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# Tyrosine hydroxylase-positive cells and dopaminergic neuronal function in human embryonic stem cells

## *An electrophysiological validation**☆

**Tianran Song[1, 2], Yue Wang[3, 4], Guian Chen[1, 2], Guogang Xing[3, 4]**

1*Reproductive Medicine Center and Stem Cell Research Center, Peking University Third Hospital, Beijing 100191, China*
2*Department of Obstetrics and Gynecology, Peking University Third Hospital, Beijing 100191, China*
3*Neuroscience Research Institute and Department of Neurobiology, Peking University, Beijing 100191, China*
4*Key Laboratory for Neuroscience, Ministry of Education and Ministry of Public Health, Beijing 100191, China*

### Abstract

**BACKGROUND:** Induced differentiation strategies and cytochemical properties of human embryonic stem cells (hESCs) have been investigated. However, the electrophysiological functions of tyrosine hydroxylase (TH)-positive cells derived from hESCs remain unclear.

**OBJECTIVE:** To investigate the differentiation efficiency of TH-positive cells from hESCs *in vitro* using modified four-step culture methods, including embryoid body formation, and to examine the functional characteristics of the differentiated TH-positive cells using electrophysiological techniques.

**DESIGN, TIME AND SETTING:** Neuroelectrophysiology was performed at the Reproductive Medicine Center and Stem Cell Research Center, Peking University Third Hospital, and the Neuroscience Research Institute and Department of Neurobiology, Peking University, from September 2004 to August 2008.

**MATERIALS:** The hESC line, PKU-1.1, a monoclonal cell line derived from a pre-implantation human blastocyst in the Reproductive Medical Center of Peking University Third Hospital. The patch clamp recording system was provided by the Neuroscience Research Institute and Department of Neurobiology, Peking University.

**METHODS:** The hESC line was induced to differentiate into TH-positive cells *in vitro* using a modified four-step culture method, including the formation of embryoid body, as well as the presence of sonic hedgehog and fibroblast growth factor 8. The cell karyotype was assessed by G-banding karyotype analysis techniques and specific markers were detected immunocytochemically. Whole-cell configuration was obtained after obtaining a tight seal of over 1 GΩ. Ionic currents were detected by holding the cells at −70 mV and stepping to test voltages between −80 and 40 mV in 10-mV increments in voltage-clamp configuration.

**MAIN OUTCOME MEASURES:** We measured the cell karyotype, specific cell markers, and the electrophysiological properties of the voltage-gated ion channels on the cell membrane of TH-positive dopaminergic cells differentiated from our hESCs line *in vitro*.

**RESULTS:** The differentiated cells had a consistent appearance, and the majority of cells (> 90%) expressed TH and β-tubulion, as well as the neural progenitor marker, nestin. Cell karyotype analysis demonstrated that all of the hESCs had a stable and normal karyotype (46, XX) after differentiation. In addition, patch clamp recording showed that the 10 recorded TH-positive cells exhibited a fast inward current when the test voltage depolarized to −30 mV, and a delayed outward current when the test voltage depolarized to −10 mV. The peak of inward current was obtained at voltage between −10 mV and 0 mV, while the peak of outward current was obtained at 40 mV. The average peak of inward current density was (−50.05 ± 15.50) pA/pF, and the average peak of outward current density was (41.98 ± 13.55) pA/pF.

**CONCLUSION:** More than 90% of the differentiated hESC-derived cells induced by the modified four-step culture method exhibit dopaminergic neuronal properties, including general electrophysiological functional properties, such as functional potassium and sodium channels.

**Key Words:** human embryonic stem cell; induced differentiation; dopaminergic neurons; patch clamp recording; Parkinson's disease

Tianran Song☆, Doctor, Reproductive Medicine Center and Stem Cell Research Center, Peking University Third Hospital, Beijing 100191, China; Department of Obstetrics and Gynecology, Peking University Third Hospital, Beijing 100191, China

Corresponding author: Guogang Xing, M.D., Ph.D., Associate professor, Neuroscience Research Institute and Department of Neurobiology, Peking University, Beijing 100191, China; Key Laboratory for Neuroscience, Ministry of Education and Ministry of Public Health, Beijing 100191, China; Guian Chen, M.D., Professor, Reproductive Medicine Center and Stem Cell Research Center, Peking University Third Hospital, Beijing 100191, China; Department of Obstetrics and Gynecology, Peking University Third Hospital, Beijing 100191, China
ggxing@bjmu.edu.cn; chenguian@bjmu.edu.cn

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## INTRODUCTION

Human embryonic stem cells (hESCs), derived from the inner cell mass of the pre-implanted blastocyst, are multipotent cells that have the potential to self renew and proliferate indefinitely[1-4]. When exposed to appropriate culture conditions and/or genetic manipulation, hESCs can differentiate into multiple defined cell types, capable of forming functional synapses after

transplantation[5-7]. These properties of hESCs allow them to function as an excellent candidate for cell replacement therapy in neurodegenerative diseases such as Parkinson's disease (PD)[8-11]. However, this replacement requires the identification of a reliable source of hESCs and a workable strategy for cells to differentiate into dopaminergic (DA) neurons capable of surviving and functioning after transplantation into the brain[12].

hESCs can be differentiated into DA neurons by two methods. One is stromal cell-derived inducing activity method, by co-culturing ESCs with stromal cell lines, like MS5, S2, or PA6 (a clonal pre-adipocyte stromal cell line isolated from newborn mouse calvaria) cells, which yields up to 90% tyrosine-hydroxylase (TH)-positive DA neurons[13-16]. However, its mechanism remains unclear and the co-culture of cell lines with animal products has contamination risks[17], limiting its clinical use. The other protocol is a multiple-step method that includes the formation of an embryoid body followed by the selection of nestin-positive cells and by the addition of neurotrophic factors[6, 9, 17-18], which does not use animal products but has lower efficiency (20%–60% TH-positive cells) than co-cultures.

Studies on differentiation protocols and biochemical properties of hESC-derived TH-positive cells[6, 9-10, 13, 18-19] have generally not examined the electrophysiological functions[16-17, 20-22]. In particular, these cells should express voltage-gated potassium channels and sodium channels, as they play a key role in neuronal function. We attempted to produce high percentage of TH-positive DA neurons *in vitro* from an hESC line (PKU1.1)[23] using a modified four-step culture method, including embryoid body formation, following selective culture medium with N2 and various neurotrophic factors such as sonic hedgehog and fibroblast growth factor8 (FGF 8). The characteristics of these hESC-derived cells were further identified by morphologic, karyotypic, and electrophysiological assays.

## MATERIALS AND METHODS

### Design

Electrophysiological experiments *in vitro*.

### Time and setting

The experiment was performed at the Reproductive Medicine Center and Stem Cell Research Center, Peking University Third Hospital, and the Neuroscience Research Institute and Department of Neurobiology, Peking University from September 2004 to August 2008.

### Materials

The hESC line, PKU-1.1, a monoclonal cell line derived from pre-implantation human blastocyst, were established by the Reproductive Medical Center of Peking University Third Hospital. Patch clamp recording was provided by the Neuroscience Research Institute and Department of Neurobiology, Peking University.

Reagents and instruments are listed as follows:

| Reagent and instrument | Source |
|---|---|
| Giemsa solution | Gibco, USA |
| rabbit anti-nestin polyclonal antibody | Chemicon, Millipore, USA |
| Mouse anti-beta-tubulin isotype III (TuJ1), anti-TH polyclonal antibodies; Avidin-biotin-conjugated horseradish peroxidase, 3, 3'-diaminobenzidine substrate kit | Sigma, USA |
| Texas Red-conjugated goat anti-rabbit IgG | Jackson, USA |
| Biotinylated goat anti-mouse IgG | Pharmingen, USA |
| Pipettes | WPI, USA |
| Inverted fluorescent microscope, Cytoktype 6.2 Software | Leica, Germany |
| Prism 4.0 software | GraphPad Software Inc., USA |
| Pulse-fit 8.52 software, EPC-10 amplifier for patch clamp recording | Heka Instruments, Germany |

### Methods

#### *hESCs and embryoid body cultures*

hESC lines, PKU-1.1[23-24], were cultured on a feeder layer of gamma-irradiated mouse embryonic fibroblasts at 37 °C. The hESC medium contained 80% knock-out™ Dulbecco's modified Eagle's medium (DMEM), 20% knock-out™ serum replacer, 4 ng/mL basic fibroblast growth factor (bFGF), 0.1 mmol/L β-mercaptoethanol, 2 mmol/L glutamine, 1% nonessential amino acid stock, 50 IU/mL penicillin and streptomycin (stage 1).

On culture day 6, cells were treated with 1 mg/mL collagenase IV for 5 minutes, detached mechanically, resuspended, and cultured in bacteriological Petri dishes with embryoid body medium containing 80% DMEM/F12, 20% knock-out™ serum replacer, 0.1 mmol/L β-mercaptoethanol, 2 mmol/L glutamine, 1% nonessential amino acid stock, 50 IU/mL penicillin and streptomycin to form an aggregated embryoid body (stage 2).

#### *Differentiation and amplification of neural progenitor cells*

The EB with cystic cavities (at 5–6 days) was seeded in tissue culture dishes coated with poly-L-lysine, and N2 medium containing DMEM/F12, N2, 50 IU/mL penicillin and streptomycin with bFGF (20 ng/mL) was used to induce cell differentiation into neural progenitor cells. After 2 weeks, cells were isolated mechanically and replated. Cells were passed every 6 days by mechanical detachment and dissociation (stage 3).

#### *TH-positive cell differentiation*

The detached neural progenitor cells at a density of $(5–5.5)\times10^4/cm^2$ were plated into tissue culture dishes pre-coated with poly-L-ornithine (15 μg/mL)/laminin (1 μg/mL). N2 medium was exchanged every 2 days, and growth factors of 100 ng/mL FGF8 and 200 ng/mL sonic hedgehog were added during the first 14 days, and then 1 ng/mL transforming growth factor type $\beta_3$ (TGF-$\beta_3$), 10 ng/mL glial cell line-derived neurotrophic factor (GDNF), and 0.5 mmol/L dibutyryl cAMP were added for

6 additional days (stage 4).

***Karyotype analysis***

Karyotype analysis was performed using G-banding techniques before differentiation and at the end of stage 4 of differentiation[23, 25]. Briefly, cells were incubated with 250 ng/mL of colchicum at 37 °C for 2 hours and with 0.05% trypsin/EDTA for 3–5 minutes.

Re-suspended cells were incubated in 0.075 mol/L KCl solution for 8 minutes at 37 °C, fixed twice in 3: 1 methanol/acetic acid for 10 minutes each, dropped onto cold slides, dried, treated with 0.02% trypsin for 10 seconds, stained with Giemsa solution, and analyzed with Cytoktype 6.2 Software.

***Immunocytochemical staining***

Immunocytochemical staining was performed for the cultured cells from stage 2 to stage 4. Cultured cells were fixed overnight at 4 °C in phosphate buffered saline (PBS)-buffered 4% paraformaldehyde solution and washed three times in PBS. Endogenous peroxidase activity was blocked by incubating the fixed cells in 15% methanol/PBS plus 1% $H_2O_2$ for 20 minutes. The non-specific binding sites were blocked with 10% normal goat serum in PBS for 30 minutes, and incubated overnight at 4 °C with the following primary antibodies: rabbit anti-nestin (1: 100), mouse anti-TuJ1 (1: 1 000), or mouse anti-TH (1: 1 000). After washing in PBS three times, the cultured cells were incubated at 37 °C respectively with the following secondary antibodies for 30 minutes: for nestin staining, the Texas Red-conjugated goat anti-rabbit IgG (1: 100) was incubated and analyzed under an inverted fluorescent microscope; For TuJ1 or TH staining, the biotinylated goat anti-mouse IgG (1: 100) was incubated. After washing in PBS, the cultured cells were incubated with avidin-biotin-conjugated horseradish peroxidase for 30 minutes, followed by coloration with 3, 3'-diaminobenzidine substrate kit[24], and observation under an inverted fluorescent microscope. Negative controls were treated with PBS rather than the primary or secondary antibodies.

***Electrophysiological recording***

The electrophysiological properties of the TH-positive cells were tested using whole-cell patch clamp recording[25]. Cultured cells in stage 4 were perfused with bath solution containing 125 mmol/L NaCl, 2.5 mmol/L KCl, 2 mmol/L $CaCl_2$, 1 mmol/L $MgSO_4$, 1.25 mmol/L $KH_2PO_4$, 25 mmol/L $NaHCO_3$, 25 mmol/L D-glucose (NaOH at pH 7.3, 300 mOsm). Pipettes [(2–5) MΩ] were filled with intracellular solutions containing 130 mmol/L K-gluconate, 10 mmol/L KCl, 3 mmol/L Mg-ATP, 10 mmol/L phosphocreatine, 0.3 mmol/L GTP, 10 mmol/L HEPES, 0.2 mmol/L EGTA, and 50 IU/mL creatine phosphokinase (pH 7.2 with KOH, 295 mOsm). The whole-cell configuration was obtained after obtaining a tight seal of > 1 GΩ. Membrane current was recorded in voltage-clamp mode with 70% series resistance compensation, and membrane potential was recorded in current-clamp mode, which was switched during the experiment. Signals were amplified using an EPC-10 amplifier, filtered at 2 kHz, digitized at 10 kHz, and stored in a computer for analysis. Current traces were initially analyzed using the software Pulse-fit 8.52 and input into Prism 4.0 software for further analysis and plotting. Data were averaged and presented as Mean ± SEM. Differences were evaluated by Student's *t*-test and one-way analysis of variance. $P < 0.05$ was considered statistically significant.

**Main outcome measures**

The cell karyotype and electrophysiological properties of the TH-positive cells differentiated from hESCs.

## RESULTS

**Differentiation of hESC-derived cells into TH-positive cells *in vitro*** (Figure 1)

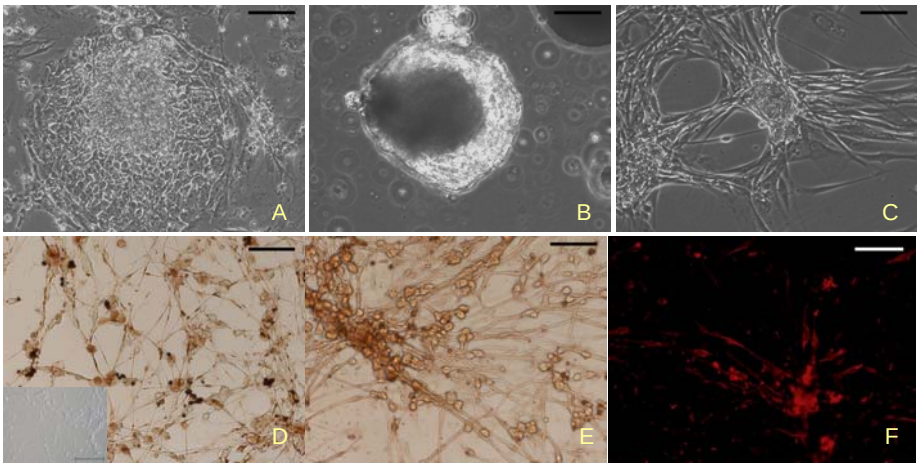



Figure 1 Differentiation of the human embryonic stem cells (hESCs) to tyrosine hydroxylase (TH)-positive dopaminergic (DA) neurons cultured *in vitro* using a modified four-step culture method (inverted fluorescent microscope, scale bar: 100 μm). (A) Undifferentiated hESC line PKU-1.1 at stage 1. (B) Formation of embryoid body at stage 2. (C) Differentiation of the hESCs into neural progenitor cells at stage 3. (D – F) Immunostaining analysis for the expression of specific cell markers in hESC-differentiated cells at the end of stage 4. The hESC-differentiated cells were positive for the neuronal marker β-tubulin/TuJ1 (D) and the DA neuronal marker TH (E); some of the cell colonies were also positive for nestin, a common neural progenitor marker (F).

The hESC line, PKU-1.1, were cultured on a mouse embryonic fibroblast feeder layer in the presence of hES medium (stage 1, Figure 1A). The embryoid body was attached after 1−2 days and expanded (stage 2, Figure 1B). By 2 days of adherence culture, most embryoid bodies had generated outgrowths of prolonged cells. By 2 weeks of culture, more cells migrated out from the colony center and formed a monolayer. After several passages, the differentiated cells expanded and organized into a meshy structure with an epithelial cell appearance (stage 3, Figure 1C). TH-positive cells had a consistent appearance, with small cell bodies and long processes (stage 4, Figure 1D). At the end of stage 3, cells were positive for nestin and negative for TuJ1 and TH. At 14 days of stage 4, cells were positive for TuJ1 and TH; by 20 days, over 90% of the differentiated cells were positive for TuJ1 and TH (Figures 1D, E). Although nestin expression was reduced (Figure 1F), most cells were still positive for nestin.

**Karyotype analysis of the TH-positive cells**

G-banding karyotype analysis showed that cell karyotype was stable during the entire differentiation, and the cell karyotype was normal (46, XX) and stable through stage 4 (Figure 2). These findings indicated that the differentiation of the hESCs into the TH positive cells by the modified four-step protocol did not change cytogenetics.

**Functional analysis of the TH-positive cells**

The functionalities of the voltage-gated sodium and potassium channels were tested in differentiated cells at the end of stage 4 using whole-cell patch clamp recording. All the 10 recorded TH-positive cells exhibited a fast inward current when the test voltage was depolarized to −30 mV and showed a delayed outward current when the test voltage depolarized to −10 mV (Figure 3A). The peak of inward current was obtained at a voltage between −10 mV and 0 mV, while the peak of outward current was obtained at 40 mV. The average peak of inward current density was about (−50.05 ± 15.50) pA/pF and the average peak of outward current density was about (41.98 ± 13.55) pA/pF (Figures 3B, C).

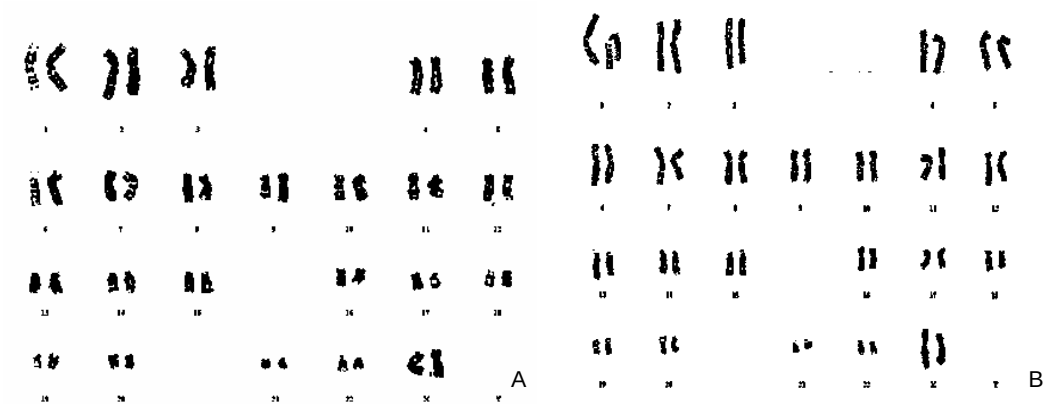
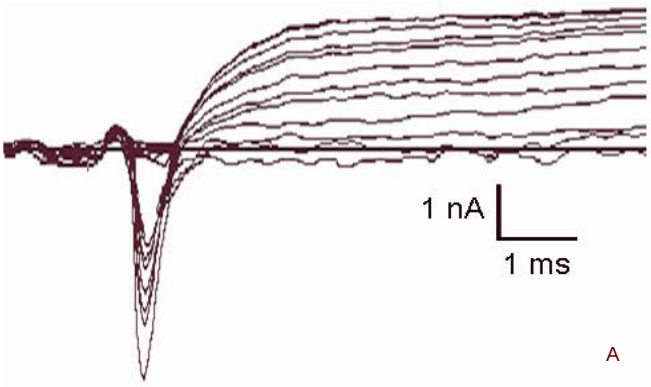


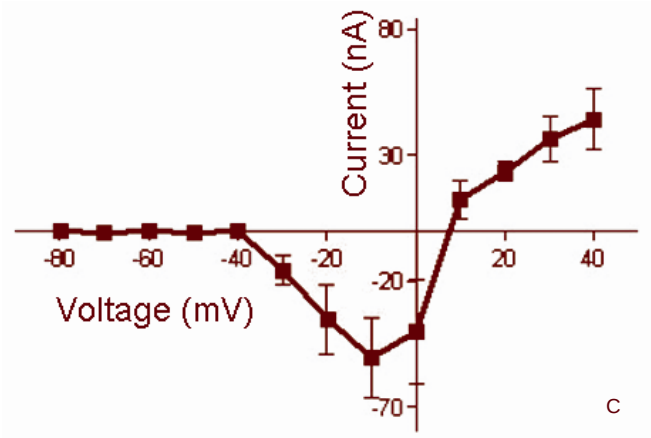

Figure 2 Karyotype analysis for the human embryonic stem cell (hESC)-derived tyrosine hydroxylase (TH) positive cells (G-banding karyotype analysis). (A) Cell karyotype of the undifferentiated hESCs at stage 1. (B) Cell karyotype of the hESC-derived TH-positive cells at stage 4 was normal (46, XX) and stable during differentiation.

## DISCUSSION

The present study used a modified four-step culture method to induce the differentiation of functional DA neurons from hESCs, and to improve the differentiation efficiency and cell survival of the differentiated DA neurons[23-24]. The differentiation protocol included several steps: the generation of embryoid bodies (stage 2), the selection and proliferation for neural progenitor cells (stage 3), and the differentiation of the progenitor cells into functional TH-positive cells (stage 4). At the beginning of stage 4, sonic hedgehog and FGF 8 were added to the culture to promote the directed differentiation of DA neurons from hESC-derived neuroepithelium[26-28]. Moreover, additional survival-promoting factors such as GDNF, TGF-β3, and db-cAMP were also added during the terminal differentiation stage to produce more mature and functional DA neurons[12, 29]. After incubation with sonic hedgehog and FGF 8 for 14 days, the majority of the hESC-derived cells expressed a neuronal marker, TuJ1, and some of them also expressed the DA neuronal marker, TH. After incubation with GDNF, TGF-β3, and db-cAMP for six additional days, more than 90% of the hESC-derived cells expressed both TuJ1 and TH, and some of the cell colonies were also positive for nestin, a common neural progenitor marker. These results suggest that the vast majority of the hESC-derived cells could differentiate into mature DA neurons, accompanied by some immature neural precursor cells. Some neural progenitor cells may remain at the end of differentiation or differentiated TH-positive cells may still express nestin. Therefore, transplantation of these differentiated cells requires further investigation.




A




C

| Holding potential (mV) | Current density (pA / pF) | |
|---|---|---|
| | mean | S.E.M |
| -80.00 | 0.168185 | 1.898723 |
| -70.00 | -0.63695 | 2.138964 |
| -60.00 | 0.043758 | 1.595654 |
| -50.00 | -0.57609 | 2.458098 |
| -40.00 | 0.172378 | 0.926121 |
| -30.00 | -15.2814 | 6.326117 |
| -20.00 | -35.0408 | 13.44465 |
| -10.00 | -50.0513 | 15.50226 |
| 0.00 | -39.7169 | 20.25169 |
| 10.00 | 9.35786 | 7.372548 |
| 20.00 | 20.30046 | 40319661 |
| 30.00 | 33.23545 | 9.516174 |
| 40.00 | 41.97607 | 13.55356 |

B

Figure 3 Electrophysiological characteristics of the human embryonic stem cell (hESC)-differentiated tyrosine hydroxylase (TH) positive cells (Whole-cell patch clamp recording). (A) Representative whole-cell current traces recorded in a hESC-differentiated TH positive cell. (B) Mean current density from 10 hESC-differentiated cells at each test voltage. (C) Plotted current-voltage curve from 10 recorded hESC-differentiated cells.

We modified the previous multi-step culture method by adding sonic hedgehog and FGF8 at the beginning of stage 4, followed by GDNF, TGF-β3, and db-cAMP during the final differentiation stage for six additional days. The generation of DA neurons requires the ventralis signal sonic hedgehog with factors that define AP patterning, such as FGF8, FGF4, and retinoic acid; therefore, FGF8 and SHH can efficiently promote the directed differentiation of DA neurons from hES cells[26-28]. GDNF is a potent survival factor for maintaining DA neurons via inhibition of apoptosis[30-31]. db-cAMP improves DA neuron yields from neural cultures and maintains genes phosphorylation or reactivation of protein kinase B to avoid apoptosis[32], while TGF-β3 at the terminal stage of ES cell differentiation can significantly enhance mRNA expression of anti-apoptotic Nurr1, TH and bcl-2[33-34]. Therefore, 90% of these cells were TH-positive, much greater than previous multiple-step culture methods using bFGF and TGF-α[6, 9, 17-18]. In addition, the lack of co-contaminating cell lines or animal products reduces the risk of contamination[14-15], although the time course for differentiation was much longer (8–9 weeks). In the intact human embryo, DA differentiation occurs at 45 days, with 56 days required for nerve process extension[35], consistent with the time course of the present study. Moreover, the cell karyotype was normal and stable during the differentiation process, indicating that the differentiation maintained stable cytogenetics.

We next measured neural function by testing voltage-gated sodium and potassium channels in stage 4 cells using whole-cell patch clamp recording. The current-voltage relationships obtained for the inward- and outward- current components were characteristic for sodium and potassium currents, respectively[21]. A few cells (3/15; data not shown) exhibited one or two action potentials with low amplitudes, and no spontaneous action potential was observed in any of the recorded cells. These results were consistent with previous findings[6-8, 11, 16].

Mouse or monkey embryonic cell-derived DA neurons function efficiently and improve motor dysfunctions in animal PD models[36]; however, few studies have observed the functional effects from transplantation of hESCs-differentiated grafts of human midbrain-like DA neurons into a rodent model of PD[5, 10] or transplantation of neural progenitor cells into the substantia nigra of 6-hydroxydopamine-lesioned rat model of PD[9]. Therefore, the clinical application of hESC-derived DA neurons remains unsatisfactory. For example, the survival rate of the hESC-derived cells is very low after transplantation *in vivo*[6, 19-22], and tumor formation has

been observed following transplantation[10]. We previously found that intra-striatal transplantation of hESC-derived TH-positive cells to PD rats could survive at least 4 weeks, improve apomorphine-induced rotation behaviors, and did not form tumors[24]. Thus, the hESC-derived DA cells derived here may also survive and function after transplant *in vivo*.

In conclusion, using a modified four-step culture method, we could differentiate hESC-derived cells into TH-positive DA neurons with stable normal karyotype and general electrophysiological properties *in vitro*. Replacement of degenerated nigrostriatal DA neurons with functional DA cells *in vivo* may be a promising strategy for clinical PD therapy.

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