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Direct and selective lineage conversion of human fibroblasts to dopaminergic precursors

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Abstract

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. Our group demonstrated previously that mouse fibroblasts can be reprogrammed into induced dopaminergic precursors (iDPs) with high differentiation efficiency. In the current study, we hypothesized that a 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. Subsequent characterization of the precursor colonies confirmed the generation of human induced dopaminergic precursors (hiDPs). These hiDPs were capable of self-renewal, proliferation, and differentiation. The hiDPs demonstrated high immunoreactivity for neural progenitor markers and high levels of gene expression for ventral mesencephalon-related neural progenitor markers such

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as Lmx1a, NIKX6.1, Corin, Otx2 and Mash1. Furthermore, the hiDPs could be differentiated into dopaminergic neurons with ~80% efficiency, which significantly increased major functionally relevant proteins such as TH, DAT, AADC, Lmx1B and VMAT2 compared to hiDPs. Additionally, hiDPs are more dopaminergic progenitor-restricted compared to those hiDP-like cells reprogrammed only by Brn2 and Sox2. Together, these results suggest that hiDPs with high differentiation efficiency can be generated by direct lineage reprogramming of fibroblasts with transcription factors Brn2, Sox2 and Foxa2. These hiDPs may serve as a safe and effective cell source for transplantation treatment of PD.

Keywords

dopaminergic precursor; cell reprogramming; Parkinson's disease

Introduction

Parkinson's disease (PD) is among the most prevalent neurodegenerative disorders, affecting 1–2% of the population that are over 70 years old[1]. Key clinical symptoms of PD can fundamentally be explained by the loss of dopaminergic neurons in the substantia nigra[2]. While existing pharmaceutical and surgical treatments can, to some extent, ameliorate the clinical symptoms of PD, there remains no cure for the disease[3, 4]. The development of stem cell-based therapeutic approaches offers hope for a cure of PD by replacing lost dopaminergic neurons, compensating for dopaminergic deficiency and preventing further neuronal dysfunction and death. For example, transplanted embryonic dopamine neurons have been shown to significantly improve motor functions in early onset PD patients[5]. Compared to fetal neurons, neural stem cells have been actively researched and may be a more suitable cell source for transplantation in PD patients[6, 7].

To generate sufficient amounts of cells for transplantation, it is more desirable to use induced neural progenitor/stem cells (iNPCs/iNSCs) that have high proliferative potentials rather than the terminally differentiated neurons[8–11]. However, iNPCs have an intrinsic potential for multilineage differentiation into astrocytes, oligodendrocytes, and dozens of neuronal subtypes, most of which will be undesirable at the site of transplantation[8–10, 12–14]. Therefore, induction of iNPCs that are restricted to dopaminergic lineage might be better suited to reverse the dopaminergic deficiency in PD patients[15]. Our previous studies have shown that mouse fibroblasts and astrocytes can be successfully converted into induced neural progenitors by two different sets of five transcription factors, including Brn2, Sox2, TLX, Bmi1 and c-Myc for fibroblasts, and Foxg1, Sox2, Brn2, Foxa2 and Lmx1a for astrocytes[11, 16]. However, the dopaminergic differentiation efficiency of 5F-iNPCs remains low (< 5%) in response to sonic hedgehog (SHH)/fibroblast growth factor 8 (FGF8) stimulation.

To improve differentiation efficiency, several research groups, including our own, have recently focused on how to directly reprogram somatic cells into functional induced dopaminergic progenitors/precursors (iDPs)[17–19]. iDPs hold an advantage over iNPCs in that cells will be restricted to dopaminergic neuronal lineage during the process of

differentiation. This approach has helped successfully generated mouse iDPs [19]. However, whether it can be successfully applied to human cells remains unclear. In the current studies, we hypothesized that somatic reprogramming can be applied to human fibroblasts to generate human iDPs for future cell therapy in PD. We selected Brn2, Sox2 and Foxa2 as the transcription factors for cell reprogramming and successfully generated iDPs from human fibroblasts. We characterized the iDPs, validated their precursor identity and differentiation potentials. These data represent an essential step to confirm that a Brn2, Sox2 and Foxa2 based reprogramming strategy holds promising applications to future PD therapies.

Materials and Methods

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, 50 U/ml penicillin, 50 µg/ml streptomycin at 37 °C in a 5% CO₂ humidified atmosphere. Fibroblasts were used within passage 2–5 to avoid replicative senescence. Human Brn2 ORF, Foxa2 ORF and Sox2 ORF (or Brn2 ORF and Sox2 ORF) were individually cloned into pMXs-retroviral vectors (Cellbiolabs, RTV-010). Retroviruses (pMXs) were generated with Plat-GP packaging cells. Plat-GP cells were seeded at $3.2 - 3.6 \times 10^6$ cells per 100 mm poly-L-ornithine (PLO)/laminin-coated dish. 24 h after seeding, 10 µg 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 & PLUSTM transfection reagent (Thermo Fisher Scientific, Waltham, MA). Specifically, 15 µg DNA was first diluted in 1ml Opti-MEM[®] Medium, then mixed with 15 µl PLUSTM Reagent and incubated for 5 min at room temperature before the addition of 15 µl Lipofectamine[®] LTX Reagent. The DNA-lipid complex was further incubated at room temperature for 5 min and then added to the cells. The medium was replaced with 5.5 ml of DMEM containing 5% FBS 24 h after transfection. Human fibroblasts (hFbbs) were seeded at $2 - 3 \times 10^5$ cells per 35 mm culture dish. At 48 h and 72 h post-transfection, 5 ml virus-containing supernatants from the Plat-GP cultures were filtered through a 0.45 µm cellulose acetate filter and collected. Equal volumes of the supernatants from 3 dishes were mixed and supplemented with 10 µg/ml polybrene. Human fibroblasts were incubated in virus/polybrene-containing supernatants overnight. The medium was changed 72 h 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.), 20 ng/ml basic fibroblast growth factor (bFGF, BioWalkersville), 20 ng/ml epidermal growth factor (EGF, BioWalkersville) and 5 µg/ml heparin (Sigma-Aldrich, St. Louis, MO). After 10 – 21 d, the predicted human iDP colonies were monitored by an inverted microscope.

2 Quantitative Real-Time reverse transcription polymerase chain reaction.

Total mRNA was isolated with TRIzol Reagent (Thermo Fisher Scientific) 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 (Thermo Fisher Scientific) with 0.5 µl of cDNA, corresponding to 1 µg of total RNA in a 15 µl final volume consisting of 7.5 µl SYBR Green, 1.5 µl H₂O and 5.5 µl oligonucleotide primer pairs (synthesized at Fisher) at 10 µM. Primers used were listed in Supplemental Table 1. PCR program: 1) 50 °C for 2 min, 2) 95 °C for 2 min; 3) 95 °C for 15 s, 4) specific annealing temperature for 15 s and 5) 72 °C for 1 min. Steps 2 to 4 were repeated 40 times. All samples were amplified in triplicate and the means were calculated and used for further analysis.

3 Immunocytochemistry.

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

4 Differentiation.

For neuronal differentiation, a SHH/FGF8 dependent protocol was used: cells were plated on PLO/laminin coated coverslips in 24-well plate with DMEM/F12 containing 1× N2 supplement (Thermo Fisher Scientific), 100 ng/ml FGF8 (Peprotech, Rocky Hill, NJ), 100 ng/ml SHH (Peprotech) and 10 ng/ml bFGF (Peprotech) for 6 days, then switched to DMEM/F12 containing 1× N2, 1× B27, 0.2 mM ascorbic acid (Sigma), 0.1 mM β-mercaptoethanol, 1.0 mM dibutyryl cAMP (Sigma), 10 ng/mL glial cell line-derived neurotrophic factor (GDNF, Peprotech), and 10 ng/mL brain-derived neurotrophic factor (BDNF) (Peprotech) for another 4 – 8 weeks. The medium was changed every 3 d.

5 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.

Results

1 Foxa2, Brn2 and Sox2 reprograms human fibroblasts into neural progenitors with midbrain identity

We previously demonstrated that Foxa2 and two other known reprogramming factors Sox2 and Brn2 can convert mouse somatic cells into dopaminergic neural progenitors[19]. To test whether this strategy can be applied to human somatic cells, we isolated human fibroblasts and use the fibroblast cultures for cell reprogramming. The fibroblasts were transfected with retroviruses encoding Brn2, Sox2 and Foxa2 following the schematic procedure in Fig. 1A. At day 10 – 21 post-transfection, we observed the colony formation in the cultures (Fig. 1B). Eight colonies were obtained in total, however, only three of them were confirmed to be expandable clones after consecutive subcultures. RT-PCR was performed to identify the relative levels of Foxa2, Sox2, and Brn2 in the resulting cells after subculture. Only the cells 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. 1C). These hiDPs (human induced dopaminergic progenitors) were further characterized through gene expression analysis of several key neural progenitor markers in real-time RT-PCR (primer sequences were listed in Supplemental Table 1). Importantly, 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. 2C). These genes were previously reported to be specifically expressed in dopaminergic neuron proliferative progenitor cells[21,24,28,29]. The hiDPs exhibited high immunoreactivity for Corin and Nestin with co-positive rates of $91.22 \pm 7.19\%$ (Fig. 2A, B). The cells also displayed high immunoreactivity for Corin and doublecortin (DCX) with co-positive rates of $94.75 \pm 3.95\%$ in the immunofluorescent staining (Fig. 2A, B). These results suggest that similar to mouse fibroblasts[19], human fibroblasts can acquire a mesencephalic regional identity and dopaminergic neural fate through the forced expression of transcription factors Foxa2, Sox2 and Brn2.

2 hiDPs express specific dopaminergic progenitor markers and have dopaminergic neuronal-restricted differentiation potentials

We further validated whether the hiDPs are restricted to the dopaminergic neuronal lineage by differentiating the hiDPs into neurons. Dopaminergic neuronal differentiation was performed using an initial SHH and FGF8 stimulation followed by treatment with BDNF, GDNF and ascorbic acid (AA) that nurtured neuronal maturation. After 4 – 8 weeks of differentiation, we tested the differentiation efficiency of hiDPs into dopaminergic neurons and found that the neurons derived from hiDPs were highly immunoreactive to the specific antibody against TH (compared to both hiDPs before differentiation and hFbbs), suggesting high levels of TH expression in hiDPs-derived neurons (Fig. 3). More specifically, approximately 91.28% of the neurons were TH/Tuj1 double positive. About 96.67% and 86.75% were TH/NeuN and TH/MAP2 double positive, respectively, indicating the mature neuron nature of the hiDP-derived cells (Fig. 3B). In addition to TH, the hiDP-derived neurons were also highly immunoreactive to the specific antibodies against vesicular monoamine transporter 2 (VMAT2) and aromatic L-amino acid decarboxylase (AADC). The VMAT2 has been suggested to be an excellent marker of presynaptic dopaminergic nerve

terminals[20]. The presence of AADC determines the ability of the neuron to make dopamine from L-DOPA[21–23]. Therefore, these data suggest that the hiDPs can differentiate into dopaminergic neurons with high efficiencies and are likely restricted to the dopaminergic neuronal lineage. Consistent with the staining data, the gene expression levels of TH, DAT, VMAT2, AADC, MAP2 and LMX1B at 4 weeks (Fig. 4A) and 8 weeks (Fig. 4B) after differentiation were significantly higher than those in fibroblasts (hFbbs) or the hiDPs before differentiation. Furthermore, the gene expression levels of TH, DAT and LMX1B increased in a time-dependent manner after hiDP differentiation for 4 weeks and 8 weeks. In contrast, the increases of VMAT2, AADC and MAP2 appeared to peak on 4 weeks (Fig. 4). Together with the staining data, these results suggest that hiDPs can effectively differentiate and generate mature dopaminergic neurons.

3 hiDPs are more dopaminergic progenitor-restricted compared to 2 factors induced human NPCs (2F-iNPCs)

To determine whether Brn2 and Sox2 are sufficient to reprogram fibroblasts into iDPs or iNPCs, we transfected human fetus fibroblasts with retroviruses encoding Brn2 and Sox2. The overexpression of Brn2 and Sox2 was confirmed by real time RT-PCR in the resulting iNPCs (Fig. 5A). Next, we compared the gene expression levels of general neural progenitor markers (Fig. 5B), telencephalon- (Fig. 5C) and ventral mesencephalon-related neural progenitor markers (Fig. 5D) among starting fibroblasts, hiDPs (3 factors) and 2F-iNPCs (2 factors). 2F-iNPCs expressed significantly higher levels of neural progenitor markers SOX1, PAX6 and telencephalon neural progenitor marker FOXG1 compared to hiDPs. In contrast, hiDPs exhibited significantly higher levels of ventral mesencephalon related neural progenitor markers including PITX3 ($p < 0.05$), LMX1A ($p < 0.0001$), NKX6.1 ($p < 0.0001$), CORIN ($p < 0.001$), OTX2 ($p < 0.01$) and Mash1 ($p < 0.01$) (Fig. 5D) compared to 2F-iNPCs. Importantly, we compared the levels of dopaminergic neuronal markers among hiDPs, hiDP-derived neurons and 2F-iNPC-derived neurons after 8 weeks of differentiation (Fig. 5E). The levels of dopaminergic neuronal markers including TH, DAT ($p < 0.0001$) and AADC ($p < 0.01$) in hiDP-derived neurons were significantly higher than those from 2F-iNPC-derived neurons (Fig. 5E).

Discussion

In the past few years, cell replacement therapy has emerged as a novel experimental intervention that offers hope for curing PD. Dopaminergic neuronal progenitors from fetal ventral mesencephalon tissues have been transplanted into the striatum of PD patients with promising results[24, 25]. However, ethical concerns of using fetal tissues limit their further utility in the growing PD patient population. In the current studies, we used direct reprogramming of human fibroblasts into hiDPs to bypasses the use of fetal tissues. The characterization and validation of hiDPs suggest that direct reprogramming with transcription factors Foxa2, Sox2 and Brn2 is a safe and efficient way to obtain desired cell types for cell replacement therapy in PD[18].

Our finding that human fibroblasts could be directly converted into iDPs adds to the rapid progress of applying cell reprogramming technology to obtain iDPs for PD therapy.

Recently, Kim and colleagues demonstrated that mouse fibroblasts can be directly reprogrammed to functional and proliferating midbrain induced dopaminergic progenitors/precursors by the forced expressions of Sox2, Klf4, c-Myc and Oct4 [18]. The reprogrammed iDPs yielded a significant higher proportion of TH+/Tuj1+ dopaminergic neurons than iNPCs (< 3%). Notably, co-inhibition of Janus kinase and glycogen synthase kinase 3 beta further improved the differentiation potentials of the dopaminergic neurons ($57.2 \pm 7.2\%$). Our research on the generation of human iDPs applied a reprogramming strategy previously optimized in mouse fibroblasts that involved three transcription factors (Brn2, Sox2 and Foxa2)[19]. Mouse iDPs achieved with this strategy have been shown to express high levels of specific neural progenitor markers and midbrain specific markers including Corin, Otx2 and Lmx1a. Compared with the mouse iDPs, we found that human iDPs were more restricted to dopaminergic neuronal lineage during the process of differentiation. After differentiation, more than 80% of the Tuj1+ neurons are TH+ dopaminergic neurons. The high expression levels of a dopamine processing enzyme (TH) and transporters of dopamine (VMAT2 and DAT) indicate that the hiDPs we obtained may have functional dopamine synthesis and processing mechanisms. However, attempts to validate the release of dopamine from the iDPs-derived neurons have not been successful due to low concentrations of dopamine that were close to the detection limit in an enzyme-linked immunosorbent assay (0.5 ng/ml, Rocky Mountain Diagnostics, Colorado Springs, CO) despite potassium chloride stimulation (data not shown). Therefore, more sensitive measurements and higher density of iDPs-derived neurons may be required for dopamine detection in future studies.

To date, at least 23 loci and 19 parkinsonism-causing genes have been identified by scientists[26, 27]. Several groups have used induced pluripotent stem cell approach to derive dopaminergic neurons that carry common PD mutations to study PD onset and its neuropathogenesis[28]. Using direct lineage reprogramming (iNPC and iDP) approach to study these Parkinson related gene mutations offers an alternative means to better understand PD pathogenesis. Although the direct lineage reprogramming bypasses pluripotency, the conversion is generally very inefficient as previously reported[29, 30]. Indeed, we have used the method on fibroblasts derived from a 39 years old PD patient. We found that generation of induced progenitors was inefficient. Even though we obtained a few colonies after transfection, none of them were confirmed to be expandable clones after subcultures (data not shown). Therefore, improvement of reprogramming efficiencies will be required for the successful direct lineage reprogramming of cells from PD patients.

To summarize, data herein indicate that direct lineage reprogramming can convert human fibroblasts to hiDPs by forced expression of transcription factors Foxa2, Brn2 and Sox2. The hiDPs express midbrain progenitor specific markers, dopaminergic progenitor markers and have dopaminergic neuronal-restricted differentiation potentials. Furthermore, the hiDPs are more dopaminergic progenitor-restricted compare to a 2-factor reprogramming approach that involves Brn2 and Sox2. The generation of hiDPs is a new and promising approach for cell-based treatment strategy in PD.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- Expressions of transcription factors Brn2, Sox2 and Foxa2 convert human fibroblasts to human induced dopaminergic progenitors (hiDPs).
- The hiDPs predominantly differentiate into dopaminergic neurons, with a differentiation efficiency higher than 80%.
- The hiDPs are able to proliferate and self-renew in continuous subcultures *in vitro*.

Figure 1. Generation of human induced dopaminergic neuronal progenitors.

(A) Strategy of hiDPs generation by ectopic expression of Sox2, Brn2 and Foxa2; (B) hiDP clone formation at 20 days post infection under an inverted light microscope; (C) The expression levels of Sox2, Brn2 and Foxa2 in hiDPs were determined through real time RT-PCR. Data were normalized to GAPDH and presented as fold change compared to hFbbs.

*** denotes $p < 0.001$, compared to hFbbs.

Figure 2. Characteristics of hiDPs.

(A) Expression of DCX, Corin and Nestin in hiDPs by coimmunostaining with hFbbs as negative controls. Scale bar: 20 μ m; (B) Quantification of Corin/Nestin and Corin/DCX double positive cells in the cultures; (C) The expression of a specific set of neural progenitor marker genes were determined through real time RT-PCR. GAPDH and human fibroblasts (hFbb) served as internal and negative controls, respectively. * denotes $p < 0.05$, *** denotes $p < 0.001$, **** denotes $p < 0.0001$ compared to hFbbs.

Figure 3. hiDPs differentiate into neurons with dopaminergic neuron identity.

(A) Expression of TH, VMAT2, AADC and neuron markers NeuN, MAP2 and Tuj1 in hiDPs-derived neurons were determined by co-immunostaining. Scale bar: 10 μ m; (B) Quantification of TH/NeuN, TH/MAP2, TH/Tuj1 and VMAT2/AADC double positive cells in the cultures.

Figure 4. Cells differentiated from hiDPs express high levels of dopaminergic neuron marker genes.

(A) The expression levels of TH, DAT, VMAT2, AADC, MAP2 and LMX1B after 4 weeks of differentiation were determined through real time RT-PCR; (B) The expression levels of TH, DAT, VMAT2, AADC, MAP2 and LMX1B after 2 months of differentiation were determined through real time RT-PCR. *denotes $p < 0.05$, ** denotes $p < 0.01$, *** denotes $p < 0.001$, **** denotes $p < 0.0001$ compared to hiDPs before differentiation and hFbbs.