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SELECTIVE GENERATION OF DOPAMINERGIC PRECURSORS FROM HUMAN FIBROBLASTS BY DIRECT LINEAGE CONVERSION

by

Miao He

A THESIS

Presented to the Faculty of

the University of Nebraska Graduate College

in Partial Fulfillment of the Requirements

for the Degree of Master of Science

Medical Sciences Interdepartmental Area

Physical Therapy Graduate Program

Under the Supervision of Professors Ka-Chun Siu and Jialin C. Zheng

University of Nebraska Medical Center Omaha, Nebraska

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Miao He

SELECTIVE GENERATION OF DOPAMINERGIC PRECURSORS FROM HUMAN FIBROBLASTS BY DIRECT LINEAGE CONVERSION

Miao He, M.S.

University of Nebraska Medical Center, 2018

Advisors: Ka-Chun Siu, Ph.D. and Jialin C. Zheng, Ph.D.

Transplantation of dopaminergic precursors (DPs) is a promising therapeutic strategy of Parkinson's disease (PD). However, limited cell source for dopaminergic precursors has become a major obstacle for transplantation therapy. In the first part of the study, we demonstrated that Foxa2 is superior to Lmx1a in inducing dopaminergic neuronal differentiation from induced neural progenitor cells (iNPCs). Based on the results above and some other published results, our group demonstrated that mouse fibroblasts can be reprogrammed into induced dopaminergic precursors (iDPs) through ectopic expression of transcription factors Brn2, Sox2 and Foxa2(1). In the current study, we hypothesized that similar strategy can be applied to generate human iDPs for future cell therapy of PD. We overexpressed transcription factors Brn2, Sox2 and Foxa2 in human fibroblasts and observed formation of neurospheres in the cultures. Subsequent characterization of the precursor colonies confirmed the generation of human induced dopaminergic precursors (hiDPs). These hiDPs were capable of self-renewal, proliferate, and differentiation. The hiDPs demonstrated high co-positive rate of midbrain neural progenitor markers DCX, Corin and neural progenitor marker Nestin by immunostaining. More importantly, the hiDPs also expressed high levels of ventral mesencephalon related neural progenitor marker genes such as Lmx1a, NIKX6.1, Corin, Otx2 and Mash1. After differentiation, those cells exhibited significantly higher levels of major functionally relevant proteins in dopaminergic neurons, including TH, DAT, AADC,

Lmx1B and VMAT2. Together, these results suggest that human iDPs can be generated by direct reprogramming of fibroblasts. These human iDPs may serve as a safe and effective cell source for transplantation treatment of PD.

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LIST OF ABBREVIATIONS

PD	Parkinson’s disease
L-DOPA	L-3, 4-dihydroxyphenylalanine
DBS	deep brain stimulation
iNPCs/iNSCs	proliferative induced neural progenitor/stem cells
iDPs	induced dopaminergic progenitors/precursors
PLO	poly-L-ornithine
VSV-G	vesicular stomatitis virus envelope G protein
PBS	phosphate buffer
MTS	[3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium]
TH	tyrosine hydroxylase
AADC	aromatic L-amino acid decarboxylase
VMAT2	vesicular monoamine transporter 2
MPTP	1-methyl-4-phenyl-1,2,3,6- tetrahydropyridine
DA	dopaminergic
SHH	sonic hedgehog
FGF8	fibroblast growth factor 8

CHAPTER 1: INTRODUCTION

Since the application of nuclei transfer technique which demonstrates the multipotency of differentiated cells, scientists have made great effort to optimize the reprogramming strategy to increase the safety and efficiency of conversion(2-6). The ultimate purpose is to provide sufficient cells for transplantation therapy or to build appropriate disease models for further study.

Parkinson's disease (PD) is among the most prevalent neurodegenerative disorders which affects 1-2% of the population over 70 years old(7, 8). Key symptoms of PD result from loss of dopaminergic neurons in the substantia nigra, including rigidity, bradykinesia, resting tremor and postural instability(9). In later stages of the disease, pathological cell death occurs in the midbrain, basal forebrain and neocortex. This progressive brain-wide degeneration is also associated with the onset of dementia, autonomic dysfunction, and severe postural instability. Even though most of the patients with PD are sporadic, there are still 15% of the patients having family history and 5-10% inherit in a monogenic fashion(10, 11). Studying Parkinson related gene mutations could also help us understand better the pathogenesis of PD. To date, at least 23 loci and 19 parkinsonism-causing genes have been identified by scientists. Familial PD has been linked to heritable autosomal dominant, recessive and X-linked mutations. Autosomal-dominant mutations in *SNCA*(12), *PARK3*(13), UCHL1(14), LRRK2(15), NR4A2(16), GIGYF2(17, 18), HTRA2(19), VPS35(20), EIF4G1(21), TMEM230(22), CHCHD2(23) and RIC3(24), as well as, autosomal-recessive mutations in PRKN(25), PINK1(26), ATP13A2(27), PARK7(28), FBXO7(29), DNAJC6(30), SYNJ1(31), PLA2G6(32) and VPS13C(33) have been implicated in the onset and progression of PD. Recently, PARK10(34), PARK16(35) and PARK12(17) with X-linked inheritance have also been found to be associated with parkinsonism. Gene locus includes PARK 1-23. PD is

incurable to date, existing pharmaceutical and surgical treatments such as L-3, 4-dihydroxyphenylalanine (L-DOPA), dopamine receptor agonists and deep brain stimulation (DBS) can only ameliorate symptoms and signs. None of these therapeutic approaches can cure or even reverse the development of disease(36, 37). Besides, although initially beneficial, the effectiveness of the above pharmaceutical and surgical treatments declines throughout disease progression. The development of stem cell based therapeutic approach brought hope of curing PD by replacing lost neurons, compensating their function and preventing further neuronal dysfunction and death. Compared to the compensatory dopamine provided temporarily by medications, dopamine-producing neurons derive from transplanted neural stem cells can produce dopamine inside patients' substantia nigra and enhance the recovery of dopamine signaling in damaged neural circuitry. It has already been proved that transplanted embryonic dopamine neurons can significantly improve motor function in early onset PD patients(38). Besides, neural stem cells are more suitable PD models compared to other somatic cells(39-42). There are two leading theories as to how these transplanted neural stem cells provide therapeutic potential. One is that those cells may directly replace damaged neurons, the other one is those cells can indirectly protect neurons after injection through release of neurotrophic and immunomodulatory factors(43-46).

For the applications, it would be more desirable to generate proliferative induced neural progenitor/stem cells (iNPCs/iNSCs), instead of neurons with no proliferation ability, from somatic cells(47-51). However, even though iNPCs hold great potential in regenerative medicine, they have an intrinsic potential for multi-lineage differentiation into astrocytes, oligodendrocytes, and dozens of neuronal subtypes(47, 49, 50, 52-54). The directed differentiation of these precursors into a highly defined state for therapeutic use might be challenging if the underlying mechanisms of differentiation are not fully

understood. Therefore, the induction of restricted neural progenitors might be better suited to regenerate specific populations of neural cells following degeneration(55). Our initial studies have shown that skin fibroblasts can be successfully converted into induced neural progenitors (5F-iNPCs) by a set of five transcription factors, including Brn2, Sox2, TLX, Bmi1 and c-Myc (51). However, the dopaminergic differentiation efficiency of 5F-iNPCs in response to sonic hedgehog (SHH)/fibroblast growth factor 8 (FGF8) stimulation remains low (<5%). Therefore, we kept on looking for more superior strategy.

Recently, several different research groups including ours have lay the focus on how to directly reprogram somatic cells to functional induced dopaminergic progenitors/precursors (iDPs)(1, 56, 57). The major potential advantage is that iDPs are restricted to dopaminergic neuronal lineage during the process of differentiation. Our lab has already successfully generated mouse iDPs through direct reprogramming(1). However, whether this strategy can be successfully applied on human cells is still unclear. We hypothesized that similar strategy can be applied to generate human iDPs for future cell therapy of PD. The transition from mouse to human cells is an essential step before this strategy can be used on real patients. This study would focus more on how to generate human iDPs from human skin fibroblasts by direct reprogramming.

CHAPTER 2: METHODS

2.1 Cell preparation, retroviral packaging, infection and direct reprogramming.

Human skin fibroblasts were isolated from the left leg of a female fetus aged 20 weeks, which was obtained from elective aborted specimens following completion of the abortion procedure through collaborative works with the Birth Defects Research laboratory at University of Washington. The protocol is in compliance with all relevant state and federal regulations and is approved by the University of Nebraska Medical Center (UNMC) Institutional Review Board (IRB#: 123-02-FB). Fibroblasts were cultured in Dulbecco's modified Eagle's medium supplemented with 10% FBS, 1×Non-Essential Amino Acid, 50U/ml penicillin, 50μg/ml streptomycin at 37 °C in a 5% CO_2 humidified atmosphere. Fibroblasts were used within passage 2–5 to avoid replicative senescence. Human Brn2 ORF, Foxa2 ORF and Sox2 ORF were cloned into pMXs-retroviral vectors respectively (Cellbiolabs, RTV-010). Retroviruses (pMXs) were generated with Plat-GP packaging cells. Plat-GP cells were seeded at 3.2-3.6×10^6 cells per 100mm poly-L-ornithine (PLO) /laminin coated dish. 24 hours after seeding, 10μg DNA of pMXs-based retroviral vectors encoding human Sox2, Brn2 and Foxa2 together with 5μg DNA of the vesicular stomatitis virus envelope G protein (VSV-G) expressing packaging plasmid (pcMV-VSV-G) were introduced into Plat-GP cells using Lipofectamine LTX & PLUS™ transfection reagent (Invitrogen). To be specific, dilute 15μg DNA in 1ml Opti-MEM® Medium, then add 15μl PLUS™ Reagent. After 5 minutes incubation at room temperature, add 15μl Lipofectamine® LTX Reagent. Add DNA-lipid complex to cells after 5 minutes incubation at room temperature. The medium was replaced with 5.5ml of DMEM containing 5% FBS 24h after transfection. Human fibroblasts (hFbbs) were seeded at 2-3 ×10^5 cells per 35mm culture dish. At 48h and 72h post-transfection, 5ml virus-containing supernatants from these Plat-GF cultures were filtered through a

0.45μm cellulose acetate filter and collected. Equal volumes of the supernatants from 3 dishes were mixed and supplemented with 10μg/ml polybrene. Human fibroblasts were incubated in virus/polybrene-containing supernatants overnight. The medium was changed 72 hours after infection to NeuroCult® human NSC Basal (Stem Cell Technologies, Inc., Vancouver, BC V5Z 1B3, Canada) Medium supplemented with NeuroCult® human NSC Proliferation Supplements (Stem Cell Technologies, Inc.), 20ng/ml basic fibroblast growth factor (bFGF, BioWalkersville), 20ng/ml epidermal growth factor (EGF, BioWalkersville) and 5μg/ml heparin (Sigma-Aldrich, St. Louis, MO). After 10-21 days, the predicted human iDP colonies were monitored by fluorescence microscope.

2.2 Quantitative Real-Time reverse transcription polymerase chain reaction.

Total mRNA was isolated with TRIzol Reagent (Invitrogen) and RNeasy Mini Kit (QIAGEN Inc., Valencia, CA) using a protocol provided by the manufacturer. The reverse transcription was performed using Transcription 1st Strand cDNA Synthesis Kit (Roche, USA). The RT-PCR analyses for the detection of human neural stem cell-specific mRNAs were performed using SYBR® Select Master Mix (Life Technologies, Los Angeles, CA) with 0.5μl of cDNA, corresponding to 1μg of total RNA in a 15μl final volume consisting of 7.5μl SYBR Green, 1.5μl H_2O and 5.5μl oligonucleotide primer pairs (synthesized at Fisher) at 10μM. PCR program: 1) 50 °C for 2 minutes, 2) 95 °C for 2 minutes; 3) 95 °C for 15 seconds, 4) specific annealing temperature for 15 seconds and 5) 72 °C for 1minutes. Steps 2 to 4 were repeated 40 times. All samples were amplified in triplicate and the means were calculated and used for further analysis.

2.3 Immunocytochemistry.

The cultured cells were fixed in 4% formaldehyde for 15 minutes at room temperature, and then washed with PBS for 3 times, 10 minutes per time. The fixed cells were simultaneously permeabilized and blocked with 0.2% Triton X-100 and 5% horse serum in PBS for 30 minutes at room temperature. Cells were incubated with primary antibodies as listed in Table 2 overnight at 4°C, and then washed with PBS for 3 times and incubated for 2h at room temperature with secondary antibodies (Table 2). Fluorescent images were obtained using a Zeiss 710 Confocal Laser Scanning Microscope (Carl Zeiss, Oberkochen, Germany).

2.4 Cell proliferation rate

The CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay (Promega) was used to determine the number of viable cells in proliferation. To be specific, cells were seeded into 96-well tissue culture plates (Fisher scientific). The reagent MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] was added to the wells in the proportion of 20 µl per 100 µl medium. After the plates were incubated at 37°C for 1-4 hours, optical density (OD) 490 values were read and calculated with BioTek Gen5 data analysis software.

2.5 Differentiation.

For neuronal differentiation, SHH/FGF8 dependent protocol was used: Cells were plated on PLO/laminin coated coverslips in 24-well plate with DMEM/F12 containing 1× N2, 100ng/ml FGF8 (Peprotech), 100ng/ml SHH (Peprotech) and 10ng/ml bFGF

(Peprotech) for 6 days, then switched to DMEM/F12 containing 1× N2, 1× B27, 0.2mM ascorbic acid (Sigma), 0.1mM β-mercaptoethanol, 1.0mM dibutyrylcAMP (Sigma), 10ng/mL glial cell line-derived neurotrophic factor (GDNF) (Peprotech), and 10ng/mL brain-derived neurotrophic factor (BDNF) (Peprotech) for another 4-8 weeks. The medium was changed every 3 days.

2.6 Statistical Analyses.

Statistical analyses were performed using GraphPad Prism 7.00 and IBM SPSS Statistics Version 22. The data presented are means with standard deviations unless specified otherwise. Normality of data was tested using the Shapiro-Wilk Test. Differences between groups were compared using the one-way ANOVA with Bonferroni correction for multiple comparisons unless specified. A P of < 0.05 was considered as significant. All assays were performed in triplicate, with triplicate samples in each experiment.

CHAPTER 3: RESULTS

3.1 Foxa2 is superior to Lmx1a in converting mouse iNPCs into neural progenitors with midbrain identity

The iNPCs (induced by 5 factors, including Brn2, Sox2, TLX, Bmi1 and c-Myc) were infected with retroviruses encoding Foxa2 or Lmx1a or both Foxa2 and Lmx1a respectively. The expression of Foxa2 and Lmx1a was engineered under the control of doxycycline (Dox) (Fig. 2A). Then we compared the growth rate of different cell lines (iNPCs, Lmx1a-iNPCs, Foxa2-iNPCs, Lmx1a-Foxa2-iNPCs) at day 1, 3, 5 and 7 (Fig. 2B). Dox negative groups were also tested as control. Our results showed that Lmx1a-Foxa2-iNPCs cell line has the lowest cell growth rate at all the time points, followed by Foxa2-iNPCs and Lmx1a-iNPCs. iNPCs cell line demonstrated the most active proliferative activity.

iNPCs exhibited the highest level of neural progenitor markers Nestin, CD133 and Mushashi compared to other cell lines (Fig 1C-E). The difference was most significant between iNPCs and Lmx1a-Foxa2-iNPCs cell line ($p<0.001$). However, Lmx1a-Foxa2-iNPCs expressed the highest levels of dopaminergic neuron proliferative progenitor cell markers Corin and Otx2, followed by Lmx1a-iNPCs (Fig. 1F, G). Foxa2-iNPCs expressed the highest level of Pitx3, which is another dopaminergic neuron proliferative progenitor marker, followed by Lmx1a-Foxa2-iNPCs ($p<0.05$, compared to iNPCs) (Fig. 1H). After 6 weeks of differentiation in the presents of SHH and FGF8 stimulation, the differentiation efficiency of different cell lines into dopaminergic neurons was tested (Fig. 2). PT-PCR was performed to identify the relative levels of tyrosine hydroxylase (TH) in the resulting cells after differentiation. Cells derived from Lmx1a-Foxa2-iNPCs cell line expressed the highest level of TH ($p<0.001$), followed by the cells derived from Foxa2-iNPCs ($p<0.05$), Lmx1a-iNPCs and iNPCs (Fig. 2A). Cells overexpressed both Lmx1a

and Foxa2 also exhibited the highest level of mature neuronal marker MAP2 ($p<0.0001$), followed by cells overexpressed only Foxa2 ($p<0.01$), Lmx1a and iNPCs (Fig. 2C). More iNPCs with overexpression of Foxa2 differentiated into dopaminergic (DA) neurons (3.6% TH and MAP2 positive cells) compared to those overexpressed Lmx1a (1.5% TH and MAP2 positive cells) according to the immunostaining results (Fig. 4). Cells from Foxa2-iNPCs expressed the highest level of another general neuronal marker Tuj1 ($p<0.0001$), followed by Lmx1a-Foxa2-iNPCs and Lmx1a-iNPCs ($p<0.01$). The results of immunostaining are consistent with that of RT-PCR (Fig. 3). On the other hand, unlike cells from iNPCs cell line, cells from Lmx1a-Foxa2-iNPCs expressed much lower levels of glial-lineage related genes, such as astrocyte marker GFAP and S100β (Fig. 2 D, E). In sum, the number of TH, Tuj1 and MAP2 positive cells were ranked in the sequence of Lmx1a-Foxa2-iNPCs, Foxa2-iNPCs, Lmx1a-iNPCs and iNPCs. In contrast, the levels of GFAP positive cells were ranked in the sequence of iNPCs, Foxa2-iNPCs, Lmx1a-iNPCs and Lmx1a-Foxa2-iNPCs (Fig. 5).

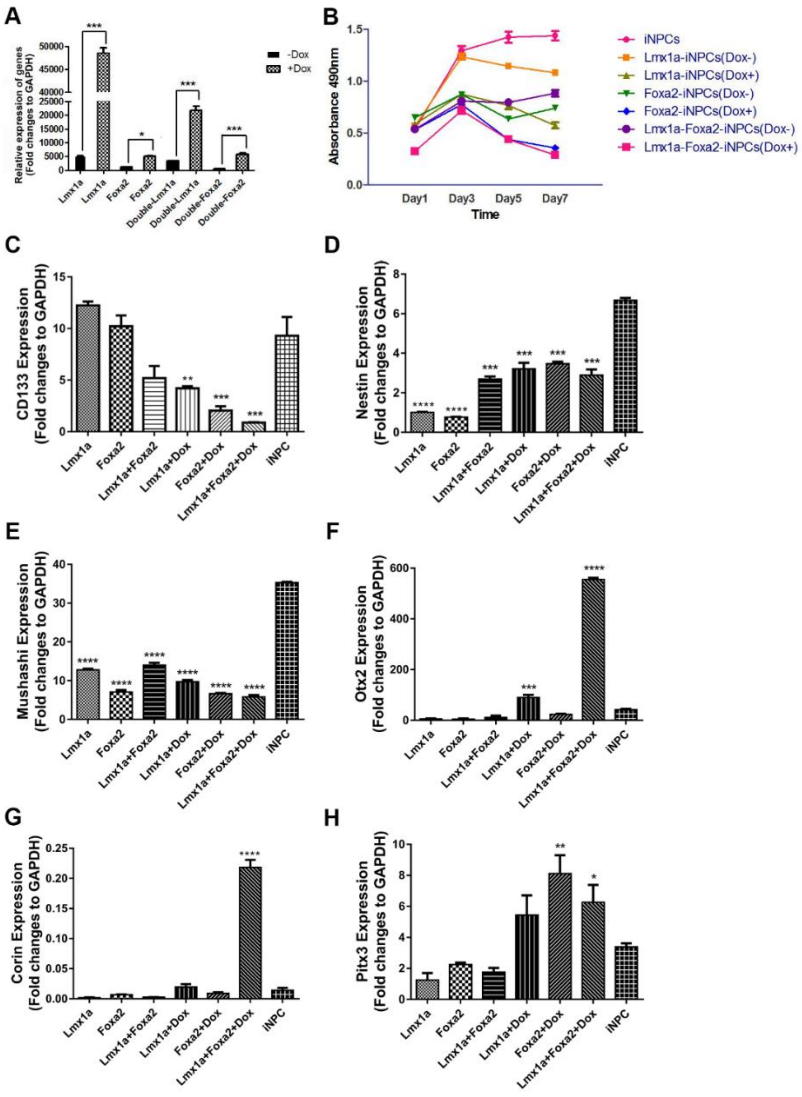

Figure 1. Characteristics of iNPCs overexpress Lmx1a, Foxa2 or both Lmx1a and Foxa2. (A) Identification of the overexpression of Lmx1a, Foxa2 or both Lmx1a and Foxa2 in 5F iNPCs by RT-PCR; (B) The growth rate of different cell lines (iNPCs, Lmx1a-iNPCs, Foxa2-iNPCs, Lmx1a-Foxa2-iNPCs) at day 1, 3, 5 and 7; (C) Comparison of CD133 expression level between different cell lines; (D) Comparison of Nestin expression level between different cell lines; (E) Comparison of Mushashi expression level between different cell lines; (F) Comparison of Otx2 expression level between different cell lines; (G) Comparison of Corin expression level between different cell lines; (H) Comparison of Pitx3 expression level between different cell lines. *denotes $p < 0.05$, **denotes $p<0.01$, ***denotes $p<0.001$, ****denotes $p<0.0001$ compared to iNPCs.

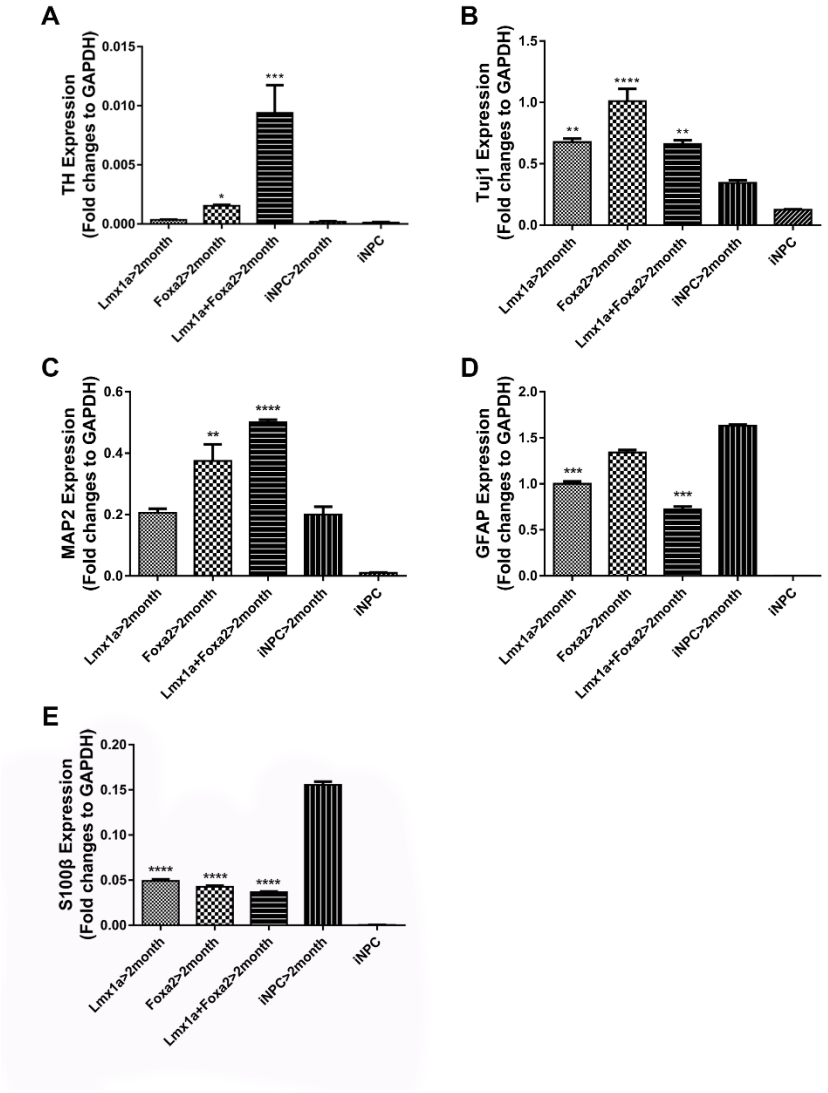

Figure 2. Differentiation efficiency of different cell lines into neurons with dopaminergic neuron identity. (A) Comparison of TH expression level between different cell lines after 2-month differentiation by RT-PCR; (B) Comparison of Tuj1 expression level between different cell lines after 2-month differentiation by RT-PCR; (C) Comparison of MAP2 expression level between different cell lines after 2-month differentiation by RT-PCR; (D) Comparison of GFAP expression level between different cell lines after 2-month differentiation by RT-PCR; (E) Comparison of S100β expression level between different cell lines after 2-month differentiation. Different cell lines include iNPCs, Lmx1a-iNPCs, Foxa2-iNPCs, Lmx1a-Foxa2-iNPCs. *denotes $p < 0.05$, **denotes $p<0.01$, ***denotes $p<0.001$, ****denotes $p<0.0001$ compared to iNPCs.

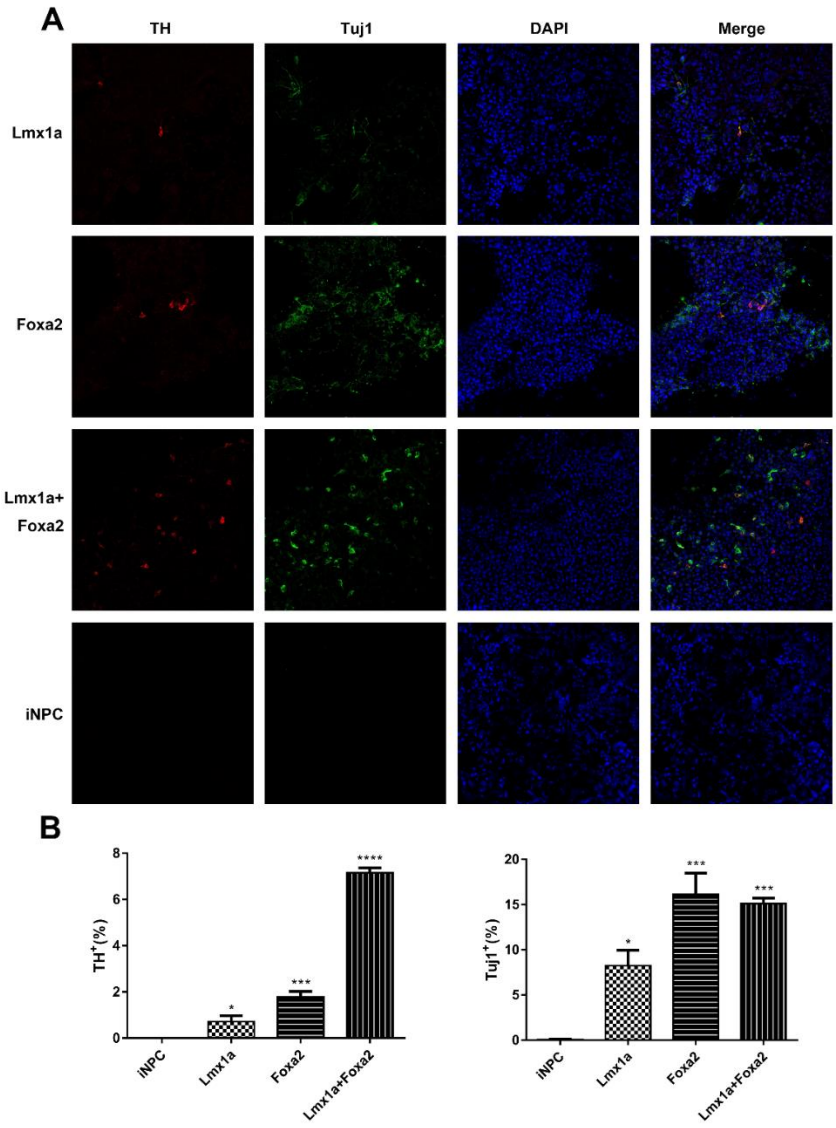

Figure 3. Comparison of the percentage of TH and Tuj1 positive neurons after differentiation from different cell lines. (A) Expression of TH and Tuj1 in cells differentiated from different cell lines by immunostaining; (B) Percentage of TH or Tuj1 positive cells differentiated from different cell lines. Different cell lines include iNPCs, Lmx1a-iNPCs, Foxa2-iNPCs, Lmx1a-Foxa2-iNPCs. *denotes $p < 0.05$, ***denotes $p<0.001$, ****denotes $p<0.0001$ compared to iNPCs.

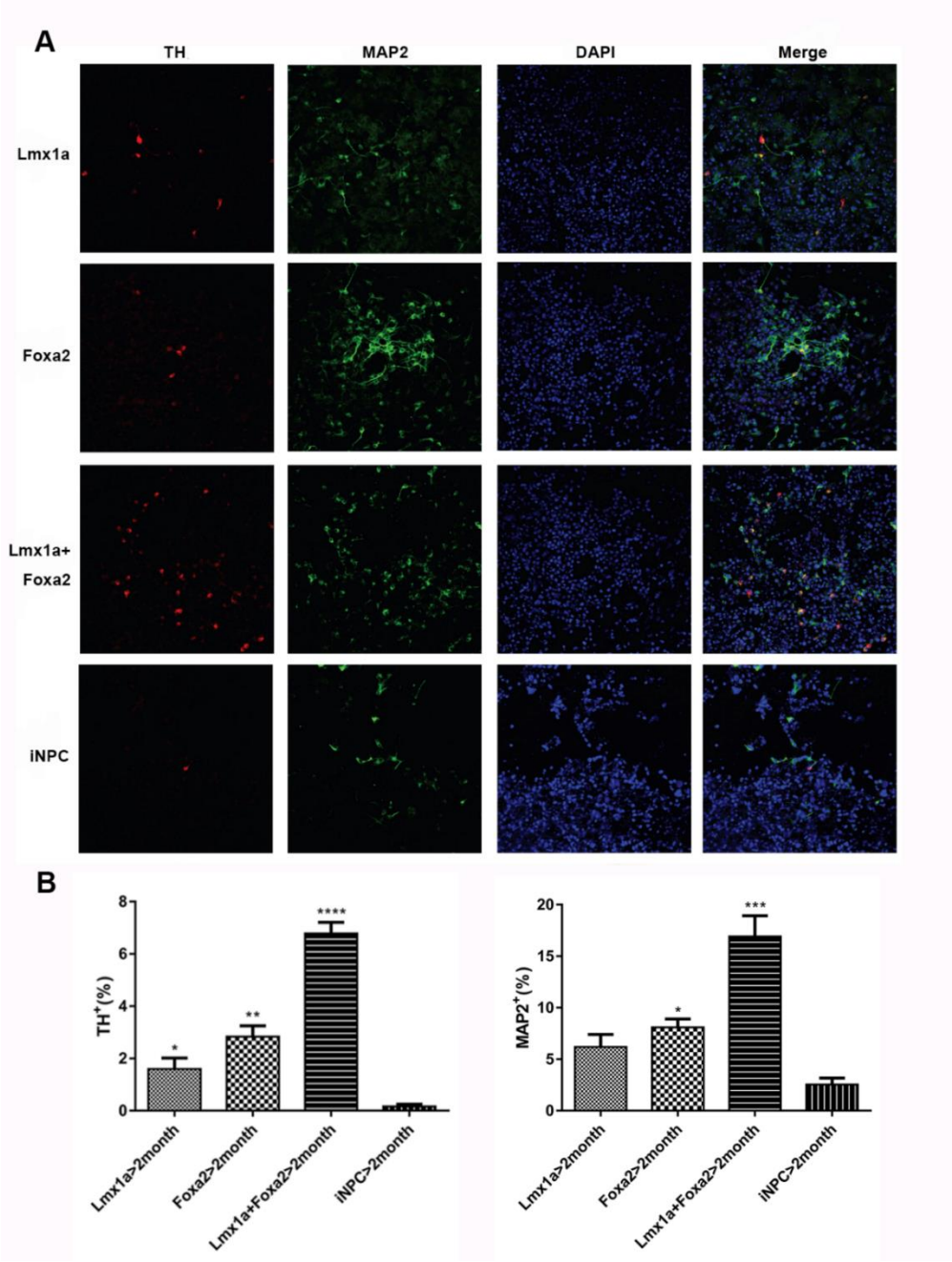

Figure 4. Comparison of the percentage of TH and MAP2 positive neurons after differentiation from different cell lines. (A) Expression of TH and MAP2 in cells differentiated from different cell lines by immunostaining; (B) Percentage of TH or MAP2 positive cells differentiated from different cell lines. Different cell lines include iNPCs, Lmx1a-iNPCs, Foxa2-iNPCs, Lmx1a-Foxa2-iNPCs. *denotes $p < 0.05$, **denotes $p<0.01$, ***denotes $p<0.001$, ****denotes $p<0.0001$ compared to iNPCs.

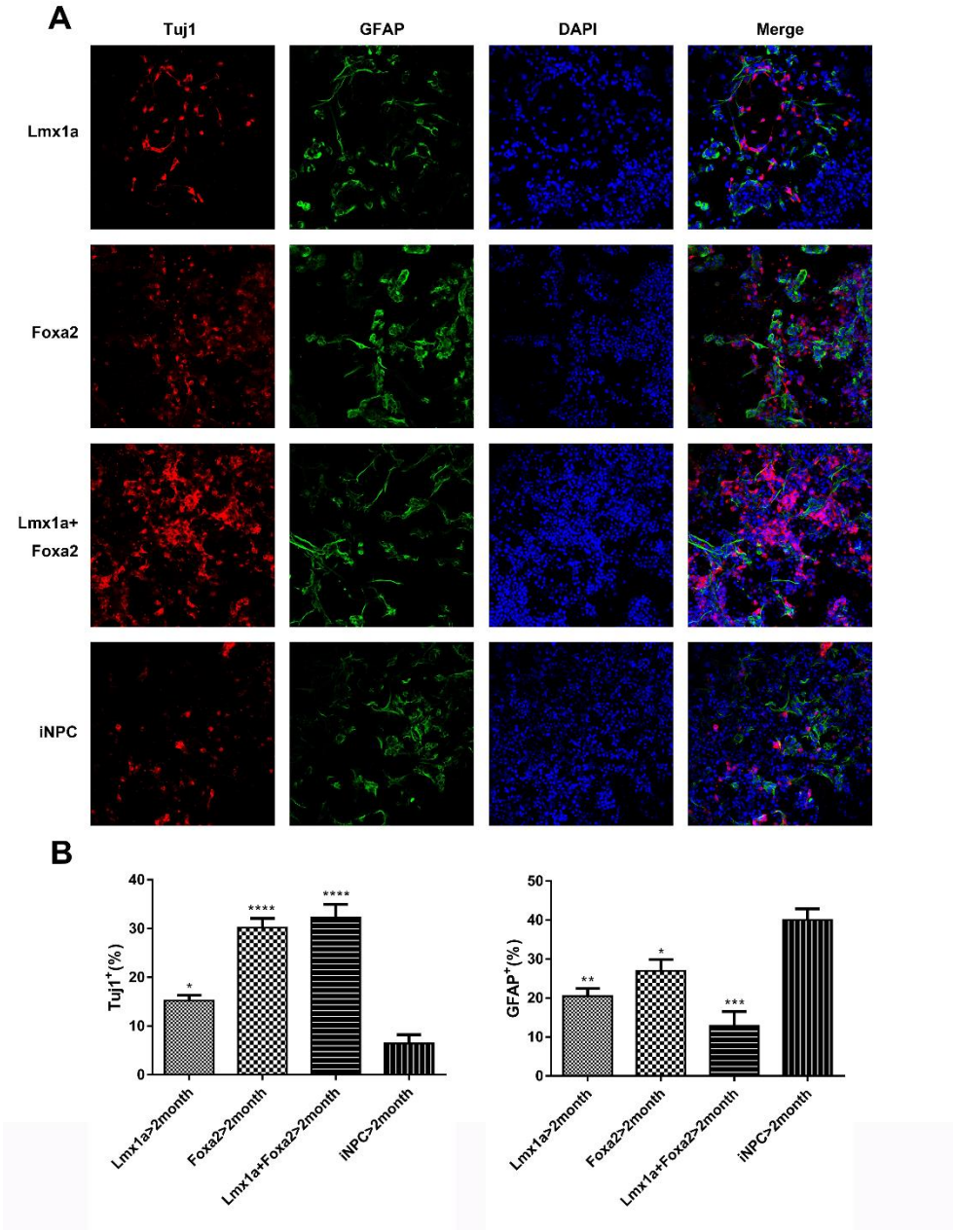

Figure 5. Comparison of the percentage of Tuj1 and GFAP positive neurons after differentiation from different cell lines. (A) Expression of Tuj1 and GFAP in cells differentiated from different cell lines by immunostaining; (B) Percentage of Tuj1 or GFAP positive cells differentiated from different cell lines. Different cell lines include iNPCs, Lmx1a-iNPCs, Foxa2-iNPCs, Lmx1a-Foxa2-iNPCs. *denotes $p < 0.05$, **denotes $p<0.01$, ***denotes $p<0.001$, ****denotes $p<0.0001$ compared to iNPCs.

3.2 Foxa2, Brn2 and Sox2 can convert human fibroblasts into neural progenitors with midbrain identity

Based on the results above and some other results that have already been published(1). Our lab initially examined the role of Foxa2 and two other known reprogramming factors Sox2 and Brn2 in the direct conversion of mouse somatic cells into dopaminergic neural progenitors and achieved success(1). Thereafter, whether this strategy can be applied on human somatic cells was tested. Human fetus fibroblasts were isolated and cultured. The fibroblasts were transfected with retroviruses encoding Brn2, Sox2 and Foxa2 following the schematic procedure in Fig. 6A. During day 10-21 post-transfection, we observed the colony formation of cells (Fig. 6B). Eight colonies were obtained in total, however only three of them were confirmed to be expandable clones after subculture. PT-PCR was performed to identify the relative levels of Foxa2, Sox2 and Brn2 in the resulting cells after subculture. Only the cells (named hiDPs, human induced dopaminergic progenitors) from one of the three expandable clones retained high expression levels of Foxa2, Sox2 and Brn2 compared to the original human skin fibroblasts (hFbbs) (Fig. 6C). Cells from the other two expandable clones only expressed high levels of Sox2 and Brn2, indicating the failure of Foxa2 transfection. The hiDPs were characterized through the expression levels of several key neural progenitor marker genes in hiDPs and hFbbs by real-time RT-PCR analysis (the sequences of primer pairs were listed in Table 1). Importantly, the hiDPs expressed high levels of dopaminergic neuron proliferative progenitor cell markers, including Corin (Lrp4), Lmx1a, Otx2, Mash1, Pitx3 and Nkx6.1 ($p<0.05$, compared to hFbbs) (Fig. 7C). These genes were previously reported to specifically expressed in dopaminergic neuron proliferative progenitor cells(20,23,27,28). The hiDPs exhibited high co-positive expression of Corin and Nestin, at the rate of 91.22±7.19% (Fig. 7A, B). The cells also displayed high co-positive rate of Corin and doublecortin (DXC) (94.75±3.95%) by

immunofluorescent staining (Fig.7A, B). These results suggest that fibroblasts could acquire the mesencephalic regional identity and dopaminergic neural fate through the forced over expression of transcription factors human Brn2, Sox2 and Foxa2. The results above are similar to what has been received from the study of mouse fibroblasts(1).

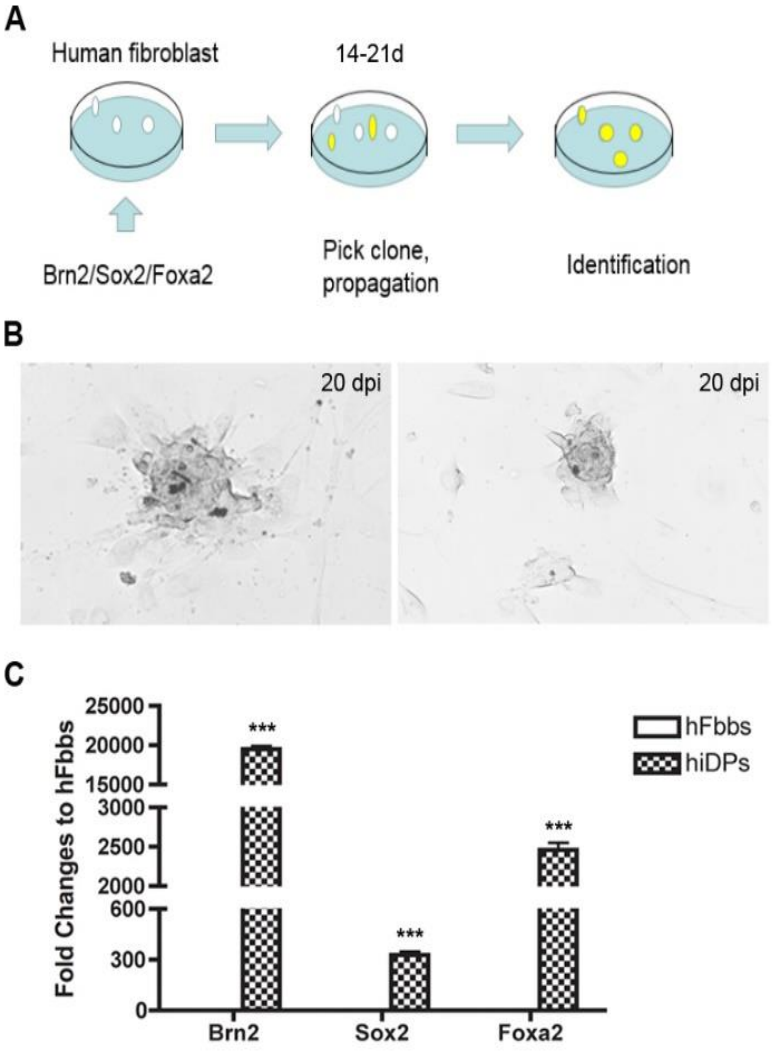

Figure 6. Generation of human induced dopaminergic neuron progenitors. (A) Strategy of hiDP generation by ectopic expression of Sox2, Brn2 and Foxa2; (B) hiDP

clone formation at 20 dpi under microscope; (C) The overexpression of Sox2, Brn2 and Foxa2 in hiDPs derived from human fibroblasts.

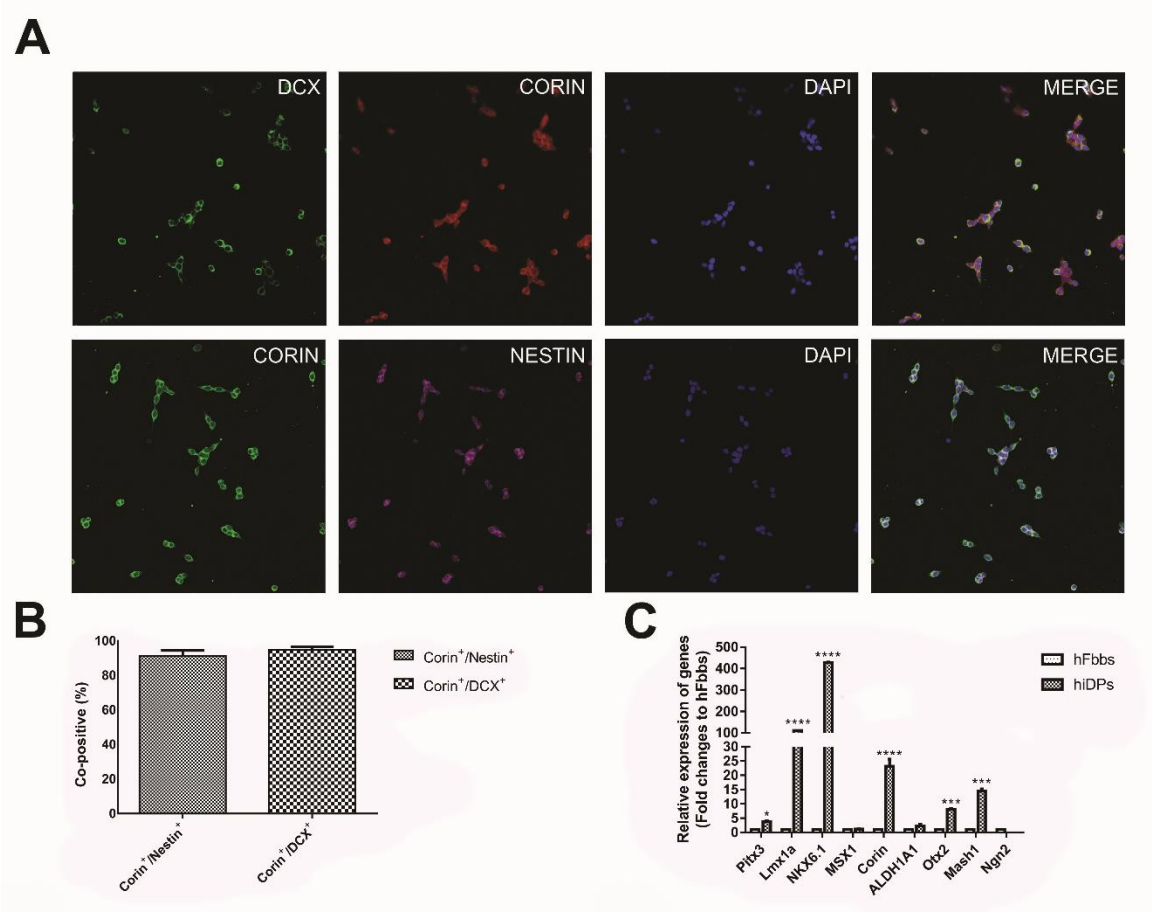

Figure 7. Characteristics of hiDPs. (A) Expression of DCX, Corin and Nestin in hiDPs cells by immunostaining; (B) Co-positive rate of Corin/Nestin and Corin/DCX; (C) The expression of a specific set of neural progenitor marker genes. GAPDH and human fibroblasts (hFbb) served as internal and negative control respectively. *denotes $p < 0.05$, ***denotes $p<0.001$, ****denotes $p<0.0001$ compared to hFbbs.

3.3 hiDPs express specific dopaminergic progenitor markers and has dopaminergic neuronal-restricted differentiation potential

We further validated whether the iDPs are dopaminergic neuronal lineage-restricted by differentiating the iDPs into neurons in the presence of SHH and FGF8 stimulation followed by the treatment of BDNF, GDNF and ascorbic acid (AA) for neuronal

maturation. After 4-8 weeks of differentiation, we tested the differentiation efficiency of hiDPs into dopaminergic neurons (Fig. 8 and 9). The results demonstrated that the neurons derived from hiDPs expressed high levels of TH after 4-week and 8-week differentiation (compared to both hiDPs before differentiation and hFbbs), suggesting dopaminergic neuronal lineage-restricted fate for the iDPs (Fig. 8 and 9). They also exhibited significantly higher levels of major functionally relevant proteins in dopaminergic neurons, including TH, DAT, AADC, Lmx1b and brain-specific isoform of the vesicular monoamine transporter (VMAT2) compared to both the original hFbb cells and hiDPs before differentiation (Fig. 9). To be specific, after 8 weeks of differentiation, the level of TH, DAT and Lmx1b in cells from hiDPs were relatively higher than those after 4 weeks of differentiation. Additionally, about 91.28% of the Tuj1+ neurons are TH+ dopaminergic neurons. About 96.67% of the TH+ neurons are NeuN positive, about 86.75% of the TH+ dopaminergic neurons are MAP2 positive neurons, indicating the mature neuron nature of the neurons derived (Fig. 8B).

Those results suggest that iDPs can effectively differentiate and generate mature and functional DA neurons.

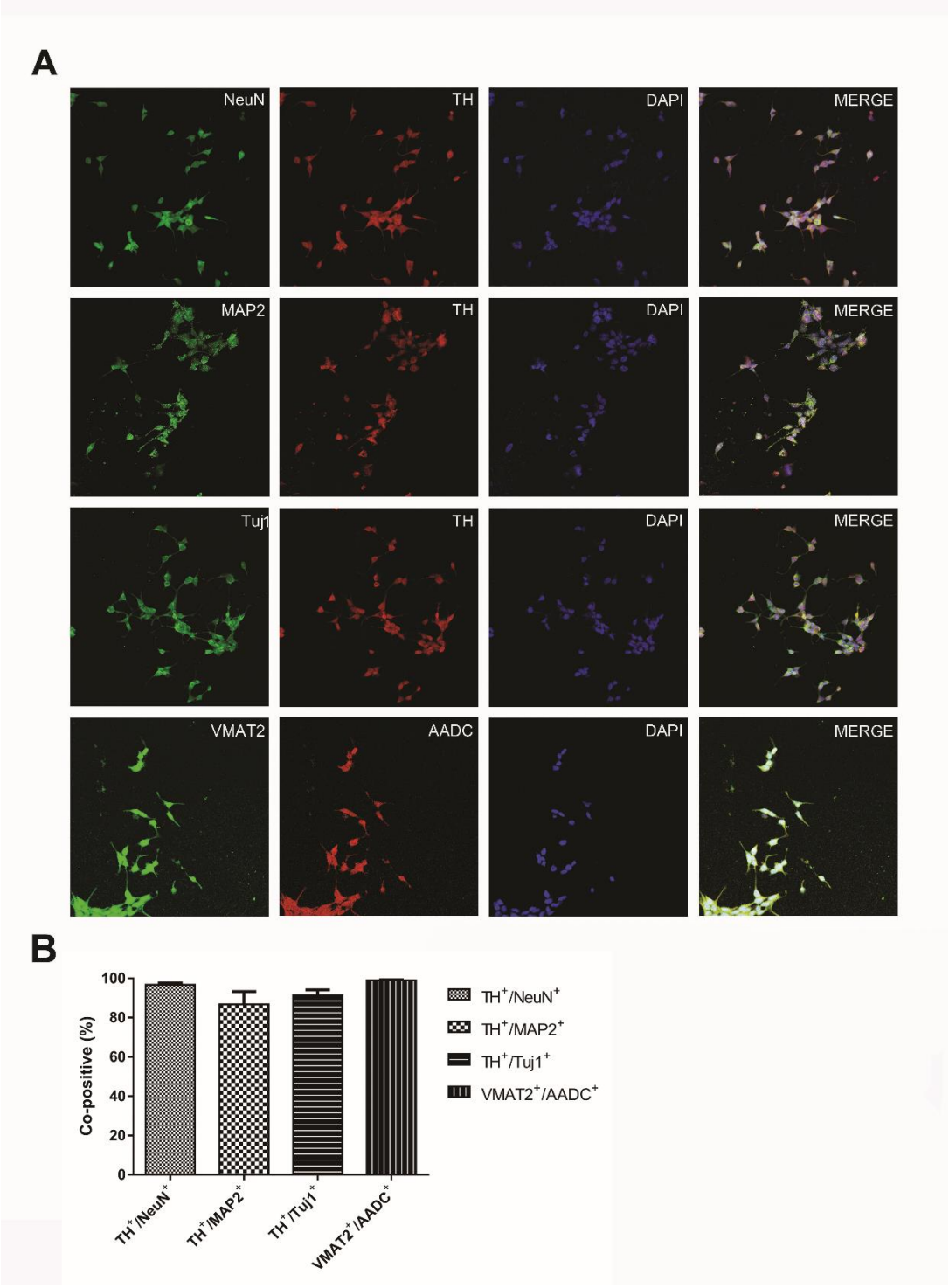

Figure 8. hiDPs differentiate into neurons with dopaminergic neuron identity. (A) Expression of TH, VMAT2, AADC and neuron marker NeuN, MAP2 and Tuj1; (B) Co-positive rate of TH/NeuN, TH/MAP2, TH/Tuj1 and VMAT2/AADC.

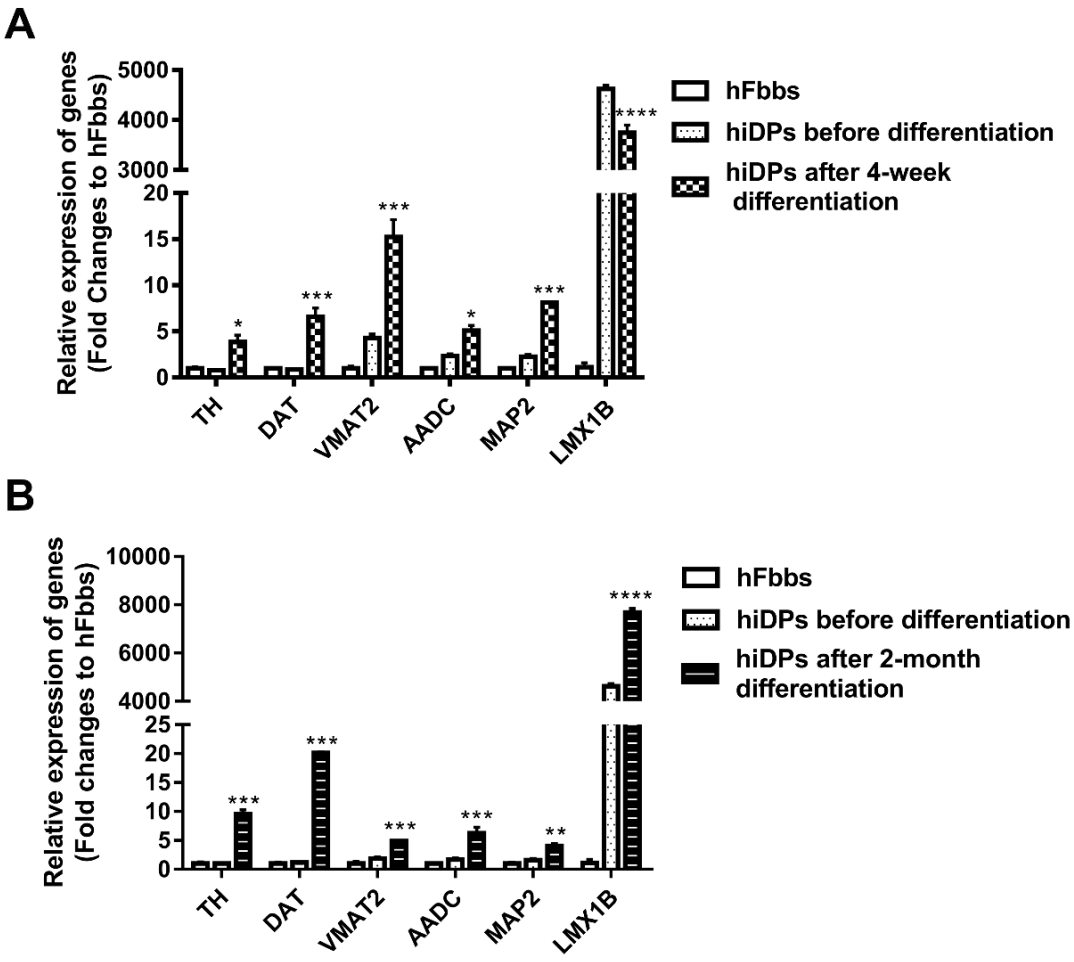

Figure 9. The expression of a specific set of dopaminergic neuron marker genes in cells differentiated from hiDPs. (A) The expression of TH, DAT, VMAT2, AADC, MAP2 and LMX1B after 4 weeks of differentiation; (B) The expression of TH, DAT, VMAT2, AADC, MAP2 and LMX1B after 4 weeks of differentiation. *denotes $p < 0.05$, **denotes $p < 0.01$, ***denotes $p<0.001$, ****denotes $p<0.0001$ compared to hiDPs before differentiation and hFbbs.

CHAPTER 4: DISCUSSION

PD is a movement disorder secondary to loss of dopaminergic neurons in the pars compacta region of the substantia nigra. Therefore, cell replacement therapy offers a potential means for curing this disease. Dopaminergic neuron progenitors from fetal ventral mesencephalon tissue have been transplanted into the striatum of PD patients with good results in several cases(58, 59). However, ethical concerns of using fetal tissues limited its use in the growing PD patient population. Direct reprogramming has been accepted as a safer and more efficient way to obtain desired type of cells(57). Several groups have previously proven that Sox2 and Brn2 are critical for the direct conversion of fibroblasts into iNPCs(49, 51, 52, 60, 61). By expressing defined transcript factors in addition to Brn2 and Sox2, we think it is possible to directly convert somatic cells to neuronal lineage-restricted progenitors. Since the cooperation of SHH-Foxa2 and Wnt1-Lmx1a pathways plays a significant role in the development of dopaminergic progenitors and DA neurons, we studied the role of Foxa2 and Lmx1a in promoting the differentiation of mouse iNPCs (5 factors) into DA neurons(62).

The fact that Lmx1a-Foxa2-iNPCs cell line has the lowest proliferation rate and expresses low level of neural progenitor markers is consistent with its tendency of differentiation. Foxa2-iNPCs and Lmx1a-iNPCs also exhibit lower proliferation rate and lower level of neural progenitor markers compared to iNPCs. In contrast, Lmx1a-Foxa2-iNPCs, followed by Foxa2-iNPCs and Lmx1a-iNPCs, express higher levels of dopaminergic neuron proliferative progenitor cell markers compared to iNPCs, indicating some mesencephalic dopaminergic progenitor characteristics of these cells. After differentiation, cells derived from Lmx1a-Foxa2-iNPCs are the most superior in DA neuron differentiation efficiency, followed by cells from Foxa2-iNPCs, then Lmx1a-iNPCs. All of the above cell lines yield more DA neurons than iNPCs after differentiation,

indicating the critical function of Foxa2 and Lmx1a in DA neuron development. iNPCs that overexpress both Foxa2 and Lmx1a demonstrate a superior DA neuron differentiation efficiency compared to iNPCs and iNPCs that only overexpress Foxa2 or Lmxa2. Recent evidence has indicated that Foxa2 is an upstream factor and can positively regulate Lmx1a/b in SHH and Wnt1 signaling which is significant in midbrain DA neuron development(62). Our results also suggest that Foxa2 is superior to Lmx1a in directing the differentiation from progenitors to DA neurons. Therefore, Foxa2, along with the other 2 known reprogramming factors Sox2 and Brn2, was used for further study to develop mouse and human fibroblasts into dopaminergic progenitors.

Recently, Kim and colleagues demonstrated that mouse fibroblasts can be directly reprogrammed to functional and proliferating midbrain induced dopaminergic progenitors/precursors (iDPs) by the induction of Sox2, Klf4, c-Myc and Oct4 and further culture in presence of SHH and FGF8(57). After differentiation, the reprogrammed iDPs yielded a higher proportion of TH+/TuJ1+ dopaminergic neurons than iNPCs (<3%), especially with the co-inhibition of Jak and Gsk3β (57.2±7.2%). Based on the above and some other experimental results, our group optimized the reprogramming strategy and proved that mouse fibroblasts could be directly converted into iDPs with forced ectopic expression of three transcription factors (Brn2, Sox2 and Foxa2)(1). The derived mouse iDPs expressed high levels of specific neural progenitor markers and midbrain specific markers including Corin, Otx2 and Lmx1a. The major advantage was that iDPs were restricted to dopaminergic neuronal lineage during the process of differentiation. After differentiation, more than 90% of the Tuj1+ neurons are TH+ dopaminergic neurons. The efficiency of differentiation remained high even in absence of the pre-patterning morphogens SHH and FGF8, strongly suggesting the dopaminergic neural properties of those mouse iDPs. Importantly, after cell transplantation into SCID mice, the majority of

grafted iDPs differentiated into mature DA neurons rather than astrocytes without tumor formation. Based on the preliminary work, we conducted direct reprogramming by combining Sox2, Brn2, Foxa2 and successfully converted human fibroblasts into hiDPs. The expression of TH, a dopamine processing enzyme, and vesicular transporters of dopamine indicate the hiDPs we obtained acquired functional dopamine synthesis and processing mechanisms. Moreover, differential gene expression analyses confirmed broad upregulation of neurogenic- and dopamine-related RNA transcripts. The high co-positive rate of Tuj1 and TH, as well as TH and MAP2/TH and NeuN, supports the high efficiency of dopaminergic neuron specific differentiation from the hiDPs. TH is an enzyme involved in the synthesis of dopamine and norepinephrine (NE), which generally used in the field as a marker for dopaminergic neurons. Tuj1, MAP2 and NeuN are general neuronal markers. MAP2 is more in the cell soma, NeuN is more in the nucleus, while Tuj1/beta III tubulin is more in the processes. MAP2 and NeuN are generally more mature markers than Tuj1. The dopaminergic neuron-like cells we derived from hiDPs also overexpress DAT, AADC, Lmx1B and VMAT2. DAT, AADC, Lmx1B and VMAT2 are widely used dopaminergic neuron markers(63-65). DAT is a transmembrane transporter that controls the re-uptake of extracellular dopamine into presynaptic neurons. Lmx1B is a transcription factor involved in a number of processes during dopaminergic neuron development. The presence of aromatic L-amino acid decarboxylase (AADC) determines the ability of the neuron to make dopamine from L-DOPA (differential expression of dopaminergic cell markers in the adult zebrafish). The vesicular monoamine transporter 2 (VMAT2) has been suggested to be an excellent marker of presynaptic dopaminergic nerve terminals(66). Overexpression of these markers indicate the functional dopaminergic neuron nature of the neurons derived.

The hiDPs we developed have great potential in basic research and clinical application. Firstly, those cells are more appropriate PD models given the pathology of PD. They can better simulate those cells primarily affected under real disease circumstance and can help us understand better the pathology of certain gene mutations. To date, several groups have used iPSCs derived dopaminergic neurons with common PD mutations to study PD onset in living cells and obtained significant findings (67).

Moreover, hiDPs generated from direct reprogramming can provide a safe cell source for the cell replacement therapy. Skin fibroblasts are relatively easy to obtain from patients. Besides, hiDPs derived from fibroblasts provide a source of autologous tissue for grafting. Without the potential for immune rejection, hiDPs can be a safe and effective cell-based treatment for PD. In addition, hiDPs circumvent the limitations including neoplasia, low efficiency of differentiation and lack of the potential to self-renew(68). According to our results, the efficiency of differentiation from hiDPs to dopaminergic neurons is above 80% with certain differentiation protocols. In addition, hiDPs, like iPSCs, benefit from its capacity of self-renewal and can serve as targeted cells for gene editing technologies(69). Last but not least, hiDPs and the neurons they differentiated into can be used as excellent tools for drug screening and helpful in finding and evaluating new drugs. In sum, derivation of hiDPs may be a promising approach that can be applied therapeutically to restore function in patients with PD, as well as tools for PD mechanism study and drug screening.

Some shortages of our study are: 1) the lack of in vivo study in a neurotoxin 1-methyl-4-phenyl-1,2,3,6- tetrahydropyridine (MPTP) mouse model of PD or other PD mouse models; 2) the lack of electrophysiological study of possible functional membrane properties of the neurons derived from hiDPs.

In this study, with the same reprogramming strategy, we successfully converted human fibroblasts directly into human iDPs by overexpressing defined transcription factors Foxa2/Brn2/Sox2 and confirmed our hypothesis. The hiDPs we developed also express high levels of midbrain progenitor specific markers including Corin (Lrp4), Lmx1a, Otx2, Mash1, Pitx3 and Nkx6.1. We believe the generation of hiDPs is a new and promising approach of cell-based treatment strategy for PD, as well as a safer and reliable way to build PD cell models. Expansion of the engineered hiDPs may be able to provide a promising cell model for etiological study and therapeutic cell source for PD.

Table 1. Sequences of primer pairs

hTH	Forward	5-AGCTGTGAAGGTGTTTGAGACGT-3
	Reverse	5-TCGAGGCGCACGAAGTACT-3
hAADC	Forward	5-GGGACCACAACATGCTGCTC-3
	Reverse	5-CCACTCCATTCAGAAGGTGCC-3
hPitx3	Forward	5-GAGCTATGCAAAGGCAGCTT-3
	Reverse	5-AGTTGAAGGCGAATGGAAAG-3
hLmx1b	Forward	5-AACTGTACTGCAAACAAGACTACC-3
	Reverse	5-TTCATGTCCCCATCTTCATCCTC-3
hDAT	Forward	5-GTATGCTCTGATGCCGTCT-3
	Reverse	5-GTATGCTCTGATGCCGTCT-3
hVMAT2	Forward	5-CTTTGGAGTTGGTTTTGC-3
	Reverse	5-GCAGTTGTGATCCATGAG-3
hMAP2	Forward	5-CAGGAGACAGAGATGAGAATTCCTT-3
	Reverse	5-GTAGTGGGTGTTGAGGTACCACTCTT-3
hNkx6.1	Forward	5-GGACTGCCACGCTTTAGCA-3
	Reverse	5-TGGGTCTCGTGTGTTTTCTCT-3
hMash1	Forward	5-CCCAAGCAAGTCAAGCGACA-3
	Reverse	5-AAGCCGCTGAAGTTGAGCC-3
hALDH1A1	Forward	5-GCACGCCAGACTTACCTGTC-3
	Reverse	5-CCTCCTCAGTTGCAGGATTAAAG-3
hFoxa2	Forward	5-CTTCAAGCACCTGCAGATTC-3
	Reverse	5-AGACCTGGATTTCACCGTGT-3
hSox2	Forward	5-CCCACCTACAGCATGTCCTACTC-3
	Reverse	5-TGGAGTGGGAGGAAGAGGTAAC-3

hBrn2	Forward	5-GGAGTAGGGACACTCCACCA-3
	Reverse	5-CAGGAAGCTGCATTTTGTG-3
hGAPDH	Forward	5-CGACCACTTTGTCAAGCTCA-3
	Reverse	5-TCTACATGGCAACTGTGAGGA-3
mPitx3	Forward	5-TGCGCTGTCGTTATCGGAC-3
	Reverse	5-GGTAGCGATTCCTCTGGAAGG-3
mLmx1a	Forward	5-ACGGCCTGAAGATGGAGGA-3
	Reverse	5-CAGAAACCTGTCCGAGATGAC-3
mCorin	Forward	5-TGGAGGTGCCTATCAGAGAGA-3
	Reverse	5-GTGAGATCCAGTAACGCATTCA-3
mOtx2	Forward	5-TATCTAAAGCAACCGCCTTACG-3
	Reverse	5-AAGTCCATACCCGAAGTGGTC-3
mGFAP	Forward	5-CTGGAACAGCAAAACAAGGCGCTGG-3
	Reverse	5-TCCAGCCTCAGGTTGGTTTCATC-3
mNestin	Forward	5-CCCTGAAGTCGAGGAGCTG-3
	Reverse	5-CTGCTGCACCTCTAAGCGA-3
mCD133	Forward	5-TGTTGTTGGCGCAAATGTGG-3
	Reverse	5-TGTTCCTTGAGCAGATAGGGA-3
mFoxa2	Forward	5-TCAACCACCCCTTCTCTATCAACAACC-3
	Reverse	5-TGGGTAGTGCATGACCTGTTCGTAGG-3
mGAPDH	Forward	5-TCACCACCATGGAGAAGGC-3
	Reverse	5-GCTAAGCAGTTGGTGGTGCA-3

Table 2. Primary and secondary antibodies used in the study

Primary antibody	Isotype	Dilution	Source
MAP2	Mouse IgG	1:1000	Millipore
TH	Rabbit IgG	1:500	abcam
Tuj1	Mouse IgG	1:1000	Sigma
GFAP	Rabbit IgG	1:1000	DAKO
Nestin	Chicken IgY	1:1000	Novus biologicals
Corin	Mouse IgG	1:1000	R&D Systems
DCX	Goat IgG	1:1000	Santa Cruz
NeuN	Mouse IgG	1:1000	abcam
AADC	Rabbit IgG	1:1000	Invitrogen
VMAT2	Mouse IgG1	1:1000	Novus biologicals
Secondary antibody	Isotype	Dilution	Source
Alexa Fluor® 488	Goat anti-Rabbit Ig G	1:1000	Molecular Probes
	Goat anti-Mouse Ig G	1:1000	Molecular Probes
	Rabbit anti-Goat Ig G	1:1000	Molecular Probes
	Rabbit anti-Mouse Ig G	1:1000	Molecular Probes
	Donkey anti-Goat Ig G	1:1000	Molecular Probes
Alexa Fluor® 594	Goat anti-Rabbit Ig G	1:1000	Molecular Probes
	Donkey anti-Rabbit Ig G	1:1000	Molecular Probes
	Donkey anti-Mouse IgG	1:1000	Molecular Probes
Alexa Fluor® 647	Goat anti-Chicken IgG	1:1000	Molecular Probes

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