Efficient derivation of functional dopaminergic neurons from human embryonic stem cells on a large scale

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Cell-replacement therapy using human embryonic stem cells (hESCs) holds great promise in treating Parkinson's disease. We have recently reported a highly efficient method to generate functional dopaminergic (DA) neurons from hESCs. Our method includes a unique step, the formation of spherical neural masses (SNMs), and offers the highest yield of DA neurons ever achieved so far. In this report, we describe our method step by step, covering not only how to differentiate hESCs into DA neurons at a high yield, but also how to amplify, freeze and thaw the SNMs, which are the key structures that make our protocol unique and advantageous. Although the whole process of generation of DA neurons from hESCs takes about 2 months, only 14 d are needed to derive DA neurons from the SNMs.

INTRODUCTION

Parkinson's disease (PD) is the second most common neurodegenerative disorder. PD is caused by the death of the neurons in the substantia nigra pars compacta[1], which leads to motor dysfunction such as bradykinesia, tremor, rigidity and postural instability. Pharmacological drugs prevailing at present, such as L-DOPA, focus on restoring DA levels in the striatum. However, the induction of side effects and decline of their effectiveness after prolonged treatment negate the usefulness of these drugs[2]. An attractive alternative approach would be restoring the damaged DA-producing system in the striatum of the PD patients. Cell therapy using hESCs offers great promise to treat PD, as hESCs could potentially become an unlimited source of DA neurons to replace the dying cells. Achieving highly efficient derivation of DA neurons from hESCs is a critical issue not only to obtain better efficacy of cell therapy, but also to minimize potential teratoma formation. Although progress, to some extent, has been made recently in differentiating hESCs into DA neurons[3–9], the proportion of TH^+ neurons among total cells is still modest. Therefore, more efficient methods for DA neuronal derivation from hESCs were in great demand.

We have recently reported highly efficient generation of functional DA neurons from both human and mouse ESCs, and these neurons induced significant behavioral recovery in a PD animal model[10–13]. In our method using hESCs[10], about 77% of the total cells were βIII-tubulin–positive neurons, and about 86% of the neurons were TH^+ neurons, which represents the highest efficiency ever achieved. Similar quantitative results were obtained by both flow cytometry and immunocytochemistry[10]. The most unique feature of our protocol includes the generation of SNMs, which are free-floating sphere-type structures originated from neural rosettes or neural tubes in our protocol. Morphologically, they resemble 'neurospheres,' which normally refer to the sphere-like structures generated during suspension culture of adult neural stem cells[14]. However, we do not know if the SNMs share exactly the same characteristics as 'neurospheres' at this moment, and detailed studies of the characteristics of SNMs and extensive comparisons between SNMs and neurospheres are currently underway.

Spherical neural masses offer several advantages in generating DA neurons: (i) SNMs can be passaged over a long period of time and, hence, provide a sufficient number of DA cells for transplantation; (ii) importantly, these long-term-passaged SNMs can still be coaxed into DA neurons with the same high efficiency; (iii) it takes only about 7–8 d to obtain committed DA neuronal precursors from SNMs for transplantation; (iv) the expanded SNMs can be conveniently stored frozen and thawed as needed. These results cumulatively suggest that a sufficient amount of DA neuronal precursors for clinical application could be easily obtained within a short period of time by our protocol. This article provides detailed step-by-step methods for deriving DA neurons from hESCs as well as handling of SNMs, such as passaging, freezing and thawing.

Our method of efficient generation of DA neurons on a large scale would be potentially of great value in bringing hESC-mediated cell therapy to clinical application to treat PD. Furthermore, the multipotent nature of the SNMs would be of great use to develop cell-based therapies and to screen new drugs for many neurological diseases. Although our protocol enabled us to generate DA neurons at the highest efficiency so far, there is still much room for improvement; there is still a portion of non-DA cells that need to be either removed or coaxed into DA neurons. With regards to this issue, further optimization and modification of our protocol are at present underway.

MATERIALS

REAGENTS

- hESC lines[15] (see REAGENT SETUP)
- STO (S, SIM; T, 6-thioguanine resistant; O, Ouabain resistant) feeder cells (ATCC, cat. no. CRL-1503, information is available online) ! CAUTION Do not use if the passage number is over 7.
- 100× N2 supplement (GIBCO, cat. no. 17502-048)
- Sonic hedgehog (R&D, cat. no. 1314-SH-025)
- Fibroblast growth factor 8 (FGF8; Peprotech, cat. no.100-25)
- Glial cell line-derived neurotrophic factor (Peprotech, cat. no. 45010)
- L-Ascorbic acid (Sigma, cat. no. A4403)
- 50× B27 supplement (GIBCO, cat. no. 17504-044)
- Collagenase type IV (GIBCO, cat. no. 17104-019)
- Matrigel (BD, cat. no. 354230)
- Basic fibroblast growth factor (bFGF; GIBCO, cat. no. 13256-029)
- DMEM/F12 (GIBCO, cat. no. 11320-033)
- Knockout serum replacement (KOSR; GIBCO, cat. no. 10828-028)
- Mitomycin C (Sigma, cat. no. M4287)
- Nonessential amino acids (GIBCO, cat. no. 11140-050)
- 100× penicillin/streptomycin (GIBCO, cat. no. 15140-122)
- 2-Mercaptoethanol (Sigma, cat. no. M7522)
- 1× Dulbecco's PBS (GIBCO, cat. no. 14040-134)
- Fetal bovine serum (GIBCO, cat. no. 16000-044) ! CAUTION Heat-inactivated at 55 °C for 30 min.
- Primocin (Amaxa, cat. no. VGA1022)

EQUIPMENT

- 35-mm tissue culture dish (Falcon, cat. no. 3001)
- T75 flask (Falcon, cat. no. 3110)
- 60-mm bacterial culture dish (SPL, cat. no. 10060)
- Pasteur pipette (Corning, cat. no. 7095B-9)
- 5-ml plastic disposable pipette (Falcon, cat. no. 7543)
- 10-ml plastic disposable pipette (Falcon, cat. no. 7551)
- Cryo 1 °C freezing container (Nalgene, cat. no. 5100-0001)
- Pipette aid (Falcon, cat. no. 7590)
- 20-p micro pipette (Gilson, cat. no. P20)
- 200-p micro pipette (Gilson, cat. no. P200)
- 1000-p micro pipette (Gilson, cat. no. P1000)
- 200-p micro pipette tip (Sarstedt, cat. no. 70-760-002)
- 1000-p micro pipette tip (Sarstedt, cat. no. 70-762)
- 22 mm × 22 mm cover glasses (Fisher, cat. no. 12-541B)
- 15-ml conical centrifuge tubes (Falcon, cat. no. 2096)
- 50-ml conical centrifuge tubes (Falcon, cat. no. 2070)
- 1.5-ml microtube (Sarstedt, cat. no. 72-694-007)
- Cryo vials (Nunc, cat. no. 375418)

REAGENT SETUP

hESC lines SNUhES1, SNUhES3 and SNUhES16 were maintained on a feeder layer of mitomycin C-treated STO cells as described previously[15]. Briefly, hESCs were cultured in ES (embryonic stem cell) medium in a standard culture condition (5% CO_2, 37 °C). The culture medium was exchanged daily and the cells were passaged onto a fresh STO layer once a week. Isolation of hESC colonies for each passage was achieved by mechanical dissection with a flame-pulled glass pipette as described previously[16].

ES medium Dulbecco's minimum essential medium/F12 medium supplemented with 20% (vol/vol) KOSR, 1 mM L-glutamine, 4 ng ml^{-1} bFGF, 1% nonessential amino acids, 50 U ml^{-1} penicillin, 50 μg ml^{-1} streptomycin and 0.1 mM 2-mercaptoethanol. ! CAUTION Both KOSR and bFGF should be added freshly and used within a week.

Embryoid body medium Omit bFGF from the ES medium. ! CAUTION KOSR should be added freshly and used within a week.

Neural precursor selection medium Modify the embryoid body (EB) medium by replacing KOSR with 0.5% N2 supplement. ! CAUTION N2 supplement should be added freshly and used within a week.

Neural precursor expansion medium Add bFGF (20 ng ml^{-1}) and more N2 supplement (final concentration, 1%) to the neural precursor (NP) selection medium. ! CAUTION Both bFGF and N2 supplement should be added freshly and used within a week.

Neuronal differentiation medium Remove bFGF from the NP expansion medium and add 2% B27 supplement instead. ! CAUTION Both N2 and B27 supplement should be added freshly and used within a week.

Cell-freezing solution Mix DMSO and the NP expansion medium in a 1:9 ratio. ! CAUTION It is recommended to make the solution fresh and keep it at 4 °C before use.

PROCEDURE

Generation of SNMs from hESCs ● TIMING 26 d

1| Overall time line of this procedure of SNM generation was shown in the schematic diagram of **Figure 1** (left, stage 1). First of all, wash the hESC colonies on a 35-mm culture dish (**Fig. 2a**) with 1 ml of 1× PBS and detach the colonies by treating them with 800 μl of 2 mg ml^{-1} collagenase in a standard incubator (5% CO_2, 37 °C) for 40 min.

▲ CRITICAL STEP The incubation time for the detachment of hESC colonies may vary depending on the collagenase used and needs to be adjusted by careful examination of the cells after treatment. When one or two colonies start being detached from the dish, it is about time to triturate using pipettes for complete detachment.

2| By pipetting with blue tips, transfer about 150–200 detached colonies (there are about 60–70 detached colonies per 35-mm dish in

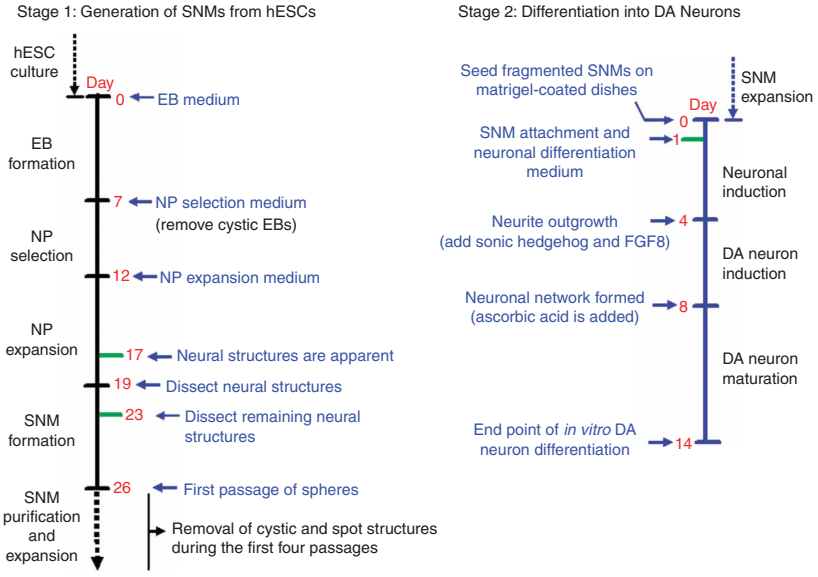

Figure 1 | Schematic diagram of differentiation of hESCs into DA neurons. (Left, stage 1) Derivation of SNMs from hESCs by going through EB formation, NP selection, NP expansion and mechanical purification of initial SNMs. (Right, stage 2) After attaching SNMs on matrigel-coated dishes, the cells were differentiated into DA neurons by adding signaling molecules, such as sonic hedgehog, FGF8 and ascorbic acid. It usually takes about 14 d to generate functional DA neurons *in vitro*.

our case) into a microtube containing 1 ml of ES medium and wait several minutes until the colonies settle down to the bottom of the tube.

3| Remove the supernatant (1 ml of ES medium) carefully by pipetting with blue tips and resuspend the remaining pellet with 1 ml of EB medium.

4| Transfer all the colonies (about 150–200) resuspended in 1 ml of EB medium to a 60-mm uncoated bacterial dish containing 5 ml of EB medium and culture them in 5% CO_2 at 37 °C for 5–7 d with change of medium every other day. At the end of the culture, EBs will be formed (**Fig. 2b–d**).

5| Transfer EBs to 35-mm culture dishes coated with matrigel (about 30–40 EBs per dish) by pipetting and culture them in 2 ml of NP selection medium for 5 d with medium change every other day. After 5 d of selection, 50–60% of the transferred EBs should remain as flat cell clumps at the bottom of the dish and many cells should be nestin positive.
▲ CRITICAL STEP Remove the EBs of cystic form (**Fig. 2d**, arrow) completely during this step by pipetting using blue tips under a stereomicroscope.
? TROUBLESHOOTING

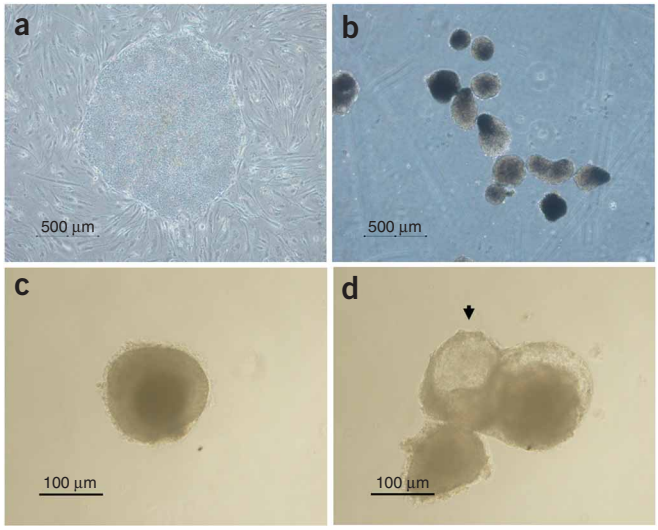

Figure 2 | Morphologies of hESCs and EBs observed during stage 1 of **Figure 1**. (**a**) A typical morphology of the hESC colony cultured for 7 d on a STO feeder layer. (**b**) The shape of EBs formed for 5 d in EB medium. (**c**) A typical shape of a normal EB after 7 d. (**d**) An example of abnormal EB with cystic structure (arrow).

6| For expansion of neural precursors, change the NP selection medium to NP expansion medium and culture the NPs with medium change every other day until neural structures appear. Neural structures take the form of either neural tube-like structures or black sponge-like clumps (**Fig. 3a**,**b**, arrows) and start appearing in about 5 d, becoming larger in 7 d.
▲ CRITICAL STEP Replating of these 3D structures gives rise to neural rosettes in 2 d (**Fig. 3c**,**d**). This step is not necessary for the subsequent steps of our protocol. Perform this step only when you want to confirm the ability of our neural structures to form neural rosettes, which are frequently observed by other differentiation protocols.
? TROUBLESHOOTING

7| Mechanically dissect out neural structures from the dishes with flame-pulled Pasteur pipettes under a stereomicroscope after 7 d. Incubate remaining neural structures after dissection for 4–5 more days and repeat mechanical dissection for the newly formed neural structures. As a rule of thumb, all the fragmented neural structures derived from three 35-mm dishes are transferred to a 60-mm dish (about 70–80 fragments in total).
▲ CRITICAL STEP Neural structures are distinct in morphology (compact clump-like structures) and easily distinguishable from the surrounding irrelevant cells.

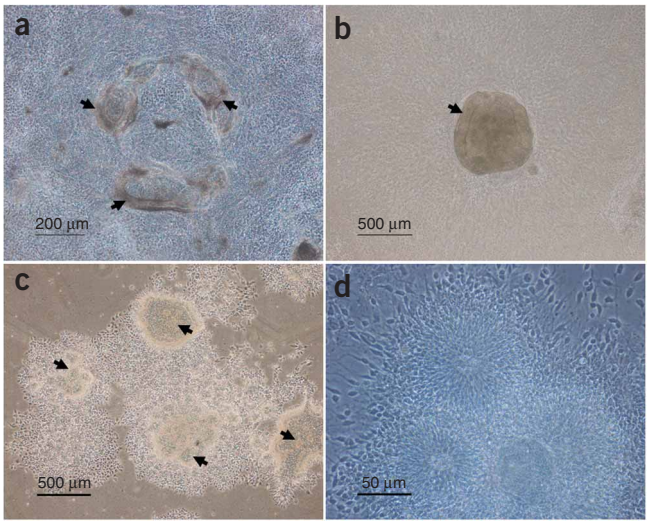

Figure 3 | Morphologies of various neural structures observed during the middle part (NP selection and expansion steps) of the stage 1 of **Figure 1**. (**a**) Neural tube-like structures (arrows). (**b**) A black sponge-like clump (arrow). (**c**) Neural rosettes (arrows) formed 2 d after replating the neural structures (**a** and **b**). (**d**) A magnified image of neural rosettes.

8| Incubate the dissected neural structures for about 2 d in 5 ml of NP expansion medium under standard culture conditions (5% CO_2, 37 °C) to generate spherical clumps.
? TROUBLESHOOTING

9| Cut the spherical clumps with flame-pulled Pasteur pipettes into smaller pieces (about 250-300 μm in diameter) with uniform size, during which cystic parts (**Fig. 4a**, arrows) are removed. The dissected spherical clumps become spheres (250–300 μm in diameter) within several hours.

10| Culture the small spheres for 5 d to form larger spheres (~500 μm in diameter), termed SNMs, by changing the NP expansion medium every other day.
▲ CRITICAL STEP Detach the spheres adhered to the bottom of the dishes by pipetting up and down with a 1,000-μl micropipette once a day.

11| When spheres grow to a certain size (~500 μm in diameter), cut them with flame-pulled Pasteur pipettes into

4–6 pieces of similar size (this step is actually the first passage of the SNMs; there are typically about 25,000–30,000 cells per SNM). During this step, remove non-neural structures such as cystic structures (**Fig. 4a**, arrows) or spot-forming fibroblast-like structures (**Fig. 4b**, arrow) from the main SNM bodies by mechanical cutting with the flame-pulled Pasteur pipettes as mentioned previously.

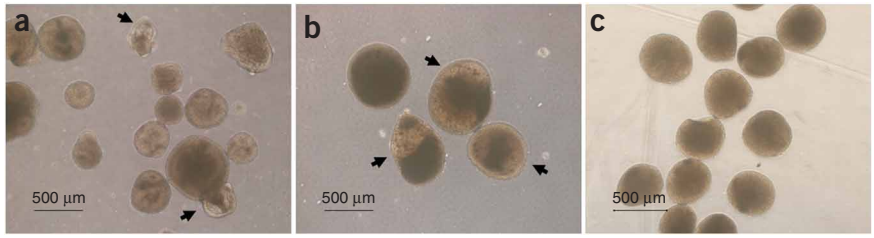

Figure 4 | Morphologies of non-neural (cystic- or spot-forming) structures frequently observed at the initial stage of sphere formation and also during the purification processes. SNMs that went through the four rounds of purification processes are also shown. (**a**) Examples of cystic structures of SNMs (arrows). (**b**) Examples of spot-forming fibroblast-like structures of SNMs (arrows). (**c**) Purified SNMs after the first four passages.

▲ **CRITICAL STEP** Cystic structures and spot-forming fibroblast-like structures of the SNMs usually form epithelia-like cells and fibroblast-like cells, respectively, at the later stage by our differentiation protocol.

12| Allow the SNM-derived fragments to grow for 5–7 d and repeat the above-mentioned purification process (Step 11) during this second passage of SNMs. This purification process should be repeated until the fourth passage of SNMs. After the four time-purification processes, a homogeneous population of SNMs is usually obtained (**Figs. 4c** and **5a**).
▲ **CRITICAL STEP** In most cases, cystic structures are mostly removed after these four times of purification processes. On the other hand, spot-forming structure can sometimes be seen even after the four-time purification. In this case, the whole SNM population containing the spots should be discarded.

13| Purified SNMs can be passaged for more than 15 passages, which corresponds to over 5 months, following the method described in Steps 14–17.

Expansion of SNMs ● TIMING 3 h

14| Initially, place about 40–50 small fragments dissected from about 10 SNMs into a 60-mm dish and culture for 7–10 d with change of NP expansion medium every 3 d.

15| When SNMs attain a certain size (**Fig. 5a**, about 500 μm in diameter) after 7–10 d in culture, passage the SNMs as follows: first, transfer half of the total number of SNMs from a 60-mm dish into a new 60-mm culture dish and allow them to adhere to the bottom of the dish by keeping them in the dish for 1–2 h in a standard culture incubator.

16| Dissect every single SNM (**Fig. 5a**) into 4–6 pieces (**Fig. 5b**) using flame-pulled Pasteur pipettes under a stereomicroscope.
! **CAUTION** Try to make the size of the dissected SNM fragments as uniform as possible.

17| Transfer the dissected fragments of SNMs into a new 60-mm bacterial dish at a density of 40–50 fragments per 60-mm dish and grow them for 7–10 d in 5 ml of NP expansion medium at a standard culture condition (5% CO_2, 37 °C).
! **CAUTION** If the SNM fragments do not grow or the structure of SNMs becomes disintegrated, contamination of micoplasma should be examined. In case of contamination, treat the SNMs with primocin at 100 μg ml^{-1} (final concentration) for more than 14 d.
! **CAUTION** It is highly recommended to cryopreserve (see **Box 1**) a portion of SNMs (**Fig. 5c**) at early stages of expansion (e.g., before the tenth passage).
? **TROUBLESHOOTING**

Differentiation of SNMs into DA neurons ● TIMING 14 d

18| Transfer the fairly homogeneous SNMs that went through the four-time purification passages as described earlier (Step 13) onto a 35-mm culture dish or a coverslip coated with matrigel at a density of either 5–6 SNMs per 35-mm dish or 2–3 SNMs per coverslip, respectively.

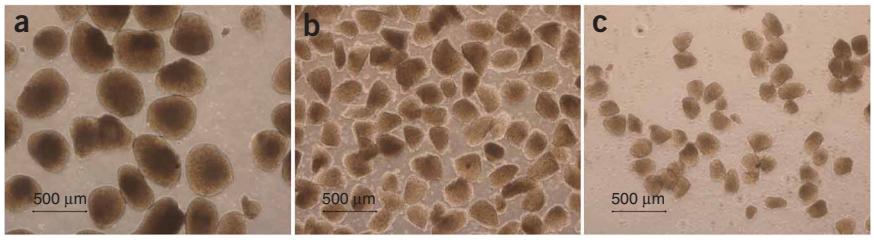

Figure 5 | The size of SNMs before and after fragmentation for either expansion or cryopreservation. (**a**) SNMs before fragmentation. (**b**) Fragmented SNMs for expansion. (**c**) Fragmented SNMs for cryopreservation. Note that the size of the SNM fragments for cryopreservation is about two-thirds of those obtained for expansion.

19| After 1- or 2-h attachment period, dissect the SNMs into 40–80 small pieces per SNM using flame-pulled Pasteur pipettes.
▲ **CRITICAL STEP** To prevent possible cell loss, which might occur during cell transfer, it would be better to perform the fragmentation work in the same dish as you want the small SNM pieces to be differentiated on. Furthermore, it is recommended to use yellow tips rather than blue ones.

BOX 1 | CRYOPRESERVATION AND THAWING OF SNMS

Cryopreservation of SNMs ● TIMING **1 h per cryovial**

1. Dissect purified SNMs of a certain size (**Fig. 5a**, about 500 μm in diameter) into small pieces (170–200 μm in diameter) (**Fig. 5c**).

▲ CRITICAL STEP The size and condition of the fragmented SNMs somewhat affect the survival of the cells during and after cryopreservation. In general, best results can be obtained when the size of the SNM fragments is about two-thirds of those obtained for passaging the SNMs. Furthermore, it is better to stabilize the dissected SNM fragments at least for 2 h in the medium that the SNMs have been grown in before cryopreservation (i.e., NP expansion medium in our protocol).

2. Wash the fragmented SNMs with 1× PBS and transfer 100–150 fragments into a 1.5-ml microtube with 1 ml of PBS.

3. When the SNM fragments settle down to the bottom of the tube (~2 min), remove the supernatant carefully with 1,000-μl micropipette and resuspend the SNMs with 1 ml of freezing solution.

4. Transfer all the SNMs (100–150 fragments) along with freezing solution into a cryovial and allow the tube to remain for 5 min.

5. Place the cryovials in a cell-freezing container wherein the cryovials are placed in an isopropyl alcohol bath and transfer the freezing container directly into 80 °C freezer and then liquid nitrogen the next day.

▲ CRITICAL STEP It is recommended to keep the cell-freezing container at room temperature (25 °C) before use and have the isopropyl alcohol replaced frequently.

Thawing of SNMs ● TIMING **30 min**

6. Take a cryovial from liquid nitrogen tank and thaw the frozen fragments of SNMs rapidly in a 37 °C water bath with gentle agitation until most of the visible ice disappears.

▲ CRITICAL STEP Freeze cells slowly to limit death and thaw them quickly to prevent the same.

7. Transfer all the contents of the vial to a 60-mm bacterial dish containing 5 ml of DMEM/F12 media and wash the fragments of SNMs gently followed by the removal of the medium. The washing step is repeated once more by gently shaking the dish using blue tips under a stereomicroscope.

! CAUTION Be careful not to lose any fragments of SNMs during this step, as the cells tend to stick to the blue tips well.

! CAUTION Never mix the cell suspension by pipetting up and down during the washing steps.

8. Add 5 ml of the NP expansion media to the dish, grow the fragments of SNMs for 3 d before transfer of the enlarged fragments to a new bacterial dish with fresh NP expansion medium, and then culture for 7–10 d with medium change every 3 d.

? TROUBLESHOOTING

9. Now SNMs of reasonable size are formed and ready for a new experiment.

20| Disperse the dissected small SNM fragments evenly throughout the surface of culture dish or coverslip with a 20 μl micropipette in the presence of 2 ml of NP expansion medium.

21| Let the fragments of SNMs settle onto the culture dish surface for an additional 10 min and then culture them for 24 h at a standard culture condition (5% CO_2, 37 °C).

22| One day later, start differentiating the cells by replacing NP expansion medium with 2 ml of neuronal differentiation medium and wait for 3 d for neurites to grow out from the attached fragments. At day 3 after neuronal differentiation, vigorous extention of neurites becomes apparent (**Fig. 6a**).

23| Once neurite outgrowth reaches a high level at day 4, add both sonic hedgehog (final concentration 200 ng ml^{-1}) and FGF8 (final concentration 100 ng ml^{-1}) to the differentiation medium (2 ml) and let the cells further differentiate for 4 more days with medium change every other day.

24| At day 8 of DA neuronal differentiation (4 d after both sonic hedgehog and FGF8 treatments) (**Fig. 6b**), add

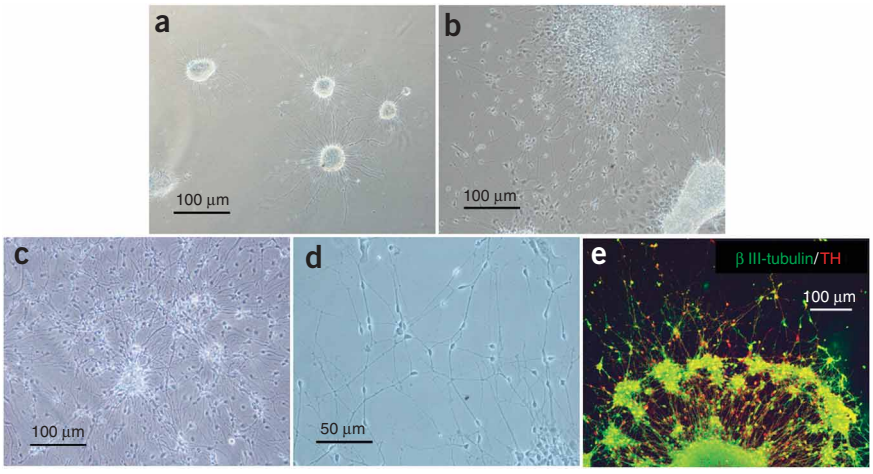

Figure 6 | Morphological changes observed during DA neuron generation from the fragmented SNMs. Formation of neuronal morphologies from the fragmented SNMs at (**a**) 3 d, (**b**) 8 d and (**c**,**d** and **e**) 14 d after neuronal differentiation. (**d**) A magnified image of **c**. (**e**) Immunocytochemical analysis of the cells generated after DA neuronal differentiation of SNMs. SNM-derived cells were stained with antibodies against tyrosine hydroxylase (TH, red) and β III-tubulin (green).

ascorbic acid (final concentration; 200 μM) to 2 ml of neuronal differentiation medium along with both sonic hedgehog and FGF8 and continue differentiation for 6 more days with medium change every 2 d (**Fig. 6c**). At the end of this protocol, which is 14 d after neuronal differentiation, about 86±1.4% of the $Tuj1^+$ neurons become TH^+ neurons (**Fig. 6d**,**e**).
▲ CRITICAL STEP In the case of significant cell loss at around 7–8 d after starting differentiation, treatment of the cells with both glial cell line-derived neurotrophic factor (20 ng ml^{-1}) and 5% (vol/vol) knockout serum replacement for about 12 h often promotes cell survival. However, long-time treatment with knockout serum replacement should be avoided, as it may induce adverse effects, such as the thickening of neurites and increase of unwanted cell populations.

● TIMING
Step 1: 1 h
Steps 2 and 3: 10 min
Step 4: 5 d
Step 6: 7 d
Step 7: 5 min per 35 mm culture dish
Step 8: 2 d
Step 9: 20 min per 60 mm culture dish
Step 10: 5 d
Step 11: 30–60 min per 60 mm culture dish
Step 12: 15–21 d
Step 13: 7–10 d per passage
Step 14: 7–10 d
Step 15: 1–2 h
Step 16: 30–60 min per 60-mm culture dish
Step 17: 7–10 d
Step 18: 1–2 h
Step 19: 30 min per 35-mm dish
Steps 20 and 21: 1 d
Step 22: 3 d
Step 23: 4 d
Step 24: 6 d

? TROUBLESHOOTING
Step 5
Problem. EBs are not attached to the matrigel-coated dishes for the following NP selection.
Possible causes and solution. If EBs are too big, they tend not to stick to the bottom of the dish well. It is always better not to grow EBs over 7 d before attachment. If the EBs do not maintain their structure tight during EB culture, you may suspect mycoplasma contamination.
If EBs do not attach to the bottom of culture dish or if there is mycoplasma contamination, discard them and try to make fresh EBs again. During this attachment step, cystic EBs should be removed completely, which, otherwise, might affect the following steps negatively. Too many cystic EBs may result from differentiated ES colonies or caused by the poor quality of KOSR.

Step 6
Problem. Neural structures are not formed properly.
Possible causes and solution. Although neural structures normally appear in about 7 d, the formation of neural structures is sometimes delayed for several days, which usually does not cause any serious problem. However, if the neural structures are not formed until 14 d, the following things need to be checked thoroughly: (i) examine if the condition of the original hESCs is normal; (ii) try to make NP selection solution fresh; (iii) check if the density and size of the EBs are within an acceptable range; and (iv) try to avoid EBs with abnormal structures including cystic form.

Step 8
Problem. At early stages, small neural structures have a tendency to stick to the bottom well and become differentiated.
Possible solution. Try to detach the small clumps from the bottom of the dish by pipetting up and down every day.

Problem. Neural structures of the SNMs are sometimes hard to see.
Possible causes and solution. This happens when SNMs have been passaged for a long-time or right after thawing of SNMs. However, this does not affect the capability of the SNMs to proliferate or differentiate (**Box 1**).

Step 17

Problem. Black spots are formed at the center of the SNMs.

Possible causes and solution. This is caused by the accumulation of dead cells and may affect the survival of the cells in the spheres. If black clumps are noticed at the center of the spheres, it is better to passage the SNMs right away no matter how small they are.

ANTICIPATED RESULTS

Although some variations have been noticed among hESC lines, our protocol produced somewhat consistent outcomes in our hands. This protocol would produce best yield of DA neurons when starting hESC lines have been well maintained, and most of them remain undifferentiated. Interestingly and usefully, the SNMs can be used to generate glial cells[17] depending on the external cues. Additionally, it will be of great interest to see if SNMs can generate other types of neurons in the presence of specific signals. As the SNMs can be easily expanded and coaxed into DA neurons with a high efficiency, our unique method described in this protocol would bring clinical application of hESC-mediated cell therapy one step closer to treat neurological diseases such as Parkinson's disease.

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