

# BMP and LIF Signaling Coordinately Regulate Lineage Restriction of Radial Glia in the Developing Forebrain

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## KEY WORDS

cortical development; restricted precursors; A2B5; 5A5; 4D4; GRP; NRP

## ABSTRACT

The earliest radial glia are neural stem cells that guide neural cell migration away from ventricular zones. Subsequently, radial glia become lineage restricted during development before they differentiate into more mature cell types in the CNS. We have previously shown that subpopulations of radial glial cells express markers for glial and neuronal restricted precursors (GRPs and NRPs) in expression patterns that are temporally and spatially regulated during CNS development. To characterize further the mechanism of this regulation in rat forebrain, we tested whether secreted factors that are present during development effect lineage restriction of radial glia. We show here that in radial glial cultures LIF/CNTF up-regulates, whereas BMP2 down-regulates GRP antigens recognized by monoclonal antibodies A2B5/4D4. These activities combined with secretion of BMPs dorsally and LIF/CNTF from the choroid plexus provide an explanation for the graded distribution pattern of A2B5/4D4 in dorso-lateral ventricular regions in vivo. The regulation by LIF/CNTF of A2B5/4D4 is mediated through the JAK-STAT pathway. BMP2 promotes expression on radial glial cells of the NRP marker polysialic acid most likely by regulating N-CAM expression itself, as well as at least one polysialyl transferase responsible for synthesis of polysialic acid on N-CAM. Taken together, these results suggest that generation of lineage-restricted precursors is coordinately regulated by gradients of the secreted factors BMPs and LIF/CNTF during development of dorsal forebrain. ©2006 Wiley-Liss, Inc.

## INTRODUCTION

Localized sources of secreted factors provide morphogenetic signals that control neural stem cell development (Briscoe et al., 2001; Panchision and McKay, 2002; Temple, 2001). Dorsally derived BMPs promote differentiation of neurons early during development and astrocytes at later stages (Samanta and Kessler, 2004). Ventrally-derived SHH promotes expression of olig2 that plays a critical role in differentiation of oligodendrocyte precursors and motor neurons (Gabay et al., 2003; Lu et al., 2002; Molne et al., 2000). Other secreted factors such as LIF and CNTF that bind to tripartite receptors including gp130 modulate neural stem cell proliferation and lineage restriction at least in vitro (Molne et al., 2000) and a recent report suggests that they have graded distributions in vivo (Gregg and Weiss, 2005).

Early expression of certain secreted factors in neural development suggests that they play critical roles in neural lineage restriction and cell differentiation. Neural stem cells appear early during development as neuroepithelial cells expressing nestin and vimentin and subsequent expression of additional markers such as BLBP and GLAST defines radial glial cells (Anthony et al., 2004; Conti et al., 2005; Noctor et al., 2002). The term radial glia has generated significant confusion in part because it has been debated whether they are glia (Gotz and Barde, 2005; Morest and Silver, 2003) or should be called radial precursors (Ever and Gaiano, 2005). However, embryonic radial glia have been defined by a combination of their morphology and distinguishing characteristics (e.g. BLBP) (Gotz and Barde, 2005) and we will refer to these cells that include neural stem cells and neural precursors as radial glia. Heterogeneity among radial glia (Kriegstein and Gotz, 2003) is likely to be influenced by differential levels of spatially-restricted morphogenetic signaling factors that is poorly understood.

Despite the wide-ranging usefulness of knockout mice, their interpretation may be complicated in cases of multiple structurally-related factors such as BMPs that can compensate functionally for each other (Chen et al., 2004). Studies of neural stem cells have also benefited from cell stabilization by expression of v-myc, which yielded clones such as C17.2 (Villa et al., 2000) that can differentiate in vivo and can influence the milieu in rodent models of injury and disease (Lu et al., 2003; Ryu et al., 2005; Yan et al., 2004; Yang et al., 2003). These cells have been studied extensively and have been proposed as a model for neural stem cells but the generality of the conclusions using C17.2 has been questioned since they express much higher levels of certain key growth factors than nonimmortalized neural progenitors (Mi et al., 2005). We have applied this approach to E14.5 rat cortical cells and derived two well characterized clones (L2.3 and RG3.6) that exhibit markers expressed in radial

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TABLE I. Primers Used for RT-PCR

	Forward	Reverse
BMP2	GTGCTCAGCTTCCATCACGA	CCTGCATTTGTTCCCGAAAA
SHH	CCCAATTACAACCCCGACAT	AGCCCTCAGTCACTCGAAGC
LIF	GCCCCTACTGCTCATTCTGC	GGCACTGCCGTTGAGTTGA
CNTF	CTCCAAGAGAACCCTCCAGGCT	GCTGGTAGGCAAAGGCAGAA
OSM	GAGCATGTCATGGGCCAAG	AGCTCTCAGGGTGAGCGTGT
LIFR	TGGCTGTGGCTGTCATTGTT	GCGTTGCTCCCTCACAGAC
CNTFRa	GCCATGTGGGACAGCAAGTT	GGAGTCACGGTGGAAACAGG
gp130	GGTCCACATGGCAGCATAACA	GAACAAGACTCCCAGCAGCG
BMPRII	TGGTCTGTGGGAGAAATCAAAA	GCAGCAAAAACGGTATGTTCC
Pax3	AAGGTGACAACGCCTGACGT	GGATCGACTGATGGAACCTCA
Pax6	TCTAACCGAAGGGCCAAGTG	GAGGAGACAGGTGTGGTGGG
ST8SiaII/STX	ATCGGGAATTCTGGAGGCAG	CTGGCTGGATGCTGTGCTT
ST8SiaIV/PST	CCTCATGTACACCCTTGCCA	TGTGAGGACTTGCGTTGGAA
NCAM	TGATGTGGTCAGCTCTCTGCC	CGGCCTTTGTGTTTCCAGAT
$\beta$ -actin	CGTAGCCATCCAGGCTGTGT	CCAGTGGTACGACCAGAGGC

glia and neural stem cells, display morphological features of radial glia in vitro and in vivo, support migration of neurons, can be induced to differentiate into neurons and glia, and can be passed extensively in culture while retaining neural stem cell characteristics (Hasegawa et al., 2005; Li et al., 2004). In recent studies using these cells as well as primary neurospheres and embryonic tissue, we found that markers (e.g. A2B5) that have been used to define glial restricted precursors (GRP) appear later than, and are likely to be derived from radial glial neural precursors (Hasegawa et al., 2005; Li et al., 2004).

In the present study, we have investigated the expression of markers for GRPs and NRPs to explore secreted factors that may be responsible for their graded expression in forebrain during development. The results indicate roles for LIF/CNTF in promoting expression of A2B5/4D4 and the dominant suppression of these markers by dorsally derived BMP2 that may account for a graded distribution of A2B5/4D4 in vivo. In addition, the expression of 5A5 in radial glia is promoted by BMP2 and it appears to be regulated by controlling expression of N-CAM, which serves as the protein substrate for the polysialic acid (PSA) residues recognized by monoclonal antibody 5A5.

## MATERIALS AND METHODS

### Dissection and Cultures of Primary Radial Glial Cells and L2.3

Forebrains of E12.5 rat embryos were dissociated into single cell suspensions and  $10^6$  cells were incubated on 10 cm dishes in DMEM/F12 (Invitrogen) with 25 mM glucose (Sigma), 2 mM glutamine (Invitrogen), penicillin/streptomycin (Invitrogen), 10 ng/mL FGF2 (BD Biosciences), 2  $\mu$ g/mL heparin (Sigma) and  $1 \times B27$  (Invitrogen). The resulting cells were initially propagated as neurospheres and passed by mild trypsinization (0.025% for 5 min) every 3–4 days. Most factor treatments were done on adherent cultures of E12.5 radial glial cells at passage 2, which is enriched for BLBP+ radial glia. Culture of radial glial-like clone L2.3 was as described previously (Li et al., 2004). Factors and drugs used in this study include BMP2 (25 ng/mL, R&D, human recombinant), LIF (10 ng/mL, Chemicon, ESGRO), CNTF (10 ng/mL, Sigma, rat recombinant),

SHH agonist (100 nM, Curis, Cur-0199567), all-trans retinoic acid (10  $\mu$ g/mL, Sigma), erythropoietin (100 U/mL, Johnson & Johnson), endothelin-1 (500 nM, Alexis) and the JAK2 inhibitor Tyrphostin AG490 (10  $\mu$ g/mL, Sigma). Experiments were performed at least three times and representative results are presented in the figures.

### Immunofluorescence

Cells were fixed with 4% paraformaldehyde and incubated with PBS, 10% normal goat serum including primary antibodies for 1 h. Triton (0.3%) was included in the staining buffer only when detecting intracellular antigens. After washing with PBS, cells were incubated with secondary antibodies, washed and mounted with Gel-Mount (Biomedica Corp.). Embryo dissection and fixation were done according to methods previously described (Li et al., 2004). For A2B5/4D4 staining, a light fixation was done using 2% paraformaldehyde. The primary antibodies used are monoclonal mouse IgMs: 4D4 (neat, Kaprielian lab), A2B5 (1:200, Chemicon), 5A5 (1:1, DSHB); monoclonal mouse IgGs: anti-vimentin (1:10, DSHB), anti-Pax6 (1:10, DSHB); polyclonal rabbit IgGs: anti-BLBP (1:1000, Heintz lab) and anti-NCAM (1:50) (Friedlander et al., 1994). Secondary antibodies included Rhodamine- or FITC-conjugated goat-anti-mouse IgM (1:200, Jackson ImmunoResearch,  $\mu$  chain specific), Oregon Green goat-anti-rabbit IgG (1:200, Molecular Probes) and Rhodamine-Red goat-anti-mouse IgG (1:200, Molecular Probes). DAPI (10  $\mu$ g/mL, Sigma) was included in the secondary antibody incubations to label nuclei.

### Conventional and Quantitative RT-PCR

Total RNA was purified from target tissue using RNeasy kit (Qiagen) and one microgram was reverse-transcribed into cDNA using oligo-dT primer and SuperScript II reverse transcriptase (Invitrogen). Conventional RT-PCR was done following standard protocol using Taq polymerase. The amplified products were analyzed in 3% agarose gels. Quantitative RT-PCR (Q-RT-PCR) was performed as described previously (Li et al., 2003) using  $\beta$ -actin to normalize the expression levels of each sample. Primers for detecting genes are listed in Table 1.

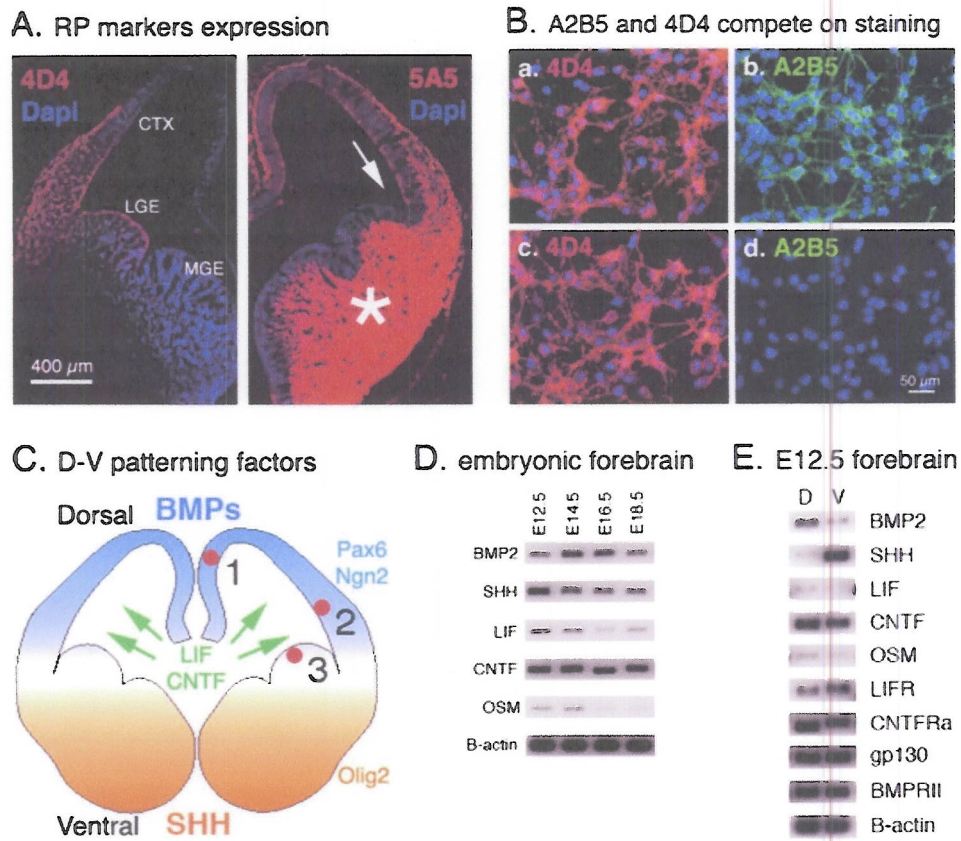


Fig. 1. Spatially regulated expression pattern of restricted precursor markers in developing forebrain. (A) Immunostaining with 4D4 for GRPs and 5A5 for NRPs on coronal sections of E15.5 forebrain. In the ventricular zone, 4D4 expression is mainly in ventro-lateral regions and LGE, attenuating in dorsal and medial regions. 5A5 is expressed uniformly in the ventricular zone from medial through dorsal and lateral region but not in the GE (the boundary is marked by arrow). Strong 5A5 staining in the GE labels SVZ progenitors and newly forming neurons (\*). Scale bar, 400  $\mu$ m. (B) Competition staining with mouse IgM antibodies, 4D4 and A2B5. Cortical cells isolated acutely from E15.5 rat were cultured, fixed and stained separately with 4D4 followed by Rhodmine-red goat-anti-mouse IgM (Ba) and A2B5 followed by FITC goat-anti-mouse IgM (Bb). Panels (Bc and d), show the same culture stained similarly with 4D4, blocked with un-conjugated goat-anti-mouse IgM, and then stained with A2B5, FITC goat-anti-mouse IgM imaged for 4D4 (Bc) and A2B5 (Bd). Nuclei were stained with Dapi. The results show that A2B5 staining was

blocked by 4D4. (C) Schematic drawing of coronal section of embryonic forebrain. Three VZ regions marked 1–3 have different immunological phenotypes (A); region 1 is 5A5 positive only; region 2 is 5A5 and 4D4/A2B5 double positive; region 3 is 4D4/A2B5 positive only. Cells in both region 1 and 2 can give rise to neurons, whereas cells in region 3 are gliogenic only (see text). (D, E) Cytokines and their signaling components are expressed at the onset of restricted precursor marker expression. (D) Gene expression of LIF, CNTF, OSM, as well as BMP2 and SHH in embryonic forebrains were determined by RT-PCR analysis at different developmental stages. (E) In E12.5 forebrain, dorsal (D) and ventral (V) forebrains were micro-dissected and subjected to RT-PCR analysis. Note that both factors and their receptors are present at the onset of restricted precursor markers expression with no dramatic differences between dorsal and ventral halves except for BMP2 and SHH, which are selectively expressed in dorsal and ventral regions, respectively.

## RESULTS

### Differential Expression of GRP and NRP Markers on Radial Glial Cells in the Developing Cortex

We showed using cell type specific marker staining that radial glia appear earlier than restricted precursors during CNS development (Li et al., 2004). Partially overlapping subpopulations of radial glia acquire markers of GRP and NRP as embryonic forebrain matures and the expression of these markers in radial glia is restricted temporally and spatially. For example, in the E15.5 dorsal forebrain, the GRP markers A2B5/4D4 are expressed at highest levels in the ventro-lateral region in a decreasing gradient toward dorsal and medial regions of the ventricular zone (Li et al., 2004) (Fig. 1A). In contrast, the NRP marker 5A5 is expressed evenly in the dorsal cortex

along the ventricular zone, and is completely absent from the ventricular zone of the rat ganglionic eminence (GE) from E12.5 to E15.5 (Fig. 1A and data not shown) (Li et al., 2004). Other cells in the GE including developing neurons (\*) are strongly labeled with 5A5, which is also a marker for embryonic neurons (Rutishauser and Landmesser, 1996). The expression of 5A5 in the ventricular zone appears to be restricted to a dorsal compartment of Pax6 expression in the forebrain (Corbin et al., 2003; Li et al., 2004). In contrast, 4D4 staining crosses from this dorsal region to extend more ventrally into the lateral GE (LGE) (Fig. 1A).

The monoclonal antibodies A2B5 and 4D4 have similar staining patterns in the developing CNS including embryonic forebrain and spinal cord (Li et al., 2004; Liu et al., 2002) (Fig. 1B). Sequential staining showed that, when

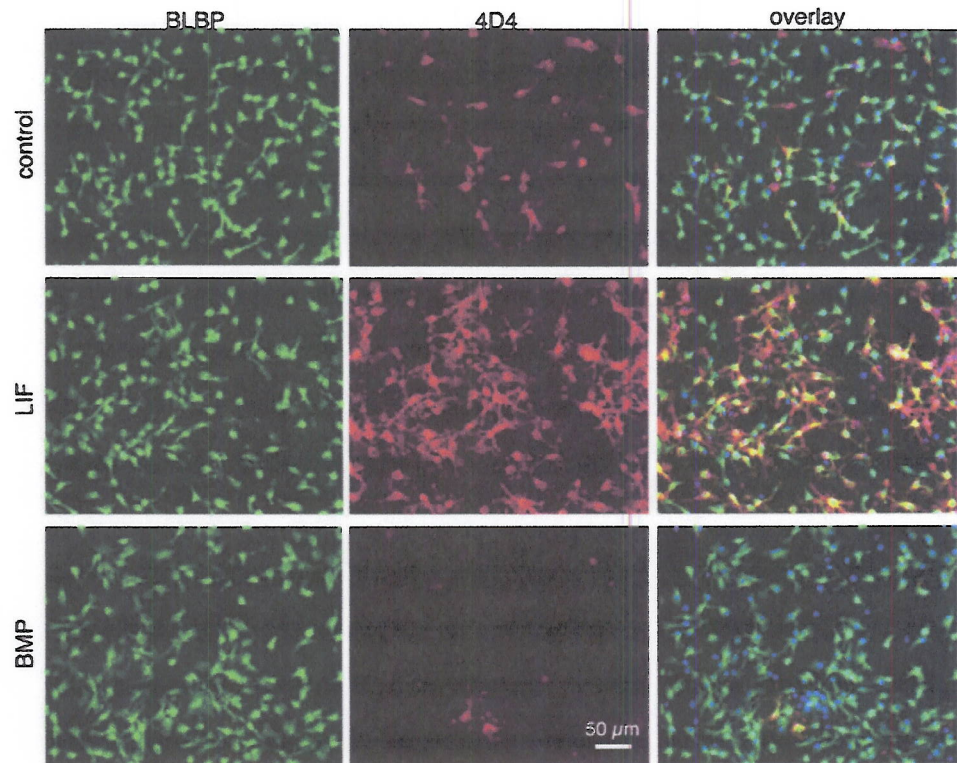


Fig. 2. Effects of secreted factors on 4D4 expression of radial glial clone L2.3. When L2.3 cells were cultured on laminin-coated substrate in FGF2 containing medium (control) for 2 days, LIF (10 ng/mL) promoted and BMP2 (25 ng/mL) suppressed 4D4 expression. These factors did not affect BLBP expression.

IgM 4D4 was applied to the cells first followed by secondary antibody and blocked further using unlabeled goat-anti-mouse IgM, subsequent IgM A2B5 staining was not detected (Fig. 1B). This result provides further evidence to support the notion that A2B5 and 4D4 recognize the same or closely related epitopes. However, A2B5 was not able to block binding of 4D4 (Supplemental Fig. 1), indicating that it binds somewhat differently to cells, perhaps to a broader set of carbohydrates including those that are recognized by A2B5. This may explain why 4D4 yielded more robust and reproducible immunostaining in tissue staining than A2B5 while the overall distributions were indistinguishable (Li et al., 2004). Moreover, there was also extensive overlap in the staining on cells in culture and the patterns were indistinguishable (Supplemental Fig. 1).

### Expression of Morphogenetic Factors in the Developing Forebrain

The radial glial marker BLBP is expressed in the rat neuroepithelium as early as E12.5 when there is little or no staining in forebrain sections for 4D4 and 5A5 (Li et al., 2004). This indicated that the appearance of radial glia precedes that of restricted precursor during CNS development, and suggests that subpopulations of radial glia transitioned to become restricted precursors in a temporally and spatially regulated manner. Therefore, E12.5 in the rat is an appropriate time to search for morphogenetic factors that may regulate generation of restricted precursors. LIF and related factors including CNTF and OSM are all present as early as E12.5 in rat forebrain (Fig. 1D).

LIF and OSM expression decrease as development proceeds, while CNTF expression stays relatively stable at least until E18.5. In addition, CNTF appears to be expressed at the highest level among the three factors examined by RT-PCR, suggesting that CNTF may be a major factor expressed *in vivo*. The corresponding receptors including LIFR, CNTFR $\alpha$ , and gp130, are also expressed at significant levels in E12.5 forebrain (Fig. 1E), indicating that these factors as well as their receptors are coordinately expressed and thus are likely to be signaling during these periods of development (Gregg and Weiss, 2005). In an attempt to explain the dorsal-ventral gradient of 4D4 expression, dorsal and ventral parts of E12.5 forebrains were micro-dissected for RT-PCR analysis. As expected, BMP2 and SHH were preferentially detected in dorsal and ventral regions, respectively, providing confirmation for the dissection techniques (Fig. 1E). No dramatic differences in expression were seen between dorsal and ventral regions for other factors and their receptors including BMPRII (Fig. 1E).

### LIF and BMPs Modulate Expression of A2B5/4D4 and PSA on Radial Glial Cells and Influence Their Differentiation in Culture

To search for potential regulators of restricted precursor marker expression in the ventricular zone, we tested the effects of various secreted factors on restricted precursor marker expression using cells in adherent cultures. Clone L2.3 has been shown previously to exhibit radial glial

properties, and it acquires increasing levels of 4D4 with increasing passage in culture (Li et al., 2004). When early passage L2.3 cells were treated for 2 days in the presence of FGF2 with LIF or CNTF there was a dramatic increase in the number of 4D4+ cells (Fig. 2 and data not shown). Most 4D4+ cells also expressed the radial glial marker BLBP. In contrast, BMP2 suppressed 4D4 expression on L2.3 cells. SHH agonist had no significant effect on expression of these markers but it promoted cell replication (data not shown). None of these factors produced noticeable changes in expression of BLBP (Fig. 2). Other factors tested including retinoic acid, erythropoietin, and endothelin-1 showed no effect on 4D4 expression.

We next tested effects of these factors on dissociated E12.5 cortical neurospheres. These primary cultures including many radial glial cells showed similar responses to factor treatments. LIF increased and BMP2 decreased expression significantly but the SHH agonist had no significant effect on marker expression as observed qualitatively (Fig. 3A–D); these results were confirmed by quantitative analysis (Fig. 3E). Most E12.5 cells express BLBP, and its expression did not appear to be regulated by any of these factors (data not shown), similar to the results obtained with the L2.3 radial glial cells (Fig. 2).

To determine whether up-regulation of lineage-restriction markers affects the differentiation of the cells, we maintained factor-treatment for 4 days. In FGF2 only treated cultures there were very few GFAP+ cells ( $3 \pm 1.5\%$ ) and some neurons ( $13.5 \pm 2.5\%$ ). However, in FGF2+LIF treated cultures, there was a drastic increase in GFAP+ cells ( $80.7 \pm 2.6\%$ ), and a moderate decrease of TuJ1+ neurons ( $7.3 \pm 1.7\%$ ). In contrast, BMP2 increased the percentage of TuJ1+ neurons  $\sim 2$  fold ( $26.4 \pm 3\%$ ). BMP2 treatment also increased GFAP+ cells ( $21 \pm 1.7\%$ ), but this effect was not as dramatic as with LIF treatment. Therefore, LIF not only up-regulated GRP markers A2B5/4D4 in 2-day treatment, but also consistently promoted generation of glia (e.g. GFAP+ astrocytes).

#### LIF Induces A2B5/4D4 Expression in Radial Glial Cells Via JAK-STAT Signaling

LIF belongs to the IL-6 cytokine family that act on the LIFR/gp130 receptor complex to signal via the JAK-STAT pathway (Segal and Greenberg, 1996; Stahl and Yancopoulos, 1994) and AG490 inhibits JAK2 (Barton et al., 2004; Kim et al., 2002; Stahl and Yancopoulos, 1994). Pre-incubation with AG490 (10  $\mu\text{g}/\text{mL}$ ) completely blocked the upregulation of 4D4 in E12.5 cortical neurosphere culture by LIF (Supplemental Fig. 2), indicating that the promoting effect of LIF on 4D4 expression is mediated by JAK-STAT pathway. The small effect of AG490 on 4D4 expression in cultures treated only with FGF2 may represent inhibition of 4D4 induction by endogenous low levels of LIF generated in the cultures; we have detected LIF mRNA in extracts of these cells by RT-PCR (data not shown). AG490 slowed cell growth but no toxicity was seen in the cultures (data not shown).

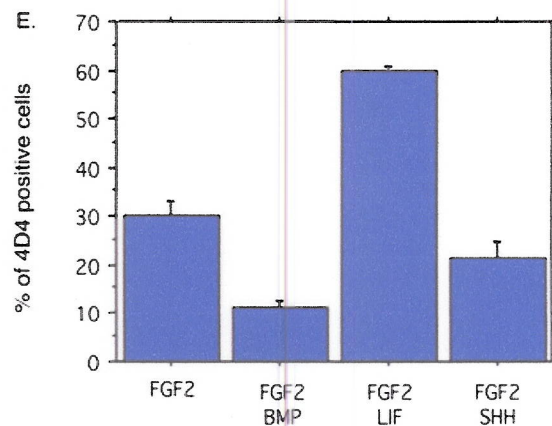
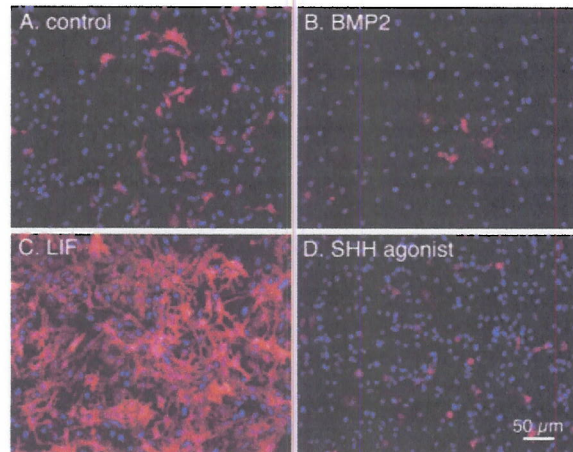


Fig. 3. Effects of secreted factors on 4D4 expression of primary radial glia. Neurospheres derived from E12.5 cortex were dissociated and cultured on laminin-coated substrate in FGF2 containing medium (control) and in the presence of LIF (10 ng/mL), BMP2 (25 ng/mL) or SHH agonist (100 nM) for 2 days and then stained with 4D4. (A–D) LIF promotes ( $P < 0.0001$ ), whereas BMP2 suppresses ( $P < 0.05$ ) 4D4 expression of primary radial glial culture compared to treatment with FGF2 alone. Addition of SHH agonist did not have a significant effect on 4D4 expression. Quantification of the results is shown in panel E.

#### GFAP and A2B5/4D4 Are Regulated Differently by LIF and BMP In Vitro

LIF/CNTF promotes, and BMP2 suppresses the expression of A2B5/4D4 in both radial glial clone L2.3 and dissociated E12.5 cortical neurosphere cultures. The pattern of 4D4 expression in vivo is graded with 4D4 being higher in ventro-lateral and lower in dorso-medial cortex. Thus dorsally derived BMPs including BMP2 exhibits a complementary graded distribution pattern with 4D4 antigen in the developing forebrain (Fig. 1), and LIF/CNTF are likely to establish a graded distribution decreasing from its source in the choroid plexus (Gregg and Weiss 2005). Expression of BMP2 is highest in dorsal regions where 4D4 antigen is absent suggesting these two molecules are present in the developing forebrain in a complementary distribution pattern (Fig. 1).

Considering that expression of A2B5/4D4 is promoted by LIF/CNTF and inhibited by BMP2, we propose that the

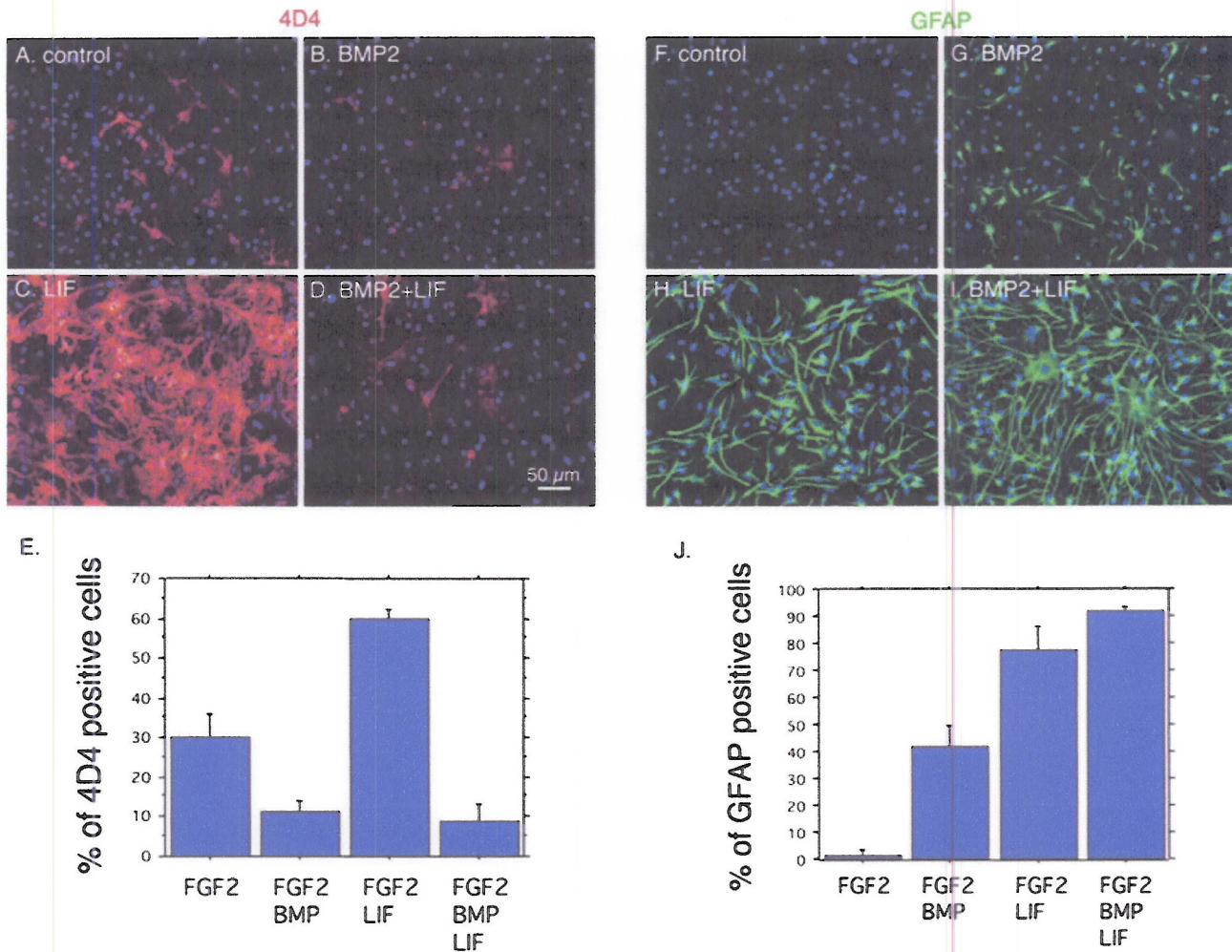


Fig. 4. Differential effects of LIF and BMP2 on expressions of 4D4 and GFAP in radial glia. Dissociated neurospheres derived from E12.5 cortex were cultured on laminin-coated substrate in FGF2 containing medium (control) and in the presence of LIF (10 ng/mL), BMP2 (25 ng/mL) or the combination of both for 2 days and then fixed and stained with 4D4 and GFAP. (A–C) LIF promotes, whereas BMP2 suppresses

4D4 expression (red) in primary radial glia in culture. BMP2 suppresses LIF induction of 4D4 when both factors were combined (D). (F–I) LIF and BMP2 each promoted GFAP expression (green) at different levels and produced at least as great an effect on GFAP expression when both factors were combined. (E) and (J) are the quantifications of the corresponding staining. DAPI (blue) was used to label nuclei.

graded expression pattern of A2B5/4D4 is inversely related to the graded distribution of BMPs. To test this hypothesis, we treated E12.5 primary dissociated neurospheres with BMP2 and LIF individually and in combination. LIF promoted and BMP2 suppressed 4D4 expression in culture (Figs. 3 and 4). However, when the two factors were combined, BMP2 suppression dominated with no detectable promotion effect of LIF on 4D4 expression (Figs. 4A–E). This result supports the hypothesis that in the presence of LIF/CNTF, BMPs play a dominant role in establishing the 4D4 gradient in vivo.

LIF and BMPs rapidly induce GFAP expression and eventually promote astrocyte differentiation when applied separately to cultures derived from E15 cortex (Molne et al., 2000). When these factors were combined they yielded more GFAP+ cells (Nakashima et al., 1999; Rajan et al., 2003). We reproduced these results in cultures from E12.5 forebrains (Figs. 4F–J); LIF and BMP2 alone upregulated GFAP

expression and when combined the effect was at least as great as with LIF alone (Fig. 4J). This is in sharp contrast to the inhibition of 4D4 expression by BMP2 in the presence of LIF (Figs. 4A–E). The differential effects of combinations of LIF and BMP2 on expression of 4D4 and GFAP in cultures of neural stem and progenitor cells suggest that the regulation of these two markers utilizes different mechanisms.

#### BMP2 Promotes PSA-NCAM Expression in Primary Radial Glial Culture

Unlike the graded pattern of the GRP marker 4D4, the expression of the NRP marker PSA recognized by 5A5 appears to be compartmentalized. For instance, along the ventricular zone of embryonic forebrains, PSA-NCAM expression is localized in dorsal, but not ventral regions (Fig. 1) (Li et al., 2004). To determine whether secreted

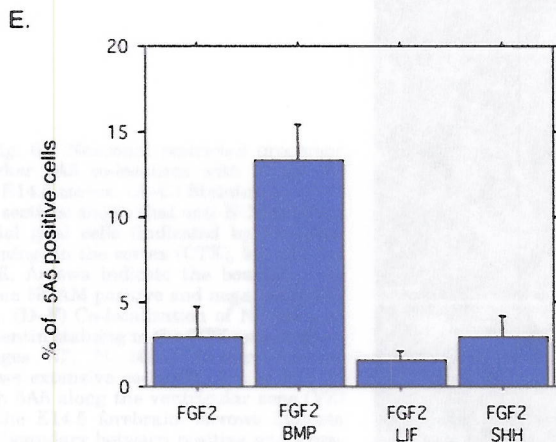
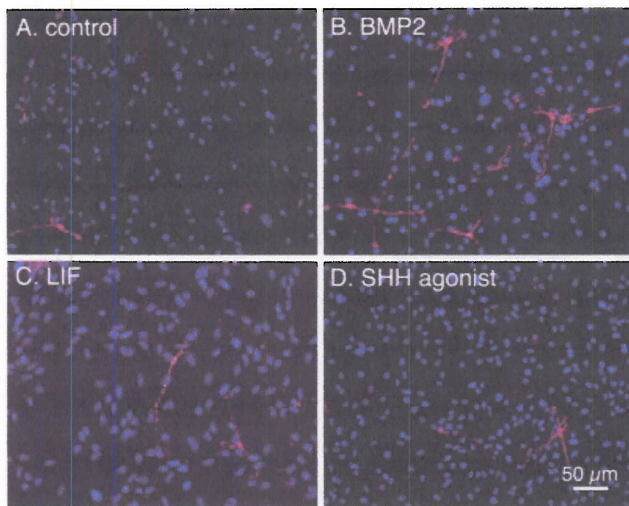


Fig. 5. Effects of secreted factors on 5A5 expression of primary radial glial cells derived from E12.5 cortex. Dissociated neurospheres derived from E12.5 cortex were cultured on laminin-coated substrate in FGF2 containing medium (control) and in the presence of LIF (10 ng/mL), BMP2 (25 ng/mL) or SHH agonist (100 nM) for 2 days. BMP2 promotes 5A5 expression on primary radial glial in culture, whereas LIF and SHH agonist did not have significant effects.

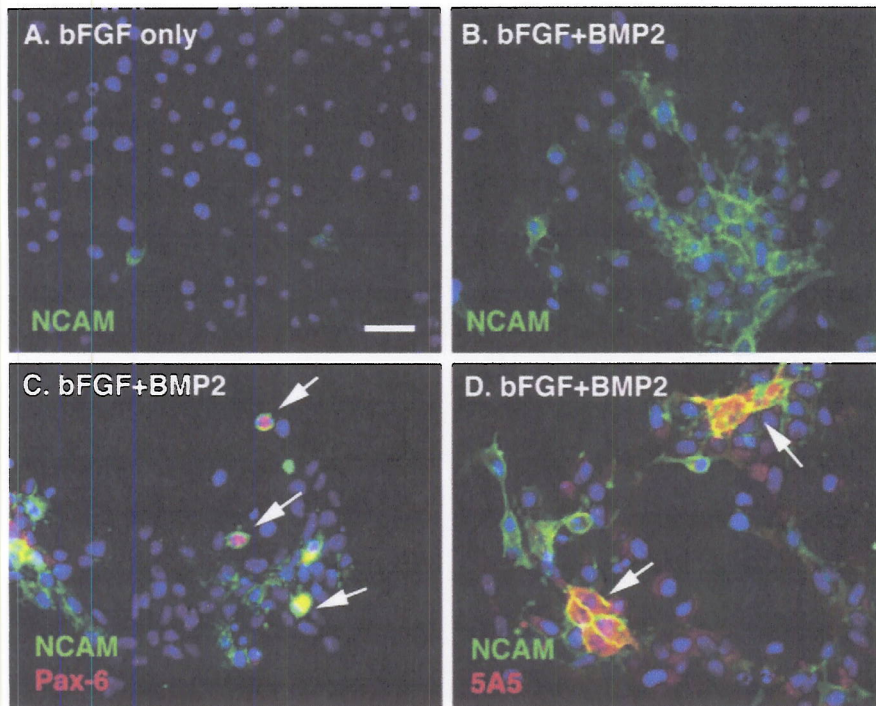
factors regulate the expression pattern of PSA-NCAM, we treated E12.5 dissociated neurospheres with factors expressed in the brain including BMP2, SHH agonist and LIF. A 2-day BMP2 treatment increased the fraction of 5A5+ cells about 4-fold, whereas neither LIF nor SHH agonist showed any detectable effect (Fig. 5). The morphology of 5A5+ cells in these cultures resembles neurons with long processes and small cell bodies and some were  $\beta$ III tubulin positive suggesting that they are becoming neurons. A 4-day factor treatment showed that BMP2 increased the percentage of TuJ1+ neurons by  $\sim$ 2 fold ( $26.4 \pm 3\%$ ) compared to FGF2 only ( $13.5 \pm 2.5\%$ ). BMP2 treatment also increased the fraction of GFAP+ cells ( $21 \pm 1.7\%$ ) compared to FGF2 only ( $3 \pm 1.5\%$ ). Thus, in dissociated cultures of E12.5 cortical neurospheres, BMP2 promotes neurogenesis and astrogliogenesis. This confirms previous results that BMPs promote neurogenesis at early times and gliogenesis at later times

during CNS development (Briscoe et al., 2001; Panchision and McKay, 2002; Temple, 2001). The dual effects of BMP2 may reflect the heterogeneity in neurosphere cultures of E12.5 cortex, which consist of a mixed populations of precursor cells at various developmental stages. Thus, BMPs up-regulate NRP markers and promote neurogenesis. Similar effects of BMP2 were observed with radial glial clone L2.3 (data not shown).

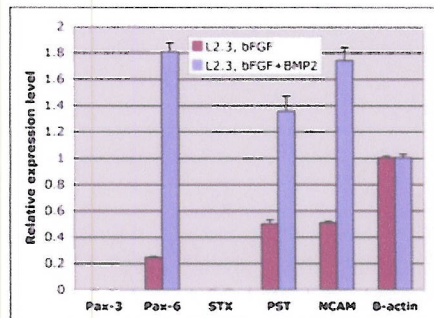
#### Regulation of PSA-NCAM Expression on Radial Glia

PSA-NCAM that is recognized by 5A5 is called the embryonic form of NCAM because it is mainly expressed during development and dramatically decreases in adults (Rutishauser and Landmesser, 1996). The two sialyltransferases known to add PSA onto NCAM, i.e. STX and PST (Angata and Fukuda, 2003), are developmentally regulated with high levels in embryonic tissue, particularly in the nervous system, and lower levels in adult (Phillips et al., 1997). Given that these two enzymes are more widely distributed than the restricted pattern observed for 5A5 (Phillips et al., 1997) it is likely that other factors are responsible for restricted expression of PSA-NCAM. Another possible explanation is that compartmentalized expression of NCAM protein itself is responsible for the 5A5 staining pattern. Since the expression of NCAM in the forebrain at this stage of development has not been described, we stained the E14.5 cortex with anti-NCAM and 5A5 antibodies and found that they exhibit very similar patterns (Fig. 6). In the embryonic forebrain, NCAM labels dorsal radial glial cells revealed by vimentin staining (Figs. 6A–C). Higher power and confocal microscopic analysis showed that 5A5 and NCAM colocalized at the cellular level in dorsal regions (Figs. 6C,D–F). There appeared to be a sharp border defining a dorsal region of NCAM staining, with vimentin staining continuing more ventrally (Figs. 6A–C). In the dorsal ventricular zone, there was nearly complete overlap of 5A5 with NCAM staining by high power and confocal microscopic analysis (Figs. 6G–L). The results suggest that the distribution of PSA-NCAM is restricted by the expression pattern of NCAM itself and is not limited by the expression of the polysialyltransferases STX and PST.

NCAM has been suggested to be one of the downstream targets of the neurogenic transcription factor Pax6 (Holst et al., 1997). The expression pattern of Pax6 mRNA and protein is restricted to a dorsal region of the cortex (Corbin et al., 2003; Stoykova et al., 2000) that is very similar to that observed for 5A5 and NCAM (Fig. 6). Pax6 expression has also been implicated under the influence of dorsalizing factors such as BMPs (Timmer et al., 2002). Pax3, which also is expressed in the dorsal neural tube (Mansouri et al., 2001), can upregulate the STX polysialyltransferase (Mayanil et al., 2001), suggesting that it might regulate STX expression in the embryonic forebrain. To explore regulation pathways that might be responsible for PSA-NCAM expression in the embryonic forebrain, we analyzed expression of these genes using



### E. Q-RT-PCR of L2.3



### F. Q-RT-PCR of tissue

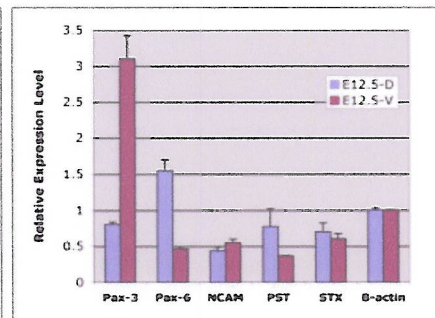


Fig. 7. BMP2 up-regulates the expression of Pax-6, NCAM and 5A5 in radial glial clone L2.3. L2.3 cells were treated with FGF2 (10 ng/mL) alone (A) or combined with BMP2 (25 ng/mL, B, C, and D) for 2 days. Cells were fixed and stained with antibodies as indicated. BMP2 promoted NCAM expression in L2.3 cells (compare B to A). Many of the NCAM-positive cells show pax-6 staining (arrows in C) and 5A5 staining (arrows in D). DAPI was used to label the nuclei. Scale bar, 20  $\mu$ m. Factor-treated L2.3 cells were harvested for Q-RT-PCR to show relative gene expression levels (E). Gene expression was also measured in extracts of E12.5 dorsal and ventral forebrain tissues (F). STX, sialyltransferase X (ST8Sia II); PST, polysialyltransferase (ST8Sia IV).

antigen in forebrain cells whereas BMP2 dominantly inhibits its expression. Ventrally-derived SHH signaling promotes expansion of progenitors but did not significantly affect expression of these restricted precursor markers. Thus, opposing dorsal-ventral gradients of BMPs and LIF/CNTF *in vivo* are likely to be responsible for generating heterogeneity of radial glia in the developing forebrain. The proximity of the ventro-lateral cortex and the LGE to the choroid plexus (Gregg and Weiss, 2005) should expose these regions to relatively high levels of LIF/CNTF resulting in expression of 4D4 antigen (Figs. 1A,B). However, the early expression of BMPs (Furuta et al., 1997) and their dominant inhibition of A2B5/4D4 expression is likely to be responsible for the lack of dorsal A2B5 expression along the neuraxis and medially in the forebrain as well as its graded increase moving ventro-laterally (Figs. 1A,C). Opposing dorsal-ventral gradients of BMPs and SHH have been suggested to promote oligodendrocyte differentiation ventrally and suppress it dorsally with BMPs acting on olig proteins (Samanta and Kessler, 2004). BMPs are

known to upregulate Pax6 that in turn can upregulate NCAM and at least one of the enzymes (PST) that catalyzes the synthesis of PSA on NCAM (Fig. 8). Thus BMPs may upregulate both NCAM and PST to sufficiently high levels in the Pax6+ dorsal cortex so that the dorsal ventricular zone radial glia express PSA-NCAM (Figs. 1A,B and 8).

### PSA-NCAM is Expressed on Neurogenic Precursors and Some Radial Glia

PSA on NCAM was first detected in ventro-lateral regions in E13.5 rat newborn neurons from the LGE (Phillips et al., 1997) that had migrated first radially and then dorsally (Fig. 1A) (Li et al., 2004). PSA is found almost exclusively on NCAM and can be synthesized by at least two enzymes (PST and STX) that act cooperatively (Angata and Fukuda, 2003). Both PST and STX are expressed widely in the rat nervous system at E15 in ventricular



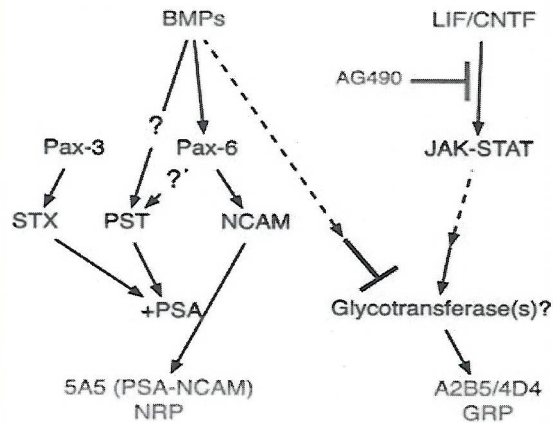


Fig. 8. Model summarizing effects of growth factors on lineage restriction markers in radial glia in E12.5 forebrain. BMPs induce expression of Pax6, which is known to regulate expression of NCAM and PST. Another polysialyltransferase STX can be regulated by Pax-3, and the two enzymes work in a complementary manner to synthesize PSA on NCAM. Both enzymes are present in radial glia and are likely to form PSA, which serves as a marker for NRP. LIF/CNTF can induce expression of the A2B5 carbohydrate epitope on radial glia in culture and its expression is blocked by AG490 implicating Jak-Stat signaling. However, this differs from the signaling that induces GFAP (see text). A2B5 and 4D4 bind to indistinguishable carbohydrate epitopes found on GRP. See discussion for differences in spinal cord.

and marginal zones (Phillips et al., 1997). A previous study that focused on ventral regions noted that PSA was not found in the ventricular zone although weak NCAM staining was detected (Phillips et al., 1997). However, they did not contrast this with expression in more dorsal regions where we found PSA-NCAM expression in the ventricular zone (Fig. 6).

BMP signaling plays a major role in PSA expression by regulating Pax6 expression in dorsal regions of the neural tube (Timmer et al., 2002) and Pax6 induces NCAM expression (Holst et al., 1997). BMPs are expressed as early as E8.5 in mouse (Furuta et al., 1997) and by E9.5 there is robust and widespread expression of Pax6 in the ventricular zone except in ventral regions (Corbin et al., 2003). Thus NCAM expression in dorsal forebrain is likely to be a major factor limiting the levels of PSA given the more widespread expression of PST and STX (Phillips et al., 1997) than NCAM (Fig. 6). Radial glia in all CNS regions including the ventral forebrain serve as neuronal progenitors (Anthony et al., 2004). Development of different types of neurons in the GE (Stenman et al., 2003) is from the subventricular zone (SVZ), which is PSA+, and the SVZ is derived from the adjacent ventricular zone that will later become gliogenic (Malatesta et al., 2000). Although radial glia in ventral forebrain do not express PSA-NCAM during development in contrast to dorsal ventricular zone, their progeny in the SVZ of the GE express PSA when they are neurogenic (Fig. 1A, see \*). This is consistent with the idea that neurogenic radial glia appear early and only transiently before becoming gliogenic but they also give rise to NRPs in the SVZ (Anthony et al., 2004; Malatesta et al., 2003).

The identification of A2B5+/5A5+ double positive cells poses a challenge for the NRP/GRP hypothesis (Noble

et al., 2003). In the spinal cord and perhaps also in the GE (Fig. 1A), progenitors that do not express A2B5/4D4 but express 5A5 (Fig. 1C, region 3) at sufficient levels to allow immunopurification behave as NRPs (Liu et al., 2002; Mayer-Proschel et al., 1997). At the other extreme, progenitors that express A2B5 but not 5A5 behave as GRPs (Liu et al., 2002; Mayer-Proschel et al., 1997). Consistent with the idea that neural precursors are first neurogenic and later gliogenic, it is possible that the double positive cells including 4D4+/5A5+ cells (Li et al., 2004) (Figs. 1A,C, region 2) represent multipotential neural precursors that are shifting from a neurogenic stage to become gliogenic radial glia in forebrain. In spinal cord and GE this transition appears to coincide with the expression of PSA on cells that do not express A2B5. However, in the dorsal forebrain, A2B5+/5A5+ double positive cells behave as multipotential cells (Noble et al., 2003).

Clonal analysis indicated that some BLBP+/PSA+ clones could be isolated from E14.5 dorsal rat forebrain but not every cell in each clone was PSA+ (Li et al., 2004) suggesting that they may give rise to PSA- progenitors. Most clones were BLBP+/A2B5+ and frequently all the cells in a clone were A2B5+ (Li et al., 2004), suggesting that they are GRPs. By E15.5 in the rat, most if not all radial glia in the dorsal ventricular zone express PSA. Thus, isolation of cells from dorsal forebrain at this stage using antibodies against PSA should yield a heterogeneous population at least with respect to A2B5/4D4. However, clonal analysis of double positive PSA+/A2B5+ cells in the cortex indicates that they give rise both to neurons and glia, suggesting that they are neural precursors (Noble et al., 2003). Similarly, we found that many acutely isolated E15.5 forebrain cells were stained by both anti-NCAM and 4D4 antibodies (data not shown). Double positive cells were not detected in the developing spinal cord (Mayer-Proschel et al., 1997). Thus, there is greater diversity of neural precursors in forebrain than in spinal cord, which may complicate isolation of lineage-restricted precursors from forebrains using these surface markers.

#### Growth Factors Regulate Expression of Markers for Restricted Precursors

BLBP protein is expressed in the ventricular zone of the rat forebrain by E12.5 one day earlier than PSA and 4D4, indicating that embryonic radial glia appear before markers for restricted precursors (Li et al., 2004). These results are consistent with other studies (Temple, 2001) demonstrating that neural precursors (including radial glia) give rise in a clonal manner first to neurons and later to glia. Precursor cells from the spinal cord that are PSA+/A2B5- are neurogenic while those that are PSA-/A2B5+ are gliogenic and may be GRPs (Liu et al., 2002; Mayer-Proschel et al., 1997). Our results suggest that BMPs and LIF/CNTF are two key growth factor signaling systems that coordinately regulate expression of PSA and A2B5 and provide a potential mechanism for generating PSA+/A2B5+ cells with different ratios of these carbohydrates.

In the developing forebrain, A2B5/4D4 expression is restricted to lateral regions adjacent to a known source of LIF/CNTF (Gregg and Weiss, 2005), which can induce A2B5/4D4 expression on radial glial cells in culture. The spinal cord differs in that A2B5/4D4 is expressed in the ventral half and it is similar to the forebrain with respect to absence of A2B5/4D4 dorsally (Liu et al., 2002; Li et al., 2004). LIF/CNTF signaling is different between forebrain and spinal cord (Gregg and Weiss, 2005) and its influence on A2B5/4D4 expression in the latter needs to be explored further. In addition, PSA expression was not observed on BLBP+ cells in the spinal cord (Liu et al., 2002). In contrast to the forebrain where Pax6 is expressed in the dorsal compartment, in the spinal cord it is expressed primarily ventrally (Timmer et al., 2002), which may explain the absence of PSA-NCAM on the dorsal radial glia in the spinal cord. These are additional differences between forebrain and spinal cord radial glia. However, as suggested in the forebrain, early and dominant BMP signaling may also restrict A2B5/4D4 expression from dorsal regions of the spinal cord. SHH signaling promotes expansion of ventral neural tube cells and we found that a SHH agonist (Gabay et al., 2003) promoted expansion of E12.5 forebrain progenitor cells (Fig. 3). Thus, in addition to opposing dorsal-ventral gradients of BMP and SHH, cortical development appears to be influenced by graded distributions of LIF/CNTF.

LIF/CNTF binding triggers activation of a family of cytoplasmic tyrosine kinases (JAKs) that are associated with gp130. The JAKs in turn phosphorylate intracellular signaling molecules including members of the STAT family of DNA binding transcriptional activators that translocate into the nucleus (Segal and Greenberg, 1996; Stahl and Yancopoulos, 1994). Our observations that LIF-induced 4D4 upregulation can be blocked by AG490 (Barton et al., 2004; Kim et al., 2002) suggests that JAK-STAT is involved in regulation of the 4D4 antigen (Fig. 8). STAT3 is a downstream component for LIF-induced GFAP upregulation, but whether or not it is also responsible for 4D4 promotion needs to be clarified. Although rat E12.5 cortical cells have functional LIF receptors they do not act to induce GFAP expression for several days until E15 (Molne et al., 2000; Viti et al., 2003). In contrast, the LIF responsiveness of E12.5 cells to express 4D4 antigen suggests that it involves different targets that perhaps are involved in progressive stages of differentiation including glial restriction. Alternatively, they may share a common pathway but GFAP expression may be controlled further either by epigenetic modifications of the GFAP promoter (Song and Ghosh, 2004) or cellular competency modulated by EGFR activation (Viti et al., 2003).

BMP binding activates phosphorylation of SMADs, which act as transcription factors to regulate downstream genes (Gross et al., 1996; Mabie et al., 1997). Crosstalk between LIF and BMP signaling pathways has been suggested based on their additive effects on GFAP expression. One model suggests that phosphorylated STAT3 and SMAD1 physically interact through a bridging protein (P300), and the resulting complex causes their additive effect on GFAP expression (Nakashima et al., 1999). Another model sug-

gests that a serine-threonine kinase FRAP associated with FK506-binding protein 12 (FKBP12) phosphorylates serine residues on STAT3 upon activation of BMP receptor, and this double phosphorylation on STAT3 could be responsible for the additive effects of LIF and BMPs (Rajan et al., 2003). However, the strikingly different effects of these two factors on 4D4 and GFAP expression indicates that they are differentially regulated in radial glial cells. The early induction of 4D4 antigen suggests that putative glycosyl transferase(s) are likely targets that are regulated by LIF signaling via the JAK2-STAT3 pathway (Fig. 8).

Both BMP and LIF/CNTF signaling in neural precursors upregulate GFAP expression but they may use different pathways and the resulting glia derived from these treatments may differ. First, BMP suppresses expression of olig genes and oligodendrocyte development (Samanta and Kessler, 2004), while LIF upregulates A2B5, which has been associated with oligodendrocyte precursors (Noble et al., 2004). Second, BMPs treatment of GRPs yielded astroglia that produce beneficial effects in spinal cord injury, which may differ from other astroglia that are gliotic (Davies et al., 2006). Thus, different precursors may yield different types astroglia.

In conclusion, we have found that LIF and BMPs regulate expression of markers for neuronal (PSA+) and glial (A2B5+) precursors in vitro. Understanding the effects of secreted factors on neural stem cell lineage restriction may help to direct the differentiation of these cells to desired cell types. Considering that carbohydrate antigens have been used to isolate neuronal precursors and GRP from embryos (Mayer-Proschel et al., 1997) and they are conserved in humans, they may be particularly useful for purifying precursor cells for therapeutic applications. These approaches may soon be feasible with neural precursors that can be generated from embryonic stem cells (Conti et al., 2005; Liour and Yu, 2003) considering that these cells can be induced to express antigens recognized by A2B5/4D4 in culture (unpublished observations).

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