Normal timing of oligodendrocyte development from genetically engineered, lineage-selectable mouse ES cells

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Summary

Oligodendrocytes are post-mitotic cells that myelinate axons in the vertebrate central nervous system (CNS). They 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 CNS stem cells that give rise to OPCs have not been purified, limiting one's 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 have genetically engineered ES cells both to positively select neuroepithelial stem cells and to eliminate undifferentiated ES cells. We have then used combinations of known signal molecules to promote the development of OPCs from selected, ES-cell-derived, neuroepithelial cells. We show that the earliest stages of oligodendrocyte development follow an ordered sequence that is remarkably similar to that observed in vivo, suggesting that the ES-cell-derived neuroepithelial cells follow a normal developmental pathway to produce oligodendrocytes. These engineered ES cells thus provide a powerful system to study both the mechanisms that direct CNS stem cells down the oligodendrocyte pathway and those that influence subsequent oligodendrocyte differentiation. This strategy may also be useful for producing human cells for therapy and drug screening.

Key words: Oligodendrocyte, Development, ES cells, Genetic selection

Introduction

Oligodendrocytes are post-mitotic 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 neuroepithelial cells of the neural tube. Oligodendrocyte development begins in the embryo, when oligodendrocyte precursor cells (OPCs) arise in very restricted regions of the ventral ventricular zone of the developing brain and spinal cord (Noll and Miller, 1993; Ono et al., 1997; Pringle et al., 1998; Timsit et al., 1995; Yu et al., 1994). The restriction of OPC development to these regions depends on localised positive and negative signals (Wada et al., 2000; Woodruff et al., 2001). The initial appearance of OPCs in the developing spinal cord, for example, depends on positive signals from the adjacent notochord: removal of the notochord in *Xenopus*, mouse or chick embryos inhibits the formation of these cells (Maier and Miller, 1997; Pringle et al., 1996), whereas transplantation of an additional notochord adjacent to the dorsal spinal cord induces their ectopic formation (Maier and Miller, 1997; Orentas and Miller, 1996). One such positive signal is Sonic hedgehog (Shh), which is secreted by both the floor plate and the notochord along the rostro-caudal axis (Echelard et al., 1993; Placzek et al., 1993; Roelink et al., 1994). Shh can induce the development of oligodendrocytes in dorsal spinal cord explants (Pringle et al., 1996; Roelink et al., 1995), and neutralising anti-Shh antibodies can block oligodendrocyte development in ventral regions of CNS explants (Orentas et al., 1999; Tekki-Kessaris et al., 2001). Furthermore, mice with a targeted deletion of the *Nkx2.1* gene lack Shh expression in the most anterior domain of the ventral hypothalamus, and OPCs fail to appear in this region at the appropriate stage of development (Sussel et al., 1999; Tekki-Kessaris et al., 2001).

The development of OPCs from neuroepithelial cells has mostly been studied in the rodent neural tube. In the mouse spinal cord, for example, the first OPCs expressing the platelet-derived growth factor receptor α (*PDGFRα*) are detected as a narrow band in the ventral neuroepithelium around E12.5-13, well after neuronal development begins at E9 (Hardy, 1997; Pringle et al., 1998). Some proteins that are characteristic of oligodendrocytes and their precursors are expressed earlier than *PDGFRα*, however, and may identify the earliest stages of oligodendrocyte lineage specification. These include the *Olig1* and *Olig2* basic helix-loop-helix gene regulatory proteins, which are expressed in the same region of the spinal cord as *PDGFRα* at E13, but are expressed as early as E9 (Lu

et al., 2000; Zhou et al., 2000). The earliest *Olig2*-expressing cells in the ventral spinal cord, however, may develop into neurons rather than OPCs (Marquardt and Pfaff, 2001; Mizuguchi et al., 2001; Novitch et al., 2001; Sun et al., 2001; Zhou et al., 2001): if *Olig2* is inactivated, neither motor neurons nor OPCs develop in the spinal cord (Lu et al., 2002; Zhou and Anderson, 2002). By E11.5-12, however, *Olig* expression is restricted to the same region, where *PDGFR*α will be expressed and OPCs emerge; it is likely, therefore, that this *Olig* expression marks the earliest stages of oligodendrocyte specification, 1-1.5 days before *PDGFR*α expression is detectable (Lu et al., 2000; Zhou et al., 2000). By E13, *Olig1*, *Olig2* and *PDGFR*α, as well as another OPC marker, the NG2 proteoglycan (Levine and Nishiyama, 1996), are all expressed in the same small region of the ventral spinal cord that generates OPCs (Lu et al., 2000; Pringle et al., 1996; Woodruff et al., 2001; Zhou et al., 2000). OPCs then migrate throughout the CNS, where they proliferate, largely in response to PDGF (Fruttiger et al., 1999) and terminally differentiate into oligodendrocytes. The first oligodendrocytes that express galactocerebroside (GC) appear in the mouse CNS around E17 (Calver et al., 1998).

The differentiation of OPCs into oligodendrocytes is better understood than the initial commitment of neuroepithelial cells to the oligodendrocyte lineage. The normal timing of OPC differentiation can be reconstituted in cultures of dissociated embryonic rat optic nerves, as long as the OPCs are stimulated to proliferate by PDGF (Raff et al., 1988), and hydrophobic signals such as thyroid hormone (TH) or retinoic acid (RA) are present (Ahlgren et al., 1997; Barres et al., 1994; Gao et al., 1998). Without PDGF, the cells rapidly stop dividing and differentiate (Temple and Raff, 1985). In the presence of PDGF, but in the absence of TH or RA, most of the OPCs tend to keep dividing and fail to differentiate (Barres et al., 1994). There is abundant evidence that TH plays an important part in timing oligodendrocyte development in vivo (Rodriguez-Pena, 1999). TH may also promote the commitment of CNS stem cells to the oligodendrocyte lineage (Johe et al., 1996).

So far, OPCs have been purified only from the rat optic nerve. Although studies of these purified cells 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 progenitor 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 (Brook and Gardner, 1997; Evans and Kaufman, 1981; Martin, 1981). They can be propagated indefinitely in culture in the presence of leukemia inhibitory factor (LIF) (Smith et al., 1988; Williams et al., 1988). When transplanted into a mouse blastocyst, ES cells integrate into the embryo and contribute to all cell lineages, including germ cells (Bradley et al., 1984). If ES cells are cultured without LIF on a non-adherent surface, they aggregate to form embryoid bodies (EBs), in which the cells form ectodermal, mesodermal and endodermal derivatives (Keller, 1995). ES cells can be produced in large numbers, can be easily genetically modified and can be induced to differentiate into various CNS cell types in vitro (Bain et al., 1995; Brustle et al., 1999; Fraichard et al., 1995; Okabe et al., 1996). They should therefore provide a powerful system for studying the early events of neural development. In the present study, we devised a strategy for using ES cells to study OPC and oligodendrocyte development.

We first modified the genome of mouse ES cells to allow us both to select positively for neuroepithelial cells (Li et al., 1998) and to select negatively against residual undifferentiated ES cells. We then studied various combinations of extracellular signal molecules to find optimal conditions for the development of OPCs from these doubly selected neuroepithelial cells. Using a variety of markers to follow the fate of the selected cells, we show that oligodendrocyte lineage cells can be efficiently produced in this way. Most important, we show that, when exposed to appropriate signal molecules, OPCs and oligodendrocytes develop on a predictable schedule that is similar to that observed in vivo, suggesting that the ES-cell-derived neuroepithelial cells follow a normal pathway of oligodendrocyte development. This model system should thus be useful for studying the early events of OPC specification and differentiation. This strategy may also be useful for producing OPCs and oligodendrocytes from human ES cells for cell therapy and drug screening.

Materials and Methods

ES cell culture

All reagents were from Gibco BRL unless otherwise indicated. The ES cell lines OS25 and OSG were grown and maintained in an undifferentiated state (Smith et al., 1988) on 0.1% gelatin-coated dishes (Costar T-25) in Glasgows' Minimal Eagle medium (G-MEM-BHK 21) containing 10% fetal calf serum (FCS, PAA Laboratories, Gmbh), non-essential amino acids, sodium pyruvate (1 mM), sodium bicarbonate (0.075%), penicillin G (100 units/ml), streptomycin (100 units/ml), L-glutamine (292 μg/ml), 2-mercaptoethanol (0.1 mM) and human recombinant LIF (1000 units/ml, Chemicon). Cells were passaged every other day and had a doubling time of around 14 hours.

Genetic engineering of ES cells

Two independently derived parental ES cell lines were used, CGR8 (Mountford et al., 1994) and E14Tg2a (Hooper et al., 1987). Both lines were subjected to sequential gene targeting in order to integrate *βgeo* into the *Sox2* locus (Li et al., 1998) and *hygromycin-thymidine kinase* (Lupton et al., 1991) into the *Oct4* (*Pou5f*) locus (Mountford et al., 1994). Linearised constructs were introduced into ES cells by electroporation, and stable transfectants were isolated by selection in G418 or hygromycin. Homologous recombinants were then identified by Southern hybridisation of genomic DNA samples using probes external to the homology regions. The *Sox2*-targeting construct and flanking probes were generously provided by Silvia Nicolis and Robin Lovell-Badge and will be described in detail elsewhere (S. Avilion and N.B., unpublished). The *Oct4* construct was generated by replacing *βgeo* in the *Oct4βgeo*-targeting vector (Mountford et al., 1994) with the hygromycin-thymidine kinase fusion gene. CGR8-derived OSG cells also carry a puromycin-selectable GFP transgene driven by the ubiquitous CAG expression unit (Pratt et al., 2000). Exposure of undifferentiated OSG or OS25 (E14Tg2a derivative) cells to gancyclovir eliminated all cells within 4 days. Following in vitro differentiation, cells became gancyclovir resistant. Neuronal differentiation and oligodendrocyte development occurred similarly in the two clones.

Differentiation protocol

Experiments with the engineered ES cell lines were performed using

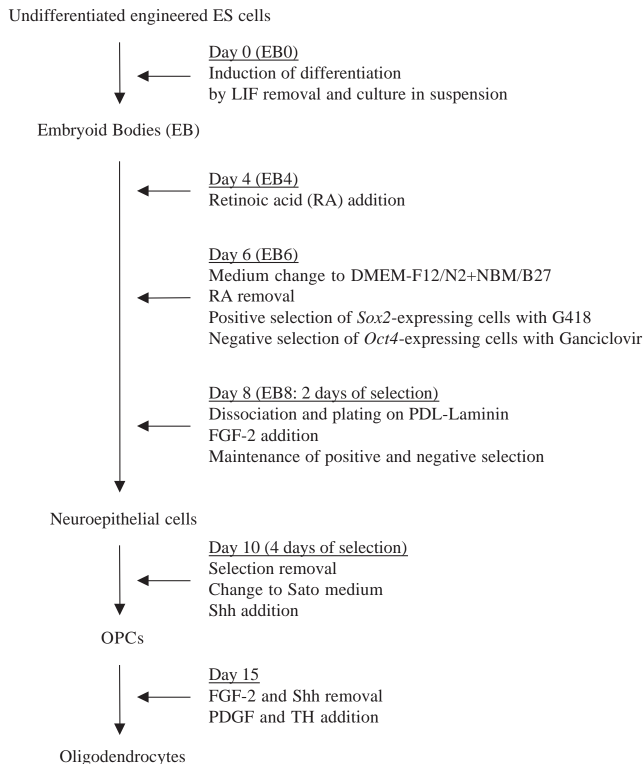


Fig. 1. Protocol for studying oligodendrocyte development from genetically engineered ES cells.

the culture protocol outlined in Fig. 1. On day 0, exponentially growing ES cells were trypsinised and 7×10^6 cells were plated on 10 cm bacterial dishes without LIF. In these conditions, ES cells form embryoid bodies (EBs). RA (10^{-6} M) was added on day 4 to promote neural development (Bain et al., 1995). After 2 days, the medium was replaced by a 50:50 mixture of DMEM-F12 containing N2 supplement and Neurobasal medium containing B27 supplement (Gibco). The N2 supplement contains insulin (25 μg/ml), transferrin (100 μg/ml), progesterone (6 ng/ml), putrescine (16 μg/ml), sodium selenite (30 nM) and BSA (50 μg/ml). In most experiments with genetically engineered ES cells, the medium was also supplemented with G-418 (100 μg/ml) and Ganciclovir (2.5 μM) to select for neuroepithelial cells and against undifferentiated ES cells, respectively. At day 8 (2 days of selection), EBs were dissociated with trypsin, and 2×10^6 cells were replated on poly-D-lysine (PDL; 10 μg/ml; Sigma) and laminin-coated (10 μg/ml; Sigma) tissue culture flasks (Falcon, T25) in the same medium, except that FGF-2 (Prepotech) was added at 20 ng/ml. Fresh G-418 and Ganciclovir were also added.

After 2 more days (day 10; 4 days of selection), the medium was changed to a modified Bottenstein-Sato medium (Bottenstein et al., 1979) to promote development along the oligodendrocyte pathway. It contained FGF-2 (20 ng/ml), NT3 (5 ng/ml), insulin (10 μg/ml), human transferin (100 μg/ml), BSA (100 μg/ml), progesterone (60 ng/ml), sodium selenite (40 ng/ml), N-acetyl-cysteine (60 μg/ml), putrescine (16 μg/ml), forskolin (5 μM), biotin (10 ng/ml), penicillin, streptomycin and L-glutamine. G418 and Ganciclovir were omitted, and, in some experiments, recombinant myristylated Shh-N (gift from Biogen, 300 ng/ml) was added.

In some experiments, 5 days after selection (when the first PDGFRα-positive OPCs were detected), FGF-2 was removed, and the cells were grown in the presence of PDGF-AA (10 ng/ml) and TH (triodothyronine, Sigma, 40 ng/ml) to promote the differentiation of OPCs into oligodendrocytes (Barres et al., 1994).

In situ hybridisation

Riboprobes were synthesised using a digoxigenin (DIG) RNA-labelling Kit (SP6/T7; Roche) according to the supplier's instructions. Probes used in this study included: *Olig1*, a 735 bp insert derived from the whole coding region of the mouse *olig1* cDNA; *Olig2*, a 630 bp insert derived from the 5′ region of the mouse *Olig2* cDNA; *PDGFRα*, a 1.6 Kb insert derived from the extracellular domain of the mouse *PDGFRα* cDNA. Sense RNA probes were prepared as controls for each Riboprobe.

In situ hybridisation with DIG-labelled Riboprobes was performed using a procedure developed by D. Anderson's laboratory (http://kclab.webprovider.com/protocols/alish.html). Briefly, following fixation with paraformaldehyde, acetylation with acetic anhydride and permeabilisation with 0.2 N HCl, cells were incubated in hybridisation buffer (50% formamide, 5× SSC, 4.8 units/ml yeast tRNA, 17 units/ml heparin, 1× Denhardt's solution, 0.1% Tween-20, 1.6 mM CHAPS, 5 mM EDTA) for 3-4 hours at room temperature. Hybridisation was carried out for 16 hours at 65°C, using 1-2 μg/ml of probe. After stringent washing with 0.2× saline-sodium citrate buffer (SSC; 3 mM sodium citrate pH 7.0, 30 mM NaCl) and blocking with 20% normal sheep serum, the signal was detected by overnight incubation at 4°C with a sheep anti-DIG antibody conjugated with alkaline phosphatase (Roche), followed by 24 hours at room temperature in a solution containing 100 mM Tris pH 9.5, 50 mM $MgCl_2$, 100 mM NaCl, 0.1% Tween 20, 5 mM Levamisole, 0.4 mM of 5-bromo-4-chloro-3-indolyl phosphate and 46 mM nitroblue tetrazolium.

RT-PCR analysis

Cells were harvested with trypsin and processed immediately for RT-PCR analysis. Poly $(A)^+$ mRNA was prepared using a QuickPrep Micro mRNA purification kit (Pharmacia Biotech). cDNAs were synthesised using AMV-Reverse Transcriptase (Promega) according to the supplier's instructions and were used as templates for the PCR reaction.

The following oligonucleotide primers were synthesised. For *glyceraldehyde-3-phosphate dehydrogenase* (*G3PDH*), the 5′ primer was 5′-ACC ACA GTC CAT GCC ATC AC-3′, and the 3′ primer was 5′-TCC ACC ACC CTG TTG CTG TA-3′; for *Oct4*, the 5′ primer was 5′-CTG CTG AAG CAG AAG AGG ATC AC-3′, and the 3′ primer was 5′-TGG TTC TGT AAC CGG CGC CAG AAG-3′; for *Sox2*, the 5′ primer was 5′-AAC ATG ATG GAG ACG GAG CTG AAG C-3′, and the 3′ primer was 5′-TAC GCG CAC ATG AAC GGC TGG AG-3′; for *Sox1*, the 5′ primer was 5′-TTA CTT CCC GCC AGC TCT TC-3′, and the 3′ primer was 5′-TGA TGC ATT TTG GGG GTA TCT CTC-3′; for *myelin basic protein* (*MBP*), the 5′ primer was 5′-AAGTACTTGGCCACAGCAAG-3′, and the 3′ primer was 5′-CAGAGCGGCTGTCTCTTC-3′.

The RT-PCR reactions were carried out as follows: 25 μl of reaction mixture contained 200 pg of template cDNA, 300 nM of 5′ and 3′ PCR primers, 0.2 mM dNTP, 1.25 mM $MgCl_2$, 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 0.1% Triton X-100, and 1.25 units of Taq DNA polymerase (Promega). The reaction mixture was denatured for 1 minute at 94°C. The PCR parameters were 94°C for 10 seconds for the denaturing step, 53°C (*MBP*), 62°C (*Sox2* and *Oct4*) or 60°C (*Olig1, Olig2, PDGFRα* and *GAPDH*) for 30 seconds for the annealing step, and 72°C for 1 minute for the elongation step. The PCR products were electrophoresed in a 1.2% agarose gel and stained with EtBr. The number of PCR cycles was 35 for *Sox-2* and *Oct4*, 26 for MPB and 25 for *G3PDH* and *Sox-1*.

Immunocytochemistry

Cells were cultured on PDL- and laminin-coated 13 mm glass cover-slips or Nunclon multidishes and fixed in 4% paraformaldehyde in PBS for 5 minutes at room temperature. After washing with PBS,

cells were incubated for 30 minutes in 10% normal goat serum to block non-specific staining. They were then incubated for 1 hour in the first antibody, washed in PBS and incubated for 1 hour in FITC-coupled goat anti-mouse Ig or goat anti-rabbit Ig antibodies (diluted 1/100; Jackson Immuno Research Laboratories) and bisbenzamide (5 ng/ml; Hoescht No. 33342; Sigma). Coverslips were mounted in Citifluor mounting medium (CitiFluor, UK) and examined with a Zeiss Axioplan 2 fluorescence microscope. Nunclon multidishes were examined with a Leica DMIRB inverted fluorescence microscope.

The following antibodies were used: monoclonal anti-galactocerebroside (GC) antibody (supernatant, diluted 1/5) (Ranscht et al., 1982); monoclonal SSEA-1 antibody (supernatant, diluted 1/5; Developmental Studies Hybridoma Bank); monoclonal anti-rat nestin antibody (diluted 1/100, Pharmingen); HA 1297 cocktail of affinity-purified rabbit antibodies that together recognize all three neurofilament proteins (diluted 1/200; Affiniti Research Products Limited); rabbit anti-NG2 chondroitin sulfate proteoglycan antibodies (diluted 1/50; Chemicon International); and O4 monoclonal antibody (supernatant, diluted 1/20) (Sommer and Schachner, 1981).

Results

Genetically engineering ES cells to enrich for neuroepithelial cells

We first used a standard protocol to encourage ES cells to develop along a neural pathway within embryoid bodies (EBs) (Bain et al., 1995; Li et al., 1998). We cultured the cells in suspension on a non-adherent surface without LIF for 8 days to induce the formation of EBs and added RA to promote neural development. After 8 days, we dissociated EBs and replated the cells on culture dishes for further development. At different stages in this protocol, we dissociated the cells and stained them for the expression of nestin protein to identify neuroepithelial cells (Brustle and McKay, 1995) and SSEA-1 antigen to identify residual, undifferentiated ES cells (Vassilieva et al., 2000). As shown in Fig. 2, this protocol resulted in a gradual increase in nestin expression and a gradual decrease in SSEA-1 expression. However, the maximum proportion of nestin-positive cells we could obtain in this way was 60% after 8 days of differentiation, and there were always 1-5% residual SSEA-1-positive ES cells present in the culture.

We therefore genetically engineered ES cells so that we could select against residual ES cells and select for neuroepithelial cells. To select against residual ES cells, we introduced a *hygromycin-thymidine-kinase* (*tk*) fusion gene into the *Oct4* locus by homologous recombination. As *Oct4* is expressed in undifferentiated ES cells (Scholer et al., 1989), we should be able to eliminate such engineered ES cells by treatment with Gancyclovir. To select for neuroepithelial cells, we introduced a *βgeo* gene into the *Sox2* locus (Li et al., 1998). As *Sox2* is specifically expressed in neuroepithelial cells (Zappone et al., 2000), these cells should selectively survive treatment with G418 (undifferentiated ES cells also express *sox2* and would be expected, therefore, to survive G418, but they should be eliminated by Ganciclovir). Thus, by treating the doubly targeted ES cells with both Ganciclovir and G418, undifferentiated ES cells could be selected against, and neuroepithelial cells could be selected for.

When we selected 6-day-old EBs derived from these doubly targeted ES cells for 4 days in G418 and Ganciclovir, no cells expressing SSEA-1 antigen (Fig. 3A) or *Oct4* mRNA (Fig. 3B) could be detected, suggesting that no residual ES cells were

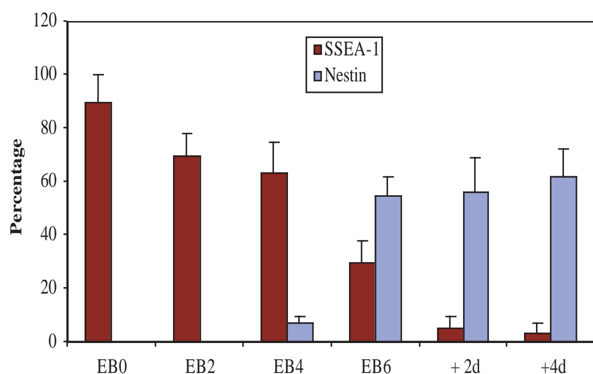


Fig. 2. Neuroepithelial cells and residual ES cells in non-selected ES-cell-derived cultures. ES cells were cultured as shown in Fig. 1 but without selection. At various times, the cells were dissociated, allowed to settle on a coverslip for 6 hours and stained for SSEA-1 or nestin by immunohistochemistry. The proportion of stained cells was determined, and the results are shown as mean±s.d. of three independent experiments.

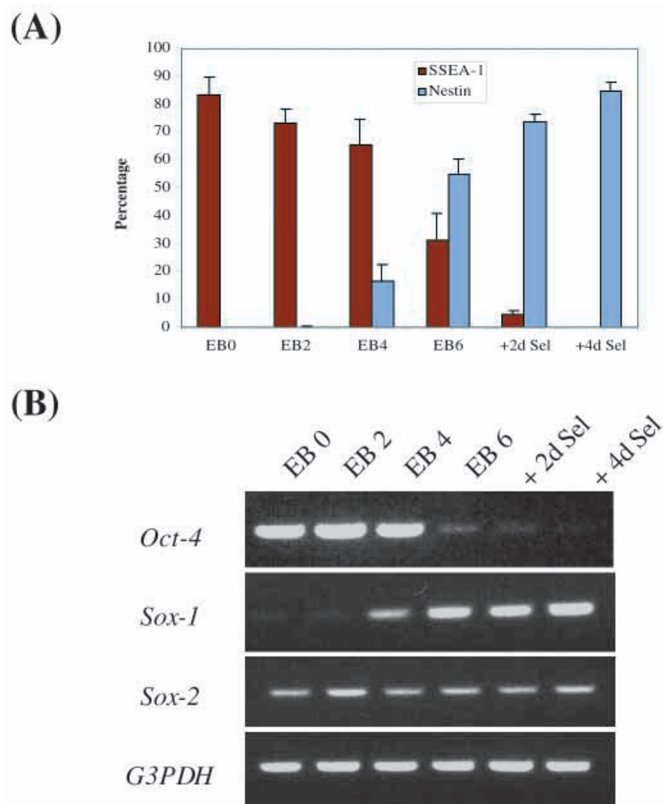
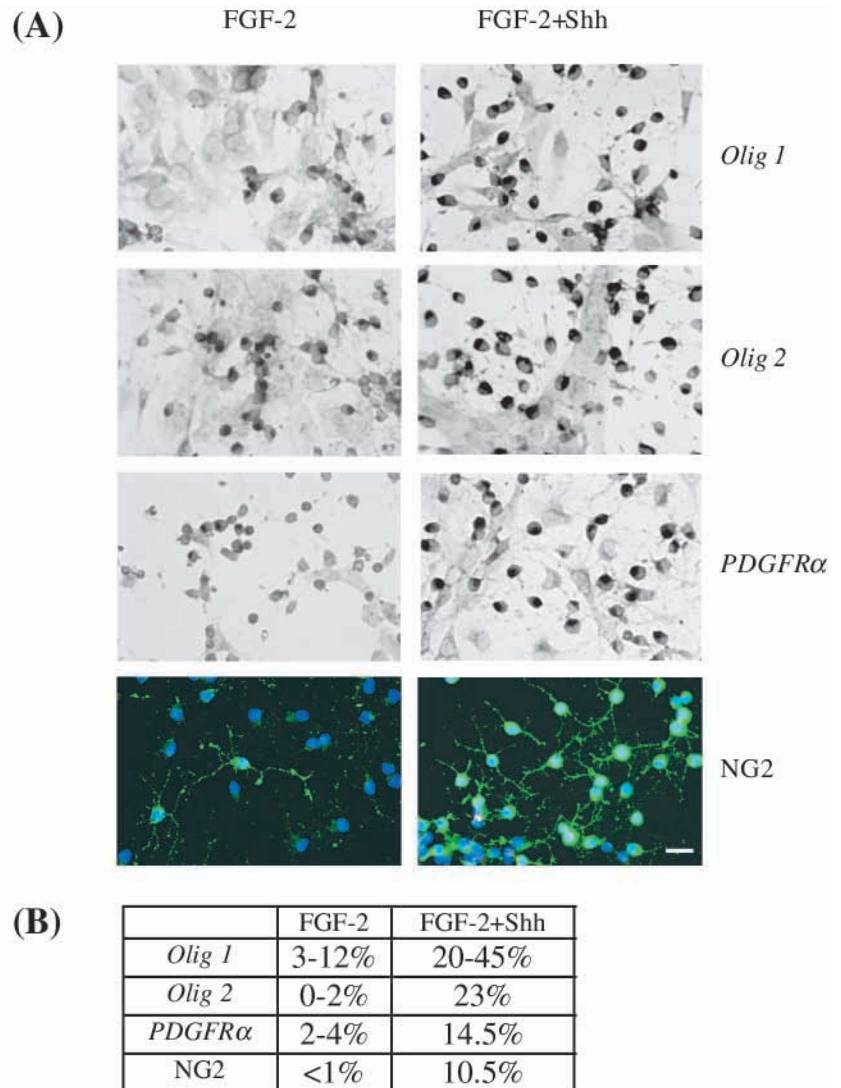


Fig. 3. Effect of combined positive and negative selection on engineered ES cells. Engineered ES cells were treated and selected as described in Fig. 1 to enrich for neuroepithelial cells and to eliminate residual undifferentiated ES cells. At various times, the cells were dissociated, and either stained for SSEA-1 or nestin by immunohistochemistry (A) or processed for RT-PCR analysis using *Oct4*, *Sox1*, *Sox2* or *G3PDH* probes (B). The results in (A) are shown as mean±s.d. of four independent experiments.

present in the culture. By contrast, 85% of the cells expressed nestin protein (Fig. 3A), and both *Sox1* and *Sox2* mRNAs were readily detected (Fig. 3B), indicating a significant enrichment in neuroepithelial cells (Brustle and McKay, 1995). Thus, combined negative and positive selection strategies proved to be extremely efficient in generating highly enriched populations of neuroepithelial cells. It is not clear why some nestin-negative cells survived the selection, although at least some of these cells are likely to be newly differentiated neurons.



	FGF-2	FGF-2+Shh
Olig 1	3-12%	20-45%
Olig 2	0-2%	23%
PDGFRα	2-4%	14.5%
NG2	<1%	10.5%

Fig. 4. Effect of Shh on the development of OPCs. Engineered ES cells were treated and selected as described in Fig. 1. On day 10, after 4 days of selection, Shh was added to some of the cultures, all of which were maintained in FGF-2, without G418 or Ganciclovir. After 5 more days, OPCs were identified either by in situ hybridization to detect *Olig1, Olig2* and *PDGFRα* mRNAs or by immunohistochemistry to detect NG2 (A). The nuclei in the bottom panels were stained with bisbenzimide. The scale bar is 20 μm. (B) Table showing the proportion of labelled cells. Randomly selected fields were counted in two experiments.

Effect of sonic hedgehog (Shh) on the production of OPCs from ES-cell-derived neuroepithelial cells

As Shh promotes the development of OPCs from neuroepithelial cells in vitro and in vivo (Orentas et al., 1999; Pringle et al., 1996; Roelink et al., 1995; Tekki-Kessaris et al., 2001), we tested its effect on the production of OPCs from our selected, ES-cell-derived neuroepithelial cells. After drug selection as before, we cultured the cells in a serum-free, modified Bottenstein-Sato medium, which is permissive for oligodendrocyte development in vitro (Bottenstein et al., 1979). We added FGF-2, which promotes the proliferation of CNS stem cells in vitro (Murphy et al., 1990) and in vivo (Wagner et al., 1999), and, in some cultures, we added Shh to promote OPC development.

We used a variety of markers to follow the fate of the neuroepithelial cells and to help identify OPCs. We used RT-PCR analysis and in situ hybridisation to detect *Olig1, Olig2* and *PDGFRα* mRNAs, and we used immunohistochemistry to detect NG2 proteoglycan. We scored cells as positive in immunohistochemical assays only if they also had the characteristic morphology of OPCs (Temple and Raff, 1986).

As shown in Fig. 4, after 5 days in the presence of FGF-2 alone, less than 12% of the cells expressed *Olig1, Olig2* or *PDGFRα* mRNAs or stained for NG2. By contrast, after 5 days in FGF-2 and Shh, the percentage of cells expressing each individual OPC marker ranged between 10 and 50%, depending on the marker (Fig. 4B); two days later, this percentage increased to 40-85% (Fig. 6A). Thus, as in the developing mouse neural tube, Shh promoted the development of OPCs from ES-cell-derived neuroepithelial cells.

Differentiation of ES-cell-derived OPCs into oligodendrocytes

To promote the differentiation of ES-cell-derived OPCs into oligodendrocytes, we first cultured selected neuroepithelial cells in FGF-2 and Shh for 5 days to enhance OPC production. We then switched the cells into PDGF-AA and TH for a further 3 to 9 days. As shown in Fig. 5A, oligodendrocytes were identified by their characteristic morphology (Temple and Raff, 1986) and by immunostaining for GC (Raff et al., 1978). About 40-50% of the cells developed into GC-positive oligodendrocytes (Fig. 6B). Interestingly, many of the oligodendrocyte lineage cells accumulated around, and extended processes to, the axons of neurons that developed in the cultures (Fig. 5B), just as they do in vivo.

Normal timing of oligodendrocyte development in ES-cell-derived cultures

Neuronal, OPC and oligodendrocyte development occur in a

predictable sequence in the developing mouse neural tube, with the first neurons appearing at around E9, the first OPCs around E12 and the first oligodendrocytes at around E17 (Calver et al., 1998; Hardy, 1997; Pringle et al., 1998).

To determine if a similar sequence occurred in our cultures, we drug-selected neuroepithelial cells for 4 days and treated them as above to optimize oligodendrocyte production (Fig. 1). We then examined the time of first appearance of various neuronal, OPC and oligodendrocyte markers. As shown in Fig. 6, neurofilament-containing neurons were present at all times tested. Their numbers peaked at 3 days post-selection (Fig. 6A) and fell rapidly thereafter (Fig. 6B). The first OPCs, as determined by morphology, staining for NG2, and *PDGFRα* mRNA expression detected by in situ hybridisation, appeared at 5 days post-selection and increased to more than 40% by 8 days post-selection (Fig. 6A). As in vivo, *Olig2* mRNA-expressing cells appeared 2 days before *PDGFRα*- and NG2-expressing cells (Fig. 6A). Moreover, the first GC-positive oligodendrocytes appeared 10 days post-selection, about 5 days after the first *PDGFRα*-expressing OPCs, just as in vivo (Fig. 6B). Finally, *MBP* mRNA was expressed from 10 days post-selection and increased thereafter. Thus, the sequence of neural cell development and the timing of oligodendrocyte differentiation in our ES-cell-derived cultures, closely resembled that in normal neural development.

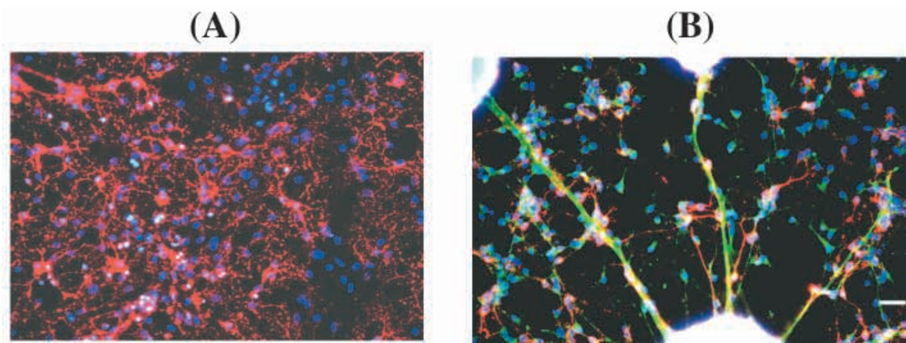


Fig. 5. Development of oligodendrocytes from ES-cell-derived OPCs. Engineered ES cells were treated and selected as described in Fig. 1. On day 10, the cells were exposed to FGF-2 and Shh for 5 days. Both FGF-2 and Shh were then removed, and the cells were cultured in PDGF-AA and TH for another 9 days (A) or 3 days (B). (A) The cells were stained with anti-GC antibody to identify oligodendrocytes and bisbenzimide to identify cell nuclei. (B) The cultures were stained with anti-neurofilament antibodies to identify neurons (green), with O4 antibody to identify oligodendrocyte lineage cells (red) and with bisbenzimide to identify cell nulei (blue). Bar, 50 μm.

Discussion

We describe here a strategy for studying oligodendrocyte development from ES-cell-derived neuroepithelial cells in culture. We have genetically engineered mouse ES cells so that we can positively select for neuroepithelial cells and negatively select against residual ES cells. We have then used various signal molecules to promote OPC and oligodendrocyte development from these selected neuroepithelial cells. Remarkably, the sequence of neural cell development and the timing of oligodendrocyte differentiation in this system closely resemble that seen in vivo. These engineered cells should therefore provide a useful model system for studying various stages of oligodendrocyte development.

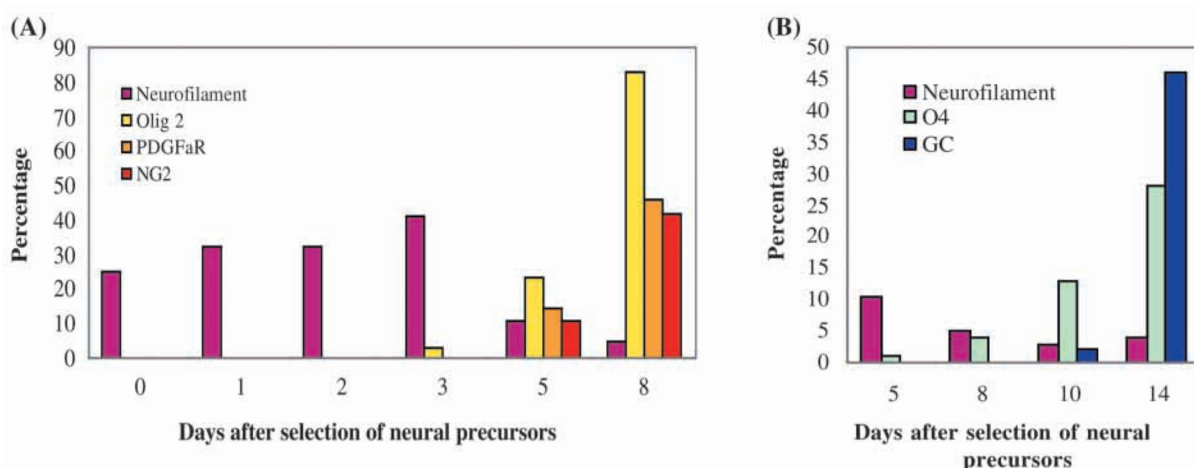


Fig. 6. Timing of neural cell development and marker expression in ES-cell-derived cultures. Engineered ES cells were treated and selected as described in Fig. 1. At various times after the end of the selection (day 10), *Olig2* and *PDGFRα* expression were analysed by in situ hybridization; neurofilaments, NG2, O4 and GC were analysed by immunohistochemistry and *MBP* expression was assessed by RT-PCR. (A) Expression of neuronal and OPC markers. (B) Expression of neuronal and oligodendrocyte markers. (C) Expression of *MBP* mRNA. Results of typical experiments are shown, but the experiments were repeated three times with similar results.

Timing of oligodendrocyte development

The different cell types in the CNS develop in a predictable sequence. It is gratifying that a similar sequence occurs in our ES-cell-derived cultures. Neurons appear first, followed by OPCs and then oligodendrocytes. More remarkably, the timing of the first appearance of various neural markers closely resembles that reported in the developing mouse neural tube. As in the developing ventral spinal cord, *Olig2* is expressed in our cultures before *PDGFRα* and NG2 are first detected. This early expression of *Olig2* might reflect the earliest stages of oligodendrocyte specification in these ES-cell-derived cultures. It could also reflect *Olig2* expression in motor neuron precursors (Lu et al., 2002; Zhou and Anderson, 2002). Again, as in vivo, *PDGFRα* expression and NG2 immunostaining precede the appearance of GC immunostaining by about 5 days (Calver et al., 1998; Lu et al., 2000; Zhou et al., 2000). The emergence of OPCs in the developing spinal cord depends on Shh produced by the notochord and the floor plate (Echelard et al., 1993; Placzek et al., 1993; Roelink et al., 1994), and we find that the treatment of our ES cell cultures with Shh increases the expression of OPC markers. It seems likely that in the absence of added Shh, endogenous Shh helps promote OPC development in these cultures.

Perhaps the most impressive and convincing normal timing that occurs in our cultures is the interval between the first appearance of OPCs, identified by their morphology and expression of *Olig2, PDGFRα* mRNAs and NG2 proteoglycan, and the first appearance of oligodendrocytes, identified by their morphology and GC staining. It is surprising that this interval so closely resembles that reported in the ventral mouse spinal cord, as the controls on the differentiation of OPCs into oligodendrocytes are thought to be complex, involving a cell-intrinsic timer (Durand and Raff, 2000), PDGF (Raff et al., 1998) and TH (Barres et al., 1994). Moreover, we treat our cultures with a high concentration of PDGF and TH, whereas PDGF (and probably TH) is limiting in the developing mouse spinal cord (Calver et al., 1998). On the other hand, it was shown previously that the time of first appearance of GC-positive oligodendrocyte is remarkably similar in the developing rat brain and in cultures of dissociated E10 rat brain cells (Abney et al., 1983). Our present finding that the sequence and timing of neural development in our cultures is similar to that observed in vivo suggests that ES-cell-derived neuroepithelial cells may follow the same pathways followed by normally developing neuroepithelial cells.

Our findings are reminiscent of those previously published for the timing of erythropoieisis in ES-cell-derived cultures, which closely resemble in vivo timing (Keller, 1995). By contrast, a recent report suggested that the development of pancreatic β cells in cultures of mouse ES cells may have followed an abnormal pathway - from neuroepithelial cells rather than from endodermal cells - although it was not shown that β cells arose from neuroepithelial cells in these ES-cell-derived cultures (Lumelsky et al., 2001).

Our culture system may be especially useful for studying the specification of neuroepithelial cells toward the oligodendrocyte lineage. During spinal cord development, OPCs are generated adjacent to the floor plate, just ventral to where motor neurons develop (Noll and Miller, 1993; Pringle and Richardson, 1993; Richardson et al., 2000; Sun et al., 1998; Yu et al., 1994). This is where *Olig2* is first expressed, presumably under the influence of Shh produced by the notochord and floorplate (Lu et al., 2000; Zhou et al., 2000). Olig2 is sequentially expressed in motor neuron progenitors (Mizuguchi et al., 2001; Novitch et al., 2001; Takebayashi et al., 2000) and OPCs (Lu et al., 2000; Zhou et al., 2000), and it is required for the development of both cell types in the spinal cord (Lu et al., 2002; Zhou and Anderson, 2002). *Olig2* apparently acts together with the homeodomain gene regulatory protein Nkx2.2 to promote OPC specification (Sun et al., 2001; Zhou et al., 2001), whereas it acts with other gene regulatory proteins to promote motor neuron specification (Mizuguchi et al., 2001; Novitch et al., 2001). Remarkably, most of the known gene regulatory proteins that act in the specification of neural cells in the ventral spinal cord, including Olig2 protein (Mizuguchi et al., 2001; Novitch et al., 2001; Sun et al., 2001; Zhou et al., 2001), seem to act as gene repressors, shutting off genes required for alternative pathways of development (Briscoe and Ericson, 2001; Marquardt and Pfaff, 2001). Our ES cells may be useful for analysing such specification mechanisms.

Prospects for human cell therapy

Human ES cells offer a potential source of cells for therapy and drug screening. For human ES cells to be useful in cell therapy, it will be necessary to induce them to develop along particular pathways, to select for the cell type of interest and to select against any residual ES cells, which are very efficient at forming tumours in the recipient (Brustle et al., 1997). The strategy we describe here should be applicable to human ES cells and may be useful in these respects, especially for producing large numbers of OPCs and oligodendrocyte for the treatment of demyelinating diseases such as multiple sclerosis. Althought it remains to be demonstrated that our ES-cell-derived oligodendrocytes are capable of myelinating axons in vivo, OPCs derived from mouse ES cells have already been shown by others to remyelinate axons in rodent models of demyelinating diseases (Brustle et al., 1999; Liu et al., 2000). The availability of human ES cells and the possibility of producing autologous human ES cells by nuclear transfer offers exciting possibilities for the treatment of such diseases in human.

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