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Standardized Generation and Differentiation of Neural Precursor Cells from Human Pluripotent Stem Cells

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Abstract

Precise, robust and scalable directed differentiation of pluripotent stem cells is an important goal with respect to disease modeling or future therapies. Using the AggreWell™400 system we have standardized the differentiation of human embryonic and induced pluripotent stem cells to a neuronal fate using defined conditions. This allows reproducibility in replicate experiments and facilitates the direct comparison of cell lines. Since the starting point for EB formation is a single cell suspension, this protocol is suitable for standard and novel methods of pluripotent stem cell culture. Moreover, an intermediate population of neural precursor cells, which are routinely >95% $NCAM^{pos}$ and Tra-1–60^{neg} by FACS analysis, may be expanded and frozen prior to differentiation allowing a convenient starting point for downstream experiments.

Keywords

Pluripotent stem cells; differentiation; neural precursor; neurons

Introduction

Human pluripotent stem cells, both embryonic (hESCs) and induced (hiPSCs) hold great promise for the generation of cell types for disease-modeling, cell-based assays or indeed for future therapies. However, much of this is limited by the lack of effective standard protocols, which can generate differentiated cell types in sufficient numbers for such applications.

The efficient differentiation of human pluripotent stem cells to neurons has been the focus of much research (reviewed in Shwartz et al. [1]) with good progress being reported recently [2–4]. Many protocols, however, rely on the formation of embryoid bodies (EBs) or involve an EB-like step [2, 4–14] which, by its subjective nature, represents a great source of variability in any differentiation protocol [15, 16]. In the area of neuronal differentiation, efforts have been made to eliminate this step [3, 17–20] or to standardize it using mechanical dissection [21] or round-bottomed 96-well plates [22]. The AggreWell™400 system (Stem Cell Technologies) is a development of the latter concept whereby each well contains 1200 microwells of 400μm diameter. This allows 1200 EBs, of specific and uniform size up to 5000 cells per EB, to be generated from a single well thus simplifying harvest.

The authors declare no potential conflicts of interest.

We have used AggreWell™400 plates to standardize the EB step in a modified version of the mouse ESC five-stage neuronal differentiation protocol of Lee et al. [23] (Fig. 1). This protocol results in a highly robust and scalable method for deriving neural precursor cells (NPCs) from hESCs or hiPSCs. Briefly, EBs are initially generated in hESC medium containing Y27632 (ROCK inhibitor) [22, 24] in an AggreWell™400 plate and subsequently cultured in low attachment plates in medium containing B27 supplement minus Vitamin A (Neuronal Precursor Medium; NPM) with noggin and basic fibroblast growth factor (bFGF) [5]. After 2 weeks, the EBs are plated on standard tissue culture plasticware in a minimal medium containing insulin, transferrin, selenium and fibronectin (ITSFn) which has previously been shown to select for nestin-positive cells [25]. Neuroepithelial cells that have emerged from the EBs are collected after 7–8 days and replated on poly-D-ornithine/laminin-coated plates in medium supplemented with NPM, bFGF and epidermal growth factor (EGF). Neural precursor cells thus generated demonstrate appropriate cell morphology (Fig. 2F) and marker expression as determined by FACS analysis (Fig. 2A–D), RT-PCR (Fig. 2E) and immunostaining (Fig. 2G). The NPCs may be expanded in this growth medium through at least 10 passages (1000 fold increase) if maintained at high density. Cells may also be frozen at this stage to generate a bank of developmentally similar cells (Supplementary Fig. 1). This provides a convenient starting point for downstream analysis of neuronal differentiation pathways, amongst others, and facilitates comparison of multiple experiments.

For subsequent differentiation of the NPCs to neurons, cells are plated at low density in growth medium for 24 hours after which the bFGF and EGF are withdrawn. After a further 48 hours, the medium is again replaced with Neurobasal medium supplemented with B27, brain-derived neurotrophic factor (BDNF) and glial cell-derived neurotrophic factor (GDNF). Under these conditions, the majority of cells exhibit positive immunostaining for the neuronal marker, β-tubulin III (TuJ1; Fig. 2H and Supplementary Fig. 2). It may be envisaged that optimization of growth factors and media may allow the selection of various neuronal subtypes. Since this protocol has been optimized for the generation of neurons using high concentrations of noggin [26], we find few cells positive for glial markers even after exposure to platelet derived growth factor (PDGF), ciliary neurotrophic factor (CNTF) and L-3,3',5-Triiodothyronine (T3).

Materials and Equipment

hESC culture medium

400ml DMEM:F12 (Invitrogen #11330-032)

100ml Knockout Serum Replacer (Invitrogen #10828-028)

5ml Non essential amino acids 100x (Invitrogen #11140-050)

2.5ml L-Glutamine (1mM; Invitrogen #25030-081)

3.5μl β-mercaptoethanol (0.1mM; Sigma #7522)

4ng/ml Fibroblast growth factor-2 (bFGF, R&D Systems Inc. #233-FB)

A stock solution of 10μg/ml is prepared in D-PBS containing 0.1% bovine serum albumin. Working aliquots are stored at −20°C and upon thaw should be stored at 4°C and used within 2 weeks.

Storage at 4°C, not to exceed 2 weeks

Neural Precursor Medium

48.5ml DMEM (Invitrogen #11965-044)

1ml B27 supplement minus Vitamin A (Invitrogen #12587-010)

0.5ml L-Glutamine (2mM; Invitrogen #25030-081)

Storage at 4°C, not to exceed 2 weeks

ITSFn Medium

49ml DMEM/F12 (Invitrogen #10565-018)

0.5ml Insulin-Transferrin-Selenium-A Supplement (100X) (Invitrogen #51300-044).

0.5ml L-glutamine (1mM; Invitrogen #25030-081)

5μg/ml human fibronectin (Roche Applied Sciences #11080938001)

Storage at 4°C, not to exceed 2 weeks

Reagents

D-PBS (Invitrogen #14190-250)

Collagenase IV (Invitrogen #17104-019)

A stock solution of 1.5mg/ml is prepared in DMEM:F12 (Invitrogen #11330-032), and frozen in working aliquots at –20°C. Aliquots are thawed immediately prior to use.

Accutase (Innovative Cell Technologies, Inc. #AT104)

Y27632 (ROCK inhibitor - Selective inhibitor of Rho-associated protein kinase ^{p160}ROCK, EMD Biosciences #688000).

A stock solution of 5mM is prepared by dissolving 0.5mg in 296μl of sterile distilled water. Working aliquots are stored at −20°C and upon thaw should be stored at 4°C and used within 2 weeks.

Recombinant human Noggin (R&D Systems #6057-NG-100)

A stock solution of 100μg/ml is prepared in D-PBS containing 0.1% bovine serum albumin. Working aliquots are stored at −20°C and upon thaw should be stored at 4°C and used within 2 weeks.

Epidermal growth factor (EGF, R&D Systems #236-EG)

A stock solution of 20μg/ml is prepared in D-PBS containing 0.1% bovine serum albumin. Working aliquots are stored at −20°C and upon thaw should be stored at 4°C and used within 2 weeks.

Brain derived neurotrophic factor (BDNF, R&D Systems #248-BD)

A stock solution of 10μg/ml is prepared in D-PBS containing 0.1% bovine serum albumin. Working aliquots are stored at −20°C and upon thaw should be stored at 4°C and used within 2 weeks.

Glial cell derived neurotrophic factor (GDNF, R&D Systems #212-GD)

A stock solution of 20μg/ml is prepared in D-PBS containing 0.1% bovine serum albumin. Working aliquots are stored at −20°C and upon thaw should be stored at 4°C and used within 2 weeks.

Distilled water (Mediatech #25-055-CM)

Poly-D-ornithine (Sigma #P3655)

A stock solution of 10mg/ml is prepared in sterile distilled water. Working aliquots are stored at −20°C and upon thaw should be stored at 4°C and used within 2 weeks.

Mouse laminin (Sigma #L2020)

Neurobasal medium (Invitrogen #21103-049)

mTeSR1 medium (Stem Cell Technologies #05850)

ENStem-A™ Neuronal Differentiation Medium (Millipore #SCM017)

ENStem-A™ Neural Freezing Medium (Millipore #SCM011)

Equipment

Cell scrapers (Costar #3010)

AggreWell™400 plate (Stem Cell Technologies #27845)

Low attachment 6-well culture dishes (Corning #3471)

Tissue culture-treated 6-well plates

Benchtop low speed centrifuge

Microplate carrier for centrifuge

Tissue culture incubator −37°C/5% CO_2

Methods

Embryoid Body (EB) Formation

1. If starting from colonies grown on feeders, wash cells once with D-PBS and add 1ml pre-warmed collagenase IV (1.5mg/ml) per well of a 6-well plate. If starting with cells on Matrigel go directly to step 6.
2. Incubate at 37°C for 20 mins at which time the plate should be tapped sharply. If the colonies do not detach at this time the plate should be incubated for a further 10–20mins and the process repeated. If the majority of colonies have not detached within 1 hour they may be removed using a cell scraper.
3. Remove colonies and transfer to a 15ml tube, wash each well with 1–2ml DMEM:F12 and add wash to the 15ml tube.
4. Allow to sediment for 3 minutes, aspirate down to the pellet, wash with 5ml DMEM:F12 without disrupting the colonies and allow to sediment again.
5. Wash with 5ml D-PBS and allow to sediment.
6. Add 1ml Accutase and incubate at 37°C for 10 mins, agitating by swirling the tube after 5 mins.
7. Add 5ml DMEM:F12 per 1ml Accutase used and pipet up and down gently.
8. Centrifuge at 200g for 5 mins.
9. Remove wash and resuspend in hESC culture medium to a density of 1.2×10^6 cells/ml in hESC medium containing 10μM Y27632 (ROCK inhibitor). This should generate approximately 1200 EBs of size 1000 cells per EB.

10. Prepare the AggreWell plate according to the manufacturer's instructions using hESC culture medium containing 10μM Y27632 (ROCK inhibitor) and add 1ml of cell suspension per well.
11. Spin at 200g for 5 mins and incubate in the tissue culture incubator for 24 hours
12. To harvest, the EBs are dislodged from the AggreWell by forceful ejection of the culture medium present in the well using a 1ml pipetman and transferred to a 15ml tube using a 2ml pipette. Do not triturate.
13. A further 1.5–2ml of medium is added and step 12 repeated.
14. Repeat until the majority of EBs are removed – no more than 5 times total.
15. Allow the EBs to sediment for 5 mins and aspirate the supernatant.
16. Transfer EBs from one well of the AggreWell plate to one well of a low attachment 6-well culture dish in 3ml hESC medium per well and return to the incubator.

Generation of Neural Precursors

1. Twenty-four hours after harvest, transfer the EBs to a 15ml tube and allow to sediment for 5 mins.
2. Aspirate the medium and replace with NPM containing 500ng/ml noggin and 20ng/ml bFGF. Return EBs to the low-attachment well.
3. Change the medium by sedimentation every 2–3 days for 2 weeks.
4. Change the medium to ITSFn and transfer to standard tissue culture plasticware at a ratio of 1 well EBs to 1 well of a 6-well culture dish. EBs should attach and neuroepithelial-like cells should emerge.
5. Change the medium every other day for 7–8 days.
6. Wash the cells once with D-PBS, add 1ml of Accutase and incubate at 37°C for 10mins.
7. Remove all detached lumps and single cells to a 15ml tube and wash with NPM. Allow the biggest aggregates to sediment briefly (30secs), then remove the upper cell suspension to a new 15ml tube.
8. Spin the cell suspension at 200g for 5 mins.
9. Resuspend in NPM containing 20ng/ml each of bFGF and EGF and plate on pre-coated poly-D-ornithine/laminin plates at $1.5–2\times10^6$ cells per well of a 6-well dish. This is considered p0 for neural precursors.
10. Cells may be passaged 1:2 or 1:3 (first passage only) with Accutase for expansion or 1:12 for differentiation.
11. Cells may be frozen using ENStem-A™ Neural Freezing Medium. We recommend the addition of 10μM Y27632 (ROCK inhibitor) upon thawing.

Poly-D-ornithine/Laminin Coated Plates

1. Add 2ml of filter-sterilized 20μg/ml poly-D-ornithine in distilled water per well of a tissue culture-treated 6-well plate.
2. Incubate overnight at 37°C.
3. Wash once with distilled water and add 2ml of 10μg/ml laminin diluted in DPBS.

4. Incubate overnight at 37°C.
5. To use, aspirate laminin immediately prior to plating cells. Do not wash.
6. For culture on glass, the concentrations of poly-D-ornithine and laminin should be adjusted to 50μg/ml and 20μg/ml respectively.
7. Precoated plates may be stored at −20°C after incubation is complete – do not remove the laminin.

Differentiation of Neural Precursor Cells to Neurons

1. Passage the neural precursor cells 1:12 onto poly-D-Ornithine/laminin coated plates in NPM containing 20ng/ml each of bFGF and EGF.
2. After 24 hours, exchange the medium for NPM without growth factors.
3. After a further 2 days, exchange the medium for Neurobasal medium supplemented with 2mM glutamine, 1× B27 supplement, 10ng/ml BDNF and 2ng/ml GDNF.
4. Exchange medium every two to three days for up to 10 days or longer if cultures remain healthy.

Notes

This protocol describes a robust, reproducible method for the generation of embryoid bodies using the AggreWell™400 system. Neural precursors may also be generated from colony-derived EBs, which does not require the use of the AggreWell™400 plate and associated reagents. Detached colonies may be cultured directly in NPM containing noggin and bFGF (Generation of Neural Precursors step 2.). Similarly, single cell suspensions may be aggregated without the use of the AggreWell™400 plate using 24-well low attachment dishes or in 96-well plates as described by Eiraku et al [22]. In 24-well plates, 1.5×10^6 cells are plated per well in hESC medium containing 10μM Y27632 (ROCK inhibitor) and incubated overnight at which time EBs from all wells may be combined for step 2 of Generation of Neural Precursors. Under these conditions, however, the uniformity and reproducibility of the AggreWell™ is lost. It should be noted that high quality cultures with a low proportion of spontaneous differentiation are essential for success with any approach.

For differentiation, cells may also be cultured in mTeSR1 medium (Stem Cell Technologies) or in Neuronal Differentiation Medium (NDM: Millipore) (Supplementary Fig. 2). Cells differentiated in mTeSR1 appear to maintain some proliferative capacity and serial passaging may enrich for neuronal precursors. After initial differentiation in mTeSR1, medium may be exchanged for other differentiation media such as NDM or Neurobasal medium containing B27, BDNF and GDNF. Cells at this stage may also be differentiated on 1.25% Matrigel (BD Biosciences) instead of poly-D-Ornithine/laminin. Precoated poly-D-Ornithine/laminin plates from BD may also be used but, in our hands, do not perform as well as self-coated plates.

This protocol has been used successfully for the differentiation of 6 hESC lines (H1/WA01, H7/WA07, H9/WA09, H14/WA14, HSF-6/UC06, SA01) and 3 iPSC lines derived in-house. Figure 2 shows representative data from H1 and HSF-6 hESCs and an iPSC line (SCU-i10) which was derived from bone marrow stromal cells [27] by lentiviral transduction of the four Yamanaka reprogramming factors [28, 29] using StemCCA (Millipore). SCU-i10 expresses pluripotent stem cell markers, SSEA-4, Tra-1–60 and Tra-1–81, is negative for SSEA-1 (Supplementary Fig. 3A) and had a normal karyotype at p40 (Supplementary Fig. 3B). Using published methods [30, 31], this line was able to differentiate not only along the ectodermal lineage, as we show here by neuronal differentiation, but also toward endoderm,

as exhibited by HNF4A and albumin immunostaining (Supplementary Fig. 3C), and mesoderm, demonstrated by spontaneously beating patches of cells (Supplementary Fig. 4D and Supplementary Movie 1).

Since the initial state of pluripotent cells required for EB formation is a single-cell suspension, the protocol may be used with cells grown under various culture conditions. Apart from standard feeder-dependent colony-based culture, cells grown under feeder-free conditions using standard substrates (reviewed by Mallon et al [32]) or novel alternative substrates such as Synthemax (Corning) and StemAdhere (Stem Cell Technologies) may also be used. On these alternative substrates, colony size and fragments recovered may be relatively small and otherwise not suitable for EB formation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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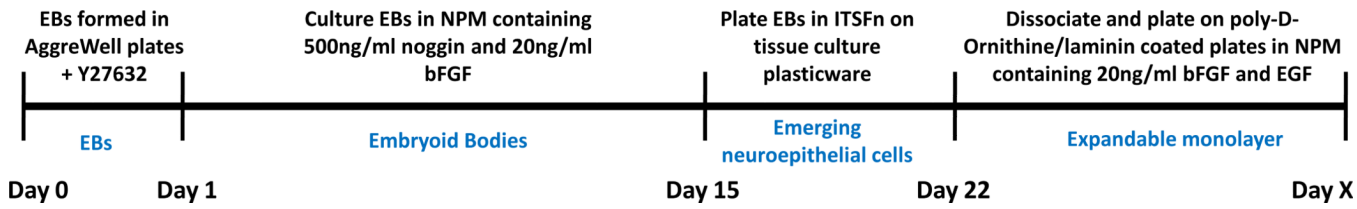

Figure 1.
Timeline of steps involved in neural precursor cell generation from human pluripotent stem cells via EB formation.

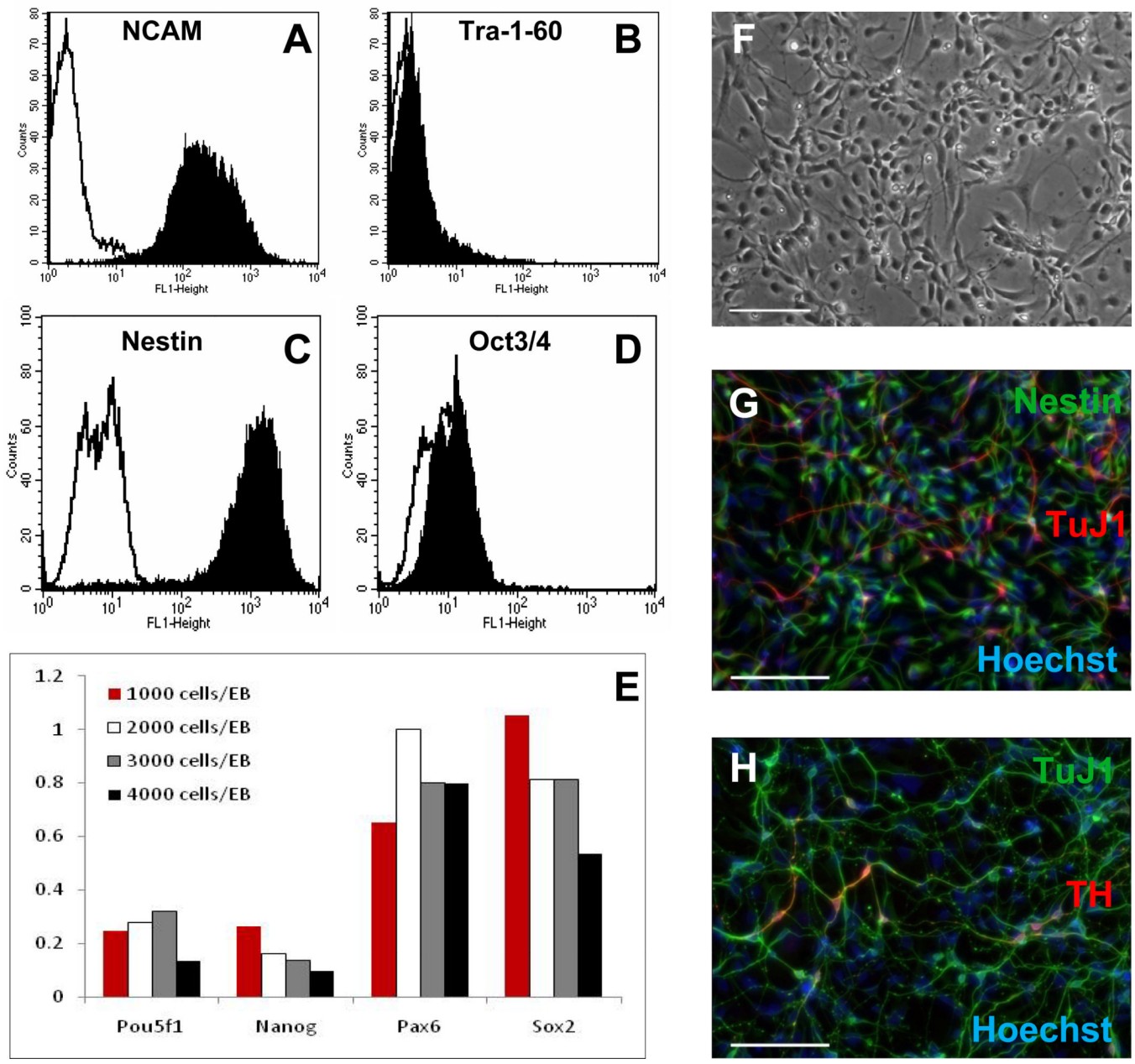

Figure 2.

Analysis of neural precursor cells. A&B) FACS analysis of neural and pluripotent stem cell marker expression in SCU-i10-derived NPCs at p3 - A) IgM isotype control (white) and NCAM (black), B) IgM isotype control (white) and Tra-1–60 (black); C&D) FACS analysis of neural and pluripotent stem cell marker expression in H1-derived NPCs at p2 – C) IgG1 isotype control (white) and Nestin (black), D) IgG2b isotype control (white) and Oct3/4 (black). E) RT-PCR analysis of neural and pluripotent stem cell marker expression in HSF-6-derived NPCs at p0 from EBs of different sizes. Data were generated using a Sabiosciences PCR array and were analyzed and normalized using the company's proprietary control gene panel and software. F) Phase image of SCU-i10 NPCs at p3, day1. Scale bar=100μm; G) Immunostaining of SCU-i10 NPCs p3, day 3 for neural precursors with anti-Nestin (green) and for neurons with anti-TuJ1 (red). Hoechst stain (blue). Scale

bar=100μm; H) Immunostaining of differentiated SCU-i10 NPCs p2 for all neurons with anti-TuJ1 (green) and for dopaminergic neurons with anti-tyrosine hydroxylase (red). Hoechst stain (blue). Cells were plated in NPM with bFGF and EGF for 4 days, growth factors withdrawn for 3 days and further differentiated in Neurobasal medium containing B27, BDNF and GDNF for 4 days. Scale bar=100μm.