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Neural progenitor cells derived from human induced pluripotent stem cells. Version 3

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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.

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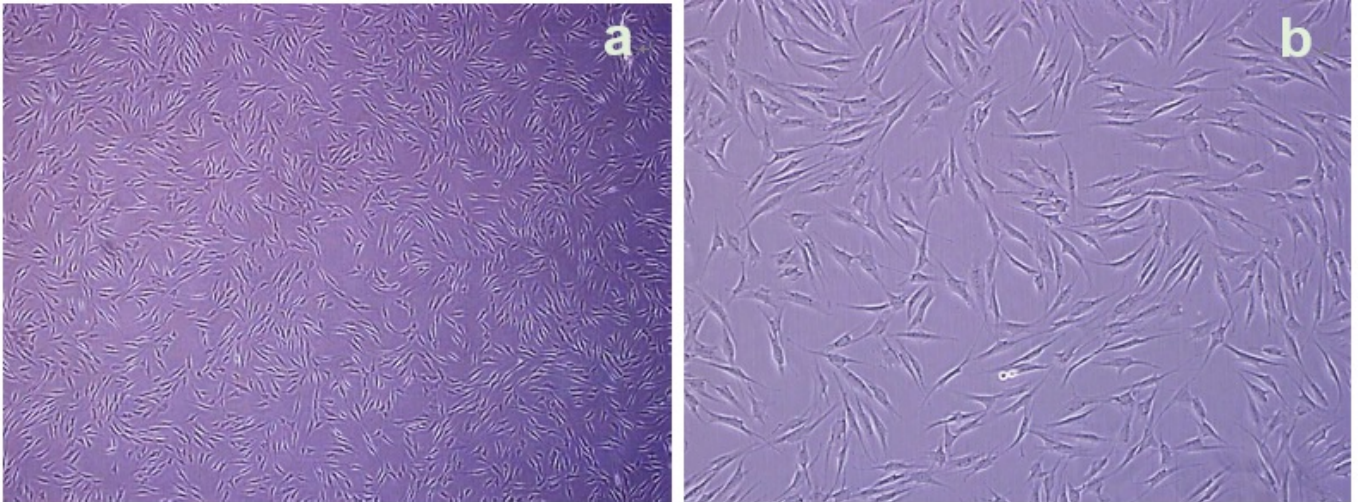
Fig.1 Bright field images of cultured human fibroblasts. Magnification = 40x, 100x respectively.

Non-integrative reprogramming

Step 2.

For reprogramming, non-integrative human iPSCs were generated following a modified Shinya Yamanaka method [1]. Briefly, 5×10^5 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 1×10^5 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).
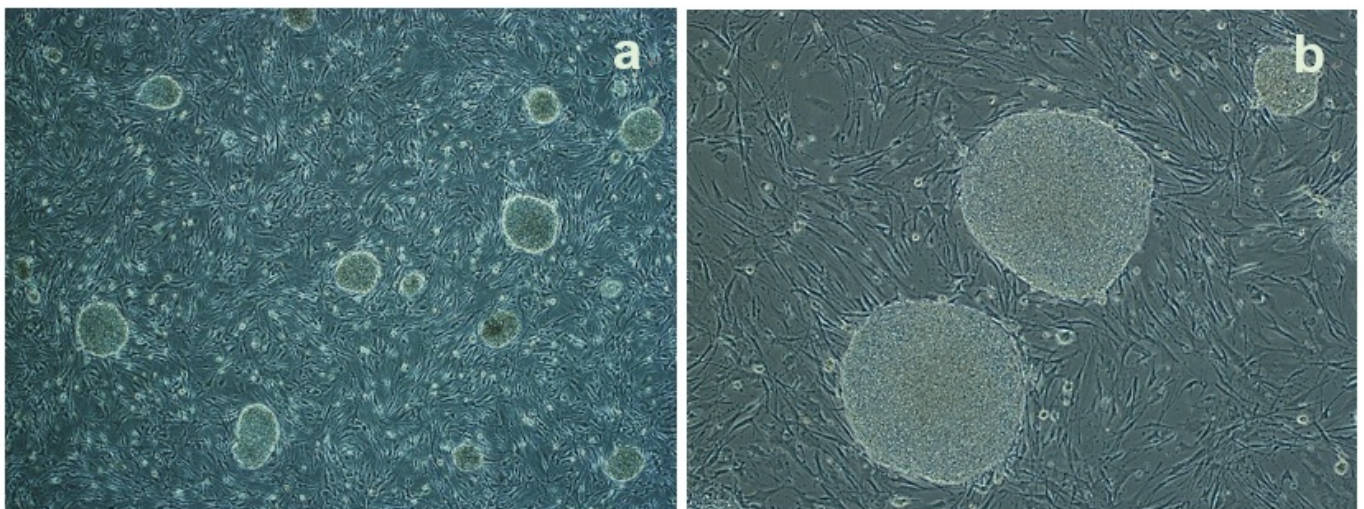

Fig.2 Bright field images of cultured human iPSCs. Magnification = 40x, 100x respectively.

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.
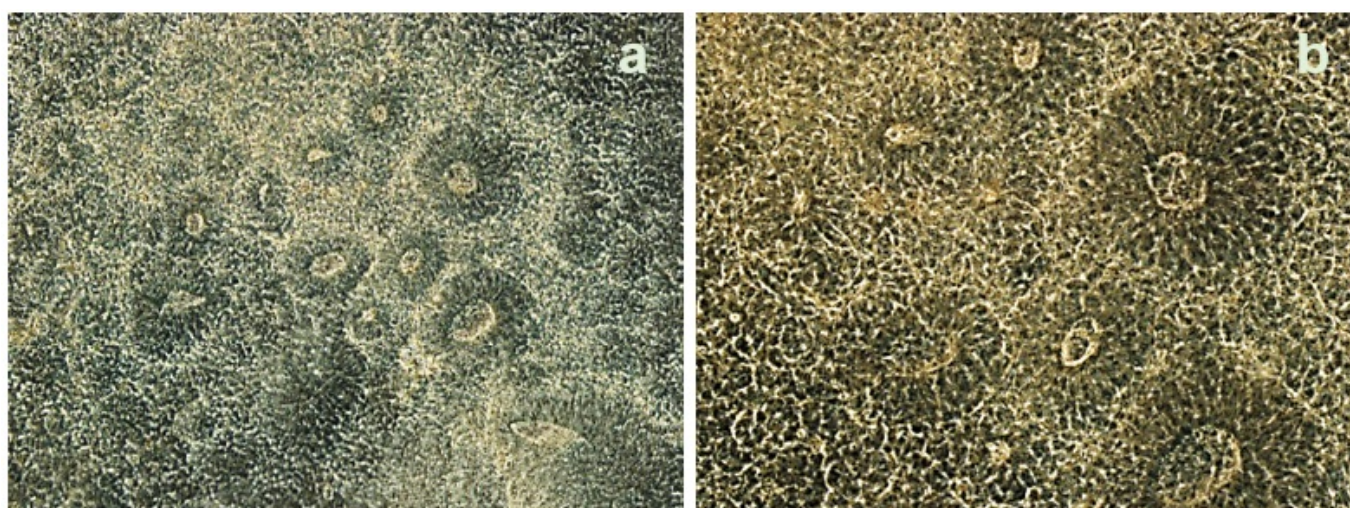

Fig.3 Bright field images of neural rosettes at post-5 days of rosettes formation. Magnification = 100x, 200x respectively.
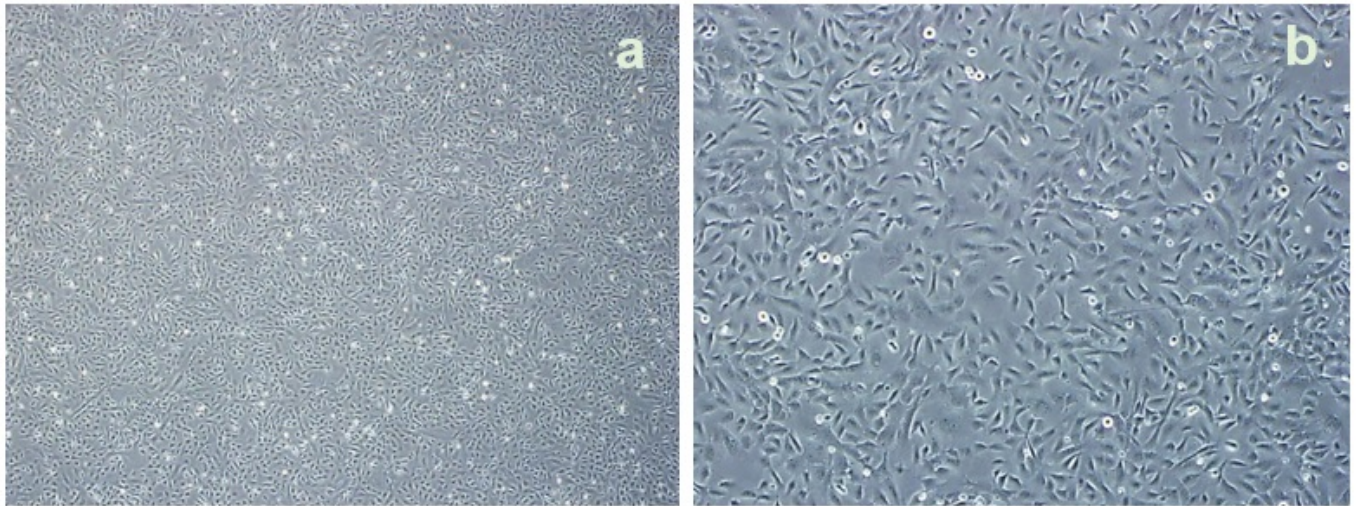
Fig.4 Bright field images of representative neural progenitor cells. Magnification = 40x, 100x respectively.

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).
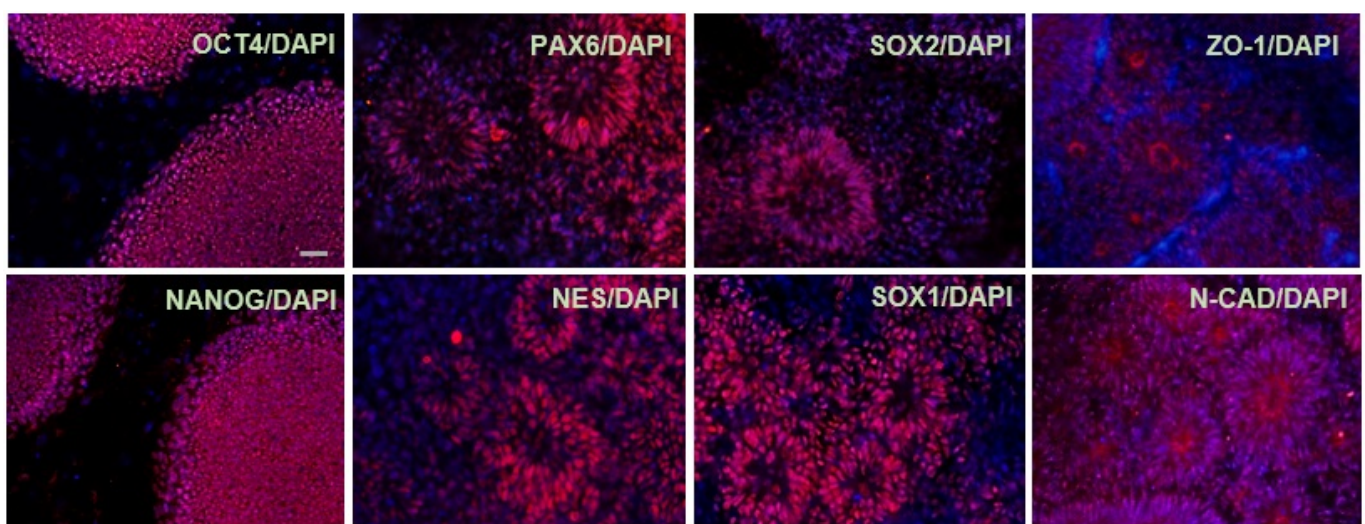

Fig. 5 Immunostaining of well-defined markers for iPSCs including OCT4 and NANOG, and for neural rosettes including PAX6, NES (NESTIN), SOX2, SOX1, ZO-1 and N-CAD (N-CADHERIN, also known as CDH2). Scale bar represents 50 µm.

Step 5.

1. Okita K, Matsumura Y, Sato Y, Okada A, Morizane A, Okamoto S, et al. A more efficient method to generate integration-free human iPS cells. Nat. Methods. 2011;8:409–12.
2. Zhang S-C, Wernig M, Duncan ID, Brüstle O, Thomson JA. In vitro differentiation of transplantable neural precursors from human embryonic stem cells. Nat Biotechnol. 2001;19:1129-33.
3. Kim DS, Lee DR, Kim HS, Yoo JE, Jung SJ, Lim BY, et al. Highly pure and expandable PSA-NCAM-positive neural precursors from human ESC and iPSC-derived neural rosettes. PLoS One. 2012;7.