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Direct conversion of human fibroblasts into dopaminergic neural progenitor-like cells using TAT-mediated protein transduction of recombinant factors

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ABSTRACT

Recent progress in the generation of induced neural progenitor cells (iNPCs) holds tremendous potential for regenerative medicine. However, a major limitation is the lack of a reliable source for cell replacement therapy in neurological diseases such as Parkinson's disease (PD). Here, we show that the combination of small molecules (SM) and TAT-mediated protein transduction of SOX2 and LMX1a in a 3D sphere culture directly convert human fibroblasts to induced dopaminergic neural progenitor-like cells (iDPCs). The generated iDPCs expressed various NPC markers (*SOX2*, *PAX6*, *NESTIN*, *OLIG2*) and midbrain progenitor markers (*EN1*, *LMX1a*, *FOXA2*, *WNT1*) as detected by immunostaining and real-time PCR. Following differentiation, the majority of cells expressed neuronal dopaminergic markers as indicated by co-expression of TH with NURR1, and/or PITX3. We found that SOX2 and LMX1a TAT-mediated protein transduction in the combination of SM could directly convert human fibroblasts to self-renewal iDPCs. In conclusion, to our best knowledge, this is the first report of generation of safe DPCs and may suggest an alternative strategy for cell therapy for the treatment of neurodegenerative disorders.

1. Introduction

Parkinson's disease (PD) is one of the most common age-related neurodegenerative diseases caused by dopamine-producing neurons in the midbrain substantia nigra. This neurodegenerative disease leads to progressive degeneration affecting millions of people worldwide [1]. To date, there is no definite cure for PD and cell replacement therapy aims to replace and repopulate the lost dopaminergic neurons (DA) with new ones. However, there is still a pressing need to identify an ideal cell source which is patient specific, easy to obtain and expandable with controlled differentiation. Stem cells have the capability to generate desired derivatives to treat these disorders [2]. In recent years, progress in reprogramming and transdifferentiation has led to the proposal of a potential patient-specific cell source for the treatment of a broad range of human neurological disorders (for review see Ref. [3]). These induced DA (iDA) have been generated directly from different mouse and human somatic cell types by ectopic expression of key transcription factors [4–8]. However, these terminally differentiated cells are not proliferative post-mitosis and inadequate for transplantation-based treatments that require large cell numbers. In this respect, hopes have been raised by the generation of induced neural progenitor cells (iNPCs) due to their ability to provide large amounts of cells once reprogrammed and their capability to give rise to differentiated cell progenies *in vitro* and *in vivo* for clinical applications. Currently, several independent groups have generated iNPCs from mouse and human somatic cells by potentially

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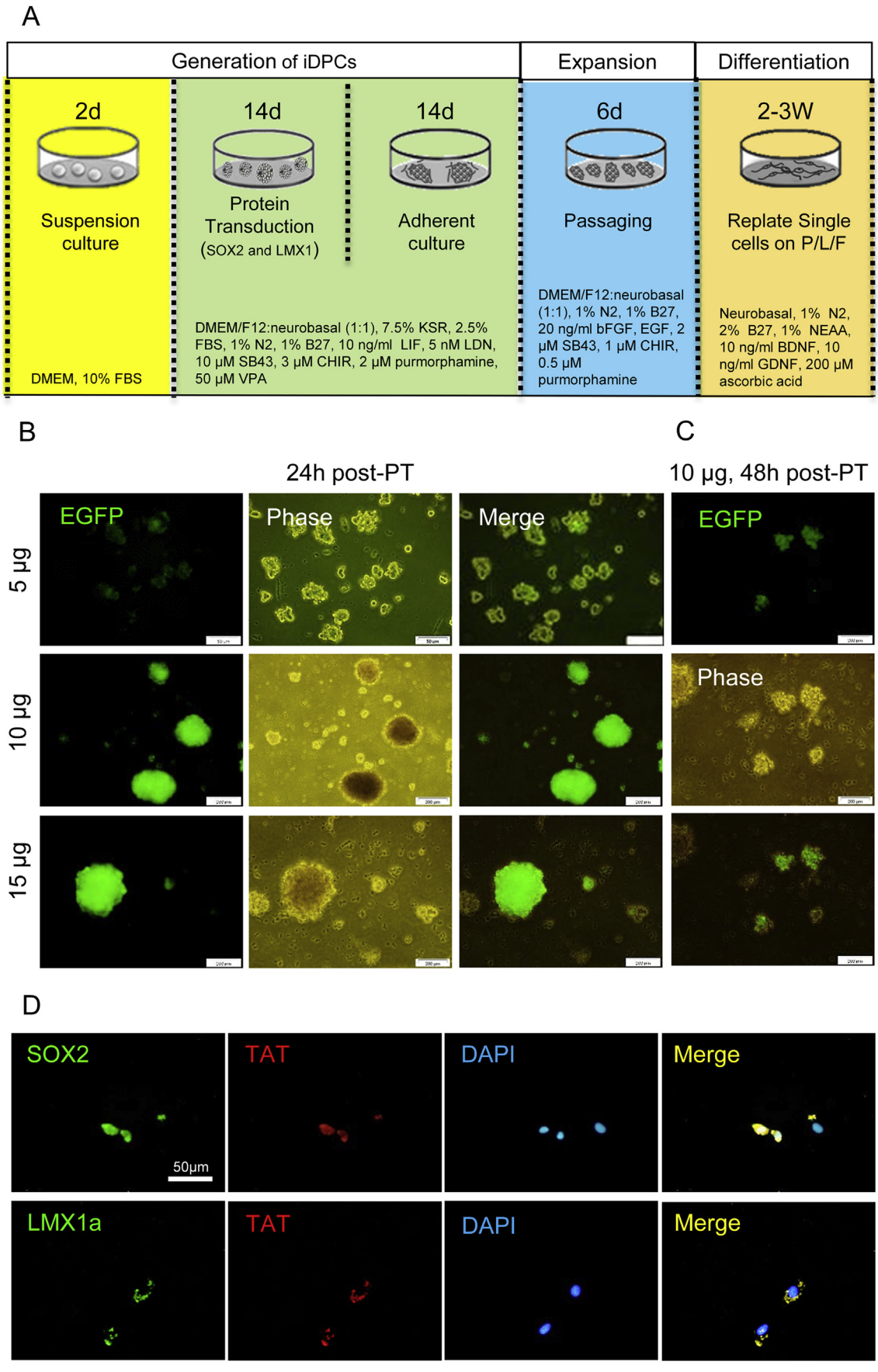
A
Generation of iDPCs
Expansion
Differentiation
2d
14d
14d
6d
2-3W
Suspension culture
Protein Transduction (SOX2 and LMX1)
Adherent culture
Passaging
Replate Single cells on P/L/F
DMEM, 10% FBS
DMEM/F12:neurobasal (1:1), 7.5% KSR, 2.5% FBS, 1% N2, 1% B27, 10 ng/ml LIF, 5 nM LDN, 10 μM SB43, 3 μM CHIR, 2 μM purmorphamine, 50 μM VPA
DMEM/F12:neurobasal (1:1), 1% N2, 1% B27, 20 ng/ml bFGF, EGF, 2 μM SB43, 1 μM CHIR, 0.5 μM purmorphamine
Neurobasal, 1% N2, 2% B27, 1% NEAA, 10 ng/ml BDNF, 10 ng/ml GDNF, 200 μM ascorbic acid
B
24h post-PT
C
10 μg, 48h post-PT
5 μg
10 μg
15 μg
EGFP
Phase
Merge
EGFP
Phase
D
SOX2
TAT
DAPI
Merge
50μm
LMX1a
TAT
DAPI
Merge

tumorigenic pluripotency-associated factors or lineage specific transcription factors via viral transduction [9–15]. However, to reduce the concerns about the safety and reduction in tumorogenesis, isolation and enrichment of homogeneous populations of desired cells, effective strategies must be developed to generate cell-type specific personalized iNPCs without viral integration for diseases such as PD that affect certain types of cells. For example, induced dopaminergic neural progenitor-like cells (iDPCs) have been generated from mouse fibroblasts through the transient expression of the four Yamanaka factors under permissive environmental cues [16]. More importantly, to date, potentially therapeutic iDPCs have not been generated from human cells.

Here, for the first time, we report that the generation of iDPCs from human fibroblasts via a novel combination of TAT (human immunodeficiency virus transactivator of transcription)-mediated protein transduction of SOX2 and LMX1a-a transcription factor determinant of midbrain DA- and small molecules (SMs). This study represents a safe strategy to provide a DPC source for cell therapy in the treatment of neurodegenerative disorders.

2. Materials and methods

2.1. Production of TAT recombinant proteins

The production of recombinant proteins was performed as previously described [17,18]. Briefly, pDest17/TAT-EGFP, TAT-SOX2 and TAT-LMX1a expression vectors were transformed into *E. coli* strain BL21 competent cells (DE3; Novagen, WI, US). The transformed cells were cultured to reach an OD 600 ~0.8, then induced by 1 mM isopropyl-beta-D-thiogalactopyranoside (IPTG, Fermentas, Lithuania). In addition, we used His6 for protein purification. The His6-TAT-SOX2 and His6-TAT-GFP proteins were purified by the Ni-NTA Fast Start Kit (Qiagen, USA) under denatured conditions. Immobilized rSOX2 proteins were eluted with 8 M urea (pH 3.5), then desalted by tris (5 mM) that contained 50% glycerol and maintained at −20 °C until use.

2.2. Cell culture and generation of induced dopaminergic neural progenitor-like cells (iDPCs)

Human foreskin fibroblasts were kindly provided by the Royan Institute Stem Cell Bank (Iran) as a primary cell culture (male donor, 7 days old). This study was approved by the Ethical Committee of Royan Institute and written consent from the donor's parents was obtained. The human fibroblasts were maintained in fibroblast medium [(DMEM, Invitrogen, CA, USA) supplemented with 10% fetal bovine serum (FBS, Invitrogen)] supplemented with 10% fetal bovine serum (FBS, Invitrogen). Briefly, for the suspension sphere culture, initial cells were seeded at a density of 1×10^6 cells/mL onto agarose coated plates [19]. After 48 h, the fibroblast medium was replaced by medium that contained [DMEM/F12:neurobasal (1:1) supplemented with 7.5% Knockout serum replacement (KSR), 2.5% FBS, 1% N2, 1% B27 (all from Invitrogen)]; 10 ng/ml human LIF (Royan Institute); and a cocktail of SMs composed of 5 nM LDN, 10 μM SB431542, 3 μM CHIR99021, 2 μM purmorphamine, and 50 μM VPA (all from Sigma–Aldrich, MO, USA). The medium was replenished every other day and protein transductions were carried out in seven repeated transduction cycles (every 48 h) using the TAT-SOX2 and TAT-LMX1a proteins with a total protein amount of 10 μg/ml per transduction cycle. After 14 days of protein transduction, cells were dissociated with trypsin-EDTA (Invitrogen) and then reseeded onto poly-ornithine/laminin/fibronectin (PLF; Invitrogen) pre-coated plates (Nunc, Germany) in NPC expansion medium that contained [DMEM/F12:neurobasal (1:1), 1% N2, 1% B27, supplemented with 20 ng/ml bFGF and EGF] supplemented with SMs (2 μM SB431542, 1 μM CHIR99021, and 0.5 μM purmorphamine).

2.3. In vitro differentiation

Dissociated cells were reseeded onto laminin coated plates in differentiation medium [neurobasal with 1% N2, 2% B27, 1% NEAA supplemented with 10 ng/ml BDNF, 10 ng/ml GDNF, and 200 μM ascorbic acid (all from Sigma–Aldrich)] for up to 3–4 weeks. Half the volume of the medium was replaced by fresh medium every 2–3 days.

2.4. Real time-PCR

RNA extraction was performed using the TRIzol reagent according to the manufacturer's instructions (Sigma–Aldrich, MO, USA). Extracted RNA was used for cDNA synthesis with a Reverse Transcription Kit (Takara). Q-PCR was performed by using the SYBR Green Kit (Takara, Japan). All primer sequences used in this study are listed in Supplementary Table 1. We used the $2^{(-\text{Delta Delta CT})}$ method and the normalizer gene for our studies was *GAPDH*. At least three biologically independent samples were analyzed.

2.5. Immunofluorescence staining

Cells were fixed in 4% neutral-buffered paraformaldehyde (PFA) for 20 min at 4 °C and permeabilized with 0.05% triton X-100 in phosphate-buffered saline (PBS) for 10 min at room temperature, then blocked with 10% secondary host serum in 1% bovine serum albumin in PBS (BSA-PBS) for 1 h at room temperature. These cells were subsequently incubated overnight at 4 °C with primary antibodies diluted in PBS that contained 1% BSA. The cells were washed three times with PBS plus 0.02% Tween 20 and incubated with their respective secondary antibodies conjugated to Alexa Fluor 568 and 546 (red) or Alexa Fluor 488 (green) in the dark for 30 min at room temperature. After washing with PBS plus 0.02% Tween 20, cells were counterstained with 4, 6-diamidino-2-phenylindole (DAPI). Finally cells were observed using a fluorescence microscope (Zeiss). All primary and secondary antibodies, sources, and dilutions are listed in Supplementary Table 2.

2.6. Statistical analysis

Values were expressed as mean ± SD. Differences between means were assessed by the *t*-test. A p-value of <0.05 was considered statistically significant.

3. Results and discussion

It has been demonstrated that *SOX2* alone can be a master regulator gene for iNSC production [11] and the overexpression of

Fig. 1. Transdifferentiation procedure and Visualization of cellular uptake of TAT recombinant proteins. (A) Schematic design of protein induced dopaminergic neural progenitor-like cell (piDPCs) generation, expansion and differentiation from human fibroblasts. The starting cells were treated with TAT recombinant proteins LMX1a and SOX2 in suspension culture in presence of small molecules. (B) Human fibroblasts were treated with different concentration (5, 10, 15 μg/mL) of TAT-EGFP proteins under suspension culture, 24 h post-transduction. (C) Human fibroblasts were treated with (10 μg/mL) of TAT-EGFP proteins under suspension culture, 48 h post-transduction. (D) Double staining TAT, SOX2 and LMX1a in transduced cells 24 h post-transduction. The nuclei were counterstained with 4-6-diamidino-2-phenylindole (DAPI).

LMX1a in combination with extrinsic factors can bias NSCs toward differentiation into DA neurons [20]. Moreover, it has been reported that iNSCs can be produced from fibroblast lines directly through an initially 3D sphere culture of the cells [19]. On the other hand, previous reports have shown that an SM cocktail can induce NSCs from human fibroblasts [21,22] and promote neural conversion efficiency [23]. Thus, we have investigated the potential effect of SOX2 and LMX1a protein transduction in combination with SMs in a 3D sphere culture of the human fibroblasts to directly induce subtype specific-neural progenitor-like cells, i.e. iDPCs. The experimental scheme for iDPCs generation is illustrated in Fig. 1A.

Optimization of protein transduction was performed by TAT-EGFP protein transduction. The fibroblast cells were treated with different concentrations of this protein (5, 10, and 15 μg/mL) under suspension culture after 24 and 48 h post-transduction (Fig. 1B, C). We observed a higher abundance of exogenous protein in cells after 24 h post-transduction (Fig. 1B). This observation was dose-dependent in cells treated with different concentration of TAT-EGFP. Since the concentrations greater than 10 μg/mL in repeated transduction cycles were lethal for human fibroblasts (data not shown), we decided to use 10 μg/mL of proteins every other day to minimize the resultant cell death. The cellular uptake of TAT-SOX2 and TAT-LMX1a proteins was confirmed using antibodies against SOX2, LMX1a and TAT (Fig. 1D). These results demonstrated that the proteins successfully penetrated into the cells. The use of chemically defined NSC medium and suspension culture has been shown to promote neural transdifferentiation [21,22,24]. Therefore, we transduced fibroblasts with proteins in a media that contained a cocktail of SM in suspension culture (Fig. 2). Interestingly, we observed that the expression of NPC related genes and DPC markers were upregulated after seven days of SOX2 and LMX1a protein transduction (Fig. 2). The expression level of NPC related genes in this group was similar to those of SOX2 treated cells and ES-NPC, as control groups. However, the levels of DPC markers, *LMX1a* and *FOXA2*, were significantly higher in the cells treated with both SOX2 and LMX1a proteins after 7 and 14 days. We observed down-regulation of the fibroblast specific marker, *FSP1* in treated cells. Since these cells expressed NPC and DPC markers we transferred them as single cells to adherent cultures with the chemical medium that favored DPC expansion [25]. After 14 days, the treated cells formed aggregates at the intersections of neural-like network structures, many of which were positive for PAX6, NESTIN, SOX2, FOXA2 and LMX1a (Fig. 3). Based on these observations, we called these cells as protein iDPCs (piDPCs). We could also expand these cells for up to 3-5 passages (Fig. 4A). During passages, cells were

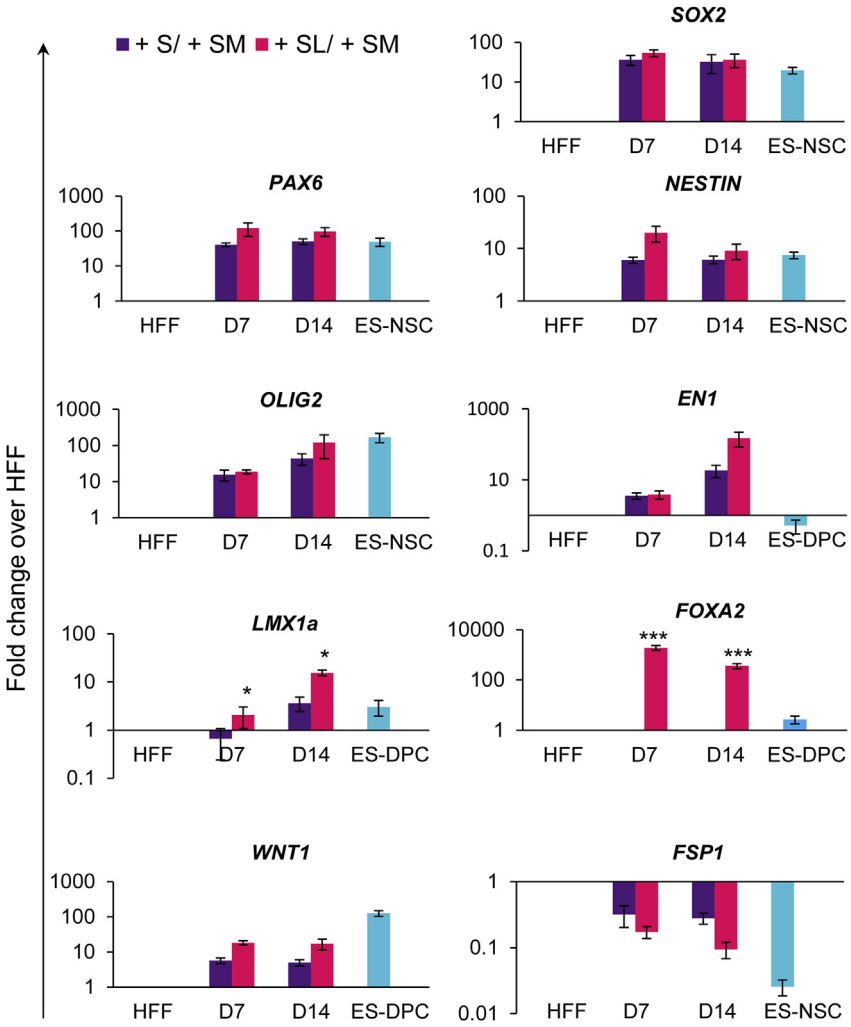

Fig. 2. Induction of dopaminergic progenitor-like cells by LMX1a and SOX2 TAT-mediated protein transduction. Real-time PCR analysis showed upregulation of NPC and DPC related genes. +S/+SM: Cells treated with TAT-SOX2 in the presence of small molecules (SMs). +SL/+SM: Cells treated with both TAT-SOX2 and TAT-LMX1a plus SMs. *: $P < 0.05$, ***: $P < 0.001$.

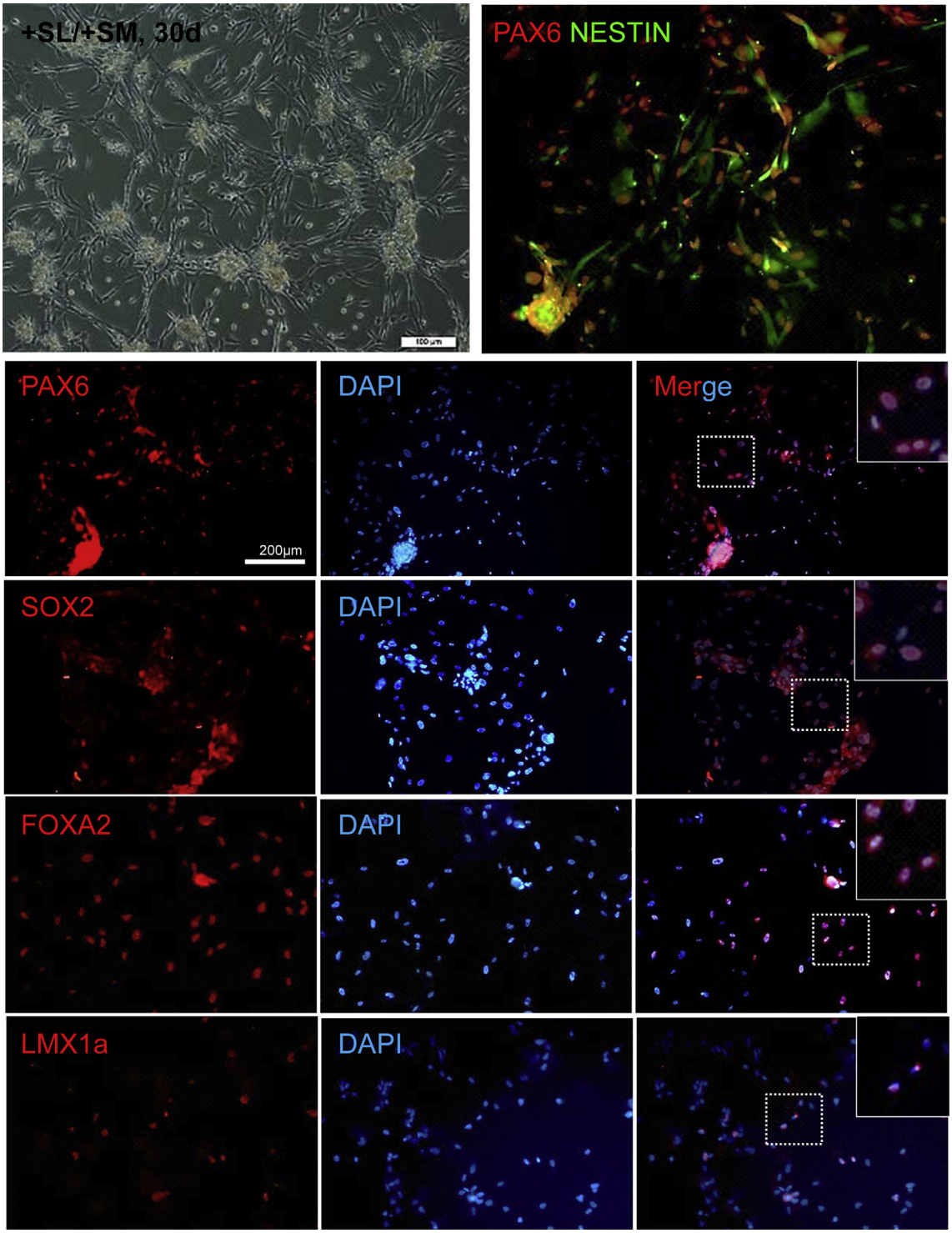

Fig. 3. Morphology of protein induced dopaminergic neural progenitor-like cells (piDPCs) and immunostaining of NPC and DPC markers. The insert represents a higher magnification of the dotted plot.

still NESTIN and SOX2 positive however a number of cells became TUJ1 positive-a marker for intermediate mature neurons (Fig. 4B). To assess the DA differentiation potential of the piDPCs, we cultured these cells under differentiation medium. The differentiated cells morphologically resembled neural cells (Fig. 4C) and were TH/MAP2 positive neurons (Fig. 4D). We also showed that TH-expressing neurons co-stained with other DA markers, NURR1 and PITX3 (Fig. 4D). These results further confirmed DPC induction by SOX2 and LMX1a proteins.

In order to increase the clinical translatability, to our best knowledge, this is first report on a more amenable viral-free protocol for induction of subtype-specific neural progenitors (iDPCs) by a TAT-mediated protein transduction system comprised of a mixture of SOX2 and LMX1a.

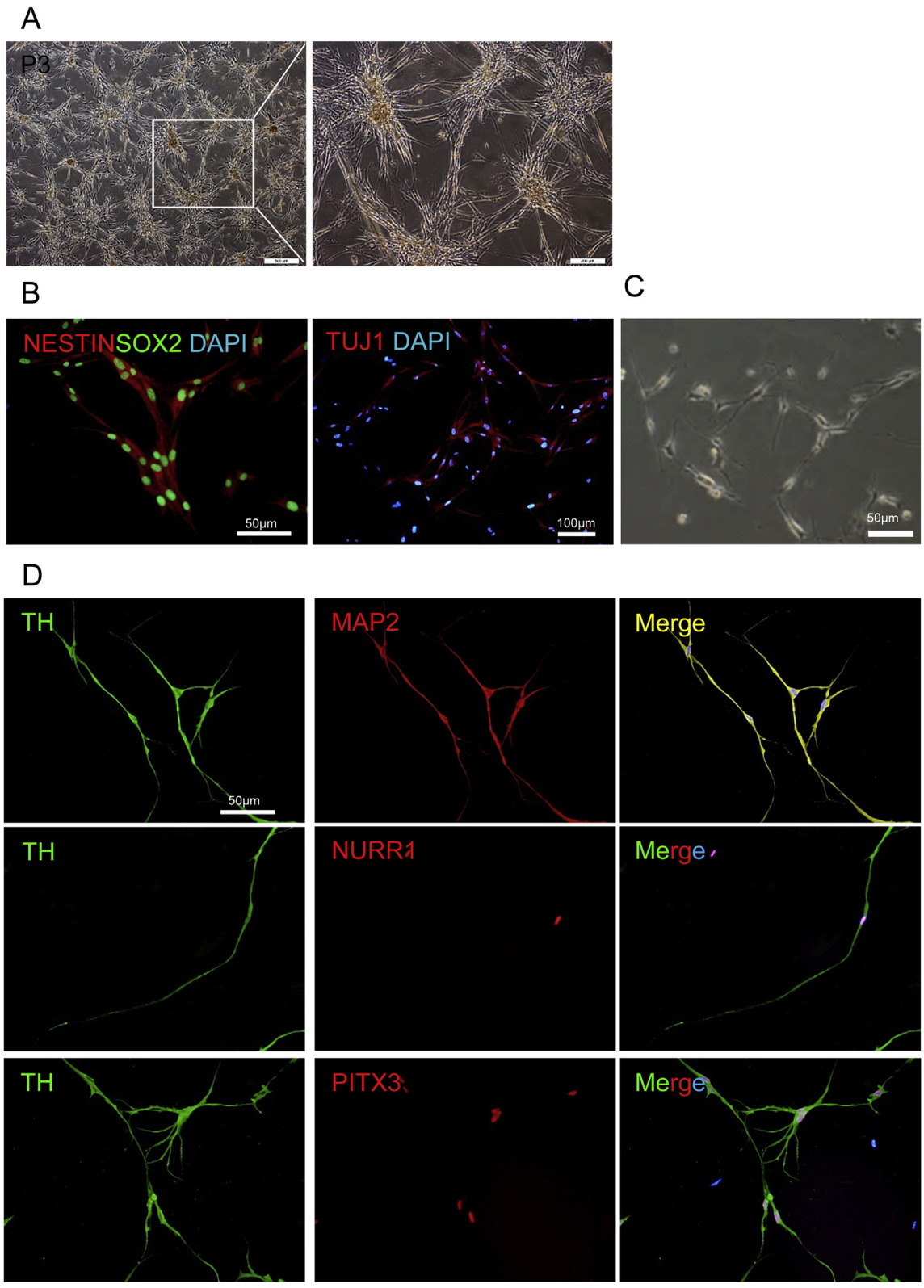

Fig. 4. Protein induced dopaminergic neural progenitor-like cells (piDPCs) can be expanded for 3–5 passages. (A) Morphology of passage 3 (P3) piDPCs. (B) piDPCs were positive for NESTIN/SOX2 and TUJ1. (C) Morphological changes of differentiated cells. (D) Co-immunostaining TH with MAP2, NURR1 and Pitx3 in differentiated piDPCs.

Conflict of interest

The authors have declared that no potential conflict of interest exists.

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Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2015.02.166.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.02.166.

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