

## Fetal Spinal Cord Tissue in Mini-Guidance Channels Promotes Longitudinal Axonal Growth after Grafting into Hemisected Adult Rat Spinal Cords

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### SUMMARY

Solid fetal spinal cord (FSC) tissue, seeded into semipermeable mini-guidance channels, was tested for the ability to promote axonal growth across the gap created by a mid-thoracic (T8) hemisection in adult rats. Fetal thoracic spinal cords, at embryonic days 13 to 15, were harvested and gently aspirated into mini-guidance channels (1.25 mm in diameter and 3.0 mm in length). Care was taken to maintain the rostral-caudal orientation of the FSC. In control rats, the FSC-channel construct was exposed to 5 freeze/thaw cycles to produce non-viable grafts before implantation into the hemisected cord. All cases revealed intact tissue cables of various diameters spanning the rostral-caudal extent of the lesion cavity, with integration of host-graft tissues at both interfaces. Immunofluorescence results indicated that numerous neurofilament-positive axons were present within the FSC tissue cable. Double-labeling of a sub-population of these axons with calcitonin gene-related peptide indicated their peripheral nervous system (PNS) origin. Descending serotonergic and noradrenergic axons were found in the proximity of the rostral host-

graft interface, but were not observed to grow into the FSC-graft. Anterograde tracing of propriospinal axons with *Phaseolus vulgaris*-leucoagglutinin demonstrated that axons had regenerated into the FSC-graft and had traveled longitudinally to the distal end of the channel. Few axons were observed to cross the distal host-graft interface to enter the host spinal cord. Cross-sectional analysis at the midpoint of the tissue cable stained with toluidine blue demonstrated a significant increase ( $P < 0.01$ ) in myelinated axons in viable FSC grafts ( $1455 \pm 663$ , mean  $\pm$  S.E.M.;  $n=6$ ) versus freeze-thaw control grafts ( $155 \pm 50$ ;  $n=5$ ). In addition to the myelinated axons, many unmyelinated axons were observed in the tissue cable at the electron microscopic level. Areas resembling the PNS with typical Schwann cells, as well as those resembling the central nervous system with neurons and central neuropil, were also seen. In freeze-thaw control grafts, neither viable neurons nor central neuropil were observed. Retrograde tracing with Fast Blue and Diamidino Yellow demonstrated that neurons within the FSC graft extended axons into the host spinal cord at least for 2 mm from both the rostral and caudal host-graft interfaces. We conclude that viable FSC grafts within semipermeable guidance channels may serve both as a permissive bridge for longitudinally directed axonal growth and a potential relay for conveying information across a lesion site in the adult rat spinal cord.

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## KEYWORDS

fetal spinal cord, spinal cord injury, neural tissue transplant, regeneration, rat

## INTRODUCTION

Traumatic spinal cord injury (SCI) produces devastating and tragic consequences by disrupting the normal anatomy and physiology of the spinal cord. Neuronal loss at the site of injury destroys segmental neural circuitry and motor output. The primary functional deficits following SCI result however, not from local neuronal or glial loss but rather from the interruption of long ascending and descending pathways within the spinal white matter. Damage to the long spinal pathways causes the loss of voluntary movement and sensory information from below the level of injury.

In both developing and adult animals, fetal spinal cord (FSC) transplantation has been identified as a possible therapeutic method for improving the recovery of function following SCI (for recent reviews see Anderson et al., 1995; Bernstein & Goldberg, 1995; Bregman et al., 1997; Jones & Harris, 1996; Reier et al., 1992; Zompa et al., 1997). Fetal spinal cord tissue has been proposed as not only a substratum for axonal growth but also a source of neurons with which to relay signals across the graft. Fetal tissue has been demonstrated to survive, to integrate into the host cord parenchyma, and to promote axonal growth in a variety of intraspinal transplantation paradigms (Anderson et al., 1995; Bregman, 1987; Giovanini et al., 1997; Pallini et al., 1989; Reier et al., 1986; Tessler et al., 1988). In neonatal animals, FSC grafts rescue immature axotomized neurons from retrograde cell death and support the growth of axons from the host central nervous system (CNS), both into and through the site of injury (Bregman & Bernstein-Goral, 1991; Diener & Bregman, 1998). In adult animals, FSC-mediated regeneration is relatively limited (Jakeman & Reier, 1991).

We have recently reported the development of

an intraspinal mini-channel implantation model in the partially lesioned spinal cord (Xu et al, 1995a; Xu et al, 1999). This model is characterized by a right spinal cord hemisection at T8, implantation of a cell- or tissue-containing mini-channel, and restoration of cerebrospinal fluid (CSF) circulation by suturing the dura. Using this model, we demonstrated that grafted Schwann cells in mini-channels form an effective bridge promoting substantial growth of proprio- and supra-spinal axons. One advantage of this model is that the guidance channels provide a "bridge-like" support spanning the injury gap. A second advantage is the capability of the guidance channel to maintain the graft orientation, allowing axons to grow longitudinally and in parallel. A third advantage is that the channel content can be easily modified by the addition of various cell and tissue types, including the use of FSC tissue.

In the present study, we examined whether FSC tissue could be used to substitute for Schwann cells in promoting axonal growth in our model system. In addition, we studied whether FSC could promote the longitudinal growth of axons while being constrained within the guidance channel environment. Finally, we investigated whether embryonic neurons could survive transplantation and whether spinal cord morphology could be maintained following FSC-channel construct implantation. A preliminary report of this work has appeared in abstract form (Bamber et al., 1997).

## METHODS

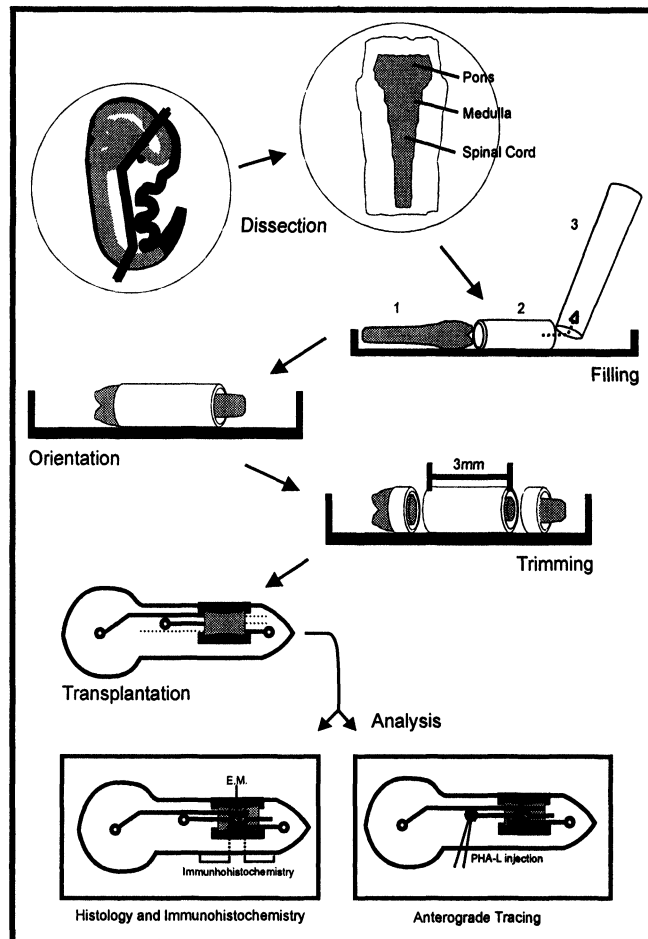
### Subjects

We used 43 adult Fischer-344 female rats, weighing 155 to 185g each (Harlan Biosciences, Indianapolis, IN) in this study. Among them, 8 pregnant females were used to obtain fetal spinal cord tissue at embryonic day (E) 13 to 15 (E0= day of insemination). The remaining rats served as hosts for grafts of viable (experimental group, n=30) or non-viable FSC (control group, n=5) tissue.

### FSC Graft Preparation

Donor dams were anesthetized with sodium pentobarbital (45mg/kg, i.p.), and the embryos were harvested and placed into a 100-mm Corning tissue culture dish (Baxter, Stone Mountain, GA) containing Leibovitz's L-15 medium (Gibco Lab., Grand Island, NY). The brainstem and spinal cord were carefully dissected out and transferred into a 35mm tissue culture dish containing fresh L-15 medium. The fetal tissue was then gently aspirated into a semi-permeable guidance channel (1.25 mm outer diameter, 5 mm length), using a glass pipette

placed at the distal end of the channel (Fig. 1). The channel is composed of a 60:40 polyacrylonitrile:polyvinylchloride copolymer (PAN/PVC; MWCO=50KDa). In the control group, the FSC-containing mini-channel was placed into a 35 mm culture dish lined with a piece of aluminum foil and then exposed to a series of 5 freeze/thaw cycles (from  $-42^{\circ}$  to  $26^{\circ}$  C) on the freezing stage of a sliding microtome, in an attempt to provide non-viable fetal tissue controls. Similar methods have been used successfully to generate acellular peripheral nerve grafts (Berry et al., 1988; Hall & Berry, 1989; Ide et al., 1983; Smith & Stevenson, 1988). To maintain the rostro-caudal orientation



**Fig. 1:** Schematic drawing showing the procedures for filling the FSC tissue (1) into a mini-guidance channel (2) by gentle suction (3). The orientation, trimming, and transplantation of the FSC-containing mini-channels, as well as analysis of the histology, immunohistochemistry, and anterograde tracing of axons that entered the FSC graft are also indicated.

of the fetal tissue within the channel environment, the lower brainstem and upper cervical cord were left intact until transplantation, at which time the fetal tissue-containing channel was trimmed to 3 mm in length by removing both the brainstem and the upper cervical cord. Thus, the FSC tissue cable within the guidance channel represents the lower cervical and thoracic levels of the cord.

### **Spinal Cord Hemisection and Graft Implantation**

While the FSC-containing channel constructs were being made, the adult Fischer rats were simultaneously prepared for channel implantation. Rats were anesthetized with sodium pentobarbital (45mg/kg, i.p.) and placed in a stereotaxic frame. Before surgery, ampicillin (Polyflex, 100mg/kg body weight, i.m.; Fort Dodge Animal Health, Fort Dodge, IA) was administered, and Duratears lubricant eye ointment (Alcon Laboratories, Inc., Fort Worth, TX) was applied to the eyes to prevent drying. During surgery, the animals were placed on a water-heated platform to maintain body temperature at  $37\pm 0.5^{\circ}\text{C}$ . A multi-level laminectomy was performed at the 7<sup>th</sup> and 8<sup>th</sup> thoracic (T) vertebral level. After a longitudinal incision was made in the dura, a spinal cord hemisection was made at T8 on the right, followed by the removal of a 2.5- to 2.8-mm hemicord segment. After bleeding was controlled with pledgets of gelfoam (Upjohn, Kalamazoo, MI), a 3mm FSC-containing mini-channel was implanted into the lesion site so that the proximal and distal stumps of the hemicord fit into the openings of the mini-channel, contacting both ends of the grafted FSC. The lower cervical portion and the thoracic portion of the FSC grafts were placed in apposition to the proximal and distal host hemi-cord stumps, respectively. The dura was sutured with a 10-0 silk suture (Ethicon, Somerville, NJ) to restore cerebrospinal fluid (CSF) flow around the graft and to decrease scar tissue invasion. The wound was then closed in two layers with 6-0 PDS II and 4-0 chromic gut sutures (Ethicon), respectively. The animals were placed on a heating pad following surgery until they recovered from the anesthesia, at which time

they were placed in recovery cages and returned to the Department of Comparative Medicine Animal Facility.

### **Post-Operative Animal Care**

Following channel implantation, the operated animals received vigilant care, including Buprenorphine (0.1mg/kg body weight, s.q.; Reckitt & Colman Pharm. Inc., Richmond, VA) as an analgesic agent twice daily for 3 days, physiologic saline (10 ml, s.q.) daily for ten days, manual bladder expression twice daily until bladder function returned, and moistened rat chow until pre-operative body weight was regained. The animals survived for approximately 30 days, with the exception of animals receiving injections of *Phaseolus vulgaris*-leucoagglutinin (PHA-L; Vector Laboratories, Burlingame, CA) for anterograde tracing or Fast Blue (FB, Sigma) and Diamidino Yellow (DY, Sigma) for retrograde tracing, which survived for an additional 7 days to allow transport of the tracer within the spinal cord. All surgical procedures and post-operative animal care were provided in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council, 1996) and the Guidelines and Policies for Rodent Survival Surgery provided by the Animal Care Committee of Saint Louis University.

### **Perfusion of Animals**

At the end of the survival period, graft recipients were anesthetized with sodium pentobarbital (60mg/kg, i.p.) and transcardially perfused with normal saline, followed by 500 ml of modified Zamboni fixative (Holets et al., 1987), using a Masterflex perfusion pump (American Medical Systems, Cincinnati, OH). Host spinal cords were dissected out and placed into 0.1 M phosphate-buffered saline (PBS). A 1 mm-thick transverse section was taken from the graft midpoint, placed overnight in a fixative solution of 2.5% glutaraldehyde and 5% sucrose in 0.1 M cacodylate buffer (pH 7.4), and then

processed for both light and electron microscopy (EM). The remaining graft tissue, along with the attached rostral and caudal spinal cord segments, was post-fixed overnight in the same perfusion fixative before being transferred to 30% sucrose buffer for immunohistochemical preparation.

### Light and Electron Microscopy

The tissue taken from the middle of the graft was immersed for 1 hr in 1% osmium tetroxide in 0.1 M cacodylate buffer. Next, the tissue was rinsed in buffer, dehydrated in graded alcohol and in propylene oxide, and then embedded in Spurr's (1969) epoxy resin. Semi-thin plastic sections (1  $\mu$ m thick) were cut and stained with 1% toluidine blue-1% sodium borate. The number of myelinated axons found in the middle of the FSC-graft was determined by plotting the axon cross-sectional profiles, using Neurolucida software (MicroBrightField, Inc., Colchester, VT) and a Leica DRMB microscope equipped with a 63 $\times$  light objective lens. The myelinated axons in FSC-containing or control cables were counted in these sections and then analyzed statistically with a Wilcoxon Rank Sum test (SigmaStat 2.0; Jandel Scientific, San Rafael, CA). For EM, the transverse ultra-thin sections of FSC-containing tissue cables were contrast-enhanced with uranyl acetate and lead citrate and examined using a Zeiss 108 transmission electron microscope.

### Immunohistochemistry

Spinal-cord segments containing the rostral and caudal FSC-graft sections were embedded in Tissue Freezing Medium (Triangle Biomedical Sciences, Durham, NC), cut horizontally on a cryostat at 15  $\mu$ m, and representatively mounted on gelatin-coated slides. The sections were placed for 15 min in a blocking solution of 0.3% Triton X-100 and 10% normal goat serum in 0.01M PBS. The antibodies used for staining included (a) monoclonal mouse SMI-31 antibody (1:2000 in Tris HCl; Sternberger/ Meyer Immunochemicals Inc., Jarrettsville, MD) to identify the phosphorylated NF-H component of

axons; (b) polyclonal rabbit anti-calcitonin gene-related peptide (CGRP) antibody (1:200; Sigma Chemical Corp., St. Louis, MO) as a marker for axons from a subpopulation of dorsal root ganglion neurons; (c) polyclonal rabbit anti-5-HT antibody (1:100; Incstar Corp., Stillwater, MN) to identify serotonergic axons; (d) polyclonal rabbit anti-DBH (dopamine  $\beta$ -hydroxylase) antibody (1:200; Eugene Tech International, Inc., Ridgefield Park, NJ) to identify noradrenergic axons; (e) polyclonal rabbit anti-GFAP (glial fibrillary acidic protein) antibody (1:100; Biomedical Technologies Inc., Stoughton, MA) to identify astrocytes; and (f) polyclonal rabbit anti-S100 protein antibody (S-100; 1:100; Dakopatts, Santa Barbara, CA) as a marker primarily for Schwann cells, but also for astrocytes. The immunostaining procedure followed previously reported protocols (Xu et al., 1995a,b). Briefly, after incubation overnight at 4°C in the primary antibody, the sections were incubated in a humidified chamber with either fluorescein-conjugated goat anti-rabbit IgG (1:100; Cappel/Organon Teknika Corp., Durham, NC) or rhodamine-conjugated goat anti-mouse IgG (1:50; Cappel/Organon Teknika Corp.) antibodies. The slides were cover-slipped with Gel/Mount aqueous mounting media (Biomedica Corp., Foster City, CA) and examined with an Olympus BX60 fluorescence microscope.

### PHA-L Anterograde Tracing

Thirty days after channel implantation, 5 animals were anesthetized with sodium pentobarbital (45 mg/kg, i.p.), and the wounds were re-opened. The lamina of the 6<sup>th</sup> thoracic vertebra was removed, and *Phaseolus vulgaris*-leucoagglutinin (PHA-L; 2.5% in 15 mM Tris buffer, pH 8.0; Vector Lab., Burlingame, CA) was injected iontophoretically into the cord bilaterally, 1.5 mm beneath the cord surface and 3 mm rostral to the implanted mini-guidance channel. The methods for PHA-L injection and subsequent tracer visualization were previously described (Xu et al., 1995b). PHA-L was introduced for 20 min (5  $\mu$ A positive current, 7s

on/7s off) per injection via a 10 to 15  $\mu\text{m}$ -tipped glass pipette, using a Midguard current device (Midguard Electronics, Inc., Newton, MA). Before the injection, the left intact hemicord was completely transected to prevent the labeling of axons entering the distal host spinal cord via that route. After 7 days, the animals were perfused with modified Zamboni fixative, as previously described. The host spinal cord and graft tissues were cut horizontally at 30  $\mu\text{m}$  on a freezing stage sliding microtome. The PHA-L reaction procedures used were modifications of the protocol suggested by Vector Laboratories (Gerfen & Sawchenko, 1985) and were reported previously (Xu et al., 1997). Briefly, the sections were washed in 0.02 M potassium phosphate buffered saline (KPBS, pH 7.4) and KPBST (KPBS + 0.3% Triton X-100), respectively. Then the sections were incubated at 4°C for 72 hr in KPBST containing 1:10,000 goat anti-PHA-L antibody and 2% normal goat serum. The sections were next rewashed and then processed with biotinylated rabbit anti-goat IgG (1:400) and Vector Avidin-Biotin-Peroxidase (ABC) Complex. The final peroxidase conjugate was reacted with hydrogen peroxide in the presence of 0.05% 3,3'-diaminobenzidine (DAB; Sigma, St. Louis, MO), and the DAB reaction was enhanced with nickel ammonium sulfate. The sections were mounted, dehydrated, and cover-slipped for examination with an Olympus BX60 microscope.

#### **Fast Blue and Diamidino Yellow Retrograde Tracing**

Thirty days after channel implantation, two animals were anesthetized with sodium pentobarbital (45 mg/kg, i.p.), and the graft implantation site was re-exposed. The lamina of the 6<sup>th</sup> and 9<sup>th</sup> thoracic vertebra were removed. After dural puncture, 0.1  $\mu\text{l}$  of Fast Blue (FB) (2%) and 0.1  $\mu\text{l}$  of Diamidino Yellow (DY) (2%) were hydraulically injected through a 50  $\mu\text{m}$ -tipped glass pipette into the rostral and the caudal host spinal cord, respectively, at a depth of 1.5 mm and a distance of 2.0 mm from the rostral

and the caudal ends of the channel. After a 7-day survival period, the animals were perfused with 4% paraformaldehyde in 0.1 M phosphate buffer. A 9 mm segment of the host spinal cord, containing FB and DY injection sites and the FSC graft, was dissected out and post-fixed overnight in the same fixative. On the second day, the samples were transferred into 30% sucrose buffer for cryoprotection. Spinal cord segments were embedded in Tissue Freezing Medium, cut horizontally on a cryostat at 40  $\mu\text{m}$ , and mounted on gelatin-coated slides. The slides were cover-slipped with DPX mounting media (Fluka Chemical Co., Milwaukee, WI) and examined with an Olympus BX60 fluorescence microscope.

## **RESULTS**

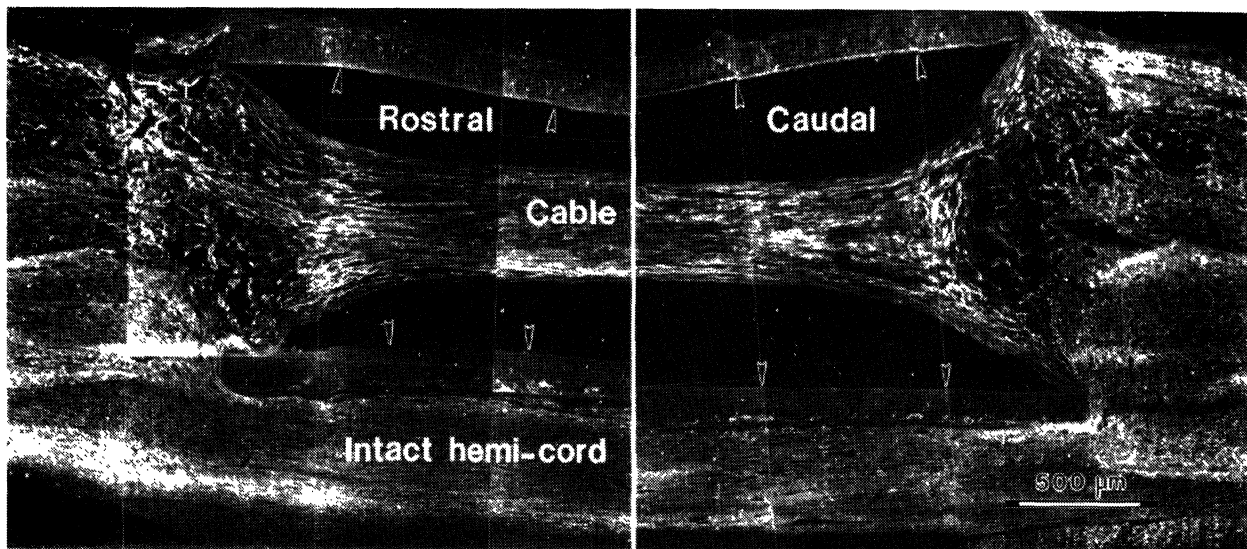
The lesion created in this model system interrupts the connectivity of ascending and descending axons within the hemisectioned side of the spinal cord. After the hemicord segment was removed, an FSC-containing mini-channel was implanted at this site. At the end of the 30 day survival period, grafts were present in all transplant cases, including the FSC grafts that were exposed to freeze/thaw cycling. FSC grafts (Fig. 2) bridged the entire rostro-caudal extent of the lesion cavity, making intimate contact with both rostral and caudal host hemicord stumps. The widest extent of typical FSC graft occurred at the interfaces between the host and graft, which then narrowed toward the center of the mini-channel (Fig. 2).

In all cases, a space, filled with CSF, existed between the mini-channel wall and the tissue cable of the FSC graft. At the transition between the host and FSC graft, mild cystic cavitation was observed in certain cases. Overall, the FSC grafts were intimately fused with the host spinal cord, although some variability in graft size was seen from case to case. The contralateral host hemicord remained intact after the lesion induction and grafting procedures with no space separating the mini-channel wall from the host cord.

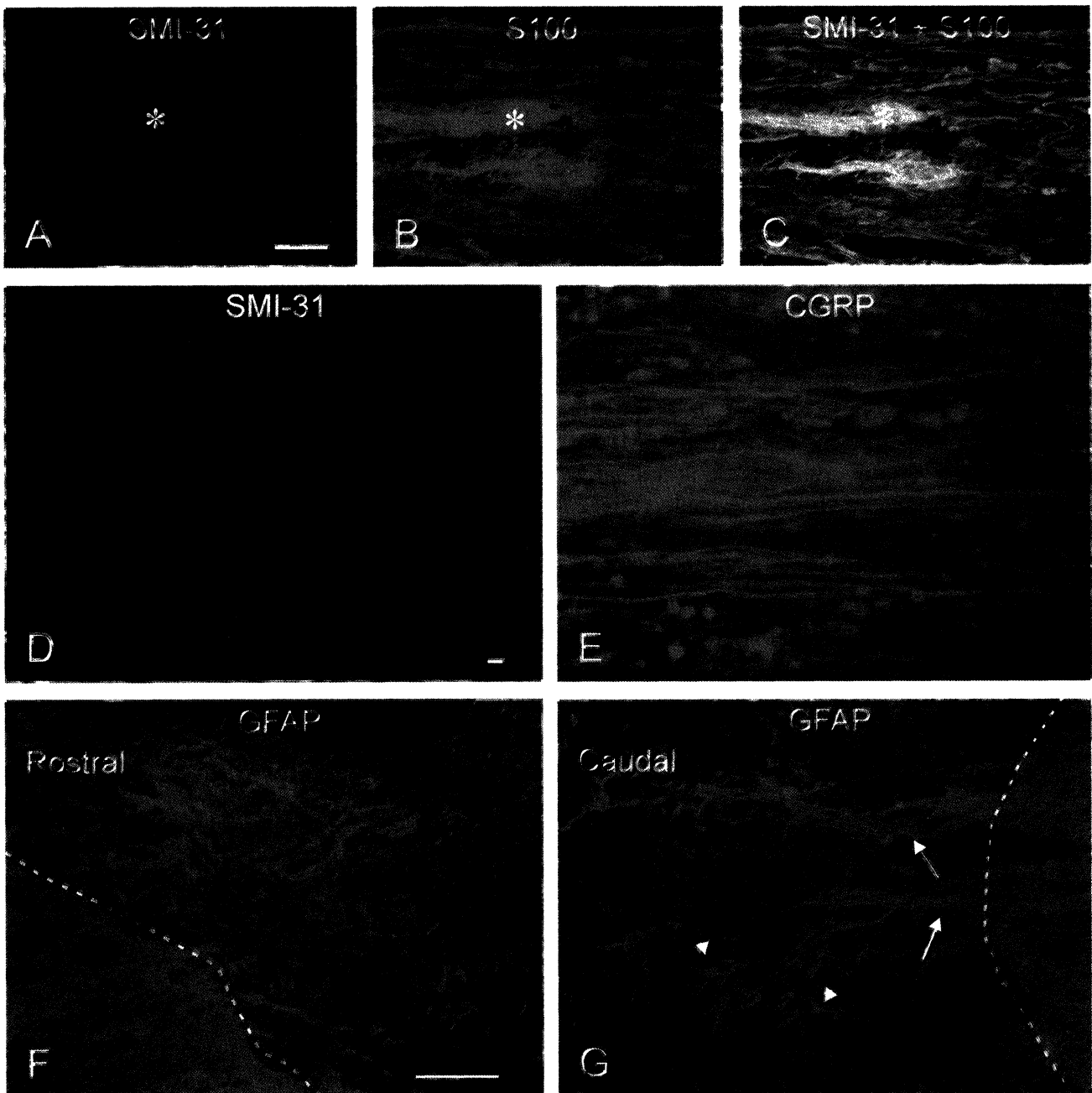
### Immunohistochemistry

In horizontal spinal cord sections containing both proximal and distal segments of the graft (Fig. 2), many immunolabeled axons (SMI-31 positive) can be seen to exit the host and enter the graft at 30 days after implantation. We observed two patterns of axonal arrangement within the graft: (1) clusters of randomly orientated axons within islands of residual fetal tissue, and (2) parallel axons forming fascicles that elongated along the longitudinal axis of the graft. Surviving fetal tissue, identified as graft areas that labeled intensively for both SMI-31 and S100 antibodies, was found as islands or clusters of cells within the tissue cable (Fig. 3A-C). In contrast, areas devoid of residual fetal tissue were occupied by SMI-31 immunopositive axons running longitudinally throughout the length of the tissue cable (Fig. 2 and Fig. 3D). A subset of ingrowing axons were CGRP positive, indicating their PNS origin (Fig. 3E). Descending serotonergic axons from brainstem raphe nuclei (5-HT positive) and nor-

noradrenergic axons from locus coeruleus and/or subcoeruleus nuclei (DBH positive) entered the interface zone between the host and the FSC tissue cable (data not shown). Nevertheless, similar to the results of earlier studies involving Schwann-cell seeded guidance channels (Xu et al., 1995c; 1997), neither 5-HT- nor DBH-positive fibers could be identified deep within the tissue cable. The host-graft interface could be clearly defined in the sections stained with anti-GFAP antibody, in which the intensity of labeling increased, indicating astrocytic gliosis (dotted line, Fig 3F, G). In many cases, we also found that GFAP-positive astrocytic processes (Fig. 3G, arrows) or individual astrocytes (Fig. 3G, arrowheads) extended from the host-graft into the FSC tissue cable. In this interface zone, the 5-HT- and DBH-immunopositive fibers were found. When compared with more recent work involving the implantation of Schwann cell-seeded, mini-guidance channels (Xu et al., 1995a; 1999), the overall intensity of the GFAP immunostaining appeared mild.



**Fig. 2:** A fluorescent photomicrographic montage showing a horizontal section of a FSC transplant. The section was immunolabeled with SMI-31 antibody to identify axons that grew into the graft longitudinally and in parallel. Note that the intact left hemi-cord was well preserved. Arrowheads - channel walls. Bar: 500  $\mu$ m.



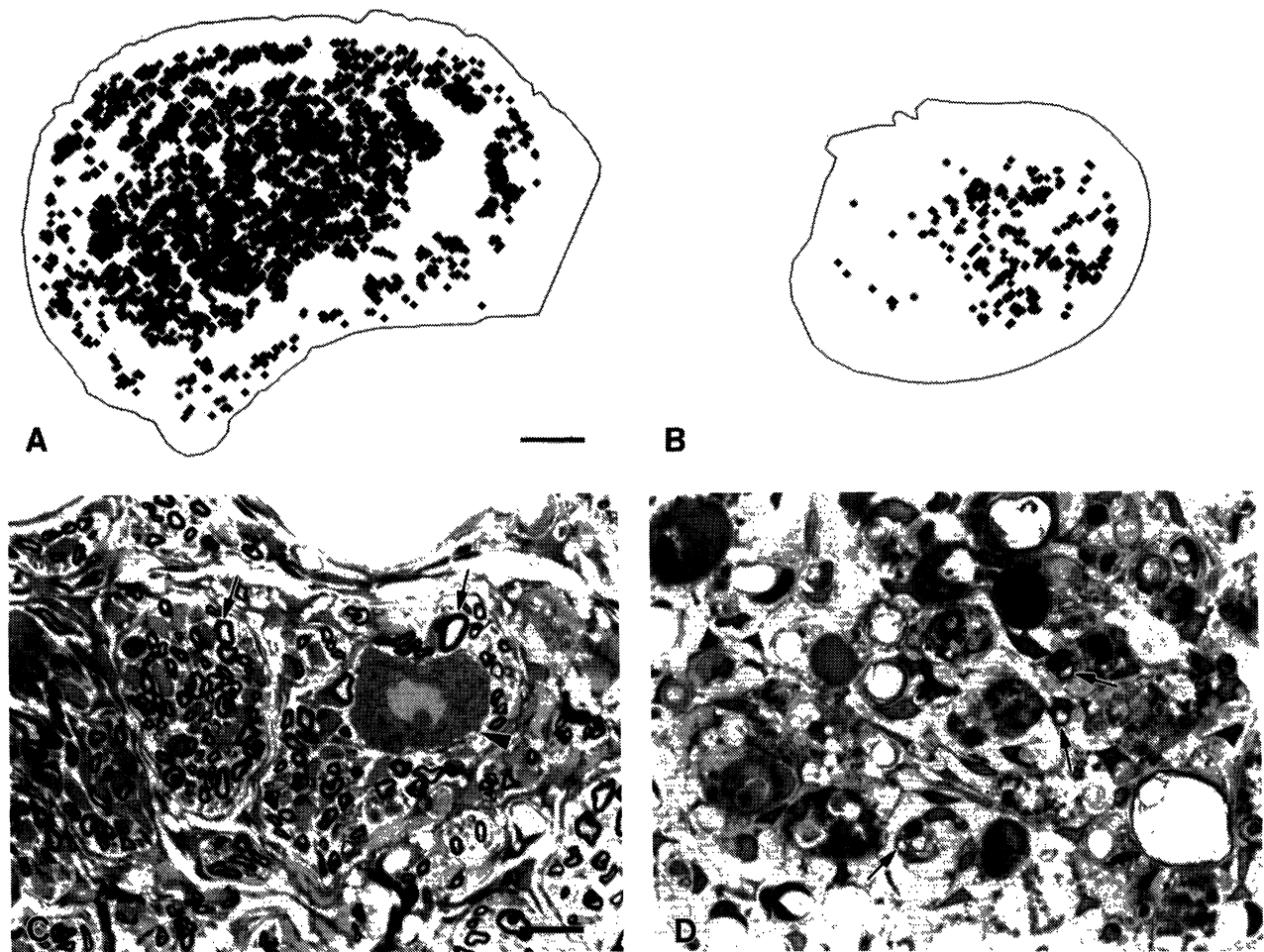
**Fig. 3:** A–C: Fluorescence photomicrographs demonstrating islands of FSC tissue (asterisks) present 30 days after transplantation. Clusters of axons recognized by SMI-31 immunolabeling (A) overlapped with S100 immunopositive astrocytes/ Schwann cells (B). Islands of tissue with intense, overlapping labeling for both SMI-31 and S100 were interpreted as surviving FSC tissue (C). D: SMI-31 immunolabeling demonstrates arrays of axons elongating longitudinally along the long axis of the FSC graft. E: A sub-population of axons within FSC grafts were immunopositive for CGRP. F–G: GFAP immunostaining demonstrates a mild astrogliotic reaction at the interface between the host and FSC graft (---). GFAP immunopositive processes from the host were observed to interdigitate with the graft tissue (G, arrows), and individual astrocytes (G, arrowheads) were seen to be present within the graft. Bars: A–C - 100 $\mu$ m; D–E - 10 $\mu$ m; F–G - 100 $\mu$ m.



### Light and Electron Microscopy

In toluidine blue-stained, semi-thin cross sections, the FSC tissue cables were surrounded by epineurial-like cells and appeared to be well vascularized. Neurolucida plots of myelinated axons were taken from semi-thin sections (Fig. 4A, B). In viable FSC grafts (Fig. 4C), tissue cables appeared to be mostly composed of a PNS-like environment, with many myelinated axons found in fascicles. Interestingly, cells with

neuron-like morphology could be seen within the fascicles of myelinated axons (Fig. 4C). In addition, within the tissue cable, areas characteristic of a CNS-like environment were also found (see EM results below). Conversely, in FSC grafts exposed to freeze/thaw cycling (Fig. 4D), far fewer myelinated axons were identified, and the tissue cables appeared to be undergoing degenerative change to a degree not seen in viable FSC grafts. The mean number of myelinated axons was  $1455 \pm 663$  (mean  $\pm$  S.E.M.;  $n=6$ ; Fig. 5)



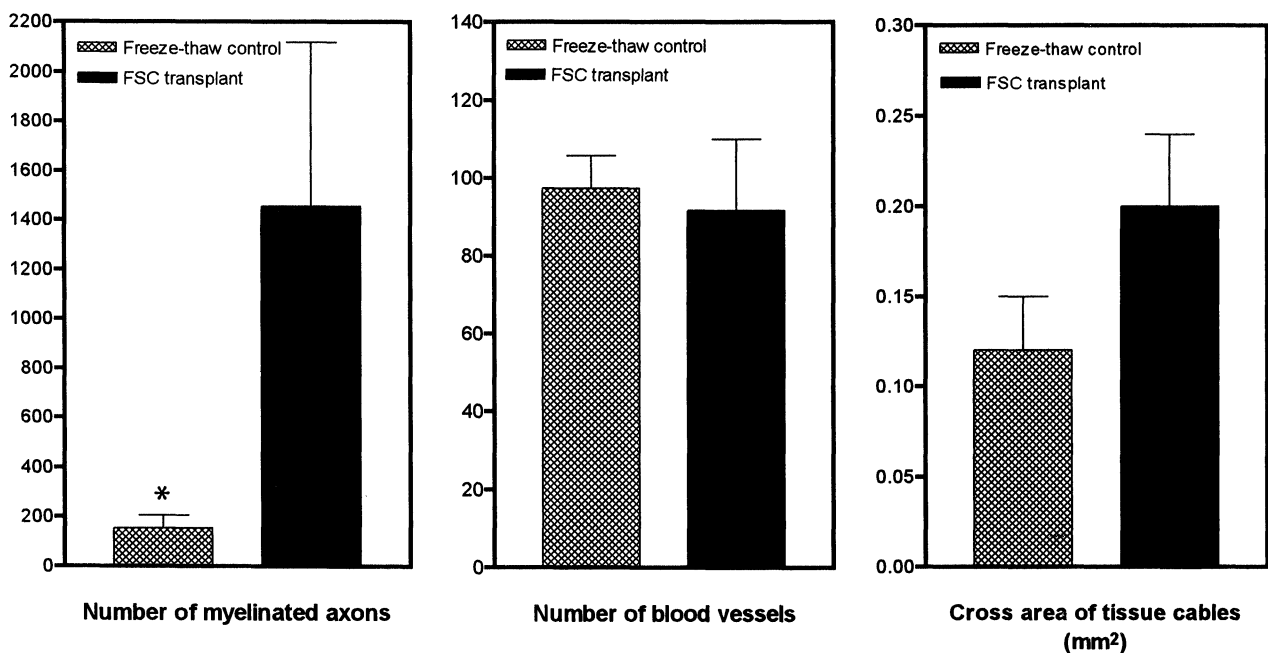
**Fig. 4:** A-B: Representative Neurolucida plots of myelinated axonal profiles taken from toluidine blue stained  $1\mu\text{m}$  semi-thin plastic cross sections of a FSC graft (A) and a freeze-thaw control graft (B). Each diamond represents a cross sectional profile of a myelinated axon. C-D High power photomicrographs of a FSC graft (C) and a freeze/thaw control graft (D). Within the FSC graft, many myelinated axons are found (C, arrows). Occasionally, neuron-like cells (C, arrowheads) are seen within the FSC graft. Note only a few axon profiles are present in the freeze-thaw control graft (D, arrows). Bars: A-B,  $100\mu\text{m}$ ; C-D,  $10\mu\text{m}$ .

in cross sections cut at the midpoint of the FSC tissue cable. In the control grafts that had been subjected to 5 freeze-thaw cycles before implantation, the number of myelinated axons was  $155 \pm 50$  ( $n=5$ ). A Wilcoxon Rank Sum test showed a statistical difference ( $P < 0.01$ ) between the numbers of myelinated axons in the experimental FSC and the freeze-thaw control groups. The non-parametric Rank Sum test was used because the unequal variance seen between the numbers of myelinated axons within the viable and non-viable FSC-grafted animals precluded the use of a parametric analysis. In cases with viable FSC grafts, the mean mid-tissue cable cross-sectional area (CSA) was  $0.200 \pm 0.043 \text{ mm}^2$ , and the number of blood vessels was  $92 \pm 18$ ; control cases averaged  $0.121 \pm 0.029 \text{ mm}^2$  and  $97 \pm 8$  for CSAs and for the number of blood vessels at the tissue cable midpoint, respectively (Fig. 5). No statistically significant difference was detected between the

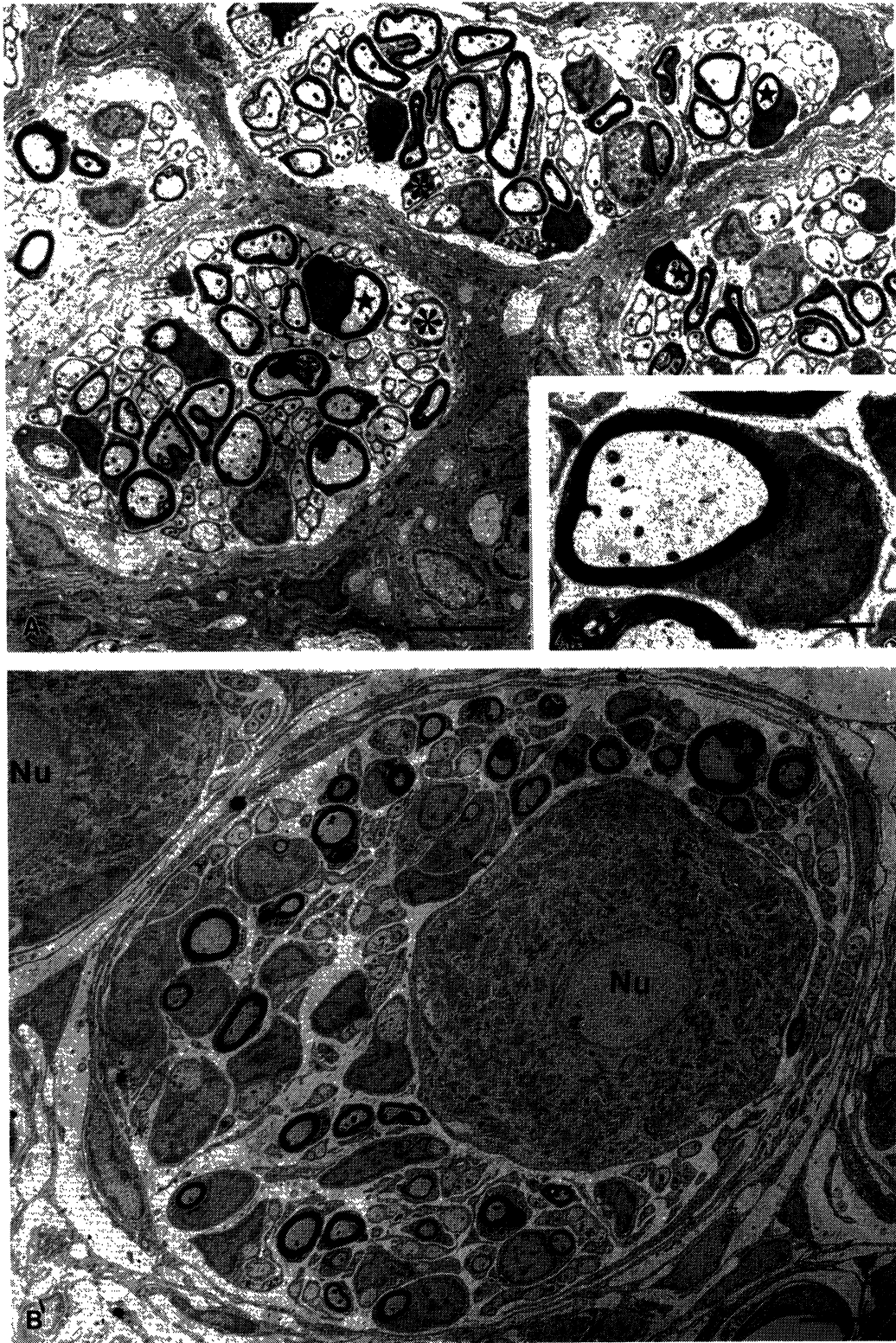
two groups in terms of numbers of blood vessels or the cross sectional area of tissue cables at the graft midpoint.

The results from EM clearly demonstrated that the FSC tissue cables consist of large areas of PNS-like tissue (Fig. 6), among which islands of CNS-like areas were found (Fig. 7). The PNS-like areas contained fascicles of axons surrounded by perineurial-like cells (Fig. 6A). Within these fascicles, single axons were myelinated by typical Schwann cells (Fig. 6A, insert), or several unmyelinated axons were ensheathed by a single Schwann cell. Occasionally, cells with neuron-like morphology (Fig. 6B) could be found in the fascicles within PNS-like areas of the tissue cable.

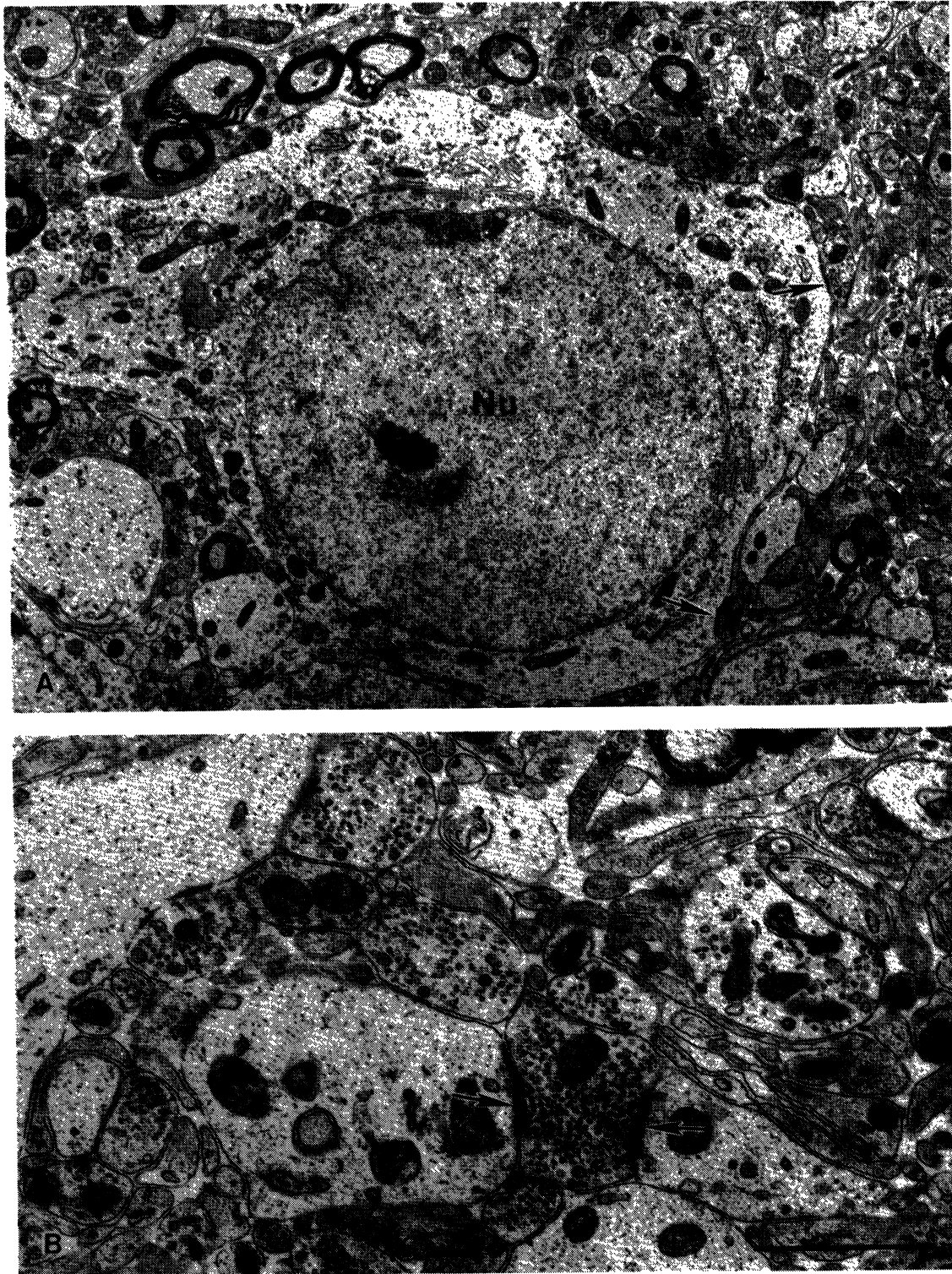
Although both myelinated and unmyelinated axons were present in the CNS-like areas of the tissue cable (Fig. 7A), their appearance was different. In these areas, the axons did not form fascicles and were not surrounded by perineurial-



**Fig. 5:** Histograms showing the numbers of myelinated axons (left), blood vessels (middle), and cross sectional areas of tissue cables (right) in animals which received either FSC grafts ( $n=6$ ; solid bar) or freeze-thaw control grafts ( $n=5$ ; hatched bar). Means and standard errors are shown. Non-parametric analysis (Wilcoxon Rank Sum test) showed a statistical difference ( $P < 0.01$ ) between the numbers of myelinated axons in experimental and control groups (asterisk).



**Fig. 6:** A: Electron photomicrograph demonstrating many PNS-like fascicles of axons either myelinated (stars) or ensheathed (asterisks) by Schwann cells. Insert: a typical Schwann cell myelinating a single axon. B: Neuron-like cells (Nu) were found to exist within a PNS-like fascicle of axons. Bars: A-B - 5 $\mu$ m; insert - 1 $\mu$ m.



**Fig. 7:** A: Electron photomicrograph showing the CNS-like environment within a FSC graft. As might be expected, neurons (Nu) are found within these CNS-like regions. Note that axo-somatic synapses ( $\searrow$ ) are found on a neuron. B: Numerous synaptic contacts were observed within the CNS-like graft tissue. All Bars: 1mm.

like cells. Further, the myelin found in these areas was thinner and was not surrounded by a layer of basal lamina. All these characteristics suggested that, in the CNS-like areas, central myelin was present, likely formed by oligodendrocytes. Finally, neurons were dispersed throughout the neuropil, in which axons, dendrites, and numerous synaptic contacts were found (Fig. 7).

#### **PHA-L Anterograde Tracing**

The anterograde tracer PHA-L was delivered into the spinal cord, 3 mm rostral to the channel, to assess the growth of axons into FSC grafts, as well as to assess the potential for the same axons to re-enter the distal host spinal cord. In viable FSC grafts (n=5), numerous anterogradely labeled axons were identified throughout the length of the FSC tissue cable (Fig. 8). Although many axons had reached the distal graft-host interface, and some of the labeled axons penetrated the interface for a short distance, long-distance axonal growth into the distal host spinal cord was not observed.

#### **FB and DY Retrograde Tracing**

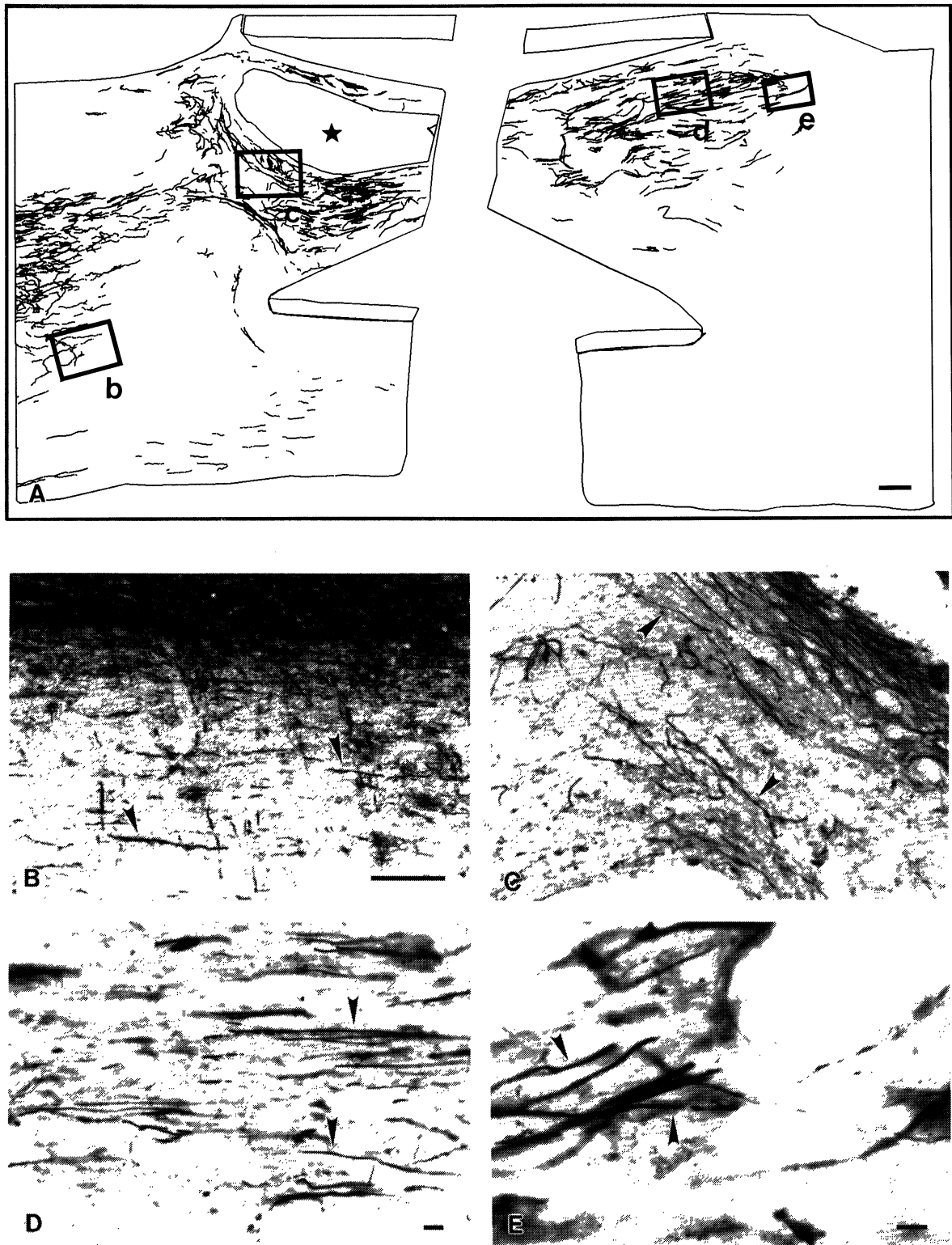
The retrograde tracers, FB and DY, were delivered into the respective rostral and caudal host spinal cord to determine if grafted fetal neurons had survived and had extended their axons into the host spinal cord at, or beyond, the site of dye injection. In both animals used for this investigation, the fluorescent dyes were confined to a small area, with no dye spreading to the host-graft interfaces. Within the FSC graft, most neurons found contained either FB or DY, indicating that axons from these neurons had penetrated through the respective rostral and caudal interfaces to arrive at, or beyond, the sites of tracer injection. These two markers are readily differentiated not only by color but also by distribution. Whereas DY labeling is confined mainly to the nucleus, FB labels the cytoplasm. Interestingly, a subpopulation of neurons in the graft were double labeled with both FB and DY (Fig. 9). The double labeled cells were interpreted as possessing axon collaterals projecting beyond

both rostral and caudal host-graft interfaces into the host spinal cord, at least 2 mm from the ends of the channel.

### **DISCUSSION**

The purpose of this study was to investigate whether a FSC graft could survive within a mini-channel transplantation environment and promote axonal growth in a partially lesioned adult rat spinal cord. This model is characterized by a mid-thoracic hemisection of the spinal cord, the implantation a neural cell- or tissue-filled mini-guidance channel, and the restoration of CSF circulation (Xu et al., 1999). We demonstrated that fetal tissue survived and bridged both the rostral and caudal ends of the lesion cavity within 30 days after transplantation. We also found substantial longitudinal axonal growth into the graft. Within the graft, large areas consisted of a PNS-like environment containing numerous fascicles of myelinated and unmyelinated axons. In addition, a smaller proportion of CNS-like areas, characterized by the presence of neurons, neurites, synaptic contacts, and CNS glia, were also observed. In control grafts in which the FSC tissue underwent repetitive freeze/thaw cycling approximately 10-fold fewer myelinated axons were present within tissue cables. We finally demonstrated that neurons within the FSC graft extended axons into the host spinal cord, at least for 2 mm from both the rostral and the caudal host-graft interfaces. In contrast, the control grafts appeared to be undergoing degenerative changes within 30 days after channel implantation.

The hemisection/mini-channel implantation model used in this study has a number of unique features that lend themselves to the investigation of tissue transplantation in the injured adult spinal cord (Xu et al., 1999). For example, suturing of the dura restores CSF circulation around the graft after transplantation, which may help to re-establish a normal CNS microenvironment and may also possibly mimic that following a closed SCI. Further, the permeability of the guidance channel wall allows a continuous flow of CSF,

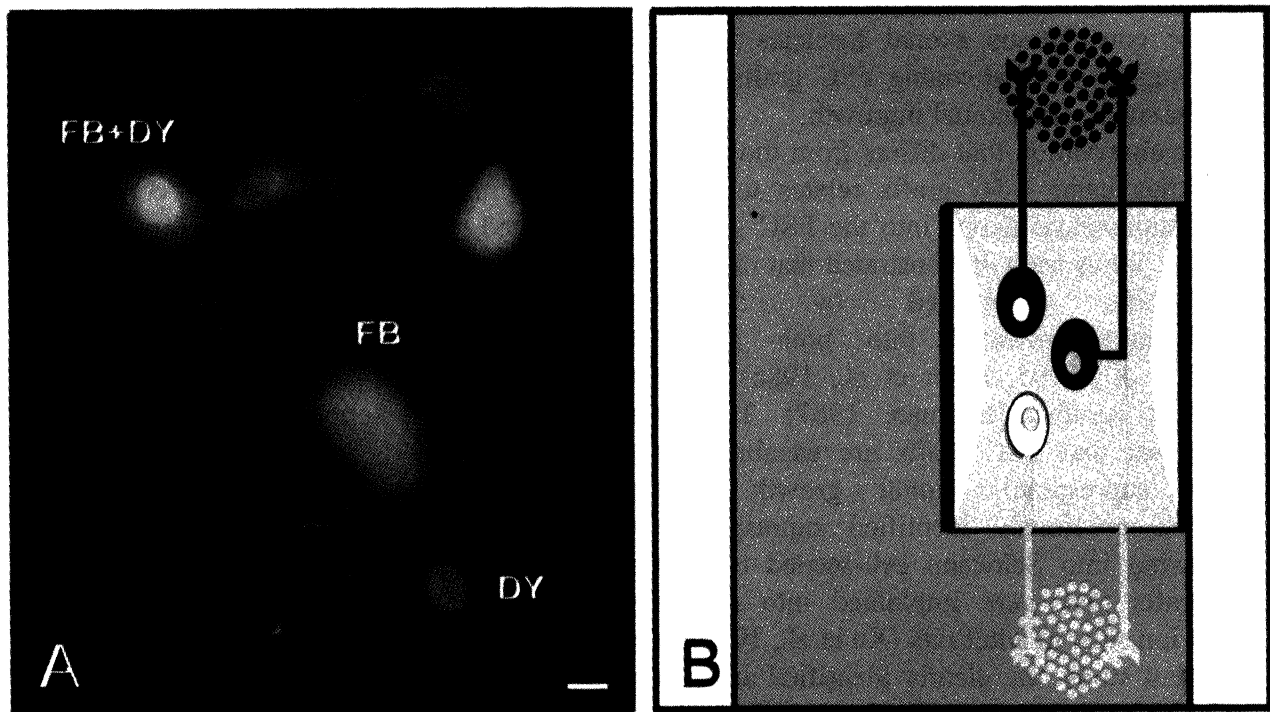


**Fig. 8:** A: Neurolucida reconstruction of a horizontal section of the graft and the host at the transplant site showing that axons anterogradely labeled with PHA-L grew into the FSC graft. Representative areas of interest are labeled (b-e) in A and are presented photographically at high magnification in B-E. Axons were identified in the rostral spinal cord (B), entering the FSC graft (C), at the distal portion of the FSC graft (D) and penetrating the distal host-graft interface for only a short distance (E). A cavity at the rostral end of the FSC graft is indicated (★). Bars: A-C - 100 $\mu$ m; D-E - 10 $\mu$ m.

containing humoral factors around the graft, providing a favorable environment for the survival of graft tissue and growing axons. Finally, this model is very flexible in that the channel contents may be easily modified to contain Schwann cells (Bamber et al., 1998; Xu et al., 1995a; Xu et al., 1999; Zhang et al., 1997), FSC tissue (the present study), and potentially other cell/tissue types. From this model, additional treatments, such as the infusion of growth factors (Xu et al., 1995b) or the administration of methylprednisolone, a steroid hormone (Chen et al., 1996), may supplement the transplantation paradigm.

Several observations made in the present study differ from those in previous studies using FSC tissue as intraspinal grafts. A notable difference

concerns the internal organization of the tissue cable itself. We have described two distinct components within the FSC grafts: a larger PNS-like region and a smaller CNS-like environment. This observation is different from those in previous reports (Iwashita et al., 1994; Reier et al., 1986), in which FSC grafts were described to have undergone an organotypic differentiation with regions of compact CNS myelinated fibers and unmyelinated, substantia gelatinosa-like areas. Graft organotypic differentiation to this degree was not seen in the current study. Our results suggest that only a portion of the FSC tissue, including some of the gray matter, survived within the channel environment, leaving islands of CNS-like tissue within the FSC graft. The solitary and randomly placed neurons within



**Fig. 9:** A: Fluorescence photomicrograph demonstrating that implanted FSC neurons projected axons beyond the host-graft interface and contained Fast Blue (FB), Diamidino Yellow (DY) or both (FB+DY). B: A schematic drawing which represents the three populations of neurons which could be found extending axons beyond the host-graft interface: (1) neurons whose axons extended beyond the rostral interface (blue cell body), (2) neurons whose axons extended beyond the caudal interface (yellow nucleus), and (3) neurons which possess axon collaterals that reach beyond both the rostral and caudal host-graft interfaces (blue cell body and yellow nucleus). Bar: A - 10 $\mu$ m.

the PNS-like fascicles indicated that isolated neurons can also survive in a PNS-like environment. Retrograde labeling demonstrated that some implanted neurons elongated axons beyond the proximity of the host-graft interface, at least for a short distance. In the PNS-like regions, most axons were either myelinated or ensheathed by Schwann cells. As they are not normally resident in the CNS, such Schwann cells are likely to originate as migrating Schwann cells that enter from the periphery. This phenomenon has been seen following contusive injuries to the adult rat spinal cord (Beattie et al., 1997). In addition, some Schwann cells from the fetal dorsal root ganglia that were occasionally attached to the FSC during preparation might contribute to the total Schwann cell population within the graft. Further studies are needed to determine the origin of Schwann cells that migrate into the graft and support ingrowing axons. In control cases in which grafts were exposed to freeze/thaw cycling neither CNS-like regions nor conspicuous axonal fascicles were observed, suggesting that viable FSC grafts are essential for successful axonal ingrowth.

Another observation that differs from those in earlier studies using FSC grafts relates to the growth of supraspinal axons into the FSC graft. For example, it has been demonstrated that raphe-spinal axons (identified by 5-HT immunostaining) projected into both adult (Anderson et al., 1995; Reier et al., 1986) and neonatal FSC grafts (Bregman, 1987). These results suggest that FSC grafts are capable of supporting supraspinal axonal growth. In addition, we previously identified supraspinal axons growing into Schwann cell-seeded mini-channel grafts that were implanted into mid-thoracic lesion sites in adult rats (Xu et al., 1999). In the present study, we clearly identified axons of supraspinal origins, specifically from raphe (5-HT-immunopositive) and locus coeruleus and subcoeruleus (DBH-immunopositive), only in the interface zone between the host and graft. The lack of axonal growth from these supraspinal regions into the graft in this study is not clearly understood. Whether axons from other

supraspinal regions grew into the FSC graft in this model remains to be elucidated. It should be noted that supraspinal axons did not grow into certain Schwann cell or into PN grafts implanted at the mid-thoracic level unless additional treatment, such as methylprednisolone (Chen et al., 1996) or trophic factors (Houle et al., 1996; Xu et al., 1995b; Ye & Houle, 1997), were applied. The distance between the site of axotomy and the involved neuronal somata may also be critical for successful axonal regrowth (Aguayo, 1985).

The mildness of the astrocytic response at the host-graft interfaces in the present study is worthy of notice. The glial boundary that forms between a spinal cord graft and the host has been considered an impediment toward axonal growth. An astrogliotic scar may (a) represent a physical barrier impassible to regenerating axons (Reier & Houle, 1988), (b) provide a point source of inhibitory molecules (McKeon et al., 1995), or (c) produce a diffusion barrier (Syková et al., 1999) against such small molecules as endogenous or exogenously administered trophic factors. Thus, to create a better interface for promoting axonal growth, transplantation paradigms within the spinal cord should be designed to minimize the development of a glial scar. In the present study, qualitatively less astrogliosis (as indicated by increased GFAP immunoreactivity) was seen at the host-graft interfaces, as compared with a similar preparation using grafts of Schwann cells (Xu et al., 1995c; Xu et al., 1999). Such a decrease in the relative amount of astrogliosis following grafting supports the concept that FSC tissue delays or reduces the glial response and negative interaction between the host and graft. This outcome has been suggested as one of the benefits of using FSC tissue in repairing CNS lesions (Hallas, 1984; Krüger et al., 1986). Quantitative analysis of glial scar formation has demonstrated that certain animals show an altered and partially disrupted glial boundary at the interfaces after grafting FSC tissue into chronically injured spinal cord lesions (Houle, 1992). Thus, grafted viable FSC tissue may attenuate glial scar formation and facilitate



axonal growth through improved host-graft interfaces (Houle et al., 1996; Reier & Houle, 1988).

The use of FSC tissue grafts in our partial lesion model offers the opportunity to investigate the mechanisms with which FSC transplantation may promote functional recovery (for review see Zompa et al. 1997). The results of PHA-L anterograde tracing (Fig. 8) indicate that FSC tissue within mini-guidance channels provided an adequate bridge for longitudinal axonal growth across the lesion site. Further application of methylprednisolone and/or addition of trophic substances may allow axonal re-entry into the host spinal cord from the FSC bridge. The islands of CNS-like regions in FSC grafts provide neuronal pools upon which ingrowing axons could make synaptic contacts. Such neurons may also project axons beyond the host-graft interface, as demonstrated by retrograde labeling (Fig. 9), providing a potential relay of information across the lesion site.

In our previous studies, we demonstrated that Schwann cells in mini-channels promoted successful axonal growth into the graft (Xu et al., 1995a; Xu et al., 1999; Zhang et al., 1997). Moreover, some regenerating axons within the SC grafts penetrated the distal graft-host interface to re-enter the host environment. Whereas Schwann cells have the advantages of (a) promoting successful axonal regeneration, (b) myelinating regenerating axons (Xu et al., 1995a; Xu et al., 1999; Zhang et al., 1997), and (c) possessing the potential for autotransplantation in SCI patients, FSC grafts have their own unique, advantageous properties. For example, the implanted neuronal population may serve as an important functional relay of information across the lesion site, a characteristic that Schwann cell grafts cannot provide. In addition, the interface that was formed between the host and the FSC graft exhibited less astroglial response than that observed with Schwann cell grafts, an indication of the integration between FSC grafts and the host.

In summary, FSC grafts placed into semi-permeable, mini-guidance channels serve as

permissive cellular bridges, promoting vigorous axonal growth into the grafts. The use of FSC tissue in this model demonstrates the great flexibility of the model system. Previously, we used this system exclusively with Schwann cell grafts (Xu et al., 1999). We now demonstrate the viability of grafting FSC tissue within mini-guidance channels. Additional manipulations, such as administering neurotrophic factors, could be used to promote further axonal growth and the re-entry of axons from the grafts back into the host spinal cord. Our long-term goal is to find a combination of cells and factors that maximizes axonal growth and promotes meaningful functional recovery in the injured adult spinal cord.

#### ACKNOWLEDGMENTS

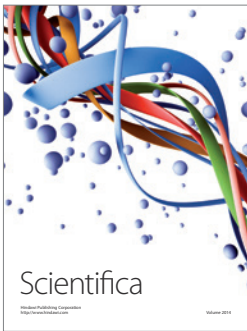
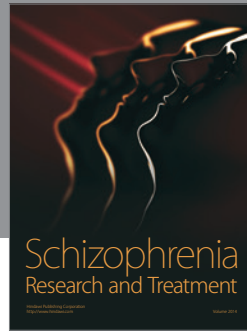
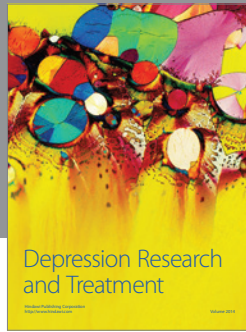
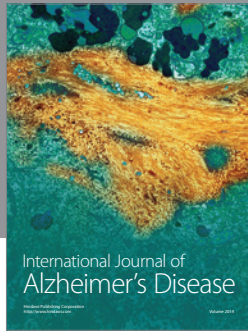
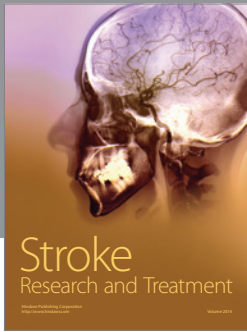
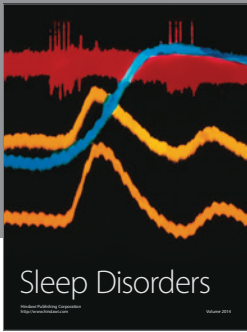
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