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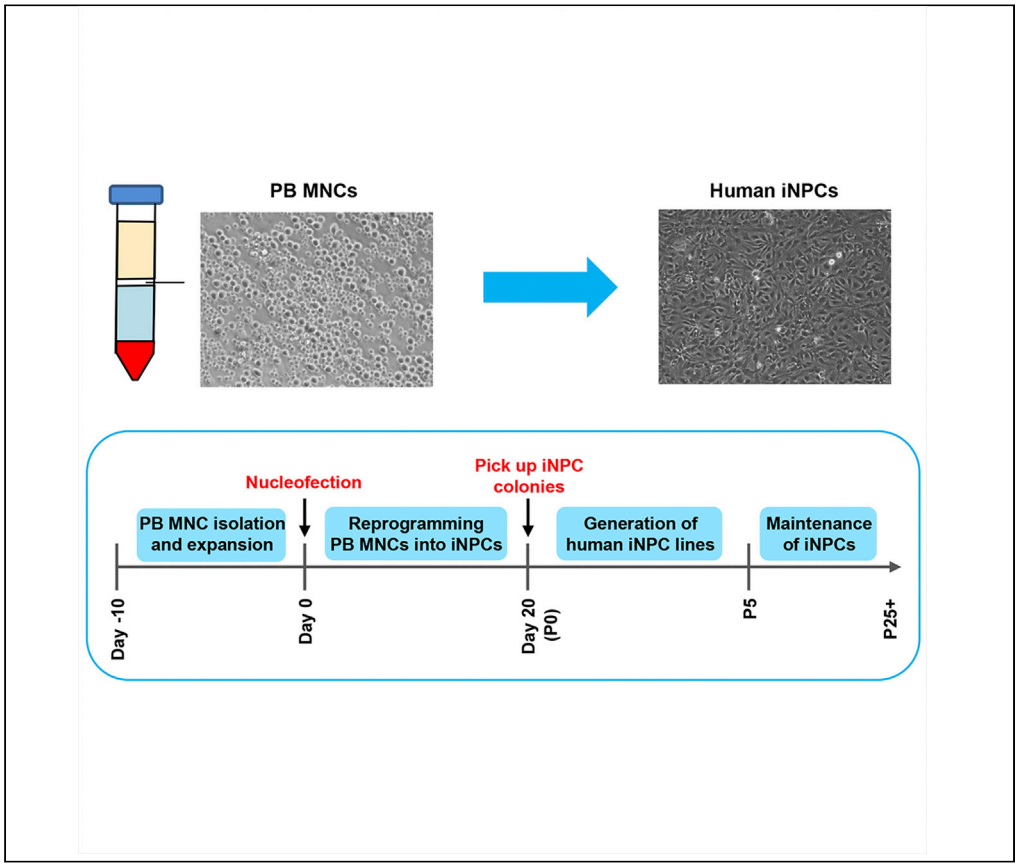
Protocol for generating human induced neural progenitor cells from immobilized adult peripheral blood



Generating induced neural stem/progenitor cells (iNPCs) from somatic cells for medical applications has remained challenging. Here, we describe a reliable protocol to make human iNPCs from a small volume of immobilized adult peripheral blood by direct reprogramming. We have verified that the integration-free human iNPCs can efficiently differentiate into mature neurons in mouse brain upon transplantation and display capacities to functionally replace the damaged neurons, suggesting their potential as donor cells in developing replacement medicine for neurodegenerative diseases.

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HIGHLIGHTS

A reliable protocol to generate human iNPCs from adult peripheral blood

Detailed procedures for establishment of human iNPC lines

Approach to systematically characterize human iNPCs and iNPC lines

Method for efficiently directing the neuronal differentiation of human iNPCs

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Protocol for generating human induced neural progenitor cells from immobilized adult peripheral blood

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SUMMARY

Generating induced neural stem/progenitor cells (iNPCs) from somatic cells for medical applications has remained challenging. Here, we describe a reliable protocol to make human iNPCs from a small volume of immobilized adult peripheral blood by direct reprogramming. We have verified that the integration-free human iNPCs can efficiently differentiate into mature neurons in mouse brain upon transplantation and display capacities to functionally replace the damaged neurons, suggesting their potential as donor cells in developing replacement medicine for neurodegenerative diseases.

For complete details on the use and execution of this protocol, please refer to Zhang et al. (2019).

BEFORE YOU BEGIN

Prepare culture medium, buffers, and chemicals

Timing: 10–30 min for each medium/buffer/chemical

1. MNC medium (50 mL; for mononuclear cell (MNC) maintenance.)

Reagent	Final concentration	Amount
IMDM	N/A	22 mL
Ham's F12	N/A	22 mL
ITS-X (100×)	1×	500 μL
Chemically defined lipid concentrate (100×)	1×	500 μL
Glutamax (100×)	1×	500 μL
L-ascorbic acid (2.5 mg/mL IMDM)	50 μg/mL	1 mL

(*Continued on next page*)

Continued		
Reagent	Final concentration	Amount
BSA (0.25 g/mL IMDM)	5 mg/mL	1 mL
1-thioglycerol (0.45 μL/mL Ham's F12)	200 μM	2 mL
SCF (100 μg/mL)	100 ng/mL	50 μL
IL-3 (10 μg/mL)	10 ng/mL	50 μL
EPO (0.5 U/μL)	2 U/mL	200 μL
IGF-1 (100 μg/mL)	40 ng/mL	20 μL
Dexamethasone (10 mM)	1 μM	5 μL
TF (20 mg/mL)	100 μg/mL	250 μL
Filter-sterilize the medium and store it at 4°C. Use the medium within 3 weeks.		

2. Red cell lysis buffer (500 mL)

Reagent	Final concentration	Amount
Ammonium chloride	8.29 g/L	4.145 g
Potassium bicarbonate	1 g/L	0.5 g
EDTA (0.5 M, pH 8.0)	0.1 mM	100 μL
ultrapure water	-	~*400 mL
*Adjust the pH to 7.2–7.4, and add ultrapure water to make the final volume 500 mL. Filter-sterilize the buffer and store it at 4°C. Use the buffer within 6 months.		

3. MEF medium (50 mL)

Reagent	Final concentration	Amount
DMEM	N/A	45 mL
FBS	10%	5 mL
Antibiotic-Antimycotic (100×)	1×	500 μL
Filter-sterilize the medium and store it at 4°C. Use the medium within 3 weeks.		

4. KSR medium (50 mL)

Reagent	Final concentration	Amount
DMEM/F12	N/A	38.5 mL
KSR	20%	10 mL
Glutamax (100×)	1×	500 μL
NEAA (100×)	1×	500 μL
β-ME (100×)	1×	500 μL
bFGF (20 μg/mL)	10 ng/mL	25 μL
NaB (0.25 M)	0.25 mM	50 μL
Filter-sterilize the medium and store it at 4°C. Use the medium within 3 weeks.		

5. N2B27 (NB) medium (100 mL)

Reagent	Final concentration	Amount
DMEM/F12	N/A	47.5 mL
Neurobasal medium	N/A	47.5 mL
Glutamax (100×)	1×	1 mL
β-ME (100×)	1×	1 mL
N2 (100 ×)	1×	1 mL
B27 (minus vitamin A, 50×)	1×	2 mL
Filter-sterilize the medium and store it at 4°C. Use the medium within 3 weeks.		

6. Reprogramming medium (50 mL)

Reagent	Final concentration	Amount
N2B27 medium	N/A	50 mL
VPA (0.5 M)	0.5 mM	50 μL
SB431542 (10 mM)	10 μM	50 μL
CHIR99021 (10 mM)	3 μM	15 μL
Forskolin (10 mM)	10 μM	50 μL
Store the medium at 4°C protecting from light and use it within 2 weeks.		

7. NPC medium[#1] (50 mL)

Reagent	Final concentration	Amount
N2B27 medium	N/A	50 mL
hLIF (10 μg/mL)	10 ng/mL	50 μL
SB431542 (10 mM)	5 μM	25 μL
CHIR99021 (10 mM)	3 μM	15 μL
Freshly prepare the medium before use.		

8. NPC medium[#2] (50 mL) 20 ng/mL bFGF and 20 ng/mL EGF.

Reagent	Final concentration	Amount
N2B27 medium	N/A	50 mL
bFGF (20 μg/mL)	20 ng/mL	50 μL
EGF (100 μg/mL)	20 ng/mL	10 μL
Freshly prepare the medium before use.		

9. B27 medium (50 mL)

Reagent	Final concentration	Amount
Neurobasal medium	N/A	48 mL
B27 (minus vitamin A, 50×)	1×	1 mL
Glutamax (100×)	1×	500 μL
β-ME (100×)	1×	500 μL
Filter-sterilize the medium and store it at 4°C. Use the medium within 3 weeks.		

10. Neural differentiation (ND) medium (50 mL)

Reagent	Final concentration	Amount
B27 medium	N/A	50 mL
Laminin (1 μg/μL)	1 μg/mL	50 μL
FN (1 μg/μL)	1 μg/mL	50 μL
BDNF (10 μg/mL)	10 ng/mL	50 μL
NT3 (10 μg/mL)	10 ng/mL	50 μL
IGF-1 (100 μg/mL)	10 ng/mL	5 μL
cAMP (10 mM)	1 μM	5 μL

Add Compound E (final concentration 0.2 μM) as required at the indicated time point in the protocol. Freshly prepare the medium before use.

11. Prepare the stock solution of chemicals using the recommended solvents.

Chemicals	Stock concentration	Final concentration
Valproic acid (VPA)	0.5 M	0.5 mM
CHIR99021	10 mM	3 μM
SB431542	10 mM	5–10 μM
Forskolin	10 mM	10 μM

Aliquot and store the stock solutions protecting from light at −20°C. Use the stock solutions within 6 months.

Prepare MEF-based feeder layer for iNPC generation

Timing: 30 min

12. Thaw an aliquot of 125 μL Matrigel on ice for 12–16 h at 4°C and dilute in 12.5 mL cold DMEM/F12 medium (to make 1:100 dilution).
13. Immediately coat a 12-well plate with 0.5 mL Matrigel/well, and place the coated plate at 25°C for at least 1 h before using.
14. Thaw irradiated MEFs (from the CF-1 strain) and seed on the Matrigel-coated plate at a density of 8×10^4 cells/well and incubate the cells in cell culture incubator at 37°C, 5% CO_2. The handling procedure for the irradiated MEFs is available at https://www.atcc.org/products/all/SCRC-1040.1.aspx#documentation.

Note: Feeder layer needs to be prepared 1 day before the nucleofection of mononuclear cells (MNCs) from peripheral blood.

Prepare plasmids for the nucleofection of peripheral blood mononuclear cells (PB MNCs)

Timing: 2–3 h

To generate integration-free human iNPCs, the oriP/EBNA1-based episomal vectors EV SFFV-OCT4-2A-SOX2 (SFFV-OS), EV SFFV-MYC-2A-KLF4 (SFFV-MK) and EV SFFV-BCL-XL are prepared and delivered into the PB MNCs via nucleofection. BCL-XL serves as an anti-apoptosis factor, and therefore can increase efficiency of reprogramming PB MNC to induced pluripotent stem cells (iPSCs) (Chou et al., 2015; Su et al., 2013). The plasmids are available through Addgene (#64120, #64121, #64122). Isolate DNA of episomal plasmids by using NucleoBond Xtra Midi kit (MACHEREY-NAGEL) according to the manufacturer's instructions at https://www.mn-net.com/

media/pdf/ed/82/0f/Instruction-NucleoBond-Xtra.pdf. Measure the quantity and quality of the plasmid DNA on Nanodrop 2000 Spectrophotometer.

⚠ CRITICAL: To minimize the adverse effect on the subsequent nucleofection, the DNA concentration for each plasmid is not lower than 1 μg/μL.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Rabbit polyclonal anti-PAX6 (1:200)	Chemicon	Cat#AB2237; RRID: AB_1587367
Rabbit polyclonal anti-SOX1 (1:200)	This paper	N/A
Rabbit polyclonal anti-SOX2 (1:200)	Abcam	Cat#ab59776; RRID: AB_945584
Rabbit polyclonal anti-NESTIN (1:200)	This paper	N/A
Rabbit monoclonal anti-Ki67 (1:50)	Abcam	Cat#ab16667; RRID: AB_302459
Rabbit polyclonal anti-FABP7 (1:50)	Abclonal	Cat# A11604; RRID: AB_2758628
Goat polyclonal anti-NANOG (1:200)	R&D	Cat#AF1997; RRID: AB_355097
Mouse monoclonal anti-OCT4 (1:200)	Santa Cruz Biotechnology	Cat#SC-5279; RRID: AB_628051
Mouse monoclonal anti-TUJ1 (1:500)	BioLegend	Cat#MMS-435P; RRID: AB_2313773
Rabbit monoclonal anti-TUJ1 (1:500)	BioLegend	Cat#MRB-435P; RRID: AB_663339
Mouse monoclonal anti-MAP2 (1:200)	Sigma-Aldrich	Cat#M4403; RRID: AB_477193
Rabbit polyclonal anti-NEUN (1:200)	Millipore	Cat#ABN78; RRID: AB_10807945
Rabbit polyclonal anti-TBR1 (1:200)	Abcam	Cat#ab31940; RRID: AB_2200219
Rabbit polyclonal anti-VGLUT1 (1:200)	SYSY	Cat#135302
Bacterial and virus strains		
DH5α chemically competent *E. coli*	Transgen	Cat#CD201-01
Biological samples		
Healthy adult peripheral blood	Zhang et al., 2019	N/A
Chemicals, peptides, and recombinant proteins		
Ficoll-Paque Premium	GE Health	Cat#17-5442-02
DPBS, no calcium, no magnesium	Thermo Fisher	Cat#14190144
IMDM medium	Thermo Fisher	Cat#A1048901
Ham's F12 medium	Thermo Fisher	Cat#11765062
DMEM/F12 medium	Thermo Fisher	Cat#1133032
DMEM medium	Thermo Fisher	Cat#10566016
Neurobasal medium	Thermo Fisher	Cat#21103049
Antibiotic-antimycotic (100×)	Thermo Fisher	Cat#15240062
Knockout serum replacement (KSR)	Thermo Fisher	Cat#10828028
N-2 supplement	Thermo Fisher	Cat#17502048
B-27 supplement, minus vitamin A	Thermo Fisher	Cat#12587010
Fetal bovine serum (FBS)	Hyclone	Cat#SH30071.03HI
Ammonium chloride	Sigma-Aldrich	Cat#A9434
EDTA (0.5 M, pH 8.0)	Thermo Fisher	Cat#15575-020
Insulin-transferrin-selenium-ethanolamine (ITS-X)	Thermo Fisher	Cat# 51500056
Human holo-transferrin (TF)	R&D	Cat#2914-HT
Dexamethasone	MPBIO	Cat#02199350.6
Recombinant human erythropoietin (EPO)	R&D	Cat#287-TC
Recombinant human interleukin-3 (IL-3)	Peprotech	Cat#200-03
Recombinant human stem cell factor (SCF)	Peprotech	Cat#300-07
1-Thioglycerol	Sigma-Aldrich	Cat#M6145
Bovine serum albumin (BSA)	Sigma-Aldrich	Cat#A9418
L-ascorbic acid	Sigma-Aldrich	Cat# A8960
Chemically defined lipid concentrate	Thermo Fisher	Cat#11905031
MEM non-essential amino acids solution (NEAA)	Thermo Fisher	Cat#11140050
Glutamax	Thermo Fisher	Cat#35050061

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
2-Mercaptoethanol (β-ME)	Millipore	Cat#ES-007-E
Matrigel hESC-qualified matrix	Corning	Cat#354277
Sodium butyrate (NaB)	Sigma-Aldrich	Cat#B5887
Valproic acid (VPA)	Selleck	Cat#S3944
CHIR99021	Selleck	Cat#S1263
SB431542	Selleck	Cat#S1067
Forskolin	Selleck	Cat#S2449
Recombinant human FGF-basic (bFGF)	Sigma-Aldrich	Cat#100-18B
Recombinant human EGF	Peprotech	Cat#100-15
Recombinant human LIF Protein (hLIF)	R&D	Cat#7734-LF-025
Accutase	Thermo Fisher	Cat#00-4555-56
Poly-D-lysine solution (PDL)	Sigma-Aldrich	Cat#A-003-M
Fibronectin (FN)	Thermo Fisher	Cat#33016015
Laminin	Sigma-Aldrich	Cat#L2020
Compound E	Calbiochem	Cat# 565790
Recombinant human b-NGF Protein	R&D	Cat#256-GF
Recombinant human/Murine/Rat BDNF	Peprotech	Cat#450-02
Recombinant human IGF-I	Peprotech	Cat#100-11
Recombinant human NT-3	Peprotech	Cat#450-03
cAMP	Sigma-Aldrich	Cat#A9501
Critical commercial assays		
Amaxa human CD34 cell Nucleofector kit	Lonza	Cat#VPA-1003
P3 primary cell 4D-Nucleofector X kit L	Lonza	Cat#V4XP-3024
NucleoBond Xtra Midi kit for transfection-grade plasmid DNA	MACHEREY-NAGEL	Cat#740410.50
Experimental models: cell lines		
MEF (CF-1), irradiated	ATCC	SCRC-1040.1
Oligonucleotides		
Primer: pEV-M2K F: 5′TGGAGCGCCAGAG GAGGAACGACGTA3′	This paper	N/A
Primer: pEV-M2K R: 5′TCGCTGACAGCCAT GTCAGACTCGCC3′	This paper	N/A
Primer: pEV-O2S F: 5′CTGTCTCCGTCACC ACTCTGGGCTC3′	This paper	N/A
Primer: pEV-O2S R: 5′GCGGCTTCAGCTCCG TCTCCATCATG3′	This paper	N/A
Recombinant DNA		
pEV SFFV-OCT4-2A-SOX2	Addgene	#64120
pEV SFFV-MYC-2A-KLF4	Addgene	#64121
pEV SFFV-BCL-XL	Addgene	#64122
Other		
NanoDrop 2000 spectrophotometer	Thermo Fisher	Cat#ND2000CLAPTOP
Swinging bucket rotor	Anke	Cat#TDL-40B
4D-Nucleofector X unit	Lonza	Cat#AAF-1002X
4D-Nucleofector core unit	Lonza	Cat# AAF-1002B
Nucleofector 2b device	Lonza	Cat#AAB-1001
TC-treated 6-well plates	Corning	Cat#CLS3516
TC-treated 12-well plates	Corning	Cat#CLS3513
TC-treated 24-well plates	Corning	Cat#CLS3524
35 mm TC-treated culture dish	Corning	Cat#430165
15 mL conical centrifuge tubes	Fisher Scientific	Cat#14-959-49B
50 mL conical centrifuge tubes	Fisher Scientific	Cat#14-432-22
Vacuum sodium heparin blood collection tube (5 mL)	Henso	Cat#E050504

STEP-BY-STEP METHOD DETAILS

Collection of peripheral blood samples

Timing: approximately 10 min

Collect 8 mL peripheral blood from each donor into sodium heparin blood collection tubes by venipuncture. Keep the blood samples at 25°C before use.

Note: The peripheral blood does not need to be mobilized. The expandable MNCs can be isolated from peripheral blood up to 24 h after collection.

⚠ CRITICAL: The collection of human peripheral blood and subsequent use of PB MNCs must be approved by ethics committee and the donors must be informed and sign the consent forms.

Isolation of MNCs from peripheral blood

Timing: approximately 3 h

This step describes how to isolate MNCs from fresh peripheral blood samples.

1. Transfer the peripheral blood from each donor into a 15 mL sterile conical tube, add equal volume of DPBS and dilute the peripheral blood gently.
2. Add 4 mL Ficoll-Paque Premium in a 15 mL sterile conical tube, carefully load half volume of the diluted blood samples onto the top of Ficoll layer by using a 10 mL pipette. Repeat this step, transfer the other half of the diluted blood samples into another 15 mL sterile conical tube.

⚠ CRITICAL: Set the power of the electric pipettor to a low pressure, tilt the tube at a 45° angle, and slowly layer the diluted blood so that the blood stays on top of Ficoll-Paque.

3. Centrifuge the tubes at 400 × *g* for 30 min at 25°C in a swinging bucket rotor.

Note: Each tube contains four layers after the centrifugation, the yellow plasma layer on the top, then the white MNC layer, the Ficoll layer, and red cell layer in the bottom.

4. Remove the upper plasma layer by aspiration, leaving a small volume of plasma (< 1 mL) on top of the opaque interface containing the MNC layer. Carefully collect the MNCs with a 1 mL tip and transfer the MNCs from two 15 mL tubes into a 50 mL conical tube. Do not stir the Ficoll layer as it will interfere with the centrifugation step.
5. Add DPBS to a final volume of 50 mL and centrifuge at 450 × *g* for 15 min at 25°C. At this step and all the subsequent steps, set the centrifuge brake to "on."
6. Carefully discard the supernatant and resuspend the pellets with 5 mL red cell lysis buffer. Let the cells incubate at 25°C for 10 min to remove the mixed red cells.
7. Add DPBS to a final volume of 50 mL and centrifuge at 450 × *g* for 10 min at 25°C.
8. Discard the supernatant and resuspend the pellets with 30 mL DPBS. Centrifuge at 450 × *g* for 10 min at 25°C.
9. Discard the supernatant and resuspend the cells with 5 mL DPBS. Aliquot 10 μL of cell suspension, mix with 10 μL of 0.4% trypan blue solution and count the viable cells using a hemocytometer. Centrifuge the rest cell suspension at 450 × *g* for 5 min at 25°C.
10. Aspirate the supernatant and resuspend the pellets with appropriate volume of MNC medium to a density of ~3 × 10^6 cells/mL for the following expansion of MNCs.

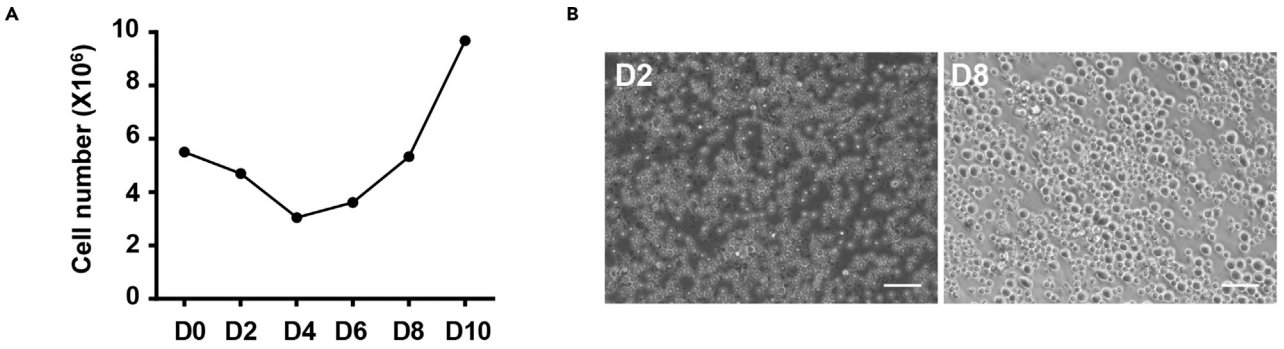

Figure 1. Typical changes in cell number and morphology during PB MNC expansion

(A) The cells are ready for reprogramming when the cell number significantly increases twice in succession.

(B) Cell body becomes larger during PB MNC expansion. Left, 2 days of culture; right, 8 days of culture. Scale bar, 100 μm.

Pause point: Alternatively, MNCs can be frozen at this step. After centrifugation at step 9, remove the supernatant and resuspend the cells in freezing medium (10% DMSO in MNC medium) to a concentration of >5 × 10^6 cell/mL/vial, and freeze the cells in a controlled-rate freezing container. Cells can be stored in liquid nitrogen for a prolonged time.

Expansion of PB MNCs

Timing: 8–10 days

This step describes how to expand MNCs, which is a modified version of a published protocol (Dowey et al., 2012). The culture conditions used in this protocol selectively enrich the erythroblasts among collected MNCs rather than the lymphocytes which may contain V(D)J DNA rearrangements (Chou et al., 2011).

11. Seed the MNCs at a density of 10^6 cells/cm^2 in an appropriate tissue culture plate. Culture the cells in the cell culture incubator at 37°C, 5% CO_2 for 2 days.
12. Collect the cells and transfer into a 15 mL conical tube.

Note: The cells are cultured in suspension, but some may loosely attach to the bottom of the tissue culture plates due to the gravity. Gently pipette the cells for several times to make cells float before collecting.

13. Centrifuge the cells at 220 × *g* for 5 min at 25°C. Discard the supernatant and resuspend the pellets in 1 mL MNC medium.
14. Count the viable cell number using a hemocytometer and seed the cells back to the tissue culture plate at a density of approximately 10^6 cells/cm^2. Culture the cells in fresh MNC medium in the cell culture incubator at 37°C, 5% CO_2 for 2 days.
15. Repeat steps 13 and 14 every 2 days until the cell number significantly increases twice in succession (Figure 1A). After expansion, the cell bodies of MNCs become much larger than those of MNCs simply isolated (Figure 1B). The expanded MNCs are ready for the subsequent nucleofection.

Reprogramming PB MNCs into human iNPCs

Timing: 3 weeks

This step describes the process of generating human iNPCs from expanded PB MNCs, which is schematically summarized in Figure 2A.

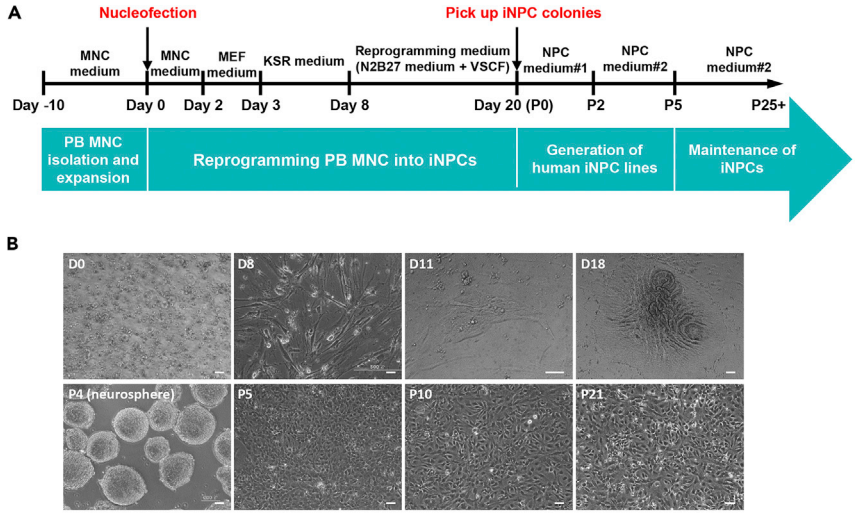

Figure 2. Schematic representation of iNPC generation from PB MNCs

(A) Timeline for the conversion of PB MNCs into iNPCs.

(B) Representative images showing cell morphology changes during the process. Scale bar, 100 μm.

16. On Day 0, perform the nucleofection of PB MNCs as follows:
    a. Turn on Lonza 4D-Nucleofector System and select program "EO-100." Bring the Nucleofector solution and Supplement in P3 primary cell 4D-Nucleofector X Kit to 25°C.
    b. Aliquot 2 × 10^6 cultured PB MNCs to a sterile 15 mL conical tube, add DPBS to a final volume of 5 mL, and centrifuge the cells at 220 × *g* for 5 min at 25°C.
    c. Add 82 μL Nucleofector Solution and 18 μL Supplement into a 0.6 mL microcentrifuge tube, mix thoroughly and keep at 25°C.
    d. Add 4 μg EV SFFV-OS, 4 μg EV SFFV-MK and 2 μg EV SFFV-BCL-XL into another 0.6 mL tube, mix thoroughly.
    e. Remove the supernatant completely, and gently resuspend the MNC pellets in 100 μL transfection solution prepared in c.
    f. Mix the cell suspension with the plasmid DNA prepared in d, and immediately transfer it to a sterile cuvette provided with the kit. Place the cuvette with closed lid into the retainer of the 4D-Nucleofector X Unit and run the program.
    g. Once nucleofection has been done, remove the cuvette, add 500 μL pre-warmed MNC medium, and incubate in a cell culture incubator at 37°C, 5% CO_2 for 10 min.
    h. Carefully aspirate the sample from the cuvette using a plastic pipette supplied with the kit, transfer, and seed the cells into one well of a 12-well-plate with 1.5 mL pre-warmed MNC medium in it. Culture in an incubator at 37°C, 5% CO_2 for 2 days.

⚠ CRITICAL: Avoid repeated aspiration of nucleofected cells to protect them from mechanical damage.

Optional: Lonza Nucleofector 2b device can also be used for MNC electroporation. Use the Amaxa Human CD34 cell Nucleofector kit and the cuvette provided with the kit. Select "T-016" program for electroporation.

17. On Day 2, transfer the recovered cells to a 15 mL sterile conical tube and centrifuge them at 220 × *g* for 5 min at 25°C.
18. Remove the supernatant and resuspend the pellet with 1 mL MEF medium, aliquot 10 μL cell suspension with 10 μL trypan blue, and count the viable cells using a hemocytometer. The cell viability varies greatly among different MNC samples but is routinely higher than 30%.

19. Plate the cells onto irradiated MEFs in Matrigel-coated 12-well-plate at a density of 2×10^5/well in MEF medium, and culture in an incubator at 37°C, 5% CO_2 for 1 days.
20. On Day 3, collect the cells by aspiration and transfer to a sterile 15 mL conical tube. Centrifuge the tube at 220 × *g* for 5 min and resuspend the pellet with 3 mL KSR medium. Seed the cells back to the wells and culture in an incubator at 37°C, 5% CO_2 for 5 days. Half-refresh the KSR medium every other day.
21. On Day 8, change the KSR medium to reprogramming medium. Refresh the medium every 2 days.
22. Check the morphological changes of the cells under an inverted microscope daily. The small cell clusters or colonies that might be reprogrammed iNPCs can be observed as early as Day 11 (Figure 2B).
23. On days 16–20, typical neural rosettes can be observed in the NPC-like colonies, which are big enough for picking. Circle the location of NPC-like colonies with a marker pen on the bottom of the plate. Under an inverted microscope, dislodge an NPC-like colony and mechanically break it into small clusters using a 200 μL sterile pipette. Plate the cells on a Matrigel-coated well in a 24-well-plate and incubate at 37°C, 5% CO_2 for further expansion. For each iNPCs, pick up at least 6 colonies and culture for the subsequent establishment of iNPC lines.

Generation of human iNPC lines

Timing: 2 weeks

24. Culture and expand the human iNPCs in NPC medium[#1] for the first two passages.
    a. To passage the iNPCs, discard the medium and add Accutase to culture plate at 1 mL/10 cm^2 surface area to detach the cells.
    b. Incubate the cells at 37°C for 4 min. Dilute the Accutase in N2B27 medium at a ratio of 1:5 and pipette the cells several times to obtain single cell suspension.
    c. Transfer the cell suspension into a 15 mL sterile conical tube, and centrifuge the cells at 200 × *g* for 2 min.
    d. Discard the supernatant and resuspend the pellets in 1 mL of NPC medium[#1]. Split the cells at a ratio of 1:3 and seed them onto Matrigel-coated dishes or plates. Passage the iNPCs every 3 days.
25. At passage 3, change the medium to NPC medium[#2] to acquire a homogeneous morphology. Follow the passaging protocol as described in step 24, except that the culture medium is changed to NPC medium[#2].
26. In case that the cells fail to show homogeneous morphology, purify the iNPCs at passage 4 by formation of neurosphere since this assay is a robust way to identify and purify the NPCs (Ahmed, 2009).
    a. Discard the medium, add Accutase at 1 mL per 10 cm^2 surface area and incubate at 37°C for 4 min to detach the cells.
    b. Dilute the Accutase in N2B27 medium at a ratio of 1:5 and pipette the cells several times into single cell suspension.
    c. Transfer the cell suspension into a 15 mL sterile conical tube, and centrifuge the cells at 200 × *g* for 2 min.
    d. Discard the supernatant and resuspend the pellets in 1 mL of NPC medium[#2]. Seed all the cells onto an equal-sized petri-dish and culture in 2 mL NPC medium[#2] per 10 cm^2 surface area in an incubator at 37°C, 5% CO_2 for 3 days to form neurospheres (Figure 2B).
    e. Collect the neurospheres and seed them directly onto Matrigel-coated tissue culture dishes at a ratio of 1:3. Incubate the cells in NPC medium[#2] in an incubator at 37°C, 5% CO_2.

Maintenance of human iNPCs

Timing: 30 min for each passage

27. After purification, maintain the established human iNPCs continuously in NPC medium[#2]. Passage the cells when they are 90% confluent following the standard passaging protocol as described in step 24 except that the culture medium is NPC medium[#2]. The human iNPC lines can be stably maintained for more than 20 passages.

Pause point: The iNPCs can be expanded and cryopreserved from passage 5 to 15. After dissociation of cells with Accutase, dilute Accutase with N2B27 medium at a ratio of 1:5 and centrifuge at 200 × *g* for 2 min. Resuspend the cells with 10% DMSO-NPC medium[#2] to a final concentration of 10^6 cells/mL and aliquot 1 mL to each cryovial. Freeze the iNPCs in a controlled-rate freezing container. Cells can be stored in liquid nitrogen for a prolonged time.

Differentiation of human iNPCs into neurons *in vitro*

Timing: 4–5 weeks

28. Dissociate the iNPCs with Accutase at 1 mL per 10 cm^2 surface area as described in step 24a-c. After the centrifugation, discard the supernatant and resuspend the pellets in 1 mL of ND medium.
29. Seed 2×10^5 cells on a PDL-Laminin coated 35 mm dish and culture in ND medium for neural differentiation.
30. On differentiation day 7, dissociate the cells using Accutase as described in step 28, and reseed 2×10^5 cells on a PDL-Laminin coated 35 mm dish and culture in ND medium supplemented with 0.2 μM Compound E up to differentiation day 19.

Note: Compound E is a Notch inhibitor and inhibition of Notch signaling can accelerate the neuronal differentiation of human NPCs (Borghese et al., 2010; Ogura et al., 2013).

31. Replace 80% of the old medium with fresh medium every 2 days during the process of neural differentiation. At differentiation days 28–35, cells can be fixed for immunofluorescence staining.

EXPECTED OUTCOMES

The reprogramming efficiency may vary among different peripheral blood samples. Routinely, you can obtain 30–500 candidate colonies from 2 million PB MNCs via reprogramming following the protocol above.

PCR analysis targeting the episomal DNA can be performed on genomic DNA of iNPCs as early as passage 5 to thoroughly detect whether the episomal vectors inserted into the genome of generated iNPCs or not. The sequences of the primers are listed in Key resources table.

For characterizing human iNPCs, we recommend that the immunofluorescence staining can be performed following Cell Signaling Technology's standard protocol at https://www.cellsignal.com/contents/resources-protocols/ immunofluorescence-general-protocol/if. The high-quality iNPCs express proliferation marker Ki67 and classical neural progenitor marker genes, such as PAX6, SOX1/2, NESTIN, and FABP7, but not pluripotency markers such as OCT4 and Nanog (Figure 3 and data not shown).

For a successful neural differentiation, majority of cells in the dish are neurons expressing neuronal markers such as TUJ1, MAP2, and NEUN as well as typical markers of cortical neurons, such as TBR1 and VGLUT1/2 (Figure 4). About 5% of cells differentiate into GFAP+ astrocytes (Zhang et al., 2019). For the whole-cell patch-clamp recording of iNPC-derived neurons, co-culture the cells on

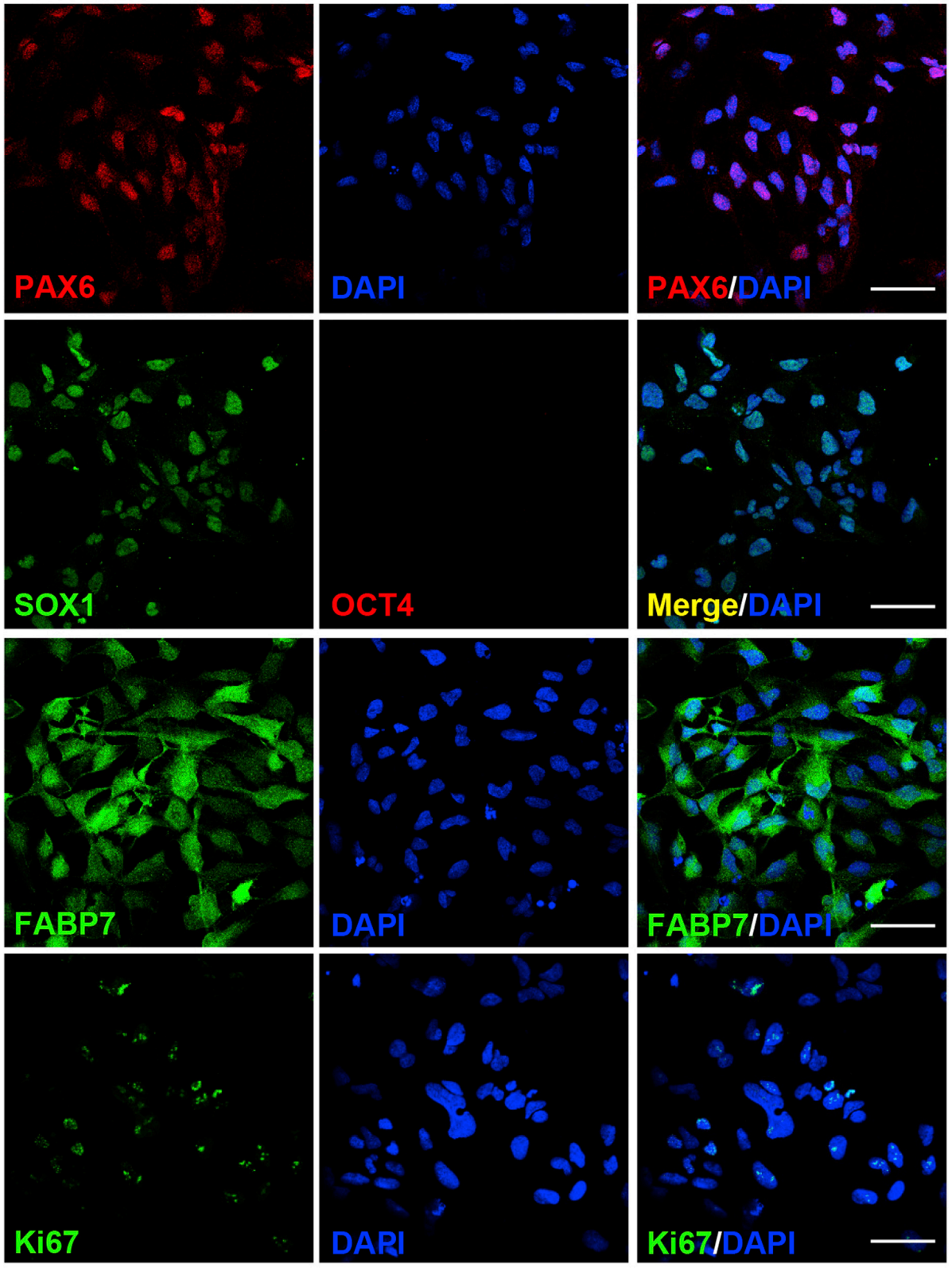
PAX6
DAPI
PAX6/DAPI
SOX1
OCT4
Merge/DAPI
FABP7
DAPI
FABP7/DAPI
Ki67
DAPI
Ki67/DAPI

Figure 3. Immunofluorescence analysis of human iNPCs at passage 15

Established iNPCs express NPC markers SOX1, PAX6, FABP7, and proliferation marker Ki67, but not pluripotency marker OCT4. Cell nuclei are counterstained with DAPI. Scale bar, 50 μm.

differentiation day 7 with mouse neonatal astrocytes and perform the recording on differentiation days 42–56, as astrocytes play a critical role in promoting functional maturation of human neurons (Tang et al., 2013).

To assess the potential of human iNPCs in the development of replacement medicine for Alzheimer's disease (AD), the generated human iNPCs can be further genetically modified by lentiviral transfection and transplanted into the brain of mice exhibiting symptoms associated with AD (Zhang et al., 2019).

LIMITATIONS

The generation of human iNPCs from PB MNCs following the protocol in this study is easy, reliable, and efficient. Most importantly, it could be done in a patient-specific way. However, the iNPCs generated in this study predominantly differentiate into cortical glutamatergic neurons as shown in Figure 4, displaying some limitations to give rise to other neurons. Therefore, the generated human iNPCs may not be a suitable donor cells for neurodegenerative diseases caused by loss or dysfunction of other subtype of neurons, such as Parkinson's disease with degenerated dopaminergic neurons in the substantia nigra (Sonntag et al., 2018). Researcher may optimize this protocol by adding extra signaling factors and try to make human iNPCs with differentiation potentials to other neuronal subtypes. Moreover, the use of MEFs as feeder cells during the process of iNPC generation may hamper the clinical translation in future. Xeno-free feeder layer or feeder-free culture system needs to be developed.

TROUBLESHOOTING

Problem 1

The number of PB MNCs fail to increase within 10 days during expansion steps (step 15).

Potential solution

Check the expiration dates for all the components of medium for PB MNCs culturing and avoid using expired products. Seed or reseed the cells at the density of 10^6 cells/cm^2 during expansion process of PB MNCs. More peripheral blood may be collected in case peripheral blood samples may contain less expandable MNCs.

Problem 2

Few colonies are generated after reprogramming (step 23).

Potential solution

This may be due to the failed delivery of episomal vectors into the PB MNCs by nucleofection. To avoid this failure, prepare plasmid DNA using commercial kits that are favorable for cell transfection. The concentration of plasmid DNA should be higher than 1 μg/μL. Be careful to avoid bubbles in cell solution and treat the cells gently during electroporation. Set up a control transfection system on Nucleofector to deliver with 2 μg pmax-GFP plasmid provided with the kit into PB MNCs. Observe the GFP^+ cells 2 days after electroporation to make sure that the Nucleofector system works well.

Problem 3

The iNPCs differentiate during passaging (steps 24–27).

Potential solution

The 4 chemicals (VPA, SB431542, CHIR99021 and Forskolin) are essential for a successful reprogramming. Without the administration of the 4 chemicals, the primary iNPCs colonies picked tend

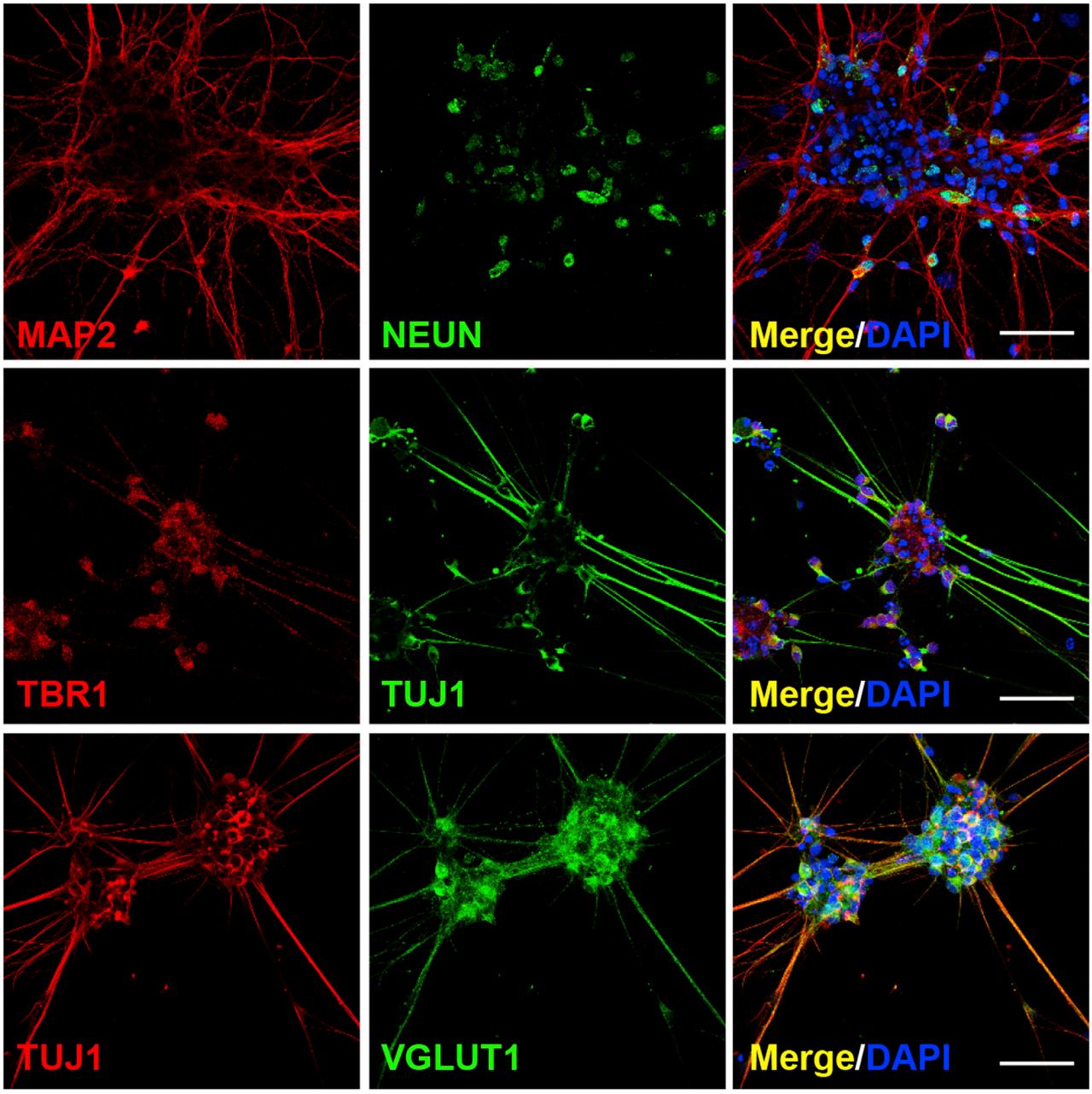

Figure 4. Immunofluorescence analysis of human iNPC-derived neurons at differentiation day 28
iNPCs predominantly differentiate into cortical glutamatergic neurons. Cell nuclei are counterstained with DAPI. Scale bar, 50 μm.

to differentiate and lose the progenitor properties during the first several passages, which is consistent with previous observation on induced neural cells from PB MNCs (Castano et al., 2014). It is also critical to pick up the compact colonies with typical neural rosette morphology instead of the loose clusters for a successful reprogramming and subsequent expansion (Figure 5).

Problem 4

Cells become detached during neuronal differentiation (steps 28–31).

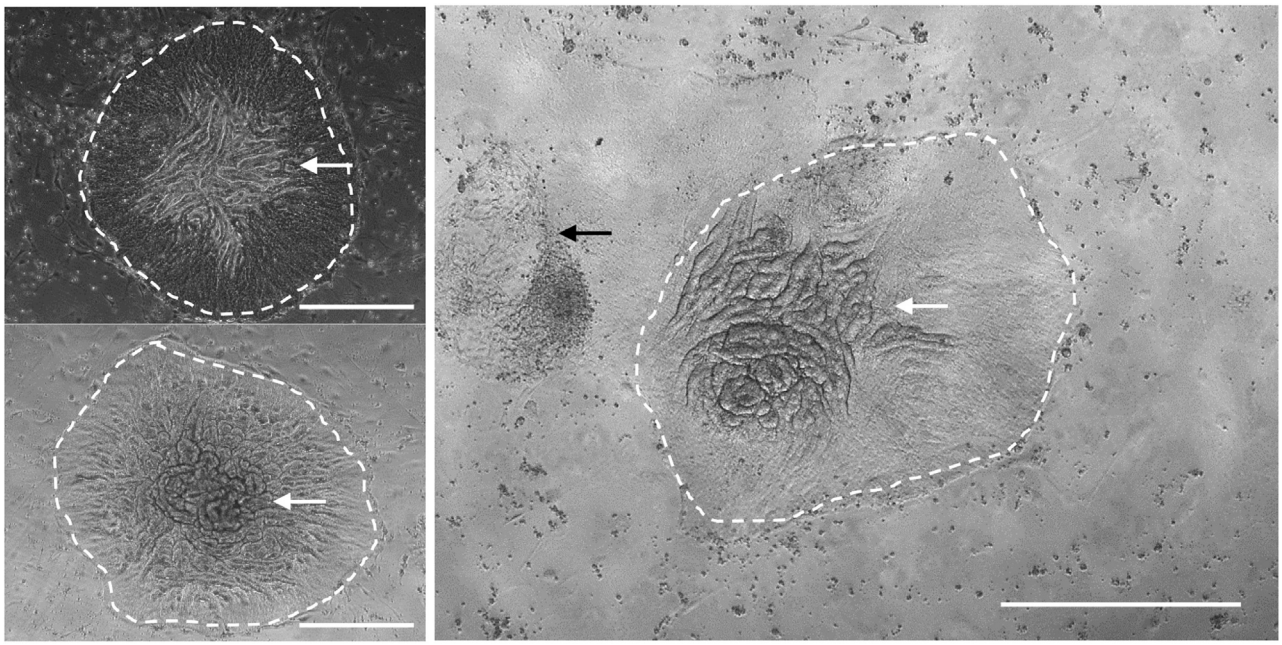

Figure 5. Representative images showing the morphology of ideal iNPC colonies with typical neural rosettes (white arrows)
The regions that should be picked are outlined by white dotted lines. Avoid picking the loose clusters of cells as shown in the same image (black arrow). Scale bar, 1 mm.

Potential solution

Differentiating neurons detach very easily in the absence of astrocytes. When refreshing medium, do not disturb the cells with pipette tips. Remove ~80% of the old medium and add fresh medium gently toward the wall of culture dishes. For long-term culture of differentiated neurons (> 5 weeks), we recommend seeding the cells on differentiation day 7 with mouse neonatal astrocytes to promote survival and maturation of neurons.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Naihe Jing, njing@sibcb.ac.cn.

Materials availability

This study did not generate new unique reagents.

Data and code availability

This protocol generates no datasets or code.

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AUTHOR CONTRIBUTIONS

N.J. and C.Y. initiated the study. T.Z., R.T., and C.Y. performed the experiments and analyzed the data. T.Z. and C.Y. wrote the manuscript. N.J. supervised the study.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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