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# PROLIFERATION, MIGRATION, AND DIFFERENTIATION OF ENDOGENOUS EPENDYMAL REGION STEM/PROGENITOR CELLS FOLLOWING MINIMAL SPINAL CORD INJURY IN THE ADULT RAT

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**Abstract—Ependymal cells of the adult mammalian spinal cord exhibit stem/progenitor cell properties following injury. In the present study, we utilized intraventricular injection of 1,1′-dioctadecyl-6,6′-di(4-sulfophenyl)-3,3,3′,3′-tetramethylindocarbocyanine (DiI) to label the ependyma lining the central canal to allow tracking of the migration of endogenous ependymal cells and their progeny after spinal cord injury (SCI). We developed a minimal injury model that preserved the integrity of the central canal and did not interfere with ependymal cell labeling. Three days following SCI, there was an 8.6-fold increase in the proliferative labeling index of the ependymal cells at the level of the needle track based on bromodeoxyuridine labeling, compared with 1 day post-injury. Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) positive cells were not detected in the ependyma or surrounding gray matter, indicating that ependymal cells do not undergo apoptosis in response to minimal injury. Nestin was rapidly induced in the ependyma by 1 day and expression peaked by 7 days post-injury. We quantitated the number and distance of ependymal cell migration following minimal injury. The number of ependymal cells migrating from the region of the central canal increased by 3 days following minimal injury and DiI-labeled glial fibrillary acidic protein expressing cells were detected 14 days post-SCI, most of which migrated within 70 μm of the region of the central canal. These results show that a minimal SCI adjacent to the ependyma is sufficient to induce an endogenous ependymal cell response where ependymal stem/progenitor cells proliferate and migrate from the region of the central canal, differentiating primarily into astrocytes.** 

**Key words: carbocyanine tracer, ependyma, GFAP, nestin, spinal cord, TUNEL.**

The ependyma in the spinal cord plays a significant role in regeneration after injury in lower vertebrates such as amphibians and lizards (Simpson, 1968; Nordlander and Singer, 1978; Beattie et al., 1990; Chernoff, 1996). After spinal cord injury (SCI), ependymal cells rapidly proliferate, migrate, and differentiate to regenerate the cord. Proliferation of ependymal cells is common during embryonic and early postnatal periods of development in most species; however, ependymal cell turnover declines significantly postnatally (Bruni, 1998). In adult mammals, limited proliferative activity persists in the ependyma under normal conditions (Adrian and Walker, 1962; Rakic and Sidman, 1968; Bruni, 1998; Horner et al., 2000). However, after SCI, there is an increased proliferative response of ependymal cells. Ependymal cell proliferation has been observed in response to several types of trauma, including compression injury (Vaquero et al., 1981; Wallace et al., 1987; Namiki and Tator, 1999), contusion injury (Beattie et al., 1997), dorsal funiculus incision (Johansson et al., 1999), and spinal cord transection (Matthews et al., 1979). The ependyma also responds to mitogenic growth factor stimulation. Previously, we have shown that intrathecal administration of the combination of epidermal growth factor and fibroblast growth factor 2 for 14 days resulted in a significantly higher and more prolonged increase in ependymal cell proliferation and improvement in behavioral recovery after clip compression injury (Kojima and Tator, 2000, 2002).

In the present study, we labeled the ependymal cell layer with the carbocyanine tracer 1,1′-dioctadecyl-6,6′-di(4-sulfophenyl)-3,3,3′,3′-tetramethylindocarbocyaninez (DiI) in order to track the proliferation, migration, and differentiation of ependymal cells after injury. Johansson et al. (1999) similarly labeled the ventricular neuraxis and showed that a precursor cell population expressing nestin exists around the central canal that can proliferate and migrate to a SCI site, and they suggested that this precursor cell population ultimately contributed to the glial scar. Recently, neural stem cells in the spinal cord were shown to reside close to the central canal since multipotential, self-renewing neurospheres were generated only when the cultured tissue included parts of the central canal (Martens et al., 2002). In the present study, we developed a minimal SCI model with preservation of the integrity of the central canal and surrounding cells that allowed us to examine in greater detail the post-traumatic ependymal cell response than was possible with the previously used clip compression model. The proliferative activity of ependymal cells was assessed by comparing the bromodeoxyuridine labeling indices. Here we show that even a minimal injury adjacent to, but not directly involving, the central canal induces an ependymal cell response where endog-

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*Abbreviations:* ANOVA, analysis of variance; BBB, Basso, Beattie, and Bresnahan; BrdU, 5-bromo-2′-deoxyuridine; CFDA-SE, 5- (and 6-) carboxyfluorescein diacetate, succinimidyl ester; CSF, cerebrospinal fluid; DAB, 3,3′-diaminobenzidine; DAPI, 4′,6-diamidino-2-phenylindole; DiI, 1,1′-dioctadecyl-6,6′-di(4-sulfophenyl)-3,3,3′,3′-tetramethylindocarbocyanine; GFAP, glial fibrillary acidic protein; H&E, hematoxylin and eosin; LFB, Luxol Fast Blue; LI, labeling index; PBS, phosphate-buffered saline; SCI, spinal cord injury; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling; 30 g, 30 gauge.

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enous ependymal stem/progenitor cells proliferate and express nestin, migrate from the region of the central canal, and differentiate to an astrocytic phenotype. This is the first study to demonstrate a temporal profile of endogenous ependymal cell migration and differentiation following injury in the mammalian spinal cord.

## EXPERIMENTAL PROCEDURES

### Animals

All animal procedures were performed in accordance with the Guide to the Care and Use of Experimental Animals (Canadian Council on Animal Care) and approved protocols from the Animal Care Committee of the Research Institute of the University Health Network. The Canadian Council on Animal Care conforms to the international guidelines on the ethical use of experimental animals. Any animal discomfort was minimized by subcutaneous administration of the analgesic byprenorphine (0.3 mg/kg) and thorough post-surgical care including daily observations of animal behaviour and nutritional supplementation if required. Adult female Sprague–Dawley rats (250–300 g; Charles River, St. Constant, QC, Canada; $n=72$) were anesthetized by inhalation of 5% halothane which was reduced to 2% during surgery, in combination with a mixture of nitrous oxide and oxygen (1:2, v/v).

### Labeling of ependymal cells

To label the ependymal cells, 10 μl of a 0.2% (w/v) solution of DiI (SP-DiIC$_{18}$; Molecular Probes Inc., Eugene, OR, USA) in dimethylsulfoxide was stereotactically injected through a burr hole into the right or left lateral ventricle 24 h prior to injury. The injection coordinates were 0.9 mm posterior and 2 mm lateral to bregma and 3.6 mm below the dura mater. To verify the specificity of ependymal cell labeling, 10 μl of a 10 mM (v/v) solution of CFDA-SE (5- (and 6-) carboxyfluorescein diacetate, succinimidyl ester; Molecular Probes Inc.) in dimethylsulfoxide was injected intraventricularly according to the same method described above for DiI.

### Minimal injury model

Twenty-four hours after DiI or CFDA-SE injection, the rats were re-anesthetized as described above and the spinal cord was exposed by laminectomy at thoracic level T8. The minimal injury was performed bilaterally using a 30 gauge (30 g) sterile needle that was bent with the tip angled approximately 45 ° from the shaft (Fig. 1A). Prior to performing the minimal injury, the halothane concentration was transiently increased to 4% from 2% to slow the rat's respiration to avoid movements of the needle due to respiration. With the aid of the operating microscope, the needle was manually inserted into the cord 0.5 mm from the midline using the dorsal midline vein as a landmark and obliquely into the lateral columns in a caudal to rostral direction with the needle tip moving approximately 2 mm rostrally. As shown in Fig. 1A, the initial angle of the needle was estimated with the operating microscope to be approximately 75° to the surface of the cord and then moved caudally so the final angle of the needle was approximately 135° to the surface of the cord before it was withdrawn. Rats were killed 1, 3, 7, and 14 days following injury. This SCI resulted in minimal functional deficits. According to the Basso, Beattie, and Bresnahan (BBB) locomotor rating scale (Basso et al., 1996) with which normal rats attain a score of 21, the rats averaged an initial BBB score of 16 at 1 day post-injury and an average BBB score of 19 at 1 week following minimal injury. Fifty milligrams/kilogram of 5-bromo-2′-deoxyuridine (BrdU; Sigma, St. Louis, MO, USA) was administered intraperitoneally after surgery three times per day for the 1 day (1d) and 3 day (3d) survival groups. In the 7 day (7d) and

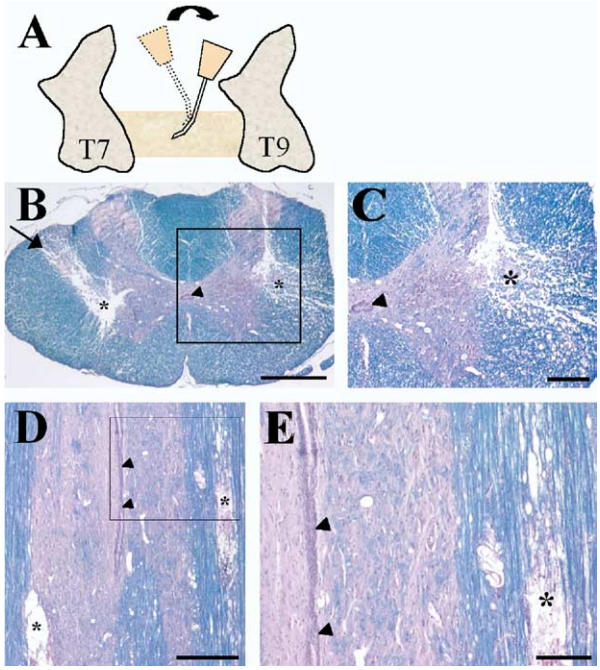


**Fig. 1.** Minimal injury model. The minimal injury model is illustrated in the schematic diagram in panel A. The spinal cord was exposed at level T8 and a bent 30 g needle was inserted bilaterally into the lateral columns. As shown by the curved arrow, the needle was inserted in a caudal to rostral direction and the tip moved approximately 2 mm rostrally. Panel B shows the histology at 1d after minimal injury in a cross-section of the spinal cord stained with H&E/LFB. The bilateral needle injury is evident in the lateral columns of the cord (indicated by asterisks) while the region of the central canal (arrowhead) remains intact. The needle track is indicated by the arrow. The boxed area is shown at higher magnification in panel C, demonstrating the lesion site (asterisk) relative to the central canal (arrowhead). Panel D shows a longitudinal section of the spinal cord 1d after minimal injury indicating the bilateral lesion (asterisks) in relation to the central canal (arrowheads). The boxed area is shown at higher magnification in panel E. Scale bars=500 μm in B and D; C and E=200 μm.

14 day (14d) survival groups, 50 mg/kg of BrdU was administered once daily until kill. BrdU administration was performed as previously described (Kojima and Tator, 2002), so that we could compare the BrdU labeling profile obtained in a clip compression SCI model to the minimal needle injury model described in the present study. Sham injury animals for each time point were injected with DiI and BrdU as described above and underwent a laminectomy but no SCI.

### Histology and immunohistochemistry

Animals were deeply anesthetized with i.p. injection of sodium pentobarbital and were transcardially perfused with 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS), pH 7.4. Tissue was cryoprotected in 30% sucrose in 0.1 M PBS for at least 24 h and a segment of the spinal cord 1.0 cm in length encompassing the injury site was removed, embedded in Tissue-Tek OCT embedding compound (VWR, Mississauga, ON, Canada), and cryosectioned transversely into 20 μm serial sections. Sections were thaw-mounted and the DiI signal was viewed immediately following sectioning. For detection of BrdU incorporation, sections were washed in 0.1 M PBS and incubated in 2 N HCl for 20 min at 60 °C to denature cellular DNA. The sections were washed for 30 min and incubated with mouse monoclonal anti-BrdU antibody (1:100; Becton Dickinson Biosciences, Missis-