig. 5b).

Discussion

It is of great importance to efficiently generate hES-NP cells that have the potential to differentiate into all neural lineages and especially into functional DA neurons. Most studies to generate hES-NP cells involved neurosphere culture (Reubinoff *et al.* 2001; Zhang *et al.* 2001; Ben-Hur *et al.* 2004; Li *et al.* 2005) or adherent co-culture (Park *et al.* 2004; Perrier *et al.* 2004; Tabar *et al.* 2005; Shin *et al.* 2006; Sonntag *et al.* 2007) systems. Neurosphere culture systems maintain cell–cell contacts, implying that neurosphere may contribute to the survival, proliferative, and differentiation potential of NP cells (Campos 2004). It is however, not easy to efficiently quantify cells within the neurospheres even after attaching them to the substrates of the culture dish (Chung *et al.* 2006). In addition, unlike undifferentiated mES cells, hES cell culture is very difficult to work with because of its poorly defined components of culture environment. The addition of LIF and bFGF to the medium does not allow the culture of human ES cells in the absence of feeder cells (Thomson *et al.*

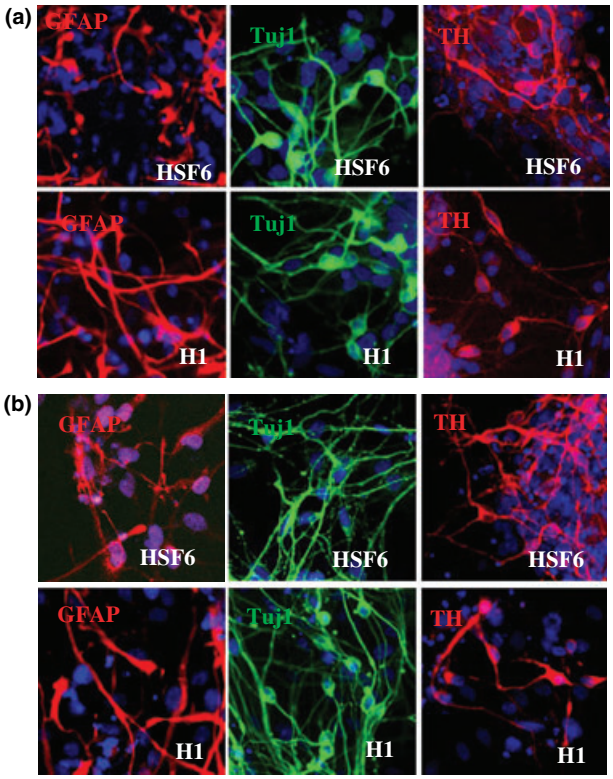

Fig. 5 hES cells-derived NP cells maintain their ability to differentiate into glial and neuronal cells as well as dopaminergic neurons after freeze–thaw cycles and long-term storage. (a) NP cells derived from both HSF6 and H1 hES cells were immunostained with neuronal marker Tuj1, dopaminergic marker TH, and astrocytes marker GFAP. (b) NP cells derived from both HSF6 and H1 hES cells after long-term storage and freeze–thaw cycles were immunostained with neuronal marker Tuj1, dopaminergic marker TH, and astrocytes marker GFAP. DAPI counter-staining (in blue) shows nuclear morphology.

1998; Daheron *et al.* 2004; Sato *et al.* 2004). Furthermore, preparation of feeder layers for both self-renewal and differentiation of hES cells is very labor intensive and time-consuming, and the variation between batches of feeder layers and passaged hES cells can introduce undesirable complexity to the experiments. In our system, we undertook the adherent co-culture system to generate hES-NP cells and plated them on dishes as single cells to characterize them efficiently.

Our hES-NP cells expanded *in vitro* have several notable features. First, hES-NP cells could be exponentially expanded up to a 380 000-fold increase in numbers after only 12 weeks without significant changes in growth rate (Fig. S1a). Second, regardless of the number of passages, hES-NP cells maintain the potential to differentiate into all three neural lineages, i.e., astrocytes, oligodendrocytes, and neurons, including functional DA neuron. Third, these properties of hES-NP cells were found even after very long-term storage in liquid nitrogen (up to 2 years) and freeze–thaw cycles. Our results demonstrate that hES-NP cells can serve as reliable and unlimited cell source for basic and therapeutic application studies including high throughput drug screening. In addition, our data also suggest that long-term passaged hES-NP cells (P10) exhibit similar properties compared to short-term passaged cells in gene expression pattern (Fig. 2i) and cell fate determination (Figs 3c, 4d and e) during development.

Our systematic analysis and comparison of the developmental potential of hES-NP cells revealed consistent results in all the passages we tested, P2, P5, P7, and P10. In contrast, it has been reported that long-term passaged, embryonic forbrain-derived NP cells showed marked changes in their growth properties and gene expression (Morshead *et al.* 2002). hES-NP cells consistently proliferate in the presence of mitogens and can be easily cryopreserved without losing their developmental potential, which makes them an even more convenient cell source. Importantly, the difficult, labor-intensive and time-consuming work of hES cells could be made more efficient, and the variations that may be caused by the different passages of hES cells (Roy *et al.* 2006) could be reduced. However, the molecular and cellular properties of long-term passaged hES-NP cells should be carefully monitored for therapeutic applications, even though they could provide unlimited number of NP cells with stable developmental potential for research purposes.

It is worthwhile to note that hES cells and their NP cells are different from mES and their NP cells. bFGF supports the growth of undifferentiated hES cells (Xu *et al.* 2005a,b) and hES-NP cells (Itsykson *et al.* 2005; Shin *et al.* 2006), whereas LIF (Zhang *et al.* 2001; Shin *et al.* 2006) and EGF (Zhang *et al.* 2001) had no effect on the maintenance of hES cells and their NP cells. However, these results differed from data obtained with primary cells as shown by human neural progenitor cells which maintained their proliferative and differential potential by either LIF or bFGF and/or in combination of bFGF and EGF, but not EGF alone (Svendsen *et al.* 1996; Carpenter *et al.* 1999; Vescovi *et al.* 1999).

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Supplementary material

The following supplementary material is available for this article online:

Fig. S1 Growth rate of hES cells-derived NP cells during *in vitro* proliferation.

This material is available as part of the online article from http://www.blackwell-synergy.com.

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