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Derivation of telencephalic oligodendrocyte progenitors from human pluripotent stem cells

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Abstract

Oligodendrocytes are the main myelinating cell of the adult CNS and are vulnerable to injury in diverse disorders such as spinal cord injury, stroke, trauma, pharmacological and radiation toxicity, as well as neuroinflammation. Human pluripotent stem cells are attractive sources of oligodendrocyte lineage cells and provide a promising treatment strategy for exogenous myelin repair through transplantation. This unit describes a protocol for the step-wise differentiation of forebrain late oligodendrocyte progenitor cells (OPCs) from human pluripotent stem cells in defined chemical *in vitro* culture conditions. It involves a stepwise progression of oligodendrocyte progenitors through their known developmental phases, starting with the expression of appropriate transcription factors (Olig2, Nkx2.2), the upregulation of PDGFRA, followed by the appearance of O4-expressing cells, then O1 expression and finally mature myelin-binding protein (MBP) expressing cells. Validation of cell fate is performed by extensive transcriptomal profiling, as well as *in vitro* myelination essays with hESCs derived neuronal cells. Recapitulating forebrain oligodendrocyte development may generate cells more suitable for transplantation strategies for disorders primarily involving the telencephalon.

Keywords

Human embryonic stem cells (hESCs); Human induced pluripotent stem cells (hiPSCs); Oligodendrocyte; Myelin; Oligodendrocyte differentiation; *In vitro* myelination

BASIC PROTOCOL

Introduction

Human pluripotent stem cells (hPSCs) are capable of self-renewal, and can differentiate into an array of somatic cells, including oligodendrocytes. Derivation of oligodendrocyte precursor cells (OPCs) from hESCs is a valuable tool to study human oligodendrocyte development and provides an unlimited source of myelin producing cells for transplantation and repair in human primary and secondary demyelinating diseases. Here, we present a protocol for derivation of telencephalic late oligodendrocyte progenitor cells (OPCs) from

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hESCs, which are suitable for transplantation approaches and for modeling disorders primarily involving the forebrain.

The protocol here described involves several stages, including neural induction, patterning and expansion of neural precursor cells (NPCs), oligodendrocyte progenitors (OPCs) proliferation and differentiation. The first step in this protocol is the rapid conversion of hESCs into forebrain neural progenitors by applying a small-molecule-based strategy via the modified dual SMAD-inhibition method. After the conversion into neuroectoderm, cells are passaged and patterned toward neural precursors (NPCs) through the neural rosette stage. Following the NPCs proliferation step, glial media is used to enrich for oligodendrocyte progenitors cells and support OPCs proliferation and differentiation. From *in vitro* culture day 70, hESCs derived late OPCs can be purified using antibody-mediated flow cytometry sorting (FACS). To evaluate myelination capacity of hESCs derived OPCs, we have developed a novel *in vitro* assay in which both neuronal cells and oligodendrocytes are derived from hESCs. The protocol for human iPS cell differentiation is identical and has been equally tested (Piao et al., 2015).

This unit begins with the Basic Protocol, which describes the derivation of oligodendrocytes from hESCs. Support Protocol 1 details the characterization of the cells at different stages of differentiation by immunostaining. Support Protocol 2 covers the method for selective enrichment of oligodendrocyte population using Fluorescence Activated Cell Sorting (FACS). This is followed by Support Protocol 3, which describes the protocol for establishing a human embryonic stem cell based *in vitro* myelination assay. Lastly, Support protocols 4 and 5 summarize the methods to maintain hESCs and hiPSCs in co-culture with mouse embryonic fibroblasts (MEFs) and for coating the dishes with gelatin, matrigel and Poly-L-ornithine, Laminin and Fibronectin.

Note: All procedures should be performed under sterile conditions in Class II Biological Hazard Flow Hoods. All centrifugations are done for 5 minutes at $200 \times g$ unless otherwise indicated.

Materials

hESCs/hiPSCs cultured on a feeder layer (see Support Protocol 4) in 10cm culture dishes

hESC medium (see recipe)

Matrigel coated 6 well cell culture plates (see recipe)

0.05% Trypsin-EDTA (Gibco-Life Technologies)

Accutase (Innovative Cell Technologies)

MEF conditioned hESC medium (CM) (see recipe)

KSR medium (see recipe)

N2 medium (see recipe)

B27 Supplement (50 $\times$), minus vitamin A (Gibco-Life Technologies)

HBSS with 15mM HEPES (see recipe)

10µg/ml FGF2

500µM LDN193189

10mM SB431542

10mM XAV939

2mM Purmorphamine

10mM Y-27632

10µg/ml AA

20 µg/ml T3

100mM dibutyl cAMP

Growth factors: 10µg/ml BDNF, 100µg/ml FGF8, 10µg/ml PDGF-AA, 10µg/ml IGF-1

PO/Lam/FN coated 10 cm cell culture dishes (see recipe)

DPBS (no calcium, no magnesium; Gibco-Life Technologies)

P200 and P1000 pipette

1ml syringe with a 27 G needle

Glass hemocytometer

15 ml conical polypropylene centrifuge tubes

1.5 ml microcentrifuge tubes

Cell lifters

5 ml and 10 ml serological pipettes

45 µm cell strainers

Centrifuge

Inverted microscope

(I) Plating human embryonic stem cells (hESCs) for neural induction

1. hESCs and hiPSCs were cultured in 10cm dishes on mouse embryonic fibroblasts (MEFs) as described in detail in Support Protocol 4.
2. Prepare Matrigel coated 6 well cell culture plate before starting the differentiation as described in Support Protocol 5.
3. When starting differentiation, first clear away the mouse feeders cells: aspirate hESC medium (see the recipe) and add 4 ml of 0.05% Trypsin-EDTA to the cells. Shake the dish horizontally for 3 minutes and confirm the MEFs lift off the plate under the microscope, while the hESCs remain attached as intact colonies.

4. Immediately aspirate the 0.05% Trypsin-EDTA solution and add 5 ml of hESC media for washing.
5. Aspirate hESC media, add 5ml of accutase to 10cm dish (2ml/6cm dish) and incubate at 37°C (incubator) for about 20–30 minutes, until single cell suspension is obtained.
6. Collect the single cell suspension in 15 ml conical tube using 10 ml pipet, add 5 ml of hESC media and centrifuge.
7. Aspirate the supernatant, add 5 ml of hESC media, and gently dissolve the pellet by pipetting using 10 ml pipet and centrifuge (first wash).
8. Repeat step 7 (second wash).
9. After the second wash, aspirate the supernatant and resuspend the cell pellet in hESC media and filter them through a 45 µm strainer to eliminate any remaining clumps. Determine the cell concentration using a hemocytometer and spin them down.
10. Aspirate the supernatant and resuspend hESCs in MEF conditioned hESC medium (CM) supplemented with FGF2 (10 ng/ml) and Y-27632 (10µM). Aspirate the Matrigel solution from a previously prepared 6 well cell culture dish and directly plate cells at a density of 40–70×10³ cells/cm² (day 0). Next day the differentiation can be initiated if the cells are 95–100% confluent.

(II) Neural conversion of human embryonic stem cells

1. To initiate differentiation (day 1) aspirate MEF conditioned hESC medium and add KSR medium containing LDN193189 (200nM) and SB431542 (10µM).
2. On day 2 of differentiation aspirate KSR media and feed the cells with KSR supplemented with LDN193189 (200nM), SB431542 (10µM) and XAV939 (5µM).
3. On day 4 of differentiation aspirate KSR media and add a mixture of KSR medium (25%) and N2 medium (75%) with LDN193189 (200nM), SB431542 (10µM) and XAV939 (5µM).
4. On day 5 of differentiation aspirate media and add fresh mixture of KSR media (25%) and N2 medium (75%) supplemented with LDN193189 (200nM), SB431542 (10µM), XAV939 (5µM) and Purmorphamine (1µM).
5. On day 7, aspirate media and feed the cells with a mixture of KSR medium (50%) and N2 medium 50%) containing LDN193189 (200nM), SB431542 (10µM) and Purmorphamine (1µM).
6. On day 8 of differentiation add fresh mixture of KSR medium (25%) and N2 medium (75%) with LDN193189 (200nM), SB431542 (10µM) and Purmorphamine (1µM).
7. On day 10 feed cells with N2 medium containing of BDNF (20ng/ml), AA (0.2mM) and Purmorphamine (1µM).

Optional: For more efficient neural induction try sooner replacement of KSR with N2 medium: on day 2 feed cells with a mixture of 50% KSR medium and 50% N2 medium. On day 4 use the mixture of 25% KSR medium with 75% N2 medium and on day 6 switch to 100% N2 medium. Alternatively, completely avoid KSR by plating the hESCs directly in the N2 media (step (I) 10) supplemented with LDN193189 (200nM), SB431542 (10μM) and Y-27632 (10μM) (day 1).

(III) Droplet replating method for neural precursors specification (NPCs)

followed by expansion—On day 12, cells can be passaged as single cells and plated onto PO/Lam/FN coated dishes.

1. 1. Two days before replating, prepare poly-L-ornithine (PO), laminin (Lam) and fibronectin (FN) coated 10 cm dishes (see recipe).
2. 2. Aspirate Lam/FN mixture from prepared 10 cm plates and let them dry at RT for 30 minutes.
3. 3. Aspirate N2 media from the 6 well cell culture plates and add Accutase (1.5 ml per single 6 well within 6 well cell culture plates). Incubate for approximately 30 minutes at 37°C until a single-cell suspension is obtained.
4. 4. Transfer the cell suspension into a 15 ml conical centrifuge tube and gently mix with 5–8 ml of filtered DMEM/F12 medium using a 10 ml pipette. Spin down the cells.
5. 5. Aspirate the supernatant, add 5 ml of filtered DMEM/F12 medium, dissolve the pellet by pipetting up and down, using 10 ml pipette and spin the cells down (first wash).
6. 6. Repeat step 5 (second wash).
7. 7. After the second wash, resuspend and triturate the cells in N2 medium, filter the cell suspension using 45μm cell strainer and count the cells using a hemocytometer.
8. 8. Spin the cells down and resuspend them in N2 medium containing B27 Supplement (1×), minus vitamin A (termed N2/B27) and supplemented with BDNF (20ng/ml), AA (0.2mM), Purmorphamine (1μM) and Y-27632 (10μM), at a concentration of 150,000–250,000 cells/15 μl. Plate 15 μl droplets individually onto the dried PO/Lam/FN coated 10 cm dishes and let them stand at RT for 20 minutes.

Note: Approximately, 30 to 40 droplets can be placed in one 10cm dish.

9. 9. After 20 minutes, carefully, without disturbing the integrity of the separate droplets, add 20 ml of N2/B27 with the above mentioned supplements and incubate at 37 °C.

Note: This is the most critical part of protocol. Replating the cells at high density is important for successful specification of CNS neural cells. Low replating

density will result in the appearance of large numbers of predominantly neural crest cells. Since there is a big difference in cell survival (after replating) among different hESCs and hiPSCs lines, optimal replating densities have to be adjusted for each line separately.

Optional: If cell survival after passaging on day 12 is poor, you may postpone replating until day 16.

10. After replating, cells are patterned toward neural precursors for two weeks. During the first week of rosette patterning, N2/B27 media containing BDNF (20ng/ml), AA (0.2mM) and Purmorphamine (1μM) is used to feed the cells every second day.
11. After seven days, switch to N2/B27 media supplemented with BDNF (20ng/ml), AA (0.2mM), Purmorphamine (1μM) and FGF8 (100ng/ml). Feed the cells every second day for another five to seven days, at which point, they will be passaged by mechanical picking (P2 stage).
12. Two days before passaging the cells, prepare freshly PO/Lam/FN coated 10 cm dishes.
13. Passage the cells by mechanical picking of CNS clusters between day 25 and day 30 (termed passage 2, P2). Mechanical cluster picking is done in a laminar flow hood with an embedded dissecting microscope. Place the 10 cm dish under the microscope and, using a 27 G needle attached to a 1ml syringe, gently cut out and lift up mostly the central part of each plated droplet, avoiding the droplet edges which are usually enriched in neural crest cells. Collect the lifted droplets with a P1000 pipette in a 15 ml conical centrifuge tube.

Note: Approximately, combine droplets picked from two 10 cm dishes into one new 10 cm dish.
14. Add 5–6 ml of N2 medium and triturate droplets gently, with a 5 ml pipette, to break them up in smaller cell clusters.
15. After breaking the clusters, fill up the tube volume up to total of 8–10ml with N2/B27 medium supplemented with BDNF (20ng/ml), AA (0.2mM), Purmorphamine (1μM) and FGF8 (100ng/ml).
16. Aspirate Lam/FN solution from already prepared 10cm dishes and directly plate the cell clusters. Shake the dish several times horizontally to equally distribute the cell clumps and move dish to the incubator.

Optional: Alternatively, mechanical picking can be done using 1ml syringe with a 27 G needle. Under the dissecting microscope, gently cut out, lift up and aspirate the central parts of droplets into 1 ml syringe. After filling up the syringe, slowly pull out the cell clusters directly into PO/Lam/FN coated dish from which, just prior, Lam/FN solution was aspirated. Add up to 8–10 ml of N2 media containing above-mentioned factors. In this way dissociation of cell clusters into smaller cell aggregates can be more efficient by simply aspirating them and pulling them out of the 1 ml syringe.

17. The NPCs are allowed to proliferate for the next 10 days (day 35–day 40) in the media supplemented with BDNF (20ng/ml), AA (0.2mM), Purmorphamine (1μM) and FGF8 (100ng/ml). Media change is every two days. (Fig. 2A, B)

(IV) Oligodendrocyte proliferation and differentiation

1. To enrich for oligodendrocyte progenitors, cells from the day 35–40 stage described in section III) are cultured in N2/B27 medium containing platelet-derived growth factor (PDGF; 20 ng/ml), insulin-like growth factor 1 (IGF-1; 20 ng/ml), triiodothyronine (T3; 60 ng/ml), dibutyryl cAMP (0.1mM–0.2mM). This is referred to as “glial media”. Change the media every two days.

Note: Purmorphamine, a Smoothed agonist, can be included in the glial media, since it has a positive effect on OPCs survival and proliferation although it is often not necessary.

Optional: Try switching to glial media earlier, immediately after mechanical picking of the NPCs, from P2, but keep the FGF8 (100ng/ml) until day 40.

2. If the cultures are relatively free of neural crest cells, they are passaged every two weeks, starting from P2, using $\text{Ca}^{2+}/\text{Mg}^{2+}$ - free HBSS with 15 mM HEPES (see recipe). The splitting ratio is 1:2.

Passaging: Incubate culture dish with HBSS with 15 mM HEPES (3ml/6cm dish; 6ml/10cm dish) for 45 minutes to 1 hour at RT. Using a cell lifter, detach the cells from dish surface and transfer them to a 15 ml tube. When the pellet settles down, aspirate the HBSS, add fresh glial medium and break up the clusters into smaller aggregates by pipetting up and down with a 5 ml pipette. Remove the Lam/FN solution from a previously PO/Lam/FN-coated dish and directly plate the cells in the total volume of 8–10 ml of glial medium.

If there is a significant percentage of neural crest cell contaminants (more than 30%), cultures must be cleaned by mechanical picking of CNS clusters.

Note: CNS clusters (Fig. 2C) can be easily distinguished from peripheral neural crest clusters according to the morphology of the cells migrating out from the cluster. Neural crest clusters appear as flat cells at the edges of the clumps (Fig. 2D).

Mechanical cluster picking of CNS clusters is done using a dissection microscope in a laminar flow hood. As mentioned above, open the 10 cm dish under the microscope and using 1ml syringe, collect the CNS clusters. After filling up the syringe, slowly pull out the cell clusters directly into a new 10 cm PO/Lam/FN coated dish from which, just prior, Lam/FN solution was aspirated. Add up to 8–10 ml of glial media and move the plate to incubator.

SUPPORT PROTOCOL 1

Characterization of the cells at different stages of differentiation by immunostaining

Materials

NPCs/OPCs derived from hESCs/hiPSCs (see Basic Protocol)
 Accutase (Innovative Cell Technologies)
 24 well cell culture plates coated with PO/Lam/FN (see recipe)
 1× PBS, pH 7.4 (containing calcium and magnesium; Gibco-Life Technologies)
 1% Bovine serum solution (1% BSA; see the recipe)
 0.1% Triton-X 100 (v/v) in 1% BSA (see the recipe)
 0.3% Triton-X 100 (v/v) in 1% BSA (see the recipe)
 Appropriate primary antibody (see Reagents)
 Fluorophore-conjugated secondary antibody (see Reagents)
 DAPI stain (Invitrogen)
 P200 and P1000 pipette
 1ml syringe with a 27 G needle
 Glass hemocytometer
 1.5 ml microcentrifuge tubes
 45 µm cell strainers
 Centrifuge
 Inverted microscope

The phenotype of pluripotent cells, neural precursor cells (NPCs), early and late oligodendrocyte precursor cells (OPCs) and mature oligodendrocytes can be analyzed by immunostaining (Fig. 1 B–I). Characterization of the cells with specific markers at different stages makes it possible to track the progression of cell differentiation and maturation.

Prepare cells for immunocytochemical analysis

1. Two days before immunocytochemical analysis coat 24 well cell culture plate with PO/Lam/FN (see the recipe).
2. Gather the cell clusters from 10 cm culture dishes that you want to analyze, either by lifting them up with 1 ml syringe and collecting them with a P1000 pipette or by picking up the clusters using 1ml syringe (both approaches are described in Basic Protocol). Spin down the collected clusters (Basic Protocol) in 1.5 ml microcentrifuge tube.
3. Aspirate the supernatant and resuspend the clusters in 300–500µl of Accutase. Incubate 40 minutes to one hour at 37°C.

4. Add 300–500 μ l of N2 media and spin down the cells.
5. Aspirate the supernatant and wash the cells two times and centrifuge in N2 media.
6. Aspirate the supernatant and resuspend the clusters in 200 μ l of N2 media. Pipet the clusters up and down with P200 pipette to dissociate them into a single-cell suspension, filter them through a 45 μ m strainer.
7. Determine the cell concentration using a hemocytometer and spin them down.
8. Aspirate the supernatant and resuspend cells in N2/B27 medium supplemented with appropriate growth factors. Plate the cell suspension on completely dry 24 well plate pre-coated with PO/Lam/FN, in a droplet fashion at a density of $20\text{--}30 \times 10^3$ cells/cm².
9. 24 hours after plating fix the cells by aspirating the media and adding 1 ml of 4% paraformaldehyde (PFA) per single well within 24 well cell culture plate. Incubate 15 min at room temperature.
10. Aspirate the 4% PFA solution from the 24 well plate and wash three times, each time for 5 min with 1 ml of 1 $\times$ PBS buffer.

Immunostaining of cells

11. Incubate the cells with 500 μ l of 1% Bovine serum solution (1% BSA; see the recipe) for 30 to 60 minutes at RT, to prevent the nonspecific binding of the primary antibodies. For immunostaining of intracellular cytosolic markers incubate cells with 1% BSA solution containing 0.1% Triton. For immunocytochemical analysis of the nuclear markers, use 0.3% Triton in 1% BSA.
12. Aspirate the supernatant from the single wells within the 24-well cell culture plate and incubate the cells with the desired primary antibody diluted, according to the manufacturer's recommendations, in 200–300 μ l of 1% BSA solution with or without Triton, overnight at 4°C.
13. Aspirate the supernatant from the wells and wash three times, each time for 5 min with 1 ml of 1 $\times$ PBS.
14. For primary antibody visualization incubate cells with an appropriate fluorophore-conjugated secondary antibody diluted in 1% BSA solution according to the manufacturer's instructions, for 1 hour at room temperature.
15. Aspirate the solution with a secondary antibody and wash three times for 5 min with 1 ml of 1 $\times$ PBS buffer.
16. After the third wash, incubate the cells for 5 min with 200–300 μ l DAPI stain (1 μ g/ml DAPI in 1 $\times$ PBS) to label the cell nuclei.
17. Aspirate the DAPI solution and wash three times for 5 min with 1 ml of 1 $\times$ PBS buffer.

SUPPORT PROTOCOL 2

Selective enrichment of oligodendrocyte population using Fluorescence Activated Cell Sorting (FACS)

Materials

Late OPCs derived from hESCs/hiPSCs (see Basic Protocol)
 Accutase (Innovative Cell Technologies)
 24 well cell culture plates coated with PO/Lam/FN (see recipe)
 5% FBS-HBSS (see recipe)
 Anti-O4 Antibody (EMD Millipore)
 Anti-Myelin Basic Protein Antibody (MBP; EMD Millipore)
 Anti-Olig-2 Antibody (EMD Milipore)
 DAPI stain (Invitrogen)
 P200 and P1000 pipette
 N2 medium (see recipe)
 B27 Supplement (50×), minus vitamin A (Gibco-Life Technologies)
 10µg/ml BDNF
 10µg/ml AA
 20 µg/ml T3
 100mM dibutyl cAMP
 24 well cell culture plates coated with PO/Lam/FN (see recipe)
 1ml syringe with a 27 G needle
 5 ml and 10 ml serological pipettes
 Glass hemocytometer
 Centrifuge
 FACS tubes
 1.5 ml microcentrifuge tubes
 45 µm cell strainers
 Flow cytometer

Late oligodendrocyte progenitors are characterized by the expression of the surface marker O4 (Fig. 1G); they can be enriched by sorting between day 70 and day 100 (Fig. 3A, B).

1. Start preparing the cells for FACS by aspirating the medium and adding the Accutase (2ml/6cm dish; 5ml/10cm dish). Incubate cells for 1hour at 37°C.

2. Rinse the cells from the dish surface with 5ml of filtered DMEM/F12 medium, transfer them to a 15 ml conical centrifuge tube and spin the cells down.
3. Aspirate the supernatant, add 5 ml of filtered DMEM/F12 medium, dissolve the pellet by pipetting up and down, using 10 ml pipette and spin the cells down (first wash).
4. Repeat step 3 (second wash).
5. Using a P1000 pipette, resuspend the cells in 1–2 ml of 5% FBS-HBSS solution and vigorously pipet the cells up and down to break all cell clusters and get a single cell suspension. If needed, use P200 pipette for making smaller the cell aggregates. Add 5–10 ml of 5% FBS/HBSS and filter the cells through a 45 μ m cell strainer.
6. Count the cells using a hemocytometer and separate 1 million cells each for the unstained and secondary antibody only (Alexa-Fluor 488) FACS controls.
7. Spin the cells down and resuspend in 5% FBS-HBSS at the concentration of 10 million cells/ml suspension.
8. Incubate the cells on ice for 15 minutes for antibody blocking and then add 5 μ l of O4 primary antibody per 10 million cells/ml suspension to the sorting sample and incubate for 20 minutes on ice.
9. After the incubation time is done, add the same volume of filtered DMEM/F12 medium to the cell suspension, gently pipet with a 10 ml pipet and centrifuge the cells.
10. Aspirate the supernatant and resuspend the cells in 5% FBS-HBSS at the concentration of 10 million cells/ml suspension. Add 1 μ l of Alexa Fluor 488 secondary antibody per 10 million cells/ml suspension to the proper samples (sorting sample and secondary antibody stained only FACS control). Incubate for 20 min on ice in the dark.
11. Add the same volume of filtered DMEM/F12 medium to the cell suspension, gently pipet with a 10 ml pipet and centrifuge the cells.
12. Repeat the step 11 (second wash).
13. After the wash, aspirate the supernatant and resuspend 10 million cells in 1ml of 5% FBS-HBSS and transfer the samples (unstained control, 488-secondary antibody stained only control and sorting sample) to FACS tubes.
14. Add DAPI (1 μ g/ml) to exclude dead cells from the FACS analysis as well from sorted population.
15. For the collection of sorted cells prepare FACS tubes with 1 ml of 100% FBS.
Note: Make sure to keep everything on ice at all times.
16. Using a flow cytometer (*i.e.* MoFlo, Cytomation, Fort Collins, CO) adjust the sorting gates according to the unstained and 488-secondary only control with

exclusion of the DAPI positive cell population. Collect O4 positive cells for further culture.

17. Following cell sorting, wash cells twice with N2 media and centrifuge them down.
18. These cells can be differentiated into myelin-expressing mature oligodendrocytes. For this assay, plate O4 sorted cells on a PO/Lam/FN coated 24 well plate, in a droplet fashion at a density $10\text{--}15 \times 10^3$ cells/cm² in N2/B27 media supplemented with BDNF (20 ng/ml), AA (0.2 mM), T3 (60ng/ml) and cAMP (0.2μM). Change the media every two days for the total of two weeks.
19. After 2 weeks of terminal differentiation characterize the cells by immunocytochemistry (Support Protocol 1) using Anti-Myelin Basic Protein (MBP) and Anti-Olig-2 antibodies (Fig. 11).

Optional. Progenitor cells can also be subjected to FACS enrichment at other stages. PDGFRA sort can be used for early oligodendrocyte progenitors at days 70 (~15%) and day 100 (~25%). O1 sorting can be used for more mature oligodendrocytes, though they are present at lower density: day 70 (~6%) and day 100 (~9%).

SUPPORT PROTOCOL 3

Human embryonic stem cell based *in vitro* myelination assay

Materials

hESCs/hiPSCs cultured on a feeder layer (see Support Protocol 4) in 10cm culture dishes

hESC media (see recipe)

Matrigel coated 6 well cell culture plates (see recipe)

0.05% Trypsin-EDTA (Gibco-Life Technologies)

Accutase (Innovative Cell Technologies)

MEF conditioned hESC medium (CM) (see recipe)

KSR medium (see recipe)

N2 medium (see recipe)

B27 Supplement (50×), minus vitamin A (Gibco-Life Technologies)

10μg/ml FGF2

500μM LDN193189

10mM SB431542

10mM XAV939

2mM Purmorphamine

10mM Y-27632
 10µg/ml BDNF
 10µg/ml AA
 20 µg/ml T3
 100mM dibutyl cAMP
 10mM CHIR99021
 10mM DAPT
 24 well cell culture plates coated with PO/Lam/FN (see recipe)
 P200 and P1000 pipette
 45 µm cell strainers
 Glass hemocytometer
 1.5 ml microcentrifuge tubes
 Centrifuge
 Inverted microscope

Human embryonic stem cell based *in vitro* myelination assay

To model myelination in the CNS, we established an *in vitro* myelin assay in which both neurons and oligodendrocytes were derived from hESCs. The protocol requires the derivation of mature human neurons from hESCs or hiPSCs, followed by co-culture with the OPCs. To generate neurons, the dual-SMAD inhibition protocol was used and the initial steps are the same as listed in *Basic Protocol*, Part (I) of the oligodendrocyte differentiation protocol.

Derivation of forebrain neuronal cells

- 1** To initiate differentiation (day 1) aspirate conditional media and add KSR medium containing LDN193189 (200nM) and SB431542 (10µM) and XAV939 (2µM).
- 2** On day 2 of differentiation, aspirate the KSR and feed the cells with fresh KSR medium supplemented with LDN193189 (200nM), SB431542 (10µM) and XAV939 (5µM).
- 3** On day 4 of differentiation, aspirate the KSR and add a mixture of KSR medium (25%) and N2 medium (75%) with LDN193189 (200nM), SB431542 (10µM) and XAV939 (5µM).
- 4** On day 6 of differentiation, aspirate the media and add fresh mixture of KSR media (50%) and N2 medium (50%) supplemented with LDN193189 (200nM) and SB431542 (10µM).

- 5 On day 8 of differentiation, aspirate the media and add fresh mixture of KSR medium (25%) and N2 medium (75%) with LDN193189 (200nM) and SB431542 (10 μ M).
- 6 On day 10 aspirate the media and add N2 (100%) containing LDN193189 (200nM) and SB431542 (10 μ M).
- 7 On day 12 aspirate the media and feed the cells with N2 media with CHIR99021 (3 μ M).
- 8 On day 14 prepare PO/Lam/FN coated 24 well tissue culture plates as described in supporting protocol, but double the concentrations of Laminin (4 μ g/ml) and Fibronectin (4 μ g/ml).
- 20 On day 15 aspirate N2 media from differentiation wells and add Accutase (1.5 ml per single well within 6 well cell culture plate). Incubate approximately for 30 minutes at 37°C until a single-cell suspension is obtained.
- 21 Transfer the cell suspension into a 15 ml tube, mix with equal volume of N2 media using 5 ml pipette and spin down the cells.
- 22 Wash and centrifuge (200 $\times$ g for 5 minutes) the cells twice in N2 medium.
- 23 After the second wash, resuspend and triturate the cells in N2 medium, filter the cells using a 45 μ m cell strainer and count the cells using a hemocytometer.
- 24 Spin the cells down and resuspend them in N2 medium containing B27 Supplement without vitamin A (1 $\times$) (N2/B27) and DAPT (10 μ M). Plate the cells in 24 well plates at densities 30–50 $\times$ 10³ cells/cm².
- 25 Neurons were allowed to mature for the next 15 days before adding oligodendrocyte progenitors to the culture. Medium change is every second day until day 30.

OPC-neuronal co-culture

- 26 On day 30, plate FACS-enriched O4+ OPCs on top of the neuronal cells, 50 $\times$ 10³ OPCs/cm². N2/B27 media is supplemented with BDNF (20 ng/ml), AA (0.2 mM), T3 (60ng/ml) and cAMP (0.2 μ M), and is referred to as the “myelination medium”.
- 27 Feed the co-cultures every other day with myelination medium, for total of 5 weeks.
- 28 After 5 weeks of co-culture fix the cells with 4% paraformaldehyde solution (PFA) for 15 minutes, wash 3 times with PBS and analyze by immunocytochemistry using Anti-Myelin Basic Protein (MBP) and Anti-Neurofilament (SMI312) antibodies (Fig. 3C).

SUPPORT PROTOCOL 4

Maintenance of hESCs and hiPSCs in co-culture with mouse embryonic fibroblasts (MEFs)

Materials

Mitotically inactivated mouse embryonic fibroblasts (MEFs; GlobalStem)

hESCs/hiPSCs cultured on a feeder layer in 10cm culture dishes

hESC media (see recipe)

10 cm cell culture dishes coated with gelatin (see recipe)

Dispase in Hank's Balanced Salt Solution (Stem Cell Technologies)

10µg/ml bFGF

Inverted microscope

hESCs and hiPSCs are cultured in 10cm dishes on mitotically inactivated mouse embryonic fibroblasts (MEFs,) plated on gelatin coated dishes at $12\text{--}15 \times 10^3$ cells/cm². The hESC-medium supplemented with 6–10 ng/ml FGF2 is changed daily. The cells should be split using dispase every 6–8 days.

Splitting hESCs with dispase

1. Remove hESC media and add the appropriate volume of dispase (e.g., 5ml for 10cm dish).
2. Incubate for 8–10 minutes at 37°C, in the incubator. Once the edges of colonies start to detach, remove dispase, add 6 ml of hES media, detach colonies from the dish surface and collect them in a 15ml conical tube.
3. Let the colonies settle with gravity for 5 minutes in the hood. Aspirate the media and repeat the wash.
4. After the second wash, aspirate the media; add 5–10 ml of hES media with FGF2; and pipet up and down to disperse the colonies into smaller clusters. Plate 1 ml to a new dish (Split ratio: 1:5–1:10).

SUPPORT PROTOCOL 5

Recipes for coating of tissue culture dishes

Coating dishes with gelatin—Use 0.1% gelatin to coat the bottom of the 10 cm dish. Allow gelatin to incubate for at least 15 min prior to plating mouse embryonic fibroblasts (MEFs).

Coating dishes with Matrigel Basement Membrane Matrix

1. Thaw the frozen vial of Matrigel from the manufacturer on ice, overnight at 4 °C. Prepare 1 ml aliquots in a chilled 50 ml centrifuge tube using chilled 5 ml pipette and freeze at –20 °C.

2. Add 19 ml of cold and filtered DMEM/F12 media to a freshly thawed 1ml Matrigel aliquot, and pipette up and down until completely dissolved. Work quickly with Matrigel because it can polymerize at temperatures above 4 °C. Add 2 ml of Matrigel solution per single well within 6-well cell culture plate. Incubate the dishes at least for 1hour at RT.

Coating dishes with Poly-L-ornithine, Laminine and Fibronectin (PO/Lam/FN)

—Coat the dishes over night at 37 °C with PO (15 µg/ml) dissolved in 1× PBS. Next day wash the plates three times with 1× PBS and then add Laminin (1–2 µg/ml) and Fibronectin (2 µg/ml) dissolved in 1× PBS. Incubate the dishes over night at 37 °C. Coated plates with PO/FN/Lam solution can be kept in the incubator for up to 1 week before use.

MATERIALS

GENERAL EQUIPMENT

Cell culture dishes, multiwell plates, pipettes, pipette tips, centrifuge tubes, FACS tubes, cell lifters Polyethylene, 45 µm nylon mesh cell strainers.

1. Cell culture incubator with CO₂, humidity, and temperature control.
2. Bench-top cell culture biosafety laminar flow hood.
3. Cell culture laminar flow hood with dissecting microscope.
4. Inverted microscope.
5. Cell culture centrifuge.
6. Glass hemocytometer.
7. Cell sorting machine.

REAGENTS AND SOLUTIONS

Solutions – Media preparation—All media should be sterilized directly in 0.22-µm filtered bottles.

hESC medium for maintenance of undifferentiated ES cells (1 liter)—Mix DMEM/F12 (800 ml), 20% (v/v) KSR (200 ml), 0.5% (v/v) L-glutamine (5 ml), 0.5% (v/v) penicillin-streptomycin (5 ml), 1% (v/v) 10 mM MEM minimum non-essential amino acids solution (10 ml), 0.1% (v/v) 2-mercaptoethanol (1 ml) and 6–10 ng/ml FGF2. Keep medium at 4°C and use within 2 weeks.

DMEM with 10% FBS for MEFs (1 liter)—Combine DMEM (885 ml) supplemented with 10% (v/v) FBS (100 ml), 1% (v/v) penicillin-streptomycin (10 ml) and 0.5% (v/v) L-glutamine (5 ml). This medium should be kept at 4°C and used within 1 month.

KSR medium for hESC differentiation (1 liter)—Mix KnockOut DMEM (820 ml), 15% (v/v) KSR (150 ml), 1% (v/v) L-glutamine (10 ml), 1% (v/v) penicillin-streptomycin (10 ml), 1% (v/v) 10 mM MEM minimum non-essential amino acids solution (10 ml) and

0.1% (v/v) 2-mercaptoethanol (1 ml). This medium should be kept at 4°C and used within 1 month.

N2 medium for hESC differentiation (1 liter)—Combine Dist. H₂O (985 ml) with 1.2% (w/v) DMEM/F12 powder (12 g), 0.15% (w/v) glucose (1.55 g), 0.2% (w/v) NaHCO₃ (2 g), 0.0025% (w/v) insulin (25 mg), 0.01% (w/v) apo-transferrin (100 mg), 100 mM putrescine, 0.5 mM sodium selenite, 1mM progesterone.

MEF conditioned hES-cell media (CM)—MEF conditioned media (CM) is harvested from MEF-coated dishes. Plate MEFs at a density of 50×10³ cells/cm² in 10 cm dishes (2–3 dishes) using DMEM with 10%FBS. The next day, remove media, wash once with 1×PBS and add 20 ml of hES media. Incubate media with MEFs for 24 hours and then remove the media (conditioned media), filter sterilize it and keep it at 4°C up to 10 days or alternatively store it at –20°C. The same dishes with feeders can be used up to ten days for conditioning of the additional hES media.

0.1%(w/v) gelatin solution—Dissolve 0.5 g of gelatin in 500 ml of Milli-Q water and autoclave for 30 minutes. Store at room temperature indefinitely.

1%(w/v) BSA solution—Add 1 g of BSA to 100 ml of 1×PBS (pH 7.4; containing calcium and magnesium) and allow to dissolve. Store at 4°C.

0.1% Triton-X 100 (v/v) in 1%BSA—For 100 ml, add 0.1 ml of Triton-X 100 in 100 ml of 1% BSA solution. Store at 4°C.

0.3% Triton-X 100 (v/v) in 1%BSA—For 100 ml, add 0.3 ml of Triton-X 100 in 100 ml of 1% BSA solution. Store at 4°C.

HBSS with 15mM HEPES—To prepare Ca²⁺/Mg²⁺ – free HBSS containing 15 mM HEPES, add 7.5 ml of HEPES (1M; Gibco-Life Technologies) to 500 ml of Ca²⁺/Mg²⁺ – free HBSS (Gibco-Life Technologies). Store at 4°C.

5% FBS-HBSS solution—For 50ml of solution, add 2.5 ml of 100% FBS to 48 ml of Ca²⁺/Mg²⁺ – free HBSS containing 15 mM HEPES. Store at 4°C.

Reagents

- hESCs and hiPSCs
- Mitotically inactivated mouse embryonic fibroblasts (MEFs; GlobalStem, cat. no. GSC-6105M)
- Milli-Q water
- DPBS, no calcium, no magnesium (Gibco/Invitrogen, cat. no. 14190-250)
- Phosphate buffered saline (PBS) liquid, pH 7.4 (Gibco-Life Technologies, cat. no. 70011-044)

- $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free Hanks balanced salt solution (HBSS; Gibco-Life Technologies, cat. no. 14170-112)
- HEPES (Gibco-Life Technologies, cat. no. 1563-080)
- Trypsin-EDTA (0.05%) (Gibco-Life Technologies, cat. no. 25300-054)
- Dispase in Hank's Balanced Salt Solution, 5 U/ml (Stem Cell Technologies, cat. no. 7013)
- Accutase (Innovative Cell Technologies, cat. no. AT104)
- Dulbecco's modified Eagle's medium (DMEM; Gibco-Life Technologies, cat. no. 11965-092)
- Fetal bovine serum (FBS; Gibco-Life Technologies, cat. no. 16140-071)
- DMEM/F12 (Gibco-Life Technologies, cat. no. 11330-032)
- Knockout DMEM (Gibco-Life Technologies, cat. no. 10829-018)
- Knockout serum replacement (KSR; Invitrogen, cat. no. 10828-028)
- Penicillin/streptomycin, 100 $\times$ solution (Pen/Strep; Gibco-Life Technologies, cat. no. 15140-122)
- L-Glutamine (Gibco-Life Technologies, cat. no. 25030-081)
- MEM nonessential amino acids, 100 $\times$ solution (MEM NEAA; Gibco-Life Technologies, cat. no. 11140-050)
- β -Mercaptoethanol (Gibco-Life Technologies, cat. no. 21985-023)
- DMEM/F12 powder (Invitrogen, cat. no. 12500-062)
- Glucose (Sigma, cat. no. G7021)
- Sodium Bicarbonate (NaHCO_3 ; Sigma, cat. no. S5761)
- Human apo-Transferrin (Sigma, cat. no. T1147)
- Insulin (Sigma, cat. no. I6634)
- Putrescine (Sigma, cat. no. P5780)
- Sodium Selenite (Bioshop Canada, cat. no. SEL888)
- Progesterone (Sigma, cat. no. P8783)
- B27 Supplement (50 $\times$), minus vitamin A (Gibco-Life Technologies, cat. no. 125870-01)
- Bovine Serum Albumin (BSA; Sigma, cat.no. A9418)
- Triton X-100 (Sigma, cat. no. T8787)
- Matrigel basement membrane matrix (BD, cat. no. 354234)
- Gelatin (Sigma, cat. no. G1890)

- Poly-L Ornithin hydrobromide (PO; Sigma, cat. no. P3655)
- Mouse Laminin-I (Lam; R&D Systems, cat. no. 3400-010-01)
- Fibronectin (FN; BD Biosciences, cat. no. 356008)
- Paraformaldehyde Solution, 4% in PBS (4% PFA, Affymetrix, cat.no. 19943 1 LT)
- LDN-193189 (StemCell Technologies, cat. no. 72144)
- SB 431542 (Tocris Bioscience, cat. no. 1614)
- XAV 939 (Tocris Bioscience, cat. no. 3748)
- Y-27632 dihydrochloride (Tocris Bioscience, cat. no. 1254)
- Purmorphamine (Stemgent, cat. no. 04-0009)
- Recombinant human FGF2 basic (R&D Systems, cat. no. 233-FB)
- Recombinant Human BDNF Protein (R&D Systems, cat. no. 248-BD)
- Sodium L-ascorbate (AA; SIGMA cat. no. A4034)
- Recombinant mouse FGF8 (R&D Systems, cat. no. 423-F8)
- Dibutyryl cAMP sodium salt (cAMP; Sigma, cat. no. D0260)
- 3, 3', 5-Triiodo-L-thyronine sodium salt (T3; Sigma, cat. no. T5516)
- 4',6-Diamidino-2-Phenylindole, Dihydrochloride (DAPI; Invitrogen, cat.no. D1306)

Primary and Secondary antibodies

- Anti-Olig-2 Antibody (1:500, EMD Milipore, cat. no. AB9610)
- Nkx2.2 antibody (1:100, DSHB, cat. no. 74.5A5)
- Nestin Mouse Antibody (1:500, Neuromics, cat. no. MO15012)
- Anti-TRA-1-60 Antibody (1:100, EMD Milipore cat. no. MAB4360)
- Anti-Pax-6 Antibody (1:500, BioLegend, cat. no. 901301)
- Anti-O4 Antibody (1:200, EMD Millipore, cat. no. clone 81 MAB345)
- Anti-O1 Antibody (1:200, EMD Millipore, cat.no. clone 59 MAB344)
- Anti-Myelin Basic Protein Antibody (MBP; 1:200, EMD Millipore, cat.no. MAB386)
- BF1 antibody (1:500, Neuracell, cat. no. NC-FAB)
- Anti-ZO-1 antibody (1:100, Zymed, cat. no. 61-7300)
- Anti-Lin28 antibody (1:100, R&D, AF3757)
- Anti-Neurofilament 200 antibody (1:100, Sigma-Aldrich, cat. no. N4142)

- PE Mouse Anti-Human CD140a (1:20, BD Pharmingen, cat. no. 556002)
- Alexa Fluor 488 goat anti-rabbit IgG (1:700, Invitrogen, cat. no. A11034)
- Alexa Fluor 568 Goat anti-Rabbit IgG (1:700, Invitrogen, cat. no. A11034)
- Alexa Fluor 568 Goat Anti-Mouse IgG2b (1:700, Invitrogen, cat.no. A-21144)
- Alexa Fluor 488 goat anti-mouse IgG1 (1:700, Invitrogen, cat. no. A-21121)
- Alexa Fluor 568 Goat anti-Rabbit IgG (1:700, Invitrogen, cat. no. A11034)
- Alexa Fluor 488 goat anti-mouse IgM (1:700, Invitrogen, cat. no. A21042)
- Alexa Fluor 568 goat anti-mouse IgM (1:700, Invitrogen, cat. no. A21043)
- Alexa Fluor 568 goat anti-rat IgG (1:700, Invitrogen, cat. no. A11077)
- Alexa Fluor 488 donkey anti-goat IgG (1:700, Invitrogen, cat. no. A-11055)
- Alexa Fluor 488 donkey anti-goat IgG (1:700, Invitrogen, cat. no A-11055)
- Alexa Fluor 568 donkey anti-rabbit IgG (1:700, Invitrogen, cat. no. A10042)
- Alexa Fluor 488 donkey anti-mouse IgM (1:700, Invitrogen, cat. no. A-21043)

Reagent stock solutions—LDN-193189 (BMP signaling inhibitor; StemCell Technologies, cat. no. 72144) dissolved in DMSO to 500 μ M stock solution. Store at -20°C . Use 200nM as final concentration.

SB 431542 (TGF β inhibitor; Tocris Bioscience, cat. no. 1614) dissolved in ethanol to 10mM stock solution. Store at -20°C . Use 10 μ M as final concentration.

XAV 939 (Wnt signaling inhibitor; Tocris Bioscience, cat. no. 3748) dissolved in DMSO to 10mM stock solution. Use 2 μ M –5 μ M as final concentration.

Y-27632 dihydrochloride (ROCK inhibitor; Tocris Bioscience, cat. no. 1254) dissolved in sterile water to 10mM stock solution. Store at -20°C . Use 10 μ M as final concentration.

Purmorphamine (Smoothened agonist; Stemgent, cat. no. 04-0009) dissolved in DMSO to 2mM stock solution. Store at -20°C . Use 1 μ M as final concentration.

Recombinant human FGF2 basic (R&D Systems, cat. no. 233-FB) dissolved in PBS with 0.1% BSA to 10 μ g/ml stock solution. Store at -20°C . Use at 10 ng/ml as final concentration.

Recombinant Human BDNF Protein (R&D Systems, cat. no. 248-BD) dissolved in PBS with 0.1% BSA to 10 μ g/ml stock solution. Store at -20°C . Use 10 ng/ml as final concentration.

Sodium L-ascorbate (AA; SIGMA cat. no. A4034) dissolved in PBS with 0.1% BSA to 10 μ g/ml stock solution. Store at -20°C . Use 10 ng/ml as final concentration.

Recombinant mouse Fgf-8b (FGF8; R&D Systems, cat. no. 423-F8) dissolved in PBS with 0.1% BSA to 100 μ g/ml stock solution. Store at -20°C . Use 100 ng/ml as final concentration.

Dibutyl cAMP sodium salt (cAMP; Sigma, cat. no. D0260) dissolved in sterile water to 100mM stock solution. Store at -20°C . Use 0.2mM as final concentration.

Recombinant Human PDGF-AA Protein (PDGF-AA; R&D Systems, cat. no. 221-AA) dissolved in PBS with 0.1% BSA to 10 $\mu\text{g}/\text{ml}$ stock solution. Store at -20°C . Use at 20 ng/ml as final concentration.

Recombinant Human IGF-I Protein (IGF-1; R&D Systems, cat. no. 291-G1) dissolved in PBS with 0.1% BSA to 10 $\mu\text{g}/\text{ml}$ stock solution. Store at -20°C . Use at 20 ng/ml as final concentration.

3, 3', 5-Triiodo-L-thyronine sodium salt (T3; Sigma, cat. no. T5516)

To prepare 20 $\mu\text{g}/\text{ml}$ stock solution: add 1 ml 1N NaOH per mg of 3,3',5-Triiodo-L-thyronine sodium salt and 49 ml of sterile water. Gently swirl to dissolve. Store at -20°C . Use 60 ng/ml as final concentration.

CHIR99021 (Wnt signaling pathway agonist; Stemgent, cat.no. 04-0004-10) dissolved in DMSO to 10mM stock solution. Store at -20°C . Use 3 μM as final concentration.

Poly-L Ornithin hydrobromide (PO; Sigma, cat. no. P3655) dissolved in PBS with 0.1% BSA to 15mg/ml stock solution. Store at -20°C . Use at 15 $\mu\text{g}/\text{ml}$ as final concentration.

Fibronectin (FN; BD Biosciences, cat. no. 356008) dissolved in PBS with 0.1% BSA to 1mg/ml stock solution. Store at -80°C . Use at 2 $\mu\text{g}/\text{ml}$ as final concentration.

4', 6-Diamidino-2-Phenylindole, Dihydrochloride (DAPI; Invitrogen, cat.no. D1306) dissolved in ultrapure water to 1mg/ml stock solution. Stock solution is stored protected from light at -20°C . For DAPI working solution, dilute the stock solution in PBS up to 1 $\mu\text{g}/\text{ml}$.

N-(N-(3,5-difluorophenacetyl)-L-alanyl)-S-phenylglycine t-butyl ester (DAPT; Notch signaling inhibitor, Torcis Bioscience, cat.no. 2634) dissolved in DMSO to 10mM stock solution. Use 10 μM as final concentration.

Commentary

Human embryonic stem cells (hESCs) and induced pluripotent stem cells (hiPSCs) represent a unique cell population considering their properties of self-renewal, pluripotency and capacity of differentiation into all the tissues of the embryo *in vivo*, and many cell types *in vitro* (Thomson et al., 1998; Takahashi et al., 2007). The generation of hiPSCs from individual patients and their differentiation into tissue and organ-specific cells offers great hope for the development of cell and regenerative therapies for the treatment of a variety of human diseases (Tabar & Studer, 2014). Demyelination, which involves the loss or dysfunction of oligodendrocytes, occurs in many severe neurological disorders, including multiple sclerosis, hereditary pediatric leukodystrophies but also appears as a consequence of CNS injury and radiation. In this context, the aforementioned pathological conditions might represent suitable targets for replacement strategies using oligodendrocyte progenitor

cells as potential therapeutic agents (Franklin et al., 2008; Goldman et al., 2012). Due to the perceived need for oligodendrocytes for regenerative medicine, in the last decade various protocols have been developed to generate oligodendrocytes from hESCs (Nistor et al., 2005; Izrael et al., 2007; Kang et al., 2007; Hu et al., 2009; Sundberg et al., 2010; Stacopole et al., 2013 and hiPSCs (Major et al., 2011; Wang et al., 2013; Douvaras et al., 2015; Goldman & Kuypers, 2015). Although these protocols have attempted to recapitulate embryonic oligodendrogenesis, they differ in several ways including culture methods, combination of growth factors and the duration of differentiation procedure (Czepiel et al., 2015). While the majority of the published approaches use Shh for oligodendrocyte derivation, several groups do not add Shh or its agonists at any stage of differentiation (Nistor et al., 2005; Izrael et al., 2007; Kang et al., 2007; Sundberg et al., 2010). In fact, this is compatible with *in vivo* development where there are Shh dependent (ventral) and Shh independent (dorsal) waves of oligodendrocyte specification. With the exception of a few groups describing derivation of forebrain oligodendrocytes (Major et al., 2011; Stacopole et al., 2013) most of the published protocols have reported hindbrain or spinal cord patterning of oligodendrocytes.

Here we detailed a novel feeder free protocol for *in vitro* differentiation of forebrain oligodendrocytes from hESCs that mimics oligodendrocyte specification turned on during telencephalic development. In the mammalian embryonic CNS, in any particular progenitor domain, the first cell type to arise is neuronal (neurogenesis), while gliogenesis occurs at a later stage. The oligodendrocyte precursors (OPCs) appear in discrete regions of the neural tube and their specification is tightly controlled by transverse gradients of Sonic hedgehog (Shh) and bone morphogenetic protein (BMP) in the developing telencephalon and spinal cord (Rowitz & Kriegstein, 2010). In the forebrain the earliest OPCs emerge in progenitor domains of the ventricular and subventricular zone and subsequently migrate throughout the CNS to populate the emerging white matter (Jakovcevski and Zecevic, 2005). In both, the spinal cord and forebrain, Sonic hedgehog (Shh) is required for the specification of ventrally derived OPCs that express Olig2, Nkx2.2 and Sox10 transcription factors (Tekki-Kessaris et al., 2001; Rowitch, 2004). In developing telencephalon, independently from Shh, Fgf8 signaling generates ventral OPCs by directly promoting their specification (Kuschel et al., 2003). After migration to the developing white matter, early bipolar OPCs, which express the Platelet-derived growth factor receptor alpha (PDGFR-A), proliferate under the influence of platelet-derived growth factor (PDGF-AA), produced by astrocytes and neurons. Maturation towards late OPCs is accompanied by the loss of mitotic capacity, morphological change and the expression of more mature oligodendrocyte specific markers (e.g. O4 and O1 markers, CNPase). At the final stage of oligodendrocyte development, under the influence of axonal soluble (thyroid hormone; T3) and cell mediated signals (e.g. Neuroregulins, N-cadherin), cells acquire the expression of myelin basic protein (MBP), myelin associated glycoprotein (MAG), myelin oligodendrocyte glycoprotein, and myelin proteolipid protein (PLP), all involved in the molecular architecture of myelin (Zhang et al., 2001).

The first step in modeling telencephalic oligodendrocyte development in the culture dish is the induction of ventral forebrain neural progenitors. For the efficient neural conversion of hESCs we used the dual-SMAD inhibition method, based on pharmacological inhibition of two signaling pathways that utilize SMADs: bone morphogenetic protein (BMP) and

transforming growth factor- β (TGF- β) pathways. To accomplish this, we used two small molecule inhibitors: LDN-193189 (LDN) and SB431542 (SB) respectively (Chambers et al., 2009) (Fig. 1A). Modulation of developmental signaling pathways, such as Shh and Wnt, can be used to assign the dorso-ventral and anterior-posterior identity of hESCs derived oligodendrocytes. Ventralization of the forebrain neural precursors was achieved using the Sonic Hedgehog (Shh) agonist Purmorphamine (Pur). The Wnt signaling has long been recognized as the main pathway that defines the anterior-posterior cell phenotypes in the developing neural tube, with anterior cells expressing Wnt antagonists and posterior expressing Wnt proteins (Leyns et al., 1997; Hashimoto et al., 2000). Therefore, to potentiate the forebrain identity through the inhibition of Wnt signaling we used a small molecule inhibitor XAV939 (XAV) (Huang et al., 2009). The combined application of LDN, SB, XAV (termed as LSBX) and Purmorphamine resulted in robust induction of forebrain fates (Fig. 1) (Maroof et al., 2013; Nicoleau et al., 2013). Patterned neuroectoderm was then passaged to become rosettes indicating the specification of forebrain neural precursors defined by the coexpression of Pax6, BF1 and Nestin cell markers (Elkabatz et al., 2008) (Fig. 1). After the patterning stage in the presence of Purmorphamine, neural progeny was kept in media supporting proliferation with FGF8 growth factor. Around day 45–50 early OPCs start appearing in the culture, which are characterized by the co-expression of Olig2 and Nkx2.2 transcription factors (Fig. 1B–F). To promote their proliferation and differentiation glial media was used, resulting in the appearance of late OPCs that express more mature markers (Fig. 1G–I).

Our method offers specification of ventral telencephalic oligodendrocytes within a reasonably short time frame (70–100 days). High purity ($95.97\% \pm 1.74$) of derived anterior cells is achieved by fluorescence activated cell sorting using O4 oligodendrocyte specific marker, starting from day 70 (21.8%) until day 100 (35.7%). The derived cells are capable of acquiring myelin protein expression as fast as by day 85 *in vitro* in high percentage (85.5%). We believe that our protocol, in addition to being a source of ventrally-derived OPCs suitable for transplantation, can also be useful for the purpose of modeling of CNS demyelinating disorders primarily involving the forebrain, considering anterior identity of derived cells and their ability to readily myelinate after transplantation into demyelinated rat brains (Piao et al., 2015).

The therapeutic potential of highly purified populations of hESC and hiPSC derived oligodendrocytes can be tested through their capacity to efficiently myelinate axons. Transplantation in the *shiverer* mouse, which lacks compact myelin, is one of the most common *in vivo* model systems to test myelination capacity of cell grafts. (Hu et al., 2009; Izrael et al., 2007; Nistor et al., 2005; Wang et al. 2013; Douvaras et al., 2015). We established a reliable *in vitro* myelin formation assay by co-culturing oligodendrocytes with neuronal cells, both derived from hESCs. Forebrain neuronal cells were also produced using the dual-SMAD inhibition protocol. After the initial neural induction, application of small molecule CHIR99021, a Wnt/ β -catenin signaling pathway activator, increased neuronal differentiation (Chambers et al., 2012). Final maturation of neuronal progeny was achieved by the addition of small molecule DAPT, the Notch signaling inhibitor, which causes a cell cycle arrest and enhances neuronal differentiation (Borghese et al., 2010). The *in vitro* myelination assay was initiated by adding oligodendrocytes to the culture of fully developed

neurons (30 days old). During 5 weeks of co-culture hESCs derived oligodendrocytes matured and expressed MBP as they aligned along the axons (Fig. 3C). Therefore, this human embryonic stem cell based *in vitro* myelination assay can serve as a useful experimental system for studying the mechanisms of myelination in the CNS and to test new therapeutic strategies for treating demyelinating diseases. As a final validation, we have demonstrated that hESCs derived oligodendrocyte progenitors will migrate widely upon grafting in vivo, and remyelinate axons in an anatomically correct manner, including reorganization of nodal and paranodal proteins. In addition to restoring structural integrity, the grafting resulted in behavioral amelioration (Piao et al., 2015).

Critical parameters and Troubleshooting

1. Initial cell plating density (*Basic Protocol*, Step (I) 11) is crucial since it determines the relative amounts of central (CNS) versus peripheral (neural crest) cells produced. Higher densities are needed for efficient conversion to CNS cells (95–100% confluence) while lower densities will result in more abundant neural crest derivatives (50–60% confluence and below). In the case when 95–100% confluence is not achieved the following day (day 1), the cells can be maintained with MEF conditioned hESC medium supplemented with FGF2 until they reach desired confluence. Alternatively, the initial number of plated cells can be increased. Ideal starting plating densities vary between different pluripotent cell lines and they should be optimized for each line.
2. If the addition of 5μM XAV939, Wnt inhibitor, from day 2 to day 6 (*Basic Protocol*, Step (II) 2) causes significant cell death, try with lower concentration of XAV939, using 2μM, from day 2 to day 10.
3. Replating the cells at day 12 on PO/Lam/FN coated dishes (*Basic Protocol*, Step (III) 8) is the most critical part of the protocol. Maintain high density at passaging to achieve successful progression to CNS neural cells. Low replating density will result in high cell death and eventual survival of only neural crest cell population. Alternatively, if the cell survival after the passaging on day 12 is poor, postpone replating until day 16.
4. If there is a significant percentage of neural crest clusters (more than 30%), the culture should be cleaned as early as possible (P2, P3) by mechanical picking of the CNS clusters (*Basic Protocol*, Step (IV) 2). Neural crest cells have a high proliferative capacity and can overtake the culture resulting in lower final percentage of derived oligodendrocytes.
5. It is important to have controls of hESC-derived cells that are unstained and stained with appropriate secondary antibodies (*Support Protocol 2*, Step 6) for defining sorting gates in the FACS machine.
6. Low survival of sorted cells (*Support Protocol 2*, Step 18) can be overcome by adding Y-27632 (10μM) to N2/B27 media, which will improve cell survival. Alternatively, pre-conditioned medium can be used for initial plating of sorted cells.

7. Coating the dishes for *in vitro* myelination co-cultures (*Support Protocol 3*, Step 8) is a critical point since the assay is 5 weeks long and strong cell attachment is needed. Use double concentration of Laminin (4 $\mu\text{g}/\text{ml}$) and Fibronectin (4 $\mu\text{g}/\text{ml}$) for coating. Additionally, gentle media change is required since the cells can start detaching.

Anticipated results

The percentage of generated O4+ late OPCs using H9 hESC line is 21% ($21.8\% \pm 4.90$) on day 70 and enriched to 35% ($35.7\% \pm 10.62$) by day 100. This protocol should reliably yield highly purified populations of late OPCs (between day 70 and day 100), after a simple antibody-mediated cell sorting strategy. Final yield depends on the specific hPSC line used, the efficiency of neural induction and CNS rosette formation. According to our general experience hiPSCs may be somewhat less efficient than H9. Sorted O4 positive cells co-express Olig2 and Nkx2.2 transcription factors (Fig. 3B). The capacity of hES derived late OPCs to produce MBP *in vitro* can be tested by exposing FACS O4 sorted OPCs to terminal differentiation conditions for an additional two weeks. By day 85, 85.5% ($85.5\% \pm 0.07$) of O4 sorted cells will fully mature and express myelin basic protein (MBP) (Fig. 1B, I). Alternatively, FACS sorted O4 expressing OPCs (70 days old) can be plated with hESC derived neurons for glial-neuronal co-culture. After five weeks of *in vitro* myelination assay, oligodendrocytes will mature, acquiring the expression of myelin basic protein (MBP), and becoming highly branched. After 35 days of oligodendrocyte co-culture with neuronal cells, active myelination will take place as there will be an increased number of regions of co-localization between MBP-positive oligodendrocyte processes and NF-positive neurites (Fig. 3C). The length of myelin segments (co-labeled by MBP/NF) can be measured with the NIH ImageJ software.

Time Consideration

The oligodendrocyte differentiation takes ~70 days with a further enrichment for O4 positive cells requiring up to 100 days. Neural induction takes initial 10 days while the NPCs patterning takes ~15 days. Oligodendrocyte precursor cell propagation and later differentiation takes ~45 – 95 days. The *in vitro* myelination assay takes 14 days after the OPCs are FACS sorted and exposed to terminal differentiation conditions. For the *In vitro* myelination co-culture assay, time frame covers derivation of neuronal cells from hESCs, their maturation and co-culture with FACS purified oligodendrocytes. Neuronal differentiation from hESCs and maturation takes ~30 days. *In vitro* co-culture takes additional 5 weeks.

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Significance Statement

Oligodendrocytes, a key cellular component of the central nervous system, provide crucial structural and functional support to neurons and their dysfunction or loss often results in disruption of the layers of axonal myelin sheath. Enhancing remyelination through transplantation of myelin-producing cells is a promising therapeutic approach to restore neurological function. Human embryonic stem cells (hESCs) and induced pluripotent stem cells (hiPSCs) can provide endless sources of oligodendrocyte progenitor cells (OPCs) for transplantation. Most protocols describing oligodendrocyte derivation from hESCs mimic spinal cord oligodendrogenesis and lack a strategy for isolation of highly enriched oligodendrocyte progenitors, a prerequisite for future clinical trials. Here, we present a protocol for the derivation of forebrain late OPCs from hESCs and a method for their efficient purification.

Figure 1. Differentiation of hESCs into telencephalic oligodendrocytes

(A) Protocol scheme for differentiation of hESCs into telencephalic oligodendrocytes. (B) Immunophenotyping of hESC progeny during differentiation into oligodendrocytes. Undifferentiated hESC colony expressing Tra1-60, a human pluripotent stem cell marker. (C) Around day 20 of differentiation, neural precursor cells form typical rosette-like structures with expression of neural stem cell progenitor marker Nestin and rosette-associated marker ZO1 in the center of the rosettes. (D) In addition to positive staining for another rosette marker LIN28, homogenous expression of the forebrain-associated marker BF1 confirms the anterior identity of the NPCs (E) Patterned NPCs express early neuroectodermal markers such as Pax6 (F) On day 50 early OPCs appear in the culture and co-express Olig2 and Nkx2.2. (G) Late OPCs present on day 70 express O4 together with Olig2. (H) Later OPCs acquire the expression of the more mature O1 oligodendrocyte marker. (I) Using flow-cytometry, O4 expressing late OPCs can be purified from the culture on day 70, then exposed to terminal differentiation condition for two weeks. By day 85, cells will fully mature and express myelin basic protein (MBP). Scale bars represent 100µm.

Figure 2. Propagation of NPCs in clusters

(A, B) NPCs are propagated as clusters and passaged every two weeks. (C) CNS clusters and (D) Neural crest clusters exhibit different morphology, best seen in the cells emerging from the clusters. Predominance of flat cell morphology is typical for neural crest clusters. Scale bars represent 100 μ m.

Figure 3. Enrichment of late OPCs via FACS and co-culture with hESCs derived neurons
 (A) FACS sort plot for purification of late OPCs using the O4 surface marker on day 70. The final sorting gates are adjusted according to the unstained control and the secondary antibody-only control, resulting in 18.9% O4 positive cells. (B) Immunohistochemistry for oligo-specific markers of FACS sorted O4+ OPCs. Late OPCs co-express Nkx2.2 and Sox10. Scale bar represent 100 μ m. (C) *In vitro* myelination assay based on co-culture of neurons and oligodendrocytes both derived from hESCs. FACS sorted O4 positive OPCs (day 70) were co-cultured with hESC (H9)-derived neurons for 5 weeks. Immunocytochemistry shows co-labeling of MBP and SMI312, an axonal marker, confirming functional interaction between neurons and oligodendrocytes. High magnification panels show anatomic co-localization. Scale bar represents 10 μ m.