Development/Plasticity/Repair

Adult NG2+ Cells Are Permissive to Neurite Outgrowth and Stabilize Sensory Axons during Macrophage-Induced Axonal Dieback after Spinal Cord Injury

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We previously demonstrated that activated ED1+ macrophages induce extensive axonal dieback of dystrophic sensory axons *in vivo* and *in vitro*. Interestingly, after spinal cord injury, the regenerating front of axons is typically found in areas rich in ED1+ cells, but devoid of reactive astrocyte processes. These observations suggested that another cell type must be present in these areas to counteract deleterious effects of macrophages. Cells expressing the purportedly inhibitory chondroitin sulfate proteoglycan NG2 proliferate in the lesion and intermingle with macrophages, but their influence on regeneration is highly controversial. Our *in vivo* analysis of dorsal column crush lesions confirms the close association between NG2+ cells and injured axons. We hypothesized that NG2+ cells were growth promoting and thereby served to increase axonal stability following spinal cord injury. We observed that the interactions between dystrophic adult sensory neurons and primary NG2+ cells derived from the adult spinal cord can indeed stabilize the dystrophic growth cone during macrophage attack. NG2+ cells expressed high levels of laminin and fibronectin, which promote neurite outgrowth on the surface of these cells. Our data also demonstrate that NG2+ cells, but not astrocytes, use matrix metalloproteases to extend across a region of inhibitory proteoglycan, and provide a permissive bridge for adult sensory axons. These data support the hypothesis that NG2+ cells are not inhibitory to regenerating sensory axons and, in fact, they may provide a favorable substrate that can stabilize the regenerating front of dystrophic axons in the inhibitory environment of the glial scar.

Introduction

Injury to the spinal cord results in significant cellular, molecular, and architectural changes which constitute the formation of the glial scar (Silver and Miller, 2004). Reactive astrocytes deposit large quantities of inhibitory chondroitin sulfate proteoglycan (CSPG) as they migrate away from the lesion core, which becomes filled with activated macrophages (Fitch et al., 1999; Horn et al., 2008). It is well documented that an immature NG2-expressing cell type migrates toward and proliferates within the astrocyte-free core of the lesion (Zai and Wrathall, 2005; Lytle et al., 2006); however, the effects of this cell population following injury are uncertain. NG2 is a CSPG expressed on the surface of glial precursor cells in the developing and mature CNS and is upregulated for months after dorsal column injury (Levine et al., 1993; Jones et al., 2002).

CSPGs are extracellular matrix (ECM) molecules known to restrict neurite outgrowth and contribute to regeneration failure after CNS injury (Snow et al., 1990; McKeon et al., 1991; Fitch and Silver, 1997; Davies et al., 1999). These observations lead to the hypothesis that NG2 would also exert detrimental effects on injured axons (Fidler et al., 1999). Indeed, NG2 has been shown to be inhibitory to neurite outgrowth of embryonic dorsal root ganglion (DRG) and neonatal cerebellar neurons *in vitro* (Dou and Levine, 1994) and to possess several domains capable of inducing growth cone collapse in neonatal neurons (Ughrin et al., 2003). Additionally, early postnatal-derived NG2+ oligodendrocyte precursor cell membranes are inhibitory to axonal growth from cerebellar explants (Chen et al., 2002a). Monoclonal antibodies against NG2 applied to the adult rat spinal cord after dorsal column injury have been shown to promote axonal growth into the lesion (Tan et al., 2006).

Despite these findings, the influence of NG2+ cells following CNS injury has become controversial, with several studies attributing beneficial effects to the presence of an NG2-expressing cell type in the adult spinal cord. Axons regrowing through neurotrophin-secreting fibroblast-containing grafts within a spinal cord lesion preferentially associate with areas containing NG2 (Jones et al., 2003). Interestingly, eliminating NG2 expression does not effect corticospinal tract regeneration after hemisection or axon growth after dorsal root injury (Hossain-Ibrahim et al., 2007), and NG2 has been shown to enhance serotonergic axon sprouting (de Castro et al., 2005). Furthermore, NG2-expressing cells facilitate growth of early postnatal neurons

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(Yang et al., 2006) and appear to support regenerating axons (McTigue et al., 2006).

Here we examined the effects of adult spinal cord-derived NG2+ cells, rather than the isolated NG2 proteoglycan itself, *in vitro* and *in vivo*. Our results suggest that NG2+ cells are not inhibitory to adult sensory axon outgrowth and that the permissive nature of these cells is largely laminin dependent. We also provide evidence that these cells have the capacity to invade the lesion environment through the use of matrix metalloproteases (MMPs). We have confirmed that NG2+ cells associate with injured fibers and demonstrate for the first time that they can provide a stabilizing substrate for axons against macrophage-mediated dieback in the injured spinal cord.

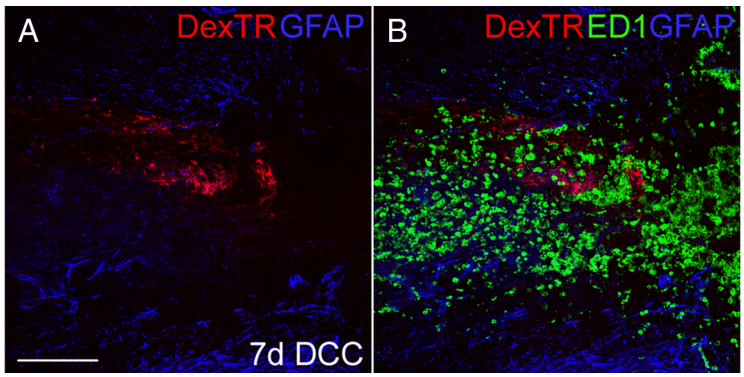

Figure 1. Injured sensory axons undergoing axonal dieback are found in the macrophage-filled lesion core. ***A***, Seven days after injury, Texas Red-conjugated dextran 3000 MW (DexTR)-labeled axons (red) are located in areas devoid of GFAP+ astrocyte processes (blue) that have withdrawn from the lesion core. ***B***, Axons are associated with ED1+ cells (green), which have been previously shown to cause axonal dieback following injury. Scale bar: ***A***, ***B***, 50 μm.

Materials and Methods

Time-lapse dish preparation. Delta-T cell culture dishes were prepared as previously described (Horn et al., 2008). Culture dishes were rinsed with sterile water and then coated with poly-L-lysine (0.1 mg/ml, Sigma-Aldrich) overnight at room temperature, rinsed with sterile water, and allowed to dry. Aggrecan spot gradients were created by pipetting 2.0 μl of aggrecan solution (2.0 mg/ml, Sigma-Aldrich) in calcium and magnesium-free HBSS (HBSS-CMF, Invitrogen) onto the culture surface. Six spots were placed per dish and when the aggrecan spots were dry, the entire surface of the dish was bathed in laminin solution (10 μg/ml; Invitrogen) in HBSS-CMF for 3 h at 37°C. The laminin bath was removed and cells were plated immediately without allowing the dish surface to dry.

DRG dissociation. DRGs were harvested as previously described (Tom et al., 2004). Briefly, DRGs were gently dissected from adult female Sprague Dawley rats (Harlan) and incubated in a solution of Collagenase II (200 U/ml, Worthington Biochemical) and Dispase II (2.5 U/ml, Roche Diagnostics) in HBSS. Cells were centrifuged at a low speed, washed, and gently triturated in HBSS-CMF three times. The dissociated DRGs were then resuspended in Neurobasal-A media supplemented with B-27, GlutaMax, and penicillin/streptomycin (Invitrogen) and counted. DRGs were plated on Delta-T dishes (Fisher) at a density of 6000 cells/dish in 2 ml of media. The anti-mitotic fluorodeoxyuridine (FudR, Sigma-Aldrich) was used in the first 24 h after plating of DRG cultures to minimize the number of satellite cells in the preparation.

Purification of adult NG2+ cells from mouse spinal cord. Highly purified NG2+ cells were obtained using a novel isolation protocol (L. Bai, A. Belkadi, A. Caprariello, and R. H. Miller, unpublished work). For each isolation, spinal cord from two adult (>8 weeks) C57BL/6J mice were dissected in Modified Eagle's Medium (MEM) and chilled. Surface blood vessels were carefully removed and tissue was dissociated for 30 min. Digested tissue was spun at 800 × *g* for 5 min, and cells were washed three times before layering on a Percoll gradient (GE Healthcare, catalog No. 17-0891-02). Following centrifugation at 2000 rpm for 30 min, the cellular fractions were collected, washed, and resuspended in 1 ml of DMEM/F-12 medium including 10% FBS at a density of 1×10^6 cells/ml in a 75 cm^2 flask and grown at 37°C in 5% CO_2 for at least 4 weeks with a 50% medium change every 2 d. Cells were passaged at least once before use, and 99% expressed NG2. The cells had a variable morphology with islands of flat cells with large cell bodies interspersed with process-bearing cells with smaller cell bodies. The majority of the cells (87%) expressed PDGF-α receptor, and 84% of the cells expressed Olig2. No detectable mature oligodendrocytes expressing myelin markers CNP or MBP were found in the cultures, and the number of GFAP+ astrocytes was <3%.

Astrocyte preparation. Astrocytes were harvested from the cortices of postnatal day 0 (P0) to P1 rats as previously described (Horn et al., 2008). Cortices were finely minced and treated with 0.5% trypsin in EDTA. Cells were plated in DMEM/F-12 (Invitrogen) with 10% FBS (Sigma-Aldrich) and 2 mM GlutaMax on T75 flasks coated with poly-L-lysine and shaken after 4 h to remove nonadherent cells. Astrocytes were allowed to mature in culture for at least 4 weeks, and used within 2 weeks of maturity. Astrocytes were harvested with trypsin and plated at a density of 25,000 cells per coverslip.

Macrophage cultures. NR8383 cells, an adult Sprague Dawley alveolar macrophage cell line (ATCC #CRL-2192, American Type Culture Collection), were cultured as described previously (Yin et al., 2003). Briefly, cells were cultured in F-12K media (Invitrogen) supplemented with 15% FBS, GlutaMax, penicillin/streptomycin (Invitrogen), and sodium bicarbonate (Sigma-Aldrich) using uncoated tissue culture flasks (Corning). Macrophages were prepared for time-lapse microscopy experiments as described previously (Horn et al., 2008). Before addition to time-lapse dishes, macrophages were harvested with EDTA and a cell scraper and resuspended at a density of 2.5×10^5/70 μl in Neurobasal-A supplemented as above with the addition of HEPES (1:500; 50 μM, Sigma-Aldrich). Macrophages were visualized using ED1 (Millipore Bioscience Research Reagents).

Time-lapse microscopy studies. Adult DRG neurons were incubated at 37°C for 48 h before time-lapse imaging. Neurobasal-A media with HEPES (50 μM, Sigma-Aldrich) was added to the cultures before transfer to a heated stage apparatus. Time-lapse images were acquired every 30 s for at least 3 h with a Zeiss Axiovert 405M microscope using a heated 100× oil-immersion objective. Growth cones were chosen that extended straight into the spot rim with characteristic dystrophic morphology. Neurons were observed for 30 min to determine baseline behavior before the addition of macrophages ($N = 5$). Growth cones were observed for 150 min after macrophage addition. We tracked extension/retraction, and rate of growth with MetaMorph software. Following time-lapse imaging, DRG cultures were fixed in 4% PFA and immunostained with anti β-tubulin-type III (1:500; Sigma-Aldrich), anti-chondroitin sulfate (CS-56, 1:500, Sigma-Aldrich), ED1 (1:500; Millipore Bioscience Research Reagents), or NG2 (1:500; Millipore Bioscience Research Reagents) and incubated with appropriate secondary antibodies (Invitrogen).

Quantification of neurite crossing of the spot rim. The average number of β-tubulin+ neurites crossing the spot rim (visualized by CS56) on GFAP+ astrocytes or NG2+ cells were counted ($N = 12$ spots per group). For quantification of digestion of the proteoglycan spot rim on NG2+ cell-containing coverslips, images of the spot rim were obtained with a constant exposure time. A set area of the spot was selected, and the average integrated pixel intensity was calculated using MetaMorph software ($N = 8$ spots per group, 4 pictures per spot).

Metalloproteinase inhibition. Preparation of aggrecan-laminin spot gradient coverslips was performed as described previously (Tom et al.,

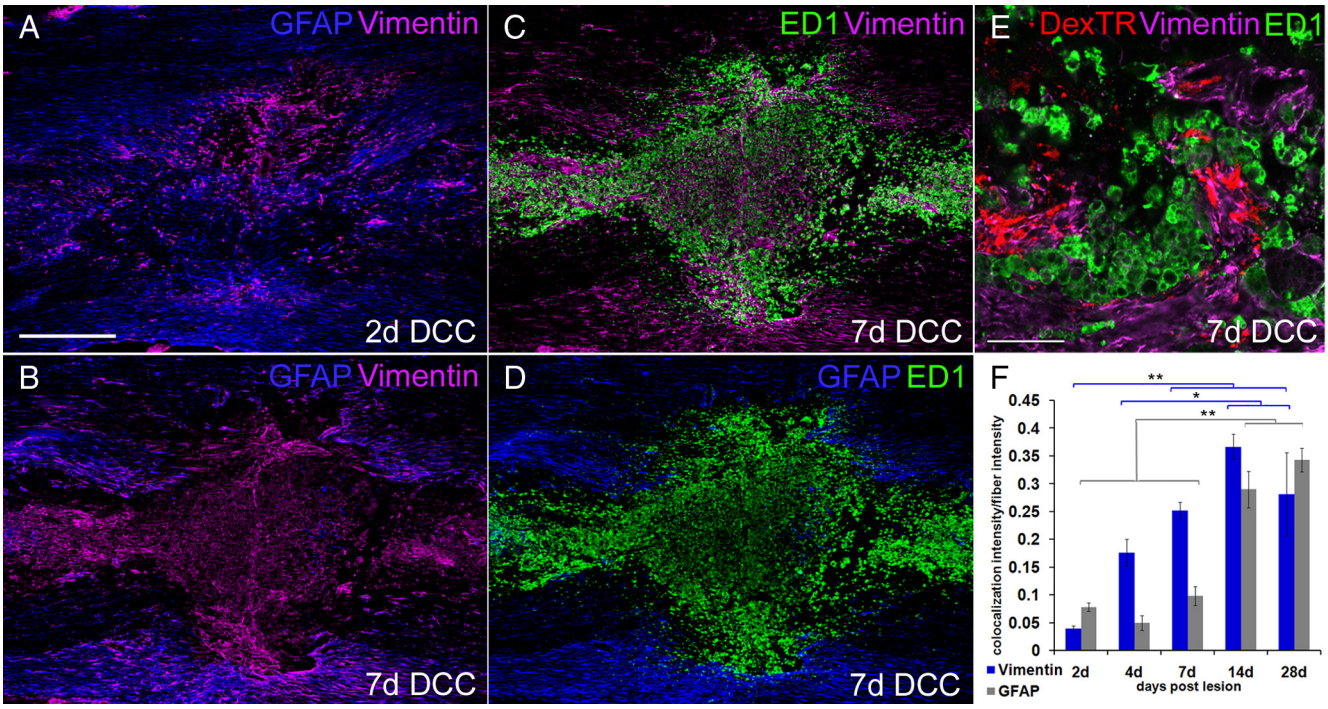

Figure 2. Dystrophic axons initially associate closely with vimentin+ cells. ***A–D***, Confocal montages of longitudinal sections (10×) of animals receiving a dorsal column crush (DCC) spinal cord injury. The section is oriented so that the caudal end is on the left. ***A***, Two days after injury, GFAP+ astrocytes (blue) and vimentin+ cells (purple) commingle in the lesion center. ***B***, Seven days after injury, there is a distinct alteration in the distribution of vimentin+ and GFAP+ cells, as astrocytes pull away from the lesion center. ***C***, Seven days after injury, ED1+ macrophages (green) have infiltrated the lesion and are found in high numbers in the lesion center, an area also populated by vimentin+ cells (purple). ***D***, ED1+ macrophages are located in the center of the lesion in a pattern opposite that of GFAP+ cells (blue). ***E***, Confocal image (40×) of DexTR-labeled fibers associating with a vimentin+ cell population. ***F***, Colocalization of fibers and vimentin or GFAP. Vimentin and GFAP are significantly different (2-way ANOVA, $F_{(1,170)} = 7.33$, Tukey's *post hoc* test, $p < 0.01$). Overall, 2 d is significantly different from 7 d, and 2 d, 4 d, and 7 d are significantly different from 14 d and 28 d ($F_{(4,170)} = 31.87$). For vimentin, 2 d is significantly different from 4 d, 7 d, 14 d, and 28 d; 4 d is significantly different from 14 d and 28 d ($F_{(4,170)} = 5.28$). For GFAP, 2 d, 4 d, and 7 d are significantly different from 14 d and 28 d ($F_{(4,170)} = 5.28$). $^{**}p < 0.001$, $^{*}p < 0.01$. Scale bars: ***A–D***, 200 μm. ***E***, 50 μm.

2004). Briefly, glass coverslips coated with poly-lysine and nitrocellulose were spotted with 2 μl of a solution of aggrecan (0.7 mg/ml; Sigma) and laminin (5 μg/ml; Invitrogen) in CMF-HBSS. Twenty-five thousand adult NG2+ cells or mature astrocytes were plated on each coverslip with DMEM-F-12 media for control or in 25 μM GM6001 (Calbiochem) in DMSO (Sigma-Aldrich). Control media or media containing the inhibitor was replaced every 24 h. Cultures were maintained for 5 d before fixation in 4% paraformaldehyde and stained with NG2 or GFAP, and CS56 to visualize the spot rim. The total number of cells crossing each spot rim was counted and compared to total number of cells adhering to each spot.

Function-blocking antibody experiments. Coverslips were coated with poly-L-lysine (1 mg/ml) overnight, rinsed several times with sterile water, and allowed to dry. A monolayer of Adult NG2+ cells was made by plating 60,000 cells in 60 μl of DMEM-F-12 at the center of the coverslip for 30 min at 37°C to allow the cells to adhere. Then, 1 ml of media was added with anti-laminin (10 μg/ml; Biomedical Technologies), anti-fibronectin (10 μg/ml; Dako), or anti-rabbit IgG as a control (RIgG) (10 μg/ml, Millipore Bioscience Research Reagents). After 24 h, the media was removed and 1500 dissociated DRG neurons in Neurobasal A were seeded onto the adult NG2+ cells monolayer with their respective function blocking antibodies. The cultures were maintained for an additional 24 h before fixation in 4% paraformaldehyde.

Dorsal column crush lesion model. All animal procedures were performed in accordance with the guidelines and protocols of the Animal Resource Center at Case Western Reserve University. Adult female Sprague Dawley rats (250–300 g) were anesthetized with inhaled isoflurane gas (2% in oxygen) for all surgical procedures. Dorsal column crush injuries were performed as described previously (Horn et al., 2008). Briefly, the dorsal aspect of the C8 spinal cord segment was exposed by performing a T1 laminectomy. A dorsal column crush lesion was then made by inserting the tines of Dumont #3 jeweler's forceps 1.5 mm apart to a depth of 1.0 mm into the dorsal spinal cord at C8. The forceps were completely closed, holding pressure for 10 s. This crushing procedure was the repeated two additional times, and gel film was placed over the lesion. The muscle layers were closed with 4-0 nylon sutures, and the skin was closed with surgical staples. Upon completion of the surgery, animals received Marcaine (1.0 mg/kg) subcutaneously along the incision as well as buprenorphine (0.1 mg/kg) intramuscularly. Animals were kept warm postoperatively with a heating lamp during recovery from anesthesia and allowed access to food and water *ad libitum*. Animals were perfused at 2, 4, 7, 14, or 28 d after lesion ($N = 6$ per group).

Axon labeling. Two days before perfusion, the dorsal columns were labeled unilaterally with Texas Red-conjugated 3000 MW dextran. Briefly, the sciatic nerve of the right hindlimb was exposed and isolated. The sciatic nerve was crushed with Dumont #3 forceps for 10 s and repeated two additional times. A hole was made in the epineurium with a 30 gauge needle, and then 1.0 μl of 3000 MW dextran-Texas Red 10% in sterile water was injected via a Hamilton syringe into the sciatic nerve at the crush site. The muscle layers were closed with 4-0 nylon sutures and the skin with surgical staples. Upon closing of the incision, animals received Marcaine (1.0 mg/kg) subcutaneously along the incision as well as buprenorphine (0.1 mg/kg) intramuscularly. Postoperatively, animals were kept warm with a heating lamp during recovery from anesthesia and allowed access to food and water *ad libitum*. Animals were killed 2 d following labeling with an overdose of isoflurane and perfused with PBS followed by 4% PFA. Tissue was harvested and postfixed in 4% PFA and processed for immunohistochemistry.

Immunohistochemistry. Tissue was postfixed overnight in 4% PFA, submersed in 30% sucrose overnight, frozen in OCT mounting media, and sectioned (20 μm) with a cryostat. Tissue sections were then stained with anti-GFAP (Accurate Chemical and Scientific), anti-vimentin (Sigma), anti-laminin (Sigma), or anti-ED1 (Millipore) and incubated with either Alexa Fluor 405, 488, or 633 (Invitrogen) secondary antibod-

ies, and then imaged on a Zeiss Axiovert 510 laser-scanning confocal microscope.

In vivo *fiber-association quantification.* Images of labeled fibers (red) and either GFAP+ cells or vimentin+ cells (both green) were merged in Adobe Photoshop, and areas where green and yellow pixels overlapped became yellow. All yellow pixels were selected and saved as a new image. The number of yellow pixels in each image was quantified using MetaMorph software.

Statistical analysis. Data were analyzed by the Student's *t* test or two-way ANOVA with Tukey's *post hoc* test using Minitab 15 Software, or with the Kruskal–Wallis test followed by the Mann–Whitney *U* test with SPSS, as appropriate.

Results

Injured fibers are found in a region lacking astrocyte processes, surrounded by activated macrophages

Our laboratory has shown that macrophages induce lengthy axonal dieback following dorsal column crush injury (Horn et al., 2008). Here, using the same model of spinal cord injury, we characterized the cellular components of the lesion and the association of NG2+ cells with regenerating axons. Seven days after injury, GFAP+ glia and their processes had pulled away from the lesion core, leaving large areas devoid of reactive astrocytes (Fig. 1*A*). This phenomenon occurred concurrently with macrophage infiltration into the lesion (Fig. 1*B*). The endings of the labeled ascending sensory axons were most often found in the caudal end of the lesion in a region lacking GFAP+ processes, but containing high numbers of activated macrophages as visualized by ED1 staining (Fig. 1*B*).

Dystrophic axons associate closely with vimentin+ cells during the time of macrophage-induced axonal dieback

We sought to demonstrate the presence of a permissive, stabilizing substrate to explain the fact that the fibers only retract a limited distance from the epicenter of the lesion despite the massive influx of macrophages. Two days after injury, GFAP+ and vimentin+ cells were found throughout the lesion site, and their patterns were not clearly segregated (Fig. 2*A*). By seven days after injury, GFAP+ astrocytes had either died or pulled away from the lesion center, and the vimentin+ cell population had dramatically expanded within the lesion core (Fig. 2*B*). The GFAP and vimentin staining patterns were nearly mutually exclusive, with the vimentin+ cells surrounded by reactive astrocytes at 7 d after lesion (Fig. 2*B*). This suggests that the GFAP+ and vimentin+ cells were two distinct cell populations. Fibers associated preferentially with these vimentin+ cells at 7 d after lesion (Fig. 2*E*). Although the ED1+ macrophage population cohabited with vimentin+ cells in the lesion core, fibers associated almost exclusively with vimentin+ cells (Fig. 2*C*,*E*). Conversely, GFAP+ processes did not overlap with the area of ED1 staining (Fig. 2*D*) and did not associate with the front of severed axons. We quantified the association of injured fibers with GFAP+ or vimentin+ cell types after 2, 4, 7, 14, or 28 d (Fig. 2*F*). Fibers were strongly associated with vimentin+ cells at 4 and 7 d after injury and continued to preferentially associate with vimentin+ cells at 14 d after injury.

Macrophages induce axonal dieback to the caudal end of the lesion by 28 d after injury

By 28 d after injury, vimentin+ cells typically formed an irregular meshwork in the lesion core and fibers retracted several hundred micrometers from the epicenter into the narrow, most caudal end of the lesion (Fig. 3*A*,*B*). At this time, NG2+/vimentin+ cells were still present in the vicinity of axons in the area of injured

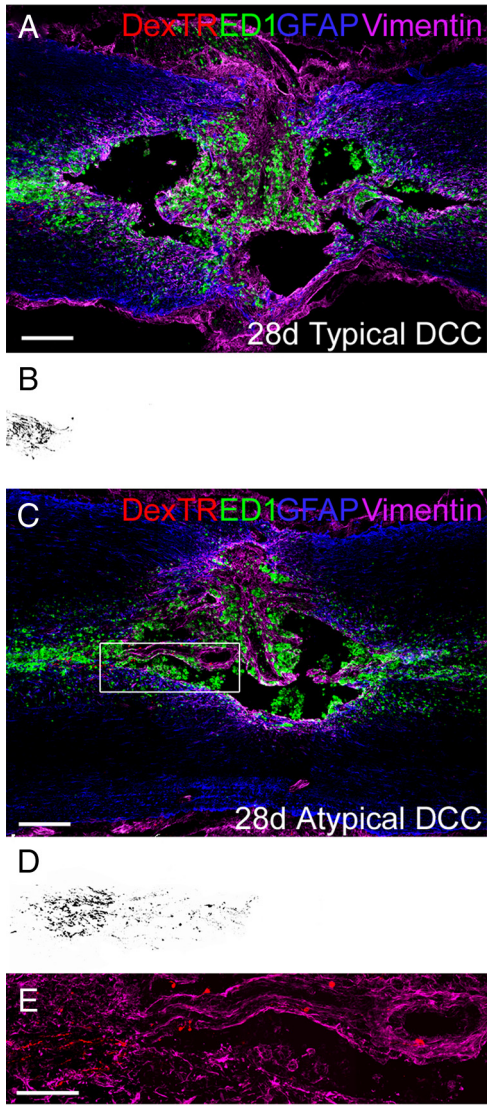

Figure 3. Macrophages typically induce axonal retraction to the caudal end of the lesion which contains GFAP+ and vimentin+ cells by 28 d postlesion. ***A***, Typical outcome of DCC 28 d after injury. Fibers (red) have retracted a considerable distance and rest on GFAP+ (blue) and vimentin+ (purple) cells. Macrophages (green) still remain in the lesion at this time point. ***B***, Inverted grayscale image of superimposed fiber tracings of three sections from the representative animal in ***A***. ***C***, Atypical outcome of DCC 28 d after injury. DexTR fibers associate with vimentin+ cells and remain in the lesion core. ***D***, Inverted grayscale image of superimposed fiber tracings of three sections from the representative animal in ***C***. ***E***, Higher magnification of vimentin (purple) and fibers (red) as seen in ***C***. Scale bars: ***A–D***, 200 μm; ***E***, 50 μm.

fibers (Fig. 3*A*,*B*; supplemental Figs. 1, 2, available at www.jneurosci.org as supplemental material), but axons were also associated with GFAP+ processes (Fig. 3*A*,*B*; supplemental Fig. 1, available at www.jneurosci.org as supplemental material). In the relatively rare instance that a bridge of vimentin+ cells protruded well into the lesion core, fibers also persisted on these cells despite being surrounded by ED1+ macrophages (Fig. 3*C–E*). The observation of a contiguous vimentin+ bridge of this length into the lesion center was seen only once in 12 animals observed at 28 d after injury.

The vimentin+ cell population expresses the progenitor markers NG2 and nestin

Vimentin is expressed by immature astrocytes and invading fibroblastoid cells (Conrad et al., 2005), macrophages (Kim et al.,

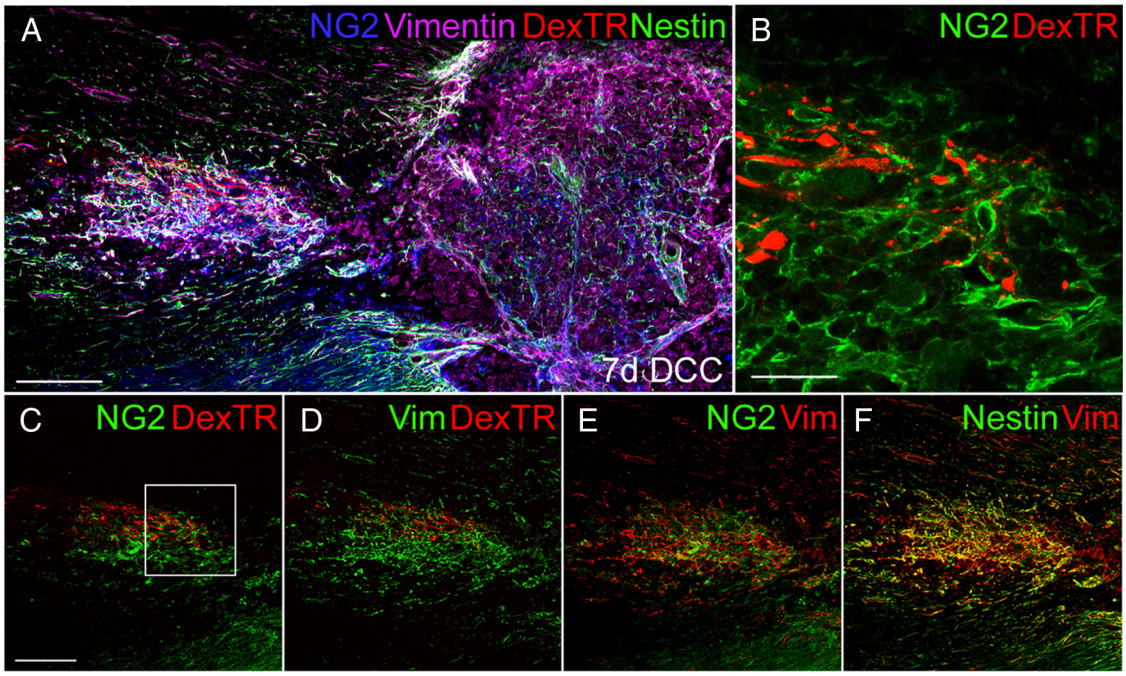

Figure 4. The vimentin+ cell population expresses the progenitor markers NG2 and nestin. ***A***, Seven days after injury, fibers associate with vimentin+ (purple), nestin+ (green), and NG2+ (blue) cells. ***B***, Confocal image (40×) of NG2- (green) and DexTR- (red) labeled axons, higher magnification of box from ***C***. ***C***, DexTR fibers (red) also associate with an NG2+ (green) cell population. ***D***, DexTR-labeled fibers associate largely with a vimentin+ (green) cell population. ***E***, There is considerable overlap between the NG2+ (green) and vimentin+ (red) populations. ***F***, Nestin+ (green) cells express vimentin (red). Scale bars: ***A***, ***C–F***, 100 μm; ***B***, 50 μm.

2003; Shin et al., 2003), tanycytes (Prieto et al., 2000), invading meningeal cells (Wang et al., 1997), blood vessels (Farooque et al., 1995), and ependymal cells (Bodega et al., 1994). We sought to identify the predominant cell population expressing vimentin in the region containing injured neurites. We observed that injured fibers closely associate with vimentin+ cells and NG2+ cells (Fig. 4*B–D*). We found substantial overlap of the neural stem/progenitor-associated markers nestin and vimentin with the pattern of NG2 staining (Fig. 4*A*,*E*,*F*; supplemental Fig. 3, available at www.jneurosci.org as supplemental material). Cells expressing NG2 and vimentin likely represent a subset of multiple heterogeneous populations. While NG2 was present extracellularly, in an area containing numerous vimentin+ cells, vimentin, an intermediate filament marker, was localized intracellularly, explaining subtle differences in the staining patterns (Fig. 4*E*). However, nestin is also an intermediate filament protein and highly colocalized with vimentin (Fig. 4*F*).

DRG axons grow freely on adult NG2+ cells *in vitro*

To more closely examine the interactions between NG2+ cells and adult neurons, we isolated primary NG2+ cells derived from the adult mouse spinal cord. These cells have a variable morphology and 99% express NG2 (supplemental Fig. 4, available at www.jneurosci.org as supplemental material). NG2+ cells were added to 1 d *in vitro* (DIV) adult DRG neuron preparations grown on a bidirectional spot gradient of the inhibitory proteoglycan aggrecan and the growth-promoting substrate laminin. The cocultures were incubated for an additional day. After 1 DIV, the NG2+ cells did not cross the inhibitory spot rim (Fig. 5*A*). Adult DRG axons grew freely on NG2+ cells *in vitro*, but were able to leave them to extend into, but not across, the inhibitory rim, where they became dystrophic. These NG2+ cells from the adult spinal cord also expressed the progenitor cell markers vimentin and nestin (Fig. 5*B*,*C*). As we observed *in vivo*, NG2 was clearly visible on the cell surface, while vimentin and nestin were localized intracellularly, suggesting that these vimentin/nestin+ cells were depositing NG2 extracellularly on their cell membranes. Interestingly, satellite cells from the dorsal root ganglia were positive for nestin and vimentin, but negative for NG2 (Fig. 5*B*,*C*). We considered the possibility that these NG2+ cells could be vascular pericytes, as they also express NG2 (Ozerdem and Stallcup, 2004). While we have not fully explored all potential pericyte markers, these cells were not positive for the CD73, which is known to be expressed on cells of mesenchymal origin (data not shown).

In vitro, NG2+ cells stabilize axons following macrophage attack

Our observations of adult DRG neurons in coculture with adult NG2+ cells suggested that the NG2+ cell population *in vivo* may be permissive for axonal outgrowth. We next sought to test the effects of these cells in our *in vitro* model of macrophage-induced axonal dieback (Horn et al., 2008; Busch et al., 2009). In this model macrophages typically induce long-distance retraction of dystrophic adult sensory growth cones. NG2+ cells were added to 1 DIV DRG neuron preparations grown on inverse gradients of proteoglycan and laminin and were incubated in coculture for an additional day. We chose to image axons that originated on NG2+ cells and extended into the inhibitory rim. The antimitotic fluorodeoxyuridine was used in the first 24 h after plating of DRG cultures to minimize the number of satellite cells in the preparation and enable us to image NG2+ cell-associated axons that were not in contact with satellite cells. Following a 30 min period of observation of the behavior of the growth cone, NR8383 macrophages were added to the culture and their interactions with the axon were monitored (Fig. 6*A*,*B*; supplemental Movie 1, available at www.jneurosci.org as supple-

mental material). Macrophages formed extensive and lasting connections to dystrophic axons in coculture with NG2+ cells. Long retraction fibers between the dystrophic endball and the substrate often formed as the growth cone rapidly retracted (Fig. 6*A*). Retractions occurred 80% of the time following macrophage contact even when the proximal portion of the axon was intimately associated with an NG2+ cell (Fig. 6*C*). An arrowhead indicates the position at which the axon has retracted to an NG2+ cell. We did not observe axons leaving NG2+ cells after retraction; however, if this were to occur, it is likely that macrophage-induced dieback of the reformed dystrophic tip could occur repeatedly.

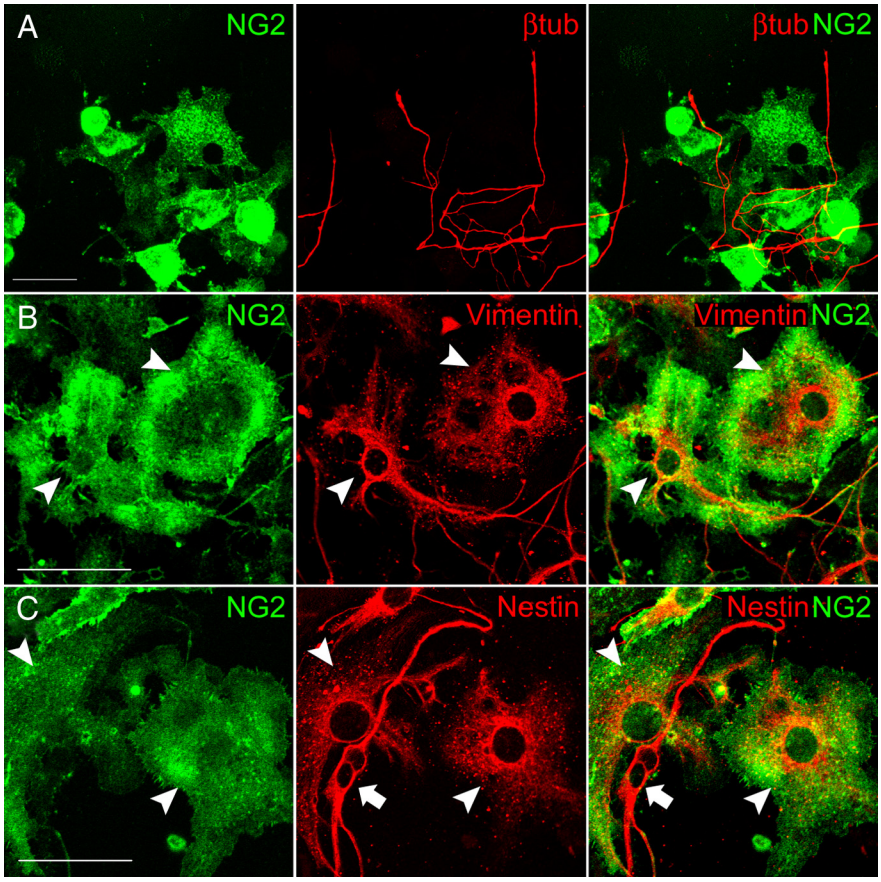

Figure 5. NG2+ cells are permissive to axon outgrowth and express vimentin and nestin. ***A***, Confocal image (40×) showing the association of axons of β-tubulin+ (red) axons with NG2+ (green) cells on a gradient of aggrecan and laminin after 2 d *in vitro*. Arrowheads indicate NG2+ cells and arrows indicate NG2 negative satellite cells. ***B***, NG2+ cells express vimentin (red). ***C***, NG2+ cells express nestin (red). Scale bars: ***A–C***, 50 μm.

NG2+ cells can provide a bridge for injured axons

After injury, NG2+ cells are able to traverse a terrain laden with astrocyte-produced CSPGs (Dow and Wang, 1998; Tan et al., 2005; Nishiyama et al., 2009). *In vitro*, NG2+ cells were initially restricted to the center of the spot, but progressed into the inhibitory rim over a period of 5 d (Fig. 7*A*). Mature astrocytes, however, remained within the center of the spot and surrounded the outside of the spot, but did not invade the rim (Fig. 7*B*). Interestingly, astrocytes in coculture with NG2+ cells were unable to cross the inhibitory rim (supplemental Fig. 5, available at www.jneurosci.org as supplemental material). Once NG2+ cells crossed the rim, adult DRG neurons could follow, using these cells as a bridge (Fig. 7*A*,*C*). An average of 37.25 ±5.56 DRG axons crossed the inhibitory rim on NG2+ cells, and an average 4.08 ± 0.70 DRG axons cross on astrocytes (NG2 and GFAP are significantly different from each other, Student's *t* test, $t_{(11.323)} = 5.919$, $p < 0.001$).

NG2+ cells use MMPs to cross an inhibitory proteoglycan rim

We next asked how adult NG2+ cells, but not astrocytes, were able to enter the inhibitory proteoglycan rim (Fig. 8*A*,*C*). We hypothesized that one or more proteases could have been released by the NG2+ cells that favorably modified the inhibitory substrate. MMPs are known to enable cell migration via cleavage of ECM such as proteoglycans and could be responsible for mediating this phenomenon (Yong, 2005). Treatment with the broad spectrum MMP inhibitor GM6001 prevented NG2+ cells from crossing the spot rim, but had no effect on astrocytes (Fig. 8*B*,*D*,*G*). We observed a decrease in CS56 immunoreactivity of the spot rim on coverslips containing NG2+ cells after 5 DIV (Fig. 8*E*), and this decrease was not observed on coverslips treated with GM6001 (Fig. 8*F*). We examined this difference by quantifying the staining intensity using average integrated pixel intensity of the spot rim. We found a significant difference between the two conditions with NG2+ cell-containing spots alone averaging 8.3×10^6 average integrated pixel intensity units versus 13.9×10^6 average integrated pixel intensity units for NG2+ cell-containing spots with GM6001 treatment (Student's *t* test, $t_{(50)} = -11.01$, $p < 0.001$).

NG2+ cells express the permissive molecules laminin and fibronectin

In vivo, laminin was expressed in abundance within the lesion, in a pattern closely associated with that of vimentin (Fig. 9*A*) and NG2 (Fig. 4*A*). Although DRG axons have been shown to regenerate short distances in association with blood vessels (Dray et al., 2009), at 14 d after injury, laminin was associated only minimally with RECA-1+ blood vessels (supplemental Fig. 6, available at www.jneurosci.org as supplemental material). ED1+ macrophages occupied the entire central lesion territory as well, but NG2+ cells expressing laminin decreased gradually toward the rostral end of the lesion (Fig. 9*B*). The changing balance between the permissiveness of laminin and the inhibition of activated macrophages may be a major factor that allows axon growth into the caudal end of the lesion core, but not further.

Given the observation of Yang et al. (2006) that immature NG2+ glia express laminin and fibronectin, we sought to determine whether these growth-promoting molecules were present on the surface of adult spinal cord-derived NG2+ cells, and whether they were actually mediating their growth-permissive effects on adult neurons. Adult NG2+ cells grown on PLL did, indeed, express high levels of laminin on their surface and throughout their processes (Fig. 9*F*), and a lower amount of fibronectin

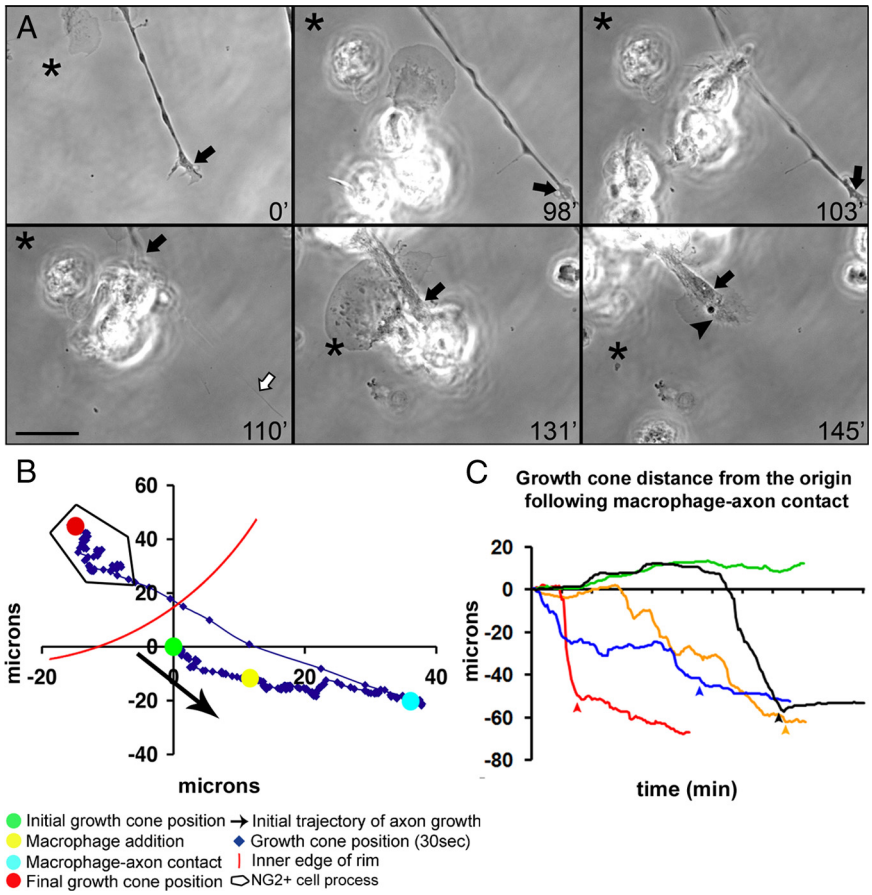

Figure 6. NG2+ cells can stabilize axons during macrophage-mediated axonal dieback *in vitro*. ***A***, Six representative frames from a time-lapse movie illustrating macrophage/axon interactions on an aggrecan/laminin gradient in the presence of adult mouse spinal cord NG2+ cells. NR8383 macrophages are added to a 2 DIV culture of adult DRG neurons. Times for each frame are given in the lower right of each image, and an asterisk marks a consistent point on the culture dish as a reference for position during frame shifts. An arrow denotes the central domain of the grown cone. Macrophages are added following a 30 min period of observation, and first contact occurs at 103 min. The axon has already undergone a long distance retraction by 110 min and an open arrow indicates the presence of a retraction fiber. The open arrow indicates the presence of a retraction fiber. ***B***, Graph of growth cone position for each frame (30 s) of the time-lapse movie shown in ***C***. The red arc represents the location of the inner rim of the spot. The arrow indicates the initial trajectory of growth. ***C***, Distance from the origin of six dystrophic axons in coculture with NG2+ cells on the aggrecan/laminin spot gradient following contact with macrophages. An arrowhead indicates the position at which the axon has retracted to an NG2 cell. Scale bar: ***A***, 20 μm.

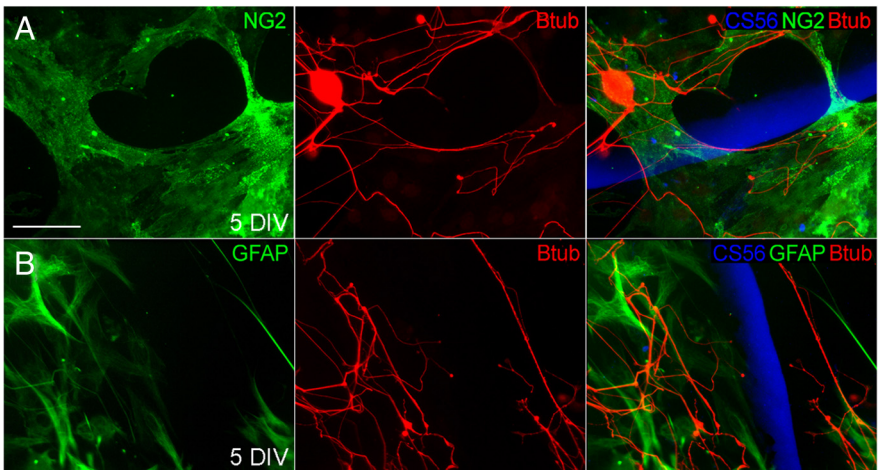

Figure 7. NG2+ cells can provide a bridge for regenerating axons. ***A***, NG2+ cells (green) derived from adult mouse spinal cord can cross a proteoglycan gradient, visualized with CS56 (blue), after 5 d *in vitro*. Adult DRGs, visualized with β-tubulin (red), use adult NG2+ cells to bridge the inhibitory gradient. ***B***, Mature GFAP+ astrocytes (green) do not cross the proteoglycan gradient, and adult DRGs cannot use them as a bridge. Scale bar: ***A***, ***B***, 20 μm.

located more centrally (supplemental Fig. 7, available at www.jneurosci.org as supplemental material). Satellite cells from the DRG neuron preparation expressed laminin, but not fibronectin, and were small in number compared to the NG2+ cells present in the monolayer (data not shown). We wanted to determine whether laminin or fibronectin were responsible for the degree of neurite outgrowth observed on the NG2+ cell surface. We blocked laminin or fibronectin with specific function-blocking antibodies on a layer of confluent adult NG2+ cells and added adult DRGs to this culture after 1 d *in vitro* and observed neurite outgrowth after 24 h. Control antibody-treated DRGs extended an average of 326 ± 8 μm. Blocking of fibronectin decreased neurite outgrowth on NG2+ cells to an average of 238 ± 10 μm, and blocking laminin dramatically decreased neurite outgrowth to an average of 164 ± 8 μm (Fig. 9*C–E*,*G*).

Discussion

Here we sought to determine why the front of severed, dystrophic fibers stops retracting at a certain distance from the lesion center despite the presence of large numbers of activated macrophages. It is known that cells proliferate and upregulate NG2 proteoglycan in response to spinal cord hemisections, ischemia, and experimentally induced demyelination (Levine et al., 2001). The fact that NG2 is present at sites where regeneration is arrested led to the hypothesis that NG2+ cells and the NG2 proteoglycan itself are inhibitory to axon outgrowth. The influence of NG2 on regeneration failure has become a topic of great interest (Rezajooi et al., 2004; de Castro et al., 2005; Tan et al., 2006; Yang et al., 2006).

Within the lesion core, nestin/vimentin+ cells, many of which also express NG2, represent a heterogeneous population. However, determining their identity is complicated by the overlapping expression of various progenitor cell markers. Previous work has suggested that they may be oligodendrocyte precursor cells (McTigue et al., 2001; Horky et al., 2006; Tripathi and McTigue, 2007; Yoo and Wrathall, 2007), nonmyelinating Schwann cells (McTigue et al., 2006), pericytes (Ozerdem and Stallcup, 2004), meningeal cells (Shearer et al., 2003), macrophages (Jones et al., 2002), or microglia (Pouly et al., 1999). Regardless of their identity, vimentin+ glia in the vicinity of the lesion may serve to guide regenerating axons after spinal cord injury (Hsu and Xu, 2005).

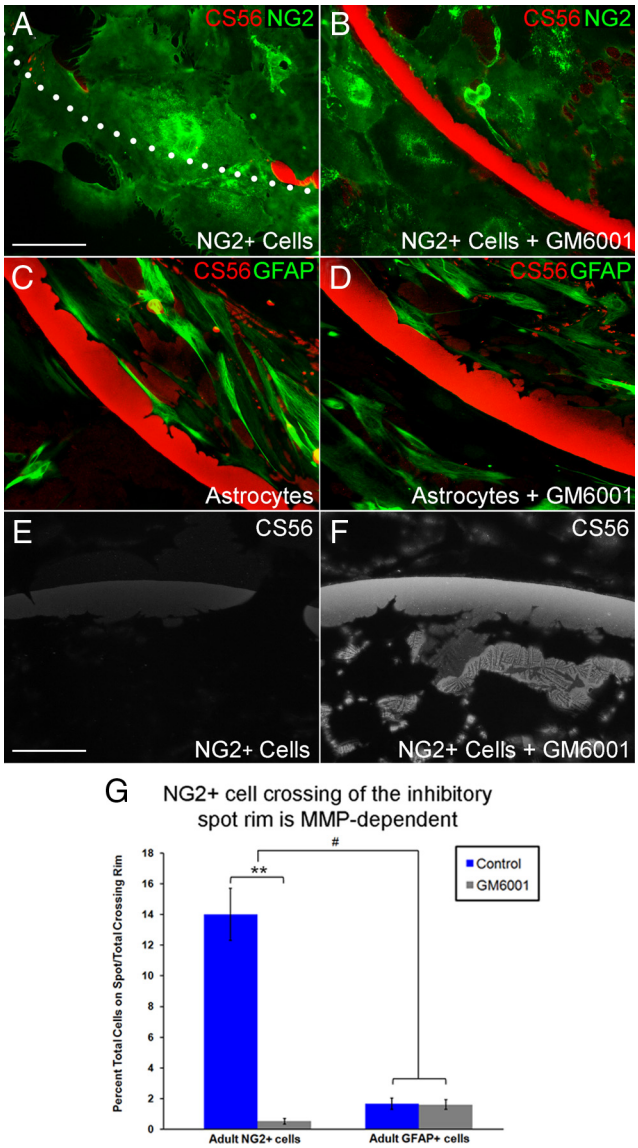

Figure 8. GM6001 prevents NG2+ cells from crossing the inhibitory proteoglycan rim over 5 d *in vitro*. ***A***, Untreated NG2+ cells (green) 5 DIV cross the inhibitory spot rim (red), denoted by the dotted line. ***B***, GM6001 prevents NG2+ cells (green) from crossing the spot rim, identified by CS56 (red). ***C***, GFAP+ astrocytes (green) do not cross the spot rim (red) over a period of 5 d. ***D***, The lack of astrocytes crossing the rim does not change upon GM6001 treatment. ***E***, Image (16×) of the proteoglycan rim of a spot after 5 d *in vitro* with NG2+ cells as visualized with CS56. ***F***, Image (16×) of the proteoglycan rim of a spot after 5 d *in vitro* with NG2+ cells and the broad spectrum MMP inhibitor, GM6001, taken using the same exposure time as ***E***. ***G***, Adult NG2+ cell crossing of the rim is MMP dependent. The Kruskal–Wallis test was used to test overall significance ($\chi^2_{(3)} = 69.621$, $p < 0.001$). The Mann–Whitney *U* test was used for pairwise comparisons: $^{\#}p < 0.02$, $^{**}p < 0.001$. Scale bars: ***A–F***, 100 μm.

We have confirmed a preferential association of regenerating axons with populations of cells expressing NG2 following spinal cord injury, during the period of axonal dieback.

Although there is extensive commingling between NG2+ cells and ED1+ macrophages, we observed very little association between GFAP+ astrocytes and macrophages. This is consistent with previous observations that astrocytes actively migrate away from macrophages in culture (Fitch et al., 1999), and retreat to the lesion penumbra as ED1+ cells infiltrate the core (Faulkner et al., 2004). After clodronate-mediated depletion of circulating monocytes, few macrophages invade the lesion and there is a concomitant decrease in astrocyte migration from the lesion center (Horn et al., 2008), further implicating the presence of macrophages as an instigator of the astrocyte response to injury. The signals that induce astrocyte migration away from the area of inflammation have not been fully elucidated, but NG2+ cells are clearly affected in a different manner as they readily associate with the population of inflammatory cells. It is known that proinflammatory cytokines can mobilize precursor cells and promote reparative processes (Ben-Hur et al., 2006), and here we found that the NG2+, vimentin+, and nestin+ cell populations expand within the lesion core concurrently with macrophage infiltration. Further studies are necessary to determine whether these cells arise from a population of resting progenitors within the spinal cord or whether they are recruited to the site following injury.

The idea that NG2+ cells may have a positive influence on regeneration has been suggested previously and supported by work using postnatally derived NG2+ cells, which still promote axon growth when surface NG2 is upregulated fivefold (McTigue et al., 2006; Yang et al., 2006). Isolation of a pure NG2+ population from the adult spinal cord allowed us to examine the interactions between adult neurons and this controversial cell *in vitro* for the first time. We have shown conclusive evidence that adult-derived NG2+ cells are not inhibitory to adult sensory neurite outgrowth. Interestingly, the presence of NG2+ cells in coculture with adult DRG neurons was unable to prevent the growth cone from becoming dystrophic, obstruct macrophage attack, or inhibit subsequent retraction of dystrophic neurons. However, when the axon reached an NG2+ cell, retraction ceased, the dystrophic growth cone flattened and stabilized. NG2+ cells appear to change the growth state of the axon, making the growth cones appear more as they would on other favorable substrates, although we do not yet fully understand this behavior. The ability of NG2+ cells to stop axonal retraction induced by macrophage attack *in vitro* suggests that these cells may also be able to stabilize axons undergoing dieback *in vivo* (Horn et al., 2008). It is possible that other cells in the lesion, such as astrocytes, can stabilize axons undergoing retraction especially at later time points. However, NG2+ cells are poised to stabilize axons due to their ability to invade the proteoglycan rich lesion core during macrophage infiltration. What allows an injured axon to finally come to rest and remain at the edge of the lesion for extended periods of time is a matter of speculation. NG2+ cells have been shown to generate action potentials and receive synaptic-like input from neurons (Bergles et al., 2000; Gallo, 2007; Káradóttir et al., 2008; Hamilton et al., 2010), and thus, it is possible that NG2+ cells could be acting as long-term synaptic partners for these injured axons.

The NG2+ cell population is the first example we found of an adult neural cell type able to tolerate the high levels of CSPG present in the outer rim of the spot gradient, and this could explain their ability to invade the lesion core. The mechanisms by which NG2+ cells migrate into the lesion have not been well characterized. It is known that MMPs can degrade various components of the ECM and regulate several repair processes in the CNS that require cell migration or invasion (Sternlicht and Werb, 2001; Yong, 2005). MMPs are known to be involved in the maturation and process extension of oligodendrocyte lineage cells (Larsen and Yong, 2004). Inhibition of MMPs with GM6001 prevented NG2+ cells from crossing the proteoglycan rim. Selective inhibition of MMP-2 or MMP-9 did not prevent NG2+ cell crossing; however, high concentrations of MMP-9 inhibitor, known to inhibit MMP-1 and -13, effectively prevented NG2+ cell crossing (data not shown). MMP-1 is upregulated after spinal cord injury (Xu et al., 2001) and known to cleave brevican and aggrecan (Nakamura et al., 2000), making it a strong candidate

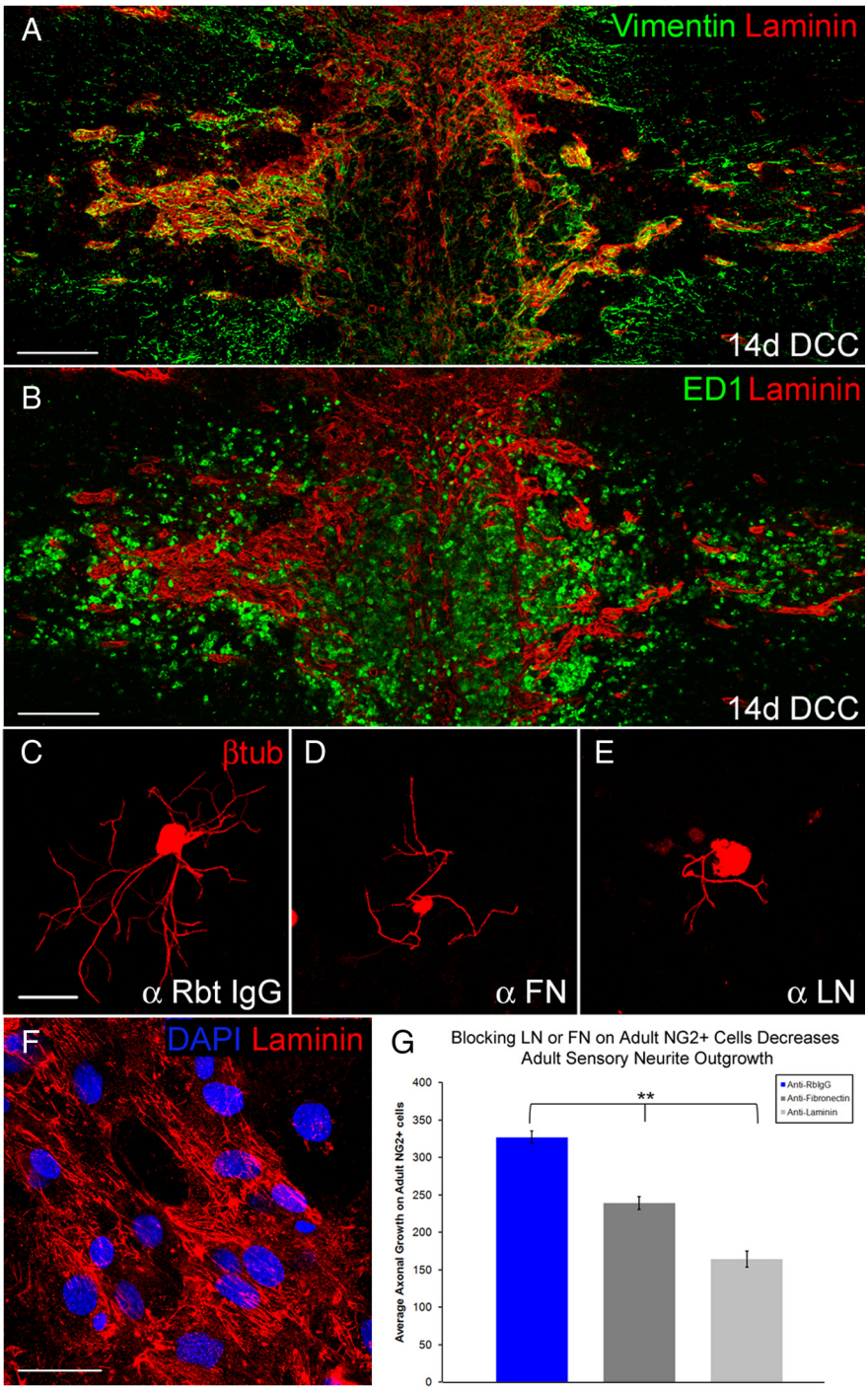

Figure 9. Laminin deposition is mostly associated with vimentin+ cells, and both laminin and fibronectin contribute to axonal outgrowth on NG2+ cells. The orientation of the longitudinal sections shown in ***A*** and ***B*** is such that caudal is on the left and rostral is on the right. ***A***, Confocal montage (10×) of a lesion 14 d after dorsal column crush injury. Laminin (red) is highly expressed in the lesion core and associated with vimentin+ cells (green). ***B***, Confocal montage (10×) of a lesion 14 d after DCC. ED1+ macrophages (green) and laminin (red). ***C–E***, Adult sensory neurons plated on a confluent culture of adult NG2+ cells for 24 h. ***C***, Adult DRGs visualized with β-tubulin (red) treated with anti-rabbit IgG control antibody for 24 h exhibit extensive process outgrowth. ***D***, Adult DRGs treated with anti-fibronectin (FN) antibody exhibit slightly diminished outgrowth. ***E***, Adult DRGs treated with anti-laminin (LN) antibody exhibit greatly diminished axonal outgrowth. ***F***, Confocal image (40×) of NG2+ cells expressing laminin (red). ***G***, Antibody blocking experiments reveal that the permissive nature of NG2+ cells is mediated by FN and LN. All groups are significantly different from each other (Kruskal–Wallis test, $\chi^2_{(2)} = 140.790$, $p < 0.001$, followed by Mann–Whitney *U* test, $^{**}p < 0.001$). Scale bars: ***A***, ***B***, 100 μm; ***C–F***, 50 μm.

for mediating NG2+ cell migration through lesion-associated proteoglycan. Another non-neural cell type, activated macrophages, can also migrate around the inhibitory rim and within the lesion core, but the mechanism these cells use to do so does not appear to involve proteoglycan digestion (Busch et al., 2009). Despite evidence that inhibition of MMPs after spinal cord injury can have beneficial effects (Noble et al., 2002; Busch et al., 2009), it is important to recognize the reparative functions of these proteases and to consider all cell types that would be affected by broad spectrum inhibition of MMPs, as this treatment could have a variety of effects on the spinal cord lesion environment.

Yang et al. (2006) suggested that the growth-promoting effects of NG2-expressing cells may be contact mediated. Indeed, we have shown that laminin and fibronectin are present in high quantities on the surface of adult NG2+ cells *in vivo* and *in vitro*, and that blocking these molecules greatly decreases neurite outgrowth. Cell-associated NG2 may provide some inhibition, but it is possible that the inhibitory region of NG2 is masked when it is expressed on the cell surface (Chen et al., 2002b), as the inhibitory properties of CSPGs can be attributed to the core protein as well as their chondroitin sulfate side chains. NG2 can be shed from the cell surface and incorporated into the extracellular matrix (Nishiyama et al., 2002), and this non-cell-associated conformation could expose the inhibitory regions of NG2 and contribute to regeneration failure *in vivo*. We have yet to fully characterize the effects of NG2 deposition on axonal regeneration, but it will be critical to determine an optimal balance and maintain the positive presence of NG2+ cells in the lesion while limiting the potentially negative effects of soluble NG2 in the ECM.

Our results suggest that spinal cord lesions that involve the white matter of the dorsal columns consist of two major compartments, the lesion core and the surrounding astrocytic scar (Fig. 10). Both compartments are flooded with a variety of sulfated proteoglycans that promote the dystrophic state in the regenerating growth cone. The core is occupied by a mixed population of cells expressing progenitor markers, many of which are NG2+, as well as activated macrophages. The second compartment consists of reactive astrocytes that have migrated from the lesion core, proliferated, and deposited proteoglycans, and presents a substantial barrier to axons. In the core, NG2+ cells are growth promoting and can counterbalance the negative effects of macrophages, which strongly inhibit forward progress of dystrophic fibers. Over time the dystrophic fiber front shifts backwards from the lesion center to a point where there is a balance between growth promotion and retraction. The three major

cell types in this compartment—neurons, NG2+ cells, and macrophages—can be altered in many ways (Bradbury et al., 2002; Neumann et al., 2002; Horn et al., 2008; Ylera et al., 2009). Despite the success of many of these strategies in driving axon growth into the lesion, this progress often ceases abruptly at the distal end. Our finding that NG2+ cells in the adult spinal cord are supportive of axonal regeneration adds to mounting evidence of considerable complexity in the lesion environment, mandating the utilization of combinatorial strategies in the treatment of spinal cord injury.

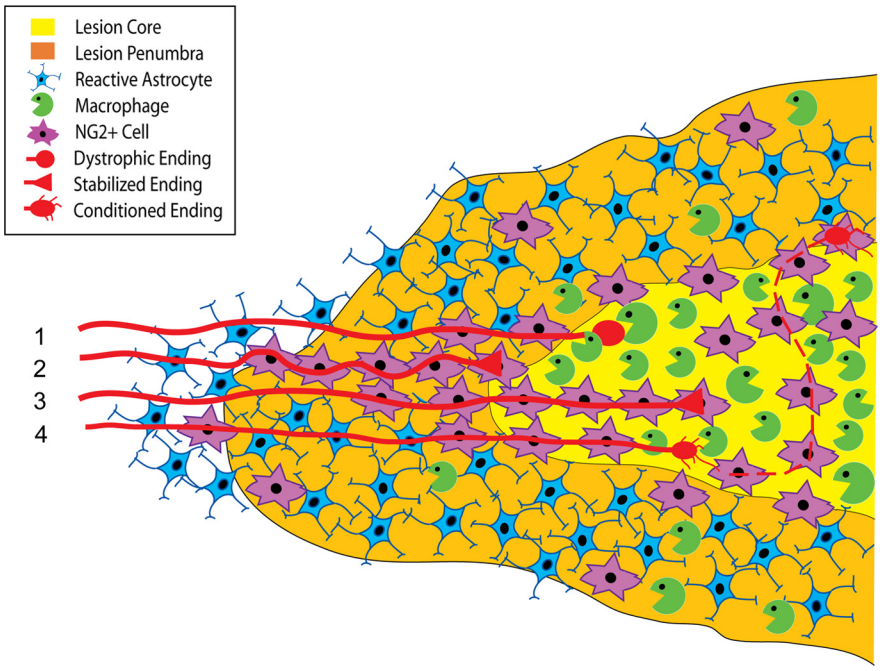

Figure 10. Schematic representation of the proximal end of a dorsal column crush lesion 7 d after injury. GFAP+ astrocytes (blue) have pulled away from the lesion core, which is now populated by NG2+ cells (purple) and phagocytic ED1+ macrophages (green). Dorsal root ganglion neurons (red) attempt to regenerate into the lesion core. 1, Typical axon with a dystrophic growth cone that has become susceptible to macrophage attack. 2, Typical axon that has undergone macrophage-mediated retraction back to NG2+ cells and stabilized. 3, Atypical axon that has stabilized further distally within the lesion core upon a contiguous bridge of NG2+ cells. 4, Growth cone of a neuron that has been stimulated or conditioned and has been able to overcome macrophage-induced axonal dieback and extend into the lesion core on NG2+ cells.

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