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Chemically-Defined Neural Conversion of Human Pluripotent Stem Cells

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Abstract

Human pluripotent stem cells (hPSCs) are characterized by their ability to self-renew and differentiate into any cell type of the human body. To fully utilize the potential of hPSCs for translational research and clinical applications, it is critical to develop rigorous cell differentiation protocols under feeder-free conditions that are efficient, reproducible, and scalable for high-throughput projects. Focusing on neural conversion of hPSCs, here we describe robust small molecule-based procedures that generate neural stem cells (NSCs) in less than a week under chemically defined conditions. These protocols can be used to dissect the mechanisms of neural lineage entry and to further develop systematic protocols that produce the cellular diversity of the central nervous system at industrial scale.

Keywords

Pluripotency; embryonic stem cell; induced pluripotent stem cell; neural induction; cell differentiation; culture medium; coating substrate; small molecules; pathway inhibition

1. Introduction

1.1. Brief Overview: Neural Induction Strategies

Since the derivation of the first human embryonic stem cell (hESC) lines in 1998 by J. Thomson and colleagues [1], a number of protocols and their variations have been published aimed at differentiating pluripotent cells, including induced pluripotent stem cells (iPSCs) [2], into the derivatives of the three primary germ layers (ectoderm, mesoderm, endoderm). Early differentiation protocols mostly relied on embryoid body (EB) formation or overgrowth of cultures that lead to spontaneous and uncontrolled differentiation of pluripotent cells into mixed lineages [3, 4]. A strategy to enrich for neural cells takes advantage of the fact that some EBs, after plating on coated substrates such as laminin or Matrigel, can give rise to neural rosettes. These neural rosette structures, which mimic some aspects of the neural tube, can be manually collected under microscopic view or enzymatically detached from surrounding cell clumps and further expanded and differentiated into the three main neural lineages (neurons, astrocytes, oligodendrocytes) [4,

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5, 6]. These protocols are difficult to standardize across laboratories, and it is apparent that different scientists employ varying preferences and practical routines when applying protocols. Investigator bias and uncontrolled strategies pose significant challenges for developing standard operating procedures (SOPs) for drug discovery and clinical applications.

Co-culture of pluripotent cells with PA6 stromal cells exerting stromal- derived inducing activity (SDIA) was reported to enhance neural induction [7, 8]. However, co-culture of human ESCs with stromal cells up to 28 days was necessary to generate efficient numbers of PAX6-positive NSCs. The protracted neural induction process and the xenogeneic origin of PA6 cells are limiting factors for streamlined and efficient use of this approach for translational purposes.

More recently, manipulation of specific cell signaling pathways that promote neural fate choice has emerged as a more controlled strategy. Consistent with knowledge accumulated on experimental model systems in developmental biology (e.g., *Xenopus laevis*), it was reported that BMP4 antagonizes recombinant Noggin (an inhibitor of BMP signaling) and promotes neural specification of human ESCs [7, 9, 10]. Importantly, activin/nodal signaling pathways contribute to pluripotency maintenance, and inhibition of these pathways promotes neural conversion of human cells [11, 12, 13]. More recently, inhibition of BMP, TGF beta, and WNT pathways was shown to direct pluripotent cells into the neural lineage although significant differences exist in protocols that use small molecule- based neural induction strategies [14, 15]. We therefore tested and compared various chemically defined conditions and small molecules that promote neural conversion of hPSCs (Figs. 1, 2, 3 and 4).

1.2. Media Formulations and Coating Substrates

Similar to mouse ESCs, the first hPSC lines were cultured in the presence of fetal bovine serum and irradiated mouse embryonic fibroblasts (MEFs) as supporting feeder layers [1]. The use of fibroblast growth factor 2 (FGF2) maintains pluripotency and supports the expansion of hESCs, which is different to mouse ESCs that rely on leukemia inhibitory factor (LIF). Over the last 20 years, the stem cell field has embarked on developing more chemically defined media, coating substrates, and alternatives to xenogeneic material as this would limit potential clinical use and regenerative medicine applications. For instance, reagents or procedures such as high concentrations of FGF2, knockout serum replacement (KSR), mTeSR, Matrigel, and laminin-521 are helpful for growing hPSCs under more defined conditions and in the absence of mouse feeders [16, 17, 18, 19, 20]. As another important step forward, J. Thomson and colleagues developed an improved chemically defined medium that consists of eight components (DMEM/F12, L-ascorbic acid, selenium, transferrin, NaHCO₃, insulin, FGF2, TGF beta-1). Of note, this culture system obviates the use of human serum albumin [21]. Using E8 medium allows successful and consistent growth of hESCs and iPSCs, and cells can be passaged using EDTA instead of enzymatic treatments, which may cause karyotype abnormalities and alter cell growth [22, 23].

Cells cultured in the presence of E8 medium are maintained on recombinant vitronectin (VTN-N), which is a truncated protein of human vitronectin corresponding to amino acids 62–478. Accordingly, for cell differentiation purposes E6 medium can be used, which is E8

medium devoid of pluripotency-promoting factors FGF2 and TGF beta-1. Based on a recent report, E6 medium alone is highly efficient in converting pluripotent cells into neural stem cells expressing PAX6⁺ [24], and we therefore performed comparison with DMEM/F12 medium (Figs. 2, 3, and 4). However, the results of this careful comparison and a recent report by Studer and colleagues [25] demonstrate the importance of inhibition of BMP and TGF beta pathways for most efficient neural induction.

1.3. Cell Density

Cell density and cell-cell contact are generally considered as important parameters for cell culture experiments using normal or cancerous cells (e.g., contact inhibition, proliferation rate, cell size, mechanical force, metabolic adaptation). Indeed, growing PSCs at high cell densities can have various negative effects including DNA damage and genome instability [26]. Another paper [14] suggested that different cell densities may control hPSC differentiation into neuroectoderm (PAX6⁺) or neural crest (SOX10⁺), but more recently the same authors could not confirm a cell density effect on cell fate determination [25]. However, the neural induction protocols we tested here (low and high cell densities) indicate a clear cell density-dependent effect on neuroectoderm generation (Figs. 3 and 4). Therefore, we believe that controlling cell behavior based on providing appropriate culture conditions, including cell density, is of importance for formulating reproducible cell differentiation protocols and developing robust and scalable assays for translational research.

2. Materials

Human embryonic stem cell line WA09 (H9 ESC line, WiCell, Madison, WI, USA); results were reproduced with an iPSC line (iPSC-GR1.1, Lonza, Walkersville, MD; cell line generated on behalf of NIH Common Fund).

1. Essential 8 (E8) medium (Life Technologies cat. no. A1516401).
2. Essential 6 (E6) medium (Life Technologies cat. no. A1517001).
3. DMEM/F12 medium (Life Technologies cat. no. 10565018).
4. N2 supplement (Life Technologies cat. no. A1370701).
5. B27 supplement (Life Technologies cat. no. 12587010).
6. Accutase (Life Technologies cat. no. A1110501).
7. Vitronectin-N (VTN-N) (Life Technologies, cat no. A14700).
8. Dorsomorphin (Tocris, cat. no. 3093).
9. LDN-193189 hydrochloride (Sigma-Aldrich, cat. no. SML0559).
10. A83-01 (Tocris, cat. no. 2939).
11. PNU-74654 (Tocris, cat. no. 3534).
12. ROCK inhibitor Y-27632 dihydrochloride (Tocris, cat. no. 1254).
13. Dulbecco's phosphate-buffered saline (DPBS; Life Technologies, cat. no. 14040133).

14. DPBS without calcium or magnesium (Life Technologies, cat. no. 14190250).
15. UltraPure 0.5M EDTA (Life Technologies, cat. no. 15575020).
16. 6-well and 24-well tissue culture plates (Corning).
17. For immunocytochemistry rabbit anti-PAX6 antibody (Biolegend, cat. no. 901301).
18. For immunocytochemistry donkey anti-rabbit secondary antibody Alexa 488 (Thermo Fisher Scientific, cat. no. A-21206).
19. For flow cytometry anti-PAX6 Alexa Fluor 488-conjugated antibody (BD Pharmingen, cat. no. 561664).
20. For flow cytometry Alexa Fluor 488 isotype control (BD Pharmingen, cat. no. 565362).
21. Paraformaldehyde (PFA, Electron Microscopy Sciences, cat. no. 15714-S).
22. Bovine serum albumin (BSA; Cell Signaling Technology, cat. no. 9998S).
23. Fetal bovine serum (FBS; Sigma-Aldrich, cat. no. F4135).
24. Triton X-100 (Sigma-Aldrich, cat. no. T9284).
25. Hoechst (Life Technologies, cat. no. H3570).
26. Tween 20 (Affymetrix, cat. no. 20605).
27. Trypan blue (Life Technologies, cat. no. 15250061).

3. Methods

3.1. Equipment

1. Incubator:
Cells are maintained at 37 °C, 5% CO₂ in Forma Steri-Cult CO₂ incubators (Thermo Fisher Scientific, model #3310) outfitted with HEPA air filtration.
2. Microscopy:
Fluorescence images were taken using LEICA DMI8 epi-fluorescence microscopy equipped with HAMAMATSU CMOS camera ORCA-Flash4.0LT, DAPI and FITC filter sets. Settings for camera exposure time and excitation intensity are kept identical for images taken from different samples. Fluorescence images from the same channels are also presented using identical contrast settings.
3. Fluorescence-activated cell sorting (FACS):
Cells were analyzed using a BD LSRFortessa flow cytometer (Model #647794L6) equipped with 405 nm, 488 nm, 561 nm, and 635 nm lasers. Sample acquisition was done using FACSDiva v6.1.3, and the data were analyzed with FlowJo v10 software.

4. Cell counter:

Cells were diluted 1:1 in trypan blue and counted using a Countess II FL automated cell counter (cat. no. AMQAF1000).

3.2. Propagation of Pluripotent Cells

1. Human ESCs and iPSCs are maintained under feeder-free and xeno-free condition using E8 medium and VTN-N following the instructions of the manufacturer.
2. Coat culture vessels with VTN-N (1:100; diluted in DPBS without calcium and magnesium). Use 1 mL of diluted VTN-N to coat one well of a 6-well, and incubate for 1 h at room temperature or overnight at 4 °C sealed with parafilm. Aspirate VTN-N immediately before plating H9 cells, and make sure that plates don't dry out.
3. H9 cells are passaged when grown to 70–90% confluence, typically every 4 to 5 days.
4. Passage cells using 0.5 mM EDTA diluted in DPBS (without calcium or magnesium). Remove E8 medium, wash once with DPBS, and then incubate with 0.5 mM EDTA for 5 min at 37 °C. When H9 cells start rounding up, remove EDTA and add E8 medium. Pipet up and down gently to dissociate pluripotent cells into small clumps. Plate cells onto fresh plates at a splitting ratio of 1:6 to 1:12.
5. To promote cell recovery after dissociation, ROCK inhibitor Y-27632 (10 µM) can be used for 24 h but is typically not necessary for standard long-term maintenance of pluripotent cells if sufficient amount of cellular material is available at every passage.

3.3. Neural Induction

1. When grown to 70–90% confluence, wash pluripotent cells with DPBS, and then treat with Accutase for 5 min at 37 °C. Use 1 mL Accutase for each well of a 6-well plate.
2. When cells start detaching, add 1 mL E8 medium to dilute Accutase. Gently detach cells from the culture dish by pipetting up and down.
3. Transfer cells into 15 mL conical tube, and centrifuge at $300 \times g$ for 5 min
4. Gently remove media by aspiration (*see* Note 1).
5. Resuspend cell pellet in E8 medium and perform cell count.
6. Add ROCK inhibitor Y-27632 (10 mM) to increase cell viability.
7. Remove VTN-N from the coated plates and plate cells at a density of 50,000 or 200,000 cells/cm².
8. Twenty-four hours after cell plating, neural induction is initiated by switching from E8 medium to the various neural induction media, including DMEM/F12 +

N2B27 or E6 medium supplemented with DA, DAP, LA, or LAP (*see* Note 2 and Subheading 3.4 for abbreviations).

9. Feed cells everyday with fresh neural induction media for 6 days.
10. In the presence of small molecular inhibitors, at day 6 this neural induction protocol will have produced more than 90% PAX6⁺neural stem cells (*see* Note 3).
11. On day 6 cells can be analyzed for neural marker expression such as PAX6 expression by immunocytochemistry and flow cytometry (*see* Note 4 and Subheadings 3.5 and 3.6).

3.4. Comparison of Neural Induction Conditions

To allow direct comparison, the following base media and conditions were tested (Fig. 1):

1. E6 + 2 μ M dorsomorphin + 2 μ M A83-01 + 2 μ M PNU-74654 (DAP)
2. E6 + 2 μ M dorsomorphin + 2 μ M A83-01 (DA)
3. E6 + 100 nM LDN-193189 + 2 μ M A83-01 + 2 μ M PNU-74654 (LAP)
4. E6 + 100 nM LDN-193189 + 2 μ M A83-01 (LA)
5. DMEM/F12 + N2B27 + DAP
6. DMEM/F12 + N2B27 + DA
7. DMEM/F12 + N2B27 LAP
8. DMEM/F12 + N2B27 + LA

3.5. Immunocytochemistry

The volume of reagents listed below applies for one well of a 24-well plate:

1. Wash cells 2 $\times$ with 1 mL DPBS, Carefully add DPBS against the side of the well to avoid detaching the cells from the well.
2. Add 500 μ L 4% PFA and fix for 30 min at room temperature.
3. Wash 3 $\times$ with 1 mL DPBS.
4. Add 0.5 mL permeabilization/blocking buffer (DPBS, 0.3% Triton X-100, 5% BSA), and incubate for 1 h at room temperature.
5. Add rabbit anti-PAX6 antibody (1:200) diluted in Permeabilization/Blocking buffer, and incubate for 1 h at room temperature or overnight at 4 $^{\circ}$ C.
6. Wash 3 $\times$ with 1 mL PBS.
7. Add Alexa 488-conjugated donkey anti-rabbit secondary antibody (1:1000) diluted in permeabilization/blocking buffer, and incubate for 1 h at room temperature.
8. Wash 3 $\times$ with 1 mL PBS.

9. Add Hoechst (1:4000) diluted in DPBS, and incubate for 30 min at room temperature.
10. Wash with DPBS, and perform microscopic analysis (*see* Note 5 and Fig. 2).

3.6. FACS

1. For flow cytometry remove culture media, rinse cells with DPBS (without calcium and magnesium), add Accutase (1 mL to each well of a 6-well plate), and incubate for 3–5 min to achieve single-cell dissociation. Wash cells off the well with 1 mL DPBS (without calcium and magnesium). Collect cells in a 15 mL conical tube, and centrifuge at $300 \times g$ for 5 min. Gently remove supernatant. Wash cells two times with DPBS (without calcium and magnesium) at 4 °C. Centrifuge at $300 \times g$ for 5 min.
2. Resuspend cell pellet in 875 μ L DPBS (without calcium and magnesium) at 4 °C. Vortex to obtain a homogenous cell suspension.
3. Dropwise add 125 μ L of 32% PFA to cell suspension while vortexing (final PFA concentration is 4%). Incubate at room temperature in fixation buffer for 30 min. Centrifuge at $300 \times g$ for 5 min. Wash cells three times with DPBS (without calcium and magnesium) at 4 °C.
4. Resuspend cell pellet in 2 mL DPBS (without calcium or magnesium). Count cells and transfer 2×10^6 cells into a new 15 mL conical tube, centrifuge at $300 \times g$ for 5 min, and gently aspirate supernatant.
5. Resuspend cell pellet in permeabilization buffer (0.2% Tween 20 in DPBS), and incubate at room temperature for 20 min. Centrifuge at $300 \times g$ for 5 min, gently aspirate buffer and wash one time with DPBS.
6. Resuspend cell pellet in 1 mL of blocking buffer (0.5% BSA and 2% FBS in DPBS), and incubate on ice for 30 min. Centrifuge at $300 \times g$ for 5 min, and carefully aspirate supernatant.
7. Resuspend cell pellet in 200 nL of sorting buffer (0.5% BSA in DPBS) containing 0.05 μ g of anti-PAX6 Alexa Fluor 488-conjugated antibody or 0.05 μ g of Alexa Fluor 488 isotype control. Incubate on ice for 30 min in the dark. Centrifuge at $300 \times g$ for 5 min, and gently remove supernatant (*see* Note 6).
8. Wash cells three times with permeabilization buffer while protecting cells from light to minimize photobleaching. Centrifuge at $300 \times g$ for 5 min, and gently aspirate supernatant.
9. Resuspend cell pellet in 500 μ L of sorting buffer, and filter cells through a 40 nm cell strainer to remove cell clumps.
10. Analyze stained isotype and unstained control cells by flow cytometry (*see* Note 7 and Figs. 3 and 4)

Notes

1. To get accurate numbers, cell densities can be calculated using ImageJ or IncuCyte (Sartorius).
2. Use swing centrifuge with swing bucket rotor for pelleting cells, and resuspend cell pellet in a minimum of 1 mL. Using a fixed-angle centrifuge will result in a higher number of cell loss throughout protocol.
3. Neural stem cells at this stage are highly proliferative and can be further expanded using mitogens such as FGF2 and EGF (epidermal growth factor) or patterned to specific progenitors using morphogens such as retinoic acid (data not shown).
4. Cells can be plated onto 24-well plates depending on experimental design or to reduce the amount of antibody required for immunocytochemistry.
5. For immunofluorescence analysis, it is critical to include a negative control to determine level of background staining. Undifferentiated H9 cells grown in E8 medium for 6 days were used as a negative control for PAX6 staining. Once the level of background staining is established, the same threshold is applied to all the samples to subtract nonspecific staining from the acquired images. This is particularly important for automated imaging and high-content imaging.
6. If using non-conjugated primary antibodies, a second incubation with a fluorescent dye conjugated secondary diluted in sorting buffer is required. Always use isotype control at the same concentration ($\mu\text{g}/\mu\text{L}$) as the primary antibody.
7. For flow cytometry analysis, use software to set gates to subtract background from unstained cells and isotype control.

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Figure 1:

Overview of neural induction strategies tested. Undifferentiated OCT4⁺ cell colonies are dissociated into single cells, counted, and plated onto dishes coated with VTN-N (50,000 or 200,000 cells/cm²). From days 0 to 5 medium was changed daily using neural induction medium containing different combinations of small molecules. On day 6, PAX6 expression was analyzed using immunocytochemistry and FACS. Representative phase (PH) contrast images are shown on days 0, 3, and 6.

Figure 2:

Immunocytochemistry for PAX6 expression in hESCs (H9) cells after a 6-day neural induction. Comparison of E6 and DMEM/F12/N2B27 media and different combinations of small molecules. See Figs. 3 and 4 for quantification. Scale bars, 100 μ m.

A Low Cell Density

Sample	% PAX6 ⁺
Unstained	0.01%
Isotype Control	1.55%
E8	12.7%
DMEM	5.62%
DMEM + LA	69.7%
DMEM + LAP	70.1%
DMEM + DA	34.0%
DMEM + DAP	33.5%

B High Cell Density

Sample	% PAX6 ⁺
Unstained	1.01%
Isotype Control	1.19%
E8	14.7%
DMEM	57.4%
DMEM + LA	85.2%
DMEM + LAP	83.4%
DMEM + DA	85.4%
DMEM + DAP	85.6%

Figure 3:

Expression of PAX6 in hESCs (H9) detected by flow cytometry in DMEM/F12/N2B27 culture media using low (a) and high (b) cell densities (50,000 or 200,000 cells/cm²). After culturing hESCs in each culture condition for 6 days, cells were dissociated and analyzed for PAX6 expression with an anti-PAX6 Alexa 488-conjugated antibody. Table shows percentage of PAX6⁺ cells in each culture condition tested. Dot plots represent PAX6 labeling for each culture condition, isotype and unstained controls. Histograms represent PAX6 labeling for each culture condition, isotype and unstained controls.

A Low Cell Density

Sample	% PAX6 ⁺
Unstained	0.01%
Isotype Control	1.52%
E8	12.6%
E6	44.5%
E6 + LA	70.4%
E6 + LAP	69.5%
E6 + DA	60.8%
E6 + DAP	58.9%

B High Cell Density

Sample	% PAX6 ⁺
Unstained	1.01%
Isotype Control	1.19%
E8	14.7%
E6	27.8%
E6 + LA	90.7%
E6 + LAP	80.8%
E6 + DA	77.3%
E6 + DAP	85.0%

Figure 4:

Expression of PAX6 in hESCs (H9) detected by flow cytometry in E6 culture media using low (a) and high (b) cell densities (50,000 or 200,000 cells/cm²). After culturing hPSCs cells in each culture condition for 6 days, cells were detached, dissociated, and analyzed for PAX6 expression with an anti-PAX6 Alexa 488-conjugated antibody. Table shows percentage of PAX6⁺ cells in each culture condition tested. Dot plots represent PAX6

labeling for each culture condition, isotype and unstained controls. Histograms represent PAX6 labeling for each culture condition, isotype and unstained controls.

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