Neural progenitor cells derived from human induced pluripotent stem cells. Version 4

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Abstract

Here we present a neural differentiation protocol for generating neural progenitor cells (NPCs) from human induced pluripotent stem cells (hiPSCs). Briefly, human dermal fibroblasts were reprogrammed into hiPSCs via non-integrative reprogramming approach. Subsequently, hiPSCs were differentiated into NPCs by forming neural rosettes *in vitro*. Characteristic morphological changes were observed and neural markers were highly enriched in the rosettes stage.

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Protocol

Cell culture of dermal fibroblasts

Step 1.

The project was approved by the Institutional Review Boards on Ethics Committee of BGI (Permit No.BGI-IRB 14057). The human fibroblast cell line was derived from the dermal skin of a healthy female donor with written informed consent. Briefly, the skin tissue was washed with DPBS 3-5 times, sliced into approximately 1mm or smaller fragment size, enzymatically dissociated in High Dulbecco's modified Eagle medium (H-DMEM, Gibco, 11965118) with 100U/ml collagenase type IV incubating in 37°C overnight, then 0.05% trypsin incubating for 5 min. The dissociation was terminated by adding 2 ml fibroblast cell culture medium (H-DMEM +10% FBS + 5ng/ml bFGF+ 2mM Gln) followed by centrifugation at 300g for 5 min. Cells were resuspended with 2ml fibroblast cell culture medium and seeded in 35mm dishes and cultured at 37°C in a 5% CO_2 incubator. The fibroblast cell culture medium was changed every 2 days until reaching 80%–90% confluence (Fig. 1a, b) at day 8. After that, cells were passaged every 3-4 days.

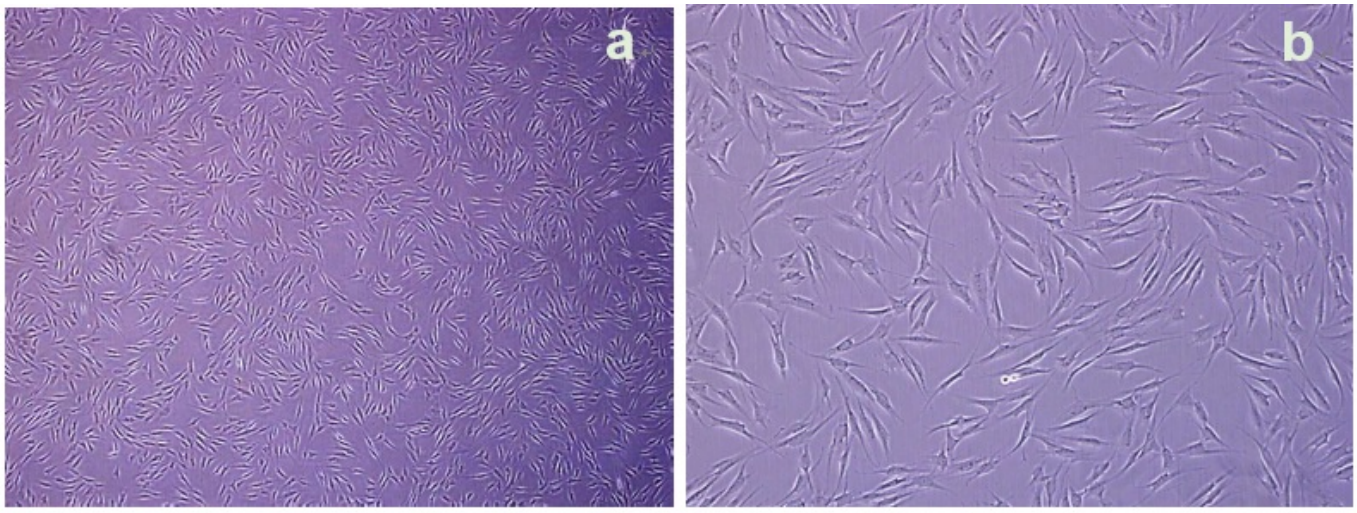

Fig.1 Bright field images of cultured human fibroblasts. Magnification = 40x, 100x respectively.

Non-integrative reprogramming

Step 2.

For reprogramming, non-integrative human iPSCs were generated following a modified Shinya Yamanaka method [1]. Briefly, $5x10^5$ human fibroblast cells at passage 4 were nucleofected with the program for human dermal fibroblast NHDF (Lonza, CC-2511) with 2.4ug episomal plasmids, including pCXLE- hOCT3/4- shp53-F (Addgene, 27077), pCXLE- hSK (Addgene, 27078), pCXLE- hUL (Addgene, 27080). Transfected cells were cultured in a six-well plate with culture medium containing H-DMEM supplemented with 10% FBS. The cells were trypsinized and $1x10^5$ cells were seeded onto a $10cm^2$ dish covered with feeder and cultured in medium containing H-DMEM with 10% FBS while reaching 80% confluence at day 5. After that, the medium was changed to hiPSCs medium containing DMEM/F12 (Gibco, 11320-033), 20% KSR (Gibco,10828-028), 2mM L-glutamine (Sigma, G8540), 0.1µM NEAA (Gibco,11140-050), 0.1µM β-Mercaptoethanol (Gibco, 21985-023) and 10ng/ml human bFGF (Invitrogen, PHG0021). The hiPSCs medium was changed every 2 days until cell colonies were observed, since then medium was changed on a daily basis. iPSCs colonies were picked mechanically under a Nikon microscope at around day 25 and maintained in hiPSCs medium until for neural differentiation at passage 15-20 (Fig. 2a, b).

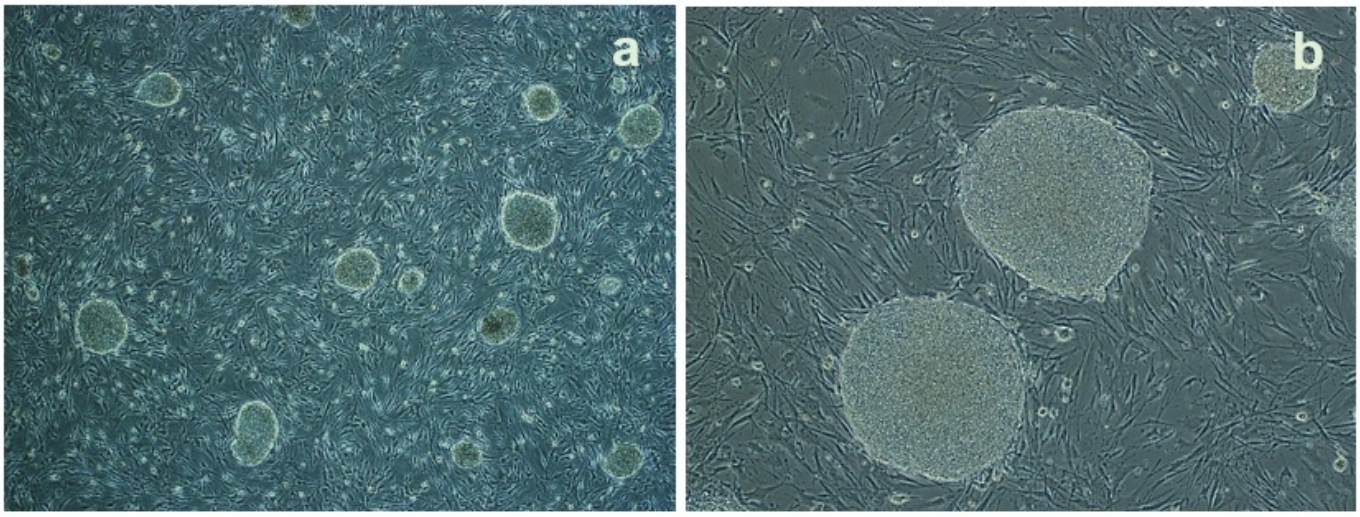

Fig.2 Bright field images of cultured human iPSCs. Magnification = 40x, 100x respectively.

Neural differentiation

Step 3.

We applied a well-adopted neural differentiation protocol [2,3]. Briefly, human iPSCs were maintained as described above. To induce neural rosettes, hiPSCs were mechanically picked and washed with DMEM/F12 twice, and then cultured for 4 days in suspension with 5μM dorsomorphin (Sigma, P5499) and 5μM SB431542 (Sigma, S4317) in hiPSCs medium without bFGF for embryoid bodies (EBs) formation, then the EBs were washed with DMEM/F12 twice and attached on matrigel (BD, 354277) coated dishes (BD, 354277) and cultured in DMEM/F12 (Gibco, 11320-033) supplemented with 20 ng/ml bFGF, 1×N2 (Gibco, 17502-048) and 2ug/ml heparin (Sigma,1304005) for an additional 3 or 5 days to harvest rosette-early (Ros-E) and rosette-late (Ros-L) cells, respectively. To collect neural progenitor cells (NPCs), rosettes structure that appeared in the center of attached colonies at Ros-L stage (Fig. 3a,b) were carefully harvested using pulled glass pipettes and seeded on matrigel-coated dishes and cultured in DMEM/F12 supplemented with 1× N2, 1× B27 (Gibco,12587-010), 20 ng/ml bFGF, 20 ng/ml EGF (Invitrogen, PHG0311) and 2ug/ml heparin (Sigma,1304005) for additional 7 days, and the medium was changed every 2 days. At day 16, the NPCs reaching approximately 80% confluence were collected (Fig. 4a, b), and all the mass or adherent cell samples were treated with TrypLE™ Express Enzyme (Gibco, 12604-021) for single cell dissociation and cryopreservation in gas-phase liquid nitrogen for further use.

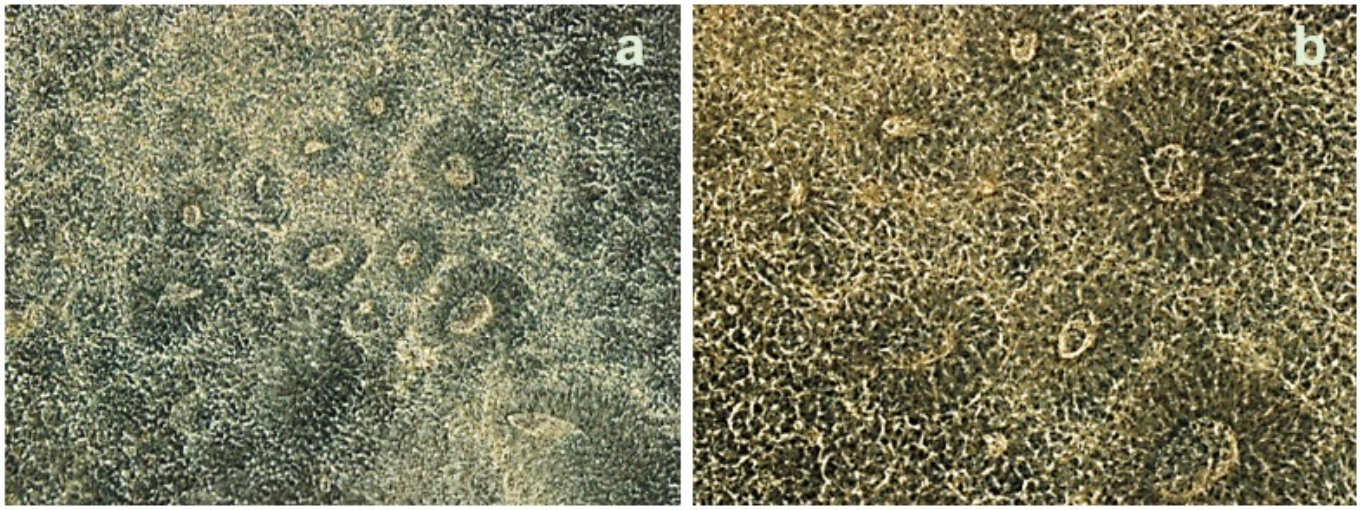

Fig.3 Bright field images of neural rosettes at post-5 days of rosettes formation. Magnification = 100x, 200x respectively.

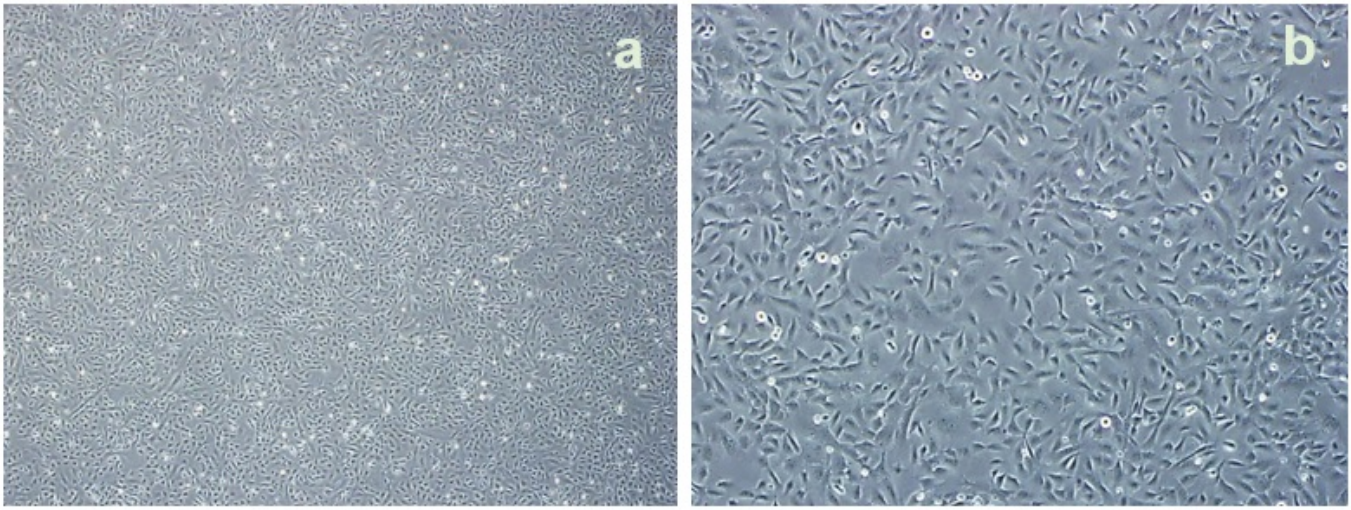

Fig.4 Bright field images of representative neural progenitor cells. Magnification = 40x, 100x respectively.

Immunofluorescence staining

Step 4.

HiPSCs and Ros-L cells were fixed in 4% paraformaldehyde in DPBS for 20 min and permeabilized with 1% Triton X-100 for 20 min at room temperature. After 60 min blocking with 2% normal goat serum, hiPSCs were incubated with primary antibodies OCT4 (1: 200, Abcam), NANOG (1: 200, Abcam), and Ros-L cells were incubated with primary antibodies PAX6 (1: 200, Abcam), SOX2 (1:200, Abcam), NESTIN (1: 200, Abcam), SOX1 (1: 200, Abcam), Zo-1 (1:100, Abcam) and N-CAD (1: 100, Abcam) overnight at 4 °C, then stained with secondary antibodies (goat anti rabbit IgG-Cy3 diluted1: 300 and goat anti mouse IgG-Cy3 diluted 1: 300) for 60 min at room temperature. DAPI (1: 500) was used as counter-staining for nuclei. The images were captured and analyzed with the Olympus IX73 and Image J (Fig. 5).

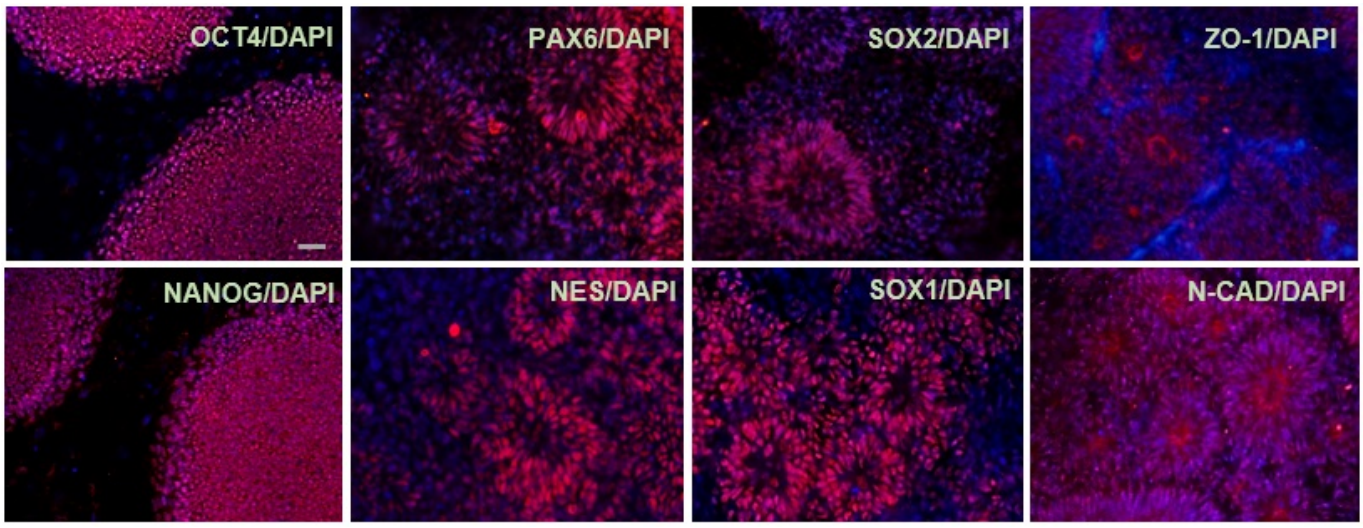

Fig. 5 Immunostaining of well-defined markers for iPSCs including OCT4 and NANOG, and for neural rosettes including PAX6, NES (NESTIN), SOX2, SOX1, ZO-1 and N-CAD (N-CADHERIN, also known as CDH2). Scale bar represents 50 µm.

References

Step 5.

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