Progenitors that express NG2-proteoglycan are the predominant self-renewing cells within the CNS. NG2 progenitors replenish oligodendrocyte populations within the intact stem cell niche, and cycling NG2 cells are among the first cells to react to CNS insults. We investigated the role of NG2 progenitors after spinal cord injury and how bone morphogen protein signals remodel the progressive postinjury (PI) niche. Progeny labeled by an NG2-specific reporter virus undergo a coordinated shift in differentiation profile. NG2 progeny born 24 h PI produce scar-forming astrocytes and transient populations of novel phagocytic astrocytes shown to contain denatured myelin within cathepsin-D-labeled endosomes, but NG2 progenitors born 7 d PI differentiate into oligodendrocytes and express myelin on processes that wrap axons. Analysis of spinal cord mRNA shows a temporal shift in the niche transcriptome of ligands that affect PI remodeling and direct progenitor differentiation. We conclude that NG2 progeny are diverse lineages that obey progressive cues after trauma to replenish the injured niche.

Introduction

The microenvironments that regulate stem cells/progenitors serve to maintain homeostasis in adult tissue. The support cells and molecular cues within these microenvironments comprise the niches that instruct progenitors within the CNS (Jordan et al., 2007; Ninkovic and Götz, 2007). Although the importance of niches has been realized, little information has been presented to describe how injuries feedback and stimulate progenitors, or how cellular deficits are replenished within the damaged nervous system to facilitate niche succession (Fuchs et al., 2004).

Despite limited posttraumatic regeneration, the spinal cord is known to contain endogenous neural progenitor cells (NPCs) that support plasticity in the mammalian CNS (Weiss et al., 1996; Johansson et al., 1999; Namiki and Tator, 1999; Horner et al., 2000; Shihabuddin et al., 2000). Stem cell niches have been characterized for several CNS regions (Doetsch et al., 1999; Palmer, 2002; Kempermann et al., 2004). Within these regions, niche succession is characterized by asymmetric cell division of stem cells to produce one stem cell and one daughter cell. The daughter cell progresses to differentiate terminally and replenish tissue requirements as directed by the intact niche (Alvarez-Buylla and Lim, 2004). Pigment epithelium (Ramirez-Castillejo et al., 2006) promotes self-renewal, whereas sonic hedgehog (Shh) (Ahn and Joyner, 2000) and bone morphogen protein (BMP) (Lim et al., 2000) signals maintain stemness as molecular cues evolve within the neural stem cell (NSC) niche to direct differentiation within neurogenic versus gliogenic regions (H. Song et al., 2002; H. J. Song et al., 2002; Shen et al., 2004). Thus, the NSC niche is a refined microenvironment evolved to facilitate communication between tissues and stem cells to sustain homeostasis. Analogous processes occur in hematopoiesis, hair follicle, and germ cell niches. Yet, a stem cell niche has not been defined within the spinal cord, despite the observation of proliferative zones (Johansson et al., 1999; Horner et al., 2000).

Spinal cord injury (SCI) produces a structural perturbation to the homeostasis of a NSC niche known to stimulate the proliferation of reactive cells and produce an inhibitory scar that restricts regeneration (Silver and Miller, 2004). Numerous studies have examined how injuries to the brain or spinal cord affect NSCs and how NSCs respond to growth factors injected into the CNS after injury. Although informative, intervention studies fail to examine how the niche evolves postinjury (PI) to rebuild damaged tissue: the process of PI niche succession. To investigate how a progressive niche recovers after injury, we targeted CNS progenitors marked by NG2-proteoglycan expression. Cycling cells that express NG2 are the most abundant progenitor in the rodent CNS (Horner et al., 2002; Dawson et al., 2003), and NG2 cells are known to respond to injury and are thought to be restricted oligodendrocyte precursor cells (OPCs) (Levine and Reynolds, 1999). However, a clear impediment to conducting lineage studies within the injured niche is the dramatic deposition of proteoglycans throughout the injury zone making the determination of the cellular source and the phenotypic fate of NG2-expressing cells equivocal. Therefore, we engineered a retroviral reporter virus to specifically target NG2-expressing cells to determine how traumatized microenvironments feedback to instruct progenitors after injury.
Materials and Methods

SCI (hemisection) and tissue processing. Adult mice (8 weeks) were anesthetized with avertin (12.6% tribromo-ethanol, 0.6% tert-amyl alcohol; Sigma) to perform a T9–T10 laminectomy. After exposure of the dorsal cord, iridectomy scissors were used to create a hemisection lesion by cutting the dorsal spinal cord tissue until the central canal could be visualized (~300 μm deep). After a successful lesion, the muscle and skin were sutured in layers to close the wound. Postoperatively, lactated Ringer’s solution was administered subcutaneously to rehydrate the animals. Analgesics (0.05 mg/kg buprenorphine) and antibiotics (4.8 μg/g gentamicin; Abaxis Pharmaceutical Products) were administered as needed, and animals recovered from surgery in warmed cages.

To harvest spinal cords, anesthetized mice were killed via intracardiac perfusion. Animals were exsanguinated with saline and fixed with 4% paraformaldehyde (containing 4% sucrose). Perfused tissue was equlillated in 30% sucrose for cryoprotection in OTC medium and serially sectioned (1.6, 20 μm coronal/cross sections) with a Leica SM1850 cryostat.

For BMP ligand injection and progenitor transplantation, similar methods were followed. Each study was coded and blinded. BMP4 and noggin (carrier free; R&D Systems) were administered by intraspinal injection (1 μg/g H9262 injection administrated by intraspinal cryostat.

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Table 1. Real-time PCR primer sets and amplicon size

| Gene          | 5′ primer sequence | 3′ primer sequence | Base pairs |
|---------------|--------------------|--------------------|-----------|
| noggin        | cagggcgccgagcatctt | ctgctgaggaagctcgc | 185       |
| BMP4          | gttgtagaaccggaggt | tggtaggaagctagaga | 131       |
| Olig1         | gaaacggcctgcgcgac | gagcggatcttcggcc  | 178       |
| Olig2         | cagactcgctagccgta | gqgctactcgctgctt | 168       |
| P0GFlx        | cagctctgagcagagcc | gacctactcaacctcg | 143       |
| Nkx2.2        | gaaagagctggctcgcgc | gaggccctcgctggcca | 180       |
| BMP2          | cggctgtgccgctccca | gcggctggctgccct  | 187       |
| GAPDH         | gggagaaacctcctac | gagggagatagctgctg | 161       |
| lid1          | ctctagtgctctgaggg | aagcctctcgctggca | 180       |
| lid2          | caggctgagcggtctag | tggtaagcagacatctg | 99        |
| lid3          | cgcctggcggcgtctc | gaggattccacgctga | 114       |
| Shh           | tcaaaacactctggaaggatt | gatgtccactgcctaggc | 248       |

Data. For all statistical analyses, significance was accepted at a p value of 0.05 and lower for a one-tailed t test.

**Results**

Cycling NG2\(^+\) cells produce progeny that differentiate into multiple cell types

Ectopic gene expression by promoter-specific retroviruses has been used for decades to trace cell lineages (Brand and Perrimon, 1993; Golden et al., 1995; Cepko et al., 1998). Since cells that express NG2-proteoglycan divide after CNS insult, the regulatory sequence upstream of the NG2 protein-coding sequence was cloned (GenBank accession number DQ241507) (supplemental material, available at www.jneurosci.org) and used to construct a bicistronic retrovirus. The NG2-reporter virus was engineered such that the NG2 promoter sequence regulated the expression of GFP (EGFP), whereas the generic viral (LTR) promoter regulated AP expression. Therefore, EGFP expression marks cells that actively express NG2-proteoglycan, AP marks dividing cells generically, and cycling NG2\(^+\) cells are analyzed (EGFP\(^+\)) and compared against other progenitors (EGFP\(^-\)) that respond to traumatic injury (Fig. 1A). In vitro analysis showed 91% of NG2\(^+\) cells coexpress GFP after infection (Table 2).

An intraspinal injection of high-titer NG2-reporter virus (\(≥1 \times 10^9\) infection particles per milliliter) was injected rostral to a midthoracic hemisection of the spinal cord at 24 h PI, and the spinal cord tissue was harvested 3 d PI (Fig. 1B). The specificity of the reporter virus was verified by colocalization of NG2-proteoglycan on EGFP\(^+\) cells and quantified as a percentage of EGFP-labeled cells (Fig. 1C). NG2-proteoglycan was colocalized on >95% of EGFP-labeled cells (>100 cells per animal; n = 4) (Fig. 1E). Therefore, colocalization of NG2 immunofluorescence, with EGFP, demonstrated stringent regulation of EGFP expression by the cloned NG2 promoter.

Since the NG2-reporter virus showed stringent regulation of EGFP expression, the NG2 promoter virus was used to investigate the fates adopted (molecular phenotype) by cycling NG2\(^+\) cells after SCI, in vivo. As shown previously, NG2 expression marked a large majority of dividing cells that respond to injury (Levine and Reynolds, 1999). EGFP was expressed by 32.6% of virally labeled cells (Fig. 1D). The proportion of EGFP\(^+\) AP\(^-\) versus AP\(^+\) colocalization with GFAP (glial lineage), Iba1 (microglial fate), and von Willebrand Factor (vWF; vascular fate) (Fig. 1F) was quantified to determine the phenotype of NG2\(^+\) versus NG2\(^-\) progenitors. Surprisingly, GFAP\(^+\)/EGFP\(^+\) cells comprised 15.1 ± 2.4% of the cells transfected (Fig. 1G) (five animals) by the reporter virus (Fig. 1G, green bars).

Historically, NG2-proteoglycan is believed to mark a single population of cycling progenitors, and NG2\(^+\) progenitors are postulated to be unipotent OPCs that remyelinate axons after injury (Keirstead et al., 1998). Previous studies have also correlated evidence of NG2 coexpression with GFAP\(^+\)/bromodeoxyuridine (BrdU)-positive cells (Levine and Nishiyama, 1996; Alonso, 2005) to suggest NG2 progenitors produce GFAP\(^+\) cells. However, direct evidence that cycling NG2\(^+\) cells participate in remyelination or astrogial production (or both) has yet to be established (Polito and Reynolds, 2005). Therefore, by labeling dividing cells 24 h after SCI, our experiments demonstrate immunofluorescent colocalization of phenotypic markers for astrocytes (GFAP, microglia (Iba1), and pericytes (vWF) on separate EGFP\(^+\) cytoplasmics. Since the stringency of the virus demonstrated >95% coincident EGFP–NG2 colocalization, these data reveal that cycling cells with an active NG2 promoter express molecular markers of multiple cell types. Therefore, newborn
cells with an active NG2 promoter are not fate-restricted OPCs and thus raise two possible conclusions: cycling NG2 cells are multipotent neural progenitors or NG2-proteoglycan labels several different unipotent progenitors whereby NG2-proteoglycan would not mark a single lineage. Regardless, these results demonstrate that in the setting of injury, NG2 expression is not limited to a population of fate-restricted OPCs.

Vacuolated NG2 progeny contain myelin debris within endosomes

Since EGFP expression labels the cell soma, the NG2-reporter virus was used to track NG2 progenitors after injury via GFP expression (C) and labeled ~33% of infected progenitors (D) with >95% of EGFP-expressing cells coexpressing NG2-proteoglycan (E). EGFP expression evaluated the phenotypes of cycling NG2 progeny. F, Confocal microscopy was used to determine the molecular phenotypes of progeny derived from NG2 progenitors via colocalization of EGFP with Iba1 (microglia marker), vWF (blood-vascularity pericytes), and GFAP (glial marker). Scale bar, ~10 μm. G, The virally labeled cell phenotypes were quantified as a function of AP or GFP expression (3024 cells) in adjacent serial sections stained with vWF, Iba1, and GFAP. Twenty-four hours PI, the majority of cycling NG2 cells display a reactive phenotype (GFAP+ or Iba1+), and NG2 progenitors comprise almost 50% (combined) of reactive phenotypes (AP+Iba1+ and NG2+Iba1+ vs AP+ and NG2+GFAP+; microglia and astrocytes, respectively). Error bars represent SEM.

**Table 2.** The specificity of transgene expression by the NG2 promoter was examined by retroviral infection of adult spinal cord progenitors (aSCPs) in vitro

| Transgene | Efficiency (% infected) | Specificity (% of NG2+) |
|-----------|-------------------------|------------------------|
| GFP       | 82.5 ± 10.63            | 91.8 ± 3.73            |
| CRE       | 45.1 ± 9.67             | 91.2 ± 5.12            |

aSCPs were infected with an NG2-reporter virus (10⁶ cfu/ml). Three days after infection, transgene expression was interrogated by immunofluorescence. The specificity of the NG2-promoter viruses was quantified by colocalization of GFP or CRE recombinase on NG2+ cells.
Phagocytes that are phagocytic in nature typically express markers such as Iba1 or EGFP. Interestingly, myelin debris was observed within the vacuoles of the vacuolated NG2 progeny, and a secondary stain to label the membrane of the vacuoles was performed. Confocal microscopy revealed a subset of cycling NG2+ cells that did not express GFAP (A, arrow) and possessed large vacuoles (~5 μm) within the cell body (B, arrows; inset in A). C, Vacuolated EGFP+ cells colocalized with s100β immunofluorescence but did not express ED1 (asterisk). D, Adjacent serial sections show the vacuolated s100β+ EGFP+ cells (cyan arrow) are a morphologically distinct population of phagocytic cell within the lesion, which are not Iba1+ (E, arrow). An orthogonal view of a confocal z-stack shows that vacuolated s100β+ EGFP+ cells (cyan arrows) do not label with other markers for phagocytic microglia (Iba1; magenta arrow). F, Immunofluorescence with antibodies against dMBP (MBP 81–92) revealed the s100β+ progeny (EGFP+), within the lesion epicenter, are a phagocytic phenotype and contained denature MBP within their cytoplasm. G, An orthogonal view, rotated about the z-axis (inset in F), clearly shows the MBP contained within numerous EGFP+ cell processes (arrows). H, The vacuolated s100β+ phenotypes do not persist outside the lesion epicenter where less denatured myelin is present (2 mm rostral; arrow) and are distinct from activated Iba1+ microglia. I, Immunofluorescence of CathD shows colocalization (cyan arrows) of the endosome protein in cells that contain dMBP (yellow arrow). J, The rotated view of a confocal z-stack (inset in F) shows CathD localization on vacuoles and EGFP+ processes (cyan arrows) that flank dMBP. Inset, Monochrome breakouts show distinct localization of dMBP within an EGFP process decorated with CathD (asterisks, arrows). Scale bars, ~10 μm.

Confounded by the presence of a vacuolated nonmicroglial cell, we examined the lesion epicenter with antibodies against denatured myelin (MBP 81–92) (Gibson et al., 1984; Martenson, 1984), which labeled a sea of debris throughout the epicenter. Interestingly, myelin debris was observed within the vacuoles of the EGFP+s100β+ cells (Fig. 2F, G, arrows). Confocal optical fractionation showed intensely labeled bundles of myelin debris contained within vacuoles circumscribed by EGFP+ cell bodies (Fig. 2G, arrows). Whereas nonvacuolated EGFP+s100β+ cells did not colocalize with MBP 81–92 rostral to the lesion site, nor did the cells possess large intracellular vacuoles (Fig. 2H).

Recently, a published astrocyte-transcriptome showed components of the endocytosis pathway (draper/Megf10 and Mertk) to be upregulated in astrocyte populations (Cahoy et al., 2008). Based on these findings, Cahoy et al. (2008) hypothesized that astrocytes are professional phagocytes. To determine whether the vacuolated NG2-progeny possessed characters of phagocytes, tissue was stained with antibodies against the endosome/lysosome protein cathepsin-D (CathD) (Westley and Rochefort, 1980) and denatured myelin basic protein (dMBP). As revealed by confocal microscopy, EGFP+ NG2 progeny do possess vacuoles decorated with CathD (Fig. 2J, cyan arrows). The CathD+ structures are present on distinct cytoplasmic vacuoles that contain dMBP, and CathD is seen on processes surrounding a large dMBP structure (Fig. 2J, asterisk). Therefore, EGFP+ NG2 progeny differentiate into a class of phagocyte that is an NG2+s100β+ astrocyte. Interestingly, alternate birth-dating studies (by virus injection at 3 and 7 d PI) did not label a population of cells with the phagocytic morphotype. In contrast, the population of phagocytic cells was prominent around the lesion epicenter when labeled 24 h PI.

CNS insults are known to induce reactive astrocytes, which provide trophic support to surrounding cells and facilitate recov-
EGFP⁺s100β⁺Casp3⁺ colocalization was quantified and used to determine the percentage of apoptotic cells at 3 d versus 7 d PI (Fig. 3A, B). In general, s100β⁺NG2⁺ progeny born 24 h PI show a significant increase (p = 0.027) in activated Casp3 between 3 and 7 d PI. The percentage of apoptotic EGFP⁺s100β⁺ cells increased from 3.6 ± 0.46% to 9.2 ± 1.87% (Fig. 3C) (n = 3 and 4, respectively), in conjunction with an overall increase in apoptotic cells in the same time course, from 16.77 ± 3.6% to 36.97 ± 4.4% Casp3⁺ (Fig. 3C) (p = 0.012; n = 4). The progressive increase (from 3 to 7 d) in cells marked by Casp3⁺ indicates that the s100β⁺NG2⁺ progeny born at 24 h PI is a transient population and short-lived in the PI environment. However, the interpretation that s100β⁺NG2⁺ progeny born at 24 h are transient is confounded by the lack of change observed in the EGFP⁺s100β⁺ cells between 3 and 7 d PI. Despite the increased number of cells with an activated Casp3, the EGFP⁺s100β⁺ population is sustained. Therefore, a single population of labeled cells could proliferate and persist within the lesion.

NG2 progenitor differentiation profiles shift temporally as SCI repair progresses

SCI tissue is known to undergo temporal–spatial changes in cell replacement as recovery progresses after injury (Wu et al., 2005; Zai and Wrathall, 2005; Lytle and Wrathall, 2007). As such, the microenvironmental changes that evolve PI define a progressive stem cell niche. Since the NG2-reporter virus showed cycling NG2 cells produce multiple cell types, we wanted to determine whether progeny fates, as a population, shift as the PI niche recovers/regenerates. Therefore, the differentiation profile of progeny derived from NG2 progenitors born at 24 h and 7 d PI was quantified at 3 and 14 d PI, respectively. Dividing cells were birth-dated with birthdate and correlate with temporal changes in a posttraumatic niche. Therefore, temporal–spatial changes in the stem cell niche would manifest changes in progenitor differentiation profiles as the niche recovers/regenerates, and thus define a progressive niche.

The temporal shift in NG2 progenitor differentiation was examined as a function of phenotype among cycling cells labeled by the NG2 virus, for cells born 24 h PI (analyzed 3 d PI) and cells born 7 d PI (analyzed 14 d PI) (Fig. 4A). Confocal microscopy was used to evaluate GFAP and APC expression in EGFP⁺ cells born 24 h versus 7 d PI. Relative quantification of the molecular phenotypes show the majority of cycling NG2⁺ cells born 24 h PI adopt an astroglial fate 3 d PI (EGFP⁺GFAP⁺ expression,
Figure 4. Progeny from cycling NG2 \(^+\) cells show temporal shifts in differentiation profile after SCI. A, The NG2-reporter virus was used to label cycling NG2 progenitors at 24 h PI or 7 d PI. The phenotypes of the labeled cells were examined at 3 and 14 d PI. B, Fifty-three percent of the progeny derived from NG2 cells born 24 h PI produce astrocytes (GFAP \(^+\)) compared with 24% APC \(^+\) immature oligodendrocytes. The direction of phenotype undergoes a significant temporal shift (***\(p < 0.001\)) to produce more oligodendrocytes (53%) and fewer (35%) when the progenitors are born 7 d after SCI and evaluated 14 d PI. Error bars represent SEM. C, The astrocyte progeny (born 24 h PI) are seen within the lesion epicenter expressing a gliotic phenotype (GFAP \(^+\) and CSPG \(^+\)). D, A high-resolution z-stack (tilted orthogonal view in inset in C) shows distinct colocalization of GFAP with EGFP \(^+\) processes in cells born 24 h PI (arrows). Inset, Color channel-breakouts highlight the morphology and localization of CSPG on GFAP \(^+\) cells. E, Progeny derived from progenitors cycling at 7 d PI express MBP by 14 d PI. F, A high-resolution z-stack (tilted orthogonal view in inset in E) shows distinct colocalization of MBP with EGFP \(^+\) processes (arrows) in cells born 7 d PI that encircle NF \(^+\) profiles. Color channel-breakouts show an NF \(^+\) profile circumscribed by an EGFP \(^+\) MBP \(^+\) process. Scale bars, 10 \(\mu m\).

Progenitor Fates in Progressive Niche

| Molecular Phenotype | Population (Percent of Total GFP\(^+\)) |
|---------------------|---------------------------------------|
| GFAP\(^+\)          | 0–25%                                 |
| APC\(^+\)           | 25–50%                                |
| GFAP\(^+\)          | 50–75%                                |
| APC\(^+\)           | 75–100%                               |

In contrast, a significant shift in phenotype is seen in NG2 \(^+\) progenitors cycling 7 d PI. Whereas acutely born cells adopt a reactive phenotype, NG2 progeny with a 7 d PI birthdate do not display a reactive profile after differentiation. Instead, the population dynamic of the progeny fates (derived from progenitor) undergoes a shift. Among NG2 progeny born 7 d PI, the progeny possess characters of early immature oligodendrocytes and express molecular markers of cells destined for an oligodendrocyte lineage (52.6%, APC\(^+\) versus the astroglial lineage (33%, GFAP\(^+\)) (Fig. 4B). When examined at 14 d PI, confocal micrographs of progeny derived from NG2 \(^+\) progenitors born 7 d PI show these cells coexpress MBP (Fig. 4E). When counterstained with antibodies against neurofilaments (NFs), EGFP\(^+\) MBP\(^+\) profiles are shown to encircle NF\(^+\) structures in the dorsal columns of hemisectioned spinal cords (Fig. 4E,F, arrows). The presence of MBP on EGFP\(^+\) processes demonstrates that progeny of NG2 progenitors are able to produce oligodendrocytes, which have the potential to participate in remyelination; however, additional studies that examine myelin profiles are needed to provide unequivocal evidence that NG2 progeny remyelinate axons after injury. Regardless, the temporal shift in adopted fates demonstrates that NG2 \(^+\) progenitors produce multiple cell types after injury. Furthermore, the fates adopted by endogenous cycling NG2 \(^+\) cells are influenced by temporal and spatial cues within the progressive niche, and thus the product of the progeny is contingent on the nurturing derived from the PI niche.

Instructive cues with the PI niche evolve to direct progenitor differentiation

Behavioral recovery is known to be minimal within the first 7 d after SCI. However, in the second and third weeks (7–21 d PI), animals show the greatest changes in behavioral recovery (Ma et al., 2001). Since we observed a temporal shift in the differentiation profiles of cycling endogenous NG2 \(^+\) progenitors that parallels the progression in behavioral recovery seen in SCI animals, we hypothesized that instructive cues within the niche must evolve temporally to promote astrocyte and oligodendrocyte differentiation. Therefore, we examined the transcriptional profile of molecules that direct BMP signaling and oligodendrogenesis in the SCI niche. After exsanguinations, mRNA was isolated from a 1 mm block of tissue (rostral edge of the lesion and T9 from controls). Quantitative real-time PCR was used to examine the transcriptional profile of each gene set at 24 h PI and 7 d PI (relative to GAPDH expression) to determine whether changes in the molecular cues precede concomitant temporal shifts in progeny fates after SCI.

The antagonistic action of BMP and noggin control glial differentiation (astrocytic versus oligodendrocytic fate, respectively) throughout development, and noggin maintains stemness within neurogenic regions of the adult brain (Lillien and Raphael, [52x41]ditional studies that examine myelin profiles are needed to pro-
The progression of the instructive niche from a pro-proliferation to pro-differentiation between 24 h and 7 d PI, respectively, indicate a potential niche progression to explain the observed shift in the fate of NG2⁺ progeny toward oligodendrocyte production. Previous studies have demonstrated how NG2 progenitors that express PDGFRα receptor produce oligodendrocytes and that NG2 cells must divide and differentiate to facilitate recovery after demyelination lesions (Keirstead and Blakemore, 1999; Hampton et al., 2004). In addition, the expression and subcellular localization of basic helix–loop–helix transcription factors (Olig1 and Olig2) are known to regulate oligodendrocyte differentiation to meet repair demands (Arnett et al., 2004). Therefore, the expression profile of Nkx2.2, Olig1, Olig2, and PDGFRα was evaluated to determine whether an increase in pro-oligodendrocyte cues accompanied the observed changes in differentiation cues (Id and Shh) within the instructive niche.

Real-time PCR experiments revealed reduced Olig1 (0.39-fold; p < 0.01) and PDGFRα (0.66-fold; p < 0.05) expression in the acute PI niche (24 h PI) (Fig. 5B). Expression levels recovered, and by 7 d PI, Olig1 and PDGFRα expression mirrored expression levels with the intact niche (control). However, Nkx2.2 and Olig2 expression did not change significantly within the PI niche at 24 h or 7 d PI. Combined, the observed changes in cues that affect proliferation and/or differentiation highlight changes that evolve within the progressive niche to orchestrate oligodendrocyte differentiation PI, in accord with the state of repair.

BMP signals modulate gliogenesis in the PI niche

Since BMP4 transcription showed persistent upregulation after injury, and since other studies have shown that BMP signaling maintains the subventricular zone stem cell niche (Lim et al., 2000), experiments were designed to examine how BMP signals affect the PI niche. To augment signaling, BMP4 (50 μg/ml) was injected into the 24 h PI lesion to supplement BMP4 concentrations and mimic the 7 d PI BMP transcriptional state. In the 7 d PI lesion, BMP signaling was antagonized by a single injection of noggin (50 μg/ml). Therefore, BMP4 protein would be upregulated early and suppressed 7 d PI, to oppose the PI niche (control). BMP4 injection into the SCI lesion 24 h PI significantly reduced the number of astrocytes and oligodendrocytes (APC⁺ cells) was reduced significantly (p = 0.021) in BMP4-injected animals. noggin increased astrogliogenesis (GFAP⁺) but decreased overall numbers of oligodendrocytes (APC⁺) in animals injected 7 d PI. Cell proliferation was not affected by BMP4 or noggin (statistics determined by one-tailed t test; error bars represent SEM).

2000; Lim et al., 2000). Interestingly, noggin expression is only slightly diminished at 24 h PI and increased at 7 d PI, but noggin expression does not differ significantly from levels seen within the intact spinal cord. In contrast, BMP4 expression is increased 2.57-fold (Fig. 5A) (p < 0.05) at 24 h PI and 8.9-fold 7 d PI (Fig. 5A) (p < 0.01).

BMPs are thought to regulate stemness by regulating the expression of inhibitors of differentiation and factors that promote progenitor self-renewal (Alvarez-Buylla and Lim, 2004). Since the upregulation of BMP4 persisted between 24 h and 7 d PI (Fig. 5A) (111 p < 0.0001), we quantified the secondary effects on the expression profile of inhibitors of DNA binding/differentiation (Id transcription factors) and Shh (which promotes self-renewal). Significant genomic shifts are observed for both Id1 and Id3. Id1 is elevated 4.1-fold (p < 0.05) 24 h PI and returns to control levels by 7 d PI, whereas Id3 does not become elevated until 7 d PI (3.6-fold; p < 0.05). Changes in Id2 expression are modest (1.6-fold at 24 h PI) and do not vary significantly from controls, and Shh expression is reduced to 0.23-fold at 7 d PI (Fig. 5A) (p < 0.01). Since Ids are known to affect stem cell fate (Ying et al., 2003), the expression profile suggests a possible mechanism for antagonism between Id1, Id3, and Shh expression. The orchestrated actions of Ids and Shh highlight a temporal shift for instructive cues within the niche that promote self-renewal toward cues that promote terminal differentiation of progenitors into astrocytes and oligodendrocytes to facilitate recovery (Tzeng et al., 1999).
the total number of GFAP\(^+\) cells in the PI niche at 7 d PI (Fig. 5C) (2.516 cells vs 2.337 cells/100 \(\mu\)m\(^3\); \(p = 0.032\); \(n = 4\)).

An examination of the oligodendrocyte population showed BMP4 had similar effects on the number of APC\(^+\) cells within the PI niche. In control animals, the pool of oligodendrocytes was diminished 21% between 24 h and 7 d PI (1.171 cells vs 0.929 cells/100 \(\mu\)m\(^3\); \(p = 0.02\); \(n = 4\)), which corroborates previously reported data (McTigue et al., 2001). Similarly, APC\(^+\) populations are reduced 17% in animals intraspinally injected with BMP4 at 24 h PI (Fig. 5C) (0.970 cells vs 1.171 cells/100 \(\mu\)m\(^3\); \(p = 0.046\); \(n = 4\)), which is equivalent to the APC\(^+\) population numbers at 7 d PI. In contrast to its effect on GFAP\(^+\) cells, noggin reduced the number of APC\(^+\) cells present in PI tissue at 7 d (Fig. 5C) \((p = 0.0088\); \(n = 4\)).

Since glial numbers showed a consistent reduction in BMP-injected animals, cell proliferation was quantified to ensure that the observed reduction in gliogenesis was not a secondary side effect of reduced cell replacement (via proliferation). All animals received intraperitoneal injections of BrdU (50 \(\mu\)g/kg) in conjunction with the intraspinal injections of BMP4 or noggin. BrdU immunoreactivity was quantified within the lesion epicenter and 2 mm rostral and caudal (as with GFAP and APC stereology). In each experimental group, proliferation was consistent and did not vary from controls at 24 h or 7 d PI (Fig. 5E). Therefore, the changes in gliogenesis were attributable to BMP4’s ability to affect differentiation and create shifts in cell populations rather than reduce cell numbers overall by reducing cell proliferation.

BMP signals modulate NG2 progenitor differentiation in the PI niche

The observed changes in glial populations, within the progressive niche, suggest that BMPs could serve to direct progenitor differentiation to facilitate lesion repair. Therefore, we performed transplantation experiments to determine whether BMP4 supplements induce NG2 progenitors to adopt a differentiation profile similar to naive cells transplanted 7 d PI. A CRE recombinase expression retroviral vector, constructed with the NG2 promoter, was used to infect CNS progenitors isolated from adult 129-Gt(Rosa)26Sortm2Sho/J mice (ROSA:GFP:26Sor cells) spinal cords. According to the paradigm, progenitors that express NG2-proteoglycan will express CRE and induce GFP to be expressed in neuronal phenotypes. 

**Figure 6.** NG2 progenitors respond to BMP4 when transplanted into a progressive niche. **A.** The NG2 promoter was used to drive the expression of CRE recombinase and infect spinal cord progenitors isolated from ROSA:GFP mice (26Sor cells). **B.** After infection, induced GFP expression was analyzed by activated fluorescence, and cells (26Sor* cells) were purified via FACS (EGFP high). C, mRNA isolated from 26Sor* cells confirmed CRE expression in cells that express NG2 and EGFP (shown with molecular weight markers (MW)). D, Sor26* cells were amplified in cell culture and analyzed, via FACS, before transplantation into SCI. E, The stemness of 26Sor* cells was examined under differentiation conditions to produce the s\(\alpha\)00\beta\(^+\) astrocyte, CNPase\(^+\) oligodendrocyte, and Map2\(^+\) neuronal phenotypes. F, Hemisection-SCI animals received transplants of 26Sor* cells at 24 h and 7 d PI (with and without BMP4). The differentiation profile of transplanted 26Sor* was examined 14 d after transplantation (TXP). G, BMP4 increased the percentage GFAP\(^+\) 26Sor* cells (31.3%; **I, J, cyan arrows), compared with naive controls (16.1%; J, K), to exceed the percentage of GFAP\(^+\) cells in animals that received transplants at 7 d PI (25.6%; ***p = 0.032). BMP4 did not alter the percentage of APC\(^+\) 26Sor* cells (yellow arrows) significantly (statistics determined by one-tailed t test). Rotated views of confocal z-plane stacks (I and K from H and J, respectively) showed increases in the proportions of GFAP colocalization (I) versus APC (K) on 26Sor* cells transplanted with BMP4 24 h PI. Monochrome breakouts (from insets) show distinct localization of GFAP and APC with EGFP. **H.** Processes (cyan and yellow arrows, respectively). Scale bars, –10 \(\mu\)m. Error bars represent SEM.
analysis by RT-PCR showed CRE and NG2 expression in GFP+ cells (Fig. 6C). After expansion in culture, 26Sor+ cultures maintained GFP expression (Fig. 6D). 26Sor+ cells displayed the morphology and expressed phenotypic markers of glia (GFAP), neurons (TuJ1, Map2), and oligodendroglia (CNPase) when exposed to differentiation medium (Fig. 6E). Therefore, as seen with the NG2-reporter virus in SCI, NG2- proteoglycan was expressed by progenitors with the potential to produce multiple cell types and morphologies and corroborate multiple cell types observed in vivo with the NG2-reporter virus (above).

Given the potential to produce a diverse array of progeny, akin to endogenous cycling NG2+ cells, we quantified the phenotypic profile adopted by 26Sor+ cells transplanted at 24 h versus 7 d PI. Whereas experiments discussed above (Fig. 1) quantified virus-labeled cycling NG2+ cells of a nonspecific origin, direct transplantation of 26Sor+ cells into the lesion epicenter enables niche effects on an NG2 progenitor, of a known differentiation potential and origin to be examined directly. Cells transplanted at 24 h PI had no fate bias, and an equal percentage of transplanted cells expressed GFAP (16.1 ± 4.8%) versus APC (13.3 ± 3.9%) (Fig. 6G) (n = 4 and 3, n = 508). However, 26Sor+ cells transplanted 7 d PI displayed a surprising differentiation profile. Contrary to virally labeled cycling NG2+ cells, which showed a shift toward an APC+ phenotype in cells born 7 d PI, 26Sor+ cells transplanted 7 d PI had a higher percentage of GFAP+ cells (25.6 ± 0.66%) compared with APC+ cells (18.5 ± 0.27%) 14 d after transplantation (Fig. 6G) (p = 0.0003; n = 3, n = 532).

Since BMP ligands induce changes in the cellular composition of the PI niche (Fig. 5C), in addition to the increase in BMP4 transcription (Fig. 4B) and coincident shift in the differentiation profile of progenitors born after SCI (Fig. 5A), we hypothesized that BMP ligands (administered at the time of transplantation) would shift the differentiation profiles of transplanted 26Sor+ cells. Specifically, we hypothesized that BMP4 at 24 h PI would induce a profile similar to naive 7 d transplants. Indeed, despite the split in phenotype, BMP4 induced 26Sor+ cells transplanted at 24 h PI (Fig. 6H, I) to adopt a differentiation profile that mimicked naive 7 d PI transplants, instead of 24 h PI naive transplants (Fig. 6J, K). Compared with the unbiased phenotype split among 24 h PI transplants, BMP4 significantly increased the percentage of GFAP+ cells (31.3 ± 3.4%) versus APC+ cells (19.3 ± 0.61%) (Fig. 6G) (p = 0.13, n = 3, n = 692). Together, these data demonstrate that BMP4 has an appreciable ability to remodel the PI stem cell niche and, in the process, BMP4 is able to modulate the fate choice of NG2-expressing progenitors.

Discussion

Cell genesis after spinal cord injuries is well documented (Fawcett and Asher, 1999; McTigue et al., 2001; Zai and Wrathall, 2005; Horky et al., 2006); however, surprisingly little data describe how progenitor cell fate is instructed and what signals drive the PI niche as it evolves. This has been attributable in part to the ambiguous nature of assaying progenitor cell lineage by histochemical methods alone. In an injury environment, dying cells, pro- teases, and Igs create aberrant protein distributions and nonspecific signals that confound the identification of normally detectable epitopes. This challenge has driven the development of viral and genetic methods to isolate and follow glial progenitor cell lineages based on expression of lineage-specific pathways (Belachew et al., 2003; Aguirre et al., 2007; Meletis et al., 2008; Zhu et al., 2008). The studies herein are the first to use a unique NG2-reporter virus to demonstrate a coordinated temporal shift in phenotypes adopted by progenitors that express NG2-Proteoglycan. This novel tool revealed a unique subpopulation of cells derived from NG2-expressing cells that produce phagocytic s100β+ cells shown to contain denatured myelin within endo- somes. The transcriptome of the PI niche was characterized and showed temporal fluctuations in regulatory genes, such as BMP, that coincide with shifts in the differentiation profiles observed for NG2 progenitors after SCI.

The fates adopted by cycling NG2+ cells indicate a temporal shift occurs within the PI niche to direct a transition in progenitor differentiation between 24 h and 7 d PI. By using a reporter virus to label cells actively expressing NG2-proteoglycan, these studies are the first to be able to address the fates adopted by NG2+ progenitors. Since the cytoplasm of cells with an active NG2 promoter are labeled by EGFP, cycling NG2+ cells could be identified as GFAP+ and APC+ expression by direct association with a particular cytoplasmic rather than coincident localization with NG2-proteoglycan in the extracellular milieu. As a result, the studies revealed progenitors born 24 h PI are gliotic, coexpress CSPGs, and participate in scar formation, whereas NG2+ cells born 7 d PI produce a greater proportion of cells that coexpress APC, which we hypothesize could participate in the functional recovery that coincides with the transition in behavioral recovery in SCI animals observed by Ma et al. (2001). Furthermore, the reporter virus has been the first study to provide evidence of direct colocalization of MBP on a cell with an active NG2 promoter.

As a tissue recovers from injury, we show that molecular cues, known to instruct progenitors, undergo a temporal shift between 24 h and 7 d PI. Experiments in culture have demonstrated BMPs induce Iδ expression to affect astrogliogenesis versus oligoden- drocyte lineage commitment (Yanagisawa et al., 2001; Samanta and Kessler, 2004). Our results demonstrate that Iδ and Iδ3 transcription undergoes a temporal shift that correlates with the shift in lineage commitment after SCI. The delayed increase in Olig1 and PDGFα may reflect the trend toward an oligogenic niche, which correlates with the observed shifts in fates adopted by cycling NG2+ progenitors from the acute astrocytic fate to produce oligodendrocytes. Combined, the data suggest a model for succession in a progressive progenitor niche: acute signals generated within 24 h aid the rescue response whereby endoge- nous neural progenitors respond to produce astroglia to facilitate blood–brain barrier repair and preserve tissue integrity. Subse- quent differentiation cues progress temporally to facilitate niche repair by directing latent progenitors (i.e., 7 d PI birthdate) to produce a greater proportion of oligodendrocytes to repair myelin. Thus, as the PI niche recovers, the progressive niche directs cycling NG2 cells to produce astrocytes or oligodendroglia in a regemented process.

In addition, these experiments are the first to show reactive astrocytes (derived from cycling NG2+ cells) as phagocytes within the acute PI niche. Recent analysis of the astrocyte-transcriptome showed increased expression of phagocyte genes (Cahoy et al., 2008), but for the first time, our studies show images of a class of reactive astrocyte (s100β expressing) with myelin debris contained within endosomes (CathD+). Astrocytes have a demonstrated role in tissue homeostasis (Aschner, 1998), and immature astrocytes have been shown to phagocytize boun- tons in development (Ronnevi, 1978), but how an astrocyte par- ticipates to restore homestasis in a PI environment is not known. More recently, quiescent multipotent s100β astrocytes have been shown to exist after a stab wound in the brain (Buffo et al., 2008). Although the cells identified by Buffo et al. (2008) did not express NG2, the quiescent astrocytes proliferated and participated in
reactive gliosis after injury. Our studies have revealed another class of S100β-expressing cell that does not express a phenotypic marker of peripheral cells and provides the custodial function of phagocytosis before the arrival of peripheral monocytes (Popovich and Hickey, 2001). Future work on this unique astrocyte progenitor will be needed to better understand its role in removal of debris after injury.

Subsequently, after intraspinal injection of BMP4, transplanted NG2 progenitors produce fewer APC+ cells compared with control transplants, and a larger proportion of transplanted NG2 progenitors express GFAP 14 d after transplantation. The data presented here suggests that BMP4 is a gliogenic cue in the PI stem cell niche that can be manipulated to direct progenitor differentiation. In conjunction, previous studies have also demonstrated that glial populations can be augmented by inhibiting noggin within the CNS, which indirectly upregulates BMP signaling without an effect on cell proliferation (Hampton et al., 2007). Alternately, the action of BMP4 could be to delay differentiation after transplantation since BMP signals have been demonstrated to regulate the "stemness" of adult progenitors and function to preserve an astrocyte stem cell (Alvarez-Buylla and Lim, 2004) and GFAP+ progenitors are proposed to be more stem like (Ma et al., 2005). Whether sustained increases in BMP4 expression act to preserve the stemness of latent progenitors, or whether BMP4 promotes stemness (as indicated by increased GFAP+ cells), BMP ligands could be administered to remodel the PI niche to direct recovery. Together, the coordinated instruction of proliferation and differentiation would act to promote the maintenance of stem cell populations and differentiation to reestablish a stem cell niche.

Using the combination of a viral NG2 reporter and transplanted NG2-expressing progenitor cells, we have shown that instructions within the PI niche change temporally to produce both expected and unique cell lineages from neural progenitors after a hemisection SCI. We focused this study on the hemisection because it does not have the wave of inflammation and demyelination that follows a contusion injury. Hence, it is easier to demarcate the border zones of the injury site. The effect on the niche or the mediators of the niche (e.g., blood, cell death, inflammation, etc.) would likely be similar at the lesion epicenter of a hemisection or a contusion. However, the secondary complications are more prolific after a contusion injury despite the cut meninges that coincide the laceration of a hemisecion (Popovich and Hickey, 2001; Donnelly and Popovich, 2008). Therefore, a contusion injury is larger and has a widespread inflammatory response, which is a result of the mechanical trauma to the vascular system and shearing of axons over larger distances. Understanding the process and the mechanism by which progenitor fates are directed by PI niches will be important to develop therapeutical tools that are (1) at the mercy of the inherent cues within progressive niche, (2) designed to augment recovery from a post-traumatic injury through transplantation of exogenous stem cells, and (3) do not introduce acute injuries after intervention. Additionally, understanding how niche succession is coordinated will elucidate how endogenous and transplanted progenitors respond to injury and how best to therapeutically manage their participation in tissue regeneration.

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