Clinically compatible differentiation protocol for human pluripotent stem cell-derived dopaminergic progenitor cells

Daisuke Doi
Center for iPS Cell Research and Application https://orcid.org/0000-0003-0992-1220
Tetsuhiro Kikuchi
Center for iPS Cell Research and Application https://orcid.org/0000-0003-2332-4967
Asuka Morizane
Center for iPS Cell Research and Application
Jun Takahashi (jbtaka@cira.kyoto-u.ac.jp)
Center for iPS Cell Research and Application https://orcid.org/0000-0003-3642-9186

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Abstract

Cell replacement therapy with human pluripotent stem cells has the potential to be a new therapy for Parkinson's disease (PD). This protocol induces human induced pluripotent stem cells (iPSCs) to dopaminergic progenitor cells (DAPs) as clinically compatible donor cells in 30 days. The protocol includes starting with high density culture, cell sorting by using a cell surface marker for floor plate, and a maturation culture to form floating aggregates. The DAPs differentiated with this protocol were used in a pre-clinical tumorigenicity and efficacy study aiming for approval to start a clinical trial in Japan.

Introduction

Parkinson's disease (PD) is a neurodegenerative disease caused by a loss of dopaminergic (DA) neurons in the substantia nigra-striatal pathway. The neuronal cell death results in mainly motor symptoms including tremors, rigid muscles, slower movement, and impaired posture and balance. Cell therapy for PD is a concept in which the lost dopaminergic neurons are replaced by surgery. As donor cells, authentic neural progenitors with ventral mesencephalic (VM) phenotype are needed. Developmentally, dopaminergic neurons in substantia nigra are derived from fetal VM. Accordingly, aborted fetal midbrain tissues were used in clinical trials in the 1990s and showed some clinical benefit. Because fetal tissue is in short supply and its use has ethical issues, human pluripotent stem cells (PSCs) are being investigated as an alternative cell source for transplantation. There have been many reported protocols for mesencephalic DA induction from human PSCs at the laboratory level[1–4]. Most of them cannot be applied to the clinic as they are, however. To meet the criteria of clinical application, xeno-materials and genetic modifications to the cells must be avoided, the prevention of contamination and GMP compatibility must be confirmed, and regulations with respective countries must be met. We have established a differentiation protocol for dopaminergic progenitor cells (DAPs) from human PSCs[5, 6] and modified it for the purpose of a preclinical study and clinical application. The reagents and equipment are changed to clinical grade products to comply with the requirements by a Japanese regulatory agency.

Reagents

Reagents

- Feeder-free human induced pluripotent stem cells (hiPSCs) cultured on a 90-mm dish
- iMatrix-511MG (Nippi, cat. no. 892005)
- D-PBS without Ca and Mg (nacalai tesque, 14249-24)
- TrypLE™ Select CTS™ (Thermo Fisher, cat. no. A12859)
- 0.5 mol/L-EDTA (pH8.0, nacalai tesque, 06894-14)

- CultureSure® DMSO (FUJIFILM Wako, cat. no. 031-24051)
- OTSUKA DISTILLED WATER (Otsuka Pharmaceutical, 1751)
- OTSUKA NORMAL SALINE (Otsuka Pharmaceutical, 0613)
- Glasgow's MEM (GMEM, Thermo Fisher, cat. no. 11710)
- KNOCKOUT™ Serum Replacement for ESCs/iPSCs (KSR, Thermo Fisher, cat. no. 10828), 30 kGy gamma-ray irradiated
- MEM Non-Essential Amino Acids Solution (NEAA, Thermo Fisher, cat. no. 11140)
- Sodium Pyruvate, 100 mM (Sigma, S8636)
- StemSure® 10 mmol/L 2-mercaptoethanol solution (2-ME, FUJIFILM Wako, cat. no. 198-15781)
- CultureSure® 10 mmol/L Y-27632 solution, animal-derived free (FUJIFILM Wako, cat. no. 039-24591)
- Stemolecule™ LDN-193189 (REPROCELL, cat. no. 04-0074)
- CultureSure® A-83-01 (FUJIFILM Wako, cat. no.039-24111)
- FGF8, human, recombinant, animal-derived-free (FUJIFILM Wako, cat. no. 067-06231)
- Purmorphamine (FUJIFILM Wako, cat. no. 166-23991)
- CultureSure® CHIR99021 (FUJIFILM Wako, cat. no. 034-23103)
- Neurobasal® medium (1×), liquid -L-Glutamine (Thermo Fisher, cat. no. 21103)
- B27 supplement (50×) without vitamin A (Thermo Fisher, cat. no. 12587), 30 kGy gamma-ray irradiated
- Cell Therapy Systems GlutaMAX™- I CTS™ (100×), 200 mM (Thermo Fisher, cat. no. A12860)
- BDNF, human, recombinant, animal-derived-free (FUJIFILM Wako, cat. no. 028-16451)
- GDNF, human, recombinant, animal-derived-free (FUJIFILM Wako, cat. no. 070-06261)
- Ascorbic acid (TOWA Pharmaceutical)
- ACTOSHIN for injection (dibutyryl cyclic AMP, Daiichi-Sankyo Pharmaceutical)
- Gentacin® 40 (gentamycin, Takata Pharmaceutical)

- Fetal bovine serum gamma irradiated by SER-TAIN™ process, sourced in Australia (FBS, SAFC, cat. no. 12007C)

- D-glucose solution, 45% in H_2O, sterile-filtered (Sigma, cat. no. G8769)

- PE-anti-CORIN antibody (not commercially available) or human CORIN antibody (R&D, cat. no. MAB2209) and a fluorescent secondary antibody for cell sorting

Reagent setup

- 0.5×TrypLE Select solution

Ø Mix 90 mL of TrypLE Select CTS with 90 mL of D-PBS(-) and 90 µL of 0.5 mol/L-EDTA

Ø Store at room temperature and use within 6 months

- 8GMK media (8% KSR-GMEM)

Ø Mix 500 mL of GMEM with 45 mL of KSR, 5.6 mL of NEAA, 5.6 mL Sodium Pyruvate, and 5.6 mL of 2-ME

Ø Store at 5 °C and use within 1 month

- NB/B27 media

Ø Mix 500 mL of Neurobasal medium with 10 mL of B27 supplement without vitamin A and 5.1 mL of GlutaMAX™- I CTS™

Ø Store at 5 °C and use within 1 month

- LDN193189 reconstitution

Ø Add 4.515 mL of DMSO to 2 mg of Stemolecule™ LDN-193189 to obtain a concentration of 1 mM

Ø Mix well to completely reconstitute the powder

Ø Freeze the aliquots at -30 °C (stable for 6 months after reconstitution)

Ø Avoid repeated freeze/thaw cycles

Ø Once the aliquot is thawed, store at 5 °C (stable for 1 week)

- A83-01 reconstitution

Ø Add 0.949 mL of DMSO to 2 mg of CultureSure® A-83-01 to obtain a concentration of 5 mM

Ø Mix well to completely reconstitute the powder

Ø Freeze the aliquots at -30 °C (stable for 6 months after reconstitution)

Ø Avoid repeated freeze/thaw cycles

Ø Once the aliquot is thawed, store at 5 °C (stable for 1 week)

- Recombinant FGF8 reconstitution

Ø Add 250 µL of distilled water to 25 µg of human recombinant FGF8 to obtain a concentration of 100 µg/mL

Ø Mix well to completely reconstitute the powder

Ø Freeze the aliquots at -30 °C (stable for 3 months after reconstitution)

Ø Avoid repeated freeze/thaw cycles

Ø Once the aliquot is thawed, store at 5 °C (stable for 1 week)

- Purmorphamine reconstitution

Ø Add 0.96 mL of DMSO to 5 mg of purmorphamine to obtain a concentration of 10 mM

Ø Mix well to completely reconstitute the powder

Ø Freeze the aliquots at -30 °C (stable for 6 months after reconstitution)

Ø Avoid repeated freeze/thaw cycles

Ø Once the aliquot is thawed, store at 5 °C (stable for 1 week)

- CHIR99021 reconstitution

Ø Add 3.58 mL of DMSO to 5 mg of CHIR99021 to obtain a concentration of 3 mM

Ø Mix well to completely reconstitute the powder

Ø Freeze the aliquots at -30 °C (stable for 6 months after reconstitution)

Ø Avoid repeated freeze/thaw cycles

Ø Once the aliquot is thawed, store at 5 °C (stable for 1 week)

- Recombinant GDNF reconstruction

Ø Add 1 mL of distilled water to 10 µg of recombinant human GDNF to obtain a concentration of 10 µg/mL

Ø Mix well to completely reconstitute the powder

Ø Freeze the aliquots at -30 °C (stable for 3 months after reconstitution)

Ø Avoid repeated freeze/thaw cycles

Ø Once the aliquot is thawed, store at 5 °C (stable for 1 week)

- Recombinant BDNF reconstruction

Ø Add 100 µL of distilled water to 10 µg of recombinant human GDNF to obtain a concentration of 100 µg/mL

Ø Mix well to completely reconstitute the powder

Ø Freeze the aliquots at -30 °C (stable for 3 months after reconstitution)

Ø Avoid repeated freeze/thaw cycles

Ø Once the aliquot is thawed, store at 5 °C (stable for 1 week)

- Ascorbic acid dilution

Ø Mix 2 mL (500 mg) of ascorbic acid with 12.2 mL of distilled water to obtain a concentration of 200 mM

Ø Freeze the aliquots at -30 °C (stable for 3 months after reconstitution)

Ø Avoid repeated freeze/thaw cycles

Ø Once the aliquot is thawed, store at 5 °C (stable for 1 week)

- ACTOSIN (dbcAMP) reconstitution

Ø Add 1.526 mL of distilled water to 300 mg of dbcAMP to obtain a concentration of 400 mM

Ø Mix well to completely reconstitute the powder

Ø Freeze the aliquots at -30 °C (stable for 3 months after reconstitution)

Ø Avoid repeated freeze/thaw cycles

Ø Once the aliquot is thawed, store at 5 °C (stable for 1 week)

- Sorting buffer

Ø Mix 500 mL of D-PBS (-) with 4 mL of D-glucose solution, 10 mL of FBS, and 1 mL (40 mg) of gentamycin

Ø Store at 5 °C (stable for 3 weeks)

Equipment

Equipment

- 6-well cell culture plates (Sumitomo Bakelite, MS80060)
- PrimeSurface® Plate 96U U-bottom 96-well cell culture plates (Sumitomo Bakelite, MS-9096U)
- 15 mL Centrifuge Tubes (Sumitomo Bakelite, MS-56150)
- 50 mL Centrifuge Tubes (Sumitomo Bakelite, MS-56500)
- 1.5 mL tubes (WATSON, T119-7155CS)
- Reagent Reservoir (Sumitomo Bakelite, MS-62800)
- Falcon® 5 mL round bottom polypropylene tubes (Corning, 352063)
- Falcon® 5 mL round bottom polystyrene tubes with a cell strainer snap cap (φ 35 μm, Corning, 352235)
- Fluorescence-activated cell sorter (BD biosciences, FACSAria^{TM} III)
- Tube rotator (WAKENYAKU, WKN-2210)
- 8-channel pipettor (Eppendorf, 3122 000.051)
- Cell container (JMS, custom made, 2 mL)

Procedure

*Culture scale: one 6-well plate (culture area = 9.2 cm^2/well)

Plate coating with iMatrix (0.5 μg/cm^2)

1. Dilute 61.3 μL of iMatrix with 10 mL of D-PBS (-) and add 1.5 mL of the solution per well. Incubate the plate with iMatrix for at least 1 hour (plates can be left overnight) at 37 °C, 5% CO_2 in an incubator.

2. Add 1 mL of 8GMK media and mix, and then aspirate the media and iMatrix solution. Do not allow the plate to dry.

3. Add 4 mL/well of 8GMK+LAY media (see step 5 in DAP differentiation: Day 0.) and return the plates to the incubator.

DAP differentiation: Day 0

4. Warm the following reagents at room temperature.

a) 8GMK media

b) Y-27632

c) LDN193189 solution

d) A83-01 solution

5. Mix 8GMK media with Y-27632 (final 10 μM), LDN193189 (final 100 nM), and A83-01 (final 500 nM) (8GMK+LAY media).

6. Remove the media from two confluent* 90-mm dishes of undifferentiated iPSCs and wash the cells two times with 4 mL of D-PBS (-) per dish.

*confluent means “state that is suitable for passage”, not the state that cells cover all area of the wells.

7. Add 3 mL per 90-mm dish of 0.5×TrypLE Select solution and incubate for 15 minutes at 37 °C, 5% CO_2 in an incubator.

8. During the incubation, prepare 12 mL of 8GMK+LAY media in a new 50 mL tube.

9. Remove the dishes from the incubator and pipette the cells 10 times to fully dissociate.

10. Collect the cells in 50 mL tubes with the 12 mL of 8GMK+LAY in step 8 and mix.

11. Centrifuge the cells at 190 × g, 4 °C for 5 minutes.

12. Aspirate the supernatant, add 1 mL of 8GMK+LAY media, and pipette 10 times.

13. Add 1.5 mL of 8GMK+LAY media and mix.

14. Count the cells with a sample diluted 10 times with D-PBS (-).

15. Remove the 6-well plate coated with iMatrix from the incubator.

16. Add 5×10^6 cells per well to the 6-well plate and culture the plate at 37 °C, 5% CO_2 in an incubator.

Day 1

17. Mix 8GMK media with LDN193189 (final 100 nM), A83-01 (final 500 nM), FGF8 (final 100 ng/mL), and purmorphamine (final 2 μM) (8GMK+LAFP media).

18. Aspirate the media from the well and wash the cells with 2 mL of D-PBS (-).

19. Add 4 mL of the 8GMK+LAFP media and culture the plate at 37 °C, 5% CO_2 in an incubator.

Day 2

20. Prepare 8GMK+LAFP media.

21. Aspirate the media from the well and add 4 mL of the 8GMK+LAFP media and culture the plate at 37 °C, 5% CO_2 in an incubator.

Days 3-6

22. Mix 8GMK media with LDN193189 (final 100 nM), A83-01 (final 500 nM), FGF8 (final 100 ng/mL), purmorphamine (final 2 μM), and CHIR99021 (final 3 μM) (8GMK+LAFPC media).

23. Aspirate the media from the well, add 6 mL of the 8GMK+LAFP media, and culture the plate at 37 °C, 5% CO_2 in an incubator.

Days 7-11

24. Mix 8GMK media with LDN193189 (final 100 nM) and CHIR99021 (final 3 μM) (8GMK+LC media).

25. Aspirate the media from the well, add 8 mL of the 8GMK+LC media, and culture the plate at 37 °C, 5% CO_2 in an incubator.

Day 12

Labeling with anti-CORIN antibody

Samples:

a) unstained sample

b) negative control (isotype control)

c) CORIN stained sample

26. Mix sorting buffer with Y-27632 (final 10 μM) (sorting buffer + Y).

27. Mix sorting buffer + Y with PE-conjugated anti-CORIN antibody (antibody solution).

28. Mix sorting buffer + Y with PE isotype control (isotype control solution).

29. Mix 8GMK with Y-27632 (10 μM) and gentamycin (final 80 μg/mL) (8GMK+Y+GM).

30. Wash the cells with 2 mL per well of D-PBS (-) two times.

31. Add 1 mL per well of 0.5×TrypLE Select solution and incubate for 15 minutes at 37 °C, 5% CO_2 in an incubator.

32. Remove the dishes from the incubator and pipette the cells 10 times to fully dissociate.

33. Collect the cells in one well into one 15 mL tube with 2 mL of sorting buffer + Y and mix.

34. Centrifuge the cells at 190 × g, 4 °C for 3 minutes.

35. Add sorting buffer +Y and pipette 10 times.

36. Filtrate through a cell strainer (Corning 352235) and count the cell number (1:10 dilution if needed).

37. Split the cells into two groups of 1×10^6 cells per 1.5 mL tube for unstained and negative control samples, and the remainder to 1×10^7cells per 1.5 mL tube for the CORIN stained sample.

38. Centrifuge the cells at 190 × g, 4 °C for 3 minutes.

39. Aspirate the supernatant and add 1 mL of sorting buffer + Y with the antibody solution to the CORIN stained samples and 1 mL of isotype control solution to the negative control sample.

40. Set the tubes to a rotator and react for 20 minutes at 4 °C.

41. Centrifuge the cells at 190 × g, 4 °C for 3 minutes.

42. Wash the cells with 1 mL of sorting buffer + Y and centrifuge at 190 × g, 4 °C for 3 minutes. Repeat this step two times.

43. Add 1 mL of sorting buffer + Y and suspend the cells.

44. Add 7-AAD (1:100) and react for 5 minutes at room temperature in the dark to exclude dead cells.

45. Analyze the cells with a FACS machine and sort the CORIN positive cells.

46. Collect the CORIN positive cells in a chilled 15 mL tube with 6 mL of cold 8GMK+Y+GM media to avoid cell death after sorting.

Post-sort procedures

47. Mix NB/B27 media with GDNF (final 10 ng/mL), ascorbic acid (final 200 μM), BDNF (final 20 ng/mL), dbcAMP (final 400 μM), Y-27632 (final 30 μM), and gentamycin (final 80 μg/mL) (NB/B27+GABA+Y+GM).

48. Centrifuge the cells at 190 × g, 4 °C for 15 minutes.

49. Aspirate the supernatant, resuspend the cells with 1 mL of NB/B27+GABA+Y+GM and count the cell number.

50. Seed the cells onto 96-well plate with 3×10^4 cells, 150 μL per well. The cells should form aggregate spheres on next day.

Media change on days 15, 18, 21, 24, and 27

51. Mix NB/B27 with GDNF (final 10 ng/mL), ascorbic acid (final 200 μM), BDNF (final 20 ng/mL), and dbcAMP (final 400 μM) (NB/B27+GABA).

52. Aspirate 65 μL of media from the 96-well plate with an 8-channel pipettor and add 75 μL of NB/B27+GABA media.

Formulation on day 30

53. Collect aggregate spheres in 2 mL cell container tubes.

54. Wash with 2 mL of saline four times.

55. Fill with 1 mL of saline and keep at 2–8 °C until shipping.

Troubleshooting

Step 9: if the cells do not detach from the dish, use a cell scraper.

Steps 17-25: if the cells detach from the well, try coating the plate with iMatrix at the concentration 1.0 μg/cm^2.

Step 50: the size of the formed aggregates can be controlled by the number of seeding cells.

Time Taken

Day -1: plate coating (0.5 h)

Day 0: cell seeding onto iMatrix-coated 6-well plates (2 h)

Days 1 to 11: medium change (0.5 h)

Day 12: labeling cells (2.5 h), cell sorting (> 4 h; depends on the pre-sorted cell number)

Days 15 to 27: medium change (1 h; depends on the number of sorted cells)

Anticipated Results

This protocol can generate enriched dopaminergic progenitor cells.

Flowcytometry analysis on day 12 shows CORIN$^+$ cells >10% pre-sort and >90% post-sort, and TRA-1-60$^+$ cells <1%.

Flowcytometry analysis on day 26 shows FOXA2$^+$TUJ1$^+$ dopaminergic progenitor cells >80%, OCT4$^+$TRA-2-49$^+$ undifferentiated iPSCs <0.1%, and SOX1$^+$PAX6$^+$ early neural stem cells <0.1%.

Transcriptional analysis shows induction of authentic dopaminergic progenitor markers such as *FOXA2*, *LMX1A*, *NURR1*, and *TH* and the down regulation of pluripotent stem cell markers *POU5F1* and *LIN28*.

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Figures

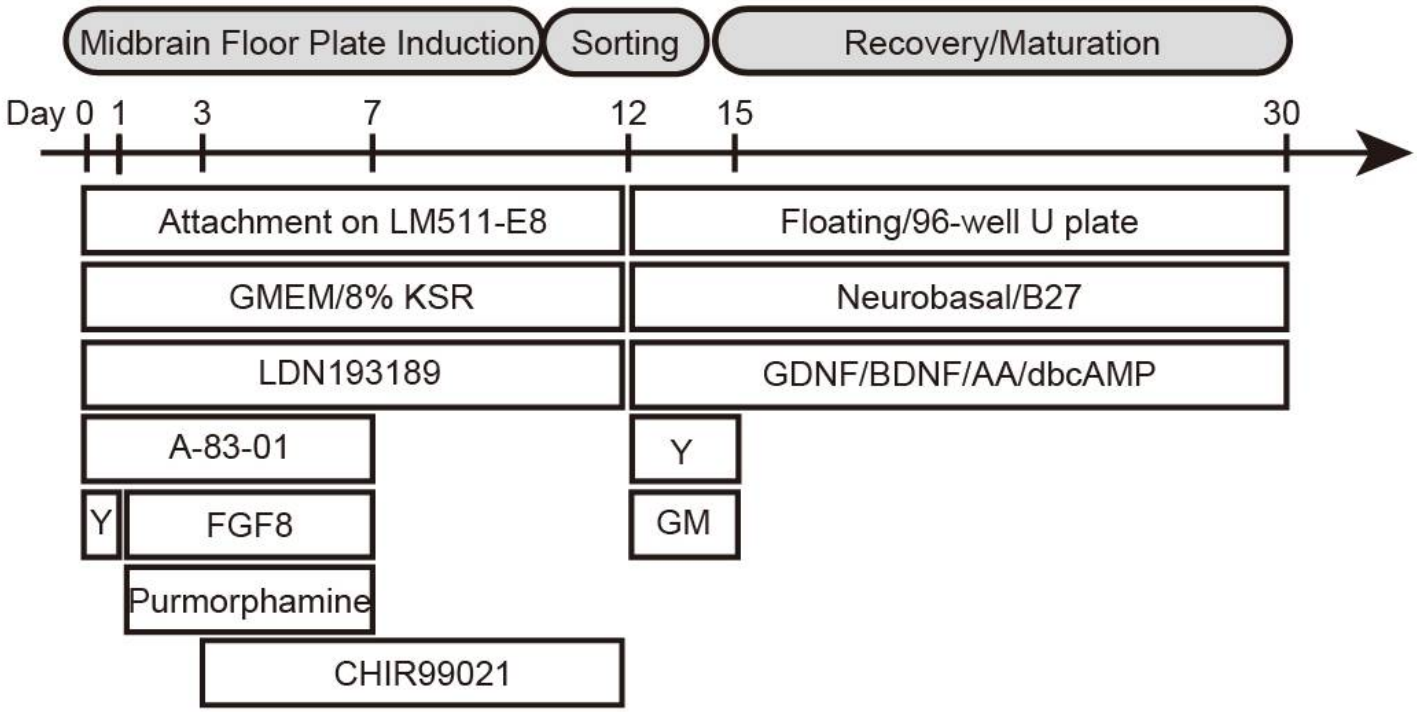

Figure 1

Differentiation protocol.

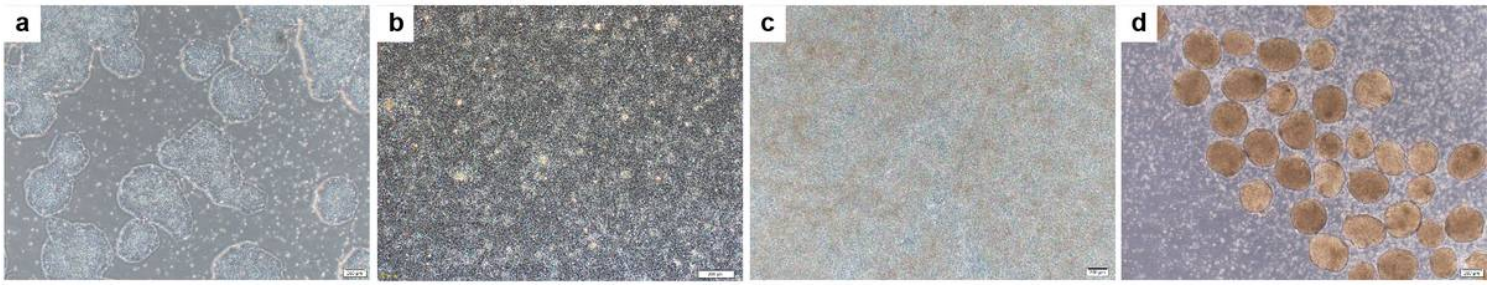

Figure 2

Bright field images of a) undifferentiated iPSCs, b) differentiation day 1, c) day 12, and day 30. Bars=200 µm.

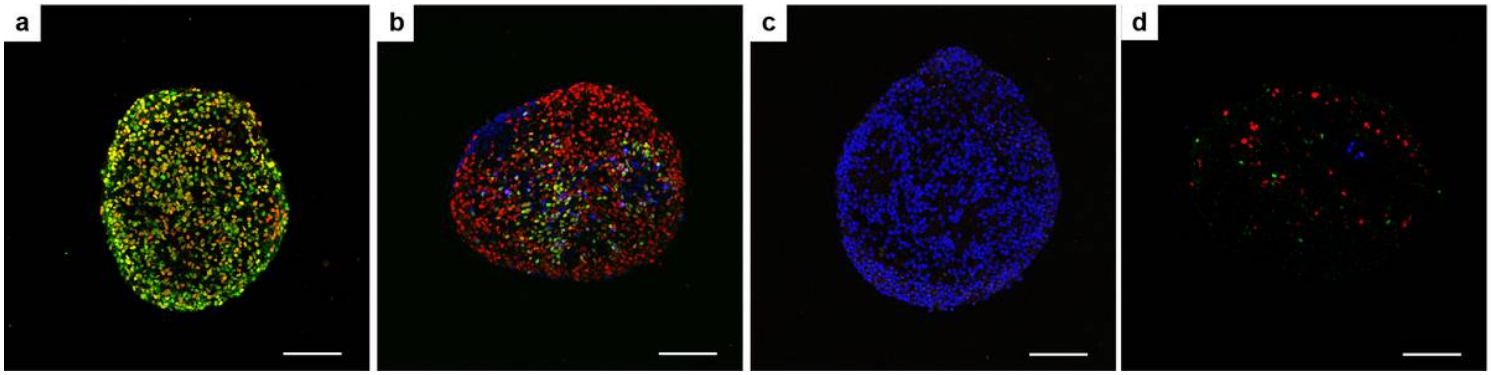

Figure 3

Immunofluorescence images of a) LMX1A(green) and FOXA2 (red), b) NURR1 (green), FOXA2 (red), and TH (blue), c) OCT3/4 (green), NANOG (red), and DAPI (blue), and d) SOX1 (green), KI67 (red), and PAX6 (blue) at differentiation day 26. Bars=100 μm.