FGF-dependent generation of oligodendrocytes by a hedgehog-independent pathway

Siddharthan Chandran1,*, Hidemasa Kato2, Dianne Gerreli3, Alastair Compston1, Clive N. Svendsen4 and Nicholas D. Allen2,†

1Cambridge Centre for Brain Repair, University of Cambridge, ED Adrian Building, Forvie Site, Robinson Way, Cambridge CB2 2PY, UK
2The Babraham Institute, Babraham, Cambridge CB2 4AT, UK
3Neural Development Unit, Institute of Child Health, 30 Guilford Street, London WC1N 1EH, UK
4Waisman Center Stem Cell Research Program, University of Wisconsin-Madison, 1500 Highland Avenue, Madison, WI 53705, USA

*Author for correspondence (e-mail: sc222@cam.ac.uk)
†Present address: School of Biosciences, Museum Avenue, PO Box 911, Cardiff CF10 3US, UK

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Summary
During development, spinal cord oligodendrocyte precursors (OPCs) originate from the ventral, but not dorsal, neuroepithelium. Sonic hedgehog (SHH) has crucial effects on oligodendrocyte production in the ventral region of the spinal cord; however, less is known regarding SHH signalling and oligodendrocyte generation from neural stem cells (NSCs). We show that NSCs isolated from the dorsal spinal cord can generate oligodendrocytes following FGF2 treatment, a MAP kinase dependent phenomenon that is associated with induction of the obligate oligogenic gene Olig2. Cyclopamine, a potent inhibitor of hedgehog signalling, did not block the formation of oligodendrocytes from FGF2-treated neurosphere cultures. Furthermore, neurospheres generated from SHH null mice also produced oligodendrocytes, even in the presence of cyclopamine. These findings are compatible with the idea of a hedgehog independent pathway for oligodendrocyte generation from neural stem cells.

Key words: Stem cell, Oligodendrocyte, FGF2, Spinal cord, Sonic hedgehog, Olig2, NKX2.2

Introduction
Neural stem cells (NSC) may be defined as self-renewing cells isolated from germinal regions of the developing and adult brain that can generate both neurones and glia (Gage, 2000). Improved understanding of the genetic and signalling requirements that underlie the generation of differentiated progeny from NSC may enable therapeutic application of such cells (Svendsen and Smith, 1999). An area of particular interest is the potential of stem cells to generate oligodendrocytes. Such knowledge will inform clinical interventions utilising stem cells for the promotion of repair in demyelinating diseases such as multiple sclerosis.

The spinal cord has served as a valuable model for the study of oligodendrocyte origins. During normal development, oligodendrocytes originate from restricted foci within the ventral neural tube under the influence of ventral midline-derived (notochord and floorplate) SHH signalling (Pringle and Richardson, 1993; Orentas et al., 1999). Graded SHH signalling patterns the ventral neural tube by influencing the expression of various transcription factors, including the basic helix-loop-helix (bHLH) genes Olig1 and Olig2 (Lu et al., 2000; Zhou et al., 2000; Takebayashi et al., 2000). Olig1 and Olig2 represent the earliest cell type specific genes within the oligodendrocyte lineage, and their expression precedes markers of the oligodendrocyte precursor cell (OPC), including PDGF receptor α (PDGFRα) and DM20/PLP (Lu et al., 2000). In addition to SHH, increasing evidence suggests that other factors are important in determining cell fate along the dorsoventral axis of the developing spinal cord (McMahon et al., 1998; Barth et al., 1999; Pierani et al., 1999; Liem et al., 2000; Ishibashi and McMahon, 2002). More specifically, two recent studies have examined the idea that dorsally derived signals play a role in controlling OPC specification (Wada et al., 2000; Mekki-Dauriac et al., 2002). Mekki-Dauriac and co-workers provide evidence that bone morphogenetic proteins (BMPs) negatively regulate spinal cord oligodendrocyte specification (Wada et al., 2000; Mekki-Dauriac et al., 2002). Mekki-Dauriac and co-workers provide evidence that bone morphogenetic proteins (BMPs) negatively regulate spinal cord oligodendrocyte specification (Mekki-Dauriac et al., 2002).

Several lines of evidence suggest that, within the spinal cord, OPCs arise exclusively from the ventral ventricular zone, and that oligodendrocyte development in the dorsal cord results only from a ventral to dorsal OPC migration (Warf et al., 1991; Pringle and Richardson, 1993; Yu et al., 1994; Timsit et al., 1995; Hall et al., 1996; Pringle et al., 1998). However, it remains possible that the lack of OPCs and oligodendrocytes in isolated rat embryonic day 14 (E14) dorsal spinal cord-derived cultures results from a failure to provide signals necessary for the generation of oligodendrocytes from neural precursors that are intrinsic to the dorsal cord. This interpretation is consistent with the demonstration that dorsal-derived cell populations have the capacity to generate...
oligodendrocytes in vitro upon exposure to notochord or SHH, and following transplantation (Trousse et al., 1995; Hardy and Friedrich, 1996; Orentas et al., 1999). In addition, dissociated E14 dorsal cultures stimulated with FGF2 and EGF generate oligodendrocytes following mitogen withdrawal (Chandran et al., 1998). The latent oligodendrogial potential of E14 dorsal cord suggests that stimulation and proliferation of a dorsal-derived cell may generate oligodendrocytes in response to these and other mitogens, without the need for inward migration of cells from the ventral cord.

Here, we characterise the relationship of the rat E14 dorsal-derived precursor to oligodendrocytes, and the role of SHH in mediating oligodendrogenesis following expansion of these precursors in response to FGF2. We show, using clonal analysis, that the embryonic dorsal spinal cord contains an FGF2-responsive stem cell, and provide evidence for the existence of a SHH-independent pathway for the generation of oligodendrocytes. Analysis of uncultured and FGF2-treated embryonic spinal cords from mice homozygous for a null mutation of SHH reveals a hedgehog-independent pathway for the induction of Olig2 and Nkx2.2 genes and oligodendrocytes. Finally, we show that FGF2-mediated induction of oligodendrocytes requires MAP kinase signalling and is inhibited by BMP4.

Materials and methods

Primary cell culture

Dorsal spinal cord segments were removed from E14 Sprague Dawley rats or E12.5 CD1 mice (Charles River). SHH-knockout mice were obtained from the UCL breeding colony (Chiang et al., 1996). Developmental age of the embryos was estimated using a standard protocol (Dunnnett and Bjorklund, 1992). Tissue was enzymatically dissociated in trypsin (0.1%), washed with DNAse (0.001%, Sigma) and trypsin inhibitor (Sigma), and resuspended in B27 medium containing 1% FCS prior to plating upon poly-L-lysine coated coverslips at a density of 5×10³ cells/coverslip. Cultures were supplemented with the following growth factors: PDGF-AA, EGF, FGF2 (all at 20 ng/ml), SHH (100-250 ng/ml), noggin (25-600 ng/ml), chordin and follistatin (R & D, Minneapolis), and BMP4 (0.1-10 ng/ml; Genetics Institute, Cambridge, MA). Explant cultures were embedded in collagen gels (Tessier-Lavigne et al., 1988) (2 mg/ml; Roche). All cultures were incubated at 37°C in 5% CO₂ and stained at various time points (see Results).

Precursor and clonal cultures

Following enzymatic dissociation of dorsal spinal cord (rat E14 or mouse E12.5) or whole SHH-null cord (E12.5), tissue was seeded into uncoated T25 culture flasks (Nunclon) at a density of ~2×10³ live cells per ml of proliferation medium [DMEM/F12 (3:1) (Gibco) containing B27 supplement with FGF2 and heparin (5 µg/ml)]. Characteristic multicellular aggregates or neurospheres formed by 7 days, at which time cells were harvested by centrifugation at 1000 g for 3 minutes, then re-suspended and dissociated using mechanical trituration to a single cell suspension. Aliquots of cells were plated, as described above, in the absence of mitogens to observe differentiation. Hedgehog requirement for oligodendrogenesis was assessed using cycloamine and the cycloamine derivative 3-keto, N-aminomethyl aminocaproyl dihydrocinnamoyl cycloamine (KAAD; Toronto Research Chemicals) (Taipale et al., 2000). The pathway inhibitors LY294002 (PI3-kinase) and U0126 (MAP kinase) were obtained from Sigma, UK and Calbiochem. For clonal studies, primary cultures were enzymatically and mechanically dissociated to a single cell suspension at 24 hours after seeding in proliferation medium. The resulting cell suspension was filtered and sorted (Becton Dickinson FACS Vantage SE, San Jose, CA) into single live cells in uncoated 96-well plates containing 200 µl of clonal proliferation medium composed of DMEM/L15 (1:1) FGF2, B27 and N2. Cultures were maintained for up to 21 days and then plated in control medium supplemented with B27. Cultures were immunostained for phenotypic potential at 7 days post-plating.

PCR analysis

The yolk sac was removed from each embryo, washed in phosphate-buffered saline and stored frozen before genotyping. Genomic DNA was extracted and PCR amplification performed using primers for the neomycin resistance gene and SHH as previously described (Chiang et al., 1996). Semi-quantitative cross comparison of cultures or tissues was performed by RT-PCR using SMART technology (Clontech). Gene expression comparisons were made using matched samples normalised for the level of GAPDH expression, quantified by SYBR Green (Molecular Probes) staining and band intensity measurements using FLA-3000 Imager System (Fujifilm). PCR conditions for all primer pairs used in this study were optimised for cDNA derived from foetal tissue: nestin, 5’-agtccagcagcaatgaa-3’ and 5’-agaacaagactgacagc-3’; Shh, 5’-ccaccccaacatcacc-3’ and 5’-aagtaagctcttcaac-3’; Ihh, 5’-cagatgtgtaattcttcc-3’ and 5’-tagatgctcttcaac-3’; Dhh, 5’-gctctgatacacaac-3’ and 5’-tgatacagcagtaacc-3’; Nkx6.1, 5’-gctcttacacttcccacag-3’ and 5’-gctatgccccagagatgc-3’; Olig1, 5’-gacctctggaatacctc-3’ and 5’-taacacatcagtgattgaac-3’; Olig2, 5’-tcagacagcagacagac-3’ and 5’-aagacacagaaagaaaaag-3’; Nkx2.2, 5’-cttcgtcaacagtagaagc-3’ and 5’-agagccgagagaaaaag-3’; Nkx2.9, 5’-gagaccaacctctgacact-3’ and 5’-gggctgtaattgctgac-3’; Pax7, 5’-cggatttctcattgacagc-3’ and 5’-cagacaggtactcagac-3’; Hh9, 5’-gcaacctctgacag-3’ and 5’-ataacactctcactac-3’; Lhx3, 5’-ccagacaacataccgag-3’ and 5’-gcagaaaggaaaaatc-3’; and Gapdh, 5’-accacagctgcagc-3’ and 5’-tccacacagttggtga-3’.

Immunohistochemistry

Live cells were stained for the surface markers O4 (supernatant diluted 1:5) and galactocerebroside (GC; supernatant diluted 1:10) (Sommer and Schachner, 1981) (IC-07 cell line, ECACC, UK) to assess glial lineages. Intracellular antigens studied were: glial fibrillary acidic protein (GFAP) as an astrocyte marker (rabbit polyclonal, 1:250; Dako, UK); β-tubulin type III as a neuronal marker (1:250; Sigma); and myelin basic protein (1:500, Serotec) and actin (1:250, Sigma) to identify smooth muscle. All antibodies were made up in 5% serum and DMEM. The surface antigens were stained with primary antibody for 60 minutes at 37°C, followed by the appropriate conjugated secondary antibody for 60 minutes. After fixation with 4% PFA for 10 minutes and permeabilisation with absolute methanol at ~20°C for 15 minutes, the primary antibodies to intracellular antigens were added for 60 minutes at 37°C, followed by the appropriate secondary conjugated antibody. All secondary antibodies (Sigma and Harlan Sera-Lab) were used at a dilution of 1:100, and Hoechst nuclear stain (1:5000) was included in the final antibody application. Cryostat sections of PFA fixed neurospheres were also stained for OLIG2 (1:3000; kindly provided by Dr Takebayashi) and NKX2.2 (1:50; Developmental Hybridoma Study Bank). Removal of primary antibodies resulted in no specific staining. Coverslips were mounted on glass slides using Vectashield (Vector Labs, UK), and viewed under a Leitz inverted microscope with appropriate filters for cell identification and counting. Using a grid, five consecutive fields were counted for each coverslip. The number of positive cells was
Hedgehog-independent generation of oligodendrocytes

expressed as a mean±s.e.m. from 2-4 coverslips and from a minimum of three separate experiments, unless stated otherwise. Statistical significance was assessed by ANOVA with post hoc Neuman-Keuls and Student's t-test using a standard statistical package (Graphpad, Prism).

Results

The rat E14 dorsal spinal cord contains an FGF2 responsive stem cell

Cells derived from E14 rat primary dorsal spinal cord did not generate galactocerebroside (GC)-positive oligodendrocytes when plated in the absence of growth factors and left for either 7 or 14 days (data not shown), as previously reported (Warf et al., 1991; Pringle et al., 1996; Chandran et al., 1998). This observation is consistent with in vitro and in vivo evidence for the absence of PDGFRα-positive OPCs in the E14 dorsal cord (Pringle and Richardson, 1993; Hall et al., 1996). In the presence of FGF2, but not EGF, rat primary dorsal spinal cord gave rise to neurospheres, which, after 7 days of growth, were also able to generate oligodendrocytes upon plating and differentiation for a further 7 days (14.4±1.0%). Together with the exclusive ventral location of OPCs at E14, these findings are compatible with the existence of more immature FGF2-responsive cells within the dorsal spinal cord (Pringle and Richardson, 1993; Yu et al., 1994; Hall et al., 1996).

Clonal analysis was next undertaken to characterise the developmental relationship of the FGF2-responsive cell and the GC-positive oligodendrocyte, in particular whether this cell represents a stem cell or a fate-committed cell of the oligodendrocyte lineage. Twenty-four hours after seeding of primary dorsal cultures in proliferation medium, individual cells were FACS sorted into a single well of a 96-well plate. Clonal neurospheres were generated in response to FGF2 under substrate and serum free conditions, and assessed for phenotypic potential. Expression of GFAP (astrocyte), β-tubulin III (neurones), and O4 or GC (oligodendrocytes) was used to identify the three principal CNS phenotypes. Twenty-two clones were studied: the predominant clonal cell type was macroglial, with 16 clones generating oligodendrocytes and astrocytes. Six clones generated neurones, oligodendrocytes and astrocytes, and could be considered to be stem cells (Fig. 1A). These findings provide in vitro evidence for the existence of an FGF2-responsive CNS stem cell within the population derived from E14 dorsal spinal cord.

Neural stem cells do not require SHH for the generation of oligodendrocytes

The initial ventral localisation of OPCs is believed to be a consequence of the requirement for oligodendrocyte formation of SHH signalling, SHH being necessary and sufficient for oligodendrocyte generation (Poncet et al., 1996; Pringle et al., 1996; Orentas et al., 1999; Nery et al., 2001). SHH alone was sufficient to induce O4-positive oligodendrocytes from primary (non-expanded) E14 dorsal-derived explants (1 out of 7 control explants produced 4 O4-positive cells, whereas 13 out of 15

Fig. 1. Immunomicrograph showing the CNS potential of E14 rat dorsal spinal cord-derived clones. (A) A single clone can generate β-tubulin+ neurones, O4+ oligodendrocytes and GFAP+ astrocytes. (B) Hedgehog signalling is not necessary for the generation of oligodendrocytes from FGF2-treated E14 rat dorsal cultures. Addition of cyclopamine or KAAD during the 7 day exposure to FGF2 does not significantly reduce numbers of oligodendrocytes generated upon mitogen withdrawal, plating and subsequent differentiation for a further 7 days (P>0.05). (C) Immunofluorescent micrograph showing representative labelling of differentiating cultures for O4-, GC- and MBP-positive cells from E14 dorsal-derived cultures following treatment with FGF2 in the presence of cyclopamine (1 μM).
explants produced 2-41 positive cells in the presence of SHH). The requirement of SHH for oligodendrogenesis from primary cultures raised the question of whether SHH was also necessary for the generation of oligodendrocytes from FGF2-treated cultures.

In order to establish whether endogenous hedgehog was responsible for oligodendrocytes generated from dorsal neurosphere cultures following FGF2 stimulation, we performed experiments with cyclopamine. Cyclopamine is a plant-derived teratogen that acts on the general hedgehog signal transducer smoothened to inhibit hedgehog signalling, and also the generation of oligodendrocytes from primary embryonic culture (Incardona et al., 1998; Taipale et al., 2000; Tekki-Kessaris et al., 2001; Alberta et al., 2001). Primary cultures derived from E14 dorsal spinal cord were grown in the presence of both FGF2 and cyclopamine. Differentiation of these cultures demonstrated that cyclopamine did not inhibit the appearance of O4- and GC-positive oligodendrocytes (Fig. 1B,C). Importantly, following cyclopamine treatment, longer-term cultures (14-21 days in vitro) express the mature oligodendrocyte marker myelin basic protein (Fig. 1C). In order to further examine the contribution of hedgehog signalling to the generation of oligodendrocytes from FGF2-stimulated cultures, we studied the effect of the addition of KAAD to FGF2 expansion medium. KAAD is a cyclopamine derivative of significantly greater potency in blocking the activation of the hedgehog signalling pathway (Taipale et al., 2000). KAAD had no significant effect on oligodendrocyte generation from the FGF2-treated cultures (Fig. 1B). By contrast, cyclopamine and KAAD at a concentration of 1 μM did result in a significant reduction in oligodendrocytes derived from dissociated primary (without FGF2 treatment) E12 rat whole spinal cord cultures, an observation consistent with the requirement of hedgehog for the induction of spinal cord oligodendrocytes (Orentas et al., 1999; Soula et al., 2001) (Table 1). The generation of a significant number of oligodendrocytes despite the hedgehog signalling blockade following FGF2 treatment suggests that an additional hedgehog-independent pathway for oligodendrocyte formation may exist.

In order to explore this possibility further, and to specifically address the requirement for early developmental exposure to SHH, embryonic spinal cords from mice homozygous for a null mutation of SHH were examined. The murine E12.5 spinal cord shows developmental equivalence to the E14 rat spinal cord, with respect to restricted ventral localisation of OPCs (Yu et al., 1994; Timsit et al., 1995). The developing SHH null cord was more friable, and can also be considered dorsalisé on account of the lack of ventral specification (Chiang et al., 1996). The whole E12.5 null spinal cord was therefore examined for constitutive oligodendrocyte potential in primary culture, following stimulation by FGF2. In primary culture (without FGF2 stimulation), no O4- or GC-positive cells were demonstrated after 8 days in vitro, a finding consistent with that reported by Alberta and co-workers (Alberta et al., 2001). However, over the same period, heterozygous cultures showed significant numbers of oligodendrocytes (data not shown). The null spinal cord gave rise to neurospheres in the presence of FGF2 that subsequently generated significant numbers of oligodendrocytes upon differentiation (Fig. 2A,B). Thus, even in the complete absence of SHH, oligodendrogenesis could still occur from FGF2-responsive neurospheres. Next, we wanted to exclude the possibility that the lack of oligodendrocytes in primary dorsal spinal cord cultures was simply the consequence of delayed maturation. Primary cultures were left to mature for 13 days, at which time no oligodendrocytes were found (data not shown). Thus, delayed maturation cannot account for lack of oligodendrocytes in the SHH-null dorsal spinal cord cultures. Importantly, SHH null-derived cultures grown in the presence of FGF2 and cyclopamine also generated oligodendrocytes, suggesting that redundant hedgehog signalling was not responsible for the emergence of oligodendrocytes (Fig. 2C). In order to examine whether oligodendrocyte generation from E12.5 SHH null cultures was arising from a stem cell and/or a more fate-restricted progenitor cell, clonal analysis was next undertaken. Ten clones were examined. Eight were glial clone producing only astrocytes and oligodendrocytes. Two were stem cell clones, which generated rare β-tubulin III-positive neurones, demonstrating the presence of SHH-independent stem cells within the developing null cord (Fig. 2D).

Table 1. Hedgehog-dependent development of oligodendrocytes from primary spinal cord cultures

<table>
<thead>
<tr>
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<th>Number of O4+ cells/20 fields</th>
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<tr>
<td>Control</td>
<td>557.3±49.40</td>
</tr>
<tr>
<td>Cyclopamine</td>
<td>74.3±1.80</td>
</tr>
<tr>
<td>KAAD</td>
<td>29.25±12.80**</td>
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Dissociated cells from E12 rat whole spinal cord were cultured in defined medium for 9 days, in the presence or absence of cyclopamine (1 μM) or KAAD (1 μM). Following live labelling with O4, the number of O4+ cells per 20 random fields was counted. Generation of oligodendrocytes was significantly inhibited by both cyclopamine and KAAD (*, **P<0.05; n=2).

Olig1, Olig2 and Nkx2.2 genes are induced by FGF2 in the absence of sonic hedgehog signalling

Olig2 expression is required for SHH to induce oligodendrocytes (Lu et al., 2000). This represents a ventral neurone oligodendroglia switch observed during development (Kessaris et al., 2001; Qi et al., 2001; Briscoe et al., 1999; Novitch et al., 2001; Mizuguchi et al., 2001; Zhou et al., 2001; Lu et al., 2002; Zhou and Anderson, 2002). Nkx2.2 co-expression with Olig2 is associated with the switch in activity of Olig2 to generate oligodendrocytes in the developing chick spinal cord, and Nkx2.2 is also present in differentiating rodent oligodendrocytes (Xu et al., 2000, Zhou et al., 2001; Kessaris et al., 2001). Untreated dorsal cultures express the dorsal precursor marker Pax7, but not ventral markers. Following expansion in FGF2, Pax7 is downregulated concomitant with the upregulation of ventral markers. No expression of Olig2 or Nkx2.2 was evident in untreated SHH null cord or in E12.5 wild-type dorsal cord (Fig. 3). However, low levels of Olig1 were present in untreated E12.5 wild-type dorsal cord. Following FGF2 treatment, Olig1, Olig2 and Nkx2.2 were significantly upregulated. Immunostaining of sectioned dorsal expanded sphere cultures grown in the presence of cyclopamine revealed OLIG2+/NKKX2.2+ co-expression (Fig. 3B; OLIG2+ cells represent 35±13.1% of total cell population, with 27±9.4% of OLIG2+ cells being also NKKX2.2+). All NKKX2.2+ cells co-expressed OLIG2.
Hedgehog-independent generation of oligodendrocytes

As oligodendrocytes in vivo share a lineage relationship with progenitor cells of the pMN domain, which earlier in gestation give rise to motoneurones, it was of interest to determine whether dorsal cord cultures were induced to express other lineage determinants of the pMN domain (Richardson et al., 2000; Tekki-Kessaris et al., 2001; Zhou and Anderson, 2002; Lu et al., 2002). Nkx6.1 is a crucial class II homeodomain protein involved in pMN domain specification, and is required to induce Olig1 and Olig2 gene expression within that domain (Novitch et al., 2001). Interestingly Nkx6.1 and Nkx6.2 genes were absent in all conditions, including in FGF2-treated cultures, as were more mature motoneurone markers, such as Hb9 and Lim3 (Fig. 3). This indicates that the observed oligodendrogenesis is not dependent on the earlier pMN domain genetic programme, despite their lineage relationship with motoneurone (MN) progenitors in vivo.

Recent observations of unexpected Indian hedgehog (IHH) expression in dissociated embryonic cultures led us to look for Ihh and Dhh transcripts (Alberta et al., 2001; Tekki-Kessaris et al., 2001). Ihh was present in some conditions, notably wild-type untreated dorsal cultures, and was consistently found to be differentially regulated by FGF2 in SHH mutant compared with wild-type tissue. Significantly the pattern of Ihh expression did not correlate with gliogenic gene expression. Nonetheless, in order to exclude the possibility that IHH may account for Olig and Nkx2.2 expression in dissociated cultures, both SHH null E12.5 whole cord and wild-type E12.5 dorsal mouse spinal cord were grown in the presence of FGF2 and cyclopamine. Significantly, Olig1, Olig2 and Nkx2.2 expression was again evident following FGF2 exposure, a finding consistent with the demonstration of O4+ oligodendrocytes in cyclopamine-treated cultures (Fig. 2C, Fig. 3). Taken together, these findings demonstrate a hedgehog-independent pathway for induction of the oligodendrogenic genes Olig2 and Nkx2.2 in the presence of FGF2.

**FGF2 mediated oligodendrocyte induction is dependent on MAP kinase signalling and is inhibited by BMP4**

In order to explore whether FGF2 has an active role in the generation of oligodendrocytes from dorsal spinal cord precursors, or whether oligodendrogenesis results from a passive process – for example, by dilution of inhibitory factors present in primary dorsal cultures – we next cultured dorsal-derived cells in the presence of both FGF2 and the respective PI3-kinase and MAP kinase pathway inhibitors LY294002 and UO126. MAP kinase inhibition resulted in a significant reduction of oligodendrocytes, suggesting an active role for...
FGF2 in the derivation of oligodendrocytes from the cultures (Fig. 4). PI3-kinase inhibition had no significant effect on oligodendrocyte numbers.

The absence of hedgehog signalling in our system and the dorsal origin of the cell population raises the possibility that FGF signalling may be negatively interacting with BMP signalling. BMPs are known to regulate dorsoventral pattern formation by inducing the expression of dorsal fate determining genes and antagonising the induction of opposing ventral fate by SHH (Basler et al., 1993; Liem et al., 1995; Liem et al., 1997; Lee and Jessell, 1999; Liem et al., 2000; McMahon et al., 1998; Mekki-Dauriac et al., 2002). Interestingly, FGF2 treatment did not influence endogenous BMP expression levels, although a reduction in noggin was apparent (Fig. 5). However, inhibition of BMP signalling by the addition of noggin, chordin and follistatin, either individually or together, to dissociated primary (untreated) dorsal-derived cultures was insufficient to induce oligodendrocyte formation (data not shown).

BMPs can also inhibit oligodendrocyte formation from embryonic cortical and striatal neural precursors (Gross et al., 1996; Mabie et al., 1999). To our surprise, BMP4-treated cultures contained reduced numbers of astrocytes, in contrast to cultures treated with FGF2 alone (Fig. 4). On account of the dorsal inductive capacity of BMPs we also examined cultures for the expression of smooth muscle actin, a marker of neural crest derivatives (Liem et al., 1995; Shah et al., 1996; Mujtaba et al., 1998; Panchision et al., 2001). Significantly, BMP4-treated cultures generated smooth muscle actin expressing cells, although a large number of cells remained negative for the studied markers (Fig. 4).

The respective phenotypic profiles of FGF2 cultures treated with BMP4 (10 ng/ml) or U0126 were similar, and revealed negligible numbers of oligodendrocytes, reduced astrogenesis and induction of smooth muscle actin upon differentiation. These findings are suggestive of crosstalk between FGF2-activated MAP kinase and BMP signalling.

**Discussion**

The developing embryonic spinal cord was investigated in order to characterise the signalling requirements for the generation of oligodendrocytes from immature neural precursors. We demonstrate, in vitro, the existence of an FGF2-responsive rat E14 dorsal spinal cord-derived stem cell, in addition to a glial-restricted precursor cell. Collectively, these are considered ‘neural precursors’. Separate lines of evidence are provided for a hedgehog-independent pathway for the generation of oligodendrocytes from FGF2-responsive neural precursors.
The E14 dorsal spinal cord contains an FGF2-responsive CNS stem cell

FGF2 alone was able to stimulate the generation of neurospheres from rat E14 dorsal spinal cord, and these neurospheres in turn were able to produce significant numbers of oligodendrocytes following differentiation. This observation is consistent with previous reports showing FGF2-dependent oligodendrocyte formation from the embryonic whole spinal cord (Kalyani et al., 1997). Single-cell analysis of E14 dorsal cultures revealed cells that were capable of generating clones. Significantly, monoclonal derivation of neurons, astrocytes and oligodendrocytes was confirmed. The observation of multipotent cells within the E14 dorsal spinal cord is compatible with reports of FGF2-responsive stem cells isolated from both the embryonic and adult whole spinal cord (Rao and Mayer-Proschel, 1997; Shihabuddin et al., 2000). Rao and co-workers have characterised the potential of whole spinal cord-derived stem cells, and have further identified neural- and gial-

restricted progenitors derived from the developing spinal cord (Rao and Mayer-Proschel, 1997; Mayer-Proschel et al., 1997). A recent study has extended these observations, and has identified differences between glial-restricted progenitors derived from the developing ventral and dorsal spinal cord (Gregori et al., 2002). However, the E14 dorsal stem cell described in this study and the dorsal glial-restricted progenitor reported by Gregori and co-workers (Gregori et al., 2002) self-evidently differ in their capacity to generate neurones, although both require FGF2. Further characterisation requires a more extensive range of markers than is presently available.

Hedgehog-dependent and -independent pathways for oligodendrocyte formation

The restricted ventral origin of spinal cord oligodendrocytes in vivo reflects the importance of notochord and floor plate-derived signals. The morphogen SHH represents the primary ventralising signal and is sufficient to induce ectopic oligodendrocyte formation (Pringle et al., 1996). Blocking SHH inhibits the induction of oligodendrocytes (Orentas et al., 1999; Soula et al., 2001; Alberta et al., 2001). Recent studies have extended our understanding of the genetic requirements underlying spinal cord oligodendrocyte specification and its relationship to neurogenesis. Oligodendrocytes emerge from the same area of the ventral spinal cord that initially generate MNs and/or V3 interneurones (Richardson et al., 2000; Tekki-Kessaris et al., 2001; Soula et al., 2001). SHH-regulated OLIG2 specifies MNs and, subsequently, oligodendrocytes...
Fig. 5. Expression of Bmp4, noggin and Egfr genes in isolated mouse E12.5 dorsal spinal cord cultures following FGF2 treatment in the presence or absence of cyclopamine (1 μM). BMP4 expression is evident in both primary and FGF2-treated cultures. Noggin expression is downregulated upon FGF2 treatment, in contrast to the induction of EGFR.

(Novitch et al., 2001). This is dependent on the differential temporal association of Olig2 with neurogenin2 and later with NKX2.2 (Mizuguchi et al., 2001; Zhou et al., 2001; Qi et al., 2001). Analysis of mice lacking Olig1 or Olig2, and double-homozygous Olig1/Olig2 mutants, has further shown that Olig expression is obligatory for oligodendrocyte and motoneurone formation (Lu et al., 2002; Zhou and Anderson, 2002).

We provide separate lines of evidence that support an FGF-dependent SHH-independent pathway for the generation of oligodendrocytes. Analysis of the SHH null mouse spinal cord showed that at E12.5, an equivalent stage of development to the E14 rat, no oligodendrocytes were identified from primary cultures by comparison with wild type, an observation consistent with the known requirement of SHH signalling for oligodendrocyte generation. However, significant numbers of oligodendrocytes did develop in FGF2-stimulated E12.5 SHH-null spinal cord cultures. This is unlikely to be due to redundancy with IHH or DHH for two reasons (Tekki-Kessaris et al., 2001; Albert et al., 2001). First, the absence of constitutive oligodendrocytes (and Olig2) in isolated mouse E12.5 dorsal cultures that contain Ihh transcripts suggests that IHH is insufficient to induce oligodendrocytes. Second, the induction of Olig2 and Nkx2.2 genes, and the subsequent generation of oligodendrocytes in SHH null cultures following FGF2 treatment in the presence of cyclopamine, provides evidence for an in vitro hedgehog-independent pathway for the induction of oligodendrocytes. These observations are also compatible with recent studies showing hedgehog-independent generation of oligodendrocytes from mouse embryonic stem cell cultures in the presence of FGF2 (H.K., S.C. and N.D.A., unpublished), and from SHH null forebrain progenitors (Nery et al., 2001).

Overall, our findings are consistent with recent studies that provide evidence for factor(s) additional to SHH having a role in determining ventral cell fates (Pierani et al., 1999). The partial rescue of ventral neuronal subtypes in double-mutant SHH/GLI3 and SHH/opb (RAB23 – Mouse Genome Informatics) mice suggests that other signalling pathways contribute to ventral specification (Litwingt and Chiang, 2000; Eggenschwiler et al., 2001). The absence of pMN and ventral interneurone progenitor domain derivatives in noggin mutant mice, despite the presence of normal SHH expression and upregulation of patched transcription, suggests that, in addition to SHH, modulation of BMP signalling is necessary for ventral patterning (McMahon et al., 1998).

Specification of motoneurones and oligodendrocytes from the same progenitor pool is temporally regulated (Lu et al., 2002; Zhou and Anderson, 2002). In the present case, the induction of Nkx2.2 and Olig1/Olig2, but not Nkx2.9, Nkx6.1, Nkx6.2, Hb9 or Lim3, in expanded dorsal cultures suggests the generation of cells with late pMN- but not early pMN-domain characteristics. The precise regulation of Olig genes is unclear. A recent study provides evidence that, in the early pMN domain, SHH regulates Olig2 expression through the class II protein Nkx6.1 (Novitch et al., 2001). However, our finding of Olig gene induction in the absence of Nkx6.1 and Nkx6.2 expression, following FGF2 treatment, provides indirect support for an additional SHH-independent pathway that regulates Olig gene expression. This idea is supported by the identification of co-expressing Olig2 and Nkx2.2 cells in dorsal neurospheres, in numbers consistent with GC-positive cells observed in differentiating cultures. Furthermore, although oligodendrocyte and motoneurone progenitors share a lineage relationship in vivo, this relationship is not obligate for the generation of oligodendrocytes in other contexts (Fig. 6).

FGF2-mediated oligodendrocyte induction is dependent on MAP kinase signalling and is inhibited by BMP4

The precise mechanism underlying FGF2-mediated oligodendrogenesis in our system is unclear. FGF signalling is mediated by receptor tyrosine kinases (RTKs) that can activate various pathways, including MAP kinase, that lead to the phosphorylation and activation of the kinases ERK1/ERK2 (MAPK3/MAPK1 – Mouse Genome Informatics) (for a review, see Boilly et al., 2000). The loss of oligodendrocytes following inhibition of ERK1/ERK2 signalling strongly suggests that FGF2 has an active role in the generation of oligodendrocytes from dorsal spinal cord precursors.

In addition to promoting dorsal cell fate, BMPs are thought to influence ventral patterning of the neural tube by modulating the hedgehog signalling pathway (Basler et al., 1993; McMahon et al., 1998; Liem et al., 2000). BMP antagonism of SHH also negatively regulates spinal cord OPC specification (Mekki-Dauriac et al., 2002). However, in the present study addition of noggin, follistatin and chordin to primary dorsal cultures was insufficient to generate oligodendrocytes. This argues against the idea of a loss or dilution of extracellular inhibitory factors in FGF2-derived expanded dorsal cultures.

The absence of oligodendrocytes in our study following co-treatment with FGF2 and BMP4 is consistent with reports of BMPs suppressing oligodendrocyte differentiation from neural precursors (Zhu et al., 1999). The comparable levels of expression of BMP4 in primary (uncultured) and FGF-treated cultures, and the dose-response nature of BMP4 suppression of oligodendrocytes, is compatible with a balance between inductive and negative signalling requirements for oligodendrogenesis in our system.

BMPs can also promote astrocyte formation from embryonic neural precursors (Gross et al., 1996; Mabie et al., 1999). However, in our dorsal spinal cord cultures an elimination of oligodendrocytes was accompanied by a reduction in astrocyte numbers. The reason for this reduction in astrocyte numbers is unclear. Multiple mechanisms appear to underly the generation of astrocytes from neural precursors (Johe et al., 1996; Gross et al., 1996; Rajan and McKay, 1998). Evidence suggests that CNTF/LIF-mediated instruction of astrogenesis is through
activation of the JAK-STAT pathway, in contrast to BMP4 and EGF. Recent studies showing the opposing actions of FGF and BMP in positively and negatively regulating EGF receptors (EGFR) in neural precursors may help to explain our findings (Lillien and Raphael, 2000). Following FGF2 treatment there was a significant induction of EGFR expression, consistent with previously reported observations (Ciccolini and Svendsen, 1998). Interestingly a recent study demonstrated that BMP4 suppresses oligodendrocyte formation from the developing spinal cord without an increase in astrocyte formation (Mekki-Dauriac et al., 2002). In our study, treatment with BMP4 also resulted in the generation of smooth muscle actin, a neural crest derivative, a finding consistent with the demonstration of a peripheral neural potential of spinal cord neural precursors following BMP treatment (Mujtaba et al., 1998; Panchision et al., 2001).

An interpretation of our findings is that FGF is opposing inhibitory effects of endogenous BMP signalling, a recognised interaction in other developmental processes, including limb, tooth, lung and submandibular gland morphogenesis (Niswander and Martin, 1993; Neubuser et al., 1997; Weaver et al., 2000; Hoffman et al., 2002). The mechanism of such antagonism is unclear. In the developing vertebrate limb there is evidence that FGF antagonism of BMP signalling is mediated in part through SHH (Zuniga et al., 1999). However, recent studies suggest alternative mechanisms that could underly the hedgehog-independent FGF antagonism of BMP observed in our system (Kretzschmar et al., 1997; Lo et al., 2001).

In summary, we describe a novel in vitro developmental pathway by which FGF-mediated signalling can influence dorsoventral neural precursor cell fate, and specifically oligodendrogenesis, by mechanisms that are independent of SHH. In future, it will be important to clarify the extent to which hedgehog-independent oligodendrocyte generation contributes to normal developmental myelination, and also to re-myelination, as this is likely to have clinical implications for the design of strategies to enhance myelin repair following disease in the adult CNS.

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References


Hedgehog-independent generation of oligodendrocytes


