

Video Article

Directed Dopaminergic Neuron Differentiation from Human Pluripotent Stem Cells

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Abstract

Dopaminergic (DA) neurons in the substantia nigra pars compacta (also known as A9 DA neurons) are the specific cell type that is lost in Parkinson's disease (PD). There is great interest in deriving A9 DA neurons from human pluripotent stem cells (hPSCs) for regenerative cell replacement therapy for PD. During neural development, A9 DA neurons originate from the floor plate (FP) precursors located at the ventral midline of the central nervous system. Here, we optimized the culture conditions for the stepwise differentiation of hPSCs to A9 DA neurons, which mimics embryonic DA neuron development. In our protocol, we first describe the efficient generation of FP precursor cells from hPSCs using a small molecule method, and then convert the FP cells to A9 DA neurons, which could be maintained *in vitro* for several months. This efficient, repeatable and controllable protocol works well in human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs) from normal persons and PD patients, in which one could derive A9 DA neurons to perform *in vitro* disease modeling and drug screening and *in vivo* cell transplantation therapy for PD.

Video Link

The video component of this article can be found at <https://www.jove.com/video/51737/>

Introduction

Dopaminergic (DA) neurons can be found in several brain regions, including the midbrain, hypothalamus, retina, and olfactory bulbs. The A9 DA neurons in the substantia nigra pars compacta (SNpc) control behavior and movement by projecting to the striatum in the forebrain and forming the extrapyramidal motor system. Degeneration of A9 DA neurons leads to Parkinson's disease (PD), which is the second most common human neurodegenerative disorder and currently incurable. Cell replacement therapy is one of the most promising strategies for the treatment of PD¹, therefore, there has been a great interest in deriving A9 DA neurons from human pluripotent stem cells (hPSCs), including both human embryonic stem cells (hESCs) and the recent human induced pluripotent stem cells (hiPSCs).

Much research has tried to derive A9 DA neurons from hPSCs using various methods. The earliest reports all generated DA neurons through a neural rosette progenitor stage. By co-culturing with MS5² or PA6³⁻⁵ stromal cells with or without external growth factors for 2-4 weeks, L. Studer and colleagues and three other groups successfully induced hESCs to produce neural rosettes. They then enriched these rosettes by mechanical dissection or enzymatic digestion for further differentiation. In other reports, researchers generated neural rosettes through embryoid body (EB) floating culture differentiation⁶⁻⁹. Later, researchers established a monolayer based differentiation method^{10,11}, where they plated hESCs and hiPSCs on extra cellular matrix, added different growth factors in the culture to induce the differentiation of hPSCs to DA neurons, which mimics the *in vivo* embryonic DA neuron development. Although all these studies obtained tyrosine hydroxylase (TH)-expressing cells with some characteristics of DA neurons, the entire differentiation process is time and labor consuming, generally inefficient, and more importantly, the A9 identity of these neurons were not demonstrated in most studies except the one with LMX1a ectopic expression¹². Recently, a new floor plate (FP)-based protocol was developed¹³⁻¹⁶, in which the FP precursors with DA neuron potential were first generated by activation of the sonic hedgehog and canonical Wnt signaling pathways during the early stage of differentiation, and then these FP cells were further specified to DA neurons. Although this protocol is more efficient, there are still some problems; for example, the whole differentiation process takes long time (at least 35 days) and is feeder cell dependent¹⁵, or is EB dependent¹⁶ or the A9 identity was not demonstrated¹⁴.

Here, based on the knowledge from *in vivo* embryonic DA neuron development and other researchers' published results, we have optimized the culture conditions for the efficient generation of DA neurons from both hESCs and hiPSCs. We first generated FP precursor cells by activation of the canonical Wnt signaling with small molecule CHIR99021 and sonic hedgehog signaling with small molecules SAG and purmorphamine. These FP cells express FOXA2, LMX1a, CORIN, OTX2 and NESTIN. We then specified these FP cells to DA neurons with growth factors including BDNF, GDNF, etc. The generated DA neurons are of A9 cell type as they are positive for GIRK2 while negative for Calbindin¹⁷. This

protocol is feeder cell or EB independent, highly efficient and reproducible. Using this protocol, one can derive DA neurons in less than 4 weeks from hESCs or hiPSCs of normal persons for cell transplantation study, or from hiPSCs of PD patients for *in vitro* modeling of PD or testing potential therapeutic agents for PD.

Protocol

1. Preparation of Culture Media

1. Prepare mouse embryonic fibroblast (MEF) medium by combining the following: 445 ml DMEM, 50 ml fetal bovine serum (FBS), and 5 ml 100x penicillin/ampicillin stock solution. Keep filter sterilized medium at 4 °C for no more than 14 days.
2. Prepare serum-containing hPSC culture medium by combining the following: 385 ml DMEM/F12, 100 ml knockout serum replacement (KSR), 5 ml 100x non-essential amino acid stock solution, 5 ml 100x penicillin/ampicillin stock solution, 5 ml 100x -mercaptoethanol stock solution, and 10 ng/ml bFGF. Keep filter sterilized medium at 4 °C for no more than 10 days.
3. Prepare mTeSR1 serum-free medium by combining the following: 400 ml mTeSR1 basal medium, 100 ml 5x supplement, and 5 ml 100x penicillin/ampicillin stock solution. Keep medium at 4 °C for no more than 10 days.
4. Prepare 10x collagenase IV stock solution: weigh out 0.5 g collagenase IV power, and dissolve it with 50 ml DMEM/F12 and filter sterilize. Make aliquots and stock them at -20 °C for months.
5. Prepare 0.1% gelatin solution: weigh out 0.5 g gelatin (from bovine skin, type B) power and dissolve it with deionized water. Keep autoclave sterilized solution at room temperature for weeks.
6. Prepare KSR differentiation medium by combining the following: 410 ml DMEM, 75 ml KSR, 5 ml 100x non-essential amino acid stock solution, 5 ml 100x penicillin/ampicillin stock solution, 5 ml 100x -mercaptoethanol stock solution. Keep filter sterilized medium at 4 °C for no more than 10 days.
NOTE: KSR varies from lot to lot, which may affect the differentiation efficiency. It is therefore better to test several batches of KSR to find the best one for differentiation.
7. Prepare N2 differentiation medium by combining the following: 98 ml DMEM, 1 ml 100x N2 supplement and 1 ml 100x penicillin/ampicillin stock solution. Keep filter sterilized medium at 4 °C for no more than 10 days.
8. Prepare B27 differentiation medium by combining the following: 480 ml neurobasal medium, 10 ml 50x B27 supplement, 5 ml 100x glutamax stock solution, and 5 ml 100x penicillin/ampicillin stock solution. Keep filter sterilized medium at 4 °C for no more than 10 days.

2. Culture of hESCs and hiPSCs on MEF Feeder Cells

The H9 hESCs were obtained from WiCell Research Institute and hiPSCs were established in the Reijo Pera laboratory through retrovirus-mediated transduction of Yamanaka factors OCT3/4, SOX2, KLF4, and c-MYC¹⁸.

1. At least one day before cell passage, prepare some gelatin plates by adding 1 ml 0.1% gelatin to 1 well of 6-well plates, and incubate plates at 37 °C for at least 30 min.
2. After incubation, aspirate gelatin from plates, and seed irradiation inactivated MEF cells onto the plates at the density of 1.6×10^5 cells/well in MEF medium using a hemocytometer to count the cells.
NOTE: Optionally prepare MEF feeders as early as 3 days before cell passage.
3. Passage cells one day after plating MEF feeders: aspirate medium from hPSCs, add 1 mg/ml collagenase IV to cells at 1 ml/well, and incubate cells at 37 °C for 1 hr.
4. During this incubation, wash MEF feeders once with PBS, and then add warm (37 °C) serum-containing hPSC culture medium at 1.5 ml/well.
5. After 1 hr, tap the culture plate a couple of times so that most of the undifferentiated colonies will detach from the plates, while the differentiated colonies remain attached. Carefully collect the floating colonies, and transfer them into 15 ml tube.
6. Gently wash the plate once with warm (37 °C) DMEM/F12 and transfer DMEM/F12 into the same 15 ml tube.
7. Centrifuge the tubes at 179 x g for 3 min.
8. Aspirate medium from the tubes, being careful not to disturb the pellet. Add warm hPSC culture medium, pipette the cells up and down several times to break them into small clusters and mix them well.
9. Transfer the cells onto new MEF feeders at 1:3-1:6 ratio.
10. Change the medium every day and passage the cells every 5-6 days.

3. Preparation of Cells for Differentiation

1. At least one day before preparation of cells, prepare some Matrigel plates (typically 3 wells of a 6-well plate). Dilute the Matrigel with cold (4 °C) DMEM/F12 at 1:40 and add 1 ml Matrigel per well of 6-well plates. Incubate Matrigel plates at 4 °C overnight. NOTE: One can seal Matrigel plates with Parafilm and stock them at 4 °C for as long as one week.
2. Use cells (see Step 2) 4 days after passage. Remove all the differentiated colonies from the plates. Aspirate medium from the culture plate, add 1 ml accutase per well, and incubate cells at 37 °C for 5 min.
3. During the incubation, prepare some gelatin plates by adding 1 ml gelatin to 1 well of 6-well plates. Place these plates and the Matrigel-plates prepared one day before in incubator.
4. After the 5-min incubation or when cells have become semi-floating, pipette cells up and down several times to make them into single cells.
5. Collect single cells into 15 ml tubes, and centrifuge at 258 x g for 3 min.
6. Resuspend cells with appropriate amount of serum containing hPSC culture medium, and add Thiazovivin to the final concentration of 2 μ M.
7. Transfer cells onto gelatin-coated plates at 1:1 ratio, and incubate cells at 37 °C for 30 min to remove MEF feeders.
8. Transfer the cells to a 15 ml conical tube, add appropriate serum containing hPSC medium and count the cells with a hemocytometer.
9. Centrifuge the cells at 258 x g for 3 min.
10. During centrifuge, add Thiazovivin to appropriate mTeSR1 medium to make a concentration of 2 μ M.

11. After centrifuge, aspirate the medium and add appropriate mTeSR1 medium with Thiazovivin so that there are 1.8×10^5 cells/ml medium.
12. Take out the Matrigel plates from the incubator, aspirate the Matrigel and add 2 ml of the mixed cells onto 1 well of the 6-well plates.
NOTE: The cell density should be 3.6×10^4 cells/cm² of surface area.
13. Return the plates back to the incubator, and let the cells attach for 24 hr.
14. 24 hr after plating the cells, aspirate old medium and add 2 ml new mTeSR1 medium per well and let cells grow for another 24 hr before starting the differentiation.

Cell Differentiation

1. Start differentiation 48 hr after replating the cells onto Matrigel plates.
2. Aspirate culture medium from the plate, wash cells once with PBS, and add 2 ml of the following differentiation medium per well: KSR differentiation medium supplemented with 10 μ M SB431542 and 100 nM LDN-193189. Mark this day as D0 of differentiation.
3. Change medium every day from D0 to D20.
NOTE: One can prepare medium for use no more than 2 days at a time.
4. Use the following medium for D1 and D2: KSR differentiation medium supplemented with 10 μ M SB431542, 100 nM LDN-193189, 0.25 μ M SAG, 2 μ M purmorphamine, and 50 ng/ml FGF8b.
5. Use the following medium for D3 and D4: KSR differentiation medium supplemented with 10 μ M SB431542, 100 nM LDN-193189, 0.25 μ M SAG, 2 μ M purmorphamine, 50 ng/ml FGF8b, and 3 μ M CHIR99021.
6. Use the following medium for D5 and D6: 75% KSR differentiation medium plus 25% N2 differentiation medium supplemented with 100 nM LDN-193189, 0.25 μ M SAG, 2 μ M purmorphamine, 50 ng/ml FGF8b, and 3 μ M CHIR99021.
7. Use the following medium for D7 and D8: 50% KSR differentiation medium plus 50% N2 differentiation medium supplemented with 100 nM LDN-193189 and 3 μ M CHIR99021.
8. Use the following medium for D9 and D10: 25% KSR differentiation medium plus 75% N2 differentiation medium supplemented with 100 nM LDN-193189 and 3 μ M CHIR99021.
9. Use the following medium for D11 and D12: B27 differentiation medium supplemented with 3 μ M CHIR99021, 10 ng/ml BDNF, 10 ng/ml GDNF, 1 ng/ml TGF3, 0.2 mM ascorbic acid and 0.1 mM cAMP.
10. Use the following medium for the rest of differentiation: B27 differentiation medium supplemented with 10 ng/ml BDNF, 10 ng/ml GDNF, 1 ng/ml TGF3, 0.2 mM ascorbic acid and 0.1 mM cAMP.
11. At about D16 of differentiation, prepare some Poly-L-ornithine/Laminin/Fibronectin plates. Dilute Poly-L-ornithine stock solution with cold (4 °C) PBS to 15 μ g/ml, add 1 ml to 1 well of 6-well plates, and incubate the plates at 37 °C overnight.
12. Aspirate the Poly-L-ornithine solution, wash the plates 3 times with sterile water and then air dry the plates.
13. Dilute laminin stock solution with cold (4 °C) PBS to 1 μ g/ml, add 1 ml to one well of 6-well plates, and incubate the plates at 37 °C overnight.
14. Aspirate the laminin solution, wash the plates 3 times with sterile water and then air dry the plates.
15. Dilute fibronectin stock solution with cold (4 °C) PBS to 2 μ g/ml, add 1 ml to 1 well of 6-well plates, and incubate the plates at 37 °C overnight.
16. Seal plates with Parafilm and place them at 4 °C for as long as two weeks.
17. After 20 days of differentiation, replat the cells to the Poly-L-ornithine/Laminin/Fibronectin plates. Aspirate differentiation medium, add 1 ml warm (37 °C) accutase to 1 well of 6-well plates, and incubate cells at 37 °C for 5 min.
18. During the incubation, place the Poly-L-ornithine/Laminin/Fibronectin plates in the incubator.
19. After the 5-min incubation or when cells have become semi-floating, gently pipette cells up and down several times to make them into single cells.
20. Collect the single cells into 15 ml conical tubes, and add the appropriate amount of neurobasal medium for cell counting, which will vary depending on expansion (after 11 days of differentiation, cells may expand 15-20 fold). Count the cells using a hemocytometer.
21. Centrifuge the cells at 258 x g for 3 min. Aspirate supernatant, and resuspend cells with B27 differentiation medium so that the cell concentration is 1×10^6 cells/ml medium.
22. Aspirate fibronectin from the plates, wash twice with PBS, and add 2 ml cells to 1 well of 6-well plates.
NOTE: The cell density for replating should be 2×10^5 cells/cm² of surface area.
23. Change medium 24 hr later to remove any unattached dead cells.
24. Change medium every other day until the desired time points for a given experiment.

Representative Results

An overview of the differentiation protocol is shown in **Figure 1**. The efficiency of the differentiation protocol presented here relies upon the status of the starting cells. Therefore, it is critical to make sure that, first one removes all the differentiated colonies before dissociating hPSCs into single cells for differentiation, and second, one depletes most, if not all, the MEF feeder cells by incubating the cells on gelatin-coated plates for 30 min, and third, one plates the hPSC single cells at the appropriate density onto Matrigel plate. As illustrated in **Figure 2**, replated hPSC single cells will expand as clusters during the 48 hr before differentiation, forming around 70% confluence in the plates. Then after differentiation, these cells reach full confluence in as short as 3-4 days, and starting from around D16-D17, these confluent cells start to make some spaces in the plates, and some axons/neurites could already be observed in these spaces and under the cell layers. After being replated at D20, the differentiated cells attached and grew as small clumps. Then during the next 5 days, much more intensive and morphologically intact axons/neurites come out from the clumps, and axons/neurites from different clumps form some connections. During long-term culture, neurons in one clump generate more extensions, get more mature and have more connections with neurons in neighboring clumps.

After 11 days of differentiation, hPSCs could be efficiently converted to FP precursors, and as illustrated in **Figure 3**, almost all the cells express the FP precursor cell markers NESTIN and FOXA2, and over 90% of the cells express another three markers CORIN, OTX2, and LMX1a. Then after more days of differentiation, the FP precursor cells are specified to DA neurons as they express TUJ1 and TH (**Figure 4A**). These DA neurons are of A9 identity as they express GIRK2 while are negative for Calbindin (**Figure 4B**). Immunostaining experiments also show that there are no GFAP+ astrocytes and very few GABAergic neurons (**Figure 4C**), indicating that the differentiation is exclusively specified toward the DA neuron lineage.

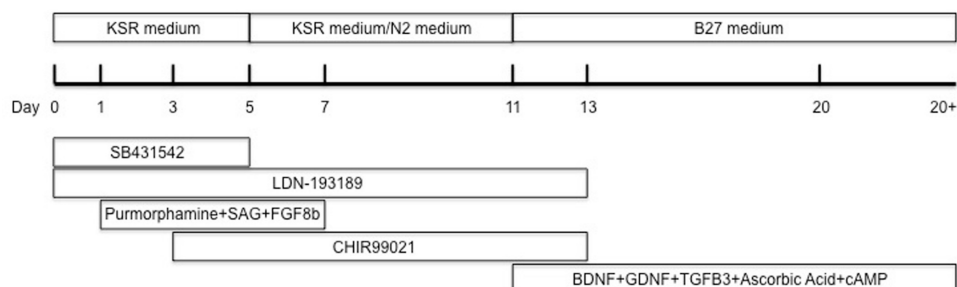


Figure 1. Overview of the differentiation protocol, with growth factors/small molecules and culture medium used for each day. [Please click here to view a larger version of this figure.](#)

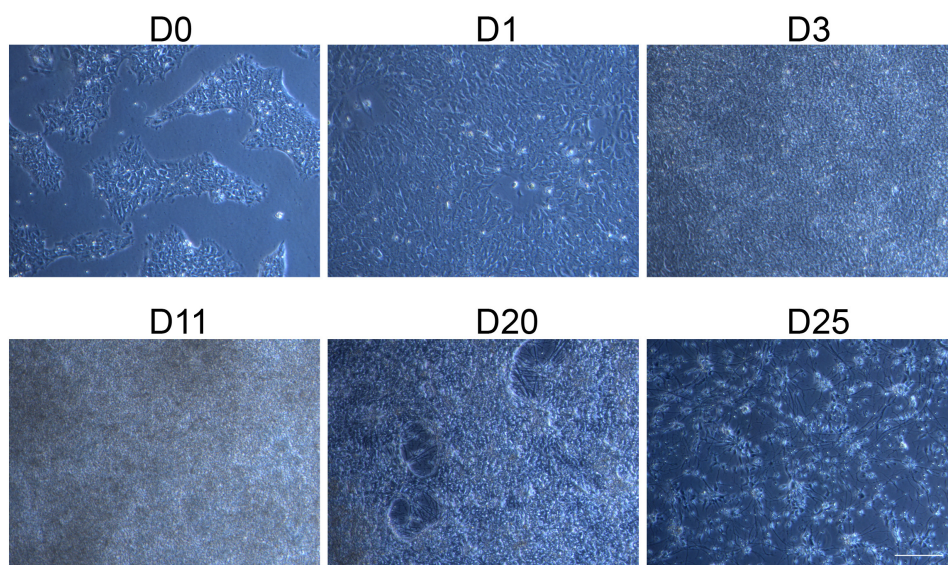


Figure 2. Morphological changes during differentiation. H9 hESCs were plated as single cells in the appropriate cell density as described, and 48 hr after that, these cells reach about 70% confluence in the plate as colonies (**D0**). During the first a few days of differentiation, the cells expand rapidly, reaching over 90% confluence after 24 hr of differentiation (**D1**), and then become confluent after 48 more hr (**D3**). However, most of these cells still exhibit typical hESC morphology with high nucleus to cytoplasm ratio (**D3**). As differentiation goes on, cells expand more and gradually lose hESC morphology. At the end of D11 of differentiation, there are more than one cell layers in many parts of the plate, as evidenced by darker color of cells in some areas than the others (**D11**). Starting from about D17 to D20, some cells died, leaving some spaces in the plate, and some neuron projections could already be observed in these spaces and sometimes under the cell layer (**D20**). After cell replating at the end of D20 to make more spaces for cells to grow and differentiation, cells aggregate as small clusters, a lot more axons/neurites emerge from these clusters, and axons/neurites from different clusters form some connections in as short as 4-5 days (**D25**). Scale bar, 200 μ m.

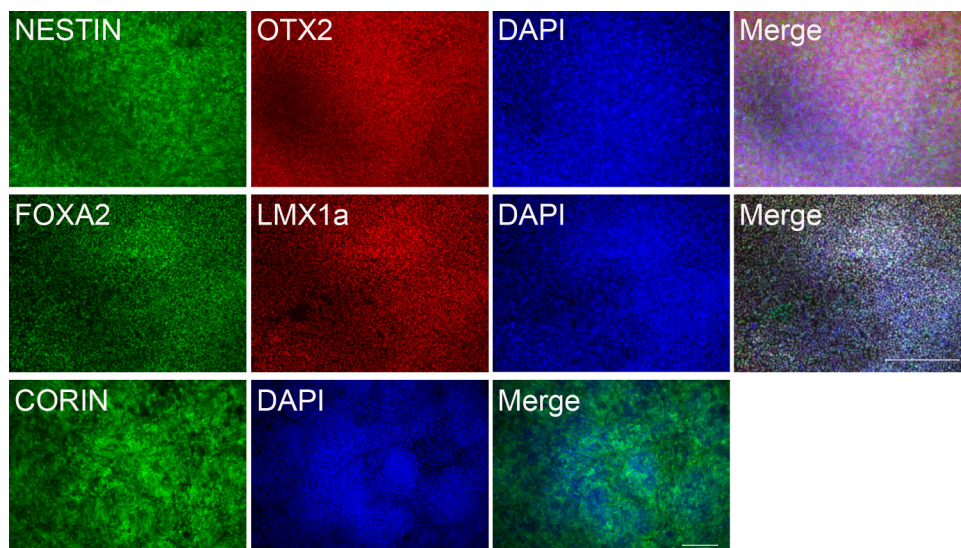


Figure 3. Immunostaining for FP markers at early stage of differentiation. H9 hESCs were differentiated for 11 days using the protocol described here. Cells were then fixed with 4% paraformaldehyde, permeabilized with Triton X-100, blocked with donkey serum and then stained for FP precursor cell markers. As shown here, almost all the cells express FOXA2 and NESTIN, and over 90% of the cells express LMX1a, CORIN, and OTX2, indicating a very high efficiency conversion from hESCs to FP cells. Scale bar, 200 μ m.

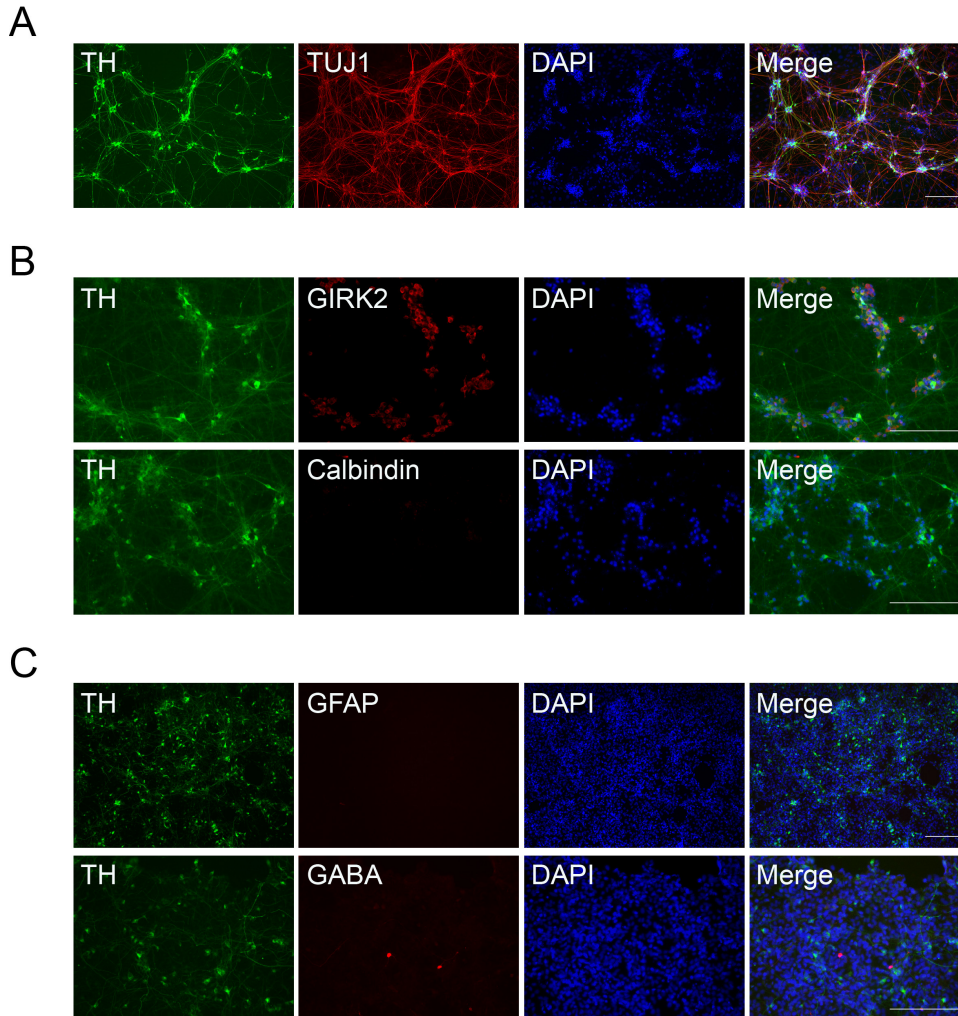


Figure 4. Immunostaining for DA neuron markers at late stages of differentiation. (A) H9 hESCs were differentiated for 25 days, and cells were subject to immunostaining for the pan-neuron marker TUJ1 and the commonly used DA neuron marker TH. As shown here, although there are more TUJ1 positive cells than TH positive cells, all the TH-expressing cells reside in the TUJ1-expressing cells, and the TH-expressing cells represent at least half of all the cells. (B). The H9 hESCs were further differentiated for another 25 days (totally 50 days) and then cells were subject to immunostaining. The results showed that there is higher percentage of TH-expressing cells than that at d25. Almost all of these TH-expressing cells also express GIRK2 while most of them are negative for Calbindin, indicating the A9 subtype identity of the generated DA neurons, which is the cell type that is lost in PD. (C) Immunostaining for cells at D25 of differentiation showed that there are no cells expressing GFAP, and very few cells express GABA. Scale bar, 200 μm.

Discussion

Human pluripotent stem cells (including both hESCs and hiPSCs) can be differentiated *in vitro* to generate most, if not all, cell types of our body including DA neurons, which has been demonstrated in previous studies¹⁹. Here, based on knowledge from embryonic dopaminergic neuron development²⁰ and published protocols from other laboratories^{14,15}, we optimized the culture conditions for the generation of DA neurons from hESCs and hiPSCs. This protocol is efficient, reproducible and we have successfully applied this protocol described here to H1 and H9 hESC lines and to the hiPSC lines from both the PD patients and unaffected controls.

In our protocol, there are three key steps to obtain the high differentiation efficiency: First, there should be no differentiated hPSC colonies in the cells before directed differentiation. As spontaneous differentiation to all three germ layers is often seen in hPSC culture, it is important to remove these differentiated colonies before dissociating these hPSCs into single cells. Second, MEF feeders should be depleted from the dissociated hPSCs, as previous reports have shown that MEF cells can secrete factors that promote self-renewal and inhibit differentiation²¹. For this purpose, incubating the hPSC and MEF cell mixtures on gelatin-coated plates for 30 min should be enough to remove almost all the MEF cells. Third, the single hPSCs should be plated at the appropriate cell density for differentiation. Increasing the cell density will lead to the death of most cells at around D11 of differentiation, while lowering the cell density will result in the detachment and death of cells in the center of the plate in as short as less than 7 days, although cells on the edge will differentiate well (data not shown). If one observes all these three criteria, it is easy to efficiently obtain DA neurons from hPSCs with our stepwise protocol.

The protocol presented here is mainly based on a previous report by L. Studer and colleagues¹⁴ with some modifications. First, the differentiation here was started 48 hr after single cell plating when cells reach only about 70% confluence, while in their report, differentiation was started

when cells reach full confluence (3 or more days of culture after single cell plating). In our experience, starting differentiation from confluent cells without a cell passage until day 20 leads to severe cell death during the differentiation process. Second, we replace the expensive SHH protein in their study with SAG, a chlorobenzothiophene-containing small molecule agonist for HH pathway²². Therefore, compared with previously published protocols, this one described here is feeder cell and neural rosette independent, and based mainly on small molecules for FP specification; it is thus less expensive and less time- and labor-consuming. Of course, the limitation of this protocol is the use of KSR for the first days of differentiation. KSR varies from lot to lot, which may affect the differentiation efficiency. Therefore, one should test different batches of KSR to get the one that works best in inducing FP precursors after 11 days of differentiation.

In summary, the differentiation protocol described here should facilitate: (1) the generation of DA neurons from hESCs or hiPSCs of normal persons for cell replacement therapy for PD; (2) the generation of DA neurons from PD patient iPSCs for *in vitro* modeling and testing potential therapeutic drugs for PD; (3) the study of the genes/signaling pathways regulating human DA neuron development.

Disclosures

The authors declare that they have no competing financial interests.

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