

Research report

# Axon growth and recovery of function supported by human bone marrow stromal cells in the injured spinal cord exhibit donor variations

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## Abstract

Bone marrow stromal cells (MSC) are non-hematopoietic support cells that can be easily derived from bone marrow aspirates. Human MSC are clinically attractive because they can be expanded to large numbers in culture and reintroduced into patients as autografts or allografts. We grafted human MSC derived from aspirates of four different donors into a subtotal cervical hemisection in adult female rats and found that cells integrated well into the injury site, with little migration away from the graft. Immunocytochemical analysis demonstrated robust axonal growth through the grafts of animals treated with MSC, suggesting that MSC support axonal growth after spinal cord injury (SCI). However, the amount of axon growth through the graft site varied considerably between groups of animals treated with different MSC lots, suggesting that efficacy may be donor-dependent. Similarly, a battery of behavioral tests showed partial recovery in some treatment groups but not others. Using ELISA, we found variations in secretion patterns of selected growth factors and cytokines between different MSC lots. In a dorsal root ganglion explant culture system, we tested efficacy of conditioned medium from three donors and found that average axon lengths increased for all groups compared to control. These results suggest that human MSC produce factors important for mediating axon outgrowth and recovery after SCI but that MSC lots from different donors vary considerably. To qualify MSC lots for future clinical application, such notable differences in donor or lot–lot efficacy highlight the need for establishing adequate characterization, including the development of relevant efficacy assays.

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## 1. Introduction

Treatments that enhance axonal growth and regeneration of damaged axons in the central nervous system (CNS) have a potential for improving recovery following spinal cord injury (SCI). The adult, and especially the injured CNS, is inhibitory to axonal growth. Therefore, effective repair strategies for SCI require the creation of a permissive

environment within the injured spinal cord that protects damaged neurons from the effects of secondary injury and also facilitates axonal regeneration. Cell transplantation is among the most promising therapeutic approaches for treating SCI. Ideally, cell transplants would be readily obtainable, easy to expand and bank, and capable of surviving long enough to facilitate sufficient and appropriate axonal regeneration [14].

Bone marrow stromal cells (MSC) are connective tissue progenitor cells that are distinct from hematopoietic stem cells [45]. While MSC can be easily expanded *ex vivo* from raw bone marrow, there is no generally accepted method for MSC isolation, propagation, and characterization. As a

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result, the phenotype of culture-expanded MSC can vary considerably when derived by different methods [44] or from different sources [42,43].

Recent studies proposed a more extensive differentiation potential of MSC showing phenotypic plasticity that appears to cross the boundaries of the traditional germ layers including cardiac cells [41], skeletal muscle [31], and neural cells [30]. Whether this apparent plasticity represents transdifferentiation, a pool of persistent pluripotent stem cells, cell fusion, or artifacts of culturing remains controversial [21,25,34,53].

Because of their ability to differentiate into a variety of cells, the ease of their isolation and expansion, and their potential use for clinical application, efforts have increased to better understand the biology of MSC. In the injured CNS, MSC transplantation has been shown to improve recovery after stroke or traumatic brain injury [8]. In animal models of SCI, grafts of MSC have been shown to promote remyelination [1] as well as partial recovery of function [9,23,60]. While previous studies have suggested that MSC can differentiate into cells with neural characteristics *in vitro* [11,28,47] and *in vivo* [9,23,30], it is unclear whether such differentiation contributes to recovery of function in animal models of neurotrauma.

There is growing evidence that MSC produce a variety of neurotrophic factors as well as chemokines and cytokines *in vitro* and *in vivo* (for review, see [8]). Kinnaird et al. [29] found that paracrine signaling of MSC is an important therapeutic mechanism in the treatment of ischemia. A recent study [54] showed that MSC secrete brain natriuretic peptide (BNP), a peptide with diuretic and vasodilatory effects *in vitro*, suggesting that MSC could facilitate recovery by reducing edema and improving perfusion. In addition, Chen et al. [6] showed that the secretion profile of MSC is responsive to the environment with increased secretion of certain growth factors (e.g., BDNF, NGF) in the injured brain. Zhong et al. [62] demonstrated that neural cell death in response to oxygen-glucose deprivation was reduced in hippocampal slices co-cultured with MSC, suggesting a neuroprotective effect possibly mediated by diffusible factors released by MSC. Thus, the cells may create a permissive environment for axon outgrowth and axonal guidance mediated by their release of trophic factors, thereby improving self-repair in the damaged CNS.

In the present study, we investigated the efficacy of different lots of MSC, each obtained from the bone marrow aspirate of a different donor, by evaluating their ability to support axonal growth following engraftment in a rat model of subtotal cervical hemisection. Functional recovery was evaluated by an array of motor and sensory tests. In addition, we showed variations in the secretion profiles for selected growth factors and cytokines of MSC from different donors, and the ability of MSC-conditioned medium to promote axon outgrowth in an *in vitro* dorsal root ganglion (DRG) culture system independent of the donor.

## 2. Materials and methods

### 2.1. Isolation and expansion of human MSC

Human MSC were isolated from bone marrow aspirates taken from the iliac crest of four healthy adult human volunteers under informed consent. Donors were tested for various chronic diseases (heart, kidney or liver disease, ulcer, cancer, diabetes, epilepsy) as well as for bacterial or viral infections. Vital signs, hematological lab values and donor weight were within normal range and donors were not currently taking prescription medication. Donor age ranged between 18 and 45 years. We took great care that harvest and culture of MSC from different donors were performed in a very similar manner so as to limit harvest- and culture-dependent variation.

Red blood cells (RBC) were first removed from the bone marrow aspirate by adding (1:20 v/v) ammonium chloride buffer consisting of 155 mM ammonium chloride, 10 mM potassium bicarbonate, and 0.1 mM EDTA (ethylenediaminetetraacetic acid), at pH 7.2. The resulting cell suspension was centrifuged for 10 min at  $500 \times g$  to remove the RBC fraction. The mononuclear cell pellet was then re-suspended and washed twice in complete medium consisting of Minimal Essential Medium- $\alpha$  ( $\alpha$ -MEM, Invitrogen, Carlsbad, CA) supplemented with 4 mM glutamine and 10% fetal bovine serum (FBS, Atlanta Biologicals, Norcross, GA) and centrifuged for 10 min at  $500 \times g$ . Cells were counted by trypan blue exclusion and seeded in tissue culture-treated flasks at a density of approximately 50,000 viable cells/cm<sup>2</sup> and placed in a 37 °C humidified cell culture incubator. On day 5, the non-adherent cells and spent medium were removed from the flasks and the adherent cells re-fed with fresh, complete medium. The adherent cells were then cultured for an additional 5 days prior to the first passage. Seeding density for subculture of MSC at all subsequent passages was approximately 5000 cells/cm<sup>2</sup>. Cells were grown to confluency before each passage (3–4 days). Medium was exchanged every second day. All cells used for transplantation (passage 4) were derived from frozen stocks. Cryopreserved MSC were thawed quickly at 37 °C and plated in  $\alpha$ -MEM (Invitrogen) supplemented with 20% FBS and 4 mM L-glutamine (Invitrogen) on 100-mm plastic dishes. The same lot of serum was used for all experiments. The serum lot chosen allowed consistent, rapid growth and proliferation of MSC. Twenty-four hours prior to grafting, cultures were incubated with 2  $\mu$ M PKH26 (Sigma-Aldrich, St. Louis, MO). The following day, cells were washed several times with HBSS and then removed from the culture plates using 0.1% trypsin/EDTA (Cellgro, Herndon, VA). MSC were then re-suspended in complete medium at 50,000 cells/ $\mu$ l and maintained on ice during transplantation surgery. After surgery, a sample of remaining cells was re-plated overnight to verify viability.

## 2.2. Grafting of human MSC into a subtotal cervical hemisection

A total of 42 female Sprague–Dawley rats (225–250 g; Taconic, Germantown, NY) were used for this study. All procedures were approved by the institutional animal welfare committee and conformed to *NIH Guidelines for the Care and Use of Laboratory Animals*. All animals were immune suppressed with a single daily injection of Cyclosporine A (CsA; Sandoz Pharmaceuticals Co., East Hanover, NJ) administered subcutaneously at a dose of 1 mg/100 g/24 h starting 3 days before transplantation procedures and continued for 2 weeks following surgery. After this 2-week period, and for the duration of the experiment, CsA was administered orally via drinking water (Neoral, Sandoz, 50 µg/ml).

Eight rats per donor (4 different donors) received a right subtotal cervical hemisection and a transplant of MSC; another eight rats received only gelfoam as a transplant. Ten rats served as uninjured behavioral controls. Rats were anesthetized by intraperitoneal (i.p.) injection of a cocktail of acepromazine maleate (0.7 mg/kg; Fermenta Animal Health Co., Kansas City, MO), ketamine (95 mg/kg; Fort Dodge Animal Health, Fort Dodge, IA), and xylazine (10 mg/kg; Bayer Co., Shawnee Mission, KS). A laminectomy was carried out at the C3–4 level to expose one spinal cord segment. The dura over the right dorsal root entry zone was opened with a micro-scalpel and a shallow incision was made in the right dorsal spinal cord. A glass-pulled fine-tipped micro-aspiration device was used to extend the lesion laterally and ventrally. The rostrocaudal extent of the lesion cavity was about 2 mm. The lesion completely disrupted the dorsal and lateral funiculus and dorsal gray matter but spared some ventral gray matter and the ventral funiculus. After hemostasis was achieved, a piece of gelfoam that had been immersed in growth medium alone or in growth medium containing a suspension of MSC ( $5 \times 10^5$ ) was implanted into the cavity, followed by injection of another 5–10 µl of cells ( $5 \times 10^4/\mu\text{l}$ ) suspended in growth medium onto the gelfoam using a 10-µl Hamilton syringe attached to a 27-gauge needle. Presoaking the gelfoam permits expansion of the matrix before it is implanted, thereby preventing potential additional injury. The dura was closed with interrupted 10-0 silk sutures, and muscle and skin were closed in layers. Within 10 min of the spinal cord lesion, all rats received a bolus injection of methylprednisolone (30 mg/kg; Pharmacia and Upjohn Company, Kalamazoo, MI) through the tail vein. A second bolus injection of the same dose was given 2 h later. After surgery, animals were kept on heating pads and closely observed until awake, and then returned to their home cages.

## 2.3. Tissue preparation

Of the eight rats that had received cells from donors 1, 2, 3, or 4, two were chosen at random and perfused 2 weeks

following surgery. Of the remaining rats, four died during the course of the experiment (one rat each with cells from donors 1, 2, and 3, and one with a gelfoam graft) and were not included in the analysis. Therefore, five rats that had received MSC from donor 1, five with MSC from donor 2, five with MSC from donor 3, six with MSC from donor 4, and seven with the gelfoam transplants were analyzed after 11 weeks. At the end of the experiment, animals were anesthetized with an i.p. injection of sodium pentobarbital (100 mg/kg, Abbott Laboratories, North Chicago, IL) and perfused transcardially with 200 ml of normal saline solution followed by 500 ml of ice cold 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Brain and spinal cord were dissected out and immersed overnight in 0.1 M phosphate buffer (PB) at 4 °C followed by cryoprotection in 30% sucrose for 3–5 days. The lesion site was identified, blocked, embedded in OCT compound (Fisher Scientific, Pittsburgh, PA) and kept at –80 °C. Using a cryostat, sagittal sections (20 µm) of the lesion site were obtained. Sections were mounted onto gelatin-coated slides and processed for histological or immunocytochemical staining using standard protocols from our laboratory.

## 2.4. Histology and quantification

Every fifth section through the lesion site was stained to demonstrate myelin sheaths and counterstained with cresyl violet acetate [22]. Selected sections were immunolabeled with one of three antibodies: a human-specific mitochondria antibody (MAB1273, Chemicon International, Temecula, CA) was used to identify the MSC; an antibody that recognizes phosphorylated epitopes of neurofilament (RT-97; Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA), and an antibody that recognizes axonal growth associated proteins (GAP-43 [5], kindly provided by Dr. Larry Benowitz) were used to identify host axons that had grown into the graft. The human mitochondria and RT-97 antibodies were used at a dilution of 1:100. GAP-43 was used at a dilution of 1:2500. All primary antibodies were diluted in 0.1M PBS, pH 7.4 containing 2% goat serum (GS-PBS). Controls omitting the primary antibody were routinely included. Sections were incubated at room temperature overnight in a humidified chamber. After several rinses in PBS, secondary antibodies, diluted 1:200 in GS-PBS, were applied (FITC-conjugated goat anti-mouse IgG, Texas Red-conjugated goat anti-mouse or goat anti-rabbit IgG; Jackson Immunoresearch Labs, Inc, West Grove, PA). Following several washes in PBS, sections were coverslipped using Vectashield (Vector Laboratories, Burlingame, CA).

A second series of every fifth section was stained using the RT-97 antibody for quantitative evaluation of axon growth. The staining procedure used was as described above except that a biotinylated goat anti-mouse IgG (Jackson) was used as the secondary antibody. Since

endogenous peroxidase levels are very low in perfused CNS tissue and only very low background staining was observed in negative controls (no primary antibody), blocking of endogenous peroxidase was not necessary. After several washes in PBS, staining was visualized using a Vector ABC kit and Sigma Fast DAB tablets according to manufacturer's instructions. The sections were then dehydrated and coverslipped using DPX (Sigma-Aldrich). Between 10 and 15 sections were analyzed for each animal. Images were acquired using a Leica DMRBE Microscope with an attached Sensys model 1401 CCD camera/LCD filter system (Biovision Technologies, Exton, PA) using IPLABS software (Scanalytics, Inc., Fairfax, VA) running on an Apple Power Macintosh G4 computer. Images were captured at 100- $\mu$ m intervals through the graft. NIH image (version 1.62) was used to outline and measure the area of the graft and the area stained for RT-97 within the graft in each section. A summation of the measured area of graft, the area within the graft stained for RT-97, and the area fraction stained for RT-97 was calculated for each animal.

## 2.5. Behavioral testing

A battery of behavioral tests was administered to all rats 8 weeks post injury and transplantation to assess functional recovery. Tests were scored by trained observers who were unaware of experimental conditions with inter-rater reliability greater than 95%.

### 2.5.1. BBB test

Hindlimb motor function was assessed in an open field (5  $\times$  2 ft) using a modification of the BBB locomotor rating scale [3]. Because these rats had received unilateral subtotal cervical hemisections, only the hindlimb on the affected side was evaluated. Rats were observed for 2 min and scored from 0 (no observable movements) to 21 (normal locomotion). Similarly injured rats have shown an initial deficit followed by almost complete recovery using this assessment [27].

### 2.5.2. Limb preference (cylinder) test

Forelimb exploration was assessed in a Plexiglas cylinder (17.8 cm diameter  $\times$  35.5 cm height) for 3 min. When placed in the cylinder, animals spontaneously rear and contact the walls with their forepaws. The number of forelimb contacts (left, right, and both) with the cylinder walls were counted and expressed as a percentage of total placements. Percentages of forelimb contacts with the right forelimb and with both forelimbs were added to reflect the full usage of the affected limb. Initial baseline performance showed no bias in forelimb preference [35,49,51]. This test has been used previously to demonstrate a deficit followed by partial recovery of forelimb function after an acute subtotal cervical hemisection and transplantation of genetically modified fibroblasts in adult rats [35,49].

### 2.5.3. Postural measures

Postural adjustments and rears were also analyzed during a 3-min recorded observation period in the cylinder [51]. Postural adjustments were defined as shifts in hindlimb placement while the rats were rearing and preparing to contact the walls with their forelimbs. The number of right and left hindlimb steps was counted as the animal adjusted its position while rearing. Data are expressed as the ratio of hindlimb postural adjustments per rear on the affected side over the total.

### 2.5.4. Grid test

Paw placement of each limb on a grid bar was assessed as animals walked on a plastic-coated wire mesh grid (36 cm length  $\times$  38 cm width  $\times$  30 cm height, with 3  $\times$  2 cm openings) for 2 min. Steps where the paw gripped the grid bar and supported the animal's weight were counted as correct. The number of correct paw placements was expressed as a percentage of the total steps. Percentages of correct paw placements were calculated for each limb. In a variation of this test, deficits from an acute thoracic dorsal hemisection were ameliorated by grafts of fibroblasts secreting NT-3 [15]. Full recovery of performance of the contralateral side was observed after a complete cervical hemisection, with less recovery in the ipsilateral forelimb than hindlimb [51].

### 2.5.5. Thermal sensitivity (heat) test

Latency for limb withdrawal in response to plantar paw contact with a heat stimulus was measured (adapted from [12,20,61]). Animals were habituated for 30 min in elevated Plexiglas cages (Ugo Basile, Italy) with a moveable radiant heat source (25–29 °C) underneath. If no paw withdrawal occurred after 30 s, the heat stimulus was removed to prevent tissue damage, and the animal was assigned the maximal withdrawal latency of 30 s. Five trials were run for each paw with a 15-min interval between each trial to prevent sensitization. The last 4 trials were averaged to provide the mean latency of withdrawal. This test has been shown to reveal a reduced latency to thermal stimuli (indicating hypersensitivity or thermal allodynia) in rats with acute midthoracic hemisections [10,16–18] followed by partial recovery of function in the right forepaw and left hindpaw after a complete cervical hemisection with delayed transplantation of genetically modified fibroblasts [51]. We have interpreted this pattern of recovery as a result of sprouting associated with the dorsal roots at the level of the injury (right forepaw) with contributions from the spinothalamic tract or its descending modulation representing the left hindpaw; therefore, we collected withdrawal data from these two limbs.

## 2.6. Collection of conditioned media

Cells were quick thawed at 37 °C and added to T75 flasks each containing 15 ml of complete medium consisting

of  $\alpha$ -MEM supplemented with 10% FBS and 4 mM L-glutamine. Flasks were then placed in a 37 °C humidified cell culture incubator overnight. The following day, cultures were re-fed with fresh medium and returned to the incubator. On day 3, conditioned medium was collected and centrifuged at  $500 \times g$  for 5 min to remove cell debris. Cells were recovered from the flasks using 0.5% trypsin and counted. Aliquots of conditioned media were frozen at  $-80$  °C for short-term storage.

### 2.7. ELISA

Aliquots of conditioned media were thawed and warmed to room temperature for quantitative sandwich immunoassay. All ELISA Kits (IL-6, MCP-1, VEGF, SCF, SDF, BDNF) were purchased from R&D Systems (Minneapolis, MN) and used as per manufacturer's instructions. Microplates were read on a SpectraMax Plus plate reader (Molecular Devices Corp., Sunnyvale, CA) using SoftMax Pro software (Molecular Devices). Cytokine levels were then extrapolated from a standard curve and normalized to pg/million cells/day.

### 2.8. DRG explant culture, immunostaining, and quantitation

Dorsal root ganglia (DRG) were obtained from E10 chick embryos [26]. For each condition, three DRG were used in two separate experiments. DRG explants were cultured in 24-well dishes coated with 100  $\mu$ g/ml poly-L-lysine (Sigma-Aldrich) containing 500  $\mu$ l of unconditioned or MSC-conditioned medium from three different donors. Cells from donor 4 were not included in this experiment due to technical reasons. After 48 h, DRG were fixed in 0.25% glutaraldehyde. DRG were then immunostained with a monoclonal anti- $\beta$ III-tubulin antibody (BabCO, Richmond, CA) and the staining developed using a secondary biotinylated antibody (Jackson) and the Elite Vectastain ABC kit (Vector Laboratories) in combination with a Fast Diaminobenzidine Tablet kit (Sigma-Aldrich).

An Olympus CK40 microscope with a 20 $\times$  phase objective connected to a Nikon Coolpix 990 digital camera was used for analysis. To determine the average length of axons extended from each DRG, the distance from the edge of the explant to the main field of growth cones was measured in four places around the perimeter by using a micrometer slide. The average of the four measurements and the standard error were calculated for each experiment [57].

### 2.9. Statistical analysis

Behavioral data from each test were analyzed by a one-way factorial ANOVA comparing all four donor groups, gelfoam controls, and uninjured animals. When ANOVA was significant (indicating the injured animals did not recover to uninjured levels), we identified

individual donor groups that promoted recovery within the range of behavioral performance demonstrated between gelfoam controls and uninjured animals. Because we are interested in identifying donors that promote recovery, individual comparisons were made between the selected experimental group and the gelfoam control group by either a one-tailed Student's *t* test or a Chi-square test (for frequency data). Significance levels were set to 0.05 and it should be noted that  $P < 0.10$  is considered significant for one-tailed comparisons. Analysis of average axonal length was determined using a two-tailed Student's *t* test with significance levels set to 0.01. Correlational analysis was performed between results using simple linear regression. All data are presented as mean values  $\pm$  SEM.

## 3. Results

### 3.1. Grafts of human MSC support axon outgrowth in a donor aspirate-dependent manner

Two weeks after grafting, MSC were identified by labeling with PKH26 and antibodies against human mitochondria. The lesion cavity was filled with MSC (Figs. 1a and b) regardless of which donor was used. Most of the grafted cells remained at the lesion site but some of them migrated into the penumbra of surrounding host tissue. In no instances were the grafted MSC observed more than 500  $\mu$ m from the lesion site. Grafts of MSC supported extensive axonal growth, as evidenced by GAP43 and neurofilament staining (Figs. 1c and d). GAP43 and neurofilament labeled axons were most often located in the same regions of the graft.

After 11 weeks, the graft site was devoid of MSC indicated by the lack of staining with the hMITO antibody. Nissl staining revealed fibroblast-like cells at the graft site that were not stained with the hMITO antibody suggesting that host cells migrated into the graft site. Previous experiments using a contusion injury model from our laboratory (unpublished results) and others [40] indicated that oligodendrocytes infiltrate the graft, and astrocytes surround the graft site extending processes into the graft periphery. In addition, other previously published studies showed that immune cells [46], oligodendrocyte precursors [19,24] and Schwann cells [4] infiltrate the injury site.

Staining with the RT-97 neurofilament antibody showed numerous axons within the graft (Fig. 2a). Control animals that received only a gelfoam graft showed very little neurofilament staining through the lesion site (Fig. 2b). We quantified the area occupied by neurofilament staining as an area fraction of the total lesion area (Fig. 2c). Cells obtained from different donors supported axon growth into the graft at different levels. Whereas MSC from donors 1 (area fraction:  $0.156 \pm 0.019$ ), 2 (area fraction:  $0.155 \pm$

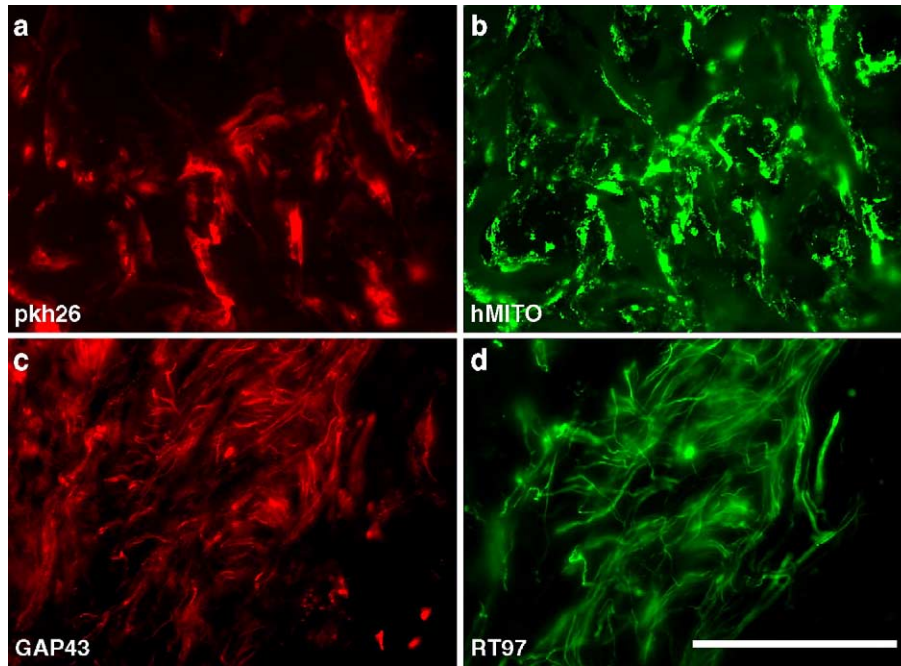


Fig. 1. Histological examination of MSC grafts in the injured spinal cord 2 weeks after grafting. MSC grafted into the injured spinal cord were identified by (a) a fluorescent membrane dye and (b) an antibody specific for human mitochondria 2 weeks after grafting. The presence of axons within the graft was demonstrated by immunostaining with antibodies against (c) GAP-43 and (d) neurofilaments (RT-97). Scale bar: 100  $\mu$ m.

0.034), and 4 (area fraction:  $0.114 \pm 0.014$ ) promoted significant ( $P < 0.01$ ) axon growth into the graft, axon growth into MSC grafts from donor 3 (area fraction:  $0.06 \pm 0.011$ ) was not significantly different from the gelfoam control ( $0.047 \pm 0.011$ ) or a fibroblast graft (data not

shown). These findings demonstrate that while MSC can promote axonal growth when grafted into the injured spinal cord, there are significant variations between cells from these different donors with respect to the ability to promote axon growth into the graft.

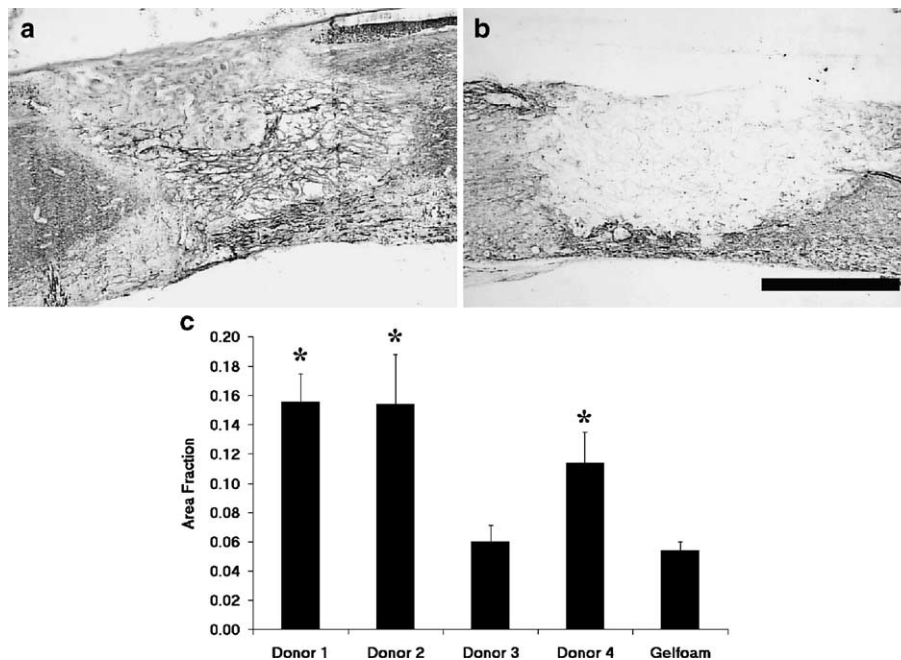


Fig. 2. Axon growth into the graft is donor-dependent 11 weeks after grafting. Sections of (a) MSC- or (b) gelfoam-grafted spinal cord were stained with antibodies against neurofilament (RT-97) to determine axon growth into the graft. (a) MSC grafts supported axonal growth, whereas (b) gelfoam grafts were negative for neurofilament staining. (c) The area of neurofilament staining was measured and calculated as a fraction of the total graft size. The histogram shows the different amounts of neurofilament staining obtained from MSC grafts of different donors. Only donors 1, 2, and 4 show significantly ( $P < 0.01$ ) more axon growth into the graft compared to the gelfoam control, while axon growth into MSC grafts from donors 3 is not significantly different from control.

3.2. Grafts of MSC from certain donors result in partial recovery of function

Motor and sensorimotor behaviors were assessed with a battery of behavioral tests at 8 weeks post injury. BBB score, which is a measure of hindlimb locomotion in an open field showed almost complete recovery in all groups with no significant differences among groups (donor 1: BBB score =  $19.6 \pm 1.4$ , donor 2: BBB score =  $19.6 \pm 1.4$ , donor 3: BBB score =  $21.0 \pm 0.0$ , donor 4: BBB score =  $21.0 \pm 0.0$ , gelfoam control: BBB score =  $18.7 \pm 1.5$ , uninjured: BBB score =  $21.0 \pm 0.0$ ). This degree of recovery is consistent with our previous results following an acute subtotal cervical hemisection [27].

Forelimb exploration in the cylinder test showed minimal recovery. Baseline performance typically results in 25% use of each forelimb independently and 50% use of both together [35,49,51]. Therefore, full recovery to baseline performance would show 75% contacts with the affected limb. In this study, rats only recovered 20–40% use of the affected forelimb (Fig. 3a). Again, this degree of recovery is consistent with our previous results following an acute

subtotal cervical hemisection in operated controls [35,49]. No differences were found among rats transplanted with MSC from these different donors, even though axon outgrowth was donor-dependent.

Postural measures (shifts in hindlimb placements while rearing) can be collected during performance in the cylinder test. Rats typically exhibit no limb preference during postural adjustment; thus, full recovery would constitute 50% use of the affected limb. Rats that had received MSC transplants from donor 2 were able to use their affected hindlimb to make postural adjustments better than the other groups (Fig. 3b). Even though MSC grafts from donor 1 supported axon outgrowth equally well, no positive effects on the use of the affected hindlimb was observed. 80% of rats that had received MSC from donor 2 used their affected limb compared with only 43% of control rats that had received gelfoam (compared with 20% of rats that had received MSC from donor 1, 20% that had received MSC from donor 3, and 17% that had received MSC from donor 4). No differences were observed among groups in the numbers or types of rears.

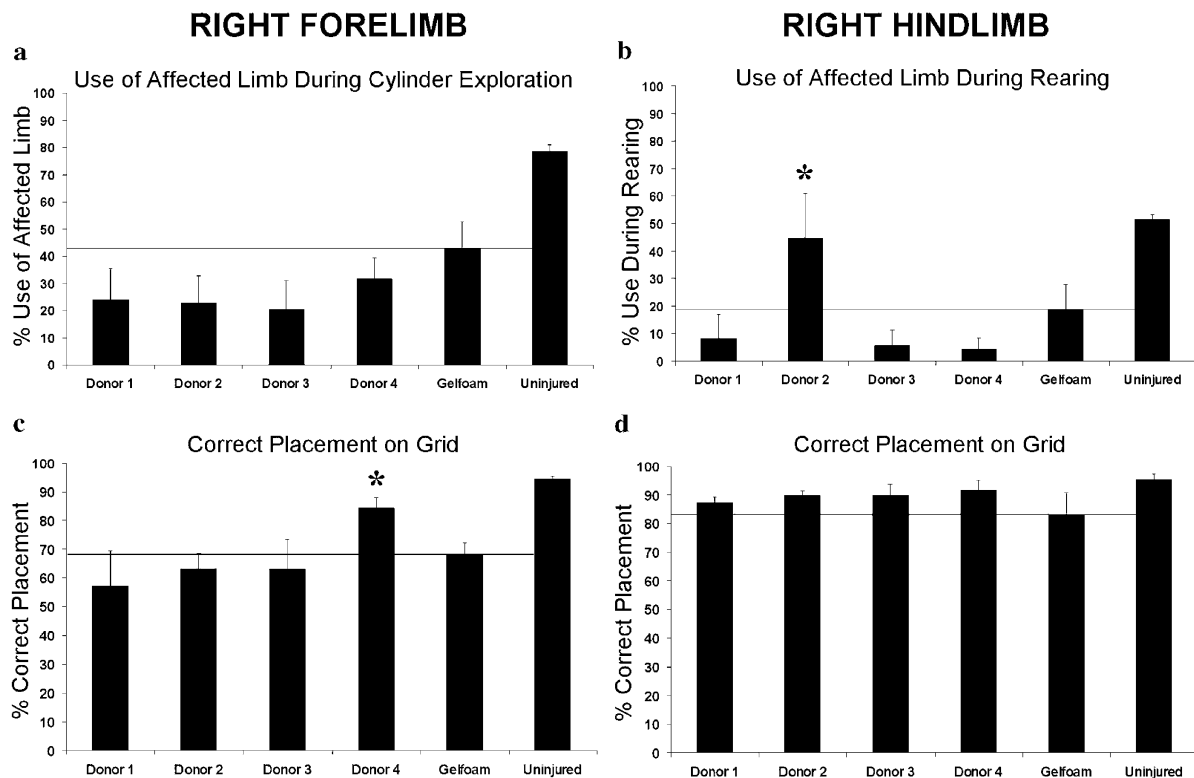


Fig. 3. Tests for the recovery of motor and sensorimotor recovery. (a) Forelimb exploration and (b) postural adjustments with the hindlimb of the affected side were evaluated in a cylinder test. (a) The number of forelimb contacts (left, right, and both) with the cylinder walls were counted and expressed as a percentage of total placements. Percentages of forelimb contacts with the right forelimb and with both forelimbs were added to reflect the full usage of the affected limb. No significant difference was observed among groups. (b) The number of right and left hindlimb steps was counted as the animal adjusted its position while rearing. Data were expressed as the ratio of hindlimb postural adjustments per rear on the affected side over the total. Only rats that had received MSC transplants from donor 2 used their affected hindlimb better than control (Chi square,  $P < 0.001$ ). (c and d) Paw placement of fore- and hindlimb of the affected side was analyzed on a grid bar. The number of correct paw placements was expressed as a percentage of the total steps. (c) Only rats that had received MSC transplants from donor 4 demonstrated more correct right forelimb paw placements than gelfoam controls (Student's  $t$  test,  $P < 0.05$ ). (d) Rats fully recovered the use of the affected hindlimb independent of the MSC donor.

Forelimb and hindlimb sensorimotor behavior associated with lateral corticospinal tract function [15] can be measured by evaluating the percentage of correct paw placements while traversing a grid. In this study, rats partially recovered the use of the affected forelimb (Fig. 3c) with only grafts from donor 4 promoting behavioral recovery, independent of the amount of axon outgrowth observed by neurofilament staining. Rats fully recovered the use of the affected hindlimb (Fig. 3d) with no differences among donor groups. As expected, the unaffected side showed full recovery (data not shown). These results following an acute subtotal cervical hemisection are consistent with the pattern of recovery seen following a complete cervical hemisection where full recovery of performance of the unaffected side, with less recovery in the affected forelimb than hindlimb, was observed [51].

Forelimb and hindlimb sensorimotor behavior associated with spinothalamic tract function can be measured by withdrawal latency following a heat stimulus [51]. Following spinal injury, rats typically show decreased withdrawal latency that slowly recovers. In this study, rats showed partial recovery of right forelimb responses with no difference among groups (Fig. 4a). Partial recovery of left hindlimb response was observed in rats that had received MSC from donor 3 (Fig. 4b), the same donor whose MSC had elicited the least amount of axon outgrowth. This could be explained by decreased regeneration of sensory fibers, thereby reducing thermal allodynia, in the case of MSC grafts from donor 3 compared to the rats that received grafts from other donors. However, as we only measured the staining for neurofilaments, we could not differentiate between motor and sensory fibers. Uninjured rats show forelimb withdrawal latencies of 7–8 s and hindlimb withdrawal latencies of 8–9 s. By using these values, we calculated that rats in all groups demonstrated about 60% recovery with the right forelimb. Control rats and rats with transplants from donors 1, 2, and 4 showed about 60%

recovery with the left hindlimb, whereas rats that had received MSC from donor 3 showed about 75% recovery.

Correlation analysis was used to determine whether the amount of axon growth into the graft as determined by neurofilament staining predicted the behavioral results. No such correlation could be found for any of the behavioral tests.

### 3.3. Human MSC secrete therapeutic factors and support axon outgrowth *in vitro*

Therapeutic factors secreted by MSC may be part of the mechanism for axon outgrowth and recovery of function. To determine if the differences described above are due to secretion of different amounts of potential therapeutic factors by MSC from different donors, we screened MSC-conditioned medium from 4 donors for the presence of certain growth factors (BDNF and VEGF) and cytokines (IL-6, MCP-1, SCF and SDF-1a) using ELISA. All protein levels were normalized to pg/ml/day/ $10^6$  cells. As shown in Fig. 5, growth factor and cytokine concentrations in the medium differed widely between donors, even though cell purification protocols were identical. Human MSC expressed 335–640 pg/ml/day/ $10^6$  cells of VEGF and 65–105 pg/ml/day/ $10^6$  cells of BDNF. Protein levels of cytokines ranged from 315 to 735 pg/ml/day/ $10^6$  cells for IL-6, 60–280 pg/ml/day/ $10^6$  cells for MCP-1, 1–13 pg/ml/day/ $10^6$  cells for SCF, and 370–810 pg/ml/day/ $10^6$  cells for SDF-1a.

To examine if MSC-conditioned medium containing these factors had any effect on axon outgrowth, we incubated chick DRG explant cultures with unconditioned and conditioned MSC medium from 3 different donors for 2 days. Average axon lengths were measured, and mean and standard error were plotted (Fig. 6a). DRG explants incubated in unconditioned MSC medium had an average axon length of 338 ( $\pm 31$ )  $\mu\text{m}$ . Treatment of DRG explants

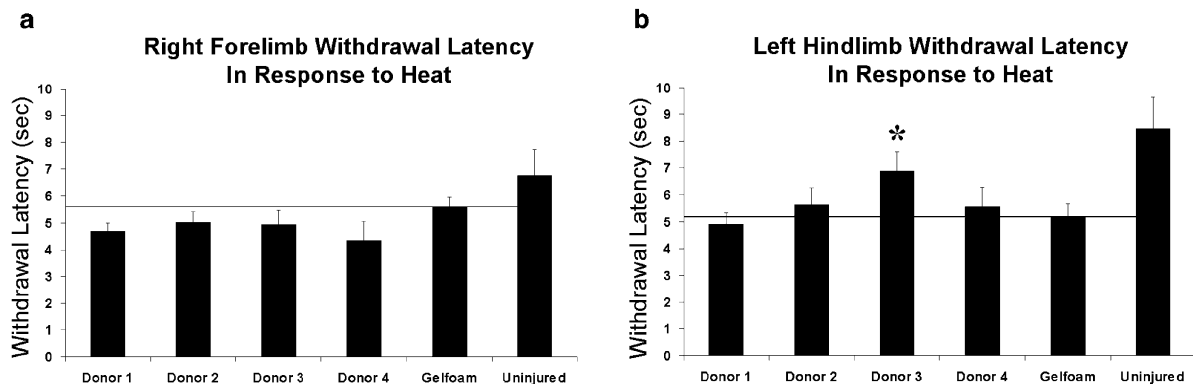


Fig. 4. Test for improvement in withdrawal latency after a heat stimulus. Latency for limb withdrawal in response to plantar paw contact with a heat stimulus was measured. Five trials were run for (a) the right forelimb and (b) the left hindlimb with a 15-min interval between each trial to prevent sensitization. The last 4 trials were averaged to provide the mean latency of withdrawal. (a) Rats showed partial recovery of right forelimb responses with no difference among groups. (b) Partial recovery of left hindlimb response was observed only in rats with MSC grafts from donor 3 compared to gelfoam controls (Student's *t* test,  $P < 0.10$ ).

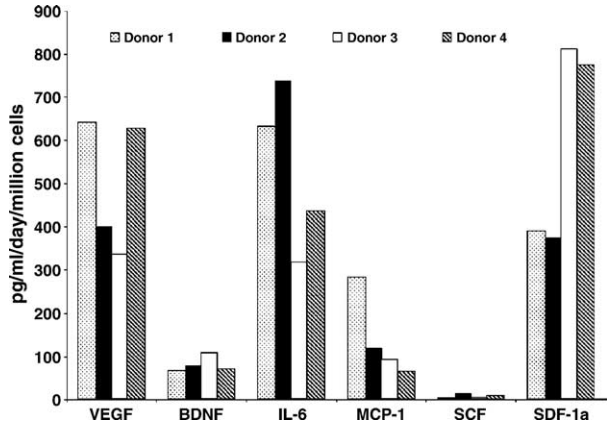


Fig. 5. Secretion of therapeutic factors by MSC. Secretion of trophic factors and cytokines is donor-dependent. Medium of MSC from four different donors was conditioned for three days and analyzed using ELISA. All cells were normalized to pg/ml/day/million cells. Secretion patterns of cells from different donors vary significantly.

with MSC-conditioned medium from all three donors significantly ( $P < 0.01$ ) increased average axon length compared to unconditioned control medium. Human MSC-conditioned medium from donor 1 resulted in a 2.3-fold increase in average axon length ( $835.42 \pm 66.93$ ), donor 2 MSC-conditioned medium resulted in a 2.6-fold increase in average axon length ( $958.33 \pm 32.18$ ), and donor 3 MSC-conditioned medium in a 3-fold increase in average axon length ( $1116.67 \pm 70.30$ ). Average axon lengths in DRG cultures treated with medium from donors 1 and 3 differed significantly ( $P < 0.01$ ) from each other.

We immunostained DRG explant cultures with antibodies against  $\beta$ III-tubulin to verify that processes were neuronal. Fig. 6b illustrates the increase in average axon length in DRG explant cultures treated with unconditioned or MSC-conditioned medium from all three donors. Interestingly, it appeared that axons extended from DRG

explants treated with MSC-conditioned medium from donor 3 were not only longer but also less fasciculated than axons from DRG explants treated with conditioned medium from other donors. This may have been due to a different composition of secreted factors that either stimulated different expression of surface molecules on axons or affected a different subtype of neurons.

#### 4. Discussion

In this study, we have demonstrated for the first time that axon growth and recovery of function in response to a human MSC graft in the injured rat spinal cord is donor-dependent. Examination of the secretion profile for certain growth factors and cytokines revealed major differences between four human MSC donors. While this secretion profile did not seem to greatly affect axon growth in vitro, axon outgrowth into MSC grafts in a subtotal cervical hemisection differed significantly depending on the cell donor. Behavioral tests also showed differences in the degree of recovery among donors. Interestingly, there was no direct correlation between the amount of in vivo axon growth supported by MSC from any specific donor and functional recovery, suggesting that additional conditions are likely to be necessary for stable functional recovery.

In our in vitro assay, MSC-conditioned medium from all three donors resulted in a significant increase in average axon length; however, there was no correlation with our in vivo results. In vitro, the effect of MSC-conditioned medium appeared to correspond to the relative amount of BDNF secreted by cells from different donors; however, amounts of BDNF in the conditioned medium were lower than BDNF concentrations typically used to support axon growth. In addition, we cannot exclude a potential effect of other neurotrophins, such as nerve growth factor or neurotrophin

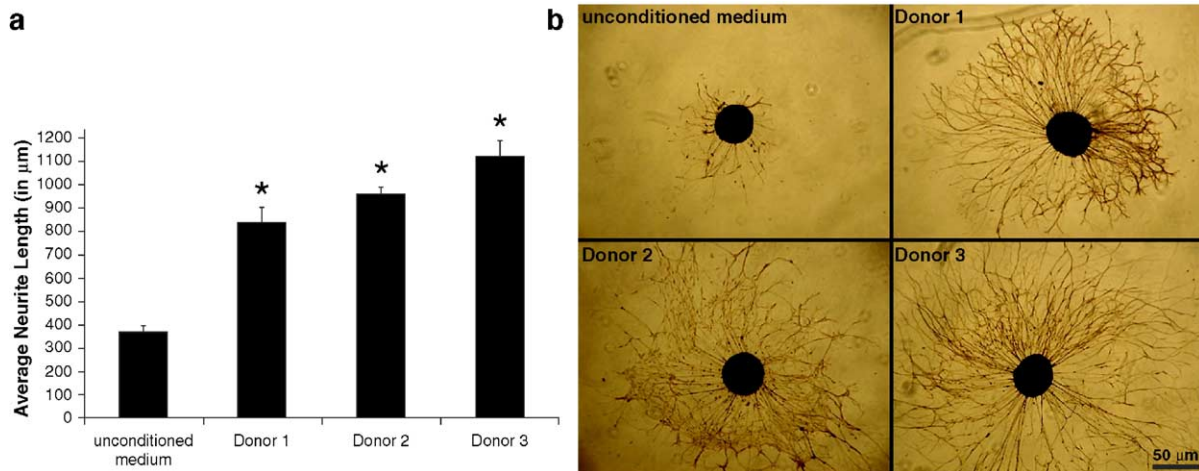


Fig. 6. Effect of MSC-conditioned medium on DRG axon length in vitro. Average axon length of DRG explants increases after treatment with MSC-conditioned medium. (a) DRG explant cultures were treated with unconditioned or MSC-conditioned medium for 48 h. Values represent average axon lengths  $\pm$  SEM (in  $\mu\text{m}$ ) of three DRG explants per condition obtained from two separate experiments. (b) Axon outgrowth from DRG explants treated with unconditioned medium or MSC-conditioned medium from three donors stained with antibodies against  $\beta$ III-tubulin.

3, which we did not evaluate in our ELISA screening. Neurotrophins including BDNF are known to stimulate axon outgrowth (for review, see [39]). Aside from secreted trophic factors, extracellular matrix molecules produced by MSC could also affect axon growth. These molecules were not present in the conditioned medium used for *in vitro* testing. *In vivo*, the presence of certain subsets of these molecules, which may be donor-dependent, could have an inhibitory effect on axon growth. Furthermore, factors secreted from astrocytes, oligodendrocytes, and immune cells may suppress the positive effects of BDNF or other neurotrophins secreted by MSC. In addition, other factors differentially secreted by MSC from different donors may alter host cell secretion to varying degrees, making the environment more or less hostile. It is also possible that factors released by the injured tissue affect the secretion profile of transplanted MSC as has been shown by Chen and colleagues [6], and that cells from different donors or lots may respond differently.

In this study, we evaluated axonal growth *in vivo* by neurofilament staining. This staining allows the visualization of processes but does not identify their source. It is likely that the RT-97-positive processes observed within the MSC graft are composed of CNS as well as dorsal root axons. Although we did not do tracing experiments in this study, in previous studies [35,37] we have shown that growth of CNS axons is supported by cellular grafts expressing neurotrophins, suggesting that at least part of the processes labeled with neurofilament are extended from CNS neurons.

To investigate if the amount of axonal growth *in vivo* correlated with functional recovery, we used different behavioral tests evaluating recovery of motor and sensory axons. However, the results obtained in these tests did not correlate with the amount of axon growth into the graft. MSC grafts release neurotrophins that can stimulate axon outgrowth; however, the stimulated outgrowth may not be associated with functionally relevant tracts (e.g., improvement in forelimb exploration in the cylinder is linked to regeneration of rubrospinal axons). Also, without activation of functionally relevant synaptic connections (e.g., stimulation through targeted exercise), axon outgrowth may not be directed toward the correct target. Considering the unstimulated release of neurotrophins by MSC grafts, it is not surprising that the amount of axon outgrowth does not correlate with behavioral recovery. Alternatively, compensatory reorganization of neural circuitry after injury may lead to synaptic reorganization to maintain function even without requiring sprouting or axon regeneration.

The modest recovery observed in motor and sensory tests indicates that the purification methods and transplantation protocols are still not optimal. We were unable to detect MSC in the injury site at the end of the study, suggesting that cell survival is limited. Poor graft survival could potentially limit the growth of axons because trophic factors would not be present for a sufficient amount of time. Also, after the disappearance of MSC, axons may lack a

permissive matrix through which to grow. Together, this may result in insufficient re-growth measured as functional recovery by behavioral testing techniques. We are currently testing improved immune suppression protocols to enhance MSC survival. Prolonged survival of MSC in the graft may result in improved behavioral recovery.

Previous studies have shown that transplantation of MSC into a contusion injury in the spinal cord promotes axon outgrowth and results in moderate recovery of function [9,23,60]; however, although several possible mechanisms have been proposed, the process by which MSC contribute to this improvement remains to be elucidated. Transdifferentiation of MSC into neural cells [30] is considered a controversial potential mechanism for recovery of function, as cell fusion or the presence of a subpopulation of pluripotent stem cells cannot be ruled out [21,25,34,53]. However, even if MSC were able to transdifferentiate, it is unlikely that the small number of MSC expressing neural markers would result in functional recovery even if the cells were integrated in neural circuitry. Aside from transdifferentiation, other potential mechanisms for the therapeutic effect of MSC depend on factors secreted by MSC or presented on the MSC surface. Co-culture of MSC with hippocampal slices results in reduced cell death after oxygen-glucose deprivation, suggesting that MSC are able to promote neuron survival [62]. Chen et al. [7] provided evidence that MSC transplantation into the infarcted brain leads to a reduction of cell death and an increase in cell proliferation. *In culture*, it has been shown that MSC, co-cultured with neural stem cells, preferentially induce neuronal differentiation [38,60]. Several growth factors released by MSC are known to affect functional recovery in CNS injury [55]. BDNF is important for the regeneration of damaged rubrospinal tract axons [35,36,56]. VEGF reduces apoptosis and promotes regeneration of the corticospinal tract [13,59]. Factors such as MCP-1 and SDF-1 $\alpha$  have been shown to be involved in the homing of MSC to injured tissue [2,58]. These factors may be important if less invasive techniques for cell delivery, like intra-thecal injection, are used. However, MSC also secrete factors, like IL-6 that may have a negative effect on recovery by increasing neutrophil and macrophage infiltration and inhibiting axonal growth [32]. MSC secrete various other cytokines that may modulate the host immune response in a positive or negative way. So far, it is agreed upon that the acute inflammation following SCI results in additional damage; however, studies have also shown a regenerative potential of certain immune cells [48]. The timing and nature of factors involved in protective as well as destructive signaling in immune cells after SCI are not yet well understood [46] so care needs to be taken in the evaluation of cytokines secreted by MSC. For an overall positive effect on recovery of function, neurotrophic factors and cytokines secreted by MSC need to be carefully balanced. Therefore, it seems logical that differences in the secretion patterns of MSC from different donors would result in different outcomes.

The promising results of previous studies [9,23,60] led to the suggestion that MSC are an ideal candidate for cell therapy in SCI. Indeed, MSC can be easily obtained from bone marrow and both expanded and stored in “universal donor” cell banks, or used for autologous transplantation. The latter possibility is exciting, as immune suppression and prevention of the host immune response would not be necessary. So far, transplantation studies have been done with MSC derived from a single donor (human or rodent), and the possibility of variation affecting axonal regeneration and efficacy of the cell transplant was not addressed. Our results suggest that such variations among donors exist, implying that not every patient’s MSC may be beneficial for autologous treatment of SCI. Similarly, MSC stored in cell banks will most likely not have identical properties.

A multitude of different factors could potentially influence variations among donors. Among these are age, gender, health status, and genetic background of donors. It is known that variations exist in the growth kinetics and the potential for osteogenic differentiation among different human donors and even within the same donor [44,50]. Similar variations have been shown for MSC derived from different strains of inbred mice [42,43]. These differences may be derived from variations in the composition of bone marrow aspirates at the time of harvest. Previous studies have also shown that yields from different bone marrow aspirates differ significantly in volume and cell composition [33,50]. However, no correlation could be made between age or gender and the above described differences. In addition, it has also been shown that proliferative activity and cell yield from human bone marrow varies with the circadian rhythm [52]. Other factors could also play a role, for instance, nutrition or stress level. As these factors are hard to control, it is essential to find specific parameters that allow quick and reliable selection of MSC with therapeutic potential.

We focused our study on variations originating in samples derived from different donors; however, it is possible that these and additional variations are due to differences in harvesting and culturing the cells. MSC consist of a heterogeneous population of cells, and culture without selection for well-defined cell types may reinforce already existing variations reflecting the properties of MSC derived from the bone marrow of different donors.

Experiments using selected subpopulations of MSC with different phenotypes will need to be conducted to determine the effects of specific parameters on recovery of function. This should lead to standardized protocols for the collection and processing of MSC that should then be strictly followed and accompanied by quality control assays as indeed required by FDA for clinical application. Additionally, use of optimized culturing protocols may result in better defined cell population and reduced variability. Future studies in our lab are aimed at defining such a protocol and characterizing MSC with proven efficacy using various parameters.

In summary, MSC for autologous or allogeneic transplantation will have to be tested for specific parameters to ensure their therapeutic potential; however, as of now, we do not know which factor(s) or factor combination(s) is/are essential. It is likely that more than one factor is involved in promoting recovery; however, it may be more complicated—factor combinations will have to be investigated and within these combinations, effects may depend on high or low expression of certain factors in relationship to others. It will be challenging to find the right parameters to determine therapeutic success, but having MSC with carefully defined secretion profiles, possibly genetically engineered to augment certain therapeutic effects, may bring us closer to a functional cell therapy for SCI.

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