Adult spinal cord progenitor cells are repelled by netrin-1 in the embryonic and injured adult spinal cord

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Adult neural progenitor cells (aNPCs) exhibit limited migration in vivo with the exception of the rostral migratory stream and injury-induced movement. Surprisingly little is known regarding those signals regulating attraction or inhibition of the aNPC. These studies demonstrate that aNPCs respond principally to a repulsive cue expressed at the embryonic floor plate (FP) and also the injured adult CNS. Adult spinal cord progenitor cells (aSCPs) were seeded onto organotypic slice preparations of the intact embryonic or injured adult spinal cord. Cell migration assays combined with genetic and molecular perturbation of FP-derived migration cues or aSCP receptors establish netrin-1 (Ntn-1) but not Slit-2, Shh, or Ephrin-B3 as the primary FP-derived repellant. When slices were prepared from injured spinal cord, aSCP migration away from the injury core was Ntn-1-dependent. These studies establish Ntn-1 as a critical regulator of aSCP migration in the intact and injured CNS.

Results

aSCPs Migrate Away from the FP. The aSCP is distributed throughout the SC and has the unique capacity of self-renewal and multipotency (21). In addition, aSCP are one of the first cell populations to respond to CNS insult and generate astrocytes and oligodendrocytes, important for tissue homeostasis (22). NSCs were isolated from whole SC preparations of adult transgenic and reporter mice. They were expanded in serum-free media containing the mitogens FGF and EGF, and multipotency was periodically confirmed [supporting information (SI) Experimental Procedures].

To explore the potential cues involved in the control of adult progenitor migration, we cocultured GFP-expressing aSCPs on embryonic day 12 (E12) organotypic SC slices. An aggregate of aSCPs was placed at the midline of the slice, at the FP that expresses many guidance molecules (Fig. 1A). By 2 days in vitro (DIV), a pattern of cell migration was apparent that became distinct by 5 DIV. The aSCPs aligned at the ventral neuroepithelium (VN) with no aSCPs persisting at the FP (Fig. 1A–D). The aSCPs established this migratory pattern in 97% of the E12SC slice–aSCP cocultures.

To better understand this pattern of migration, we repeated the E12 SC slice–aSCP coculture, but with the roof plate, another major source of guidance molecules, as a midline structure. The aSCPs did not migrate, but stayed at the roof plate even after 5 DIV (Fig. 1E). These results suggest that the migration pattern requires the FP.

Embryonic-derived SC progenitor cells (E12) also were capable of proliferation, self-renewal, and multipotency in vitro (SI Fig. 5) and resettled similarly to the aSCP, indicating a potentially common mechanism of migration.

To rule out selective cell proliferation within the VN or increased cell death (apoptosis) in FP development, we performed an analysis of bromodeoxyuridine incorporation and apoptosis detection by immunostaining. These studies demonstrated equal rates of death and proliferation across the open-book preparation. In addition, aSCP placed at the FP remained undifferentiated with the exception of cells at the VN, which exhibited a predominantly astroglial


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Abbreviations: aSCP, adult SC progenitor cell; CM, conditioned media; DIV, days in vitro; En, embryonic day n; FP, floor plate; HEK, human embryonic kidney cell; KO, knockout; Ntn-1, netrin-1; NSC, neural stem cell; SC, spinal cord; VN, ventral neuroepithelium.

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respond to guidance molecules expressed during neural tube specifically repulsed by the FP, and they have the ability to and aSCPs established the migration pattern, but only on the half dissected away one part of the FP from the SC slice (SI Fig. 7 dorsal neuroepithelium (SI Fig. 7 FN). The aSCPs migrated to alternate sides of the endogenous FP and reproduced this pattern in register with the ectopic FP (SI Fig. 7 C and D). In the second experiment, we also dissected away one part of the FP from the SC slice (SI Fig. 7E), and aSCPs established the migration pattern, but only on the half of the slice with the preserved FP (SI Fig. 7F). For the third experiment, we dissected one part of the SC slice (SI Fig. 7H) and rotated it by 180° to place the dorsal neuroepithelium adjacent to the FP (SI Fig. 7G). The aSCPs migrated to either side of the FP and did not show any preference for the VN compared with the dorsal neuroepithelium (SI Fig. 7I and J). The aSCPs are specifically repulsed by the FP, and they have the ability to respond to guidance molecules expressed during neural tube development.

Slit-2, Ephrin-B3, Shh, and Ntn-1 as Potential Migration Cues. To identify the guidance molecule(s) responsible for the migration pattern, we screened for guidance receptors expressed by aSCPs by using RT-PCR for Slit-2, Ephrins, and Ntn-1 receptors. aSCPs express two of the three Slit receptors, Robo-1 and -2, but not -3 (SI Fig. 8A). At E12, Ephrin-B3 is highly expressed at the FP (SI Fig. 8 K and K’) (23), and aSCPs express many Ephrin-B receptors: Eph-B1, -B2, -B3, -B4, and -B6 (SI Fig. 8J). aSCPs also express the Ntn receptors of the DCC family, DCC and neogenin, and the UNC5 family, UNC5A, UNC5B, and UNC5C (Fig. 2A).

Because aSCPs express receptors for both Ntn-1 and Slits, and because these ligands are all highly expressed at the FP, we used four methods to determine the responsiveness of aSCPs to these molecules: (i) a coculture of aSCPs on an isolated substrate of human embryonic kidney cells (HEKs) (cell patch); (ii) a coculture of aggregates of aSCPs and HEKs in a three-dimensional collagen matrix; (iii) a transwell migration assay with conditioned media (CM); and (iv) a stripe migration assay with recombinant Ntn-1.

Ntn-1 is a Chemorepellent for aSCPs. We used a transwell assay in which CM from HEK, HEK-Slit2, HEK-Ntn1, and recombinant Ntn-1 were placed in the lower chamber, and aSCPs were placed in the upper chamber (Fig. 2G). The presence of Ntn-1 but not Slit2 in the lower chamber almost completely blocked aSCP migration into the lower chamber and onto the underside of the filter. Only 6–7% of the cells crossed the filter with HEK-Ntn1 or recombinant Ntn-1 at 100 ng/ml and 200 ng/ml (Fig. 2H) compared with 38–39% for the HEK and HEK-Slit2.

We used the collagen-embedded explant cocultures assay (24) to determine if Slit-2 and Ntn-1 might be responsible for FP-mediated repulsion. Aggregates of aSCPs were cocultured with aggregates of HEK, HEK-Slit2, or HEK-Ntn1 cells. When the aSCPs were in contact with the HEK and the HEK-Slit2 aggregates, 50–60% migrated into the aggregates (SI Fig. 8 D and E). In contrast, <5% of aSCPs migrate into HEK-Ntn1 aggregates (Fig. 2I). We also used an assay in which the substrate for aSCP growth was a patch of HEK, HEK-Slit2, or HEK-Ntn1 cells (Fig. 2B). aSCPs grew randomly over the coverslips when the patches of cells were HEK or HEK-Slit2 (SI Fig. 8 B and C and Fig. 2 C and D). In contrast, aSCPs developed a distinctive distribution when in contact with a patch of HEK-Ntn1 cells (Fig. 2 E and F). The aSCPs were clearly repelled by the Ntn-1-expressing cells and migrated outside the patch to form a clear border between the patch and the laminin-coated coverslip.

To directly test the repellent activity of Ntn-1, we used a stripe assay (Fig. 2K) to prepare a substrate of alternating bands of recombinant Ntn-1 and laminin (Fig. 2 K and L). The aSCPs were clearly repelled by Ntn-1, as indicated by the near-total absence of cells attaching to stripes of Ntn-1. These results support the conclusion that aSCPs are not repelled in response to Slit-2 but only in response to Ntn-1. In both stripe and transwell assays, Ntn-1 repulsion appears to depend on DCC (deleted in colorectal cancer) function (SI Fig. 9 A and B).

The Repellent Action of Ntn-1 Is Not Mediated by a Proliferation or Survival Mechanism. aSCPs were cultured with CM from HEK, HEK-Slit2, HEK-Ntn1, and recombinant Ntn-1 for 3 days. The cells were pulsed with BrdU and fixed after 6 h, 12 h, 1 DIV, 2 DIV, or 3 DIV. BrdU-positive cells were observed as soon as 6 h after the pulse with HEK-Slit2 and at 12 h for HEK. The number of positive cells remained increased and was similar for both conditions until 3 DIV (SI Fig. 10). In contrast, very few BrdU-positive cells were observed with HEK-Ntn1 and recombinant Ntn-1. After TUNEL staining, very few labeled cells were observed in any of the conditions. Ntn-1 does not increase aSCP death or proliferation and further supports the central concept of Ntn-1 mediating a migratory response of the aSCPs.

Slit-2, Ephrins-B, and Shh Are Not Implicated in the FP Migration Pattern. Although these results support the conclusion that Ntn-1 functions as an effective FP-derived repellent, they do not establish whether Ntn-1 is sufficient to cause repulsion from the FP in the organotypic slice model. Therefore, we investigated the potential contributions of Slit-2, Ephrin-B3, Shh, and Ntn-1 to the repulsive
activity of the FP and to the induction of the migration pattern by repeatedly the E12SC slice–aSCP coculture with methods to perturb their function.

We first evaluated the possible involvement of Slit-2 by inhibiting its extracellular action. We added CM containing Robo/Fc with the extracellular portion of Robo fused to an Fc domain of the human Ig to the E12SC slice–aSCP coculture. Robo/Fc binds to extracellular Slits and inhibits the function of those expressed in the SC slice (25). The addition of Robo-Fc CM did not affect frequency of pattern formation (SI Fig. 8 F–I).

Next, we tested the role of Ephrin-B3 by preparing SC organotypic slices from Ephrin-B3 KO embryos. Down-regulation of ephrin-B3 in the SC slice did not affect the aSCP migration pattern. The FP pattern was still observed for 77% of Ephrin-B3+/−, 83% of Ephrin-B3+/, and 87% of Ephrin-B3+ SC slices (SI Fig. 8 L–N and Q). We also blocked the ability of the aSCPs to respond to Ephrin-B family by isolating a fresh population of aSCPs from EphB1/B3 double knockout (KO) mice (SI Fig. 11 D–F). The FP pattern was observed for 90% of EphB1/B3+−-derived aSCPs and did not significantly differ from the probability of pattern formation observed in wild-type aSCPs (97%) (SI Fig. 8 O–Q).

To determine whether Shh is required for FP repulsion, we pretreated aSCPs with cycloamine, a plant alkaloid that selectively inhibits Shh signaling (26). We also preincubated the SC slices with Shh-N antibody against the biologically active amino-terminal fragment of Shh (27). After these two treatments, aSCPs still migrated away from the FP (in 88–96% of the slices; SI Fig. 8 R–V). These experiments suggest that Slit-2, Ephrin-B3, and Shh have a limited role in the elaboration of the FP migration pattern.

**Down-Regulation of Ntn-1 Disrupts the Pattern of Migration.** To determine whether Ntn-1 signaling is required for the repulsive activity of the FP and the establishment of the migration pattern, we repeated the coculture assay under conditions of decreased Ntn-1 expression. First, when cocultured with Ntn-1−/− SC organotypic slices, aSCPs stayed at the FP and did not migrate selectively to the VN (Fig. 3 A–D). Ninety-four percent of the wild-type SC slices resulted in the typical FP migration pattern, whereas only 15% of the Ntn-1−/− SC and 63% of the Ntn-1+− exhibited the pattern.

Because Ntn-1 is absent throughout development of the Ntn-1 KO, it could, during development, conceivably influence the expression of other guidance cues downstream. We sought to control for the effects of altered development of the Ntn-1−/− mouse by creating a model to selectively down-regulate Ntn-1 expression in a wild-type organotypic slice by using an Ntn-1 siRNA (Fig. 3 E–K and Tables 1 and 2). We found that siRNA against Ntn-1 successfully and selectively down-regulated Ntn-1 gene expression in HEK-Ntn1 cells compared with nontreated (32.6%), GAPDH siRNA-treated (27.9%), and negative siRNA-treated (27.9%) cells (Fig. 3 E and Table 1). GAPDH siRNA (positive control) and the scrambled siRNA as negative control did not decrease Ntn-1 expression.

We next examined if siRNA (Ntn-1) application on E12SC slices blocked aSCP migration or prevented the development of the FP migration pattern. After 3 days of siRNA treatment, the FP no longer promoted migration, and only 28% of the slices exhibited the FP pattern compared with 97% of controls (Fig. 3 F–J). After Ntn-1 siRNA treatment, there is a corresponding decrease in β-gal activity of the FP and to the induction of the migration pattern by repeating the E12SC slice–aSCP coculture with methods to perturb their function.

We first evaluated the possible involvement of Slit-2 by inhibiting its extracellular action. We added CM containing Robo/Fc with the extracellular portion of Robo fused to an Fc domain of the human Ig to the E12SC slice–aSCP coculture. Robo/Fc binds to extracellular Slits and inhibits the function of those expressed in the SC slice (25). The addition of Robo-Fc CM did not affect frequency of pattern formation (SI Fig. 8 F–I).

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expression at the FP of slices prepared from Ntn-1+/− slices (Fig. 3I) compared with the nontreated slices (Fig. 3F). siRNA against Ntn-1 applied to the SC slices down-regulated Ntn-1 gene expression compared with nontreated (32%), GAPDH siRNA-treated (41.8%), and neg siRNA-treated (30.8%) slices. The expression of Ephrin-B3 or GAPDH was not significantly affected (Fig. 3K and Table 2). As a control for the effects of perturbations on the extent of migration from the FP, we measured the most lateral spread of aSCPs on slices and found no differences (control: 158 ± 13 μm; Net-1 KO: 171 ± 10 μm; Net-1 siRNA: 191 ± 15 μm). These data indicate that among the guidance molecules that were tested, only Ntn-1 plays a major repellent role in the migration pattern of aSCPs.

**Ntn-1 Is Sufficient and Necessary to Repel aSCPs from an Injury Core.** These data show that Ntn-1 is up-regulated at the injury site after an SC lesion. The lesion core is a zone that becomes devoid of neural elements over time. Because we demonstrated that aSCPs are repelled by Ntn-1, it could participate in the repulsion of aSCPs from the lesion site after an SC injury. We developed an *in vitro* model to study the repulsion of aSCPs observed *in vivo* (Fig. 4A). Organotypic SC slices, prepared from an adult mouse after an SC hemisection, were cocultured with aSCPs. After a hemisection, Ntn-1 expression is increased in dorsal white matter compared with other regions (Fig. 4G and H). In the uninjured preparation, aSCPs remained within the dorsal columns where they were originally placed (Fig. 4B; 606 ± 46 μm from the central canal). However, 3 days postinjury, aSCPs migrated away from the injury core that was delineated by a gliotic ring and proteoglycan deposition (Fig. 4C; 285 ± 50 μm; P < 0.0001; n = 39). The model reproduces key elements of the repulsion seen after hemisection injury and allows us to interrogate the molecular signals responsible for repulsion from the lesion core. To determine if Ntn-1 could be totally or partially implicated in the repulsive activity at the injury site, we treated the injured SC slices with Ntn-1 siRNA for 3 DIV before placing the cells at the dorsal columns. In two replicate experiments, we found that Ntn-1 siRNA treatment significantly down-regulated Ntn-1 RNA (Fig. 4F). Importantly, Ntn-1 down-regulation (Fig. 4E; 613 ± 85 μm) or blocking of DCC function (SI Fig. 9C) prevented the repulsion of aSCPs from the lesion core, whereas digestion of condroitin sulfate proteoglycans had no effect (P < 0.0001; n = 18; Table 2). As a control for the effects of perturbations on the extent of migration from the FP, we measured the most lateral spread of aSCPs on slices and found no differences (control: 158 ± 13 μm; Net-1 KO: 171 ± 10 μm; Net-1 siRNA: 191 ± 15 μm). These data indicate that among the guidance molecules that were tested, only Ntn-1 plays a major repellent role in the migration pattern of aSCPs.

**Table 1. Comparison of Ntn-1 expression after siRNA treatment on HEK-Ntn1 cells**

<table>
<thead>
<tr>
<th>Gene expression</th>
<th>Treatment</th>
<th>Ntn-1 siRNA, %</th>
<th>GAPDH siRNA, %</th>
<th>neg siRNA, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ntn-1</td>
<td>No treatment</td>
<td>32.6</td>
<td>7.3</td>
<td>19.2</td>
</tr>
<tr>
<td>Ntn-1</td>
<td>Ntn-1 siRNA</td>
<td>27.9</td>
<td>27.9</td>
<td>27.9</td>
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<tr>
<td>GAPDH</td>
<td>No treatment</td>
<td>19.2</td>
<td>30.6</td>
<td>7.5</td>
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</table>

The relative expression of Ntn-1 and GAPDH was quantified from the intensity of bands in Fig. 3E. Bold underlined text indicates a significant decrease of Ntn-1 expression after Ntn-1 siRNA treatment.

**Table 2. Comparison of Ntn-1 expression after siRNA treatment on E12 SC slices**

<table>
<thead>
<tr>
<th>Gene expression</th>
<th>Treatment</th>
<th>Ntn-1 siRNA, %</th>
<th>GAPDH siRNA, %</th>
<th>neg siRNA, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ntn-1</td>
<td>No treatment</td>
<td>32</td>
<td>16.8</td>
<td>2.7</td>
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<tr>
<td>Ephrin-B3</td>
<td>No treatment</td>
<td>10</td>
<td>10.8</td>
<td>10.7</td>
</tr>
<tr>
<td>Ntn-1</td>
<td>Ntn-1 siRNA</td>
<td>41.8</td>
<td>30.8</td>
<td>7.0</td>
</tr>
<tr>
<td>Ephrin-B3</td>
<td>No treatment</td>
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<td>0.7</td>
<td>2.1</td>
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<tr>
<td>GAPDH for Ntn-1</td>
<td>No treatment</td>
<td>23.8</td>
<td>1.2</td>
<td>5.5</td>
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<tr>
<td>GAPDH for</td>
<td>Ephrin-B3</td>
<td>13.3</td>
<td>5.2</td>
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</tr>
</tbody>
</table>

The relative expression of Ntn-1 and GAPDH was quantified from the intensity of bands in Fig. 3K. Bold underlined text indicates a significant decrease of Ntn-1 expression after Ntn-1 siRNA treatment.
aSCPs Migrate in Response to a Guidance Cue. Although studies have documented that directed progenitor migration occurs in the adult brain and SC, the underlying molecular cues of these processes are still largely unknown (14–19, 28–30). Our data indicate that Ntn-1 is a major mediator of adult NSC repulsion. Netrins remain constitutively expressed in the adult vertebrate CNS, but their normal or postinjury roles are unknown (9, 11, 12, 31, 32). Studies in the lamprey demonstrate a continued role of adult-derived Ntn-1 as an axonal injury role for Ntn-1 in the adult (9, 34). In a complex CNS injury environment, many factors other than Ntn-1 expression alone could contribute to the repulsion of aSCPs. For example, it is suggested that Ntn-1 can collaborate with other molecules such as Shh (35, 36) and Slit (25) to guide axons, and that extracellular factors such as laminin-1 can affect Ntn-1 responsiveness through effects on the intracellular level of cAMP (37). The expression of receptors by the aSCP can also affect their response to Ntn-1. By itself, DCC mediates attraction, but DCC and UNC5 receptor families can form a complex that mediates repulsion (38). The relative amount of cell-surface DCC or UNC5 protein may determine the type of cell response: attraction or repulsion (9). For example, after optic nerve injury, a down-regulation of Ntn receptor modifies the sensitivity of axons to Ntn (12, 39). Future work is needed to actively track and manipulate migration cues and their cognate receptors in an in vivo setting. In addition, the molecular pathway responsible for Ntn-1 signaling in aSCP needs to be determined to guide molecular approaches in vivo.

**Discussion**

The experiments outlined here lead to a better understanding of the cues that affect the motility of adult SC stem cells in the intact and injured CNS. We demonstrated that aSCPs migrate and form an FP pattern of resettlement. Our observations confirm that Ntn-1 alone mediates the observed aSCP repulsion and the establishment of the FP migration pattern. The aSCP expresses DCC, neogenin, UNC5A, UNC5B, and UNC5C. Manipulation of Ntn-1 in a variety of in vitro paradigms demonstrates that Ntn-1 produces a clear repulsive effect. Importantly, we also demonstrate that Ntn-1 significantly participates in the repulsion of aSCPs from an SC lesion site in an in vitro model of SC injury. These data establish Ntn-1 as a key aSCP repulsive cue and illuminate the clear dominance of this cue compared with either eph/ephrin family members, Slit-2, Shh, or injury-related inhibitors like condroitin sulfate proteoglycans. Hence, Ntn-1 is likely a key mediator influencing aSCP migration in the intact CNS but also may influence the mode of progenitor migration in settings of injury and degeneration.
iters and whether Ntn-1 expression is typical of other CNS lesions and mammalian species. Understanding the pathways involved in Ntn-1-mediated NSC guidance is likely to provide insight into the function of other guidance molecules expressed in the adult CNS and lead to strategies for therapeutically directing NSC migration within the damaged brain.

Materials and Methods

See SI Experimental Procedures for further details.

Isolating and Culture of aSCPs on Embryonic SC Slices. Embryonic (E12) SC progenitor cells and aSCPs (3 mo) were isolated from transgenic mice expressing GFP under the β-actin promoter and an Eph B1/B3 double KO. Organotypic slices were prepared from neural tubes from C57BL/6, Ntn-1, and ephrin-B3 (E11.5–12.5) embryonic KO mice.

Migration Assays. Collagen gel cocultures. Cell aggregates were prepared as previously described (47). aSCPs were aggregated 0–200 μm apart from HEK-Ntn1 or HEK-Slit2 cell aggregates for 3 DIV (25).

Strip assay. Recombinant mouse Ntn-1 (200 ng/ml; R&D Systems, Minneapolis, MN) was mixed with blue fluorescent polymer microspheres (Du Pont Scientific, Palo Alto, CA). A coverslip precoated with type IV collagen (10 μg/ml; Invitrogen, Carlsbad, CA) was put onto a silicone matrix with 90-μm-wide parallel channels (Max Plank Institute, Tübingen, Germany), and the Ntn-1 was injected in the channels (48). aSCPs were plated onto the stripes substrate for 3 DIV.

Cell patch. Cell suspensions of HEK, HEK-Slit2, and HEK-Ntn1 were plated on laminin-coated coverslips. After 1 h, aSCPs were cultured on this patch substrate of adherent HEK cells and laminin for 3 DIV.

Transwell migration assay. aSCPs were plated at a density of 4 × 10^6 cells per ml on noncoated or laminin-coated polycarbonate transwell culture inserts (Corning, Acton, MA). Cell migration was evaluated for six conditions, including CM from HEK, HEK-Slit2, HEK-Ntn1, and recombinant Ntn-1 at 50, 100, and 200 ng/ml. At 24 or 48 h, cells were scraped from the upper and lower filter and counted.

Function-blocking assay. SC organotypic slices were dissected from Ephrin-B3 (49, 50) or an Ntn-1 KO mouse (51). The Ntn-1 mutant allele is severely hypomorphic but is not a complete null. Heterozygote and homozygote slices were compared with the wild type from the same litter.

To inhibit Ntn-1 function, we used small interfering RNAs (siRNA) to down-regulate Ntn-1 expression. We also inhibited the Ntn-1 by blocking the function of the DCC receptor with a monoclonal DCC antibody [OP455P1 DCC (Ab-1)] (47) EMD Biosciences/Cabiochem, San Diego, CA). To inhibit Slit-2 function, we added Robo1/Fc-CM (2:1, 1:1) (52). We controlled the activity of Slit-2 and Robo1/Fc-CM with a motor neuron repulsion assay (25). To block the SHH signaling, we used either SHH-N (5E1 IgG; Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA) or inhibited the function of Smoother (SMO; part of SHH-receptor complex) with cycloamine; tomatidine is a negative control (5, 10, and 20 μM; Toronto Research Chemicals, Toronto, ON, Canada).

SC Injury Model. Female C57B/6 mice (3 mo) were subjected to a (T8) dorsal hemisection as previously described (22). One hour after injury, the mouse was killed, and coronal slices were prepared from the lesion core and noninjured animals and cultured for 6 DIV. siRNA and transfection agent (Ambion, Foster City, CA) was applied at 0 h, 1 DIV, and 2 DIV after the hemisection; and after 3 DIV, aSCPs aggregates were placed at the dorsal columns, representing the acute injury site. The slices were also treated with chondroitinase ABC (53).

Analysis and Image Processing. The presence of an FP pattern was defined as the accumulation of the majority (>90%) of aSCPs on each side of the FP. Statistical analysis was performed by a Fisher’s exact test with a confidence interval set at 95% and a t test (GraphPad Prism; GraphPad, San Diego, CA). In the in vitro injury model, we evaluated cell migration by determining the distance of the aSCP GFP intensity center from the central canal (Metamorph 6.2). All experiments were repeated at least three times.

We thank Dr. Jane Wu (Northwestern University, Chicago) for providing HEK-Slit2 cells; Dr. Robert Rostomily (University of Washington) for cyclomamine; and Loan Nguyen and Don Maris for technical assistance. A.P. is a Fonds de la Recherche en Sante du Quebec fellow. This work was supported by grants from the Craig H. Nielsen Foundation and National Institutes of Health Grant R01 NS546724 (to P.J.H.). P.J.H. is a member of the University of Washington Institute for Stem Cell and Regenerative Medicine and The Center on Human Development and Disability.

A

0 h 1 DIV 2 DIV
BrdU (0.5 µM) 4% PFA fixation and BrdU IHC Tunel reaction 4% PFA fixation and BrdU IHC Tunel reaction

B GFP/ BrdU

1 DIV

C GFP/ Tunnel

2 DIV

D E FP FP FP FP
<table>
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<tr>
<th>A</th>
<th>0 h</th>
<th>6 h</th>
<th>12 h</th>
<th>1 DIV</th>
<th>2 DIV</th>
<th>3 DIV</th>
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<td>CM + BrdU (0.5 µM)</td>
<td>4% PFA fixation and BrdU IHC Tunel reaction</td>
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B

**HEK CM**

C

**HEK-Slit2 CM**

D

**HEK-Ntn1 CM**

E

**HEK CM**

F

**HEK-Slit2 CM**

G

**HEK-Ntn1 CM**