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Converse control of oligodendrocyte and astrocyte lineage development by Sonic hedgehog in the chick spinal cord

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Abstract

In the developing spinal cord, oligodendrocyte progenitors (OLPs) originate from the ventral neuroepithelium and the specification of this lineage depends on the inductive activity of Sonic hedgehog (Shh) produced by ventral midline cells. On the other hand, it has been shown that OLP identity is acquired by the coexpression of the transcription factors *olig2* and *nkx2.2*. Although initially expressed in adjacent nonoverlapping domains of the ventral neuroepithelium, these transcription factors become coexpressed in the pMN domain at the time of OLP specification through dorsal extension of the Nkx2.2 domain. Here we show that Shh is sufficient to promote the coexpression of Olig2 and Nkx2.2 in neuroepithelial cells. In addition, Shh activity is necessary for this coexpression since blocking Shh signalling totally abolishes Olig2 expression and impedes dorsal extension of Nkx2.2. Although Shh at these stages affects neuroepithelial cell proliferation, the dorsal extension of the Nkx2.2 domain is not due to progenitor proliferation but to repatterning of the ventral neuroepithelium. Finally, Shh not only stimulates OLP specification but also simultaneously restricts the ventral extension of the astrocyte progenitor (AP) domain and reduces astrocyte development. We propose that specification of distinct glial lineages is the result of a choice that depends on Shh signalling.

Keywords: Oligodendrocyte; Astrocyte; Sonic hedgehog; Spinal cord; Chick embryo; Olig2; Nkx2.2

Introduction

Although oligodendrocytes (OLs), the myelinating cells of the CNS, are widely distributed throughout the adult white and grey matter, oligodendrocyte progenitors (OLPs) arise from restricted domains of the undifferentiated neuroepithelium. In the spinal cord, in particular, explant culture experiments or the use of early lineage markers have shown that OLPs are restricted to a small ventral neuroepithelial region, close to the floor plate (Ono et al., 1995; Pringle and Richardson, 1993; Pringle et al., 1996; Spassky et al., 1998; Timsit et al., 1995; Trousse et al., 1995; Yu et al., 1994). OLPs migrate out from these regions to populate the entire spinal cord, where they actively proliferate and differentiate. The ventral origin of OLPs results, at least in part, from the signalling events that also induce ventral neuronal populations. The morphogen protein Sonic hedgehog (Shh), produced by the notochord and the floor plate, induces various populations of ventral neurons (Briscoe and Ericson, 1999; Briscoe et al., 2000) and also induces OL development from undifferentiated neural progenitors (Poncet et al., 1996; Pringle et al., 1996; Trousse et al., 1995). Shh activity is required for oligodendrogenesis since absence of Shh signalling prevents OL development in the spinal cord (Orentas et al., 1999; Soula et al., 2001) and in the telencephalon (Nery et al., 2001; Spassky et al., 2001; Tekki-Kessaris et al., 2001). Recent work suggests the alternative FGF-signalling pathway for promoting OL fate from cultivated neural stem cells (Chandran et al., 2003; Hsieh et al., 2004). However, it is unclear whether this pathway also operates in neural progenitors in vivo.

Abbreviations: AP(s), astrocyte progenitor(s); BMP(s), bone morphogenetic protein(s); CNS, central nervous system; OL(s), oligodendrocyte(s); OLP(s), oligodendrocyte progenitor(s); Shh, Sonic hedgehog.

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The molecular mechanism of action of Shh in specifying ventral subclasses of neurons is now well characterized. The ventro-dorsal gradient of Shh activity induces the expression by neural progenitors of a set of homeodomain and bHLH transcription factors (Briscoe et al., 2000). These factors have been classified into two categories: class I proteins, which are repressed by Shh, and class II proteins, which depend on Shh signalling for their expression. The combinatorial expression of these factors at each dorso-ventral level of the neuroepithelium allows the identity of various classes of neurons in the ventral neural tube to be defined (Briscoe et al., 2000). Some of the transcription factors are used within the neuroepithelium both during the neurogenic and the gliogenic phases. In the chick spinal cord, OLPs are found within the ventral-most Nkx2.2-expressing domain of the neuroepithelium, and not in the Pax6-expressing domain, located just dorsally (Soula et al., 2001). In addition, a fraction of OLPs and, subsequently, most OLs, express Olig2, a bHLH transcription factor that is also expressed at earlier stages in motor neuron progenitors (Lu et al., 2000; Takebayashi et al., 2000; Zhou et al., 2000, 2001). Loss of function of *nkx2.2* drastically reduces and delays OL development (Qi et al., 2001), and loss of function of *olig2* totally abolishes it (Lu et al., 2002; Takebayashi et al., 2002; Zhou and Anderson, 2002). Thus, *olig2* appears to play a key role in OL specification. However, misexpression experiments have shown that both genes are required to promote OL specification in the chicken neural tube (Zhou et al., 2001). At the time when OLPs arise, expression of both Olig2 and Nkx2.2 undergoes dynamic changes in the neuroepithelium. These transcription factors are first expressed in mutually exclusive domains but then the expression domain of Nkx2.2 extends in a dorsal direction, resulting in partial overlap of both regions (Fu et al., 2002; Zhou et al., 2001). However, the mechanisms that initiate and control these changes in expression, in particular the extension of the Nkx2.2 domain and the coexpression of the two transcription factors, remain unknown.

Unlike that of OLs, the origin of astrocytes has not yet been elucidated. Studies of the expression of markers of the radial glia–astrocyte lineage in the mouse and chicken suggest that these cells originate from a restricted part of the neuroepithelium (Pringle et al., 2003; Shibata et al., 1997) located dorsally to OLPs (Pringle et al., 2003). In vitro studies have suggested the existence of glial-restricted progenitors (GRP) derived from multipotent neuroepithelial stem cells that give rise both to the astrocyte and the OL lineage (Rao and Mayer-Proschel, 1997). Although no common molecular mechanism underlying the emergence of the two lineages has yet been discovered, studies of the *olig1/olig2* double-knockout mouse suggest a possible link between OL and astrocyte development in vivo, since cells that normally should have expressed *olig* genes differentiate into astrocytes (Zhou and Anderson, 2002). Furthermore, in vitro studies have shown that Olig2 is able to negatively control astrocyte differentiation from neural precursors (Fukuda et al., 2004; Gabay et al., 2003).

In the present study, we have analysed the possible function of Shh produced by the floor plate in generating the appropriate pattern of expression of transcription factors required for OL specification. We show that Shh activity is sufficient for Olig2/Nkx2.2 coexpression and is required for the maintenance of Olig2 expression and for the dorsal extension of the Nkx2.2 expression domain. Furthermore, modification of the size of the Nkx2.2 domain involves repatterning of the neuroepithelium and not cell proliferation. Interestingly, Shh signalling regulates neuroepithelial expression of *Fgfr3* and *glast*, early markers of the astrocyte lineage, by limiting their ventral extension and simultaneously decreases astrocyte differentiation. Altogether, our results suggest that Shh controls the specification of both glial lineages by inducing the OL lineage and inhibiting the astrocyte lineage. We propose that neural progenitors give rise either to astrocytes or OLs depending on their dorso-ventral position.

Materials and methods

Materials

Fertilized chick eggs (White Leghorn, commercial source) were incubated at 38°C in a humidified incubator and embryos were staged according to Hamburger and Hamilton (1992).

Explants cultures

In all culture experiments, explants were grown in DMEM (Gibco BRL) supplemented with 10% FCS (Sigma). Ventral neuroepithelial explants (Soula et al., 2001) were grown on collagen gel-coated 12-mm plastic coverslips placed in 14-mm four-well dishes (Nunc). The recombinant N-terminal fragment of the human Shh protein (Biogen) was used at concentrations of 12 nM for neuroepithelial cultures. For flat-mounted spinal cords, a saturating concentration of 100 nM was used in all experiments. This later concentration very reproducibly inhibited astrocyte development in our assay. Cyclopamine, a generous gift of Dr. Frederic Rosa, was used at a concentration of 4 μM.

For embryonic chick spinal cord cultures, we used a flat whole-mount preparation already described (Mekki-Dauriac et al., 2002). Briefly, the cervico-brachial spinal cord from E4 chick embryos was dissected free of surrounding tissue, opened along the dorsal midline, and flattened on a nitrocellulose membrane (Sartorius) with the neuroepithelial layer up. The explants were then grown as organotypic cultures, at the air–medium interface, with or without Shh protein or cyclopamine for the indicated period of time. For cell-cycle-blocking experiments, E5 spinal cord flat mounts were incubated with 5 μM aphidicolin (Sigma) and fixed after 24 h.

Staining procedures

Control embryos or flat-mounted explants were fixed in 4% paraformaldehyde (PFA) for 4 h at 4°C and processed for immunohistochemistry or overnight at 4°C and processed for in situ hybridisation. The tissues were then sectioned at 60–80 μm on a vibratome (Leica) before being processed. Ventral neuroepithelial explants were fixed in 4% PFA in PBS for 30 min and stained with the appropriate antibodies.

O4 immunohistochemistry was performed as previously described (Giess et al., 1992). For the detection of intracellular antigens Nkx2.2, Olig2, Pax6, GFAP, fixed cultures were permeabilised using Triton-X 100 (0.5% in PBS) and primary antibody was applied at the appropriate dilution in 0.1% Triton-X 100/PBS and incubated overnight at 4°C. After incubation, the sections were rinsed in PBS and further incubated for 30 min with biotinylated secondary antibody directed against either mouse or rabbit Ig (Amersham, diluted 1:50). After rinsing, biotinylated antibodies were revealed by 30-min incubation with fluorescein (FITC)- or rhodamine (TRITC)-coupled streptavidin (Amersham, diluted 1:50). To detect simultaneously Olig2 and Nkx2.2, immunohistochemistry was performed sequentially, using first anti-Olig2 antiserum then biotinylated secondary antibody and fluorescein isothiocyanate (FITC)-coupled streptavidin as described above; this was followed by anti-Nkx2.2 antibody, revealed with goat antimouse Alexa-546 antibody (Molecular Probes).

For BrdU staining, organotypic cultures were incubated for 45 min with BrdU (BrdU labeling and detection kit, Roche) at the end of the culture period. Explants were then fixed for 4 h and processed for BrdU staining according to the manufacturer's instructions. TUNEL assay (Roche) was performed as recommended by the manufacturer. In some experiments, cell nuclei were stained with chromomycin A3. Sections were rinsed in PBS/$MgCl_2$ (150 mM), incubated 1 h with 100 nM chromomycin A3, rinsed and mounted with moewiol.

Antibodies

Cells of the OL lineage were identified using O4 antibody. The mouse monoclonal O4 antibody recognises sulfatides at the surface of immature and differentiated OLs (Bansal et al., 1989; Ono et al., 1995; Sommer and Schachner, 1981), and the so-called "prooligodendroblast antigen", expressed before sulfatides by OL progenitors and early differentiating OLs. Culture supernatant obtained from O4 hybridoma cells, a gift from Dr R. Bansal, was used undiluted. The anti-Olig2 antiserum (Takebayashi et al., 2000) was used at 1:2000 dilution. Astrocytes were evidenced by an antiserum directed against Glial Fibrillary Acidic Protein (GFAP; DAKO A/S, Denmark) used at 1:500. All the following monoclonal antibodies (culture supernatants) were obtained from the Developmental Studies Hybridoma Bank. Motor neurons were identified by their expression of MNR2 (81.5C10, used at 1:8; Tanabe et al., 1998). Ventricular cell subpopulations were visualized either with the anti-Nkx-2.2 antibody used at 1:2 dilution (74.5A5, Ericson et al., 1996), or the anti-Pax-6 antibody used at 1:2 dilution (Ericson et al., 1997).

Sections and neuroepithelial explants were analysed with either a Zeiss LSM-410 or a Leica SP2 confocal microscope, equipped with argon and helium–neon lasers and appropriate filter combinations for fluorescein and rhodamine, respectively. In all cultures, labelled cells were counted and results are expressed as the mean ± SEM number of labelled cells per culture or per explant. Significance of the results was analysed using the Student's *t* test.

In situ hybridisation

Olig2, *patched*, *nkx2.2*, *irx3*, *sim1*, *Fgfr3*, *GFAP*, *PLP/D20M* and *glast* transcripts were analysed using the antisense digoxigenin RNA probe. *Patched* cDNA was a gift from C. Tabin, *Nkx2.2* cDNA was a gift from J. Rubenstein, *irx3* cDNA was a gift from T. Ogura, *sim1* cDNA was a gift from J. Michaud, *Fgfr3* was a gift from O. Delapeyriere. *PLP/DM20* cDNA was a gift from B. Zalc. *GFAP* cDNA was purchased as an EST (pgp1n.pk013.g7) from Delaware Biotechnology Institute. A *glast* 595p-cDNA fragment was generated by PCR using the following primers: upstream 5′TGCTCCACTTGGAATCCTCT3′, downstream 5′CTTCAGCTCATGCCGTGATA3′. These primers were defined using the *glast* cDNA sequence (accession number, AF154672). The whole-mount in situ procedure was performed according to Wilkinson (1992).

Results

Shh induces coexpression of Olig2 and Nkx2.2 in neuroepithelial cells

We first investigated whether Shh was sufficient to drive the coexpression of Olig2 and Nkx2.2 observed in neuroepithelial cells in vivo at the time of oligodendrocyte specification, i.e., E5 to E6 in the chick cervico-brachial spinal cord (Soula et al., 2001). Since this coexpression occurs in vivo around E5.5 (Fu et al., 2002; Zhou et al., 2001), we documented the direct effect of Shh on ventral neuroepithelial explants isolated in culture at E5, free of surrounding tissues. Under these conditions, such explants never generate OL cells, even in long-term cultures, whereas 12 nM Shh rapidly induces numerous OLPs (Soula et al., 2001). After 48 h of culture, we monitored the expression of both transcription factors in control and treated explants. In control conditions, explants displayed very few Olig2-Nkx2.2 double-labelled cells (12 cells/explant, $n = 18$; Figs. 1A,C). Shh treatment caused a ninefold increase in the number of cells coexpressing both factors (105 cells/ex-

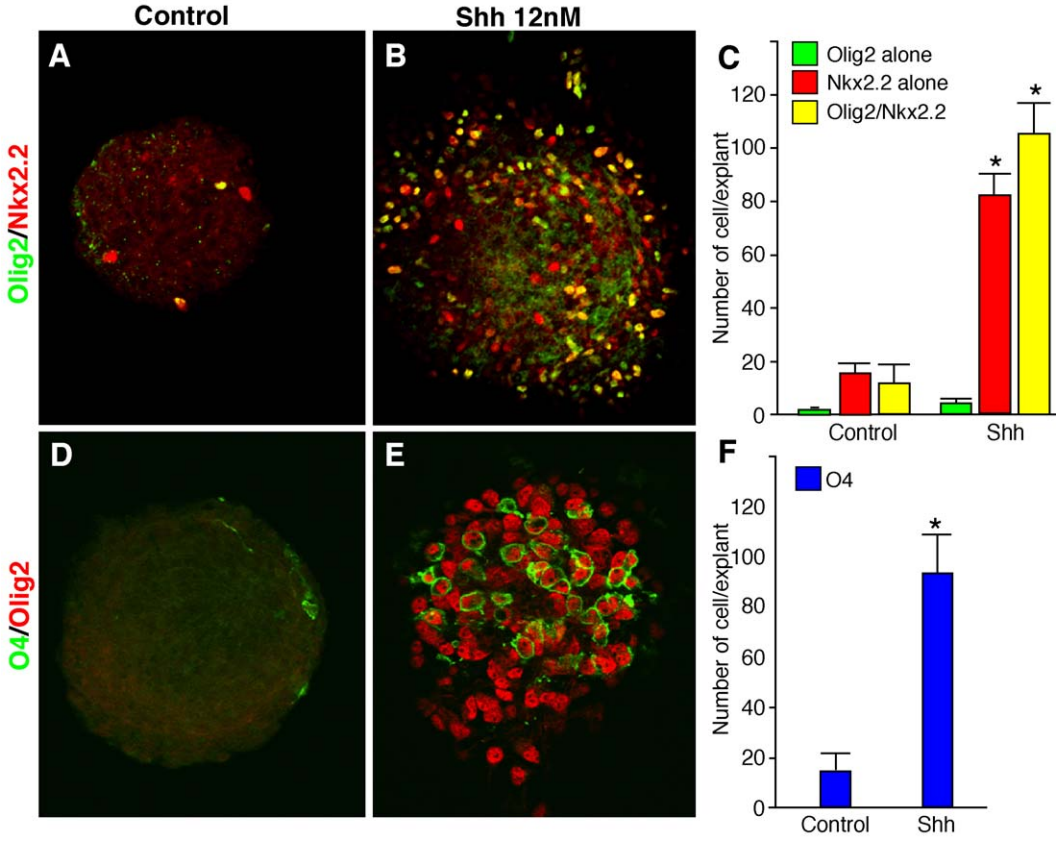

Fig. 1. Shh induces the coexpression of Olig2 and Nkx2.2. Neuroepithelial explants were dissected at E5, before OL specification, and cultivated for 2 days under control conditions (A, D) or with 12 nM Shh protein (B, E). After culture, explants were double-immunostained either with anti-Olig2 and anti-Nkx2.2 antibodies (A, B) or with anti-Olig2 and O4 antibodies (D, E). Control neuroepithelial explants contain very few cells expressing Nkx2.2, Olig2 or both (A, C) and very few $O4^+$ cells (D, F). After Shh treatment, expression of both Olig2 and Nkx2.2 is induced dramatically. All $Olig2^+$ cells also express Nkx2.2 (B, C). Concomitantly, Shh induces numerous $O4^+$ cells, all of which also express Olig2 (E, F). C: Quantification of the number of cells per explant expressing Nkx2.2, Olig2 or both under the different conditions. F: Quantification of the number $O4^+$ cells per explant. Asterisk: Significant difference between control and treated cultures ($P < 0.01$).

plant, $n = 18$; Figs. 1B,C). In addition, Shh also increased the number of cells expressing Nkx2.2 alone (a fivefold increase, from 15 to 82 cells per explant). In all cases, most cells expressing Olig2 also expressed Nkx2.2. To ascertain that, under our experimental conditions, cells coexpressing Olig2 and Nkx2.2 corresponded to OLPs, sister cultures were double-stained with Olig2 and the OLP marker O4. As previously described, we did not detect significant numbers of O4 cells in control conditions (Figs. 1D,F), whereas Shh induced numerous $O4^+$ cells (93 cells per explant; $n = 7$; Figs. 1E,F), all of which coexpressed Olig2. These results suggest that Shh is sufficient to cause the coexpression of Olig2 and Nkx2.2 in ventral neuroepithelial cells, in conjunction with OLP specification.

Shh is required for the maintenance of Olig2 expression and for the extension of the Nkx2.2 expression domain in the neuroepithelium

To further investigate the relationships between Shh, transcription factor expression and OLP specification, we developed an organotypic culture system for the spinal cord, in which the multilayered structure of the tissue is maintained over several days, enabling progenitor cells to be distinguished from differentiated cells of the mantle layer. The spinal cord was isolated at E4, i.e., before OLP specification, opened dorsally, flat-mounted and maintained in culture for 2 days or more (Fig. 2G). To verify that spinal cords were cultivated under conditions appropriate for OL induction, we first analysed O4 expression. As shown in Fig. 2H, O4 antibody decorated ventral neuroepithelial cells in bilateral groups of cells, adjacent to the floor plate, a pattern of expression similar to that described in E6 embryonic spinal cord in vivo (Ono et al., 1995; Soula et al., 2001), indicating that OLP specification had occurred normally.

We then compared the patterns of expression of Olig2, Nkx2.2 and Pax6 between in vivo spinal cords and these ex vivo preparations, by counting the number of neuroepithelial cell profiles along the D/V axis. During the period of neuronal genesis, i.e., until E5, the Nkx2.2 domain was adjacent to, but did not overlap with the dorsally located

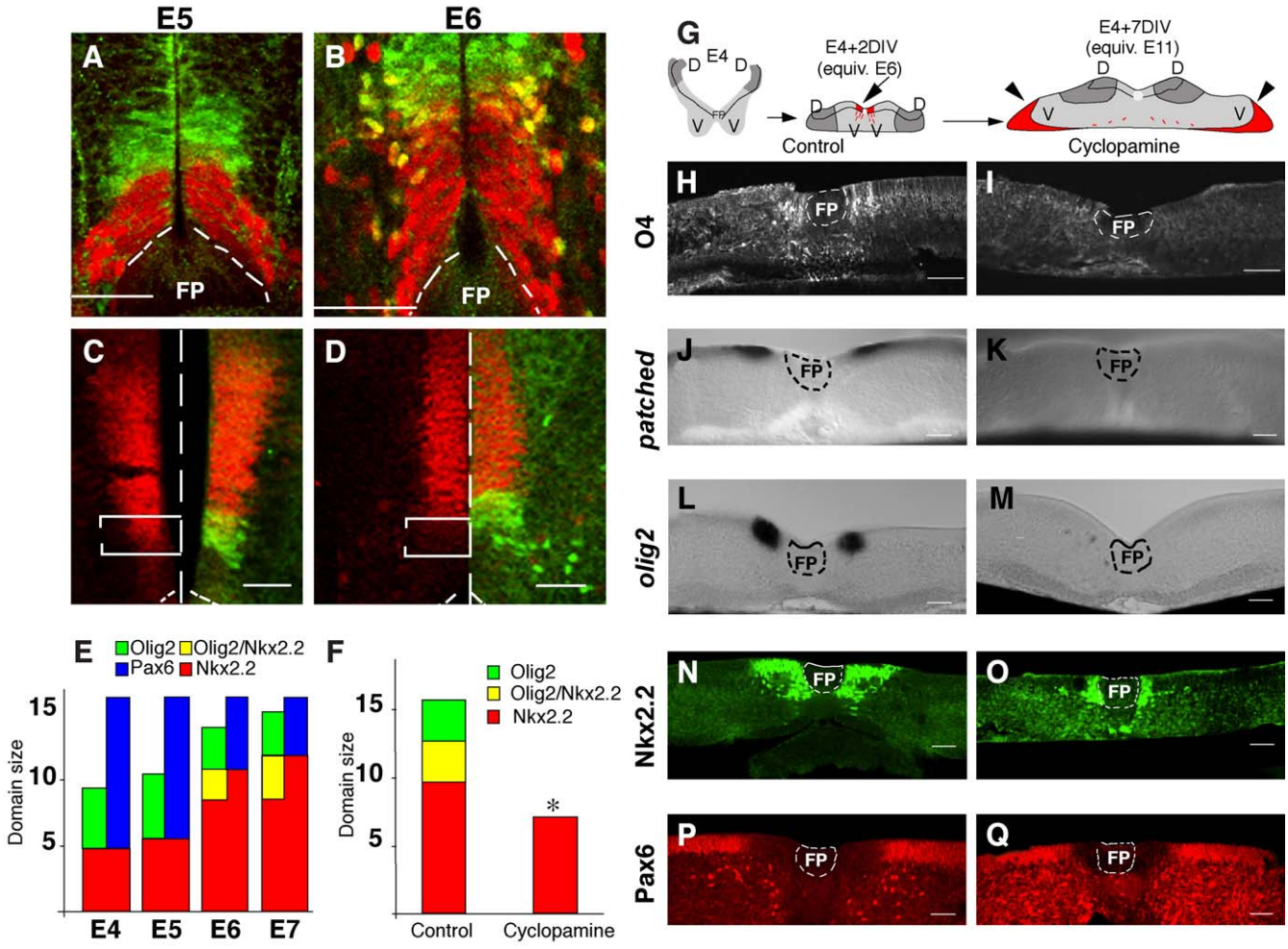

Fig. 2. Shh is required for the coexpression of Olig2 and Nkx2.2 concomitantly with OLP specification. (A–B) Double immunostaining of transverse spinal cord sections with anti-Olig2 (green) and anti-Nkx2.2 or anti-Pax6 antibodies (red). A: At E5, the Olig2-expressing domain is strictly contiguous to that of Nkx2.2. The dorso-ventral extension of each of these domains corresponds to about five cell profiles (E). B: At E6, the Nkx2.2 domain has extended dorsally to about 12 cell profiles (E) and the Olig2 domain is shifted dorsally. In addition, the two domains now overlap leading to coexpression of Nkx2.2 and Olig2 in some cells. C–D: Between E5 and E6, the Pax6 domain regresses dorsally. The left panel of each image shows Pax6 immunostaining alone, while the right panel shows Olig2/Pax6 overlay. C: At E5, Pax6 expression is constant down to the dorsal limit of the Olig2 domain and from then on diminishes as a dorso-ventral gradient. D: At E6, the Pax6 gradient has disappeared, resulting in a dorsal shift of the ventral border of Pax6 expression. Thus, only the dorsal half of the Olig2 domain coexpresses Pax6. The parallel brackets delineate the position of the $Pax6^{low}$/Olig2 domain observed at E5. G: Scheme of the organotypic culture assay. The spinal cord is dissected from the embryo at E4, cultivated for 2 days in vitro (2DIV) or 7 days in vitro (7DIV). At E4 + 2DIV, OLPs are generated in the ventral neuroepithelium (arrow) and at E4 + 7DIV, OLs have migrated in the ventral white matter, which is displaced laterally under these culture conditions (arrowheads), and express PLP-DM20. H–Q: Flat-mounted E4 spinal cords were cultivated for 2 days (equivalent to E6) under control conditions (H, J, L, N, P) or with 4 μM cyclopamine (I, K, M, O, Q). After culture, explants were sectioned and immunostained using antibodies directed against O4 (H, I), Nkx2.2 (N, O) and Pax6 (P, Q), or prepared for in situ hybridisation with probes for *patched* (J, K) or *olig2* (L, M). H: Under control conditions, the OL lineage marker O4 is expressed by neuroepithelial cells located on each side of the floor plate and by differentiating OLs that have migrated in the mantle and marginal layers. I: Cyclopamine treatment abolishes the OL lineage as shown by absence of O4 staining. H–Q: Under control conditions, the domains of expression of *patched* (J), *olig2* (L), Nkx2.2 (N), Pax6 (P) are comparable in size and position to those observed in vivo. After cyclopamine treatment, *patched* (K) and *olig2* (M) expression domains are completely abolished, the expression domain of Nkx2.2 (O) is reduced compared to that of control (N), the domain of Pax6 (Q) is extended ventrally. These images are representative of five to seven different experiments. E: Quantification of the in vivo domain size at the various stages examined, expressed as the number of cell profiles in each dorso-ventral domain. F: Quantification of domain size for the various transcription factors, expressed as the number of cell profiles after culture in control or cyclopamine-containing medium. Asterisk: Significant difference between control and treated cultures ($P < 0.05$). Dashed lines highlight the limits of the floor plate (FP). Scale bar represents 50 μm.

Olig2 and Pax6 domains (Fig. 2A), in keeping with previous observations (Fu et al., 2002; Zhou et al., 2001). The extent of the Nkx2.2 and Olig2 domains was 4.8 ± 0.2 and 4.6 ± 0.3 cells, respectively, and did not vary between E4 and E5 (Fig. 2E). The Pax6 domain was characterised by a marked dorso-ventrally decreasing gradient of expression (Fig. 2C). We observed that these expression patterns were profoundly modified after E5, confirming previous results (Fu et al., 2002; Zhou et al., 2001). In fact, the Nkx2.2 domain extended dramatically in a dorsal direction (Fig. 2B),

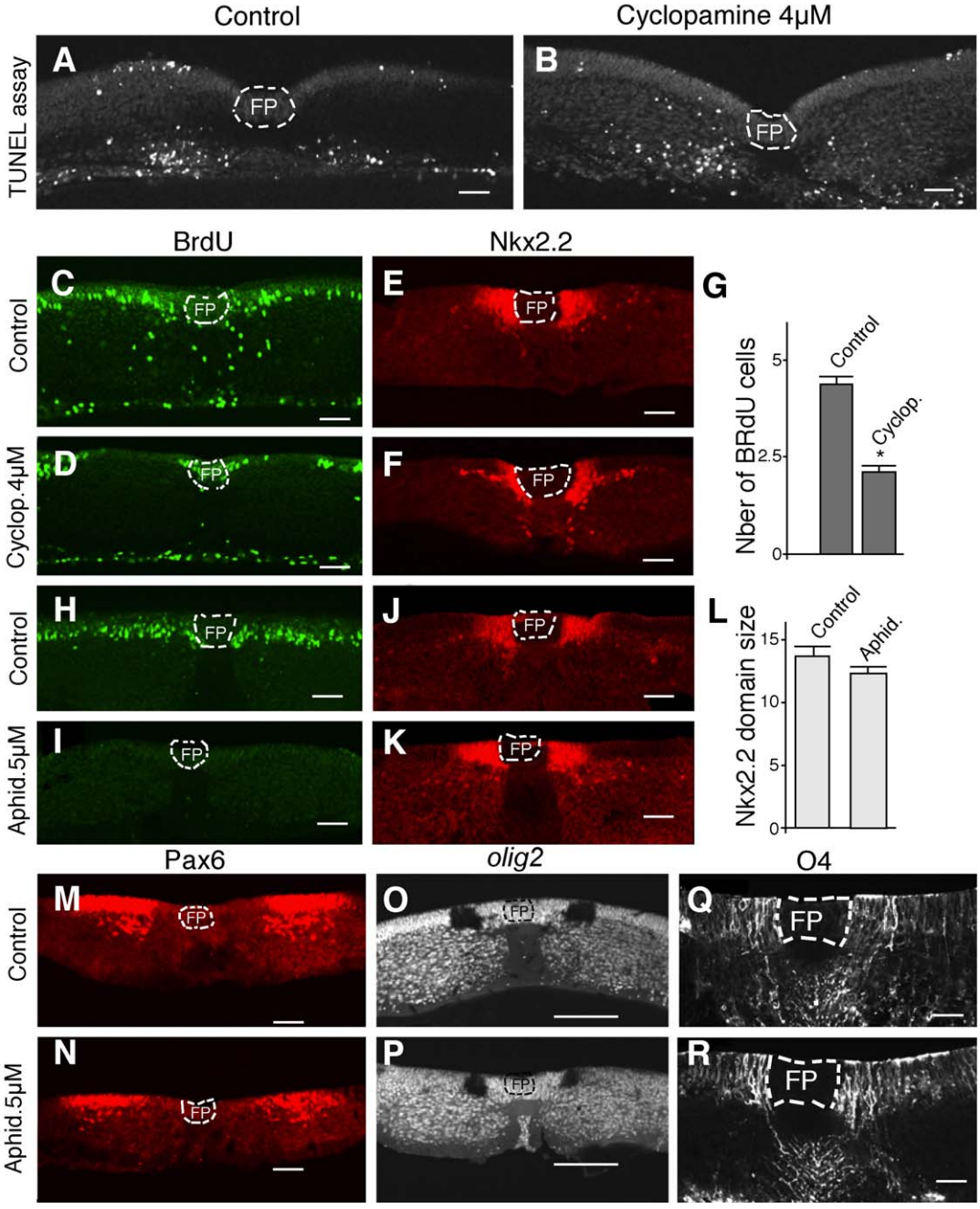

Fig. 3. Proliferation in the neuroepithelium is partly controlled by Shh signalling but this does not account for the repatterning of Nkx2.2, *olig2* and Pax6 domains. A–F: Flat-mounted E4 spinal cords were cultivated for 2 days under control conditions (A, C, E), or with 4 μM cyclopamine (B, D, F). A–B: After culture, explants were prepared for TUNEL assay. No modification of apoptosis can be observed between the control (A) and the cyclopamine-treated culture (B). C–F: Explants were incubated for 45 min in the presence of BrdU at the end of the culture period and processed for BrdU or Nkx2.2 immunohistochemistry. BrdU incorporation can be observed homogeneously distributed in the neuroepithelium of control spinal cord explants (C). After cyclopamine treatment, BrdU incorporation is reduced in the ventral neuroepithelium (D) and the size of the Nkx2.2 domain is reduced (F) compared to the control (E). G: Number of cells incorporating BrdU in the Nkx2.2 domain in control or cyclopamine-treated cultures ($n = 3$, Asterisk: $P < 0.01$). H–R: Flat-mounted E5 spinal cords were cultivated for 24 h under control conditions (H, J, M, O, Q), or with 5 μM aphidicolin (I, K, N, P, R). After culture, they were stained with anti BrdU antibody (H, I), anti-Nkx2.2 antibody (J, K), anti-Pax6 antibody (M, N), O4 antibody (Q, R) or processed for in situ hybridisation with the *olig2* probe and counterstained with chromomycin A3 (O, P). After aphidicolin treatment, BrdU incorporation is completely abolished (compare H to I). However, the size of the Nkx2.2 domain observed in aphidicolin-treated explants (K) is not modified compared to control explants (J). L: Quantification of the Nkx2.2 domain size, expressed as the number of cell profiles ($n = 4$). The expression profile of Pax6, *olig2* and O4 under control conditions (M, O, Q) is not altered after aphidicolin treatment (N, P, R). FP: floor plate, Scale bar represents 50 μm.

becoming more than twice its initial size (10.3 ± 0.6 cells, Fig. 2E). The size of the Olig2 domain, however, did not change significantly between E5 and E6 (about five cells), but its ventral border was shifted dorsally by about three cell diameters (Figs. 2B,E). Thus, the two domains were found to overlap partially (2.3 ± 0.6 cells coexpressing both genes at this stage). Concomitantly, the ventral border of the Pax6 domain was also displaced dorsally by about five cell diameters (Figs. 2D,E), through a marked reduction of the Pax6 protein gradient (Fig. 2D). Interestingly, these modifications did not continue beyond that stage since, at E7, the size and the localisation of the domains remained stable (Fig. 2E). In organotypic conditions, the kinetics of expression of the three transcription factors were identical to those observed in vivo. In E4 spinal cords cultivated for 2 days (equivalent to E6), the Nkx2.2 domain also extended dorsally over about 12 neuroepithelial cell profiles (Figs. 2N,F) and the Olig2 domain (Fig. 2L) overlapped both Nkx2.2 (Fig. 2N) and Pax6 (Fig. 2P) domains.

The inductive activity of Shh in neuroepithelial explants raised the question whether this morphogen is required for driving the coexpression of Olig2 and Nkx2.2. We analysed transcription factor expression after treatment with cyclopamine, an alkaloid that inhibits Shh signalling (Incardona et al., 1998) by preventing Smoothened–Gli interactions (Chen et al., 2002). Cyclopamine treatment (4 μM) efficiently blocked Shh signalling as seen by a total extinction of the expression of Shh receptor *patched* (compare Fig. 2K with Fig. 2J). This treatment completely prevented the emergence of $O4^+$ OLPs (Fig. 2I), confirming the requirement for Shh activity in OLP specification in this system. Strikingly, expression of *olig2* was totally abolished in spinal cord explants cultivated in the presence of cyclopamine (Fig. 2M). Additionally, we observed a drastic reduction in the size of the Nkx2.2 domain compared to control explants (Figs. 2O,N). This reduction was paralleled by a ventral shift of the ventral border of the Pax6 domain (Figs. 2Q,P). These results indicate that continuous Shh activity is required for maintaining Olig2 expression in the pMN domain and for promoting the dorsal extension of the $Nkx2.2^+$ domain and the regression of the $Pax6^+$ domain that occur between E5 and E6.

Shh repatterns the ventral neuroepithelium between E5 and E6

Since Shh is required for the survival of neuroepithelial cells during early development in vertebrates (Thibert et al., 2003), the lack of OLP generation in the absence of Shh signalling could result from apoptosis in the ventral ventricular zone. To test this possibility, cyclopamine-treated and control spinal cords were stained using the TUNEL assay. In both types of explants, few TUNEL-positive cells were observed in the neuroepithelium and we did not observe any significant difference in apoptosis between control (Fig. 3A) and cyclopamine-treated explants (Fig. 3B). This indicates that the absence of OLPs was not due to cell death.

Two mechanisms could account for the rapid dorsal expansion of the Nkx2.2 domain mediated by Shh between E5 and E6: increased cell proliferation or repatterning of the ventral neuroepithelium. Since Shh is involved in the control of neuroepithelial cell proliferation in various CNS regions (Rowitch et al., 1999), we first tested the influence of Shh signalling on cell proliferation at this stage through analysis of BrdU incorporation in spinal cord explants cultivated under control conditions or after addition of cyclopamine. In control explants, BrdU incorporation was observed within the entire neuroepithelium and in some migrating cells within the mantle layer located underneath the Nkx2.2 domain (Figs. 3C,E). Cyclopamine treatment reduced BrdU incorporation in the neuroepithelium and blocked proliferation of the cells migrating under the Nkx2.2 domain in the

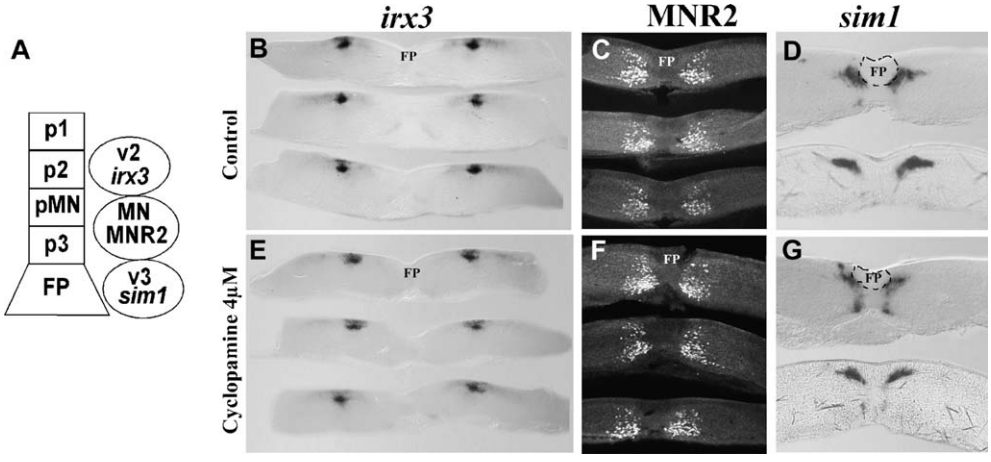

Fig. 4. Cyclopamine treatment at E4 does not modify the expression of ventral neuronal markers. A: Diagram representing ventral neuroepithelial domains and neuronal subpopulation markers. B–G: Flat-mounted E4 spinal cords were cultivated for 2 days under control conditions (B, C, D), or with 4 μM cyclopamine (E, F, G). Cyclopamine treatment induces no obvious modification of the expression of *irx3*, marker of neuron progenitors including V2 interneurons (E), MNR2, marker of motoneurons (F) and *sim1*, marker of V3 interneurons (G), compared to the corresponding controls (B, C, D).

mantle layer (Figs. 3D,F). Quantification of BrdU-positive neuroepithelial cells showed a 50% reduction after cyclopamine treatment (Fig. 3G). This indicates that cell proliferation in the neuroepithelium is partially controlled by Shh, substantiating the possibility that its effect on the expansion of the Nkx2.2 domain was due to proliferation.

To further examine this possibility, we blocked cell division with aphidicolin, an inhibitor of DNA polymerase that stops the cell cycle in the early S phase and prevents neural cell proliferation (Harris and Hartenstein, 1991). E5 spinal cords were cultured for 24 h either under control conditions or in the presence of 5 μM aphidicolin. In our system, aphidicolin efficiently blocked cell division, as shown by total inhibition of BrdU incorporation in the tissue (Figs. 3H,I). However, the drug affected neither the size of the Nkx2.2 domain (Figs. 3K,L) nor the position of the ventral border of the Pax6 domain (Figs. 3M,N) or of the *olig2* domain (Figs. 3O,P). Finally, this treatment had no effect on the emergence of $O4^+$ OLPs (Figs. 3Q,R). Thus, the expansion and regression of Nkx2.2 and Pax6 domains and the dorsal shift of the olig2 domain, observed between E5 and E6, result from repatterning of ventral neuroepithelial cells rather than from cell proliferation.

Shh controls emergence of the astroglial lineage in the spinal cord neuroepithelium

As shown above, late inactivation of Shh signalling leads to modifications of the expression pattern of ventral transcription factors, raising the question of the consequences of such inactivation upon the developmental repertoire of

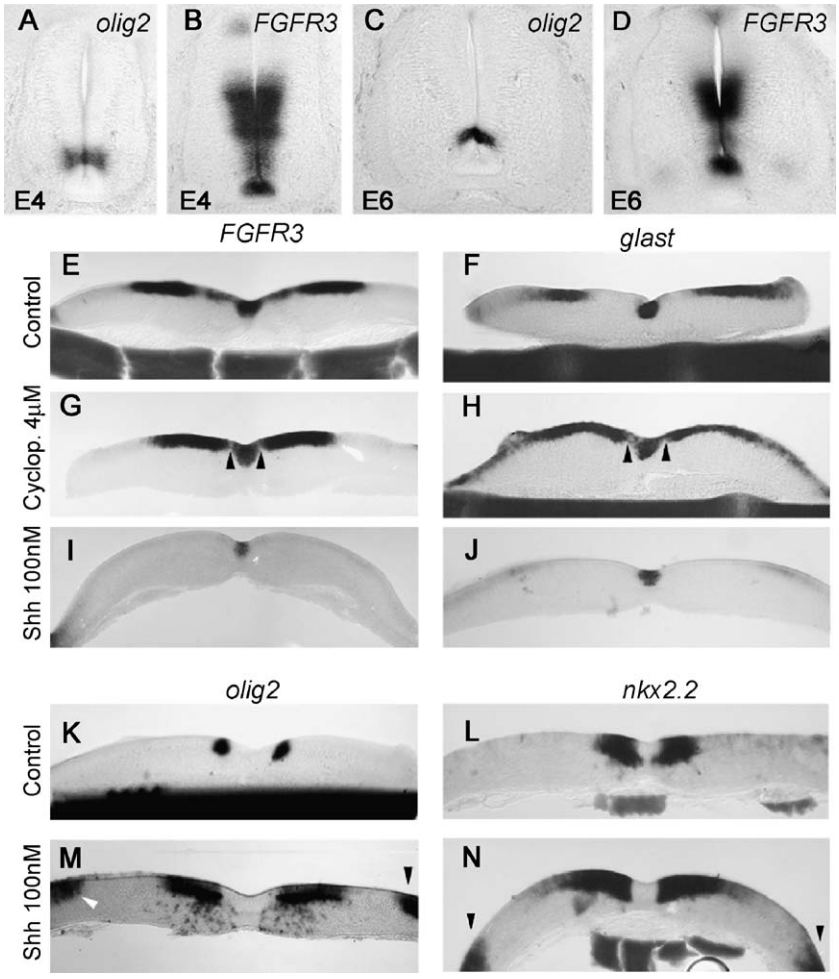

Fig. 5. Shh signalling controls the expression of early astrocyte markers in the neuroepithelium. A–D: Comparison of the expression of *olig2* (A, C) and Fgfr3 (B, D) in the E4 and E6 spinal cord. The AP marker *Fgfr3* (B, D) is strongly expressed in two domains, the floor plate and a median neuroepithelial domain, separated by a region of much lower expression. Note that *olig2* (A, C) is expressed in the domain of low *Fgfr3* (B, D). E–N: Flat-mounted E4 spinal cords were cultivated for 2 days under control conditions (E, F, K, L), treated with 4 μM cyclopamine (G–H) or with 100 nM Shh protein (I, J, M, N) and subjected to in situ hybridization for *Fgfr3* (E, G, I), *glast* (F, H, J), *olig2* (K, M) or *nkx2.2* (L, N). Cyclopamine treatment extends *Fgfr3* (G) and *glast* (H) expression ventrally. Note that a small gap of expression (arrowheads) remains at the floor plate border, which presumably corresponds to the Nkx2.2-expressing domain (compare with Fig. 2O). After Shh treatment (100 nM), *Fgfr3* (I) and *glast* (J) expression was almost abolished. The same treatment extends the domains of expression of *olig2* (M) and *nkx2.2* (N) dorsally compared to controls (K, L). In addition, Shh treatment induces strong expression of both genes in the dorsal neuroepithelium (arrowheads in M, N).

neural progenitors at this level of the spinal cord. We first determined whether blocking Shh signalling after E4 altered the development of ventral neurons. Three transcription factors, markers of different neuronal subpopulations originating from progenitor domains located immediately dorsal and ventral to the *olig2* domain, were analysed (Fig. 4A). These were *Irx3*, a marker of interneuron progenitors whose domain of expression abuts dorsally to that of *olig2* domain, MNR2 for motoneurons and *sim1* for V3 interneurons (Briscoe et al., 2000). No obvious modification in the expression of any of these markers was observed between control (Figs. 4B–D) and cyclopamine-treated spinal cord explants (Figs. 4E–G). This suggests that modification of Shh signalling at this stage no longer has any effect in ventral neuron formation.

We then tested the possibility that ventral neuroepithelial precursors, prevented from becoming OLs by blocking Shh signalling, differentiated into astrocytes instead. We first analysed two markers expressed in astrocyte progenitors (APs), the glutamate–aspartate transporter (Glast), and the FGF receptor 3 (Fgfr3). Both markers are expressed early in radial glia and their expression is maintained in APs and mature astrocytes in the vertebrate spinal cord (Hartfuss et al., 2001; Pringle et al., 2003; Shibata et al., 1997). At E4, *Fgfr3* was strongly expressed in two separate domains, the floor plate and a median neuroepithelial domain, located dorsal to the *olig2* expression domain, with a much lower level of expression between these two regions (Figs. 5A,B). *Glast* expression followed a very similar pattern (not shown). These expression patterns were not modified after E6 (Figs. 5C,D), at least up to E9 (Pringle et al., 2003).

Similar patterns of expression were found in spinal cord explants cultivated under control conditions (Figs. 5E,F). Strikingly, however, cyclopamine treatment resulted in ventral extension of the neuroepithelial domain of expression of both genes (Figs. 5G,H). However, a gap of expression still remained just above the floor plate, at the level of the Nkx2.2 domain (see Fig. 2O), suggesting that these markers were not expressed by Nkx2.2^{+} cells. These results suggest

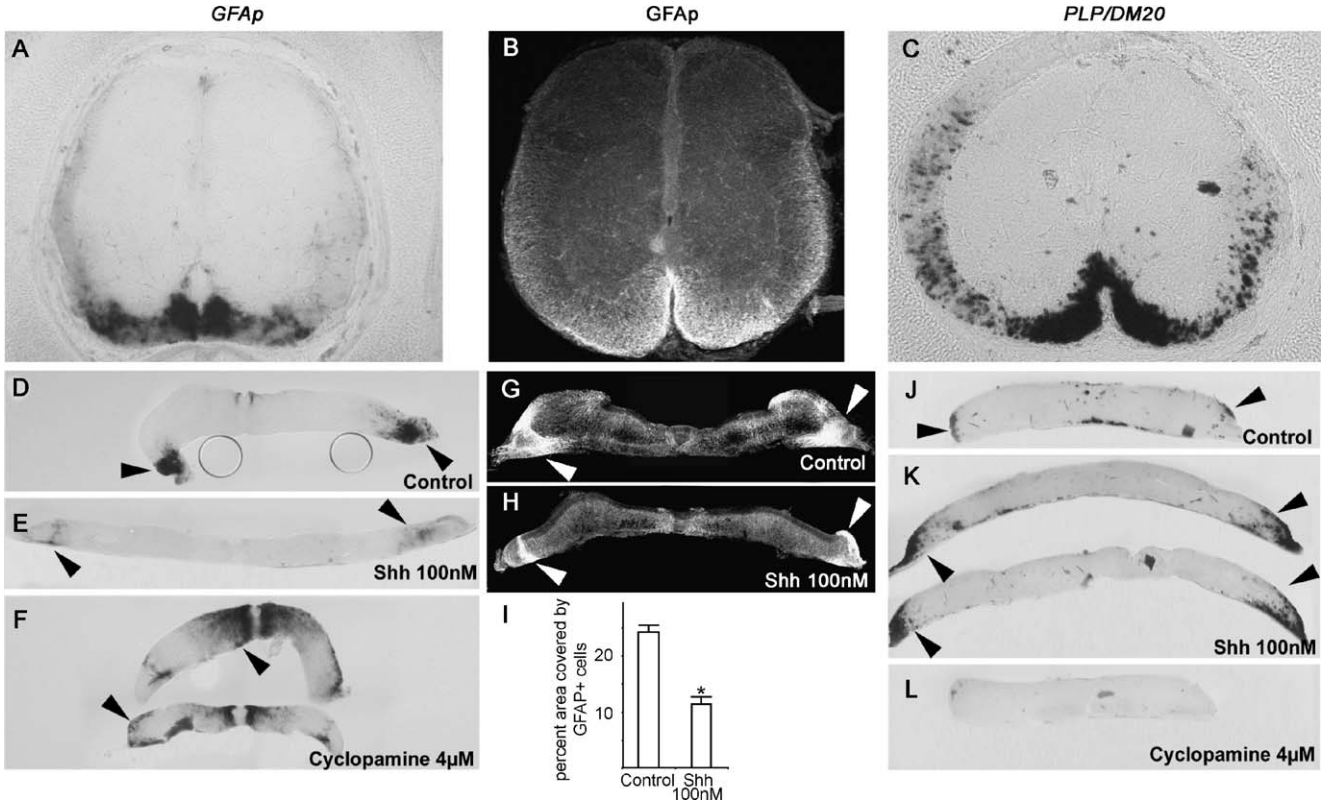

Fig. 6. Shh signalling inhibits the development of astrocytes. A–C: Comparison of *GFAP* mRNA (A), GFAP protein (B) and *PLP/DM20* mRNA (C) expression in E11 spinal cord. At this stage, both markers are expressed essentially in the ventral white matter. D–L: E4 Spinal cord explants cultivated for 7 or 8 days to allow differentiation of GFAP^{+} astrocytes and *PLP/DM20*$^{+}$ oligodendrocytes. Explants were grown under control conditions (D, G, J), in the presence of 100 nM Shh (E, H, K) or with 4 μM cyclopamine (F, L). Under control conditions, the latero-ventral part of the explants contains numerous astrocytes expressing *GFAP* mRNA (arrowheads in D) and also displaying the characteristic filamentous GFAP staining (arrowheads in G), and OLs expressing *PLP/DM20* mRNA (arrowheads in J). In the presence of 100 nM Shh, GFAP expression is drastically reduced (E, H), while OL development is concomitantly stimulated as seen by increased *PLP/DM20* mrRNA expression (K). Conversely, cyclopamine treatment stimulates *GFAP* expression (F) while it abolishes *PLP/DM20* mrRNA expression (L). I: The effects of Shh on GFAP protein expression were quantified by measuring the surface covered by GFAP immunoreactivity, using Image Tool software. Shh treatment reduced GFAP staining by 50%. Asterisk: Significant difference between control and treated cultures ($P < 0.05$).

that endogenous Shh signalling prevents the expression of *glast* and *Fgfr3*. To further address this possibility, E4 spinal cord explants were treated with Shh protein (100 nM) for 48 h. This treatment caused a drastic reduction in the expression of both markers within the neuroepithelium (Figs. 5I,J). A similar reduction was also seen after electroporation of the E4 spinal cord with a *shh*-expressing plasmid (data not shown). Interestingly, expression of both genes in the floor plate was not affected by modifications of Shh signalling, suggesting an alternative control mechanism in the latter cell type.

Since Olig2 has recently been shown to repress astrocyte formation (Fukuda et al., 2004; Gabay et al., 2003), we also analysed the consequences of Shh treatment on *olig2* expression in this system. As expected, 100 nM Shh caused marked extension of *olig2* domain in the ventral neuroepithelium (Fig. 5M) compared to untreated spinal cords (Fig. 5K). The extension of the *olig2* domain is concomitant to the dorsal extension of the *nkx2.2* domain (Figs. 5N,L) confirming that Shh is able to induce coexpression of both genes in ventral neuroepithelial cells. In addition, it induced coexpression of both factors in the dorsal neuroepithelium (arrowheads in Figs. 5M,N), in keeping with the observations that Shh can promote oligodendrocyte differentiation in dorsal spinal cord tissue (Poncet et al., 1996). However, these transcription factors were not induced along the entire neuroepithelium: a region where *glast* and *Fgfr3* are normally expressed remained deprived of *olig2* and *nkx2.2* expression (Figs. 5M,N).

Taken together, the results of our experiments suggest that Shh signalling specifically restricts the ventral expression of astrocyte lineage markers while promoting OL development in the ventral neuroepithelium.

To assess long-term effects of Shh signalling on astrocyte specification, GFAP expression, a property of mature astrocytes, was analysed under the same experimental conditions. OL differentiation was monitored in parallel by studying the expression of the myelin marker PLP/DM20. In the E11 spinal cord, both GFAP and PLP/DM20 are expressed in cells migrating in the ventral white matter (Figs. 6A,B). Under control conditions, after 7 or 8 days of culture, $GFAP^+$ astrocytes developed and accumulated in the latero-ventral aspect of the explants (arrowheads in Figs. 6D,G). Small numbers of $PLP/DM20^+$ OLs could also be detected at this stage in the same location (arrowheads in Fig. 6J). Shh treatment drastically reduced GFAP expression in the explants, both at RNA and protein levels (Figs. 6E,H). Quantification of GFAP immunostaining indicated a 50% reduction in the area covered by immunoreactive protein (Fig. 6I), suggesting impaired astrocyte development. Conversely, cyclopamine treatment clearly enhanced the number of cells expressing *GFAP* (Fig. 6F), which accumulated in the median region of the explant. As expected, Shh treatment strongly stimulated OL differentiation, as judged from increased *PLP/DM20* expression (Fig. 6K), whereas cyclopamine totally abolished it (Fig. 6L).

Taken together, these results suggest that, during the development of the chick spinal cord, Shh signalling represses specification of the astrocyte lineage in the ventral-most region of the neuroepithelium, while concomitantly stimulating the emergence of OLPs in this domain.

Discussion

Studies on the mechanisms of specification of OLs in the chick ventral spinal cord has resulted in the identification of numerous extracellular (i.e., Shh, BMPs) and intracellular (i.e., transcription factors) proteins involved in this process. In this work, we show that Shh signalling controls OL specification by tightly regulating the expression of ventral transcription factors involved in oligodendrogenesis, and it does so by repatterning the ventral neuroepithelium. This results in the appearance of a population of ventral neuroepithelial cells coexpressing *olig2* and *nkx2.2*, engaged along the OL lineage. Conversely, at the same time, Shh signalling appears to inhibit the appearance of the astrocyte lineage in the ventral-most aspect of the neuroepithelium leading to the restriction of APs dorsally to OLPs.

Shh signalling repatterns the neuroepithelium between E5 and E6

During early development, various classes of neurons are generated at defined positions in the ventral neural tube through the combined expression of a variety of transcription factors (Briscoe and Ericson, 1999). Among these, some are probably involved in gliogenesis, as shown by the fact that both neuronal and glial differentiation can be affected by their loss of function. For example, loss of function of *nkx2.2* modifies the production of ventral neuronal types (Briscoe et al., 1999) and induces an increase in the production of Olig1/Olig2-positive cells but eventually causes a reduction in the number of mature OLs (Qi et al., 2001). In Small eye (Pax6-deficient) mice, the origin of MNs and OLPs are shifted dorsally and their differentiation is delayed (Sun et al., 1998). Finally, loss of function of *olig* genes severely affects motoneuron development and totally abolishes that of OLs (Lu et al., 2002; Park et al., 2002; Takebayashi et al., 2002; Zhou and Anderson, 2002).

Expression of these transcription factors is regulated from the early stages through graded Shh secreted by the floor plate (reviewed in Briscoe and Ericson, 1999). The present results demonstrate that at later stages, during the gliogenic phase, ongoing Shh signalling is also required to (i) maintain *olig2* expression and (ii) regulate changes in the expression domains of *pax6* and *nkx2.2*. In particular, dorsal extension of the Nkx2.2 domain and simultaneous regression of the Pax6 domain appear essential for OLP specification. Shh not only controls domain repatterning but also neural precursor proliferation in certain CNS areas (Goodrich et al., 1997; Rowitch et al., 1999). Our experiments

with the cell cycle inhibitor aphidicolin allowed to dissociate the two mechanisms and showed that dorsal extension of the Nkx2.2 domain does not rely on proliferation, and thus is primarily due to repatterning. This repatterning, which occurs in the total absence of cell proliferation, leads to normal OLP development, judging by the normal number and position of OLPs developing in the presence of aphidicolin. Thus, cell proliferation at the time of Nkx2.2 domain extension does not appear to be a prerequisite for OLP specification. Our results also indicate that neuroepithelial cells expressing low Pax6 before E5 will subsequently express Nkx2.2. Whether Shh primarily controls the regression of Pax6 or the dorsal extension of Nkx2.2 remains to be investigated, since this morphogen has been shown to control, negatively and positively, the expression of both transcription factors (Barth and Wilson, 1995).

The Shh-dependent rapid change of transcription factor expression between E5 and E6 can only be explained by an abrupt change in Shh signalling. Further work is required to discriminate between increased Shh release and increased sensitivity of the Shh-target ventral neuroepithelial cells. This also leads to the possibility that Shh would not trigger itself glial fate choice, but would direct OLP specification out of a glial progenitor. In this view, other extrinsic or intrinsic mechanisms, for example the transcription factor Sox9 (Stolt et al., 2003), would be required to enable a switch between neurogenesis and oligodendrogenesis.

In any case, our experiments demonstrate that active Shh signalling is absolutely required for the maintenance of Olig2 expression. Thus, Shh signalling appears necessary and sufficient to induce the coexpression of *olig2* and *nkx2.2* genes in the ventral neuroepithelium concomitantly with oligodendrogenesis. Initially, *olig2* and *nkx2.2* are expressed in mutually exclusive domains and *nkx2.2* has been shown to repress *olig2* expression (Novitch et al., 2001). Why *olig2* is no longer repressed by *nkx2.2* during the gliogenic phase remains to be determined.

Coexpression of both *olig2* and *nkx2.2* genes is required to induce ectopic oligodendrogenesis in the chick spinal cord (Zhou et al., 2001). Additionally, Shh has been shown to induce OLs in dorsal spinal cord explants (Orentas et al., 1999; Soula et al., 2001). In our experiments, a large ectopic *nkx2.2*, *olig2* coexpression domain was observed in dorsal neuroepithelium after Shh treatment, reinforcing the conclusion that the OL-inducing activity of Shh is mediated by its ability to allow *olig2-nkx2.2* coexpression in neural progenitors. Interestingly, neither *nkx2.2* and *olig2*, nor OLs (P. Kan and P. Cochard, unpublished) can be induced in the intermediate region of the neuroepithelium. This corresponds to the high *pax6*-expressing domain that was already shown not to be affected by increased Shh signalling at early stages of development (Patten and Placzek, 2002). In fact, reduction in Pax6 expression requires both increased Shh and coexpression of *shh* with *chordin*, a BMP inhibitor (Patten and Placzek, 2002). This indicates that the transcription factor repertoire expressed by ventral neuroepithelial cells is controlled not only by Shh but also by other factors such as BMPs. It is noteworthy that a similar dual control has already been proposed to regulate OL emergence (Mekki-Dauriac et al., 2002). Thus, the finely regulated repatterning observed in the ventral neuroepithelium between E5 and E6 may involve several extracellular signalling molecules.

O4 immunostaining studies indicate that OLs arise from the entire *nkx2.2*-expressing domain (Soula et al., 2001), part of which only also expresses *olig2*. This suggests that at least two populations of OLP are produced in the ventral neuroepithelium. Studies in the mouse and the chick have provided additional data supporting this conclusion (Fu et al., 2002; Lee et al., 2003; Perez Villegas et al., 1999). In the chick, both OLP populations are induced by Shh, since OLPs identified by O4 expression are totally absent when Shh signalling is blocked (Orentas et al., 1999; Soula et al., 2001). The molecular mechanisms responsible for the emergence of the $Olig2^{-}$ population are still unknown. It is possible that a difference in the concentration or the availability of Shh is involved since dose-dependent effects of Shh are clearly important during neurogenesis (Briscoe and Ericson, 1999). Alternatively, different relay mechanisms downstream of Shh could also be involved. For example, Olig3 expression (Takebayashi et al., 2002) could be initially involved in the specification of a $Nkx2.2^{+}/Olig2^{-}$ OLP subpopulation.

Extracellular signals that regulate both astrocyte and OL lineages

Analysis of the expression of *Fgfr3* and *glast* (Pringle et al., 1998, 2003 and the present results) strongly suggests that APs and OLPs are spatially segregated in the neuroepithelium. Here we show by gain- and loss-of-function experiments that Shh not only induces OLP specification but also negatively controls the expression of early AP markers and the numbers of differentiated astrocytes, suggesting that Shh inhibits AP specification. On the other hand, studies in mice have allowed to characterize a glial-restricted progenitor with the ability to give rise to astrocytes and OLs (Rao and Mayer-Proschel, 1997). Our results suggest the interesting possibility that neuroepithelial precursors, at the stages studied here, indeed have the potential to generate both lineages, but do not actually do so because they are directed towards one or the other of these fates depending on local Shh signalling. In addition to Shh, other environmental clues may also control this choice. For example, BMPs, which in vitro can promote astroglial differentiation and concomitantly restrict the oligodendroglial fate (Mabie et al., 1997; Mehler et al., 2000), have been shown to inhibit OLP specification in vivo (Mekki-Dauriac et al., 2002) and thus may also participate in astrocyte specification in vivo.

Expression of *Fgfr3* and *glast* is excluded from the ventral neuroepithelium by Shh signalling. This raises the

question about how their expression is prevented in the dorsal neuroepithelium. A possible explanation is that cells in the dorsal neuroepithelium are more immature (Megason and McMahon, 2002). Alternatively, extracellular factors expressed dorsally could repress the expression of *glast* and *Fgfr3* in the dorsal neuroepithelium. *Wnt* genes, which are expressed dorsally and have been shown to promote proliferation and reduce differentiation (Megason and McMahon, 2002), are possible candidates for the mediation of this dorsal inhibition, although their role in astrocyte specification has not yet been documented.

Molecular mechanisms that control astrocyte marker expression

In the chick spinal cord, *olig2* and *Fgfr3/glast* are expressed in nonoverlapping neuroepithelial domains (Pringle et al., 2003 and the present results). In the *olig1/2* double mutant mice, in which OL differentiation is abolished, the cells normally fated to become OLs (i.e., expressing GFP under the control of the *olig2* promoter) differentiate along the astrocyte lineage as shown by GFAP expression (Zhou and Anderson, 2002). In addition, enforced expression of Olig1 or Olig2 in mouse telencephalic neural cells and neurospheres in vitro inhibits astrocytic differentiation (Fukuda et al., 2004; Gabay et al., 2003), indicating that Olig2 plays a direct role in preventing astrocyte development. In the present work, cyclopamine treatment, which abolished *olig2* expression, resulted in a ventral extension of astroglial marker expression. This shows that Shh signalling inhibits the ventral extension of APs by maintaining *olig2* expression. However, two different observations suggest that other molecular factors may be involved. First, after cyclopamine treatment, expression of *Fgfr3* and *glast* was still excluded from the ventral-most part of the neuroepithelium that expresses *nkx2.2*, but not *olig2*, suggesting that the former may also prevent AP development. In support of this possibility, BMP4 overexpression, which also abolishes *olig2* expression, does not affect Nkx2.2 expression and does not appear to modify the ventral border of the Glast expression domain (Mekki-Dauriac et al., 2002). Second, Shh treatment of the spinal cord totally abolished expression of *Fgfr3* and *glast* from the intermediate region of the neuroepithelium without concomitant induction of *olig2* and *nkx2.2*.

Transcription factors involved in astrocyte specification have not yet been characterized. However, the presence of E-boxes in the *GFAP* promoter (Lee et al., 2003) suggests that at least some bHLH factors are involved in either the specification or the differentiation of astrocytes. In the light of what is now known of the role of the *olig2* gene in the emergence of motoneurons and the OL lineage (Zhou and Anderson, 2002), such bHLH encoding genes may be involved in the successive specification of interneurons and astrocytes.

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