Cooperation between sonic hedgehog and fibroblast growth factor/MAPK signalling pathways in neocortical precursors

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Summary

Sonic hedgehog (SHH) and fibroblast growth factor 2 (FGF2) can both induce neocortical precursors to express the transcription factor OLIG2 and generate oligodendrocyte progenitors (OLPs) in culture. The activity of FGF2 is unaffected by cyclopamine, which blocks Hedgehog signalling, demonstrating that the FGF pathway to OLP production is Hedgehog independent. Unexpectedly, SHH-mediated OLP induction is blocked by PD173074, a selective inhibitor of FGF receptor (FGFR) tyrosine kinase. SHH activity also depends on mitogen-activated protein kinase (MAPK) but SHH does not itself activate MAPK. Instead, constitutive activity of FGFR maintains a basal level of phosphorylated MAPK that is absolutely required for the OLIG2- and OLP-inducing activities of SHH. Stimulating the MAPK pathway with a retrovirus encoding constitutively active RAS shows that the requirement for MAPK is cell-autonomous, i.e. MAPK is needed together with SHH signalling in the cells that become OLPs.

Key words: FGF, SHH, MAPK, Embryonic neural stem cells, Cell fate specification, Neural development, Oligodendrocyte progenitors, Mouse

Introduction

Sonic hedgehog (SHH) is required during development of the spinal cord for specification of ventral neurons (Briscoe et al., 2001; Wijgerde et al., 2002) and oligodendrocyte progenitors (OLPs) (Orentas et al., 1999). SHH secreted from the floor plate induces transcription of the basic helix-loop-helix transcription factors OLIG1 and OLIG2, which are initially expressed throughout the ventral half of the embryonic cord but later become restricted to a subdomain of the ventral ventricular zone (VZ) known as pMN (Lu et al., 2000; Takebayashi et al., 2000; Zhou et al., 2000) (reviewed by Kessaris et al., 2001; Rowitch et al., 2002). Neuroepithelial precursors in pMN generate motor neurons followed by OLPs, which migrate throughout the spinal cord before differentiating into myelin-forming oligodendrocytes (Richardson et al., 2000). Specification of both motor neurons and OLPs requires OLIG function, because *Olig2* null mice lack both motor neurons and OLPs in the spinal cord (Lu et al., 2002; Rowitch et al., 2002; Takebayashi et al., 2002; Zhou and Anderson, 2002). Neuroepithelial precursors in the dorsal spinal cord are believed not to generate OLPs in vivo (Pringle et al., 1998; Pringle et al., 2002) but they can be artificially induced to do so by culturing dorsal explants or dissociated cells with SHH in vitro (Poncet et al., 1996; Pringle et al., 1996; Orentas et al., 1999).

SHH is also expressed in the ventral forebrain where it induces formation of neurons (Ericson et al., 1995) and OLPs (Nery et al., 2001; Tekki-Kessaris et al., 2001). The OLPs then appear to migrate into all parts of the developing forebrain including the cerebral cortex (Tekki-Kessaris et al., 2001). There might also be local production of OLPs within the mammalian cortex (Gorski et al., 2002) (but not avian cortex: see Discussion). If so, cortical OLP production must begin after E17 in the mouse because significant numbers of OLPs are not found in the cortex before then, whereas OLPs are generated in large numbers in the ventral forebrain as early as E13. If cortical precursor cells are removed and cultured at E13, they do not generate OLPs for at least four days in vitro (DIV4) (Tekki-Kessaris et al., 2001). However, E13 neocortical precursors can be induced to generate OLPs within a couple of days of SHH treatment in vitro (Tekki-Kessaris et al., 2001; Alberta et al., 2001; Murray et al., 2002). *Olig* genes are also expressed and required for oligodendrogenesis in the ventral forebrain, because *Olig1/2* double-knockout mice lack OLPs in the forebrain, and indeed anywhere else in the central nervous system (CNS) (Zhou and Anderson, 2002).

In addition to SHH, fibroblast growth factor 2 (FGF2) also stimulates the generation of oligodendrocytes from cultured cortical precursors (Qian et al., 1997; Hall, 1999). Since SHH and FGF2 share this property, the question arises whether SHH and FGF2 use some of the same intracellular signalling pathways or operate along entirely independent lines. This is the main question addressed by the work described here. FGF2 is routinely added to neural stem cell (neurosphere) cultures derived from embryonic or adult forebrain, so understanding the interactions between FGF and other factors will help us to understand the behaviour of stem cells in these cultures.

FGF proteins often act as mitogenic growth factors but can also signal cell survival and differentiation (reviewed by Yamaguchi and Rossant, 1995; Ornitz and Itoh, 2001). SHH was originally identified as a morphogen and cell fate determinant (Roelink et al., 1995) but more recently has been shown to influence axonal outgrowth (Charron et al., 2003), cell survival and proliferation (Teillet et al., 1998; Ahlgren and Bronner-Fraser, 1999; Marcelle et al., 1999; Rowitch et al., 1999; Yu et al., 2002; Thibert et al., 2003). Moreover, over-activity of the Hedgehog signalling pathway is associated with tumour growth (Oro et al., 1997; Dahmane et al., 2001). SHH is now known to be a mitogen for neural precursors from several regions of the CNS including cerebellum (Dahmane and Ruiz i Altaba, 1999; Wechsler-Reya and Scott, 1999; Kenney and Rowitch, 2000) and retina (Jensen and Wallace, 1997).

The biochemical basis for these activities of SHH is poorly understood. SHH binds to the transmembrane receptor Patched (PTC), causing disinhibition of its co-receptor Smoothened (SMO), a seven-pass transmembrane G-protein coupled receptor (GPCR). In *Drosophila*, this eventually promotes nuclear translocation of a proteolytic fragment of the transcription factor Cubitus interruptus (Ci). The mammalian equivalents of Ci are the GLI proteins, which are transcriptionally activated as a result of SHH signalling. The downstream targets of Ci/GLI proteins include G_1- and S-phase cyclins (Kenney and Rowitch, 2000; Duman-Scheel et al., 2002) and N-MYC (Kenney et al., 2003; Oliver et al., 2003), linking directly to the cell cycle machinery. FGF (of which there are >20 known mammalian family members) triggers an entirely different set of early signalling events. FGF binds to one of four closely related receptors (FGFR1-4) at the extracellular surface, causing receptor clustering and autophosphorylation of their intracellular tyrosine kinase (TK) domains. The phosphorylated receptor then acts as a nucleation centre for signalling molecules, which bind either directly or indirectly to individual phosphotyrosine residues. The main pathways that are activated by FGFR include the mitogen-activated protein kinase (MAPK) pathway, phosphoinositol 3-kinase (PI 3-kinase) pathway, phospholipase C_γ and elevation of intracellular calcium (reviewed by Klint and Claesson-Welsh, 1999). Despite their apparently divergent signalling mechanisms there is evidence of intracellular cross-talk between Hedgehog and FGF signalling pathways. For example, the GLI proteins seem able to integrate SHH and FGF signalling in some circumstances (Brewster et al., 2000). Moreover, it is established that receptor TKs can be trans-activated inside cells by GPCRs (Schwartz and Baron, 1999; Ferguson, 2003; Wetzker and Bohmer, 2003), raising the possibility that SMO might be able to trans-activate FGFR.

Here, we investigate the signalling pathways used by SHH in cell fate specification and lineage progression of embryonic neocortical precursors. Our starting point was the apparent connection between SHH and FGF2 biology. We searched for – but found no evidence of – trans-activation of FGFR by SHH in cortical cells. However, we did find that the shared OLP-inducing activities of SHH and FGF2 involve overlapping intracellular signals. Notably, SHH has a requirement for a low basal level of MAPK phosphorylation that results from constitutive FGFR signalling – since both basal MAPK activity and SHH activity could be blocked by a selective inhibitor of

FGFR-TK. Infection of cultures with a retrovirus encoding constitutively active RAS protein demonstrated that SHH signalling and MAPK activity were required in the same cells for OLP induction. Our results raise the possibility that cooperation with receptor or non-receptor TK pathways might be a more general requirement for cell fate specification by SHH.

Materials and methods

Neocortical cultures

Cerebral hemispheres from E13.5 mouse embryos were dissected in Hepes-buffered minimal essential medium (MEM-H) (Invitrogen). Meningeal membranes were removed mechanically following gentle treatment with 1 mg/ml dispase (Roche) and the neocortical neuroepithelial cells were dissociated by incubation in 0.0125% (w/v) trypsin in Earle's balanced salt solution (EBSS, Invitrogen) for 30 minutes at 37°C in 5% CO_2. The cells were mechanically dissociated in the presence of DNaseI and seeded onto 13 mm poly-D-lysine-coated coverslips in a 50 μl droplet of Dulbecco's modified Eagle's medium (DMEM, Invitrogen) containing 4% (v/v) FCS at a density of 3×10^5 cells/coverslip. The cells were allowed to attach for 30 minutes, then 350 μl of defined medium (Bottenstein and Sato, 1979) was added (diluting the FCS to 0.5%) and incubation continued at 37°C in 5% CO_2. Human recombinant FGF2, FGF8, FGF9 and FGF10 were purchased from ImmunoKontact. The small molecule agonist of Hedgehog signalling (Cur-0188168 or SHH-Ag1.2) (Frank-Kamenetsky et al., 2002) was provided by Curis Inc. U0126, a specific inhibitor of MEK1/2, and LY294002, a PI 3-kinase inhibitor, were purchased from Calbiochem. Tyrphostin A9 was purchased from Sigma-Aldrich. Cyclopamine and the FGFR inhibitor PD173074 were kindly provided by William Gaffield and Stephen Skaper, respectively. The extracellular domains of human FGFR1α(IIIc) and FGFR1β(IIIc) fused to human IgG1 Fc were purchased from R&D Systems. Unless otherwise stated, the working concentrations of reagents were as follows: FGF2, 10 ng/ml (~0.6 nM); SHHAg1.2, 100 nM; cyclopamine, 1 μM; PD173074, 100 nM; U0126, 20 μM; LY294002, 10 μM; FGFR1α(IIIc)/FGFR1β(IIIc) extracellular domains, 600 ng/ml; tyrphostin A9, 0.5 μM.

Target specificity of PD173074

PD173074 has been described as a selective inhibitor of FGFR1 (Skaper et al., 2000) but it probably blocks all four high-affinity FGF receptors (FGFR1-4). It might conceivably inhibit other closely related RTKs or non-RTKs as well. PD173074 inhibits FGFR1 at ~25 nM but does not inhibit PDGRβ, EGFR or SRC significantly at this concentration (Dimitroff et al., 1999). Skaper et al. (Skaper et al., 2000) showed that PD173074 effectively blocked the neurotrophic action of FGF2 on cerebellar granule neurons and dorsal root ganglion neurons at nM concentrations, but had no effect on the activities of insulin-like growth factor 1, nerve growth factor, brain-derived neurotrophic factor or ciliary neurotrophic factor.

The concentration of PD173074 required for half-maximal inhibition of SHH-induced NG2 expression was ~25 nM, similar to that reported for inhibition of FGFR1 itself. This strongly suggests that the target of PD173074 in our experiments was indeed FGFR, not another related kinase. As an additional test of specificity we wanted to confirm that PD173074 does not inhibit the platelet-derived growth factor alpha-receptor (PDGFRα), since NG2-positive OLPs express PDGFRα and proliferate in response to PDGFAA (Hall et al., 1996; Fruttiger et al., 1999). We cultured E13 ventral forebrain cells in defined medium plus recombinant PDGFAA (10 ng/ml) and either PD173074 or tyrophostin A9 (a selective inhibitor of PDGFR-TK) (Levitzki and Gilon, 1991) for 48 hours before immunolabelling with anti-NG2 and counting OLPs. In the presence of PDGFAA alone, there was a large increase in the number of NG2-positive OLPs (not

shown). Tyrphostin A9 effectively neutralised PDGF-driven OLP proliferation (not shown) but PD173074 had no significant effect. Thus, PD173074 selectively inhibits FGFR over the closely related PDGFRα.

Chick neural tube cultures

Chick neural tubes from Hamburger-Hamilton stage 11 (E2) embryos (Hamburger and Hamilton, 1951) were isolated in MEM-Hepes following a gentle treatment with 1 mg/ml dispase to remove surrounding tissues. Using a flame-sharpened tungsten needle the neural tube was divided into dorsal and ventral halves. Approximately $100\,\mu m^3$ explants were cultured in three-dimensional collagen gels as previously described (Guthrie and Lumsden, 1994) in defined medium (Bottenstein and Sato, 1979) lacking transferrin but containing concanavalin A, 0.25% (v/v) foetal calf serum (FCS) and antibiotics.

Retroviral vectors

To identify transfected cells we used the pBird retroviral vector, which encodes enhanced green fluorescent protein (eGFP) driven by the cytomegalovirus (CMV) promoter. The pBird-Ras^{V12} vector co-expresses eGFP and a constitutively active form of RAS (Tang et al., 2001). Recombinant retroviruses were produced and concentrated as described previously (Kondo and Raff, 2000). Neocortical cultures were infected for 3 hours with concentrated retroviral supernatant, starting 1 day after plating the cells. Infected cells were identified by eGFP fluorescence.

Immunocytochemistry

Cells on coverslips or explants were lightly fixed in 4% (w/v) paraformaldehyde (PFA) in phosphate-buffered saline (PBS) for 5 minutes at room temperature and washed in PBS. The following primary antibodies were used: anti-NG2 rabbit serum (1:350 dilution, Chemicon) or monoclonal anti-NG2 (clone N11.4) (Levine and Stallcup, 1987; Stallcup and Beasley, 1987), monoclonal antibody O4 (Sommer and Schachner, 1981), anti-OLIG2 rabbit IgG (DF308, 1:4000 dilution, a gift from David Rowitch) and monoclonal anti-MAPK (diphosphorylated ERK1/ERK2; Sigma-Aldrich, 1:200 dilution). For OLIG2 staining, the cells were made permeable with 0.1% (v/v) Triton X-100 in PBS. Primary antibody treatments were for 1 hour or overnight in a humid chamber at 4°C. Fluorescent secondary antibodies (Perbio Science, UK) were applied for 60 minutes at room temperature. Following staining of the nuclei with Hoechst (Sigma) the cells were post-fixed for 5 minutes in 4% (w/v) PFA in PBS and mounted on slides in Citifluor (City University, UK).

Results

Oligodendrocyte progenitor cell specification by FGF2 acting through FGFR1

We first confirmed that FGF2 can cause neocortical precursors to differentiate along the oligodendrocyte pathway by establishing dense cultures of dissociated cells from E13.5 mouse cerebral cortex and incubating them in defined medium plus 0.5% foetal calf serum (FCS), with or without FGF2. We scored OLPs by immunolabelling with anti-NG2 proteoglycan. At three days in vitro (DIV3) there was a striking dose-dependent induction of NG2-positive OLPs by FGF2 (Fig. 1A-D), followed by O4-positive OLPs about 24 hours later (data not shown). OLP induction by FGF8, 9 or 10 was 100-1000 times lower (Fig. 1E). The different binding specificities of these FGF isoforms for the three FGF receptors found in the CNS (FGFR1-3) suggests that the effect is mediated predominantly through FGFR1 (MacArthur et al., 1995; Ornitz et al., 1996; Belluardo et al., 1997; Igarashi et al., 1998; Beer et al., 2000; Ohuchi et al., 2000; Ford-Perriss et al.,
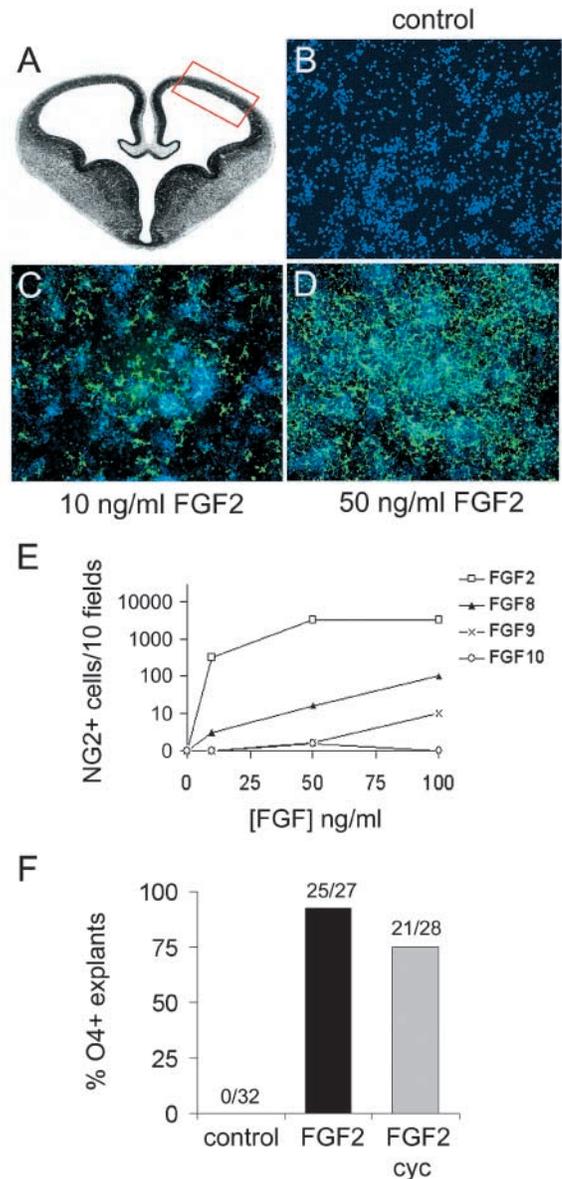

Fig. 1. FGF2 induces OLPs through FGFR1. (A) Dissociated cells from mouse E13.5 neocortex (boxed region) were cultured in defined medium in the presence or absence of different FGFs for 3 days in vitro (DIV). (B-D) The cultures were fixed and immunolabelled with polyclonal anti-NG2. Control cultures lacked NG2 immunoreactivity (B). Numerous NG2-positive cells developed in 10 ng/ml FGF2 (C) and 50 ng/ml FGF2 (D). (E) The numbers of OLPs induced by FGF8, 9 or 10 were at least 100× lower than with FGF2, suggesting that the inducing activity is mediated via FGFR1. NG2-positive cells were counted in more than 10 randomly selected fields on each of two coverslips (×63 microscope objective). At least two independent experiments gave similar results, one of which is illustrated. (F) Dorsal spinal cord explants from chick Hamilton-Hamburger stage12 (E2) developed O4-positive OLPs when cultured in the presence of FGF2, even in the presence of cyclopamine (FGF2 + cyc). The number of O4-positive explants and the total number of explants are shown above each bar.

2001). The OLP-inducing effect of FGF2 was also observed with E15.5 rat neocortical cultures (Hall, 1999) (and data not shown) and in explant cultures of chick dorsal spinal cord
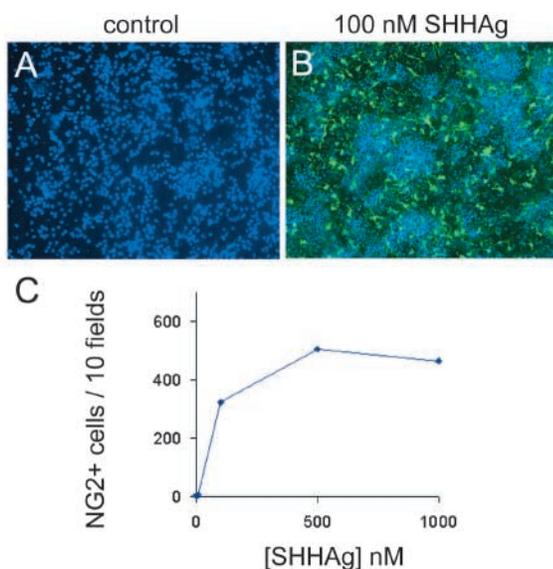
Fig. 2. Induction of OLPs by the Hedgehog agonist
SHHAg1.2.Mouse E13.5 neocortical neuroepithelial cells were
cultured in the presence or absence of SHHAg for 4 DIV. The
cultures were fixed and immunolabelled with polyclonal anti-NG2.
(A) Control cultures lacked NG2 immunoreactivity. (B) Numerous
NG2-positive cells developed in the presence of 100 nM SHHAg1.2.
(C) The dose-response curve shows induction of NG2-positive cells
at a half-maximal concentration of SHHAg1.2 of ~25 nM.

(Fig. 1F). FGF2 also induced ISL1/2-positive neurons,
presumably motor neurons, in chick dorsal spinal cord explants
(not shown). (Note that this activity of FGF2 was not inhibited
significantly by the Hedgehog inhibitor cyclopamine (Fig. 1F).
This is discussed in more detail below, in the section entitled
'FGF2 dependent induction of OLPs...'.)

   In parallel experiments we showed that the SHH agonist
Cur-0188168 (ShhAg1.2, hereafter referred to simply as
SHHAg) (Frank-Kamenetsky et al., 2002) can induce
neocortical precursors to generate OLPs in a dose-dependent
manner (Fig. 2). This confirms previous studies with full-length
recombinant SHH (Tekki-Kessaris et al., 2001; Alberta et al.,
2001; Murray et al., 2002).

   As we showed previously (Tekki-Kessaris et al., 2001),
cortical cultures maintained in defined medium eventually
generate NG2-positive OLPs if left long enough (DIV5-6)
even without added growth factors. However, most of this
endogenous activity can be neutralised by cyclopamine,
demonstrating that it derives mainly from Hedgehog proteins
made by the cultured cells (Tekki-Kessaris et al., 2001). In the
experiments reported here we used concentrations of FGF2
(10 ng/ml, ~0.6 nM) or SHHAg (100 nM) that induced NG2-
positive OLPs by DIV3-4, well ahead of endogenous
Hedgehog activity.

Rapid induction of OLIG2 by FGF2 or SHH

Activation of NG2 expression is a relatively late event in
oligodendrocyte lineage progression, an earlier lineage marker
being OLIG2. We looked at induction of OLIG2 expression in
response to FGF2 or SHHAg. OLIG2-positive cells first
appeared within 20 hours of either FGF2 or SHHAg treatment,
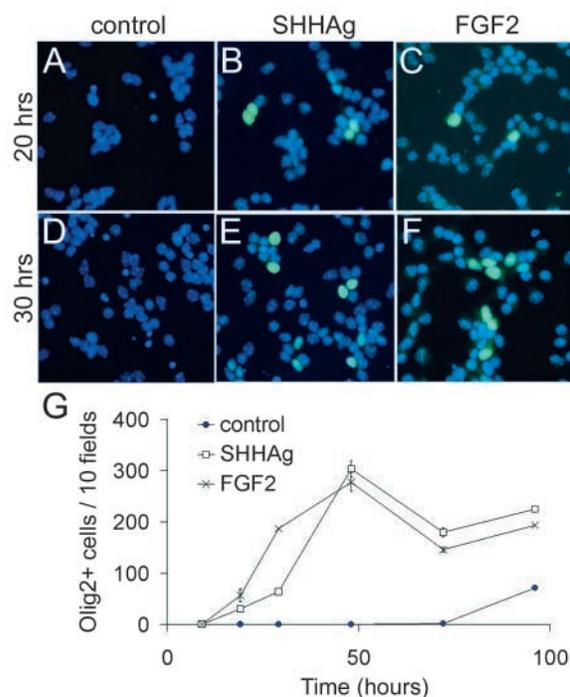peaking around 48 hours (Fig. 3). Control cultures without

Fig. 3. Rapid induction of OLIG2 by SHHAg or FGF2. Neocortical
precursors from E13.5 mice were cultured in the presence or absence
of FGF2 or SHHAg and assayed for OLIG2 immunoreactivity at
different times. OLIG2-positive nuclei appeared within the first
20 hours in both FGF2-treated (C,F) and SHHAg-treated (B,E)
cultures. The experiment was quantified (G) by counting OLIG2-
positive cells in more than 10 randomly selected fields on each of
two separate coverslips (×63 microscope objective) and are displayed
as mean±s.d.

added FGF2 or SHH did not develop any OLIG2-positive cells
for at least 70 hours (Fig. 3G).

FGF2-mediated induction of OLPs is SHH independent: SHH requires FGFR

The fact that either SHH or FGF2 can induce OLPs raises the
question: do these different factors act sequentially in the same
induction pathway or in separate, parallel pathways? As an
example of sequential action, FGF2 might stimulate cells in the
cortical cultures to synthesise SHH or a related Hedgehog
protein, which could secondarily induce OLPs. If so, one would
expect to be able to block FGF2 activity with cyclopamine, an
inhibitor of Hedgehog signalling (Cooper et al., 1998; Incardona
et al., 1998). Alternatively, SHH might stimulate synthesis or
release of FGF. In that case one would expect to block SHH-
mediated induction by PD173074, which inhibits signalling
through FGFR (Dimitroff et al., 1999; Skaper et al., 2000). If,
on the other hand, SHH and FGF2 trigger independent, parallel
pathways, one would not expect to block the SHH effect with
PD17074, or the FGF2 effect with cyclopamine.

   To test these predictions we cultured E13.5 neocortical
precursors at high density in the presence of FGF2 or SHHAg,
with or without cyclopamine or PD173074, and looked for
induction of OLIG2 at DIV2. We found that the OLIG2-
inducing activity of FGF2 (10 ng/ml) was strongly inhibited by
PD173074, as expected, but was unaffected by cyclopamine
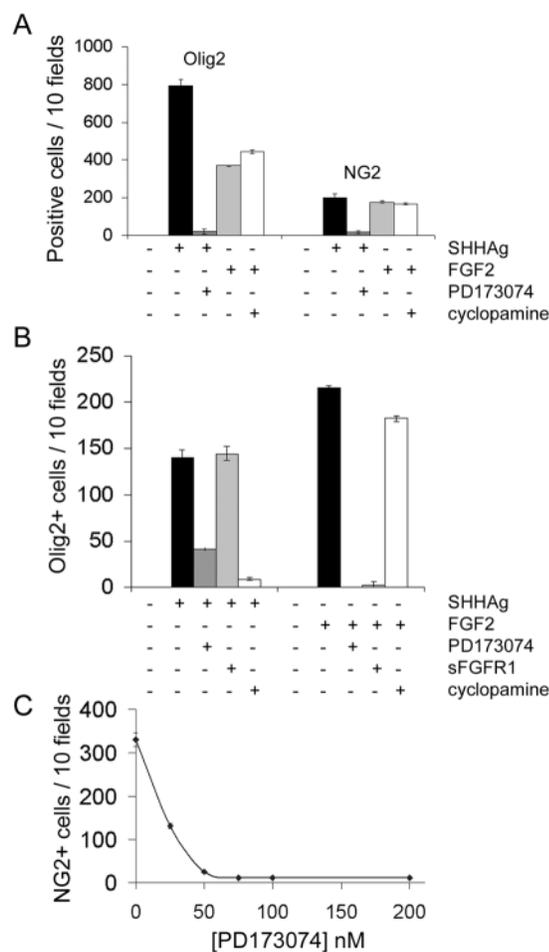(Fig. 4A,B). Moreover, cyclopamine did not inhibit the OLP-

A

Positive cells / 10 fields

Olig2

NG2

-	+	+	-	-	-	+	+	-	-	SHHAg
-	-	-	+	+	-	-	-	+	+	FGF2
-	-	+	-	-	-	-	+	-	-	PD173074
-	-	-	-	+	-	-	-	-	+	cyclopamine

B

Olig2+ cells / 10 fields

-	+	+	+	-	-	-	-	SHHAg
-	-	-	-	-	+	+	+	FGF2
-	-	+	-	-	-	+	-	PD173074
-	-	-	+	-	-	+	-	sFGFR1
-	-	-	-	+	-	-	+	cyclopamine

C

NG2+ cells / 10 fields

[PD173074] nM

Fig. 4. OLP induction by SHH requires FGFR. (A) E13.5 mouse neocortical cells were cultured for 2 DIV in the presence or absence of FGF2 or SHHAg, with or without the FGFR inhibitor PD173074 or the Hedgehog inhibitor cyclopamine. The cultures were assayed for OLIG2 immunoreactivity at DIV2 or NG2 immunoreactivity at DIV4. Induction of both OLIG2-positive and NG2-positive cells by SHH was inhibited by PD173074. The inducing activity of FGF2 was unaffected by cyclopamine. (B) Cortical cells from mouse E13.5 embryos were cultured in the presence or absence of SHHAg or FGF2, PD173074, a combination of FGFR1αIIIc and FGFR1βIIIc extracellular domains (sFGFR1) or cyclopamine. The inducing effect of FGF2 was inhibited by both PD173074 and sFGFR1 but not by cyclopamine. The effect of SHH was inhibited by PD173074 but not by sFGFR1, suggesting that SHHAg activity requires ligand-independent activation of FGFR. (C) Dose-response curve showing inhibition of OLP induction by SHHAg in the presence of increasing concentrations of PD173074 at DIV4. Half-maximal inhibition occurs at ~25 nM PD173074, as described for inhibition of FGFR1 itself (Dimitroff et al., 1999).

inducing activity of FGF2 in dorsal spinal cord cultures (Fig. 1F). In contrast, the OLIG2-inducing activity of SHHAg in cortical cultures was strongly inhibited by both cyclopamine (Fig. 4B) and PD173074 (Fig. 4A,B). We also looked at induction of NG2-positive OLPs at DIV4, with analogous results, i.e. NG2-induction by FGF was blocked by PD173074 (not shown) but not by cyclopamine (Fig. 4A), whereas induction by SHHAg was sensitive to both reagents (Fig. 4A

and not shown). The concentration of PD173074 required for half-maximal inhibition of SHHAg was ~25 nM, similar to that reported for FGFR1 itself (Dimitroff et al., 1999) (Fig. 4C). We also determined that PD173074 does not inhibit the closely related PDGFR (see Materials and methods). Our data suggest that SHH ultimately relies on activation of FGFR, either directly or indirectly, for its OLIG2- and OLP-inducing abilities.

We tried to determine whether FGFR activation in SHH-treated cells requires extracellular FGF, by sequestering FGF outside cells with recombinant, extracellular fragments of FGFR1. We used a mixture of FGFR1αIIIc and FGFR1βIIIc alternative splice isoforms, which can bind FGF2 and other FGFR1-binding isoforms at high affinity. These reagents effectively prevented induction of OLIG2 by added FGF2 but had no effect on OLIG2 induction by SHHAg (Fig. 4B). Taken together, our data suggest that SHH activity requires ligand-independent activation of FGFR. Perhaps the G-protein-coupled SHH receptor SMO trans-activates FGFR inside cells. Alternatively, SHH might not itself trans-activate FGFR, but might rely on a basal level of FGFR activity that is constitutive in our cultures.

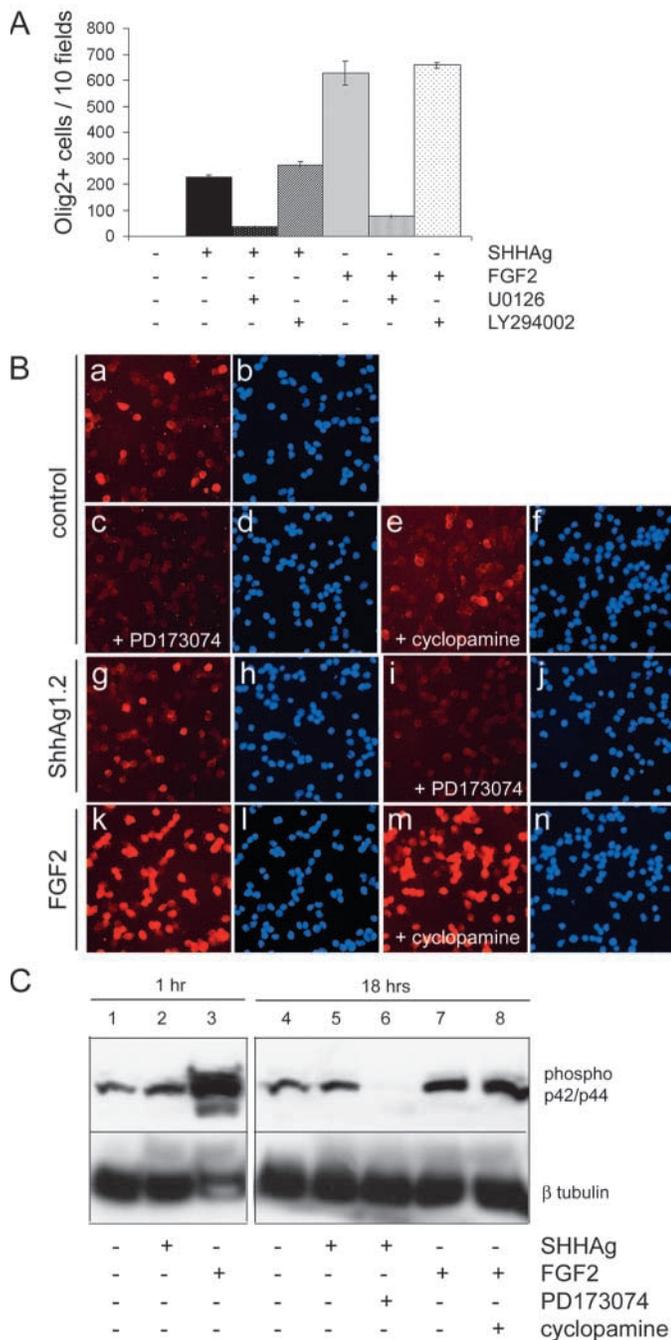### Induction of OLIG2 expression by SHHAg or FGF2 requires MAPK activity

If SHH and FGF both act through FGFR as implied above, one would expect them to trigger the same intracellular signalling pathways. FGFR activation leads to autophosphorylation of the TK domains, which in turn can initiate MAPK and pathways and elevation of intracellular calcium. We investigated the involvement of MAPK and PI 3-kinase signalling pathways, using synthetic drugs that inhibit MEK1/2 (U0126) or PI 3-kinase (LY294002).

We found that induction of OLIG2 by either FGF2 or SHHAg was strongly inhibited by U0126, but not by LY294002, at either DIV1 (Fig. 5A) or DIV2 (not shown), indicating that the MAPK pathway but not the PI 3-kinase pathway is crucial for this first step of lineage specification. We visualised MAPK activation directly by immunofluorescence microscopy with an antibody that specifically recognises the phosphorylated form of the protein. Within 1 hour of FGF2 exposure there was a marked increase in MAPK immunolabelling over control (compare Fig. 5Ba with Bk). Surprisingly (given the data of Fig. 5A), we could detect no increase in MAPK immunolabelling after SHHAg treatment (compare Fig. 5Ba and 5Bg).

We confirmed these findings by looking directly at p42/p44 (MAPK) activation by western blotting with an antibody directed against the phosphorylated forms of p42/p44 (Fig. 5C). As expected, FGF2 caused a large increase in the level of MAPK phosphorylation within 1 hour (compare lanes 1 and 3). SHH did not cause significant MAPK activation at one hour (compare lanes 1 and 2). After 18 hours incubation with FGF2 there was a small residual increase in phosphorylated MAPK compared with control (compare lanes 4 and 7). However SHH still had no effect on MAPK (lanes 4, 5).

FGFR maintains a constitutive low level of active MAPK that is required for SHH activity

The inability of SHHAg to activate MAPK argues against trans-activation of FGFR, since direct stimulation by FGF2 causes robust MAPK activation. What, then, is the essential

Fig. 5. OLP induction by SHH depends on activation of the MAPK pathway by FGFR1. (A) Neocortical neuroepithelial cells cultured for 24 hours in the presence of the MEK1/2 inhibitor U0126 and either SHHAg or FGF2 fail to develop Olig2-positive cells. The inhibitor of PI 3-kinase, LY294002, has no effect on the inducing activities of either SHHAg or FGF2. (B) To assess whether FGF2 and/or SHH activate the MAPK pathway we cultured E13.5 cortical cells in the absence (a-f) or presence of either SHHAg (g-j) or FGF2 (k-n), together with PD173074 (c,i) or cyclopamine (e,m) for 1 hour prior to immunolabelling with an anti-phospho-ERK1/2 antibody and Hoechst dye (b,d,f,h,j,l,n). FGF2 by itself caused strong activation of MAPK. SHH failed to activate MAPK above endogenous levels (compare a, g) and all MAPK activity was abolished by PD173074 (c,i). (C) Protein lysates from cortical cultures incubated with FGF2 or SHHAg and PD173074 or cyclopamine for 1 hour or 18 hours were separated by PAGE, and analysed for the presence of phosphorylated ERK1/2 (p42/p44) by western blot. SHHAg failed to activate MAPK above control levels, and PD173074 abolished all MAPK activity even in the presence of SHH.

phosphorylation is required in neocortical cultures for the OLP-inducing activity of SHH but did not distinguish between a direct or indirect effect of MAPK. For example, MAPK might stimulate release of a diffusible factor that acts secondarily on neighbouring cells to render them responsive to SHH. Alternatively, MAPK might be required within the same cells that respond to SHH.

We addressed this question by infecting neocortical precursors with a retrovirus vector encoding a mutated form of RAS that constitutively activates the MAPK pathway. The retrovirus also encodes the enhanced green fluorescent protein (eGFP) so that infected cells can be positively identified using the fluorescence microscope. Unsurprisingly, we found that constitutively active RAS was not by itself sufficient to activate OLIG2 expression in the absence of SHH signalling (added cyclopamine; Fig. 6Ab-d). However, in the presence of SHHAg and PD173074 (to block MAPK activation via FGFR) the only cells that expressed OLIG2 were those that also expressed activated RAS (Fig. 6Af-g,B). Note that not all cells that expressed active RAS also expressed OLIG2 (Fig. 6Af-h). These observations allow us to conclude, (1) the MAPK pathway is necessary but not sufficient for OLIG2 induction as SHH signalling is also required, and (2) MAPK activation is required cell-autonomously, i.e. it acts directly in the SHH-targeted cells.

Two stages of OPC induction (OLIG2, NG2) with different signalling requirements

We investigated the requirement for MAPK and PI 3-kinase in the later transition from OLIG2-positive, NG2-negative (OLIG2+, NG2−) to (OLIG2+, NG2+) OLPs. We first allowed (OLIG2+, NG2−) cells to develop until DIV2 under the influence of FGF2 or SHHAg, then added the MAPK and/or PI 3-kinase inhibitors for a further 2 days (until DIV4) before immunolabelling with anti-NG2. We found that NG2 expression was inhibited strongly by both drugs (Fig. 7), indicating that both the MAPK and PI 3-kinase pathways are important during this later stage of oligodendrocyte lineage progression. Thus, there are different signalling requirements for the initial specification event (MAPK only) compared to later lineage progression (MAPK and PI 3-kinase).
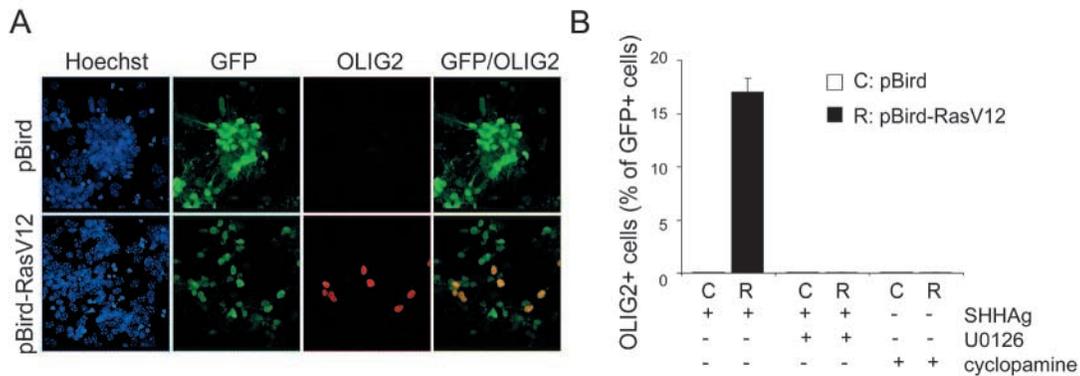
role of FGFR in the activity of SHH? In the absence of added SHH or FGF2 there is a background of active MAPK in our cultures (Fig. 5Ba and 5C lanes 1, 4), but this background is abolished by adding PD173074 (Fig. 5Bc). Even in the presence of SHH, the basal level of active MAPK is obliterated by PD173074 (Fig. 5Bi, and lane 6 in C). Therefore, it seems likely that the steady-state level of active MAPK in our cultures is caused by low, constitutive FGFR activity and that this basal activity is absolutely required for OLIG2 induction by SHH.

Cell-autonomous requirement for MAPK activation in SHH-responding cells

The experiments described above showed that MAPK

Fig. 6. Cell-autonomous activation of the MAPK pathway is required for OLIG2 induction by SHH. (A) Cultured neuroepithelial cells from E13.5 mouse embryos were infected with a control retrovirus (pBird) encoding GFP alone (a,b,c,d) or a retrovirus encoding GFP and a constitutively active form of RAS (pBird-Ras^{V12}) (e,f,g,h). Cells were then cultured for 48 hours in the presence of SHHAg. At DIV3, the cultures were assayed for OLIG2 immunoreactivity (c,g) and labelled with Hoechst dye (a,e). In control virus-infected cultures (a,b,c,d) there were no OLIG2-positive cells. However, in activated RAS virus-infected cultures, SHHAg induced OLIG2 expression in a subset of GFP-positive cells (e,f,g,h). (B) The number of OLIG2- and GFP-positive cells was counted and is presented as the percentage of all GFP-positive cells. In RAS virus-infected cultures in the presence of SHHAg, 17.0±1.3% of GFP-positive cells expressed OLIG2. No OLIG2-positive cells were induced by either in the absence of active MAPK (added U0126), or in the absence of SHHAg (added cyclopamine). In all cultures PD173074 was added to block MAPK activation via FGFR.
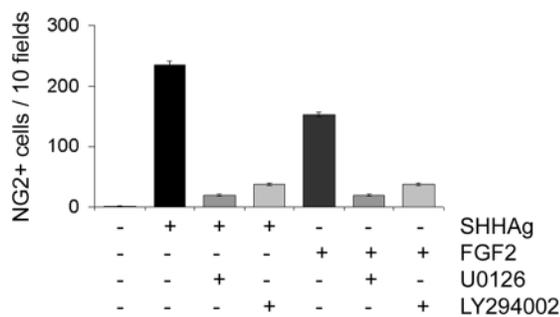

Fig. 7. The progression of cells from being OLIG2-positive to NG2-positive requires both MAPK and PI 3-kinase activation. Neocortical cells were initially cultured in the presence or absence of SHHAg or FGF2 for 48 hours until OLIG2-positive cells appeared. The inducers were then removed and the medium replaced with defined medium containing U0126 or LY294002. The appearance of NG2-positive cells in the cultures was inhibited by both drugs.

FGFR signalling is not required for SHH activity in the ventral spinal cord or forebrain

The data described above raised the possibility that induction of OLPs in the ventral neural tube in vivo, which is known to depend on SHH, might also require FGFR signalling. We cultured explants from E2 chick ventral spinal cords and cultured them in the presence of either cyclopamine or PD173074. In these experiments it is not necessary to add exogenous SHH because the cells generate OLPs even in defined medium, presumably because they have already been exposed to SHH for some time in vivo, prior to dissection, and because of continuing endogenous SHH production. Cyclopamine prevented the appearance of O4-positive OLPs, but PD173074 had no effect (Fig. 8A). We performed analogous experiments with dissociated cells from the ventral mouse forebrain and obtained similar results: induction of NG2-positive OLPs was blocked by cyclopamine but not by PD173074 (Fig. 8B-F). Therefore, it
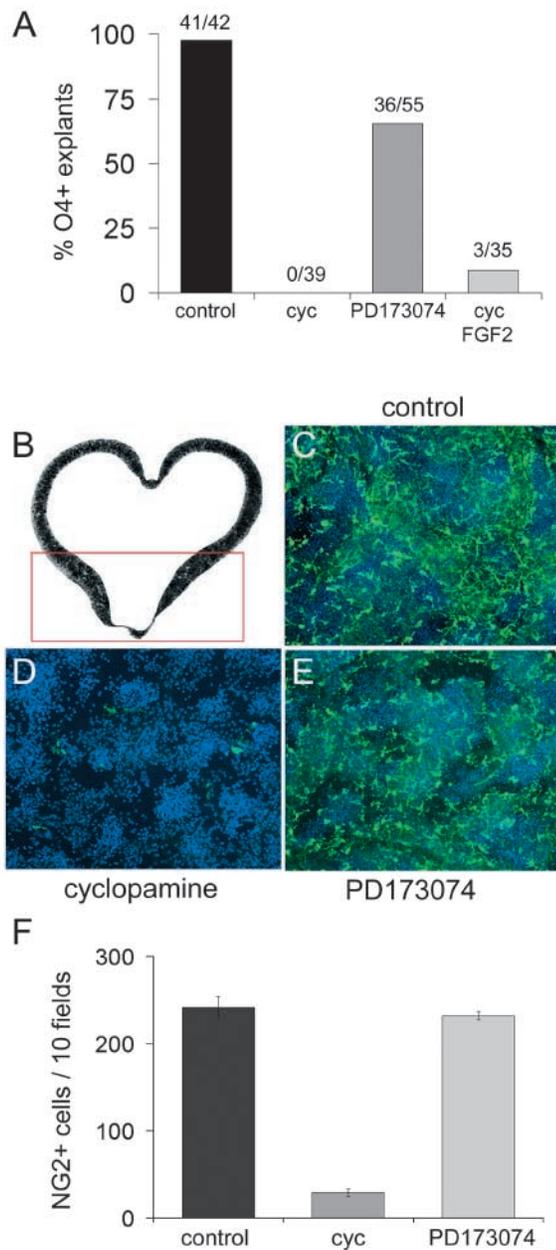
seems that the signalling requirements for SHH activity are different in the ventral spinal cord and forebrain, where OLPs are generated endogenously, compared to its mode of action in the dorsal spinal cord or neocortex.

Discussion

Cell fate specification by SHH in cortical precursors requires FGFR and MAPK

We have investigated the relationship between FGF2 and SHH signalling for specification of OLPs in cultures of embryonic neocortical precursor (stem) cells. We showed that FGF2 probably acts through FGFR1 in these cells. FGF-mediated OLP induction was not blocked by cyclopamine, a naturally occurring inhibitor of Hedgehog signalling, and so acts independently of SHH. This conclusion was also reached independently by Chandran et al. (Chandran et al., 2003). Unexpectedly, we found that OLP induction by SHH could be blocked equally well by cyclopamine and PD173074, a synthetic inhibitor of FGFR tyrosine kinase activity. This suggests that the OLP-inducing activity of SHH depends on parallel activation of FGFR. In keeping with this, we found that both SHH and FGF2 signalling require MAPK activation. These observations might be explained if SHH, through its G-protein coupled co-receptor SMO, could trans-activate FGFR. However, we found that SHH does not by itself activate MAPK, arguing against FGFR trans-activation. Instead, it seems likely that SHH needs a basal level of active MAPK in order to function, and that constitutive FGFR activity in our cortical cultures provides the necessary stimulus. We could not neutralise SHH activity with soluble, ligand-binding FGFR1 fragments, suggesting that constitutive FGFR activation is ligand independent. However, the complexity of FGF-FGFR interactions and the large number of FGF family members, not all of which are fully characterised, means that we cannot be entirely confident of this conclusion.

We previously reported that cortical precursor cell cultures have the ability to generate OLPs in the absence of added SHH

effect of FGF when added at the higher concentrations (10 ng/ml) used in our present study, because in our hands FGF-mediated OLP induction was not inhibited significantly by cyclopamine. On the contrary, we found that the OLP-inducing activity of SHHAg is dependent on FGFR and MAPK. However, MAPK alone is not sufficient to induce OLPs – SHH signalling is also required. The additional obligatory signal that is triggered by SHH is presumably also triggered by FGFR, since FGF2 can induce OLPs independently of SHH.

Despite its critical role in cortical precursors, we found that FGFR is not required for SHH-mediated cell fate specification in ventral spinal cord or forebrain, even though ventral precursors are known to express FGFR1-3 in vivo. It is possible that receptor TKs other than FGFR collaborate with SHH in non-cortical cells.

Do FGF2 and SHH act on the same population of cortical precursors?

FGF2 activated the MAPK pathway rapidly in all, or nearly all, E13 cortical precursors (Fig. 5Bk). This is consistent with the fact that FGFR1-3 are expressed in most cortical cells at this age. However, only a minority of the MAPK-active cells – around 10% – went on to express OLIG2 at DIV2 (not shown). A similar proportion of precursors expressed OLIG2 after SHHAg stimulation. What distinguishes the precursor cells that are competent to express OLIG2 from their OLIG2-incompetent neighbours is a mystery.

The FGF2 and SHH-responsive cells could belong to the same or different populations. The simplest interpretation of our data – the one we prefer – is that SHH and FGF2 act directly on the same sub-population of cortical precursors to activate OLIG2. This interpretation is strengthened by our finding that MAPK and SHH act together in the same precursors.

Is FGF involved in oligodendrocyte generation in vivo?

We and others have presented evidence that OLPs are generated in the ventral spinal cord and forebrain during embryogenesis and migrate from there into more dorsal territories including the cerebral cortex (Warf et al., 1991; Pringle and Richardson, 1993; Noll and Miller, 1993; Timsit et al., 1995; Spassky et al., 1998; Nery et al., 2001; Tekki-Kessaris et al., 2001). Chick-

or FGF2. This inherent potential takes a long time to manifest itself (DIV6) and can be blocked by cyclopamine, implying that endogenous Hedgehog activity in the cultures is largely responsible (Tekki-Kessaris et al., 2001). Consistent with this, we found that mRNAs encoding SHH and its relative Indian Hedgehog (IHH) were up-regulated in the cultures (Tekki-Kessaris et al., 2001). Recently, Gabay et al. (Gabay et al., 2003) reported that neocortical cells in monolayer or neurosphere culture up-regulate SHH in response to FGF2 (0.2 ng/ml) and that the OLIG2-inducing activity of this low concentration of FGF2 can be blocked by cyclopamine. This suggests that the up-regulation of Hedgehog transcripts that we observed previously (Tekki-Kessaris et al., 2001) might be due to endogenous FGFR activation and that part of the OLP-inducing activity of added FGF might be mediated indirectly through Hedgehog proteins. However, that cannot account for all of the

quail grafting experiments suggest that, in birds, all oligodendrocytes in the cortex develop from ventrally derived, migratory OLPs (Olivier et al., 2001). However, cell fate analysis in mice, using a *Emx1-Cre* transgene to activate a conditional *lacZ* reporter, showed that the majority of cortical oligodendrocytes were descended from *Emx1*-expressing precursors – presumably indigenous cortical precursors (Gorski et al., 2002). It is possible that as ventrally derived progenitors migrate into the cortex they turn on *Emx1*, so that the *Emx1-Cre* fate mapping experiments erroneously score them as cortex derived. Alternatively, there could be two populations of OLPs: a primitive population that is derived from ventral precursors and a later-developing population that is indigenous to the cortex. This second wave might be present in rodents but not birds, because of the need for greater numbers of OLPs in the much expanded mammalian cortex. It is conceivable that FGF signalling might be involved in the putative second wave of oligodendrogenesis in the mammalian cortex. It might be possible to address this question in future by studying OLP production in neocortex-specific *Fgfr1* knockout mice.

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