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Neurotrophic Factor-Expressing Mesenchymal Stem Cells Survive Transplantation into the Contused Spinal Cord without Differentiating into Neural Cells

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Abstract:	<p>The aim of this study was to assess the feasibility of transplanting mesenchymal stem cells (MSCs), genetically modified to express glial-derived neurotrophic factor (GDNF), to the contused rat spinal cord and to subsequently assess their neural differentiation potential.</p> <p>MSCs expressing green fluorescent protein (GFP) were transduced with a retroviral vector to express the neurotrophin GDNF. The transduction protocol was optimized by using GFP-expressing retroviral constructs; approximately 90% of MSCs were transduced successfully after G418 selection. GDNF-transduced MSCs expressed the transgene and secreted growth factor into the media (~12 ng/500,000 cells secreted into the supernatant 2 weeks after transduction). Injuries were established using an impactor device, which applied a given, reproducible force to the exposed spinal cord. GDNF-expressing MSCs were transplanted rostral and caudal</p>

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	<p>to the site of injury. Spinal cord sections were analyzed 2 and 6 weeks after transplantation. We demonstrate that GDNF-transduced MSCs engraft, survive and express the therapeutic gene up to 6 weeks post-transplantation, while maintaining an undifferentiated phenotype. In conclusion, transplanted MSCs have limited capacity for the replacement of neural cells lost as a result of a spinal cord trauma. However, they provide excellent opportunities for local delivery of neurotrophic factors into the injured tissue. This study underlines the therapeutic benefits associated with cell transplantation and provides a good example of the use of MSCs for gene delivery.</p>

For Peer Review



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3 **Neurotrophic Factor–Expressing Mesenchymal Stem Cells Survive**
4 **Transplantation into the Contused Spinal Cord without Differentiating**
5 **into Neural Cells**
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29 **Running head:** GDNF Expression after Stem Cell Transplantation

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Abstract

The aim of this study was to assess the feasibility of transplanting mesenchymal stem cells (MSCs), genetically modified to express glial-derived neurotrophic factor (GDNF), to the contused rat spinal cord and to subsequently assess their neural differentiation potential.

MSCs expressing green fluorescent protein (GFP) were transduced with a retroviral vector to express the neurotrophin GDNF. The transduction protocol was optimized by using GFP-expressing retroviral constructs; approximately 90% of MSCs were transduced successfully after G418 selection. GDNF-transduced MSCs expressed the transgene and secreted growth factor into the media (~12 ng/500,000 cells secreted into the supernatant 2 weeks after transduction). Injuries were established using an impactor device, which applied a given, reproducible force to the exposed spinal cord. GDNF-expressing MSCs were transplanted rostral and caudal to the site of injury. Spinal cord sections were analyzed 2 and 6 weeks after transplantation.

We demonstrate that GDNF-transduced MSCs engraft, survive and express the therapeutic gene up to 6 weeks post-transplantation, while maintaining an undifferentiated phenotype.

In conclusion, transplanted MSCs have limited capacity for the replacement of neural cells lost as a result of a spinal cord trauma. However, they provide excellent opportunities for local delivery of neurotrophic factors into the injured tissue. This study underlines the therapeutic benefits associated with cell transplantation and provides a good example of the use of MSCs for gene delivery.

Keywords: gene therapy; mesenchymal stem cells; neurotrophins; retrovirus; spinal cord injury

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Abbreviations

CNS, central nervous system

DAPI, 4',6-diamidino-2-phenylindole

ELISA, enzyme-linked immunosorbent assay

GDNF, glial-derived neurotrophic factor

GFAP, glial fibrillary acidic protein

GFP, green fluorescent protein

MSC, mesenchymal stem cell

NGS, normal goat serum

PBS, phosphate-buffered saline

rMSC, rat mesenchymal stem cell

SCI, spinal cord injury

TRITC, tetra-rhodamine isothiocyanate

Introduction

Spinal cord injury (SCI) results in the destruction of neurons, which constitute the ascending and descending pathways that connect the brain with the body. Sensory loss, paralysis, and loss of autonomic function result. Replacement of cells lost during a central nervous system (CNS) injury is considerably lower when compared with that of most other organs. Adult stem cell therapy may enhance the intrinsic capacity for regeneration around the injury site. The objective of this study was to examine the neural differentiation potential of mesenchymal stem cells (MSCs) within the injured spinal cord and to assess their ability to deliver a therapeutic factor to the site of injury over an extended period.

The neural differentiation potential of MSCs *in vitro* is controversial (1-5), and the literature contains conflicting reports about the ability of MSCs to generate neural cells after transplantation into the CNS. Many studies report a low percentage of MSCs differentiating into astrocytes, oligodendrocytes, and neurons after transplantation into the CNS (6-8), while other studies present no evidence for MSC differentiation (9-11).

Such variation may result from transplantation into embryonic versus adult animals or from the use of different animal models. Some concerns have also been raised about the use of 5-bromo-2-deoxyuridine and bis-benzamide as molecular labels. Such labels can be misleading because they may colocalize with endogenous cells and give a false impression of differentiation in the transplanted population (12).

Rather than relying on the neural differentiation potential of MSCs, these cells may instead be used to modify the environment around a CNS injury site (11, 13, 14). They may act as vehicles to deliver growth factors to the injured spinal cord and thereby

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3 enhance the intrinsic regenerative capacity of the CNS (15-17). Some researchers
4 attribute improvements of gait, BBB scores, or hindlimb sensitivity to MSC
5 transplantation (9, 18) following a SCI, while others report no differences in motor
6 recovery after MSC transplantation (19, 20). MSCs are easy to isolate and propagate *in*
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10 *vitro*, and some evidence suggests that they are hypo- or nonimmunogenic in an
11 allogeneic setting (21). MSCs are capable of reducing the proliferation of stimulated T-
12 cells and altering the balance of pro/anti inflammatory cytokines secreted by dendritic
13 cells, naive and effector T cells (TH1 and TH2), and natural killer (NK) cells (22, 23).
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18 These immunomodulatory characteristics may be of therapeutic value in the clinic where
19 reducing inflammation is a central aspect of current treatment for human SCI;
20 methylprednisolone is a synthetic glucocorticoid that is currently used to reduce
21 inflammation and edema at the site of injury. Indeed, bone marrow transplants are
22 already in clinical use. For instance, in graft versus host disease (24), and for bone repair
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2 a much-needed substrate for axonal growth and regeneration across the site of injury.

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4 Indeed, MSCs have been shown to form guiding strands in the injured spinal cord that

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6 promote recovery (9).

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8 The objectives of this study were to determine whether MSCs could be genetically
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10 modified, using a retroviral vector, to express GDNF, and to subsequently assess the
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12 survival and differentiation potential of MSCs after transplantation into the contused
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14 spinal cord.
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For Peer Review

Materials and Methods

Reagents

Gibco culture media were purchased from Invitrogen (Groningen, the Netherlands). Penicillin-streptomycin, EDTA, polybrene, phosphate-buffered saline (PBS), G418 disulfate salt solution, paraformaldehyde, normal goat serum (NGS), Triton X, Tween 20, anti-rabbit IgG tetrahydroamine isothiocyanate (TRITC) antibody, anti-mouse IgG TRITC antibody, hematoxylin, eosin, and mounting media with DPX were obtained from Sigma-Aldrich (Dublin, Ireland). Vectashield mounting media containing 4',6-diamidino-2-phenylindole (DAPI) was purchased from Vector Laboratories (Peterborough, United Kingdom).

Isolation of Wild-Type and Green Fluorescent Protein-Expressing MSCs

MSCs were isolated from the bone marrow of 8 to 12 weeks old male or female Sprague Dawley rats (either transgenic rats expressing GFP - green rats CZ-004 [SD TgN(act-EGFP)OsbcZ-004] (Osaka University, Japan) or from wild-type rats). After rats were euthanized, marrow was flushed from the femoral and tibial compartments, and all marrow plugs were pooled by sex. Cells were counted and plated at a density of 9×10^5 cells/cm² in complete rat MSC (rMSC) media (α MEM-F12 and 10% FBS) with 1% penicillin-streptomycin and incubated at 37°C in 5% CO₂ and 90% humidity. After 3 days, nonadherent cells were removed, and cells were refreshed with complete rMSC media. The media were changed every 3 or 4 days. Cells were ready for subculture when colonies began to show a compact appearance with multilayered growth or were loosely formed and began to merge into a monolayer (<90% confluence); this typically occurred after 16 or 17 days. Cells were resuspended in 0.25% trypsin with 1 mM EDTA and plated at a density of 5.7×10^3 cells/cm². Subsequently, cultures were passaged at 4- to 6-day intervals.

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3 All batches of MSCs were characterized by their ability to differentiate into adipocytes,
4 osteocytes and chondrocytes, and by expression of CD71, CD172 and absence of CD45,
5 as we have previously described (16).
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8 ***GFP-Retrovirus Transduction of Wild-Type MSCs and G418 Selection***

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10 We aimed to determine the percent uptake of murine leukemia virus, which conferred the
11 GFP and NEO^R antibiotic resistance genes into wild-type MSCs. This percentage was
12 used to approximate the percent uptake of GDNF-bearing murine leukemia virus into
13 these same cells.
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17 Passage 3 wild-type MSCs were plated in triplicate at a density of 3,000 cells/cm² and
18 allowed to adhere for 24 hours in complete rMSC media. Complete rMSC media also
19 were added to confluent cultures of a retrovirus-producing GP+E GFP packaging cell line
20 and incubated overnight. Packaging cell line media was sterilized by passing through a
21 0.2- μ m filter. The supernatant (containing retroviruses) was combined with polybrene to
22 a final concentration of 8 μ g/mL and added to the MSCs. Cells were centrifuged at
23 2,000 \times g for 1.5 hours at 32°C. Cells were incubated overnight, washed with PBS, and fed
24 with complete rMSC media. After 2 to 3 days, transduced cells were selected by culturing
25 with G418 disulfate salt (final concentration, 0.2, 0.5, 0.8, or 1 mg/mL). G418
26 selectively kills off any cells which have not been successfully transduced.
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30 The effect of freezing and thawing on the transduced cell population also was examined.
31 Cells were suspended in 90% serum/10% DMSO and frozen in liquid nitrogen. The
32 number of GFP-expressing cells, before and after freezing, was assessed using a
33 fluorescence-activated cell sorter (Guava Express Plus program and the Guava Easycyte
34 machine [Guava Technologies, Hayward, California]).
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38 GFP-transfected wild-type MSCs were not otherwise used in this study. GDNF
39 transduction was subsequently performed on cells from transgenic rats bearing the GFP
40 gene.
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GDNF-Retroviral Transduction of GFP-MSCs

GFP-MSCs, isolated from GFP transgenic rats, were transduced to express GDNF. We followed the same protocol that we used for GFP-retrovirus transfection of wild-type MSCs. The G418 disulfate salt concentration that was deemed optimal for selection of transfected cells was 0.8 mg/mL.

A human GDNF enzyme-linked immunosorbent assay (ELISA) (R&D Systems, Abingdon, United Kingdom), which qualitatively measures mouse and rat GDNF, was performed on the supernatant from these samples. The assay was performed according to the manufacturer's protocol. Transduction experiments were repeated in triplicate, and ELISAs were performed on samples taken 12 days after transfection and G418 selection.

Characterization of GDNF-Transfected GFP-MSCs

GDNF-transduced transgenic GFP-MSCs were plated on chamber slides, allowed to adhere for 24 hours, and fixed with 4% paraformaldehyde. Immunocytochemistry analysis was performed on the fixed cells. Nonspecific staining was blocked by incubating cells in 3% NGS for 15 minutes at room temperature. The cells were incubated with the appropriate primary antibody (Table) diluted in PBS with 1% NGS and 0.2% Triton X; incubation was maintained for 2.5 hours at room temperature. Control cells were incubated in 1% diluent with no primary antibody. Cells were washed with PBS and 0.1% Tween 20 (3 washes, 3 minutes per wash). Secondary antibodies were diluted according to manufacturer's instructions (anti-rabbit IgG TRITC, 1:50 dilution; or anti-mouse IgG TRITC, 1:50 dilution) in PBS with 1% NGS and 0.2% Triton X, and cells were incubated in the appropriate secondary antibody at room temperature for 1.5 hours in the dark. Cells were washed again in PBS (3 washes, 3 minutes per wash). Samples were mounted with Vectashield mounting medium (containing DAPI) and visualized using an Olympus IX71 fluorescent microscope (Olympus UK Ltd, London, United Kingdom).

Spinal Cord Injury

All animal experiments were approved by the Institutional Animal Care and Use Committee of Ireland. Adult female Sprague-Dawley rats were anesthetized with intraperitoneal injections of ketamine and xylazine (dosage, 100 mg/kg and 10 mg/kg, respectively). The level of anesthesia was assessed by pinching the base of the tail or hind foot and by assessing the respiratory rate. Surgery was initiated only when the animal was completely insensitive to pain. The rate and adequacy of respiration, eye color, and response to pain was monitored throughout the surgical procedure.

Hair was removed from the rodent back (above the cervical and thoracic vertebrae) using electric clippers. The incision site was disinfected with a povidone-iodine solution. Ointment containing mineral oil and petroleum jelly was applied to protect the animals' eyes. Heating pad temperature was maintained at a constant 37°C during surgery. A 2-inch incision was made on the dorsal aspect of the spine, and a laminectomy was performed at the T8 to T10 level to expose the dorsum of the spinal cord. For investigation of acute SCI, a contusion injury was produced at the level of the T9 vertebra using the Infinite Horizon impactor device (Precision Systems and Instrumentation, Lexington, Kentucky). This model has previously been shown to produce a significant force-related decline in locomotive ability following injury, with the amount of spared tissue significantly correlating with the locomotor ability (30). The variability of injury is thereby greatly reduced with the application of a constant preset force. A 200-kdyne severe contusion was introduced into the spinal cord of 18 rats. A laminectomy also was performed on several animals without contusion injury as another control. The wounds were sutured.

Acute postoperative care included the use of warming blankets for 24 hours after surgery and twice daily monitoring for wound sepsis and bladder expression. Antibiotics and analgesics were administered as follows: 0.1 mL gentamicin (50 mg/mL) was injected subcutaneously once daily throughout the course of the entire experiment. Buprenorphine

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(0.5 mg/kg) was injected subcutaneously for about 7 days after surgery to minimize pain. Saline solution (3.5 mL) was administered subcutaneously for 3 days after surgery. Food and water were provided in a manner such that injured animals would not have difficulty reaching them.

GFP-MSC and GDNF-Transduced GFP-MSC Transplantation Into the Contused Spinal Cord

Seven days after surgery, 6 rats received GFP-MSCs (isolated from GFP transgenic rats), 6 rats received GDNF-transfected GFP-MSCs, and 6 rats received buffer alone as a control. MSCs were resuspended at a concentration of approximately 50,000 cells/ μ L in sterile buffer containing F12. Animals were anesthetized as described above, and the spinal cord was re-exposed. The spinal cord was held in place using a forceps gripping system. Two μ L of cells or buffer were injected 1 mm rostral and caudal the site of injury using a Hamilton syringe with a 26-gauge needle (total injected volume, 4 μ L). The syringe was placed in a stereotaxic frame, and the needle was lowered 1.5 mm below the surface of the spinal cord. The injection was administered over a 5-minute period and left in position for 5 minutes before removal.

Functional analysis

Functional recovery of experimental and control animals was assessed by performing open field testing using the Basso, Beattie, Bresnahan (BBB) locomotor rating scale 1 day, two weeks, four weeks and 6 weeks after implantation of cells into the contused spinal cord (31). Each rat was observed in an open plastic box for five minutes, and three independent, blinded observers recorded hind limb joint movements, weight support, toe clearance, tail position, coordination of gait and paw position. Observations were used to give each rat a rating on the 21-point BBB locomotor rating scale.

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Euthanasia and Tissue Harvesting

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Animals were euthanized 2 or 6 weeks after cellular transplantation. Animals first were deeply anesthetized by an intraperitoneal injection of pentobarbital. The left ventricle was pierced using surgical scissors, and a cannula was inserted through the left ventricle into the ascending aorta. The cannula was clamped in place, and the right atrium was pierced to allow fixative to flow through the heart. After washing with at least 100 mL of perfusion wash (saline solution), 500 mL of perfusion fixative was administered; the flow was slow until stiffness of the limbs and tail was observed. The entire spinal cord, from cervical to sacral segments, was removed from the animal and fixed in 4% paraformaldehyde for 24 hours at 4°C. The spinal cord was placed in a 30% sucrose solution for 24 hours to cryoprotect the tissue. Afterward, the spinal cord was instantly frozen in liquid nitrogen–chilled isopentane and stored at –80°C until further processing.

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Spinal Cord Cryosection

Spinal cord tissue was sectioned into 20- μm slices in the rostral-to-caudal direction. Two sections were placed on 1 slide, and 4 slides were collected per sampling region (ie, 8 sections were collected per sampling region). Sampling regions were separated by 200 μm (ie, 10 section widths).

Immunohistochemical Analysis

Frozen sections were rehydrated by immersion in PBS buffer for 20 minutes, and the same immunohistochemistry protocol and antibodies (Table) were used. To counterstain all cell nuclei, DAPI was added to the sections (1 $\mu\text{g}/\text{mL}$, diluted in PBS) for 5 minutes. Sections were washed 3 times in PBS, and a drop of mounting media (Dako, Carpinteria, California) was applied to the sections before a coverslip was applied. A negative control of each immunostaining procedure was performed by substituting PBS for the primary antibody.

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Hematoxylin and Eosin Staining

Tissue sections (harvested from the contused spinal cord 48 hours after injury) were stained with hematoxylin and eosin to visualize the lesion and facilitate calculation of lesion volume. With this procedure, the nuclei stain dark blue and the cytoplasm stains pink. Sections were placed in water for 2 minutes and then stained with Mayer hematoxylin solution for 6 minutes (sufficient for the nuclei to stain). Samples were washed in water for 4 minutes and then stained with eosin for 2 minutes. After a quick rinse in tap water, samples were dehydrated with 2-minute washes in ethanol (concentrations of 50%, 75%, 90%, 100%; samples were washed twice in 100% ethanol). Dehydrant was removed by washing twice in xylene for 15 minutes per wash. DPX mounting media and a cover slip were applied to each slide, and slides were incubated at 60°C overnight to solidify.

Stereologic Analysis

Stereologic analysis was performed to estimate the volume and number of GFP-expressing cells in each spinal cord at each time-point. Volume was estimated using the Cavalieri method (Mayhew and Olsen 1991). This method involved arbitrarily placing a grid of points onto magnified images ($\times 20$) of spinal cord sections. The number of points within the boundaries of each cell (region of interest) was counted. Cell volume was calculated using the following formula:

$$V = \sum P \cdot A \cdot T,$$

where V is the volume, P is the number of points in the region of interest, A is the area associated with each point, and T is the distance between each sampled section. Area associated with each point is defined as (distance between adjacent points/linear magnification)². The total volume of GFP-MSCs transplanted into the spinal cord was divided by the average GFP-MSC volume to estimate the number of surviving GFP-MSCs.

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Results

Retroviral Transduction of Wild-Type MSCs with GFP, G418 Selection, and Retroviral Transduction of GFP-MSCs With GDNF

Optimization of transduction efficiency with retroviral vectors first was determined by transducing to express GFP using a GFP retroviral construct. GFP-positive cells were selected over a 12-day period with different concentrations of G418 (Figure 1 A). Two days after transduction, the percentage of GFP-positive cells was low (approximately 10%) without G418 selection but was approximately 20% with 0.5 or 0.8 mg/mL of G418. Without G418 selection, the percentage GFP-positive cells increased between 2 days and 6 days. This was due to the fact that the GFP was under the control of the IRES promoter, which is a very weak promoter and can take up to 7 days to see the full extent of GFP expression. The percentage GFP-positive cells without G418 treatment does not increase between 6 days and 12 days. By the 12-day time point however, the percentage of GFP-positive cells increased to approximately 90% if cells were treated with 0.8 or 1.0 mg/mL of G418. We determined that transduced MSC cultures treated with 0.8 mg/mL of G418 would yield the highest number of GFP-positive cells (Figure 1 B) and used that concentration in subsequent experiments when selecting GDNF-transfected cells.

Fluorescent microscopy showed that GFP expression had no adverse effects on cell morphology (Figure 1 C). Fewer GFP-positive MSCs were visible without G418 selection at 4 and 12 days after transfection. The freezing of cells in liquid nitrogen had no negative impact on the viability of GFP-expressing cells (assays were performed in triplicate; $P > .05$) (Figure 1 D).

GFP-MSCs were transduced with a GDNF-expressing retroviral vector. Analysis of the cell supernatant by ELISA showed GDNF secretion; 12 days after transfection and G418 selection, triplicate assays showed that GDNF levels were approximately 12 ng/500,000 cells, 48 hours after fresh media was placed on the cells (Figure 1 E).

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Characterization of GDNF-Transduced GFP-MSCs

MSCs were characterized by their ability to differentiate into adipocytes and osteocytes, as previously published (16). Further characterization of specific markers pertinent to this study was performed before transplantation of transduced cells.

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Immunocytochemistry analysis of the GDNF-MSCs (Figure 2) showed continued expression of the mesodermal marker CD90 and of GDNF. Expression of mesodermal marker CD105 also was observed (data not shown). Cells expressed the neural intermediate filament marker nestin (data not shown), β -III tubulin (Figure 2), brain lipid-binding protein (data not shown), and the astrocytic marker glial fibrillary acidic protein (GFAP) (Figure 2) but did not express the oligodendrocyte marker myelin basic protein (Figure 2).

Spinal Cord Injury Contusion Model

Hematoxylin and eosin stains were used for histopathologic examination of the wound after contusion of the spinal cord, 48 hours after inducing the injury. A laminectomy was performed without contusion as a control, and in those animals, the transverse spinal cord remained intact with clearly distinguishable areas of white and gray matter (Figure 3 A). When the spinal cord was contused, however, an area of hemorrhage was evident on the dorsal surface of the spinal cord, above the central canal (Figure 3 B).

Outcome of GDNF-Transduced GFP-MSCs in the Contused Spinal Cord

Fluorescent microscopy and stereologic analysis showed that approximately 20% of GFP-MSCs (~37,202 cells out of 200,000 transplanted) and GDNF-transfected GFP-MSCs (~42,061 cells out of 200,000 transplanted) survived transplantation into the contused spinal cord for at least 2 weeks. GDNF expression was almost completely undetectable after transplantation of GFP-MSCs (Figure 4 A-C). The appearance of very low GDNF expression may be attributable to nonspecific binding. However, GDNF expression was clearly detected 2 and 6 weeks after transplantation of GDNF-transfected GFP-MSCs (Figure 4 D-H). The GDNF and GFP did not co-localize at the 2 week time-

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point which may be the result of either secretion of the growth factor from the transplanted cells or upregulation of GDNF expression in neighboring host cells via paracrine effects. It is important to note that cells were only detected in 2 out of 6 animals in the GDNF-GFP-MSC transplanted group at 6 weeks with ~15% (~31,218 cells out of 200,000 transplanted) of the transplanted cells surviving, while none were detected in the GFP-MSC transplanted group at 6 weeks.

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Immunohistochemical analysis of the mesodermal marker CD90 showed continuous expression in GDNF-transfected GFP-MSCs after transplantation (Figure 5 A-E), indicating that differentiation did not occur. Assessment of neural markers β -III tubulin (Figure 5 F-H), growth-associated protein 43 (Figure 5 I and J), myelin basic protein (Figure 5 K), GFAP (Figure 5 L), and brain lipid-binding protein (Figure 5 M) showed that their expression patterns were associated closely with transplanted cells but were not colocalized with them, which strengthens the argument against neural differentiation of the transplanted cells. Animals that did not receive GFP-MSCs did not show green fluorescence labeling in the spinal cord or CD90-TRITC labeling in the dorsal area, where it localized in GFP-MSC-treated animals (Figure 5 N).

Functional analysis

Recovery of locomotor function was assessed using the BBB rating scale. No significant difference in BBB scores was evidenced between the groups tested (Figure 6). All scores reflect the time elapsed post-transplantation of cells into the contused spinal cord. The BBB scores for the 1 day time-point were 2.83 ± 0.66 , 1.08 ± 0.18 and 1.5 ± 0.29 for the control, GFP-MSC and GDNF-MSC groups respectively; The 2 week time-point scores were 10.41 ± 1.32 , 8.83 ± 0.63 and 9.83 ± 0.44 for the control, GFP-MSC and GDNF-MSC groups respectively; The 4 week time-point scores were 10.58 ± 1.44 , 9.58 ± 0.63 and 10.42 ± 0.69 for the control, GFP-MSC and GDNF-MSC groups respectively; and the 6 week time-point scores were 13.17 ± 0.94 , 9.75 ± 0.66 and 11.92 ± 1.02 for the control, GFP-MSC and GDNF-MSC groups respectively.

Discussion

The decrease in neurotrophic factor expression between the neonatal and adult CNS may contribute to the restricted axonal growth and regeneration observed with aging or injury (32). Provision of neurotrophic factors to injured neurons therefore may help stimulate neuronal survival and axonal regeneration. MSCs are an ideal candidate for cell-based delivery of neurotrophins into the CNS for several reasons: 1) they are easily isolated and propagated *in vitro*, 2) they may be nonimmunogenic in an allogeneic setting (21), and 3) they bypass many of the ethical concerns associated with use of embryonic stem cells in a clinical setting.

Researchers have successfully delivered brain-derived neurotrophic factor (33), nerve growth factor (34, 35), and GDNF (36, 37) to the injured nervous system by using marrow stromal cells, fibroblasts, Schwann cells, and neural progenitor cells. GDNF promotes the survival, maturation, and maintenance of developing and injured motor neurons *in vitro* and *in vivo* (26). Treatment with GDNF *in vivo* also promotes the survival of dopaminergic neurons in the striatum and midbrain in animal models of Parkinson disease (27) and promotes growth of motor and dorsal column sensory axons coupled with remyelination, after spinal cord transection (37).

We have previously successfully transduced MSCs to express nerve growth factor using an adenoviral vector (16). In this study, we clearly demonstrated that MSCs could also be transduced effectively by retroviral vectors to express the neurotrophin GDNF and that these MSCs could secrete GDNF for longer than 1 week *in vitro*. However, the concept of “physiologic levels” of growth factors is not readily modeled *in vitro*, and our objective therefore was to maintain expression of GDNF for several weeks after delivery of GDNF-transduced MSCs into the injured CNS.

Stereological analysis demonstrated that 20% of GFP-MSCs and GDNF-transfected GFP-MSCs survived 2 weeks after transplantation into the contused spinal cord. Those GDNF-transduced GFP-MSCs that were detected at 2 weeks and 6 weeks, continued to

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Deleted: We were able to efficiently generate large numbers of GDNF-secreting cells for transplantation because transduced cells showed good stability after freezing and thawing.¶

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3 express the therapeutic protein. The GDNF and GFP did not co-localize extensively,
4 which may be the result of either secretion of the growth factor from the transplanted
5 cells or upregulation of GDNF expression in neighboring host cells via paracrine effects.
6 This lack of co-localization prevented us from estimating the percentage of transplanted
7 cells which were still GDNF-positive. GDNF expression was not documented in the
8 animals that received buffer alone or GFP-MSCs alone, which indicates that the presence
9 of GDNF in the GDNF-GFP-MSC transplanted group was due to the presence of the
10 transgene in the transplanted cells. We found no evidence of MSC differentiation after
11 transplantation into the CNS, which was consistent with previous studies (9-11).

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12 Transplanted cells remained positive for the mesodermal marker CD90 and did not
13 express neural markers such as β -III tubulin, growth-associated protein 43, myelin basic
14 protein, GFAP, or brain lipid-binding protein. The latter observation was in spite of
15 detection of these proteins in MSCs *in vitro*. We therefore suggest a more cautious
16 approach when interpreting *in vitro* differentiation of cells on the basis of neural marker
17 expression alone. Although cells may express certain markers *in vitro*, such expression
18 may be attributable to the culture environment (1); our results show that cells may not
19 continue to express certain markers *in vivo*.

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20 Despite this failure to generate neural cells *in vivo*, MSCs may still have an important
21 role modifying the environment around the site of a CNS injury. The finding that MSCs
22 do not differentiate and do not proliferate over time when delivered into the contused
23 spinal cord may be a distinct advantage; in comparison, embryonic stem cells form
24 teratomas when transplanted in an undifferentiated state (38).

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25 Here, we showed that MSCs were useful stem cell candidates for delivery of trophic
26 molecules into the injured nervous system. These cells were easy to isolate and propagate
27 *in vitro* and could be genetically modified to express neurotrophins. We also showed that
28 cells isolated from transgenic animals were reliable (ie, capable of long-term expression
29 of GFP and GDNF). From a clinical perspective, MSCs are advantageous because they

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3 are available in large numbers and may be used for allogeneic transplantation. The
4 combination of stem cell therapy and gene therapy offer an integrated approach toward
5 repair. Future strategies will aim to improve function after SCI using different
6 combinations of neurotrophic factors delivered via MSCs.
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10 **Conclusion**

11 In conclusion, transplanted MSCs have limited capacity for the replacement of neural
12 cells lost as a result of a spinal cord trauma. However, they provide excellent
13 opportunities for local delivery of neurotrophic factors into the injured tissue. This study
14 underlines the limited differentiation potential of MSCs, while highlighting their potential
15 for promoting therapeutic benefits within the CNS.
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39 Scientific Publications, Mayo Clinic.
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Author Disclosure Statement

No competing financial interests exist between any of the authors.

Table Antibodies Used in Immunohistochemical Staining

Primary Antibody ^a	Isotype	Source	Dilution
<u>Anti-BLBP</u>	<u>Rabbit Polyclonal</u>	<u>Chemicon, Hampshire, United Kingdom</u>	<u>1:100</u>
Anti-β-III tubulin	Mouse IgG	R&D Systems, Abington, Oxon, United Kingdom	1:100
Anti-CD90	Mouse IgG	Santa Cruz, Heidelberg, Germany	1:50
Anti-GAP 43	Mouse IgG	Sigma-Aldrich, Dublin, Ireland	1:100
Anti-GDNF	Rabbit polyclonal	BD Pharmingen, Oxford, United Kingdom	1:50
Anti-GFAP	Rabbit polyclonal	Chemicon, Hampshire, United Kingdom	1:200
Anti-MBP	Mouse IgG	Chemicon, Hampshire, United Kingdom	1:50
Anti-nestin	Mouse IgG	BD Pharmingen, Oxford, United Kingdom	1:500

Abbreviations: GAP-43, growth associated protein-43; GDNF, glial derived neurotrophic factor; GFAP, glial fibrillary acidic protein; MBP, myelin basic protein; Ig, immunoglobulin.

^a All primary antibodies were derived from a goat host.

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Legends

Figure 1. Green Fluorescent Protein, Transduction of Rat Mesenchymal Stem Cells (GFP-MSCs), G418 Selection, and Glial-Derived Neurotrophic Factor (GDNF)

Transduction of MSCs. A, Percentage of GFP-MSCs after transduction with a mouse leukemia retrovirus and selection with G418 disulfate salt solution. Error bars represent standard deviation (triplicate assays). B, Number of GFP-positive cells harvested from the transduced populations. Error bars represent standard deviation (triplicate assays). C, Photomicrographs of wild-type MSCs, 4 or 12 days after GFP-retroviral transduction, with or without G418 selection. D, Percentage of cells expressing GFP before freezing in liquid nitrogen and after freezing and thawing. Error bars represent standard deviation (triplicate assays; $P > .05$). E, Quantification of secreted GDNF (measured by enzyme-linked immunosorbent assay) from GFP-MSCs transduced with a GDNF-expressing retroviral vector (plotted as ng/500,000 cells over a period of 48 hours). Nontransduced cells served as a control. Error bars represent standard deviation (triplicate assays).

Figure 2. Photomicrographs of GDNF-Transduced GFP-MSCs ($\times 200$). Cells were stained with 4',6-diamidino-2-phenylindole (DAPI) to visualize nuclei, and specific tetramethylrhodamine isothiocyanate (TRITC)-labeled markers were used to visualize CD90, GDNF, β -III tubulin, glial fibrillary acidic protein (GFAP), and myelin basic protein (MBP). Merged images combine TRITC, GFP, and DAPI images. Abbreviations from Figure 1 apply. MSCs were shown to express the neural markers β -III tubulin and GFAP, as well as the mesodermal marker CD90, prior to transplantation into the contused spinal cord.

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3 **Figure 3.** Photomicrographs of Sections of the Spinal Cord. A, After laminectomy only
4 (no contusion) ($\times 40$). B, Contused spinal cord 48 hours after injury ($\times 100$).

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6 **Figure 4.** Fluorescent microscopy showing TRITC-Labeled GDNF in the Contused
7 Spinal Cord. A-C, GFP-MSCs, 2 weeks after transplantation, showing little or no GDNF
8 staining (A, $\times 40$; B, $\times 100$; C, $\times 400$). D-F, GDNF-transduced GFP-MSCs, 2 weeks after
9 transplantation, showing extensive GDNF staining (D, $\times 40$; E, $\times 100$; F, $\times 400$). G-H,
10 GDNF-transduced GFP-MSCs, 6 weeks after transplantation, showing some GDNF
11 staining (G, $\times 200$; H, $\times 400$). All show merged TRITC, GFP, and DAPI images.
12 Abbreviations from Figures 1 and 2 apply.

13
14 **Figure 5.** Characterization of Spinal Cord Sections With Fluorescent Microscopy After
15 Transplantation of GDNF-Transduced GFP-MSCs Into the Contused Spinal Cord.
16 Control animals were injected with buffer only. Sections were stained with DAPI, and
17 individual markers were labeled with TRITC. A-E, GDNF-transduced GFP-MSCs
18 labeled with CD90 (A, $\times 40$; B-E, $\times 100$). F-H, GDNF-transduced GFP-MSCs labeled
19 with β -III tubulin (F, $\times 40$; G, $\times 100$; H, $\times 400$). I and J, GDNF-transduced GFP-MSCs
20 labeled with GAP 43 (I, $\times 100$; J, $\times 200$). The oval area represents the area in I which was
21 magnified in J. K, GDNF-transduced GFP-MSCs labeled with MBP ($\times 200$). L, GDNF-
22 transduced GFP-MSCs labeled with GFAP ($\times 100$). M, GDNF-transduced GFP-MSCs
23 labeled with BLBP ($\times 100$). N, Control spinal cord injected with buffer only, and stained
24 for CD90 ($\times 100$). F-N, All show merged TRITC, GFP, and DAPI images, in a dorsal-
25 ventral orientation. Abbreviations from Figures 1 and 2 apply. MSCs continue to express
26 the mesodermal marker CD90, while there was no co-localization of the CD90 positive
27 cells with any of the neural markers.
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3 Figure 6. Basso, Beattie and Bresnahan (BBB) locomotor rating scale analysis of groups
4 after transplantation of cells into the contused spinal cord. Graphic representation of
5 BBB scores in animals that received no cells (control), GFP-MSCs and GDNF-MSCs at 1
6 day, 2 weeks, 4 weeks and 6 weeks post surgery. Error bars represent standard error of
7 the mean (n=6). No significant difference was observed between groups at each time-
8 point.
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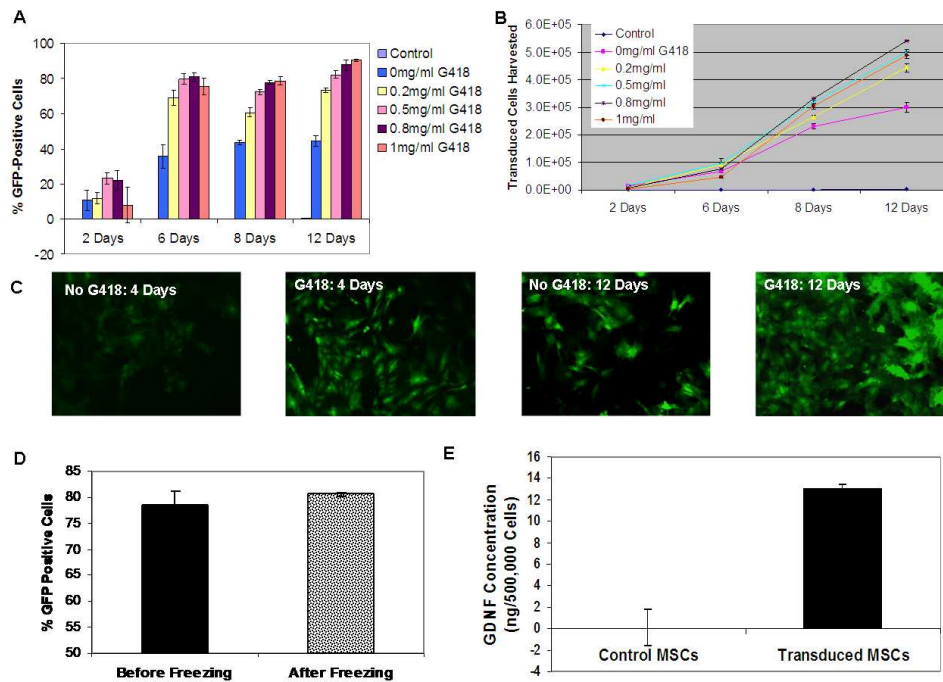
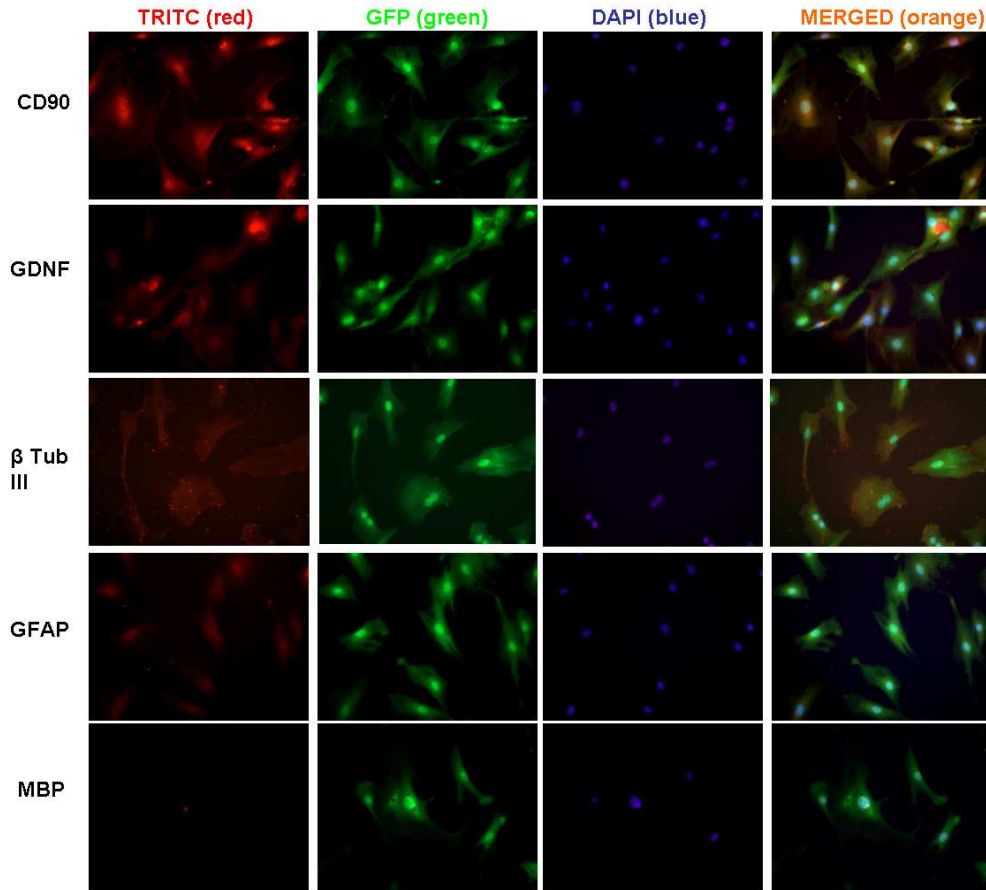


Figure 1. Green Fluorescent Protein Transduction of Rat Mesenchymal Stem Cells (GFP-MSCs), G418 Selection, and Glial-Derived Neurotrophic Factor (GDNF) Transduction of MSCs. A, Percentage of GFP-MSCs after transduction with a mouse leukemia retrovirus and selection with G418 disulfate salt solution. Error bars represent standard deviation (triplicate assays). B, Number of GFP-positive cells harvested from the transduced populations. Error bars represent standard deviation (triplicate assays). C, Photomicrographs of wild-type MSCs, 4 or 12 days after GFP-retroviral transduction, with or without G418 selection (x200). D, Percentage of cells expressing GFP before freezing in liquid nitrogen and after freezing and thawing. Error bars represent standard deviation (triplicate assays; $P < 0.05$). E, Quantification of secreted GDNF (measured by enzyme-linked immunosorbent assay) from GFP-MSCs transduced with a GDNF-expressing retroviral vector (plotted as ng/500,000 cells over a period of 48 hours). Nontransduced cells served as a control. Error bars represent standard deviation (triplicate assays).

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Figure 2. Photomicrographs of GDNF-Transduced GFP-MSCs (x200). Cells were stained with 4',6-diamidino-2-phenylindole (DAPI) to visualize nuclei, and specific tetramethylrhodamine isothiocyanate (TRITC)-labeled markers were used to visualize CD90, GDNF, β -III tubulin, glial fibrillary acidic protein (GFAP), and myelin basic protein (MBP). Merged images combine TRITC, GFP, and DAPI images. Abbreviations from Figure 1 apply. MSCs were shown to express the neural markers β -III tubulin and GFAP, as well as the mesodermal marker CD90, prior to transplantation into the contused spinal cord.
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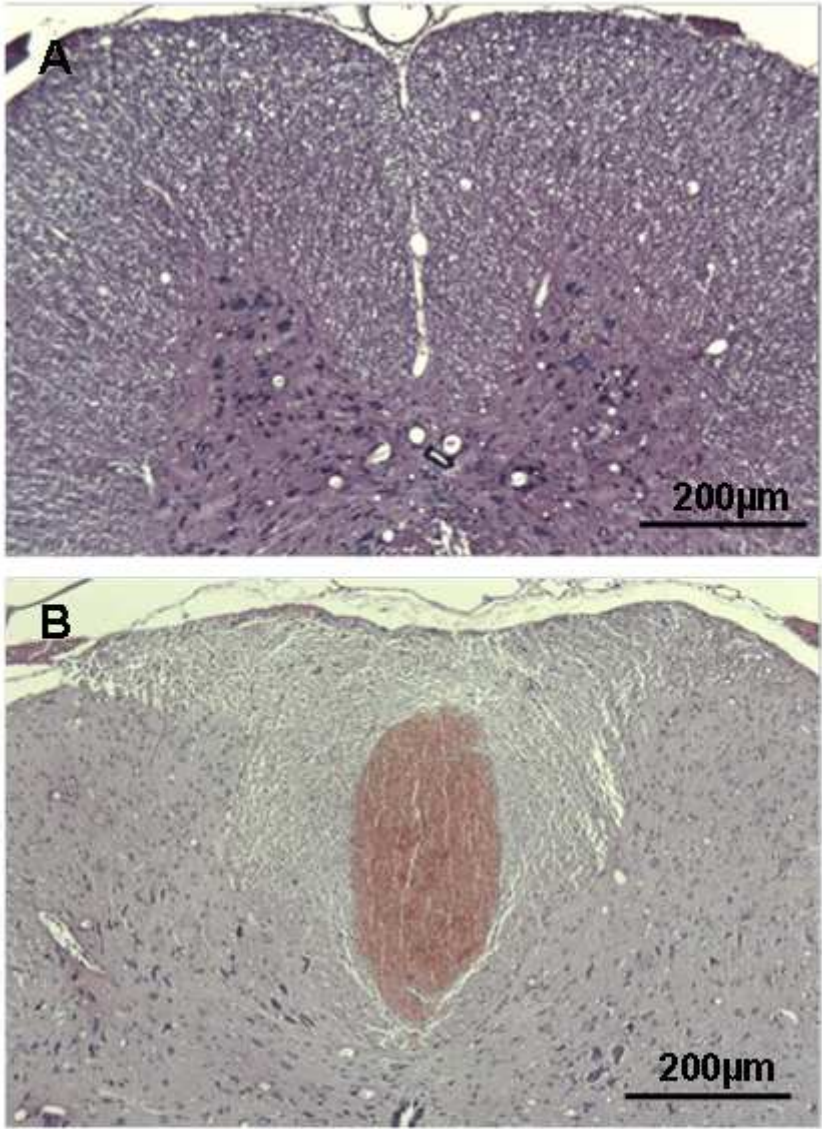


Figure 3. Photomicrographs of Sections of the Spinal Cord. A, After laminectomy only (no contusion) (x40). B, Contused spinal cord 48 hours after injury (x100).
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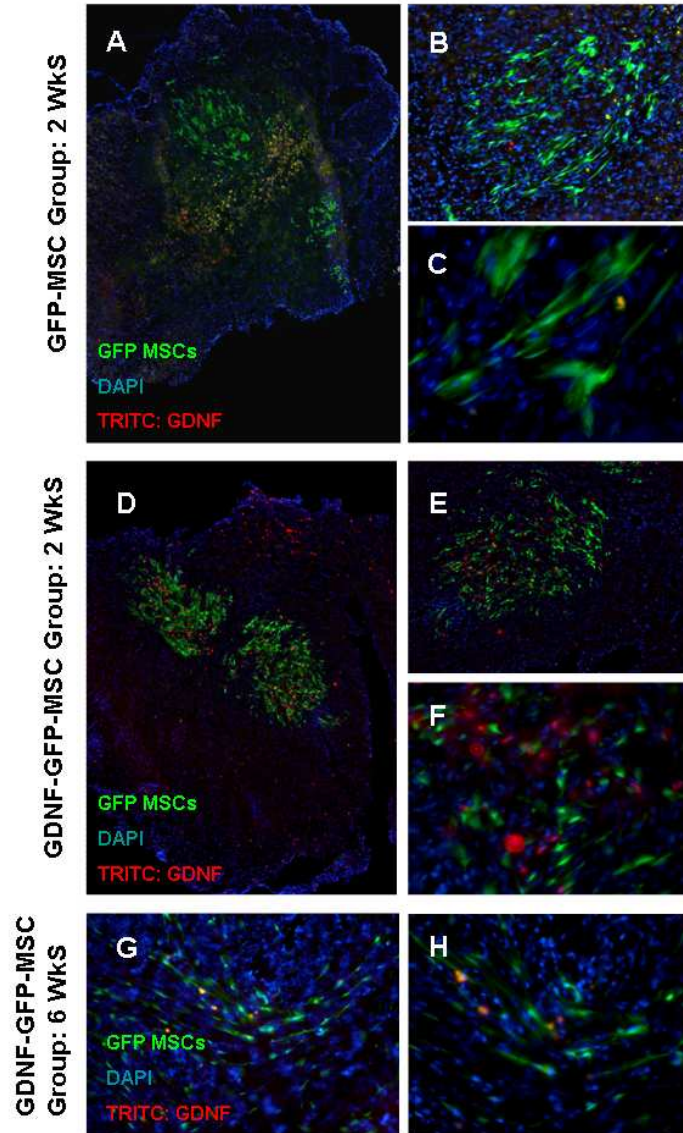


Figure 4. Fluorescent microscopy showing TRITC-Labeled GDNF in the Contused Spinal Cord. A-C, GFP-MSCs, 2 weeks after transplantation, showing little or no GDNF staining (A, x40; B, x100; C, x400). D-F, GDNF-transduced GFP-MSCs, 2 weeks after transplantation, showing extensive GDNF staining (D, x40; E, x100; F, x400). G-H, GDNF-transduced GFP-MSCs, 6 weeks after transplantation, showing some GDNF staining (G, x200; H, x400). All show merged TRITC, GFP, and DAPI images. Abbreviations from Figures 1 and 2 apply.
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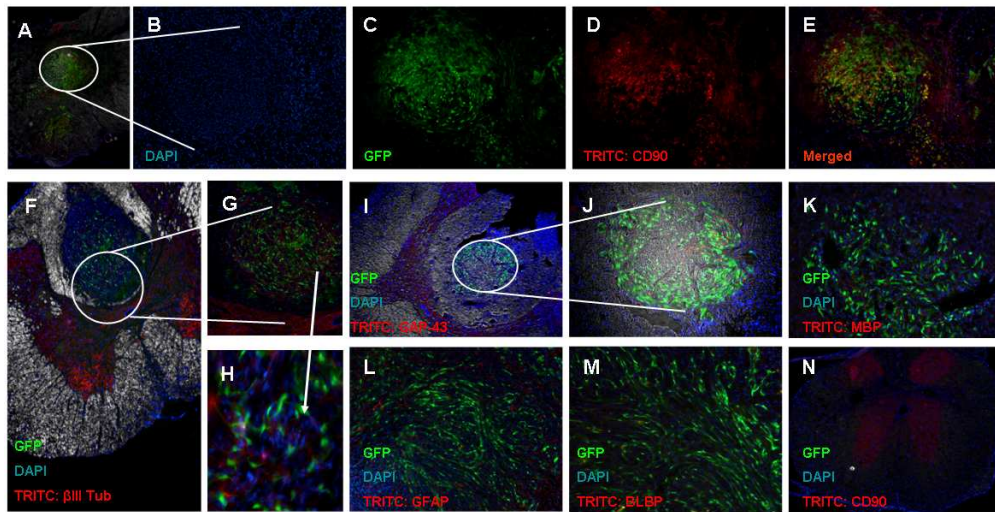


Figure 5. Characterization of Spinal Cord Sections With Fluorescent Microscopy After Transplantation of GDNF-Transduced GFP-MSCs Into the Contused Spinal Cord. Control animals were injected with buffer only. Sections were stained with DAPI, and individual markers were labeled with TRITC. A-E, GDNF-transduced GFP-MSCs labeled with CD90 (A, x40; B-E, x100). F-H, GDNF-transduced GFP-MSCs labeled with β -III tubulin (F, x40; G, x100; H, x400). I and J, GDNF-transduced GFP-MSCs labeled with GAP 43 (I, x100; J, x200). The oval area represents the area in I which was magnified in J. K, GDNF-transduced GFP-MSCs labeled with MBP (x200). L, GDNF-transduced GFP-MSCs labeled with GFAP (x100). M, GDNF-transduced GFP-MSCs labeled with BLBP (x100). N, Control spinal cord injected with buffer only, and stained for CD90 (x100). F-N, All show merged TRITC, GFP, and DAPI images, in a dorsal-ventral orientation. Abbreviations from Figures 1 and 2 apply. MSCs continue to express the mesodermal marker CD90, while there was no co-localization of the CD90 positive cells with any of the neural markers.

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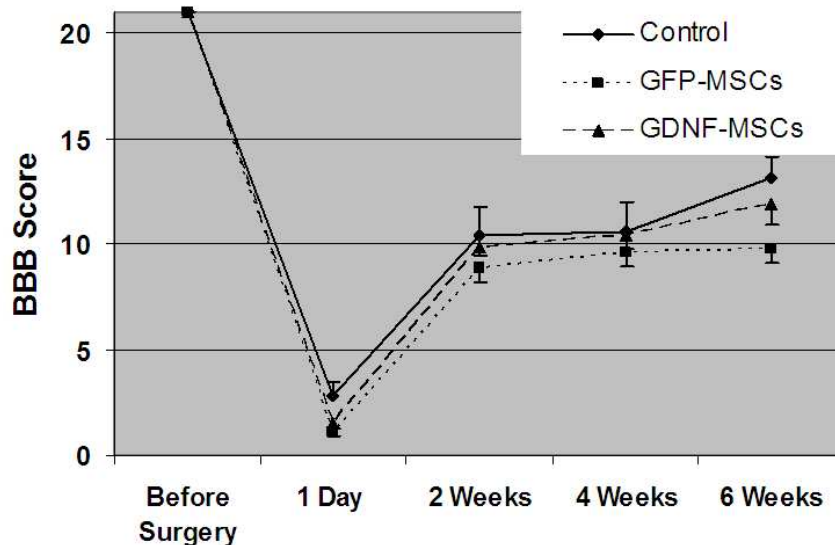


Figure 6. Basso, Beattie and Bresnahan (BBB) locomotor rating scale analysis of groups after transplantation of cells into the contused spinal cord. Graphic representation of BBB scores in animals that received no cells (control), GFP-MSCs and GDNF-MSCs at 1 day, 2 weeks, 4 weeks and 6 weeks post surgery. Error bars represent standard error of the mean (n=6). No significant difference was observed between groups at each time-point.

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