

Generation and Characterization of Oligodendrocytes From Lineage-Selectable Embryonic Stem Cells In Vitro

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Summary

Oligodendrocytes develop from proliferating oligodendrocyte precursor cells (OPCs), which arise in germinal zones, migrate throughout the developing white matter and divide a limited number of times before they terminally differentiate. Thus far, it has been possible to purify OPCs only from the rat optic nerve, but the purified cells cannot be obtained in large enough numbers for conventional biochemical analyses. Moreover, the central nervous system stem cells that give rise to OPCs have not been purified, limiting the ability to study the earliest stages of commitment to the oligodendrocyte lineage. Pluripotent mouse embryonic stem (ES) cells can be propagated indefinitely in culture and induced to differentiate into various cell types. We describe protocols for culture conditions in which neural precursor cells, OPCs, and oligodendrocytes can be efficiently produced from genetically modified ES cells. This strategy should be useful for study of the intracellular and extracellular factors that direct central nervous system stem cells down the oligodendrocyte pathway and influence subsequent oligodendrocyte differentiation. It may also be useful for producing OPCs and oligodendrocytes from human ES cells for cell therapy and drug screening.

Key Words: Differentiation; ES cells; genetic selection; oligodendrocyte.

1. Introduction

Oligodendrocytes are postmitotic cells that myelinate axons in the vertebrate central nervous system (CNS). Like the majority of other cells in the CNS, oligodendrocytes are generated from pluripotent neural precursor cells of the neural tube, which itself derives from the embryonic ectoderm. Oligodendrocyte development begins in the embryo when proliferating oligodendrocyte precursor cells (OPCs) arise in restricted regions of the ventral ventricular zone of the developing brain and spinal cord (1–5). The restriction of OPC development to these regions depends on both localized positive and negative signals (6,7). One such positive signal is Sonic hedgehog (Shh), which is secreted by both the floor plate and the notochord along

the rostrocaudal axis (8–10). In the mouse, OPC markers, such as the *Olig1* and *Olig2* transcription factors (11,12), the platelet-derived growth factor receptor (PDGFR)- α (3,13), and the NG2 proteoglycan (14) are all expressed by embryonic age 13 (E13) (6).

After they have arisen in germinal zones of the brain and the spinal cord, OPCs migrate throughout the developing white matter, where they divide a limited number of times, largely in response to PDGF (15). They then stop moving, withdraw from the cell cycle, and terminally differentiate into myelinating oligodendrocytes, which express galactocerebroside (GC) (16,17). The first GC-expressing oligodendrocytes seem to appear in the mouse CNS around E17 (18).

The differentiation of OPCs into oligodendrocytes is better understood than the initial commitment of neural precursors to the oligodendrocyte lineage. This is mostly because of the development of a powerful *in vitro* system in which purified OPCs isolated from the perinatal rat optic nerve can proliferate and differentiate in serum-free cultures on the same schedule as they do *in vivo* (19). Using this system, it has been shown that the OPCs have an intrinsic timer that regulates when they stop dividing and differentiate, and that at least two kinds of extracellular signals seem to be required for the timer to operate normally: PDGF (20–22) and thyroid hormone (23–26). This cell model has been an invaluable tool in the identification of a number of intracellular mechanisms that regulate OPC differentiation (27–32).

Although studies of purified rat OPCs have provided important insights into the control of OPC differentiation, the cells cannot be purified in large enough numbers for conventional biochemical analyses. Moreover, the neural precursor cells that give rise to OPCs have not been purified, making it difficult to study the earliest stages of OPC specification.

Mouse embryonic stem (ES) cells are proliferating, pluripotent stem cells that have been isolated from the epiblast of blastocyst stage mouse embryos (33–35). They can be propagated indefinitely in culture in the presence of leukemia inhibitory factor (LIF) (36,37). When transplanted into a mouse blastocyst, ES cells integrate into the embryo and contribute to all cell lineages, including germ cells (38). If ES cells are cultured without LIF on a nonadherent surface, then they aggregate to form embryoid bodies (EBs), in which the cells form ectodermal, mesodermal, and endodermal derivatives (39). ES cells can be produced in large numbers, easily genetically modified, and induced to differentiate into various CNS cell types *in vitro* (40–43). They should therefore provide a powerful system for studying the early events of neural development.

Here, we describe protocols for culture conditions in which neural precursor cells, OPCs, and oligodendrocytes can be efficiently produced from genetically modified ES cells. This strategy should be useful for study of the intracellular and extracellular factors that direct CNS stem cells down the oligodendrocyte pathway and influence subsequent oligodendrocyte differentiation. It may also be useful for producing OPCs and oligodendrocytes from human ES cells for cell therapy and drug screening. Oligodendrocytes derived from mouse ES cells have already been shown to remyelinate axons in rodent models of demyelinating diseases (43,44).

2. Materials

2.1. Tissue Culture

The tissue culture facility for ES cell culturing requires the following:

1. Humidified incubator at 37°C and 5% CO₂.
2. Laminar flow cabinet.
3. 37°C water bath.
4. Centrifuge.
5. Inverted microscope.
6. Coulter cell counter Z2 series.
7. Plastic pipets designated for tissue culture (5, 10, and 25 mL).
8. BD Falcon™ conical centrifuge tubes (15 and 50 mL; BD Biosciences, Cambridge, UK; cat. no. 352086 and 352196).

2.1.1. Maintenance of ES Cells

1. Genetically engineered ES cells (**45**).
2. 2% gelatin (bovine skin, Sigma-Aldrich, Gillingham, UK; cat. no. G1890). Add 2 g gelatin in 100 mL H₂O. Autoclave and store at 4°C.
3. Polystyrene, 25-cm², canted, phenolic Costar flasks (Fisher Scientific, Leicestershire, UK; cat. no. 430372).
4. Phosphate-buffered saline-ethylenediaminetetraacetic acid (PBS-EDTA): combine 8 g NaCl, 0.2 g KCl, 1.15 g disodium hydrogen orthophosphate, and 0.20 g potassium dihydrogen orthophosphate in distilled water. Adjust to pH 7.2 and complete to 1 L. Add 0.374 g EDTA. Autoclave.
5. 1X Trypsin (0.025%): add 5 mL trypsin 2.5% (Invitrogen Ltd., Paisley, UK; cat. no. 15090-046) to 500 mL PBS-EDTA. Store at 4°C.
6. Dulbecco's modified Eagle's medium (DMEM) (500-mL bottle; Invitrogen, cat. no. 31966 021).
7. Bovine serum (Invitrogen, cat. no. 26170043).
8. Penicillin-streptomycin-glutamine (PSG) (100X; Invitrogen, cat. no. 10 378-016).
9. Glasgow's modified Eagle's medium (GMEM, BHK21) (500-mL bottle; Invitrogen, cat. no. 21710-025).
10. Fetal calf serum (FCS) screened for ES cell growth (PAA Laboratories GmbH, Somerset, UK; cat. no. A15-649) (*see Note 1*).
11. β-Mercaptoethanol (Sigma, cat. no. G9391) stock solution: add 100 β-mercaptoethanol to 14.1 mL H₂O. Prepare fresh.
12. 10 mM nonessential amino acids (100X; Invitrogen, cat. no. 11140-035).
13. 100 mM sodium pyruvate (100X; Invitrogen, cat. no. 11360-039).
14. 7% sodium bicarbonate (Invitrogen, cat. no. 25080-060).
15. 10⁷ U/mL LIF (Chemicon, Hampshire, UK; cat. no. ESG1106).
16. Neutralization medium: for 500 mL, combine 500 mL DMEM, 50 mL bovine serum, and 5 mL PSG 100X.
17. ES cell medium: for 500 mL, combine 420 mL GMEM, 50 mL FCS, 5 mL PSG 100X, 0.5 mL β-mercaptoethanol stock solution, 5 mL 100X nonessential amino acid, 5 mL 100X sodium pyruvate, and 13.2 mL 7.5% sodium bicarbonate. For exponential culture, add LIF to a final concentration of 10³ U/mL (1/10⁴ dilution).

2.1.2. Differentiation of ES Cells Into the Oligodendrocyte Lineage

2.1.2.1. COATING OF THE PLATES

1. Falcon polystyrene flasks (25 cm²; BD Bioscience, cat. no. 353009).
2. Nunc four-well plates (Fisher, cat. no. 176740).
3. 1 mg/mL poly-D-lysine (PDL): dissolve 5 mg PDL (Sigma, cat. no. P 6407) in 5 mL H₂O. Aliquot (0.2 mL) and store at -20°C.
4. 1 mg/mL laminin (Sigma, cat. no. L 2020). Aliquot (0.2 mL) and store at -20°C.

2.1.2.2. FORMATION AND SELECTION OF NEURAL PRECURSORS

1. 10-cm Petri dishes (BD Biosciences, cat. no. 351029).
2. Universal containers for EBs (Bibby Sterilin, Staffordshire, UK; cat. no. 128PYR).
3. All-*trans* retinoic acid (RA), 5000X (5 × 10⁻³ M): dissolve RA (Sigma, cat. no. R2625) in dimethyl sulfoxide. Aliquot (30 µL) and keep at -20°C away from light. Use once and discard.
4. Neural basal medium (Invitrogen, cat. no. 20103-049).
5. 4X trypsin (0.1%): add 20 mL 2.5% trypsin (Invitrogen, cat. no. 15090-046) to 500 mL PBS-EDTA. Store at 4°C.
6. Dulbecco's modified Eagle medium/F12 (DMEM/F12) (500-mL bottle; Invitrogen, cat. no. 31331 028).
7. 50X B27 (Invitrogen, cat. no. 17504).
8. 100X N2 (Invitrogen, cat. no. 17502).
9. 2000X geneticin sulfate (G-418) (200 mg/mL): dissolve G418 (Roche Diagnostics, Hertfordshire, UK; cat. no. 1464990) in sterile water to have 200 mg/mL active units (the activity is written on each tube of G418). Aliquot (0.5 mL) and store at -20°C.
10. 1000X fibroblast growth factor (FGF) (20 µg/mL): dilute 10 µg human FGF-2 (Peprotech, London, UK; cat. no. 100-18B) in 0.5 mL 0.01 M HCl. Aliquot (25 µL) and keep at -80°C.
11. 1000X ganciclovir (2.5 mM): dissolve 6.4 mg ganciclovir (Sigma, cat. no. G2536) in 10 mL DMEM. Sterilize through a 0.2-µm filter, aliquot (1 mL), and keep at -20°C.
12. DMEM/F12+N2 medium: add 1 mL N2 to 100 mL DMEM-F12.
13. Neural basal+B27 medium: add 2 mL B27 to 100 mL neural basal medium.
14. Neural differentiation medium: combine DMEM/F12+N2 medium and neural basal+B27 medium in equal volumes (1:1) to obtain F12/N2/NB/B27 medium. This medium can be kept at 4°C for up to 2 wk. Add growth factors and drugs when needed.

2.1.2.3. OPC DIFFERENTIATION

1. Earle's balanced salt solution (EBSS; Invitrogen, cat. no. E2888).
2. 0.005% trypsin: add 0.1 mL 2.5% trypsin (Invitrogen, cat. no. 25090-028) in 50 mL EBSS. Aliquot (5 mL) and store at -20°C.
3. Dulbecco's modified Eagle's medium (DMEM), high in glucose, pyruvate, pyridoxine (500 mL; Invitrogen, cat. no. 31966 021).
4. 100X penicillin-streptomycin-glutamine (PSG; Invitrogen, cat. no. 10 378-032).
5. Apo-transferrin (Sigma, cat. no. T1147).
6. Crystalline bovine serum albumin (Sigma, cat. no. A4161).
7. Progesterone stock solution (25 mg/mL): add 2.5 mg progesterone (Sigma, cat. no. P8783) to 100 µL ethanol (EtOH). Prepare a fresh stock each time Sato medium is to be made.
8. Putrescine (Sigma, cat. no. P5780).
9. Sodium selenite stock solution (0.4 mg/mL): add 4 mg sodium selenite (Sigma, cat. no. P8783) to 10 mL DMEM and 100 µL 0.1 N NaOH. Prepare a fresh stock each time Sato medium is to be made.

10. 100X *N*-acetyl-cysteine (40 mM): add 50 mg *N*-acetyl-cysteine (Sigma, cat. no. A9165) in 8.33 mL sterile water. Aliquot (200 μ L) and keep at -20°C .
11. 1000X biotin (10 $\mu\text{g}/\text{mL}$): add 9 mg biotin (Sigma, cat. no. B4639) in 9 mL DMEM, then add 20 μ L of this solution in 2 mL DMEM. Aliquot (25 μ L) and keep at -20°C .
12. 5000X forskolin (25 mM): add 10 mg forskolin (Sigma, cat. no. F6886) in 975 μ L dimethyl sulfoxide. Aliquot (50 μ L) and keep at -20°C .
13. 1000X insulin (5 mg/mL): add 20 mg insulin (Sigma, cat. no. I6634) in 4 mL 0.01 M HCl. Sterilize with a 0.20- μ M filter. Aliquot (25 μ L) and keep at -20°C .
14. 1000X NT3 (5 $\mu\text{g}/\text{mL}$): dilute 2 μg human NT3 (Peprotech, cat. no. 450-03) in 0.4 mL sterile PBS. Aliquot (25 μ L) and keep at -80°C .
15. 1000X Shh (N-term) (0.3 mg/mL) (R&D systems, Oxon, UK; cat. no. 461-SH-025).
16. Sato 100X stock solution: combine 20 mL DMEM, 200 mg transferrin, 200 mg crystalline bovine serum albumin, 5 μ L progesterone stock solution, 32 mg putrescine, and 200 μ L sodium selenite stock solution. Aliquot (0.2 mL) and keep at -20°C .
17. OPC differentiation medium (Sato base): combine 20 mL DMEM, 200 μ L PSG, 200 μ L 100X Sato stock solution, 200 μ L 100X *N*-acetyl-cysteine, 4 μ L 5000X forskolin, 20 μ L 1000X biotin, 20 μ L 1000X insulin, 20 μ L 1000X NT3. Keep this medium for 1–2 wk at 4°C . Add growth factor and drugs when needed.

2.1.2.4. OLIGODENDROCYTE DIFFERENTIATION

1. 1000X PDGF-AA (10 $\mu\text{g}/\text{mL}$): dilute 2 μg human PDGF (Peprotech, cat. no. 100-13A) in 0.2 mL filter-sterilized 10 mM acetic acid. Aliquot (20 μ L) and store at -80°C .
2. 1000X T3 (triiodothyronine, thyroid hormone) (40 $\mu\text{g}/\text{mL}$): prepare a solution of 4 mg/mL T3 (Sigma, cat. no. T6397) in 0.1 N NaOH. Dilute this solution 100X in DMEM to make a 1000X solution. Aliquot (20 μ L) and store at -80°C .

2.2. Analysis of Differentiation

2.2.1. Reverse Transcriptase Polymerase Chain Reaction

1. Thermal cycler.
2. Agarose gel apparatus and reagents.
3. Diethylpyrocarbonate-water.
4. RNeasy Mini Kit (Qiagen, West Sussex, UK; cat. no. 74103).
5. Oligo dT₁₂₋₁₈ (Invitrogen, cat. no. 18418-012).
6. Avian myeloblastosis virus (AMV) reverse transcriptase (Promega, Southampton, UK; cat. no. M5101).
7. 10 mM dNTP mix (dATP, dCTP, dGTP, dTTP, each at 10 mM) (Promega, cat. no. C1141).
8. RNasin ribonuclease inhibitor (Promega, cat. no. N2511).
9. Polymerase chain reaction (PCR) 5' and 3' primers diluted to 3 μ M with sterile water.
10. Platinum *Taq* DNA polymerase high fidelity (Promega, cat. no. 11304-011).

2.2.2. Immunohistochemistry

1. PDL-coated 13-mm glass cover slips.
2. 4% paraformaldehyde: dilute 16% stock solution (Electron Microscopy Sciences RT 157-10) 1:4 in PBS and keep at 4°C for no more than 1 wk.
3. 10% goat serum: dilute goat serum (Invitrogen, cat. no. 16210072) 1:10 in PBS. Keep at 4°C for no more than 1 wk.
4. 0.1% Triton in 10% goat serum.
5. Monoclonal stage-specific embryonic antigen (SSEA) 1 antibody (diluted 1/5; Developmental Studies Hybridoma Bank, Iowa City, IA; cat. no. MC-480).

6. Monoclonal antirat nestin antibody (diluted 1/100, Pharmingen, San Diego, CA; cat. no. 556309).
7. Rabbit anti-NG2 chondroitin sulfate proteoglycan antibodies (diluted 1/50; Chemicon, cat. no. AB5320).
8. Monoclonal anti-GC antibody (supernatant, diluted 1/5) (16).
9. Conjugated secondary antibodies. Fluorescein isothiocyanate-coupled goat antimouse immunoglobulin or goat antirabbit immunoglobulin antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA; cat. no. 111095003 and cat. no. 115225003).
10. 500X Hoechst cat. no. 33342 (4 mg/mL): dilute 4 mg Hoechst (Sigma, cat. no. B2261) in 1 mL H₂O. Aliquot (100 μ L) and store at -20°C away from light.
11. 95% EtOH-5% acetic acid (-20°C).
12. Citifluor mounting medium (Electron Microscopy Sciences, London, UK; cat. no. 17970).
13. Microscope slides.
14. Tissues.
15. Nail polish.
16. Microscopes. Cover slips were examined with a Zeiss Axioplan 2 fluorescence microscope. Nunclon multidishes were examined with a Leica DMIRB inverted fluorescence microscope.

3. Methods

To obtain a homogeneous population of neural precursor cells from which oligodendrocyte lineage cells originate, we used genetically engineered ES cells that allow the selection of such committed cells. Specifically, we used doubly targeted ES cells so that, after induction of differentiation, we could select negatively against residual ES cells and select positively for neural precursor cells. Genetic engineering of ES cells was done in A. Smith laboratory (Centre for Genome Research, Edinburgh, UK), and only the theoretical fundamentals of it are briefly described here: to select against residual ES cells, a *hygromycin-thymidine-kinase* (*tk*) fusion gene was introduced into the *Oct4* locus (46). As *Oct4* is expressed in undifferentiated ES cells (47), such engineered ES cells should be eliminated by treatment with ganciclovir; to select for neural precursor cells, a β *geo* gene was introduced into the *Sox2* locus (45). As *Sox2* is specifically expressed in neural precursor cells (48), these cells should selectively survive treatment with G418 (*see Note 2*). Thus, by treating the doubly targeted ES cells with both ganciclovir and G418, undifferentiated ES cells should be selected against, and neural precursor cells should be selected for. Two independently derived parental ES cell lines were used for genetic engineering: CGR8 (49) and E14Tg2a (50). Both lines support neural and oligodendrocyte differentiation and should be available on request from the Smith lab.

3.1. Tissue Culture

3.1.1. Maintenance of ES Cells

CGR8 and E14Tg2a ES cells can be grown on gelatin-coated TC dishes in GMEM supplemented with 10% serum and LIF. ES cells must be passaged every other day, and they should never reach more than 90% confluence to obtain good neural differentiation afterward. Serum can affect ES cell growth and differentiation, so it should be batch tested (*see Note 1*). Mycoplasma contamination can also have profound effects

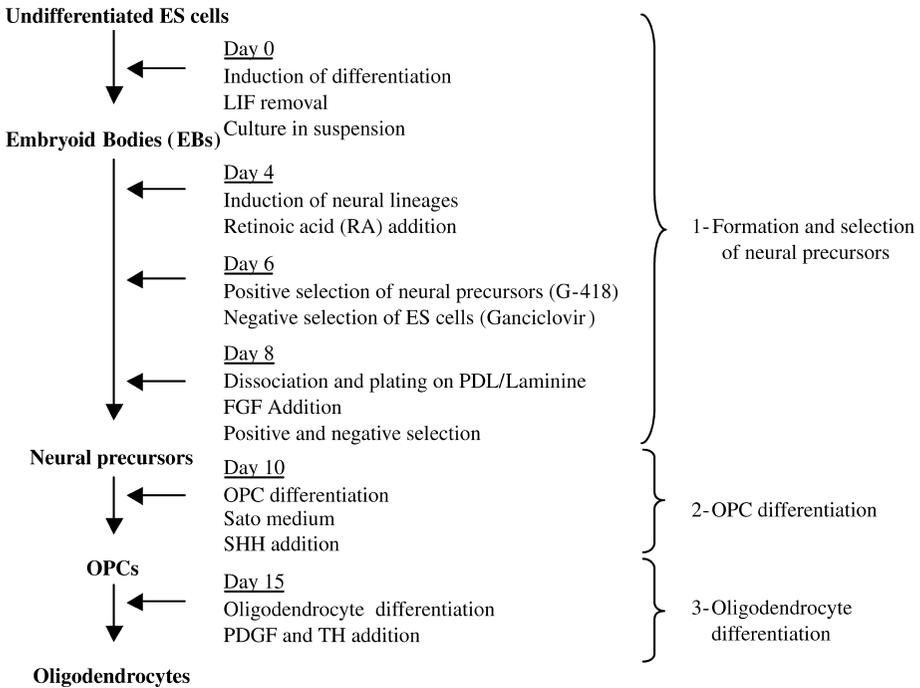


Fig. 1. Schematic view of the differentiation protocol.

on ES cell growth and differentiation, so it is recommended that cells are tested routinely for mycoplasma (*see Note 3*).

To subculture ES cells:

1. Prepare a 0.1% gelatin solution by diluting 2% gelatin in PBS, 37°C. Add 5 mL of this solution to a 25-cm² flask and incubate for about 20 min.
2. During this time, passage the cells. Gently remove medium and wash with 10 mL PBS-EDTA. Remove PBS and add 1.5 mL trypsin. Return flask to incubator for 5 min.
3. Gently agitate the flask. When the cells are floating, neutralize trypsin with 8.5 mL neutralization medium. Pipet up and down several times to make a single-cell suspension and transfer the cells into a universal tube.
4. Collect the cells by centrifugation at 700g for 5 min.
5. Resuspend in 10 mL ES cell medium plus LIF (10³ U/mL).
6. Count cells (usually 10⁷ cells can be obtained from a 25-cm² flask).
7. Remove gelatin from the flask and plate 1.5 × 10⁶ cells/flask in 5 mL ES cell medium plus LIF.

3.1.2. Differentiation of ES Cells Into the Oligodendrocyte Lineage

Our differentiation protocol consists of three distinct stages, outlined in **Fig. 1**.

Stage 1: Formation and selection of neural precursors. We have used a standard way to induce ES cells to differentiate, which is to allow them to aggregate in vitro in the absence

of LIF to form EBs. Treatment with RA at d 4 encourages the cells in the EBs to differentiate into neural cells, which can then be selected by the addition of G418 at d 6. Furthermore, undifferentiated ES cells are removed by adding ganciclovir to the medium. At d 8, a good proportion of neural precursors have formed within the EBs, and they are dissociated and encouraged to develop further by plating out on a PDL-laminin substrate in the presence of FGF-2, which has been shown to be important for neural precursors proliferation *in vitro* (51) and *in vivo* (52). Positive and negative selections are maintained for another 2 d to enrich for neural precursors.

Stage 2: OPC differentiation. By d 10 of differentiation, more than 85% of ES cell-derived cells are neural precursors, and undifferentiated ES cells have been eliminated. The purified population of neural precursors can then be induced to differentiate through the oligodendrocyte lineage. This is achieved by culturing the cells in a serum-free, modified Bottenstein-Sato medium known to be permissive for oligodendrocyte development *in vitro* (53) in the presence of Shh, which has been shown to promote the development of OPCs from neural precursors *in vitro* and *in vivo* (54–57).

Stage 3: Oligodendrocyte differentiation. By d 15 of differentiation, OPCs have formed, and they can be induced to differentiate by PDGF and thyroid hormone, which have been shown to promote OPC differentiation in cultures of purified rat OPCs (23–26).

3.1.2.1. COATING OF THE PLATES

At some point during the first 8 d of differentiation, make sure to coat Falcon 25-cm² flasks and Nunc four-well plates with PDL:

1. Prepare a 10- μ g/mL PDL solution by diluting 1 mg/mL 100X PDL in sterile water. This diluted solution can be kept for up to 2 wk at 4°C.
2. Cover the bottom of the plates with this solution and leave under the hood for 5–15 h.
3. Remove PDL solution and wash the plates three times with sterile water.
4. Let the plates dry under the hood (1–12 h).
5. Use the plates immediately or store them for up to 2 wk at room temperature.

3.1.2.2. FORMATION AND SELECTION OF NEURAL PRECURSORS

1. At 1 d before differentiation, passage the cells as described in **Subheading 3.1.1**. Replate the cells at $3\text{--}5 \times 10^6$ cells/flask so that they reach 90% confluency on the day differentiation is started.
2. On d 0 of differentiation, trypsinize the cells as in **Subheading 3.1.1**. At **step 3**, add 10 mL neutralization medium and resuspend cell pellet by gently shaking the tube (no extensive pipetting). At this stage, it is recommended to leave small aggregates of two to five cells instead of single cells as it seems to promote further differentiation.
3. Collect cells by centrifugation and gently resuspend them (as in **step 2**) in 5 mL ES cell medium (without LIF). Count cells (a single-cell suspension can be obtained by pipetting a small aliquot with a 200- μ L pipetboy).
4. Put 7×10^6 cells in a 10-cm bacterial dish and complete to 10 mL with ES cell medium.
5. Check for small cell aggregates under the microscope. If there are too many single cells, then repeat centrifugation and gently resuspend the pellet as in **step 2**.
6. Return cells to the incubator and check for the formation of EBs every day under the microscope (they should be visible from d 1) (*see Note 4*).
7. Change ES cell medium every other day. To change the medium, gently transfer EBs into a universal container with a 10-mL plastic pipet. When EBs have settled (5 min),

gently remove supernatant by aspiration. Add fresh medium and transfer EBs into a new bacterial dish.

8. On d 4, when changing the medium, add 10^{-6} M RA (2 μ L 5000X stock in 10 mL ES cell medium).
9. On d 6, when changing the medium, replace RA-containing ES cell medium by neural differentiation medium (F12-N2-NB-B27). Start selection of neural precursors by adding 100 μ g/mL G-418 (5 μ L 2000X stock in 10 mL medium).
10. On d 8, dissociate EBs and plate them on coated 25-cm² flasks.

3.1.2.3. COATING PDL-COATED 25-CM² FLASKS WITH LAMININ

1. Dilute 1 mg/mL laminin 1/100 in sterile PBS.
2. Put 5 mL of this solution in a 25-cm² flask precoated with PDL.
3. Put the flasks in the incubator while dissociating EBs (approx 1 h).
4. Remove laminin just before plating the cells (do not rinse).

3.1.2.4. DISSOCIATION OF EBs

1. Wash EBs with 10 mL PBS (let EBs settle and then remove PBS).
2. Add 0.5 mL warm 4X trypsin and incubate at 37°C in a water bath for 5–10 min. Carefully check for EB dissociation (*see Note 5*).
3. Neutralize trypsin with 10 mL neutralization medium and dissociate EBs into single cells by gently pipetting up and down with a 10-mL pipet.
4. Collect cells by centrifugation at 700g for 5 min.
5. Wash once with 10 mL DMEM to remove all traces of serum.
6. Collect cells by centrifugation at 700g for 5 min.
7. Resuspend cells in 5 mL neural differentiation medium by pipetting up and down three or four times with a 5-mL glass pipet.
8. Count cells. Usually, 5×10^6 to 10^7 cells can be obtained from one 10-cm bacterial dish. Flask 3×10^6 cells per 25-cm² flask (precoated with PDL and laminin) in 5 mL differentiation medium.
9. Add 20 μ g/mL FGF-2 (5 μ L 1000X stock) to promote neural precursor proliferation, 100 μ g/mL G-418 (2.5 μ L 2000X stock) to select for neural precursors and 2.5 μ M ganciclovir (5 μ L 1000X stock) to select against any residual ES cells.
10. Maintain cells in this medium for 2 d more to favor the expansion of neural precursors.

This procedure results in progressive enrichment in neural precursors together with a decrease in undifferentiated ES cells (**Fig. 2**). By d 10, it reproducibly generates a cell population containing more than 85% nestin-expressing neural precursors.

3.1.2.5. OPC DIFFERENTIATION

On d 10 of differentiation, selection can be stopped, and OPCs can be induced to differentiate from the homogeneous population of neural precursors. OPCs can be identified from d 15 by staining for NG2 (**Fig. 3**).

1. Coat PDL-coated Nunc four-well plates with laminin as in **Subheading 3.1.2.3.** and prepare OPC differentiation medium.
2. To passage neural precursors, gently wash the cells with warm EBSS and add 1 mL trypsin 0.005%. Return to the incubator for 1–5 min (check for floating cells).

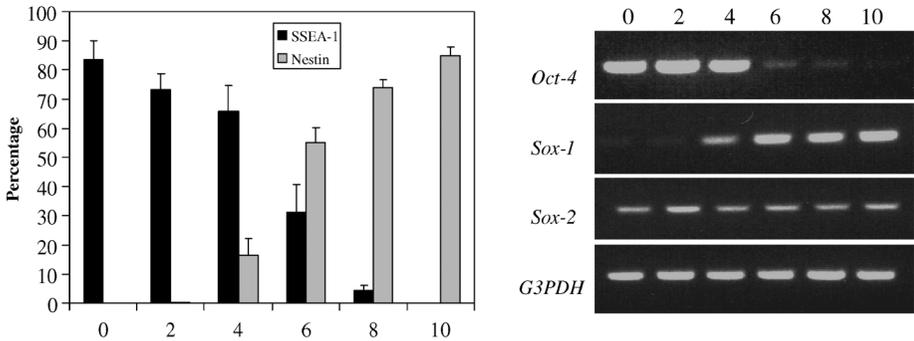


Fig. 2. Development and selection of neural precursors from engineered embryonic stem (ES) cells. Engineered ES cells were treated and selected as described in **Fig. 1** to enrich for neural precursors and to eliminate residual undifferentiated ES cells. At various times after the start of differentiation, the cells were dissociated and either stained for stage-specific embryonic antigen or nestin by immunohistochemistry (**left**) or processed for reverse transcriptase polymerase chain reaction analysis using *Oct4*, *Sox1*, *Sox2*, or *G3PDH* probes (**right**).

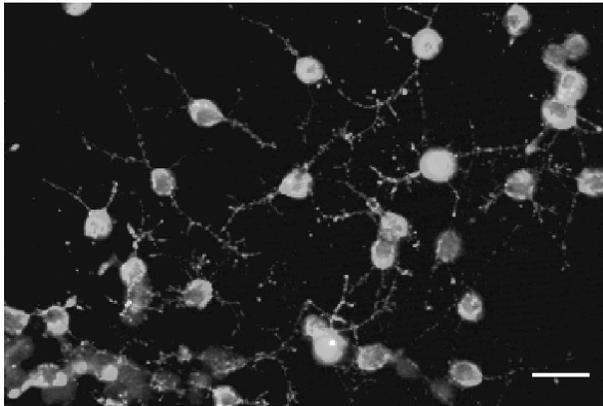


Fig. 3. Development of OPCs from embryonic stem cell-derived neural precursors. At d 15 of differentiation, the cells were stained with anti-NG2 antibody to identify oligodendrocyte precursor cells and bisbenzimidazole to identify cell nuclei.

3. When the first cells are floating, add 9 mL neutralization medium and gently pipet up and down. Collect the cells by centrifugation at 700g for 5 min.
4. Wash the cells with 10 mL DMEM to remove all trace of serum.
5. Collect cells by centrifugation and gently resuspend the cells in 5 mL Sato medium.
6. Count the cells and plate them in four-well plates coated with PDL and laminin. Put 5×10^4 cells/well (0.5 mL/well) in OPC differentiation medium containing 20 ng/mL FGF-2 and 0.3 μ g/mL Shh.
7. Change half of the medium every other day for 5 d (until d 15 of differentiation). If the cells become confluent during this time-course, then passage them as described in **steps 2–6**.

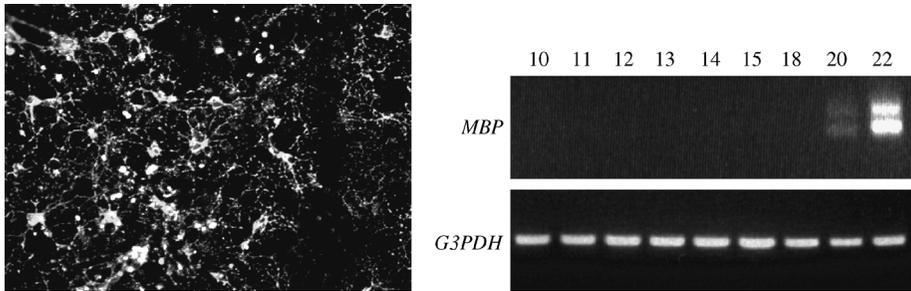


Fig. 4. Development of oligodendrocytes from embryonic stem cell-derived oligodendrocyte precursor cells. At d 22 of differentiation, the cells were stained with anti-galactocerebroside antibody to identify oligodendrocytes and bisbenzimidazole to identify cell nuclei (**left**). At various times after the start of differentiation, the cells were processed for reverse transcriptase polymerase chain reaction analysis using myelin basic protein or G3PDH probes (**right**).

3.1.2.6. OLIGODENDROCYTE DIFFERENTIATION

On d 15 of differentiation, OPCs have formed and can be induced to differentiate. Oligodendrocytes can be identified from d 22 by GC staining or expression of myelin basic protein (MBP) mRNA (**Fig. 4**).

1. Replace medium with Sato medium containing 10 ng/mL PDGF-AA and 40 ng/mL T3.
2. Change half of the medium every other day for 7 d (until d 22 of differentiation).

3.2. Analysis of Differentiation

A variety of markers can be used to follow the fate of ES cells and to help identify neural precursors, OPCs, and oligodendrocytes as outlined in **Fig. 5**. We have used reverse transcriptase (RT) PCR analysis to detect *Oct4*, *Sox1*, *Sox2*, and *MBP* mRNAs and immunohistochemistry to detect SSEA-1, nestin, NG2, and GC antigens (*see Note 6*). We scored cells as positive in immunohistochemical assays only if they also had the characteristic morphology of OPCs and oligodendrocytes (**58**).

3.2.1. Reverse Transcriptase Polymerase Chain Reaction

The sequences of the primers that we used to detect *Oct4*, *Sox1*, *Sox2*, and *MBP* mRNAs are indicated in **Table 1**.

3.2.1.1. RNA EXTRACTION

If RNA is to be extracted from EBs, then proceed to **step 1**. If RNA is to be extracted from neural precursors, OPCs, or oligodendrocytes, then trypsinize cells as in **Subheading 3.1.2.3., steps 2–6**. After neutralization of the trypsin with serum-containing medium,

1. Harvest cells by centrifugation at 700g for 5 min.
2. Remove medium and gently wash the cell pellet with 5 mL warm PBS.
3. Facultative: count cells and keep a record of cell numbers for further RNA extraction.
4. Collect cells by centrifugation at 700g for 5 min.

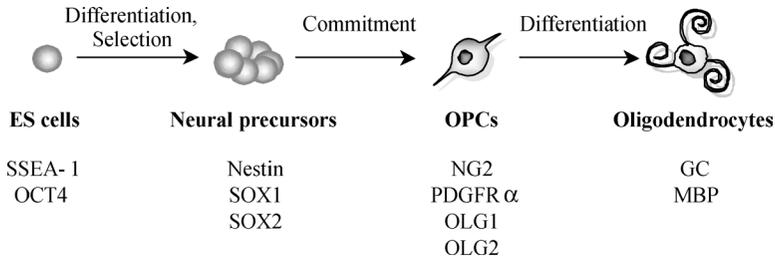


Fig. 5. Markers used to identify embryonic stem cells, neural precursors, and oligodendrocyte lineage cells.

Table 1
Primer Sequences

Gene	Forward primer	Reverse primer
Oct4	5'-CTG CTG AAG CAG AAG AGG ATC AC-3'	5'-TGG TTC TGT AAC CGG CGC CAG AAG-3'
Sox1	5'-TTA CTT CCC GCC AGC TCT TC-3'	5'-TGA TGC ATT TTG GGG GTA TCT CTC-3'
Sox2	5'-AAC ATG ATG GAG ACG GAG CTG AAG C-3'	5'-TAC GCG CAC ATG AAC GGC TGG AG-3'
MBP	5'-AAG TAC TTG GCC ACA GCA AG-3'	5'-CAG AGC GGC TGTCTC TTC-3'
G3PDH	5'-ACC ACA GTC CAT GCC ATC AC-3'	5'-TCC ACC ACC CTG TTGCTG TA-3'

5. Remove supernatant, add 1 mL fresh PBS, and transfer the cells to an Eppendorf tube.
6. Centrifuge at maximum speed for 1 min.
7. Remove supernatant and snap-freeze the cell pellet on dry ice (*see Note 7*).
8. Extract RNA using the RNeasy Mini Kit from Qiagen following the manufacturer's instructions for isolation of cytoplasmic RNA from animal cells.
9. At the last step, elute RNA in 30 μ L RNase-free water.
10. Find the optical density (OD) at A_{260} and A_{280} . Determine the concentration of the RNA sample (*see Note 8*).

3.2.1.2. REVERSE TRANSCRIPTION

1. In a PCR tube, add 1 μ g RNA diluted in water to a final volume of 4 μ L and 1 μ L Oligo dT₁₂₋₁₈ (Gibco BRL).
2. Heat at 70°C for 5 min.
3. Cool on ice for 5 min.
4. Prepare the RT mix (*see Note 9*): 5X AMV reverse transcriptase buffer, 2.5 μ L 10 mM dNTP mix, 1 μ L RNasin, AMV-RT, and 8.5 μ L H₂O.
5. Add the RT mix to the RNA and vortex briefly.
6. Incubate at 42°C for 60 min.

Table 2
Annealing Temperature and Number of Cycles for PCR Amplification

Gene	Annealing temperature	Number of PCR cycles
OCT4	62°C	35
SOX1	62°C	25
SOX2	62°C	35
MBP	53°C	26
G3PDH	60°C	25

7. Incubate at 95°C for 10 min.
8. Cool on ice for 5 min.

3.2.1.3. POLYMERASE CHAIN REACTION

1. Prepare the PCR mix (25 μ L final volume): 1 μ L template complementary DNA, 2.5 μ L 10X high-fidelity PCR buffer, 0.5 μ L 25 mM MgSO₄, 0.5 μ L 10 mM dNTP mix, 2.5 μ L 3 μ M 5' primer, 2.5 μ L 3 μ M 3' primer, 0.2 μ L *Taq* DNA polymerase high fidelity, and 15.8 μ L H₂O.
2. Centrifuge the tubes briefly to collect the contents.
3. Incubate tubes in a thermal cycler at 94°C for 2 min.
4. Perform 25–35 cycles of PCR amplification as follows: the number of cycles and the annealing temperature depends on the primers used (*see Table 2*): 94°C for 30 s, X°C for 30 s, 72°C for 90 s.
5. Incubate at 72°C for 5 min.
6. Maintain the reaction at 4°C.
7. Analyze the products by agarose gel electrophoresis and visualize by ethidium bromide staining. Use appropriate molecular weight standards.

3.2.2. Immunohistochemistry

Before starting the staining procedure, cells must be plated at low density (10⁵ cells/cover slip) on PDL-coated cover slips. They are then left on the cover slips for a minimum of 2 h before they can be fixed and stained. If staining is to be performed on EBs, **then** they first have to be trypsinized as indicated in **Subheading 3.1.2.4.** and then seeded on cover slips. If staining is to be performed on dissociated neural precursors, OPCs, or oligodendrocytes, **then** cells can be grown directly on cover slips or trypsinized and put on cover slips just before staining, as for EBs.

3.2.2.1. PDL COATING OF GLASS COVER SLIPS

1. Wash cover slips in 70% EtOH for 1 h (with agitation).
2. Replace with fresh EtOH and incubate overnight with agitation. Replace with fresh EtOH and keep until use.
3. When ready to use, wash the cover slips with sterile water about five or six times (rapid washes) and put the cover slips in four-well plates.
4. Proceed with coating of cover slips. Coat with PDL as described in **Subheading 3.1.2.1.**

3.2.2.2. STAINING PROCEDURE

1. Gently wash the cells with warm PBS.
2. Fix in 4% paraformaldehyde for 5 min.
3. Wash twice with PBS.
4. Add 10% goat serum and incubate for 30 min to block nonspecific staining.
5. When needed (nuclear or cytoplasmic antigen), permeabilize membranes by incubating the cells in 0.1% Triton (diluted in goat serum) for 30 min. For membrane antigens, directly proceed to **step 7**.
6. Wash twice in PBS.
7. Incubate with primary antibody (diluted in 10% goat serum) for 60 min.
8. Wash four times in PBS.
9. Incubate with conjugated secondary antibody (diluted 1/100 in 10% goat serum) and Hoechst 33342 (diluted 1/500, for coloration of the nucleus) for 60 min.
10. Wash four times in PBS.
11. Postfix the cells with 95%EtOH-5% acetic acid (-20°C) for 30 s (*see Note 10*).
12. Wash twice with PBS.
13. Mount cover slips on a slide: add a very small drop of Citifluor on the slide with a 200- μL tip; gently pour over the cover slip and absorb the excess of liquid with a tissue. Seal with nail polish. Wait about 45 min before looking at the slide under the microscope.

4. Notes

1. The ability of serum to support growth of pluripotent ES cells is crucial. Every time new serum is to be ordered, several lots should be tested, and suitable batches must be ordered in large quantities to avoid variability. As an indication, we usually consider a serum suitable if it allows sustained ES cell growth (10^7 cells must be obtained out of 1.5×10^6 cells after 48 h for at least three passages), and it is not toxic at a 30% concentration. A number of suppliers now sell sera that have already been tested for supporting ES cell growth.
2. Undifferentiated ES cells also express *Sox2* and would be expected therefore to survive G418; they should be eliminated by ganciclovir.
3. Several methods and kits are now available to test for *Mycoplasma* contamination. We have been successfully using kits from ATCC, Venorgem, and Gibco-BRL.
4. To give nice neural precursors, EBs should be compact with a round, three-dimensional shape, dark in the middle with a large surrounding light band, or completely light. When EBs have an irregular shape and a smooth appearance, are entirely dark (meaning that there are many dead cells), or stick to the bottom of the dish, it is usually a bad prognostic for neural differentiation.
5. EB dissociation is an important step. Overtrypsinization may result in cell death; undertrypsinization may keep EBs as cell aggregates that may not proceed correctly with neural differentiation. Therefore, it is recommended to carefully check for EB dissociation during trypsinization—when ready, the solution should become cloudy—and stop it when necessary. If too many cell aggregates are still present after dissociation, then repeat trypsinization.
6. Other proteins that are characteristic of oligodendrocytes and their precursors are expressed earlier than NG2 and may therefore be used to identify the earliest stages of oligodendrocyte lineage specification in ES cell-derived cultures. These include the *Olig1* and *Olig2* basic helix-loop-helix gene regulatory proteins (*11,12*), as well as PDGFR- α , which can be detected by *in situ* hybridization or RT-PCR (*46*).
7. Cell extracts may be stored at -80°C until RNA extraction.

8. The A_{260}/A_{280} ratio should be between 1.6 and 2. To determine RNA concentration, use the following equation: $[] \mu\text{g}/\mu\text{L} = [A_{260} \times 40 \times \text{Dilution factor}]/1000$.
9. As genomic DNA has not been removed from RNA samples, always include a control for which RT is omitted and replaced by water.
10. This allows the cells to stick better on the cover slip, and it amplifies the signal.

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