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The Tripotential Glial-Restricted Precursor (GRP) Cell and Glial Development in the Spinal Cord: Generation of Bipotential Oligodendrocyte-Type-2 Astrocyte Progenitor Cells and Dorsal–Ventral Differences in GRP Cell Function

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We have found that the tripotential glial-restricted precursor (GRP) cell of the embryonic rat spinal cord can give rise *in vitro* to bipotential cells that express defining characteristics of oligodendrocyte-type-2 astrocyte progenitor cells (O2A/OPCs). Generation of O2A/OPCs is regulated by environmental signals and is promoted by platelet-derived growth factor (PDGF), thyroid hormone (TH) and astrocyte-conditioned medium. In contrast to multiple observations indicating that oligodendrocyte precursor cells in the embryonic day 14 (E14) spinal cord are ventrally restricted, GRP cells are already present in both the dorsal and ventral spinal cord at E13.5. Ventral-derived GRP cells, however, were more likely to generate O2A/OPCs and/or oligodendrocytes than were their dorsal counterparts when exposed to TH, PDGF, or even bone morphogenetic protein-4. The simplest explanation of our results is that oligodendrocyte generation occurs as a result of generation of GRP cells from totipotent neuroepithelial stem cells, of O2A/OPCs from GRP cells and, finally, of oligodendrocytes from O2A/OPCs. In this respect, the responsiveness of GRP cells to modulators of this process may represent a central control point in the initiation of this critical developmental sequence. Our findings provide an integration between the earliest known glial precursors and the well-studied O2A/OPCs while opening up new questions concerning the intricate spatial and temporal regulation of precursor cell differentiation in the CNS.

Key words: glial-restricted precursor cell; GRP cell; oligodendrocyte; O2A progenitor cell; OPCs; spinal cord development; ventral origin; neuroepithelial stem cells

Understanding how the differentiated cell types of the body are generated is a central challenge in developmental biology. Multiple components contribute to this process, including signaling molecules and transcription factors that cause precursor cells to progress along different developmental pathways. Central to understanding cell generation, however, is identification of the precursor cell from which a given cell type arises, for it is the specific precursor cell that represents the actual target for exogenous influences.

The creation of specific precursor cells and differentiated cell types proceeds through a sequence of lineage restrictions but also may involve a phenomenon of lineage convergence. Through lineage restriction, the totipotent stem cells of the earliest embryo generate progeny that are more restricted in the range of cell types they generate. For example, totipotent embryonic stem cells give rise to tissue-specific stem cells. Tissue-specific stem cells proceed to produce differentiated cell types via intermediate lineage-restricted precursor cells. These lineage-restricted precursor cells ultimately generate a subset of the differentiated cell types in a particular tissue. Lineage restriction is complemented in development by the process of lineage convergence, by which different lineages give rise to the same cell type. One example of such convergence is seen in the formation of cartilage from both mesenchymal and cranial neural crest lineage (Baroffio et al., 1991).

Studies on CNS development are revealing a rich diversity of precursor cells that can give rise to the same cell type, particularly with respect to glial development. For example, it is well established that oligodendrocytes can be generated from oligodendrocyte-type-2 astrocyte progenitor cells (Raff et al., 1983; Skoff and Knapp, 1991), which also are referred to as oligodendrocyte precursor cells (Raff et al., 1983; Skoff and Knapp, 1991) and abbreviated here as O2A/OPCs. More recent studies on embryonic rat spinal cord have led to the isolation of a new and distinct population, called tripotential glial-restricted precursor (GRP) cells, that also can generate oligodendrocytes *in vitro* and *in vivo* (Rao et al., 1998; Herrera et al., 2001). GRP cells and O2A/OPCs differ in several characteristics. For example, GRP cells and O2A/OPCs differ in their responses to mitogens, survival factors, and inducers of differentiation (Rao et al., 1998). GRP cells and O2A/OPCs also express distinct differentiation potentials *in vitro*: GRP cells are able to generate oligodendrocytes and two distinct astrocyte populations, whereas O2A/OPCs can generate oligodendrocytes and only one kind of astrocyte. Moreover, GRP cells readily generate astrocytes when transplanted into the neonatal or adult brain (Herrera et al., 2001), a cell type not generated from primary O2A/OPCs, after transplantation into the normal CNS (Espinosa de los Monteros et al., 1993).

Several critical questions arise from the fact that it now is possible to isolate two distinct precursor cell populations (i.e., GRP cells and O2A/OPCs) from the developing animal, each of

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Table 1. Differentiation potential of GRP-derived O4$^+$ and O4$^-$ cells

Culture condition	O4$^+$ cells		O4$^-$ cells	
	PDGF/TH	10% FCS	PDGF/TH	10% FCS
	Number of clones that contain specific cell types/number of all scored clones			
Type-2 astrocytes only	0/15	21/22	0/29	1/21
Type-1 astrocytes only	0/15	1/22	0/29	3/21
Type-1 and type-2 astrocytes	0/15	0/22	0/29	17/21
Oligodendrocytes and progenitors	15/15	0/22	29/29	0/21

Individual GRP cell-derived O4$^+$ and O4$^-$ cells were expanded to a clonal size of 5–10 cells before being exposed to 10% FCS to induce astrocytic differentiation or exposed to PDGF and TH to promote oligodendrocyte differentiation. After 5 or 10 d, respectively, clones were stained with anti-GFAP, A2B5, and anti-GalC antibodies. In serum-containing medium, all but one of the clones derived from O4$^+$ cells contained only astrocytes with the antigenic phenotype of type-2 astrocytes (i.e., GFAP$^+$ and A2B5$^+$). In contrast, clones generated from O4$^-$ cells contained a mixture of type-1 (i.e., GFAP$^+$A2B5$^-$) and type-2 astrocytes. Although some clones derived from O4$^+$ or O4$^-$ cells contained one or two progenitor cells, none of the clones contained oligodendrocytes at that time point. All clones exposed to PDGF and TH, regardless of being derived from O4$^+$ or O4$^-$ cells, contained A2B5$^+$ progenitor cells and GalC$^+$ oligodendrocytes. None of these clones contained astrocytes.

which can generate oligodendrocytes. Is the relationship between these two populations one of lineage restriction or lineage convergence? If GRP cells and O2A/OPCs are related, what signals promote the generation of one from the other and how can the existence of both populations be integrated with existing studies on the generation of oligodendrocytes during spinal cord development?

MATERIALS AND METHODS

Cell culture. A2B5$^+$ GRP cells were isolated from embryonic day 13.5 (E13.5) Sprague Dawley rat spinal cords by positive selection on immunopanning dishes coated with A2B5 antibody (Rao et al., 1998). GRP cells were then grown in the presence of 10 ng/ml basic FGF (bFGF) and indicated supplements for various time points on fibronectin/laminin-coated coverslips at 3000 cells/well for mass culture experiments or on coated grid dishes for clonal analysis. Cultures were fed every other day with the factors indicated. At the end of the experiment, cells were stained with O4 (Sommer and Schachner, 1981) or A2B5 antibodies to detect precursor cells, anti-galactocerebroside (GalC) antibody (Gard and Pfeiffer, 1990; Gard et al., 1995) to identify oligodendrocytes, and anti-GFAP antiserum to identify astrocytes (Norton and Farooq, 1993; Morita et al., 1997; Gomes et al., 1999) followed by appropriate fluorochrome-conjugated secondary antibodies (Southern Biotechnology, Birmingham, AL). The number of cells of each type relevant to each experiment was calculated, as was the total cell number. As originally defined, GFAP$^+$ cells were scored as type-2 astrocytes if they were stellate and A2B5$^+$ and as type-1 astrocytes if they were fibroblast-like in morphology and were A2B5$^-$.

Rationale for use of the O4 antibody in analyzing generation of O2A/OPCs from GRP cells. To determine whether one cell type gives rise to another, it is useful to identify a marker that is expressed by one cell type but not by the other. This is particularly problematic for analysis of GRP cells and O2A/OPCs. Freshly isolated GRP and O2A/OPCs both label with the A2B5 monoclonal antibody. We have shown previously that GRP cells can express receptors for platelet-derived growth factor (PDGF) without losing their tripotentiality (Rao et al., 1998). Our ongoing studies have revealed that tripotential GRP cells also label with anti-GD3 and anti-NG-2 antibodies (C. Pröschel, D. Gass, and M. Mayer-Pröschel, unpublished observations). Thus, none of these markers, which have been used by many others to study development of O2A/OPCs (Hart et al., 1989; Yim et al., 1995; Nishiyama et al., 1996), allow a distinction to be made between GRP cells and O2A/OPCs.

At this stage, the only remaining candidate marker for investigating whether GRP cells can generate O2A/OPCs is the O4 monoclonal antibody (Sommer and Schachner, 1981). This antibody can be used to define a secondary stage of O2A/OPC development, in which A2B5$^+$O4$^-$ O2A/OPCs give rise to cells that are A2B5$^+$ and also O4$^+$. The great majority of O2A/OPCs isolated from the p7 optic nerve are O4$^+$ (M. Noble, unpublished observations), whereas GRP cells are O4$^-$ (Rao and Mayer-Pröschel, 1997; Rao et al., 1998). In addition, it has been shown that development of GalC$^+$ oligodendrocytes in the O2A/OPC lineage is preceded by the appearance of cells that are O4$^+$ but GalC$^-$ (Schachner et al., 1981; Sommer and Schachner, 1981; Bansal et al., 1989; Gard and Pfeiffer, 1990, 1993). Critically, O4$^+$GalC$^-$ cells isolated from many regions of the postnatal CNS, including spinal cord, are bipotential cells capable of differentiating into both oligodendrocytes and type-2 astrocytes (Trotter and Schachner, 1989; Barnett et al., 1993; Grzenkowski et al., 1999). O4$^+$GalC$^-$ cells also can be induced to proliferate *in vitro* and in this respect are not terminally differentiated (Small et al., 1987; Trotter et al., 1989; Gard and Pfeiffer, 1990; Reynolds and Wilkin, 1991; Warrington and Pfeiffer, 1992; Avossa and Pfeiffer, 1993; Barnett et al., 1993; Gard et al., 1995). Thus, although some authors have preferred to consider O4$^+$GalC$^-$ cells (isolated from postnatal animals or derived from O2A/OPCs) as more committed "oligodendroblasts," the O4$^+$GalC$^-$ cells studied thus far express those characteristics (in particular, bipotentiality *in vitro* and ability to divide) that are most important in defining a cell as being a bipotential O2A/OPC.

Clonal analysis of E13.5 GRP cell-derived O4$^+$ cells. We confirmed the differentiation potential of a cell by clonal differentiation analysis, as used in our previous studies on GRP cells (Rao and Mayer-Pröschel, 1997; Rao et al., 1998) and extensive studies on O2A/OPCs (Ibarrola et al., 1996; Smith et al., 2000); this is the only technique that allows the differentiation characteristics of individual precursor cells to be unambiguously ascertained. The basic strategy used to conduct such analyses in the present studies was as follows: GRP cells were isolated from E13.5 spinal cord as described previously and grown either for 24 hr or for 21 d in the presence of bFGF (10 ng/ml) before being exposed to the condition most effective at generating O4$^+$GalC$^-$ cells (i.e., chemically defined medium supplemented with 10 ng/ml PDGF–A chain homodimer; Peprotech, Rocky Hill, NJ) and thyroid hormone (TH; Sigma, St. Louis, MO). It is critical to note that GRP cells grown for 24 hr in FGF do not express PDGF receptor-α (PDGFR-α), whereas long-term cultured GRP cells express this receptor. We have determined that when grown in the presence of FGF, GRP cells remain tripotential regardless of their PDGF receptor status (Rao et al., 1998). After periods of additional *in vitro* growth indicated in Results, cultures were labeled with both O4 and anti-GalC antibodies, followed by appropriate fluorescein- and rhodamine-conjugated secondary antibodies. Fluorescence-activated cell sorting was then used to obtain populations of O4$^+$GalC$^-$ cells. O4$^+$GalC$^-$ cells were plated at clonal density and single O4$^+$GalC$^-$ cells were identified and circled. Cells were induced to divide for 5 d (in PDGF/bFGF at 10 ng/ml), and clones were switched to PDGF plus TH or 10% FCS when they reached a density of 5–10 cells. After 10 or 3 d, respectively, clones were stained with the A2B5, anti-GFAP, and anti-GalC antibodies. Control cells were switched to PDGF plus TH or 10% FCS without previous proliferation and stained after an additional 10 and 3 d, respectively. The results of our clonal analyses are shown in Tables 1 and 2.

Immunostaining of clones. Staining procedures were as described previously (Rao and Mayer-Pröschel, 1997). Briefly, the A2B5 and anti-GalC antibodies were grown as hybridoma supernatants (American Type Culture Collection, Manassas, VA) and used at a dilution of 1:2. The O4 hybridoma cell line was a generous gift from Ilse Sommer (University of Glasgow, Glasgow, UK), and its supernatant was also used at a 1:2 dilution. Anti-GFAP (polyclonal, rabbit anti-cow; purchased from Dako, Glostrup, Denmark) was used at a 1:100 dilution and applied overnight. All secondary antibodies [i.e., goat anti-mouse IgM-biotin, IgG3-tetramethylrhodamine B isothiocyanate, goat-anti-rabbit Ig (heavy and light chain)-FITC (Southern Biotechnology), and streptavidin (Molecular Probes, Eugene, OR)] were used at a 1:100 dilution. Anti-NG2

Table 2. NSC-derived A2B5$^+$ cells represent GRP cells

	Ventral E10.5 spinal cord-derived					Dorsal E10.5 spinal cord-derived				
Antigenic phenotypes of cells within clones	GFAP$^+$ A2B5$^+$ and GFAP$^+$ A2B5$^-$	GFAP$^+$ A2B5$^-$ only	GFAP$^+$ A2B5$^-$ only	A2B5$^+$ only	GalC$^+$	GFAP$^+$ A2B5$^+$ and GFAP$^+$ A2B5$^-$	GFAP$^+$ A2B5$^-$ only	GFAP$^+$ A2B5$^+$ only	A2B5$^+$ only	GalC$^+$
Percentage of clones (total number) after 7 d in 10% FCS/bFGF	70 (59)	0	0	30 (26)	0	83 (68)	0	0	17 (14)	0
Percentage of clones (total number) after 10 d in TH/bFGF	29 (33)	0	0	36 (40)	35 (39)	34 (23)	0	0	41 (29)	25 (18)

NSCs were isolated from dorsal and ventral regions of the E10.5 spinal cord and expanded in nondifferentiation conditions. After 3 d, dorsal and ventral cultures were allowed to differentiate, and the appearing A2B5$^+$ cells were harvested and replated at clonal density. Expanded clones were exposed either to 10% FCS or to TH to promote astrocytic and oligodendrocytic differentiation, respectively (both conditions also contained FGF). Both ventral- and dorsal-derived clones generated two types of astrocytes in the presence of 10% FCS and GalC$^+$ oligodendrocytes in the presence of TH. All clones that contained oligodendrocytes also contained A2B5$^+$ progenitor cells. We did not see any clones that contained only one type of astrocytes. The numbers shown refer to the number of clones containing different cell types but not the relative composition of the entire culture. For example, the great majority of astrocyte-containing clones in TH only had 1–5% GFAP$^+$ cells, suggesting that the presence of FGF allows some astrocyte differentiation to occur even when TH is present. Numbers in parentheses refer to the total number of clones, whereas numbers without parentheses indicate the percentage of clones in each category.

antiserum was a generous gift from Dr. W. Stallcup (Burnham Institute, La Jolla Cancer Research Center, CA) and was used at a 1:100 dilution.

RESULTS

Tripotential GRP cells, which are O4$^-$ cells, generate bipotential O4$^+$GalC$^-$ cells when grown in the presence of PDGF and thyroid hormone

The first question we addressed was whether tripotential GRP cells can generate cells with the antigenic and differentiation characteristics of bipotential O2A/OPCs. This question was investigated by a combined analysis of antigen expression and of differentiation potential at the clonal level. The requirement to use the O4 antibody (Sommer and Schachner, 1981) as a potential marker of O2A/OPCs is explained in Materials and Methods. Briefly, both GRP cells and O2A/OPCs label with the A2B5 antibody, the NG-2 antibody (Stallcup and Beasley, 1987), and the anti-GD3 antibody (Seyfried and Yu, 1985), and both populations can express PDGF receptors while maintaining their characteristic differentiation potential. Thus, of all of the markers that have been used to study the ancestors of oligodendrocytes, it was only the O4 antibody that remained potentially useful in this context. We designed experiments that would allow us to answer the following questions: (1) are there *in vitro* growth conditions that promote the generation of O4$^+$GalC$^-$ cells from O4$^-$ GRP cells, and (2) do GRP cell-derived O4$^+$GalC$^-$ cells still behave like tripotential GRP cells or do they now behave like bipotential O2A/OPCs?

We first examined the effects on GRP cells of a wide variety of conditions (see Materials and Methods) shown previously to induce generation of oligodendrocytes in cultures of O2A/OPCs. Although astrocyte-conditioned medium in combination with TH was the most effective condition for inducing the appearance of oligodendrocytes over a 3 d time period (data not shown), it was growth in the presence of FGF plus PDGF plus TH that was associated with the generation of the greatest proportion of O4$^+$GalC$^-$ cells.

In cultures of freshly isolated GRP cells that were grown for 24 hr in the presence of FGF and then additionally exposed to PDGF plus TH (with FGF still present), 78 $\pm$ 9% of the cells were O4$^+$GalC$^-$ after 3 d in culture. In addition, we noticed that 20 $\pm$ 5% of all cells were O4$^+$GalC$^+$ oligodendrocytes and a small percentage (2 $\pm$ 0.7%) of cells represented GFAP$^+$ astrocytes. We never observed the appearance of any cells that were GalC$^+$ but O4$^-$, consistent with previous observations that passage through an O4$^+$ stage is required before the expression of GalC immunoreactivity (Schachner et al., 1981; Sommer and Schachner, 1981; Bansal et al., 1989; Gard and Pfeiffer, 1990, 1993). GRP cell cultures that were grown in the presence of FGF alone contained no O4$^+$ cells, and previous studies have demonstrated that GRP cells expanded in this manner retain the ability to generate oligodendrocytes, type-1 astrocytes, and type-2 astrocytes.

Although previous studies have shown that O4$^+$GalC$^-$ cells isolated from postnatal animals or derived from bipotential O2A/OPCs are bipotential *in vitro* (Trotter and Schachner, 1989; Barnett et al., 1993; Grzenkowski et al., 1999), it cannot be assumed that such differentiation characteristics necessarily apply to O4$^+$GalC$^-$ cells derived from tripotential GRP cells. To determine the differentiation potential of GRP cell-derived O4$^+$GalC$^-$ cells, we cultured expanded GRP cells in the presence of FGF for several days, grew them in the additional presence of PDGF plus TH for 3 more days, purified the O4$^+$GalC$^-$ cells, and analyzed their differentiation potential in clonal cultures. Extending the previous expansion period in FGF in this manner resulted in a higher percentage of the cells in the culture remaining O4$^-$, thus allowing the study of this population also.

Cloned O4$^+$GalC$^-$ cells derived from GRP cells expressed the bipotential differentiation characteristics associated with O2A/OPCs. When grown in conditions that induced generation of astrocytes, O4$^+$GalC$^-$ cells derived from GRP cells exhibited the typical differentiation response of O2A/OPCs. In the presence of 10% FCS, the only astrocytes generated in 21 of 22 clones derived from O4$^+$GalC$^-$ cells were type-2 astrocytes (i.e., A2B5$^+$GFAP$^+$ stellate cells; Table 1 and Fig. 1*A*). Only one clone generated type-1-like astrocytes (i.e., A2B5$^-$GFAP$^+$ cells with a fibroblast-like morphology), a frequency low enough to be consistent with the possibility that this one clone had been mislabeled at the beginning of the experiment. This outcome was very different from that obtained with GRP cells themselves, clones of which generate a combination of type-1 and type-2 astrocytes in these conditions (Rao et al., 1998). Moreover, the O4$^-$GalC$^-$ cells that remained after the purification process were still tripotential, emphasizing that the acquisition of bipo-

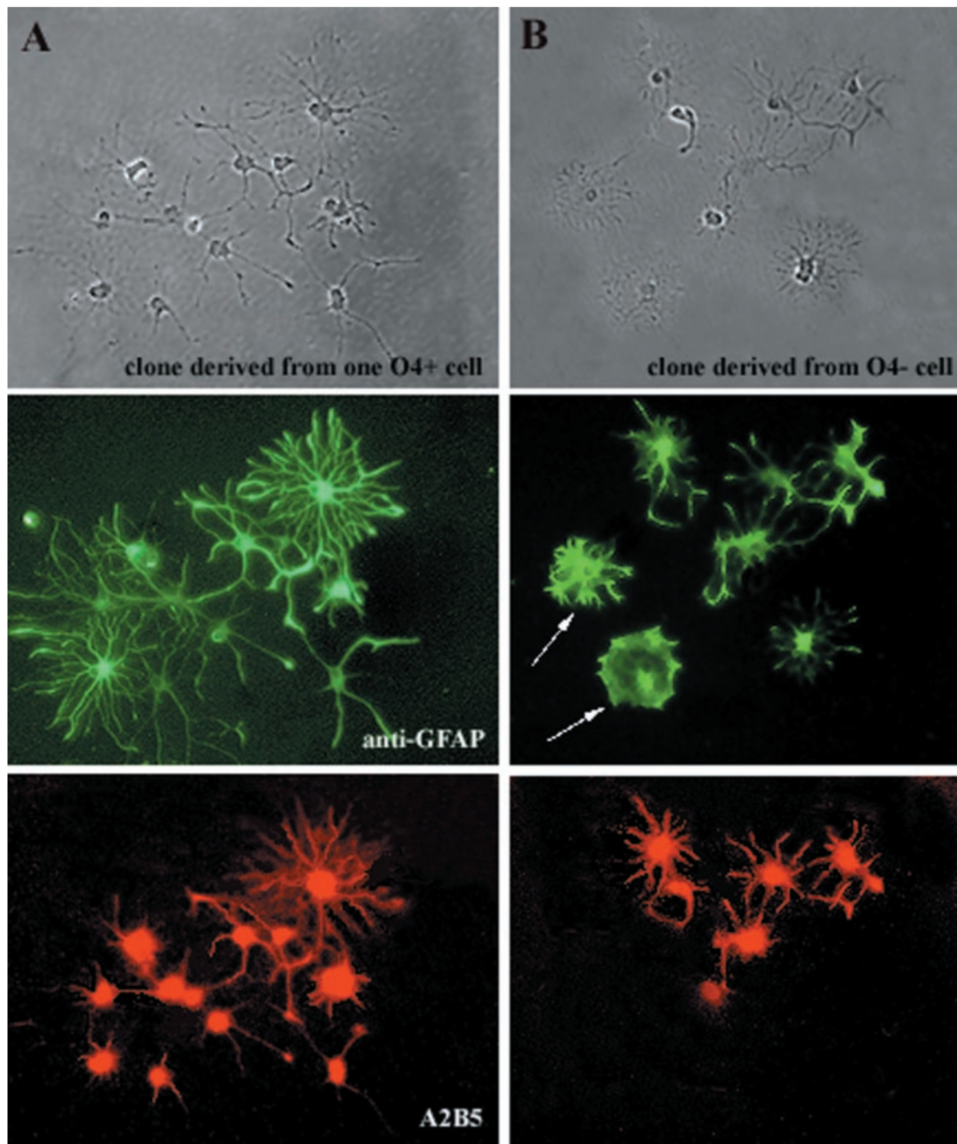

Figure 1. GRP-derived O4$^+$ cells are bipotential and represent O2A/OPC-like cells. Freshly isolated GRP cells were grown for 3 weeks in defined medium in the presence of bFGF and then switched to a medium supplemented with bFGF and TH; after 5 d, cultures were stained with the O4 antibody (see Materials and Methods). Cells were then dislodged from the surface and plated at clonal density in poly-L-lysine-coated dishes. Single O4$^+$ cells were circled. After 3 d in culture, cells were exposed to medium supplemented with bFGF and 10% FCS. (Parallel experiments using BMP4 instead of FCS yielded identical results.) After 5 d, clones were stained with A2B5 (rhodamine), anti-GFAP (fluorescein), and anti-GalC (coumarin) antibodies. The coumarin staining is not shown because none of the clones contained any GalC$^+$ oligodendrocytes in this condition. *A*, Clone derived from a single O4$^+$GalC$^-$ cell. The progeny from O4$^+$ founder cells consists exclusively of A2B5/GFAP double-positive cells, consistent with the antigenic phenotype of type-2 astrocytes. *B*, Clone derived from a single O4$^-$GalC$^-$ cell. The progeny from O4$^-$ founder cells consists of A2B5/GFAP double-positive type-2 astrocytes and A2B5$^-$GFAP$^+$ cells, representing type-1 astrocytes (indicated by *arrows*).

tentiality was a specific event and not merely associated with aging, even in conditions that promote the transition to a bipotential phenotype. When grown in the presence of 10% FCS, O4$^-$GalC$^-$ cells generated clones containing a mixture of type-1 and type-2 astrocytes (Table 1 and Fig. 1*B*), and thus behaved as GRP cells. In contrast to this difference with respect to astrocyte induction, both O4$^+$GalC$^-$ and O4$^-$GalC$^-$ cell-derived clones contained oligodendrocytes when grown in the presence of PDGF plus TH.

The simplest explanation of the data obtained in the above experiments is that GRP cells can generate O4$^+$GalC$^-$ cells that exhibit *in vitro* the defining bipotential differentiation restriction of O2A/OPCs. The results also indicate that generation of such bipotential cells is an environmentally regulated differentiation event, for which PDGF and TH represent potent inducing agents.

GRP cells can be isolated from both ventral and dorsal E13.5 spinal cord

A critical component of the current understanding of oligodendrocyte development *in vivo* is that specific precursor cells for oligodendrocytes first appear in the ventral spinal cord (Warf et al., 1991; Pringle and Richardson, 1993; Fok-Seang and Miller, 1994; Timsit et al., 1995; Hall et al., 1996; Miller, 1996; Rogister et al., 1999; Richardson et al., 2000; Spassky et al., 2000). We subsequently determined whether GRP cells are selectively localized in the ventral spinal cord at or before the time when putative oligodendrocyte precursor cells first appear ventrally. Because previous studies have shown that GRP cells are already present at E13.5 (Rao et al., 1998), we microdissected dorsal and ventral portions of the E13.5 cord to determine the regional distribution of GRP cells; this is a half day earlier than the earliest reported appearance of specific oligodendrocyte precursor cells, as defined by expression of the PDGF receptor (Hall et al., 1996). Freshly isolated cells from dorsal and ventral cord were immunolabeled with A2B5 antibody, purified by fluorescence-activated cell sorting, and plated at clonal density on grid dishes in different conditions as described below. In three independent experiments, the dorsal spinal cord consistently contained an average of 19 ± 8% A2B5$^+$ cells, whereas the ventral portion contained an average of 52 ± 7% A2B5$^+$ cells. Thus, although the ventral cord contained a higher proportion of A2B5$^+$ cells than did the dorsal cord, such cells were found in both regions of the cord.

To determine whether dorsal- and ventral-derived A2B5$^+$ cells

were GRP cells, the A2B5$^+$ clones were first grown in the presence of bFGF until they reached a size of 10–20 cells. Astrocytic differentiation was then induced by exposing cultures for 3 d to 10% FCS. All clones contained both A2B5$^-$GFAP$^+$ type-1 astrocytes and A2B5$^+$GFAP$^+$ type-2 astrocytes independent of their site of isolation. Thus, these cells were typical of GRP cells in their ability to generate two distinct astrocyte populations. Generation of oligodendrocytes was also possible with both ventral- and dorsal-derived cells, as discussed in the following section.

GRP cells derived from both the ventral and dorsal E13.5 spinal cord can generate O2A/OPCs, oligodendrocytes, and astrocytes

Because expression of PDGF receptor-α in the E14 spinal cord has been interpreted to be an indication of a preferential ventral origin of oligodendrocytes (Pringle and Richardson, 1993; Hall et al., 1996), we asked whether ventral- and dorsal-derived GRP cells differed in their ability to generate O2A/OPCs and/or oligodendrocytes. GRP cells were isolated from ventral or dorsal E13.5 spinal cord as described in the preceding section. Freshly isolated cells were plated at a low density on coverslips in the presence of FGF and exposed to conditions (PDGF plus TH) that would induce the transition into O2A/OPCs (as determined previously) or to conditions that would potentially inhibit a transition into O2A/OPCs. As a potential inhibitor molecule, we used bone morphogenetic protein-4 (BMP4), which has been shown to inhibit oligodendrocyte generation (Mabie et al., 1997; Grinspan et al., 2000; Mehler et al., 2000; Zhu et al., 2000) and is present in the embryonic neural tube (D'Alessandro and Wang, 1994; Barth et al., 1999; Grinspan et al., 2000; Liem et al., 2000). After 3 d of *in vitro* growth in the condition discussed, cells were stained with the O4 monoclonal antibody and with anti-GalC and anti-GFAP antibodies.

In the presence of PDGF and TH, cells from both the dorsal and ventral spinal cord were able to generate O4$^+$GalC$^-$ cells with equal frequencies but differed with respect to oligodendrocyte generation (Fig. 2). Specifically, 88 ± 6% of dorsal-derived cells were O4$^+$GalC$^-$, 3 ± 2% were GalC$^+$ oligodendrocytes, and 3 ± 2% were GFAP$^+$ astrocytes. In contrast, 74 ± 9% of ventral-derived cells were O4$^+$GalC$^-$, 28 ± 5% were GalC$^+$ oligodendrocytes, and 2 ± 1% were GFAP$^+$ astrocytes. Thus, although both dorsal and ventral cells were able to generate O2A/OPCs, only ventral-derived cells generated a significant number of GalC$^+$ oligodendrocytes over a 5 d time period. The lack of oligodendrocytes in dorsal cultures is not likely to be attributable to preferential cell death. because the total number of cells was not different in dorsal and ventral cultures (327 ± 8 and 326 ± 38. respectively). In addition, dorsal-derived cells demonstrated an equal ability to eventually generate oligodendrocytes. If cultures were examined after 10 d in the presence of TH, instead of after 5 d, then 69 ± 15% of the ventral cells and 73 ± 3% of dorsal cells were oligodendrocytes (data not shown).

Differences between dorsal- and ventral-derived GRP cells were also observed in response to BMP4. When dorsal- or ventral-derived GRP cells were grown in the presence of BMP4 in concentrations ranging from 1 to 100 ng/ml over 3 d, we observed that BMP4 promoted the generation of astrocytes in both dorsal and ventral cells (Fig. 3). At a low BMP concentration (1 ng/ml), ventral cells were more likely to differentiate into astrocytes than were dorsal cells (45 ± 4% vs 14 ± 4%, respectively). The preferential generation of GFAP$^+$ cells in ventral cultures was a transient phenomenon, in that only in dorsal-derived cultures did these numbers increase over the next several days (as discussed in the following paragraph). The addition of 10 ng/ml BMP had an identical effect on ventral and dorsal cells (48 ± 3% and 54 ± 7% astrocytes, respectively). The most dramatic difference between ventral and dorsal cells was observed at high BMP doses (100 ng/ml). In this condition, ventral cells responded with cell death rather than cell differentiation. In contrast, dorsal-derived GRP cells differentiated almost completely into astrocytes when exposed to 100 ng/ml BMP4.

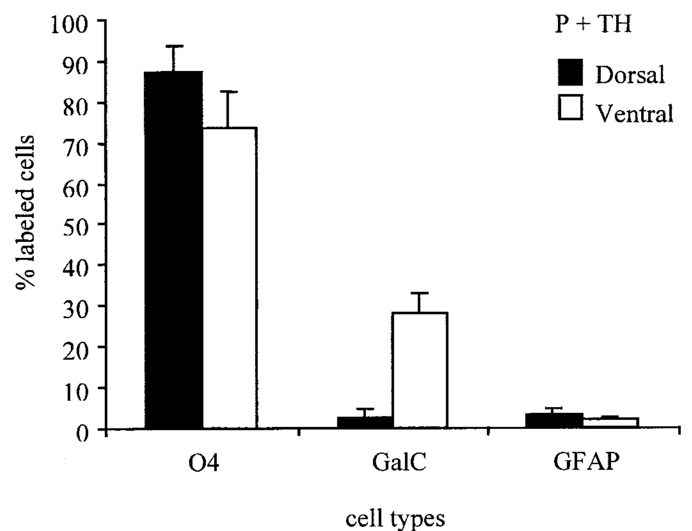

Figure 2. Both dorsal- and ventral-derived GRP cells generate O4$^+$GalC$^-$ cells. A2B5$^+$ cells were isolated from either the dorsal or ventral spinal cord of E13.5 rat embryos and plated in the presence of bFGF supplemented with PDGF plus TH (*P + TH*) for 7 d. Dorsal and ventral cultures were then stained with O4, anti-GalC, and anti-GFAP antibodies. Both dorsal- and ventral-derived cultures generated comparable numbers of O4$^+$ precursor cells. However, GalC$^+$ oligodendrocytes were found predominantly in ventral-derived GRP cell cultures. Only a small fraction of both dorsal and ventral cultures gave rise to GFAP$^+$ astrocytes. Two independent experiments examining six data points for each condition revealed comparable results.

Because BMP4 at 1 ng/ml revealed differences between dorsal- and ventral-derived GRP cells in the absence of toxicity, we subsequently examined the generation of O4$^+$GalC$^-$ cells in this culture condition (Fig. 4*A*). Cells were plated at a low density on coverslips in the presence of FGF and BMP4 (1 ng/ml) and examined after 7 d to allow for the generation of O4$^+$GalC$^-$ cells and/or GalC$^+$ oligodendrocytes. In cultures of GRP cells derived from dorsal spinal cord, the majority of cells (87 ± 8%) differentiated into GFAP$^+$ astrocytes, and only 12 ± 7% of the cells were O4$^+$GalC$^-$. We did not observe any GalC$^+$ oligodendrocytes in these cultures. In contrast, when ventral-derived cells were exposed to 1 ng/ml BMP for 7 d, 47 ± 8% differentiated into astrocytes (as observed for 3 d time point discussed previously) and 52 ± 7% of the cultures consisted of O4$^+$GalC$^-$ cells. Again, we did not observe any GalC$^+$ oligodendrocytes. Thus, BMP4 exposure was associated with a strikingly more significant decrease in the number of O4$^+$GalC$^-$ cells in dorsal- than in ventral-derived GRP cells.

We subsequently determined whether the addition of TH, a potent inducer of the generation of O4$^+$GalC$^-$ cells and/or oligodendrocytes, could counteract the effects of BMP4 (Fig. 4*B*). Dorsal and ventral cells were exposed to BMP at 1 ng/ml in the presence of TH at 50 nM for 7 d before the cultures were labeled

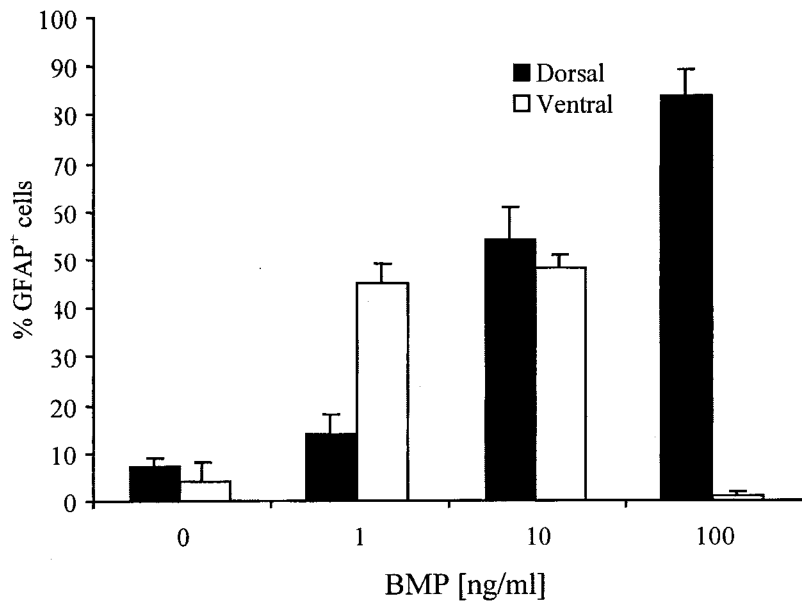

Figure 3. BMP4 induces differentiation of astrocytes from dorsal- and ventral-derived A2B5$^+$ cells in a dose-dependent manner. A2B5$^+$ cells, isolated from either the dorsal or ventral spinal cord of E13.5 rat embryos, were plated at a low density in the presence of bFGF and increasing concentrations of BMP4 (0.1–100 ng/ml). After 3 d, cultures were labeled with anti-GFAP antibodies and the number of astrocytes was determined. Whereas dorsal cultures exhibited a continuous, dose-dependent increase in the number of GFAP$^+$ astrocytes, ventral-derived GRP cells generated significantly more astrocytes at lower doses of BMP (1 ng/ml) at this time point, and higher doses of BMP4 (100 ng/ml) proved to be lethal to ventral-derived GRP cells.

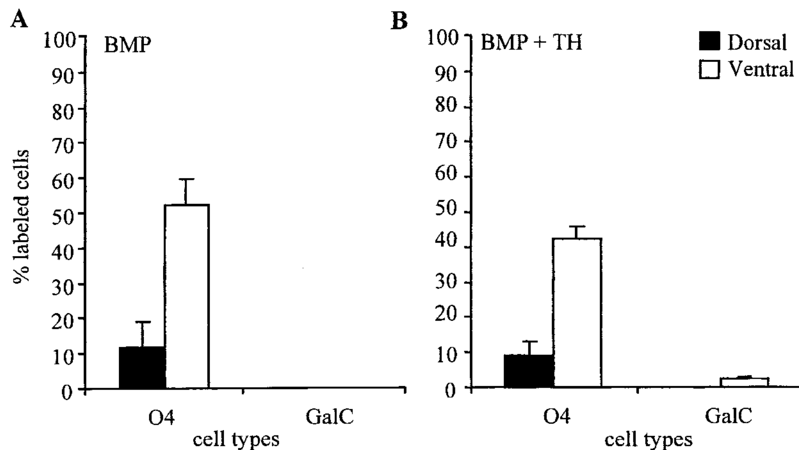

Figure 4. Differential effects of BMP4 on dorsal- and ventral-derived GRP cells. GRP cells were isolated from either the dorsal or ventral spinal cord of E13.5 rat embryos and plated at a low density in the presence of FGF and BMP4 (*A*) (1 ng/ml) or FGF, BMP4 (1 ng/ml), and TH (*B*). To allow for oligodendrocyte generation, cultures were examined after 7 d for the presence of O4$^+$GalC$^-$ precursor cells or GalC$^+$ oligodendrocytes. Although GalC$^+$ oligodendrocytes were only found in ventral GRP cell cultures containing TH, both dorsal- and ventral-derived cultures contained O4$^+$GalC$^-$ precursor cells. In the presence of BMP, the ability of dorsal GRP cells to generate O4$^+$GalC$^-$ precursor cells was lower than that of ventral-derived cultures; this was not changed by the addition of TH.

with O4, anti-GalC, and anti-GFAP antibodies. As shown in Figure 4*B*, the addition of TH had little or no effect on the generation of O4$^+$GalC$^-$ cells in both dorsal and ventral cultures. However, we did detect a small but significant increase ($p < 0.002$) in the number of GalC$^+$ oligodendrocytes specifically in the ventral-derived cells. This effect was not seen in dorsal-derived cultures.

GRP cells can be generated from dorsal and ventral neuroepithelial stem cells of the E10.5 spinal cord

Our results thus far demonstrate that there is a dorsal–ventral gradient in GRP cell distribution in the spinal cord of the E13.5 rat, and that dorsal- and ventral-derived GRP cells are dissimilar in their abilities to generate oligodendrocytes over short time periods *in vitro*. In addition, these two populations differ in their response to BMP. Because GRP cells themselves are derived from neuroepithelial stem cells (NSCs) (Rao and Mayer-Pröschel, 1997), we subsequently determined whether dorsal- and ventral-derived NSCs differed in their capacity to generate GRP cells.

In these experiments, E10.5 spinal cord [at which time point all cells are NSCs (Kalyani et al., 1997)] was microdissected into dorsal and ventral regions. Dissociated cells were plated at clonal density on fibronectin/laminin-coated grid dishes in the presence of 10 ng/ml bFGF and embryonic chick extract (CEE), a condition that prevents differentiation of NSCs (Kalyani et al., 1997). After 3 d in culture, when clones reached a size of 20–50 cells, CEE was removed to allow the clones to differentiate into lineage-restricted precursor cells (Kalyani et al., 1997; Mayer-Pröschel et al., 1997; Rao and Mayer-Pröschel, 1997). After 5 d in the absence of CEE, clones were stained with A2B5 antibody and the number of clones containing A2B5$^+$ cells was determined.

Both dorsal- and ventral-derived NSCs generated A2B5$^+$ cells with a similar efficiency. From a total number of 175 ventral-derived NSC clones, 152 (i.e., 87%) contained A2B5$^+$ cells after 5 d of *in vitro* growth. Similarly, 200 of 213 (84%) dorsal-derived NSC clones cell clones contained A2B5$^+$ cells at this time point. Analysis of the differentiation potential of A2B5$^+$ cells derived from dorsal and ventral NSCs confirmed that these cells expressed the differentiation characteristics of GRP cells (Table 2). These experiments were performed as described previously (Rao and Mayer-Pröschel, 1997). Briefly, clones were stained with A2B5 as live cells and single clones were picked and replated into grid dishes. Single A2B5$^+$ cells were marked and expanded in the presence of bFGF. After clones reached a size of 20–40 cells (5 d), they were switched to 10% FCS to generate astrocytes. After 7 d, clones were stained with A2B5 and anti-

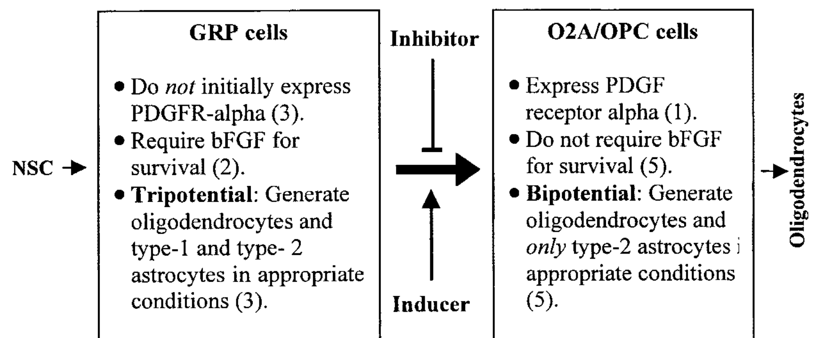

Figure 5. Sequential lineage restriction in the glial lineage of the CNS. A side-by-side comparison of the salient features of two lineage-restricted glial precursors of the CNS is shown. The evidence presented here strongly suggests a progressive and sequential transition from the tripotential GRP cell to the bipotential O2A/OPC. In the developing spinal cord, it currently seems most likely that this transition is controlled in a temporal and spatial pattern and is regulated by cell-extrinsic signaling molecules (Pringle et al., 1992; Rao and Mayer-Pröschel, 1997; Rao et al., 1998).

GFAP antibodies. All astrocyte-containing clones always contained a mixture of type-1 and type-2 astrocytes, regardless of whether they were generated in dorsal- or ventral-derived cultures and whether they were generated in response to FCS (or BMP, data not shown). A smaller number of clones consisted of A2B5$^+$ cells only, and none of these clones contained oligodendrocytes. (Table 2). In contrast, exposure of clones to PDGF plus TH for 10 d was associated with oligodendrocyte generation in all clones. Although differences were not striking, significantly more ventral clones generated oligodendrocytes than did dorsal clones over this time period (35 ± 7% vs 25 ± 2%, respectively; $p < 0.02$).

DISCUSSION

One of the essential challenges that arises with the discovery of any new precursor cell population is to determine how these cells might be integrated into (or might alter) existing views on tissue development. In the present studies on the tripotential GRP cell of the embryonic rat spinal cord, we have found that this recently discovered novel glial precursor cell can generate progeny with the antigenic phenotype and differentiation characteristics of bipotential O2A/OPCs. This process is regulated by cell-exogenous signaling molecules, with growth in the presence of PDGF plus TH being particularly effective in promoting such differentiation. In contrast to previous suggestions that putative oligodendrocyte precursor cells are localized in ventral regions of the E14 spinal cord (Warf et al., 1991; Pringle and Richardson, 1993; Fok-Seang and Miller, 1994; Timsit et al., 1995; Hall et al., 1996; Miller, 1996; Rogister et al., 1999; Richardson et al., 2000; Spassky et al., 2000), GRP cells could be isolated from both the dorsal and ventral cord of E13.5 rats. However, there were differences between dorsal- and ventral-derived GRP cells in their response to conditions that promote or inhibit generation of O2A/OPCs or oligodendrocytes, with ventral-derived GRPs exhibiting a greater propensity to differentiate along the oligodendrocyte lineage.

The demonstration that GRP cells can yield O2A/OPCs integrates these two glial precursor cell populations for the first time and indicates that their relationship is one of sequential lineage restriction rather than being independent precursors that generate oligodendrocytes. In light of our present studies, the simplest model of oligodendrocyte generation that appears to be consistent with all available data would be that production of these cell types requires the initial generation of GRP cells from NSCs followed by the generation of O2A/OPCs from GRP cells (Fig. 5). Our previous studies (Rao and Mayer-Pröschel, 1997; Rao et al., 1998) indicated strongly that GRP cells are a necessary intermediate between NSCs and differentiated glia, and our present studies raise the possibility that O2A/OPCs are a necessary intermediate between GRP cells and oligodendrocytes. Despite the fact that both GRP cells and O2A/OPCs are A2B5$^+$, it seems unlikely that the O4$^+$GalC$^-$ cells studied in our *in vitro* experiments were derived from a subset of A2B5$^+$O4$^-$ bipotential O2A/OPCs present in the original GRP cell culture. In our previous characterizations of GRP cells derived from E13.5 spinal cords, we consistently failed to find clones that gave rise exclusively to type-2 astrocytes when exposed to 10% FCS, even when cells were serially recloned three times over a period of several weeks (Rao et al., 1998). Moreover, analysis of hundreds of putative GRP cell clones thus far has failed to reveal clones that generate only type-2 astrocytes when exposed to FCS or BMPs (Mayer-Pröschel, unpublished observations). Thus, it appears that the generation of cells with the characteristics of O2A/OPCs is a differentiation event that requires exposure of GRP cells to appropriate inductive signals, such as PDGF plus TH. Moreover, we could find no GalC$^+$O4$^-$ oligodendrocytes in any conditions, which would have at least raised the possibility that oligodendrocytes might be generated directly from GRP cells. Such results are consistent with previous observations that passage through an O4$^+$GalC$^-$ stage of development is required for oligodendrocyte generation from bipotential O2A/OPCs (Gard and Pfeiffer, 1990, 1993; Gard et al., 1995). Our data are also consistent with other studies indicating that O4$^+$GalC$^-$ cells are bipotential (Trotter and Schachner, 1989; Barnett et al., 1993; Grzenkowski et al., 1999).

It remains formally possible that GRP cells might be able to generate oligodendrocytes without passage through an intermediate O2A/OPC stage, or that NSCs could generate O2A/OPCs without going through a GRP cell stage. Nonetheless, it is important to stress that no data exist to support the possibility that O2A/OPCs are directly generated from NSCs or that oligodendrocytes are directly generated from either NSCs or GRP cells. Thus, the developmental pathway we suggest is at present the only one supported by experimental observations.

It is of particular interest to find that ventral-derived GRPs seem to differ from dorsal cells in such a manner so as to have an increased probability to generate O2A/OPCs and/or oligodendrocytes, even in the presence of BMP. Thus, it may prove necessary not only to study GRP cells but also to focus attention on ventral-derived GRP cells to understand the mechanism of action of those factors that eventually lead to oligodendrocyte generation. It will be of considerable interest to determine whether these differences are intrinsic to ventral- or dorsal-derived GRP cells or are acquired as a consequence of exposure to particular environmental signals. It also will be of interest to determine whether the O2A/OPCs generated from dorsal and ventral GRP cells themselves differ in their responsiveness to

inducers of oligodendrocyte generation, an interpretation that would be consistent with our data (Fig. 4*B*). In addition, our observation that the responsiveness of GRP cells to PDGF plus TH as promoting signals of O2A/OPC and oligodendrocyte generation may decrease with increased GRP cell expansion *in vitro* is reminiscent of our previous findings that O2A/OPCs expanded for continued periods become less responsive to PDGF as a mitogen (Bogler et al., 1990). Although the biological implications of this observation with respect to GRP cell biology require additional investigation, this result does emphasize the importance of expanding precursor cell populations *in vitro* as minimally as possible in studies on the function of exogenous signaling molecules.

It is important to consider the question of whether all previous studies attempting to define the early origin of the oligodendrocyte lineage have in fact been describing early differentiation events affecting GRP cells. It is clear from our previous work that GRP cells can express the PDGFR without losing their tripotential character (Rao et al., 1998). In addition, our ongoing work (Pröschel, Gass, and Mayer-Pröschel et al., unpublished observations) is demonstrating that GRP cells can also be NG-2$^+$ and GD3$^+$, two other antigens that have been used in studies on O2A/OPCs (Mayer-Pröschel, unpublished observations). Moreover, it currently appears that GRP cells are the dominant (if not exclusive) A2B5$^+$ cell population in the spinal cord until as late as E17 (Mayer-Pröschel, unpublished observations). Thus, it is beginning to seem likely that events such as expression of PDGFR in ventral A2B5$^+$ cells may reflect a differentiation process in GRP cells rather than the transition to being an O2A/OPC. Analyzing the early stages of generation of O2A/OPCs from GRP cells, whether *in vitro* or *in vivo*, will require identification of a marker that can be used to antigenically distinguish GRP cells from the A2B5$^+$O4$^-$ stage of O2A/OPCs. As indicated, none of the markers currently available seem to enable this distinction.

The field of developmental neurobiology is in the early stages of determining the relationship between different lineage-restricted precursor cells in the CNS, and our present experiments represent a critical step in determining whether GRP cells may be the ancestors of all glial populations of the spinal cord. Our present observations are consistent in two ways with such a suggestion. First, if this hypothesis were to be correct, then GRP cells should be able to give rise to O2A/OPCs (as we have found). We also would anticipate that GRP cells would be found in both the dorsal and ventral cord, although they may generate different progeny in these two regions. In future studies, it will be important to discover whether precursor cells with the properties of GRP cells also exist in other regions of the CNS. In addition, it will be important to determine whether other progeny of GRP cells include the A2B5$^+$ astrocyte precursor cells present in embryonic (E17) spinal cord and originally described by Fok-Seang and Miller (1992, 1994), the putative astrocyte precursor cells from the embryonic mouse cerebellum described by Seidman et al. (1997), the astrocyte precursor cells described by Mi and Barres (1999), or the pre-O2A progenitor cell described by Grinspan et al. (1990). In addition, it is of importance to determine whether the developmental inter-relationships that seem to exist in the spinal cord also apply to development of the brain. By identifying the relationship between these developmental pathways and the signals responsible for these transitions, we will move closer to a comprehensive understanding of glial development in the CNS.

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