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TITLE:

Generation of Induced Neural Stem Cells from Peripheral Mononuclear Cells and Differentiation Toward Dopaminergic Neuron Precursors for Transplantation Studies

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SUMMARY:

The protocol presents the reprogramming of peripheral blood mononuclear cells to induce neural stem cells by Sendai virus infection, differentiation of iNSCs into dopaminergic neurons, transplantation of DA precursors into the unilaterally-lesioned Parkinson's disease mouse models, and evaluation of the safety and efficacy of iNSC-derived DA precursors for PD treatment.

ABSTRACT:

Parkinson's disease (PD) is caused by degeneration of dopaminergic (DA) neurons at the substantia nigra pars compacta (SNpc) in the ventral mesencephalon (VM). Cell replacement therapy holds great promise for treatment of PD. Recently, induced neural stem cells (iNSCs) have emerged as a potential candidate for cell replacement therapy due to the reduced risk of tumor formation and the plasticity to give rise to region-specific neurons and glia. iNSCs can be reprogrammed from autologous somatic cellular sources, such as fibroblasts, peripheral blood mononuclear cells (PBMNCs) and various other types of cells. Compared with other types of somatic cells, PBMNCs are an appealing starter cell type because of the ease to access and expand in culture. Sendai virus (SeV), an RNA non-integrative virus, encoding reprogramming factors including human *OCT3/4*, *SOX2*, *KLF4* and *c-MYC,* has a negative-sense, single-stranded, non-segmented genome that does not integrate into host genome, but only replicates in the cytoplasm of infected cells, offering an efficient and safe vehicle for reprogramming. In this study, we describe a protocol in which iNSCs are obtained by reprogramming PBMNCs, and differentiated into specialized VM DA neurons by a two-stage method. Then DA precursors are transplanted into unilaterally 6-hyroxydopamine (6-OHDA)-lesioned PD mouse models to

evaluate the safety and efficacy for treatment of PD. This method provides a platform to investigate the functions and therapeutic effects of patient-specific DA neural cells in vitro and in vivo.

INTRODUCTION:

Parkinson's disease (PD) is a common neurodegenerative disorder, caused by degeneration of dopaminergic (DA) neurons at the substantia nigra pars compacta (SNpc) in the ventral mesencephalon (VM), with a prevalence of more than 1% in population over 60 years of age[1,2]. Over the past decade, cell therapy, aimed at either replacing the degenerative or damaged cells, or nourishing the microenvironment around the degenerating neurons, has shown potential in treatment of PD[3]. Meanwhile, reprogramming technology has made significant progress[4], which provides a promising cellular source for replacement therapy. Human induced pluripotent stem cells (iPSCs) and embryonic stem cells (ESCs) have been proven to be able to differentiate into DA neural cells, which could survive, maturate, and improve the motor functions when grafted into rat and non-human primate PD models[5-8]. iPSCs represent a milestone in cellular reprogramming technologies and have a great potential in cell transplantation; however, there is still a concern about the risk of tumor formation from the incompletely differentiated cells. An alternative cellular source for cell transplantation is lineage-committed adult stem cells obtained through direct reprogramming, such as induced neural stem cells (iNSCs), which can be derived from the unstable intermediates, bypassing the pluripotency stage[9-11].

Both iPSCs and iNSCs can be reprogrammed from autologous cellular sources, such as fibroblasts, peripheral blood mononuclear cells (PBMNCs) and various other types of cells[12-14], thus reducing the immunogenicity of transplanted cells to a great degree. Moreover, compared with iPSCs, iNSCs are inherent with reduced risk of tumor formation and lineage-committed plasticity, only able to differentiate into neurons and glia[11]. In the initial studies, human or mouse iPSCs and iNSCs were generated from fibroblasts obtained from skin biopsies, which is an invasive procedure[14,15]. With this respect, PBMNCs are an appealing starter cell source because of the less invasive sampling process, and the ease to obtain large numbers of cells within a short period of expansion time[16]. Initial reprogramming studies employed integrative delivery systems, such as lentiviral or retroviral vectors, which are efficient and easy to implement in many types of cells[17]; however, these delivery systems may cause mutations and reactivation of residual transgenes, which present safety issues for clinical therapeutic purposes[12]. Sendai virus (SeV) is a non-integrative RNA virus with a negative-sense, single-stranded genome that does not integrate into host genome, but only replicates in the cytoplasm of infected cells, offering an efficient and safe vehicle for reprogramming[18,19]. Recombinant SeV vectors are available that contain reprogramming factors including human *OCT3/4*, *SOX2*, *KLF4* and *c-MYC* in their open reading frames. In addition, SeV viral vectors can be further improved by introducing temperature-sensitive mutations, so that they could be rapidly removed when the culture temperature is raised to 39 °C[20]. In this article, we describe a protocol to reprogram PBMNCs to iNSCs using the SeV system.

Many studies have reported derivation of DA neurons from human ESCs or iPSCs using various methods[6,8,21]. However, there is a shortage of protocols describing the differentiation of DA

neurons from iNSCs in details. In this protocol, we will describe the efficient generation of DA neurons from iNSCs using a two-stage method. The DA neuronal precursors can be transplanted into the striatum of PD mouse models for safety and efficacy evaluations. This article will present a detailed protocol that covers various stages from generation of induced neural stem cells by Sendai virus, differentiation of iNSCs into DA neurons, establishment of mouse PD models, to transplantation of DA precursors into the striatum of the PD models. Using this protocol, one can generate iNSCs from patients and healthy donors and derive DA neurons that are safe, standardizable, scalable and homogeneous for cell transplantation purposes, or for modeling PD in a dish and investigation of the mechanisms underlying disease onset and development.

PROTOCOL:

All procedures must follow the guidelines of institutional human research ethics committee. Informed consent must be obtained from patients or healthy volunteers before blood collection. This protocol was approved by the institution's human research ethics committee and was performed according to the institution's guidelines for care and use of animals.

1. Collection, isolation and expansion of PBMNCs

1.1. Collection of PBMNCs

1.1.1. Collect 10–20 mL of donor's peripheral venous blood by venipuncture with a sodium heparin preservative vial.

NOTE: Blood samples should be stored or shipped at room temperature (RT). Process the blood samples within 24 h.

1.2. Preparation of culture medium

1.2.1. Prepare serum free medium (SFM) by combining the following components: 245 mL of Iscove's modified Dulbecco's medium (IMDM), 240 mL of Ham's F-12, 5 mL of insulin-transferrin-selenium-X supplement (ITS-X), 5 mL of 100x glutamine stock solution (**Table of Materials**), 5 mL of chemically defined lipid concentrate, 2.5 g of fetal bovine serum, 0.025 g of ascorbic acid and 9 µL of 1-thioglycerol. Filter the medium and store it at 4 °C.

CAUTION: Ascorbic acid and 1-thioglycerol are toxic by skin contact and inhalation.

1.2.2. To prepare mononuclear cell (MNC) medium, supplement the SFM medium with 10 ng/mL human interleukin 3 (IL-3), 2 U/mL erythropoietin (EPO), 100 ng/mL human stem cell factor (SCF), 40 ng/mL human insulin-like growth factor 1 (IGF-1), 100 µg/mL holo-transferrin and 1 µM dexamethasone. Filter the medium and store it at 4 °C.

NOTE: Prepare the medium immediately before use.

1.3. Isolation of PBMNCs

1.3.1. Ultraviolet-sterilize a clean bench prior to use. Sterilize all surfaces and equipment with 75% alcohol. Sterilize all tips by using an autoclave.

1.3.2. Transfer the peripheral blood (PB) into a 50 mL conical tube and dilute the PB with an equal volume of sterile Dulbecco's phosphate-buffered saline (D-PBS).

1.3.3. Prepare 15 mL of sterilized density gradient medium (**Tables of Materials**) in another 50 mL conical tube.

NOTE: Keep the density gradient medium and PB at RT to allow for better isolation of PBMNCs.

1.3.4. Tilt the conical tube containing the density gradient medium at a 45° angle, and then slowly and carefully lay 30 mL of diluted PB onto the density gradient medium.

NOTE: Take care and allow the PB to slowly run down the side of the conical tube onto the density gradient medium layer. Red blood cells will deposit to the bottom of the tube. Tilt the tube carefully to minimize disruption of the layer interface.

1.3.5. Centrifuge the tubes at 800 x *g* for 15 min at RT with the centrifuge brake set at "off" position. Aspirate the yellow, upper plasma layer and discard it. Then transfer the white cloudy thin film layer containing MNCs with a 10 mL pipette to a new 50 mL conical tube.

NOTE: Switching centrifuge brake off is important for the isolation of MNCs.

1.3.6. Add 30 mL of D-PBS to the tube with MNCs and centrifuge at 600 x *g* for 10 min at 4 °C. Discard the supernatant, and then add 45 mL of D-PBS to re-suspend the cells. Centrifuge at 400 x *g* for 10 min at 4 °C.

NOTE: The centrifuge brake should be switched on for this and the following centrifugation steps. As the cell pellets are dense, add 1−2 mL of D-PBS to gently re-suspend the pellets, and then add D-PBS to 45 mL.

1.3.7. Discard the supernatant and re-suspend the cells with 5 mL of D-PBS and count the live cells with the trypan blue exclusion method.

1.3.8. After setting aside the MNCs needed for expansion, freeze the remaining cells for future use.

NOTE: At least 5 x 10^6 MNCs can be frozen in one vial with 1 mL of freezing medium (**Table of Materials**). The protocol can be paused here.

1.4. Expansion of MNCs

1.4.1. On day -14, seed MNCs at a density of 2–3 x 10^6 cells per milliliter in one well of six-well plates with 1.5 mL of pre-warmed (37 °C) MNC medium. Incubate at 37 °C, 5% CO_2 for 2 days.

1.4.2. On day -11, collect the cells and medium with a sterilized pipette and transfer to a new 15 mL conical tube. Centrifuge the cells at 250 x *g* for 5 min at RT. Discard the supernatant and re-suspend the cells in 1 mL of pre-warmed (37 °C) MNC medium.

1.4.3. Count the viable cells with trypan blue. Seed the MNCs at a density of 1 x 10^6 cells per milliliter in pre-warmed MNC medium and incubate at 37 °C, 5% CO_2 for 3 days.

NOTE: It is expected that the total number of cells may decrease on day -11.

1.4.4. On day -8, repeat steps 1.4.2–1.4.3 and culture the cells for 3 days.

1.4.5. On day -4, repeat steps 1.4.2–1.4.3 and culture the cells for 3 days.

NOTE: After 14 days of culture, an equal or greater number of MNCs should remain in the culture.

2. Reprogramming of PBMNCs to iNSCs by SeV Infection

2.1. Preparation of solution and culture medium

2.1.1. Prepare a poly-D-lysine (PDL) stock solution by dissolving 100 mg of PDL with 100 mL of H_2O to a concentration of 1 mg/mL. Store at -20 °C in 1 mL aliquots.

2.1.2. Prepare an insulin stock solution by dissolving 100 mg of insulin in 20 mL of 0.01 N HCl to a concentration of 5 mg/mL. Store at -20 °C in 1 mL aliquots.

2.1.3. To prepare 200 mL of iNSC basal medium, combine 96 mL of DMEM-F12 and 96 mL of basic medium (**Table of Materials**) with 2 mL of 100x glutamine stock solution, 2 mL of nonessential amino acid (NEAA), 2 mL of N2 supplement and 2 mL of B27 supplement. Add 10 ng/mL recombinant human leukemia inhibitory factor, 3 µM CHIR99021 and 2 µM SB431542 prior to use. Filter the medium and store it at 4 °C.

NOTE: Use the medium within 2 weeks. Add recombinant human leukemia inhibitory factor, CHIR99021 and SB431542 immediately before use.

2.2. Reprogramming of PBMNCs to iNSCs by SeV Infection

2.2.1. Ultraviolet-sterilize a clean bench prior to use. Sterilize all surfaces and equipment with 75% alcohol. Sterilize all tips using an autoclave.

2.2.2. On day 0, collect the cells in MNC medium and transfer to a 15 mL conical tube. Centrifuge the cells at 200 x *g* for 5 min. Aspirate the supernatant and re-suspend the cells with 1 mL of pre-warmed MNC medium.

2.2.3. Count the viable cells with trypan blue. Re-suspend the cells with pre-warmed (37 °C) MNC medium to a concentration of 2 x 10^5 cells per well in 24-well plates.

2.2.4. After removing the SeV tubes from -80 °C storage, thaw the tubes containing SeV in 37 °C water bath for 5−10 s, and then allow them to thaw at RT. Once thawed, place them on ice immediately.

2.2.5. Add the SeV encoding human *Klf4, Oct3/4, SOX2 and c-MyC* to the wells, at a multiplicity of infection (MOI) of 10. Centrifuge cells with plates at 1,000 x *g* for 30 min to facilitate the attachment of cells. Leave the cells and supernatant in the plates. Place the plates in the incubator at 37 °C, 5% CO_2.

CAUTION: All procedures involving SeV must be performed in a safety cabinet, and all tips and tubes should be treated with ethanol or bleach before disposal.

2.2.6. On day 1, transfer the medium and cells to a 15 mL centrifuge tube. Rinse the well with 1 mL of MNC medium. Centrifuge the cell suspension at 200 x *g* for 5 min. Aspirate the supernatant and re-suspend the cells with 500 µL of fresh pre-warmed MNC medium in 24-well plates.

NOTE: Use a low attachment 24-well plate to prevent attachment of any cells before plating on PDL/laminin.

2.2.7. On day 2, dilute 1 mL of 1 mg/mL PDL with 19 mL of D-PBS to a concentration of 50 µg/mL. Coat 6-well plates with 50 µg/mL PDL for at least 2 h at RT.

2.2.8. Dilute 200 µL of 0.5 mg/mL laminin with 20 mL of D-PBS to a concentration of 5 µg/mL. Aspirate PDL in the 6-well plates, and dry on the vertical clean bench.

2.2.9. Coat 6-well plates with 5 µg/mL laminin and incubate for 4−6 h at 37 °C. Wash with D-PBS before use.

2.2.10. On day 3, plate the transduced cells obtained in step 2.2.6 in iNSC medium on PDL/laminin-coated 6-well plates.

NOTE: Move the plates gently if needed after the cells are placed on PDL/laminin-coated plates, trying not to disturb the attachment of the cells.

2.2.11. On day 5, add 1 mL of pre-warmed (37 °C) iNSC medium in each well in 6-well plates gently.

NOTE: It is expected that the cells will undergo drastic death (>60%).

2.2.12. On day 7, add 1 mL of pre-warmed (37 °C) iNSC medium in each well in 6-well plates gently.

2.2.13. From day 9 to day 28, replace spent medium with fresh pre-warmed (37 °C) iNSC medium every day. Monitor the emergence of iNSC colonies. Pick and transfer iNSC clones for expansion in about 2−3 weeks. Pick up colonies with appropriate morphology using burned glass pipettes, excluding any possibly contaminating cells, and aspirate the colonies with 200 µL tips.

NOTE: The characterized iNSCs can be frozen for future use with 2−5 colonies in one vial. The freezing medium includes serum free basal medium **(Table of Materials)** and dimethyl sulfoxide mixed at a ratio of 9:1, which should be prepared immediately before use. The protocol can be paused here.

3. Differentiation of iNSCs to dopaminergic neurons

3.1. Preparation of solution and culture medium

3.1.1. Prepare 200 mL of iNSC differentiation basal medium by combining 192 mL of DMEM-F12 with 2 mL of 100x glutamine stock solution, 2 mL of NEAA, 2 mL of N2 supplement and 2 mL of B27 supplement.

NOTE: Use the medium within 2 weeks.

3.1.2. Prepare iNSC differentiation stage I medium by supplementing the iNSC differentiation basal medium with 1 µM SAG1 and 100 ng /mL FGF8b.

NOTE: Use the medium within 2 weeks.

3.1.3. Prepare iNSC differentiation stage II medium by supplementing the iNSC differentiation basal medium with 0.5 mM cyclic adenosine monophosphate (cAMP), 0.2 mM ascorbic acid, 10 µM DAPT, 10 ng/mL brain derived neurotrophic factor (BDNF), 10 ng/mL glial derived neutrophic factor (GDNF) and 1 ng/mL transforming growth factor βIII (TGF-βIII).

NOTE: Use the medium within 2 weeks.

3.2 Coating the culture dishes

3.2.1. Ultraviolet-sterilize a clean bench prior to use. Sterilize all surfaces and equipment with 75% alcohol. Sterilize all tips using an autoclave.

3.2.2. For PDL coating, at least one day before re-plating the cells, dilute 1 mL of 1 mg/mL PDL with 19 mL of D-PBS to a concentration of 50 µg/mL. Coat 12 mm glass coverslips that have been sterilized with 75% alcohol in the 24-well plates with 50 µg/mL PDL at RT for at least 2 h.

3.2.3. For laminin coating, dilute 200 µL of 0.5 mg/mL laminin with 20 mL of D-PBS to a concentration of 5 µg/mL. Aspirate PDL and dry the wells in the clean bench. Coat the 12 mm coverslips with 5 µg/mL laminin and incubate for 4−6 h at 37 °C. Wash with D-PBS before use.

3.3. Passage cells for differentiation.

3.3.1. When the confluence of cultured iNSCs reaches 70-90%, aspirate medium from the culture plate, and add 1 mL of D-PBS to wash the cells. Add 1 mL of pre-warmed (37 °C) cell dissociation reagent (**Table of Materials**) per well and incubate at 37 °C for 3 min to dissociate the cells.

3.3.2. After incubation for 3 min, the cells have become semi-floating; add 3 mL of pre-warmed (37 °C) DMEM-F12 medium per well, and pipette cells up and down to dissociate cell pellets into single cells.

3.3.3. Transfer cells into a 15 mL conical tube, and centrifuge at 250 x *g* for 3 min. Aspirate the supernatant, re-suspend the cells with appropriate volume of pre-warmed (37 °C) iNSC medium according to the number of cells.

3.3.4. Count the cells using the trypan blue exclusion method. Plate 5 x 10^3 cells per 12 mm glass coverslip in 24-well plates and incubate at 37 °C, 5% CO_2.

3.4. Differentiate iNSCs into dopaminergic neurons.

3.4.1. Start differentiation 24 h after re-plating the cells onto PDL/laminin-coated coverslips. Aspirate culture medium, wash cells once with D-PBS, and then add 600 µL of pre-warmed differentiation stage I medium per well in 24-well plates and incubate at 37 °C, 5% CO_2.

3.4.2. Change medium every day from day 1 to day 10 during the first stage of differentiation.

3.4.3. On day 10, aspirate the culture medium, and wash cells once with D-PBS. Add 600 µL of pre-warmed (37 °C) differentiation stage II medium per well in 24-well plates and incubate at 37 °C, 5% CO_2.

3.4.4. Change medium every other day from day 11 to day 25 during the second stage of differentiation. The differentiated cells can be fixed by paraformaldehyde at different time points for analysis.

3.4.5. For immunofluorescent staining, wash the cells with D-PBS three times gently at chosen time points within differentiation day 11 to 25.

3.4.6. Pipette 300 µL of nonionic surfactant (**Table of Materials**) into 100 mL of PBS to make a 0.3% nonionic surfactant in PBS.

CAUTION: Nonionic surfactant is toxic by skin contact and inhalation.

3.4.7. Fix the cells with 4% paraformaldehyde for 10 min at RT. Then wash with 0.3% in PBS three times.

CAUTION: Paraformaldehyde is toxic by skin contact and inhalation.

3.4.8. Block the cells by 3% donkey serum for 2 h at RT.

3.4.9. Dilute the primary antibody in 1% donkey serum at an appropriate concentration and gently triturate to mix. Add 300 µL of the primary antibody solution to each well of the 24-well plate. Incubate the cells at 4 °C overnight. Wash the cells with 0.3% nonionic surfactant in PBS three times.

3.4.10. Dilute the secondary antibody in 1% donkey serum at an appropriate concentration and gently triturate to mix. Add 300 µL of the secondary antibody solution to each well of the 24-well plate. Incubate the cells at RT for 2 h, protected from light.

3.4.11. Wash the cells with 0.3% nonionic surfactant in PBS three times. Dilute 4',6-diamidino-2-phenylindole (DAPI) with PBS with 1:500 dilution. Incubate the cells in each well of the 24-well plate with 300 µL of diluted DAPI for 15 min at RT, protected from light. Wash the cells with 0.3% nonionic surfactant in PBS three times.

3.4.12. Gently take out the coverslips from the wells of plates with forceps. Dry in dark overnight at RT. Mount under a fluorescence microscope.

4. Establishment of unilateral 6-hyroxydopamine (6-OHDA)-lesioned PD mouse models

4.1. To generate PD mouse models for cell transplantation, use adult male SCID-beige mice weighing 20−25 g for 6-OHDA injection.

4.2. Preparation of drugs for surgery

4.2.1. Prepare a 0.2% ascorbic acid solution by dissolving 0.2 g of ascorbic acid into 100 mL of sterilized saline (0.9%) and store at -80 °C until use. On the day of surgery, dilute 0.2% ascorbic acid solution by 10 times to obtain a 0.02% ascorbic acid solution.

NOTE: Ascorbic acid is added to prevent oxidation of 6-OHDA to an inactive form.

4.2.2. To prepare a 6-OHDA solution, weigh appropriate amount of 6-OHDA into a sterilized 1 mL tube, and then add some volume of 0.02% ascorbic acid to make a 5 µg/µL 6-OHDA solution. Vortex the mixture until it is dissolved. Place the 6-OHDA on ice until use.

NOTE: 6-OHDA is temperature and light sensitive. Be careful to protect the solution from light and keep it on ice before use.

4.3. Prepare sterilized surgical equipment by autoclaving before surgery. Clean all equipment and surface areas with ethanol when setting up the stereotaxic frame. Set up a mouse recovery cage under a heating lamp.

4.4 Conduct surgery to establish unilateral 6-OHDA-lesioned mouse models.

4.4.1. Weigh each mouse, record the weight and calculate the amount of drug that needs to be administered. Each mouse receives 0.5 mg/kg atropine 20 min prior to operations. Anesthetize the mouse with 80 mg/kg ketamine and 10 mg/kg xylazine.

4.4.2. Administer 0.5 mg/kg atropine by intraperitoneal injection.

4.4.3. Anesthetize the mouse with 80 mg/kg ketamine and 10 mg/kg xylazine by intraperitoneal injection 20 min after administration of atropine.

4.4.4. Put the mouse in a closed chamber. After 3−5 min, the mouse will be deeply anesthetized without response to hind leg pinch.

NOTE: It is expected that the mouse that has received anesthesia would experience an excitation period.

4.4.5. Shave the head of mouse and apply erythromycin eye ointment on the eyes of mouse for protection from developing corneal ulcers.

4.4.6. Place the mouse on the stereotaxic apparatus. Fix the mouse with incisor bars firstly. Insert the ear cups correctly to make the mouse head in a flat and secure position.

4.4.7. Sterilize the head of the mouse with povidone iodine and isopropyl alcohol. Cut a sagittal incision (~1.5 cm) on the head skin with a scalpel blade, and expose the skull. Adjust the incisor bar and ear bars to reduce the height difference between bregma and lambda to less than 0.1 mm.

NOTE: The mouse bregma is located at the intersection of coronal and sagittal sutures, and lambda is at the intersection of lambdoid and sagittal sutures.

4.4.8. Slowly move and lower the tip of the needle towards bregma and treat the bregma as a zero point. Move the tip to a position with coordinates of A/P +0.5 mm, M/L -2.1 mm relative to bregma. Retract the tip and mark the point. Burr a little hole into the skull.

4.4.9. Extract 2 µL of 5 µg/µL 6-OHDA solution into the microsyringe (**Table of Materials**). Return the needle to the point marked, and insert the needle to D/V -3.2 mm.

4.4.10. Inject 2 µL of 5 µg/µL 6-OHDA solution (10 µg total) at a rate of 1 µL/min. After injection of 6-OHDA is completed, leave the needle in place for another 5 min. Then retract the injection needle slowly.

4.4.11. Close the incision with sutures and apply erythromycin eye ointment on the eyes of mouse. Remove the mouse from stereotaxic apparatus and put it in the recovery cage. Put the mouse back and allow access to food and water until it regains consciousness.

4.4.12. Inspect the mouse daily post-surgery.

5. Behavioral assessment after unilateral 6-OHDA lesioning

5.1. Two to three weeks following surgery, conduct behavioral assessment to estimate PD symptoms. Weigh each mouse, record their weight and calculate the amount of drug that should be administered (0.5 mg/kg apomorphine prior to assessment).

5.2. Administer 0.5 mg/kg apomorphine by subcutaneous injection before assessment and place the mouse in a glass cylinder.

5.3. After a 5 min habituation period, count the number of contralateral rotations relative to the lesion side per minute and record their activity with a video camera.

5.4. Mice with contralateral rotations >7 rpm/min are considered successfully lesioned and selected as candidates for cell transplantation experiments. Return the mice to the housing cages after a 30-min rest.

5.5. Conduct the behavioral assessment one week before and 2, 4, 6, 8, 12, 16 weeks after cell transplantation.

6. Cell transplantation of DA precursors

6.1. Prepare cell suspension for transplantation. For cell engraftment, suspend 2 x 10^5 DA precursors mixed by D10 and D13 DA precursors at a ratio of 1:7 in 4 µL of 5 g L^{-1} glucose in balanced salt solution (**Table of Materials**) of transplantation buffer.

6.2. Perform cell transplantation surgery following the procedures described in section 4.4, except that 6-OHDA is replaced by DA precursor cell suspension or buffer.

6.3. Perform the behavioral assessment 2, 4, 6, 8, 12, 16 weeks after cell transplantation of DA precursors following the procedures described in section 5. Count the number of apomorphine-induced contralateral rotations relative to the lesion side per minute and record their activity with a video camera.

6.4. At 4, 8 ,12, and 16 weeks after cell transplantation, perfuse the mouse under deep anesthesia with 4% paraformaldehyde until the body of mouse becomes stiff and the liver of mouse becomes pale.

6.5. Separate the brain of mouse gently and put the brain in 4% paraformaldehyde at 4 °C overnight.

6.6. On the second day, put the brain in 30% sucrose for dehydration until the brain sinks to the bottom.

6.7. Slice the brains at 40 μm thickness by using a freezing microtome.

6.8. Perform immunostaining as previously described in steps 3.4.5−3.4.12.

REPRESENTATIVE RESULTS:

Here, we report a protocol that covers different stages of iNSC-DA cell therapy to treat PD models. Firstly, PBMNCs were isolated and expanded, and reprogrammed into iNSCs by SeV infection. A schematic representation of the procedures with PBMNC expansion and iNSC induction is shown in **Figure 1**. On day -14, PBMNCs were isolated by using a density gradient medium (**Table of Materials**). Before centrifugation, blood diluted with PBS and the density gradient medium were separated into two layers. After centrifugation, four gradient layers appeared (from bottom to top): the bottom layer contained granulocytes and erythrocytes; the second layer contained density gradient medium; the third contained PBMNCs (red arrow); the top layer contained platelet-rich plasma (**Figure 2A**). After 14 days of expansion, PBMNCs were infected with SeV as day 0. On day 1, medium with SeV was removed (**Figure 2B**). As illustrated in **Figure 2B,** it is expected that the number of cells was reduced gradually. Cells underwent a drastic death (>60%) until day 5 (**Figure 2B**). iNSC colonies emerged on day 12 at the earliest (**Figure 2B**). After picking and transferring iNSC clones for expansion for a number of passages, the morphology of cells is shown in **Figure 2C**. The iNSCs showed a good morphology and could self-renew stably in iNSC medium, either in a monolayer form or as spheres (**Figure 2C**).

iNSCs could give rise to DA neurons using a two-stage method (**Figure 1**). During stage one which lasted 10 days, iNSCs were treated with SAG1 and FGF8b to induce specification of VM floor plate cells with neurogenic potentials. Then the cells were treated with ascorbic acid, BDNF, GDNF, cAMP, DAPT, TGF-βIII in the second stage (**Figure 1**). With this two-stage method, DA precursors could be obtained towards the end of the first stage, and more mature DA neurons could be generated in the end of the second stage. After 24 days of differentiation, iNSCs could be efficiently specified to DA neurons as a majority of them expressed forkhead box A2 (FOXA2), neuron-specific class III β-tubulin (TUJ1) and tyrosine hydroxylase (TH) (**Figure 3**).

Three weeks after establishment of unilateral 6-OHDA-lesioned PD mouse models, behavioral assessment was conducted to estimate PD symptoms (**Figure 4A**). Then one week later, dopaminergic precursors were transplanted to the PD mouse models (**Figure 4A**). The behavioral assessment was performed one week before and 2, 4, 6, 8, 12 weeks after cell transplantation

(**Figure 4A**). The mice that had received cell transplantation showed significant improvement in motor function (**Figure 4B**). The extent of 6-OHDA-induced lesioning can be verified by post-mortem immunofluorescent staining for TH at the striatum, medial forebrain bundle (MFB) and SNpc (**Figure 4C**). The TH-positive signals in engrafted mice were greatly recovered in the striatum where cells were implanted and mildly recovered at SNpc (**Figure 4C**). Three months after transplantation, about 13.84% were TH^+ DA neurons among surviving cells (**Figure 4D,E**). About 91.72% and 86.76% of the TH^+ cells were expressed orphan nuclear receptor (NURR1) and FOXA2, respectively (**Figure 4D,E**). About 98.77% of the TH^+ cells were co-labeled with G-protein-coupled inward rectifier potassium (GIRK2) (**Figure 4D,E**).

FIGURE LEGENDS:

Figure 1: A schematic representation of procedures regarding PBMNC expansion, iNSC induction and differentiation of iNSCs into DA precursors. PBMNCs were isolated and expanded in MNC medium for over 14 days, and then infected with SeV encoding human *SOX2, OCT3/4, c-MYC* and *KLF4*. iNSC colonies emerged as early as 12 days after SeV infection. After 3−4 weeks, iNSC colonies were picked and differentiated into DA precursors by a two-stage method. PBMNCs: peripheral blood mononuclear cells; MNC: mononuclear cells; iNSCs: induced neural stem cells; SeV: Sendai virus; DA: dopaminergic; PDL: poly-D-lysine; BDNF: brain-derived neurotrophic factor; GDNF: glial cell line-derived neurotrophic factor; AA: ascorbic acid; cAMP: dibutyryladenosine cyclic monophosphate; TGF: transforming growth factor.

Figure 2: PBMNCs are isolated and expanded, and then reprogrammed into iNSCs by SeV infection. (**A**) An example of PBMNCs before and after gradient centrifugation. Before centrifugation, diluted blood samples and density gradient medium were separated into two layers. After centrifugation, four density gradient layers formed from bottom to top: the bottom layer contained granulocytes and erythrocytes; the second layer contained the density gradient medium; the third layer contained PBMNCs (red arrow); the top layer contained platelet-rich plasma. (**B**) Images of the typical morphology of cells after PBMNCs were infected with SeV on days 1, 2, 5, and 12. After PBMNCs were infected with SeV, some of the cells died and the number of cells was reduced gradually. A small number of cells remained on day 5. On day 12, iNSCs colonies emerged. Scale bar, 100 μm. (**C**) The typical morphology of iNSCs of passage number 20 in monolayer and sphere culture. Scale bar, 100 μm.

Figure 3: Differentiation of iNSCs to dopaminergic neurons. Immunofluorescent staining for FOXA2, TH, TUJ1, DAPI and merged images on day 24 on cells differentiated from iNSCs. Scale bar, 50 μm.

Figure 4: Transplantation of iNSC-differentiated DA precursors into unilateral 6-OHDA-lesioned PD mouse models. (**A**) Timeline for cell transplantation and behavioral tests. (**B**) The results of behavioral tests at different time points from the 6-OHDA+cells group (n = 10), 6-OHDA+buffer group (n = 8) and control group (n = 3). Data are presented as mean ± standard error of the mean (SEM). ***$p < 0.001$ by two-way analysis of variance (ANOVA) with Dunnett's multiple comparison test. (**C**) Post-mortem immunofluorescent staining for TH at the striatum, medial forebrain bundle (MFB) and substantia nigra (SN) of 6-OHDA-leisioned hemisphere, 6-

OHDA+cells hemisphere, and control group. Scale bars, 100 μm. (**D**) Percentages of FOXA2/TH, NURR1/TH, GIRK2/TH, TH/HNA in engrafted mice 3 months after transplantation. HNA: human nuclei antibody. Data are presented as mean ± SEM. (**E**) Immunofluorescent staining for FOXA2, NURR1, GIRK2, TH and HNA on brain slices from PD mice 12 weeks after cell transplantation. HNA: human nuclei antibody. This figure has been modified from Yuan et al.[11].

DISCUSSION:

Here we presented a protocol that covered different stages of iNSC-DA cell therapy for PD models. Critical aspects of this protocol include: (1) isolation and expansion of PBMNCs and reprogramming of PBMNCs into iNSCs by SeV infection, (2) differentiation of iNSCs to DA neurons, (3) establishment of unilateral 6-OHDA-lesioned PD mouse models and behavioral assessment, and (4) cell transplantation of DA precursors and behavioral assessment.

In this protocol, the first part involves collecting and expanding PBMNCs in a serum-free medium (MNC medium), which preferentially expands erythroblasts and does not support lymphocyte proliferation. In previous studies, several somatic cell types have been reprogrammed to iPSCs or iNSCs[12-14]. In comparison with other types of somatic cells, PBMNCs possess several advantages. The most significant advantage is their favorable gene expression patterns and epigenetic profiles. PBMNCs are short-lived in vivo and replenished frequently from activated hematopoietic stem cells, and may accumulate fewer mutations than skin fibroblasts do. Also, the way to sample PBMNCs is less invasive than that for fibroblasts, and it takes a shorter period of time to expand PBMNCs (around 14 days) versus fibroblasts (around 28 days)[16,22]. After centrifugation using a density gradient medium, four density gradients will be formed from bottom to top; the bottom layer contains granulocytes and erythrocytes, the second lower layer contains the density gradient medium, the third layer contains MNCs, and the top layer contains plasma. The yield of PBMNCs normally varies between individuals, particularly those of different ages. Generally, younger people tend to have a greater number of PBMNCs than older people. With the described protocol, about 1.8−3.4 x 10^7 PBMNCs could be isolated from 15 mL of PB. Among PBMNCs, $CD34^+$ hematopoietic stem cells are relatively prone to reprogramming, and MNC medium may enrich $CD34^+$ hematopoietic stem cells to some extent. It is expected that an equal or greater number of visible cells cultured with MNC medium remain after 14 days of expansion. SeV, an RNA non-integrative virus, has a negative-sense, single-stranded, non-segmented genome that does not integrate into host genome, but only replicates in the cytoplasm of infected cells[18,19]. Recombinant SeV encoding reprogramming factors *OCT3/4*, *SOX2*, *KLF4* and *c-MYC*, can be generated as a temperature-sensitive mutant, which could be removed easily at 39 °C[20]. With this protocol, we derived 8−20 iNSC colonies by reprogramming PBMNCs from one healthy volunteer or patient using 15 mL of PB. Then researchers could select colonies with good morphologies for further passaging and line establishment. In the published report, iNSCs of passage numbers 10, 20, and 30 showed similar proliferation rates, and could be passaged more than 50 times, showing a good self-renewal and proliferative capacity[11]. iNSCs could be characterized by differentiation assays and immunostaining for neural stem cell markers SOX2, PAX6, NESTIN and OLIG2, and the proliferative marker Ki67. iNSCs should express those neural stem cell markers and possess a differentiation ability to become $TUJ1^+$, $MAP2^+$ neurons (after 6 weeks), $GFAP^+$ astrocytes (after 6 weeks) and $OLIG2^+$, $O1^+$ oligodendrocytes (after 7−8

weeks)[11]. However, one limitation of this protocol is that MNC medium is preferentially favorable to erythroblast expansion, which may render it not suitable for generating iNSCs from patients who are deficient in erythroblast development. Besides, the efficiency of reprogramming PBMNCs to iNSCs is not high. One may enhance reprogramming efficiency by using certain small molecules or increasing the yield by using more starting PBMNCs[23].

The method about the differentiation of iNSCs into DA neurons described here builds upon a great number of protocols for neural differentiation. As PD is mainly caused by degeneration of DA neurons located in the midbrain[1,2], the aim of the protocols is focused on the derivation of specialized VM DA neurons, which arise from floor plate cells during development[24]. Induction of VM floor plate cells with neurogenic potentials depends on two key morphogens, SHH and FGF8b[6,25,26]. SHH is a ventralizing morphogen, secreted by notochord[26,27]. Here we replaced SHH with the small molecule SAG1, a SHH pathway agonist that is more economical than SHH. Also, basic neural culture medium and supplement (**Table of Materials**) are important for neuronal survival and differentiation. With this protocol, DA precursors were obtained towards the end of the first stage, and more mature DA neurons were generated at the end of the second stage. Increasing the period of time of the second stage to 40−55 days could further enhance the proportion of mature DA neurons in culture. Included in the second stage medium are the retinoic acid, BDNF, GDNF, TGF-βIII, DAPT and cAMP, which have been demonstrated to be able to promote DA neuron maturation and survival. To test the efficiency of iNSCs in differentiation into DA neurons, the differentiated cells were examined for expression of markers NURR1, FOXA2, GIRK2 and TH. In a previous study, at the end of stage I (day 10), 87.76% and 65.33% cells expressed NURR1 and FOXA2, respectively[11]. At the end of stage II (day 24), the percentage of $NURR1^+$ cells reached 95.58% and the proportion of $FOXA2^+$ cells reached 77.33%[11]. At this time point, 57.23% and 28.55% cells were positive for TH and GIRK2, respectively[11]. Similar to what has been observed for iPSC differentiation towards DA neurons, a batch to batch/line to line variation for iNSC differentiation also exists, which is influenced by factors such as cell state, the activity of small molecules used, and the plasticity of stem cells from different persons. It is also noteworthy that a key determinant of the differentiation efficiency is cell density. A seeding density of 5×10^3 cells per 12 mm glass coverslip in 24-well plates is recommended. In fact, iNSCs exhibit a good proliferative rate during differentiation stage I. The iNSCs seeded in one well of a 24-well plate would give rise to a sufficient number of DA precursors by differentiation day 10−13 for transplantation into one mouse (2×10^5 for each mouse).

This protocol presents a method for the establishment of reproducible and stable unilateral 6-OHDA-lesioned PD mouse models. The extent of 6-OHDA lesion can be estimated by behavioral assessment that measures the contralateral rotations after injection of apomorphine. Also, the degree of 6-OHDA-induced lesion can be quantified by post-mortem immunofluorescent staining for TH in the SNpc. Another type of neurotoxin used in PD modeling is 1-methyl-4-phenyl-1,2,3,6-tertahydropyridine (MPTP), which also disrupts dopaminergic pathways. Compared to MPTP, 6-OHDA could be administered uni- or bilaterally. Also, the MPTP method is more sensitive to animal age, gender and strain and thus may show a higher degree of variation between the animals[28]. The critical factors include selecting mice with matching weight, injecting freshly prepared 6-OHDA solution and performing surgery accurately and quickly.

The procedures of cell transplantation of DA precursors into the striatum of lesioned mice are basically the same as the procedures for generation of 6-OHDA-lesioned PD mouse models, except that 6-OHDA is replaced by DA precursors at the injection step. The key factor in this part is searching the optimal time window of DA cell differentiation for transplantation. It has been demonstrated that a higher degree of stemness correlates with a higher survival rate but a lower potential of specification into mature DA neurons[11]. However, a more mature stage of neural cells are more vulnerable, and show a lower survival rate after transplantation[11]. Therefore, finding a suitable time window that balances the ability of maturation and survival is of significance. In a previous study, we transplanted cells of differentiation day 10 and 13 into the striatum of immunodeficient SCID-beige mice[11]. One month after engraftment, immunofluorescent staining results revealed that about 88.63% and 93.13% were TUJ1-positive for day 10 and day 13 groups, respectively, and some TH^+ cells (5.30%) were detected from day 13 group but few TH^+ cells from day 10 group. Nevertheless, compared to day 13 cells, day 10 cells gave rise to a slightly higher overall survival rate[11]. The results revealed that day 10 to day 13 is an optimal time window of cells for engraftment[11]. In this protocol, we used a mixture of DA cells from differentiation day 10 and day 13 at a ratio of 1:7 for engraftment, which showed a good result of survival and differentiation[11]. Using a mixture of cells from day 10 and day 13 was based on a hypothesis that relatively immature and mature neural cells, when put together, may support each other, reminiscent of the in vivo situation in mice where neural stem cells are surrounded by mature neurons.

This protocol presents the method of generating iNSCs from PBMNCs by SeV infection, differentiating iNSCs into DA neurons, and transplanting DA precursors into 6-OHDA-lesioned PD mouse models. Using this protocol, one can generate iNSCs with potentials not only for treatment of PD, but also of other neurodegenerative diseases. Since the iNSCs represent a primitive NSC stage, they can be specified into different region-specific neural cells, such as spinal cord neurons or motor neurons, which may be of promising utility for treatment of amyotrophic lateral sclerosis or spinal cord injury. Besides, iNSCs derived from familial disease patients offer a platform to study mechanisms underlying disease onset and development, and to conduct drug screening tests.

There is more than one way to obtain DA neural cells for transplantation studies. DA cells can also be generated from iPSCs or by direct conversion[29]. Compared with iPSCs, iNSCs are inherent with reduced risk of tumor formation, a shorter period of reprogramming and line establishment, and lineage-committed plasticity — only able to differentiate into neurons and glia[11]. Compared with direct conversion, differentiating DA precursors from iNSCs gives rise to a higher yield and efficiency[30].

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

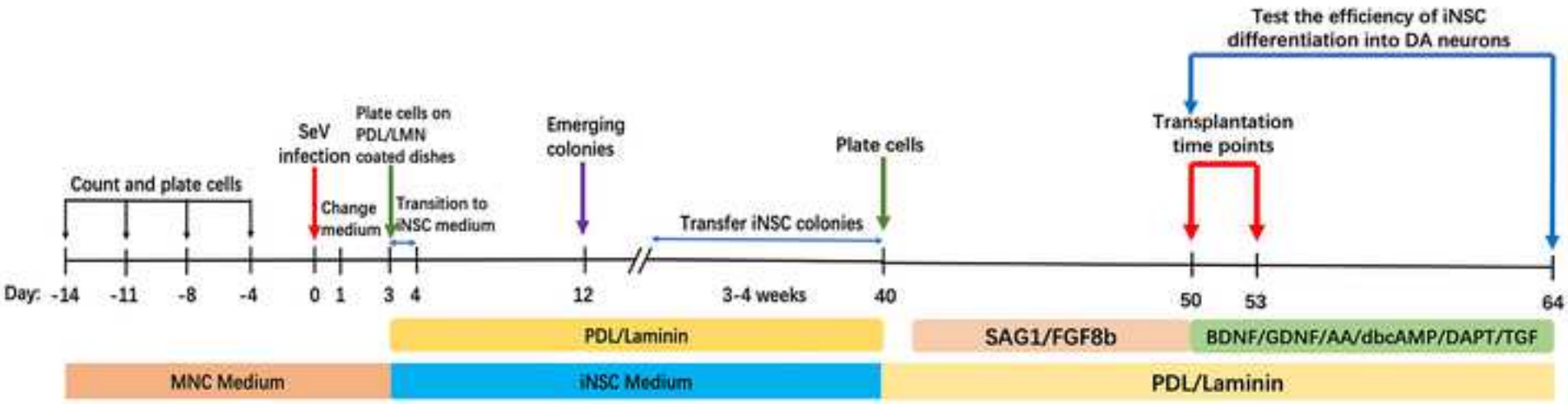

Figure 2

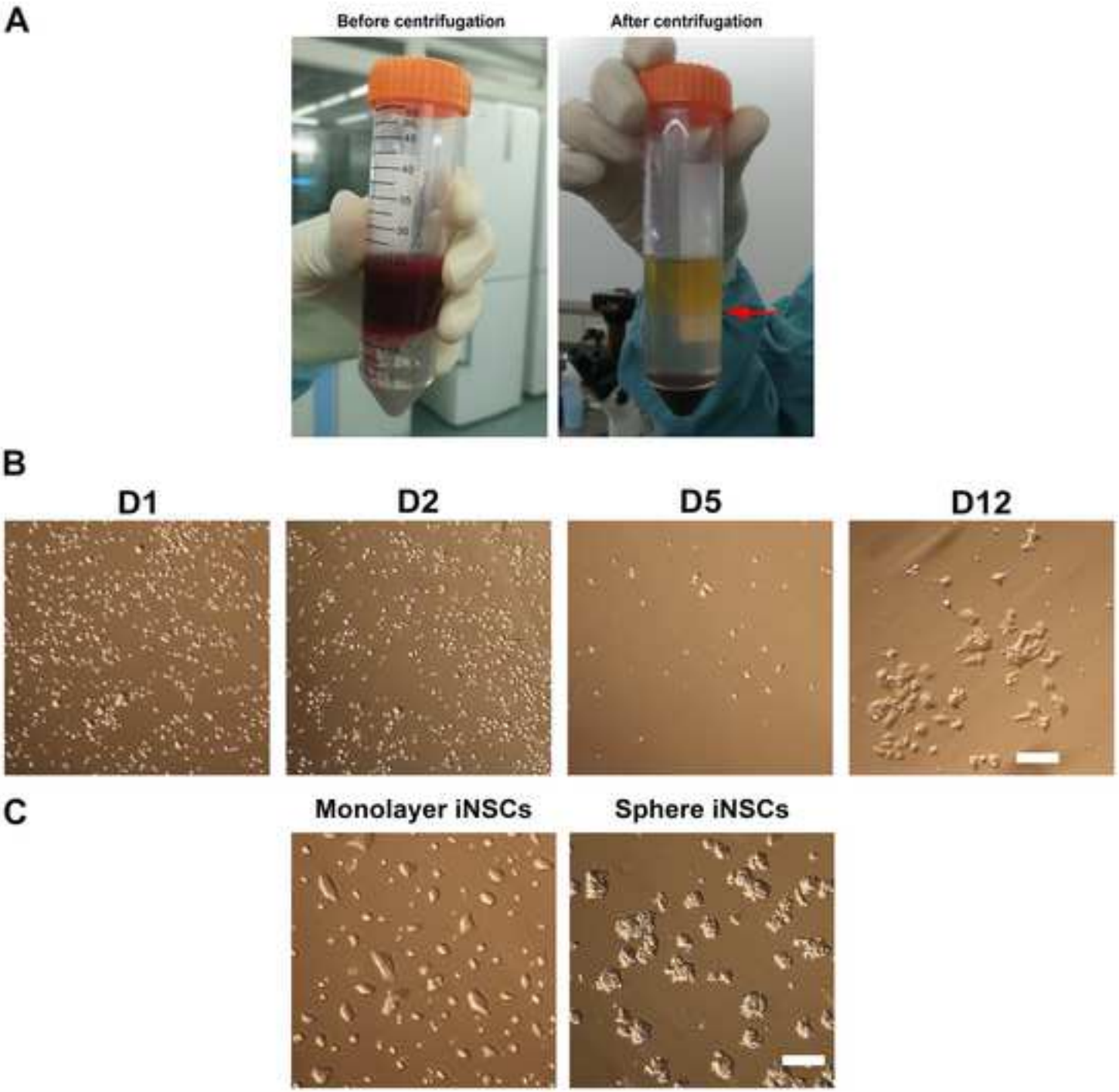

Figure 3

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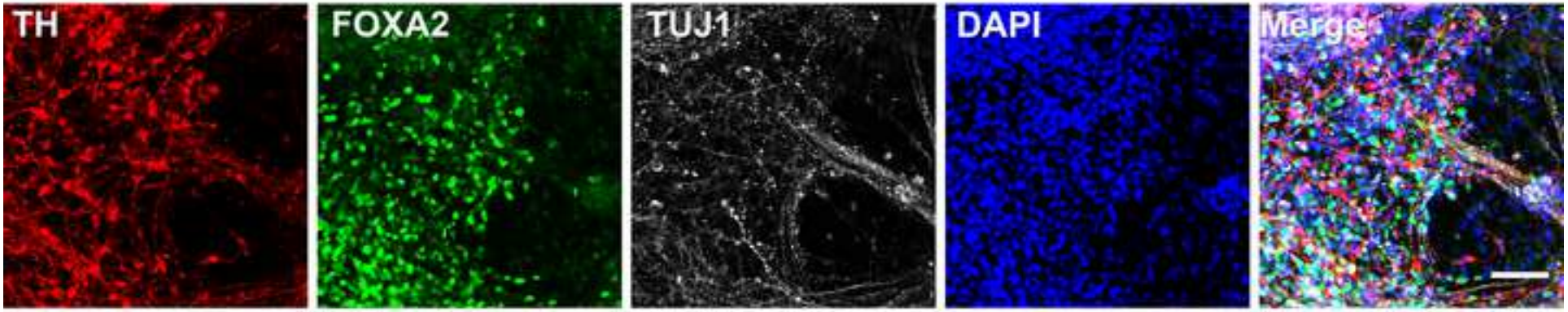
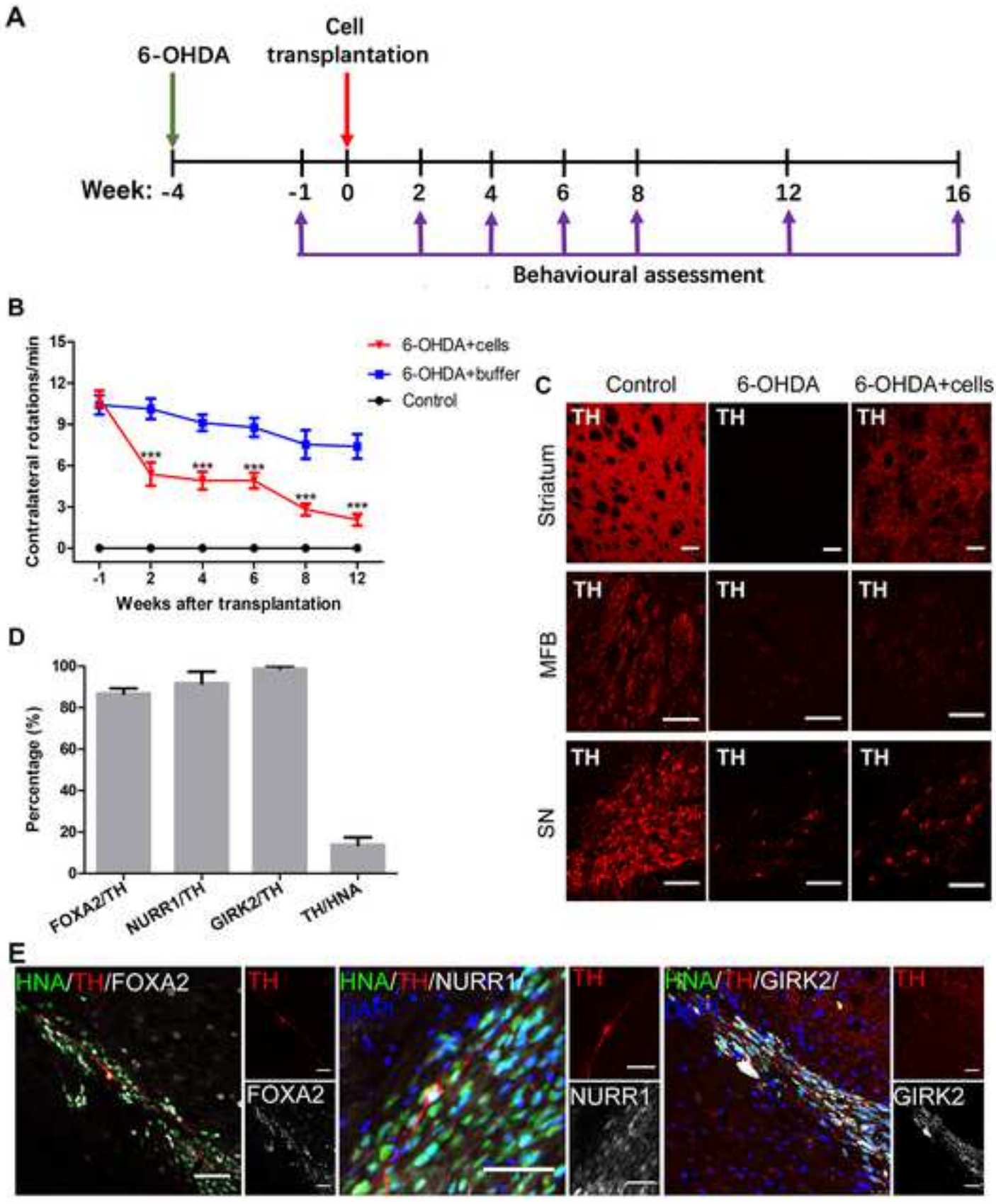


Figure 4



Material/ Equipment	Company	Catalog Number	Comments/Description
15-ml conical tube	Corning	430052	
1-Thioglycerol	Sigma-Aldrich	M6145	Toxic for inhalation and skin contact
24-well plate	Corning	3337	
50-ml conical tube	Corning	430828	
6-OHDA	Sigma-Aldrich	H4381	
6-well plate	Corning	3516	
Accutase	Invitrogen	A11105-01	Cell dissociation reagent
Apomorphine	Sigma-Aldrich	A4393	
Ascorbic acid	Sigma-Aldrich	A92902	Toxic with skin contact
B27 supplement	Invitrogen	17504044	
BDNF	Peprotech	450-02	Brain derived neurotrophic factor
Blood collection tubes containing sodium heparin	BD	367871	
BSA	yisheng	36106es60	Fetal bovine serum
cAMP	Sigma-Aldrich	D0627	Dibutyryladenosine cyclic monophosphate
CellBanker 2	ZENOAQ	100ml	Used as freezing medium for PBMNCs
concentrate	Invitrogen	11905031	
CHIR99021	Gene Operation	04-0004	
Coverslip	Fisher	25*25-2	
DAPI	Sigma-Aldrich	D8417-10mg	
DAPT	Sigma-Aldrich	D5942	
Dexamethasone	Sigma-Aldrich	D2915-100MG	
DMEM-F12	Gibco	11330	
DMEM-F12	Gibco	11320	
Donkey serum	Jackson	017-000-121	
EPO	Peprotech	100-64-50UG	Human Erythropoietin
FGF8b	Peprotech	100-25	

Ficoll-Paque Premium	GE Healthcare	17-5442-02	P=1.077, density gradient medium
GDNF	Peprotech	450-10	Glial derived neurotrophic factor
GlutaMAX	Invitrogen	21051024	100 × Glutamine stock solution
Ham's-F12	Gibco	11765-054	
HBSS	Invitrogen	14175079	Balanced salt solution
Human leukemia inhibitory factor	Millpore	LIF1010	
Human recombinant SCF	Peprotech	300-07-100UG	
IGF-1	Peprotech	100-11-100UG	Human insulin-like growth factor
IL-3	Peprotech	200-03-10UG	Human interleukin 3
IMDM	Gibco	215056-023	Iscove's modified Dulbecco's medium
Insulin	Roche	12585014	
ITS-X	Invitrogen	51500-056	Insulin-transferrin-selenium-X supplement
Knockout serum replacement	Gibco	10828028	Serum free basal medium
Laminin	Roche	11243217001	
Microsyringe	Hamilton	7653-01	
N2 supplement	Invitrogen	17502048	
NEAA	Invitrogen	11140050	Non-essential amino acid
Neurobasal	Gibco	10888	Basic medium
PDL	Sigma-Aldrich	P7280	Poly-D-lysine
SAG1	Enzo	ALX-270-426-M01	
SB431542	Gene Operation	04-0010-10mg	Store from light at -20℃
Sendai virus	Life Technologies	MAN0009378	
Sucrose	baiaoshengke		
TGFβⅢ	Peprotech	100-36E	Transforming growth factor βⅢ
Transferrin	R&D Systems	2914-HT-100G	
Triton X 100	baiaoshengke		Nonionic surfactant
Trypan blue	Gibco	T10282	
Xylazine	Sigma-Aldrich	X1126	

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Editor

JoVE

Feb. 26, 2019

RE: JoVE59690

Dear Editor,

We are grateful to the editorial and the reviewers' comments and suggestions on our manuscript, JoVE59690 "Protocols of Using Induced Neural Stem Cell Derivatives to Treat Parkinson's Disease Mouse Models: From Generation, Differentiation to Transplantation". The comments were very helpful for improving the quality of the manuscript. Based on these comments and suggestions, we have carefully revised the manuscript accordingly. The revised portion has been marked in red in the paper. Below please find the detailed point-by-point responses to the comments.

With these changes, we hope that the revised manuscript will meet the standards for publication in *JoVE*. We would be glad to respond to any further questions and comments you may have.

Thank you for your consideration.

Sincerely yours,

Zhiguo Chen

Editorial comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The *JoVE* editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

Response: Thanks for your comments. We have thoroughly proofread the manuscript and revised any spelling or grammar errors we found.

2. Please obtain explicit copyright permission to reuse any figures from a previous

publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]."

Response: In this manuscript, only Figure 4 was modified from our previous publication in *Theranostics*. And as an author of the article, there is no need to obtain permission, which was written on home page of *Theranostics* (http://www.thno.org/ms/feedback).

3. Title: Please revise it to be more concise and avoid the use of colon or dash.

Response: According to your suggestion, we have revised the title and avoided the use of colon.

4. Please include an ethics statement before the numbered protocol steps, indicating that the protocol follows the guidelines of your institution's human research ethics committee.

Response: We have added an ethics statement at the top of the protocol, indicating that the protocol follows the guidelines of institution's human research ethics committee (Line 107-109).

5. Please include an ethics statement before your numbered protocol steps, indicating that the protocol follows the animal care guidelines of your institution.

Response: According to your suggestions, we have added an ethics statement at the top of the protocol, indicating that the protocol follows institutional guidelines for care and use of animals (Line 107-109).

6. Please define all abbreviations before use (D-PBS, PDL, etc.).

Response: Thanks for your comments. We have defined all abbreviations, such as D-PBS (Line 151), PDL (Line 215).

7. Please abbreviate liters to L (L, mL, µL) to avoid confusion.

Response: We have abbreviated liters to L as you advised (Line 115, etc).

8. Please include a space between all numerical values and their corresponding units: 15 mL, 5 g, 7 cm, 37 °C, 60 s, 24 h, etc.

Response: We have included a space between all numerical values and their corresponding units (Line 192, etc.).

9. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. You may use the generic term followed by "(Table of Materials)" to draw the readers' attention to specific commercial names. Examples of commercial sounding language in your manuscript are: GlutaMAX, Ficoll, Neurobasal, etc.

Response: We have removed all commercial language from the manuscript and used generic terms instead (Line 130, 153, 225, etc.).

10. Please revise the Protocol text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

Response: We have revised the Protocol text to avoid the use of any personal pronouns according to your suggestion (Line 183-184, etc.).

11. Please revise the Protocol to contain only action items that direct the reader to do something (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "NOTE." Please include all safety procedures and use of hoods, etc. However, notes should be used sparingly and actions should be described in the imperative tense wherever possible. Please move the discussion about the protocol to the Discussion.

Response: According to your suggestions, the protocol has been described in the imperative tense, and the part that cannot be written in the imperative tense as a "NOTE" (Line 186-187, 191, 485-486, etc.). We have added safety procedures (Line 134, 254-255, 380, 388), and moved the discussion about the protocol to the Discussion part

(Line 627-630).

12. 2.2.2, 2.2.5: What happens after centrifugation, aspirate the supernatant? Please specify throughout the protocol.

Response: Thanks for your comments. We have added how to treat the pellet or supernatant after centrifugation (Line 180, 240-241).

13. What happens to mice at the end of protocol? Please specify.

Response: We have described how to treat the mice at the end of the transplantation experiments (Line 510-529).

14. In the protocol, please describe how to perform immunofluorescent staining and conduct the behavioral assessment to estimate PD symptoms as such data are shown in the Representative Results section.

Response: We have added to the protocol how to perform immunofluorescent staining and how to conduct the behavioral assessment for estimation of PD symptoms (Line 372-400, 481-496).

15. Please combine some of the shorter Protocol steps so that individual steps contain 2-3 actions and maximum of 4 sentences per step.

Response: Thanks for your suggestions, and we have combined some of the shorter Protocol steps so that individual steps contain 2-3 actions and maximum of 4 sentences per step. (Line 166-169, 173-175, 194-196, etc.).

16. After you have made all the recommended changes to your protocol (listed above), please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

Response: We have highlighted 2.75 pages of the protocol that identifies the essential steps of the protocol for the video (Line 145-205, 234-290, 448-475).

17. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative

tense. Notes cannot usually be filmed and should be excluded from the highlighting. Please do not highlight any steps describing anesthetization and euthanasia.

Response: We have highlighted complete sentences, and included at least one action that is written in imperative tense. And we have excluded notes and any steps describing anesthetization and euthanasia from the highlighting (Line 145-205, 234-290, 448-475).

18. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.

Response: We have added all relevant details that are required to perform the steps in the highlighting as you suggested (Line 145-205, 234-290, 448-475).

19. Figure 4: Please define error bars in the figure legend.

Response: According to your suggestions, we have defined error bars in the figure legend of Figure 4 (Line 601, 607).

20. Table of Materials: Please ensure that it has information on all relevant supplies, reagents, equipment and software used, especially those mentioned in the Protocol. Please sort the items in alphabetical order according to the name of material/equipment.

Response: According to your comments, we have added all relevant supplies, reagents, equipment used in Table of Materials. And we have sorted items in alphabetical order.

21. References: Please do not abbreviate journal titles.

Response: We have revised the journal titles of references.

Reviewers' comments:

Reviewer #1:

Specific comments/suggestions:

- The title should appropriately specify the source of cells used to differentiate DA

neuronal precursors (i.e. PBMNCs are used to derive iNSC through "Yamanaka" factors (OSKM) reprogramming that are further specified toward dopaminergic neuron precursors).

One possible suggestion could be: "Generation of induced neural stem cells from peripheral mononuclear cells and subsequent differentiation toward DA neuron precursors for transplantation studies"

Response: Thanks for your thoughtful comments. We have revised the title as you advised. The limit of the title is 150 characters as required by the journal. So we removed “subsequent” to meet the requirement.

- Two important missing aspects for the described protocol is a list of Quality Control criteria (e.g. list of positive markers that define the different cell types throughout the differentiation as well as markers for possible contaminant cell products as controls) and efficiency at each critical step of the protocol. QC steps should be explained along with overall expected efficiency and will certainly help researchers to verify that their experiments are proceeding according to the described technology. For example, QC and expected efficiency should be specified for the following steps: isolation of PBMNCs from PB, conversion of PBMNCs into iNSC and conversion of iNSC into mDA precursors at stage I and stage II.

Response: Thanks for your suggestions. We have added these two important aspects, Quality Control criteria (e.g. some positive markers that define the different cell types throughout the differentiation) and efficiency at each critical step of the protocol (e.g. isolation of PBMNCs from PB, conversion of PBMNCs into iNSCs and conversion of iNSCs into mDA precursors at stage I and stage II) (Line 630-633, 640-649, 669-675, etc.).

- It would be useful to have a general schema of the entire protocol, from the isolation and expansion of PBMNCs to their reprogramming into iNSCs and induction of DA fates. I suggest combining the schematics of Figure1 and Figure3A in one schema that highlight the timing of the procedure, the specific treatment at each step (including the names of the media; i.e. iNSC stageI and stageII and the relative small molecules and growth factors composition) and the critical time points for quality control steps.

Response: Thanks for your comments. We have combined the schematics of Figure 1

and Figure 3A in one schema that highlight the timing of the procedure, the specific treatment at each step and the critical time points for quality control steps.

- It is not clear the final yield of cells (as compared to the initial number of isolated PBMNCs) and for how many passages PBMNCs and iNSC can be expanded while maintaining their identity. This information could be useful for researchers in order to plan their experiments according to the number of cells needed at the end of the differentiation for the downstream applications (e.g. number of animals planned to transplant for example).

Response: As you advised, we have added the information about the yield of cells, and for how many passages PBMNCs and iNSCs can be expanded while maintaining their identity (Line 630-633, 640-645, 680-683).

- Since the entire procedure last many weeks, it would be useful to include (if known and tested) at which step of the protocol cells can be banked as frozen stocks. Also, in 1.3.10, which freezing media is used?

Response: According to your suggested, we have added information about at which steps the cells can be banked (Line 183-187, 292-294). And we have described the components of freezing media (Line 292-294).

- The temperature of the media is not specified. Should be media pre-warmed?

Response: Thanks for your comments. The media should be pre-warmed at 37 ℃. And we have added this information in the manuscript (Line 192, etc.).

- Sterility conditions should be stated in the protocol

Response: We have emphasized the importance of using sterility conditions in the protocol according to your suggestions (Line 147-148, 236-237, 325-326).

- In 2.2.13, the authors should better described the method used for picking colonies

Response: As you advised, we have described the method for picking colonies (Line 289-290).

- The authors focus on the application of their cell product to transplantation studies

and described in detail the procedures for such experiments. It would be informative to briefly include in the discussion the other potential downstream applications of their cell product and which advantages/disadvantages this method has as compared to other strategies for generated human DA-like neuronal precursors.

Response: Thanks for your valuable comments. We have added the other potential downstream applications using our cells and described the advantages of this method as compared to other strategies to generate human DA-like neuronal precursors. (Line 720-733)

Reviewer #2:

Minor Concerns:

1. Could the authors suggest if the iNSC derived could be frozen for future use differentiation.

Response: Thanks for your thoughtful comments. iNSC could be frozen for future use. And we have added the frozen media in our protocol (Line 292-294).

2. In iPSC differentiation towards dopaminergic neurons there is a big batch to batch/line to line variation in the efficiency for generating dopaminergic neurons. Is this observed in the PBMC derived iNSC.

Response: As a big batch to batch/line to line variation exists in terms of the efficiency for iPSC differentiation towards DA neurons, a batch to batch/line to line variation also exists with regard to the efficiency of iNSC differentiation towards DA neurons. This has been added to the Discussion part (Line 675-678).

3. Authors suggested that combined day10 and day 13 cells is the best for differentiation. Has the authors tried time points between the day 10-13 window and what would be the outcomes?

Response: Thanks for your valuable comments. In this protocol, we used a mixture of DA cells from differentiation day 10 and 13 at a ratio of 1:7 for engraftment, which showed a good result of survival and differentiation. Using a mixture of cells from day 10 and day 13 was also based on a hypothesis that relatively immature and mature

neural cells, when put together, may support each other, reminiscent of the *in vivo* situation in mice where neural stem cells are surrounded by mature neurons (Line 705-716).

4. For Figure 4A, the unit (week) is missing for the timeline.

Response: We have added the unit for the timeline on Figure 4A according to your suggestion.

5. There is no staining picture of the graft in the striatum shown in Figure 4. The staining for the makers quantified in Figure 4D should be shown

Response: According to your comments, we have added the staining pictures of the graft in the striatum, shown in Figure 4E.

6. For Figure 4C, it is better to state the marker stained in the picture.

Response: The marker name TH has been added in Figure 4C as you advised.