Cellular and Molecular Neurobiology, Vol. 26, Nos. 7/8, October/November 2006 (© 2006)
DOI: 10.1007/s10571-006-9093-1

Transplants of Human Mesenchymal Stem Cells Improve Functional Recovery After Spinal Cord Injury in the Rat

Daša Čížková,[1,4] Ján Rosocha,[2] Ivo Vanický,[1] Stanislava Jergová,[1] and Milan Čížek[3]

Received December 6, 2005; accepted May 23, 2006; Published online: July 29, 2006

SUMMARY

Human mesenchymal stem cells (hMSCs) derived from adult bone marrow represent a potentially useful source of cells for cell replacement therapy after nervous tissue damage. They can be expanded in culture and reintroduced into patients as autografts or allografts with unique immunologic properties. The aim of the present study was to investigate (i) survival, migration, differentiation properties of hMSCs transplanted into non-immunosuppressed rats after spinal cord injury (SCI) and (ii) impact of hMSC transplantation on functional recovery. Seven days after SCI, rats received i.v. injection of hMSCs (2×10^6 in 0.5 mL DMEM) isolated from adult healthy donors. Functional recovery was assessed by Basso–Beattie–Bresnahan (BBB) score weekly for 28 days. Our results showed gradual improvement of locomotor function in transplanted rats with statistically significant differences at 21 and 28 days. Immunocytochemical analysis using human nuclei (NUMA) and BrdU antibodies confirmed survival and migration of hMSCs into the injury site. Transplanted cells were found to infiltrate mainly into the ventrolateral white matter tracts, spreading also to adjacent segments located rostro-caudaly to the injury epicenter. In double-stained preparations, hMSCs were found to differentiate into oligodendrocytes (APC), but not into cells expressing neuronal markers (NeuN). Accumulation of GAP-43 regrowing axons within damaged white matter tracts after transplantation was observed. Our findings indicate that hMSCs may facilitate recovery from spinal cord injury by remyelinating spared white matter tracts and/or by enhancing axonal growth. In addition, low immunogenicity of hMSCs was confirmed by survival of donor cells without immunosuppressive treatment.

KEY WORDS: mesenchymal stem cells; spinal cord trauma; cell therapy; regeneration.

INTRODUCTION

The experimental studies dealing with adult bone marrow (BM) derived mesenchymal stem/progenitor cells (MSCs) isolation, propagation, and differentiation have been considered very important and intriguing after having shown that these

[1] Institute of Neurobiology, Center of Excellence, SAS, Šoltésovej 4, Košice 04001, Slovakia.
[2] Associated Tissue Bank, Faculty of Medicine and University Hospital, P.J. Šafárik University, Trieda SNP 1, Košice 04011, Slovakia.
[3] UVM, Komenského 72, Košice, Slovakia.
[4] To whom correspondence should be addressed at Institute of Neurobiology, Center of Excellence, SAS, Šoltésovej 4, Košice 04001, Slovakia; e-mail: cizkova@saske.sk.

cells can be expanded ex vivo and give rise not only to the population of the mesenchymal lineage (cartilage, bone, adipocytes) (Pittenger *et al.*, 1999) but also differentiate into cells derived from other embryonic layers, probably due to their ability to lose their linage restriction (Krause *et al.*, 2001). Transplanted bone marrow derived stem cells may contribute to endothelium (Jackson *et al.*, 2001), skeletal muscles (Gussoni *et al.*, 1999), cardiomyocytes (Orlic, 2003), acquire properties of hepatocytes (Schwartz *et al.*, 2002), lung, gut, and skin epithelia (Krause *et al.*, 2001) as well as neuroectodermal cells (Sanchez-Ramos *et al.*, 2000; Mezey *et al.*, 2003). Furthermore, their pluripotency and ectodermal differentiation properties were described in recent studies, where BM derived multipotent adult progenitors (MPC) injected in the blastocyst or embryonic brain contributed to the majority of somatic cell lineages including brain tissue (Jiang *et al.*, 2002; Munoz-Elias *et al.*, 2004). The widespread use of stem cell therapy (Racekova *et al.*, 2000, 2003; Kim *et al.*, 2004) has shown that transplantation of MSCs can improve recovery after stroke (Chopp and Li, 2002), promote remyelinization (Akiyama *et al.*, 2002) as well as contribute to partial recovery of locomotor function in animal models of spinal cord injury (SCI) (Chopp *et al.*, 2000; Hofstetter *et al.*, 2002; Wu *et al.*, 2003; Zurita and Vaquero, 2004). While there is growing evidence that MSCs can give rise to cells with neural characteristics in vitro (Kim *et al.*, 2002) and in vivo (Chopp *et al.*, 2000; Jendelova *et al.*, 2004), it is still unclear whether neural differentiation or other mechanisms such as neurotrophic factors (Zhong *et al.*, 2003; Yang *et al.*, 2003), vascular factors secretion (Hamano *et al.*, 2000) or even immunomodulatory effects (Aggarwal and Pittenger, 2005) have a dominant influence on recovery of function following spinal cord trauma.

Therefore, in attempt to further understand the in vivo processes associated with proposed MSCs therapeutic value, we have investigated effectiveness of systemic hMSCs therapy in the model of spinal cord compression injury (SCI) (Vanicky *et al.*, 2001). Bone marrow derived mesenchymal cells obtained from human donors were intravenously injected into recipients without any additional immunosuppressive treatment. The improvement of functional recovery together with graft survival and differentiation capacity was evaluated by means of behavioral tests, immunocytochemical and quantitative analyses. Accordingly, the goal of the present study was to evaluate, whether systemically infused hMSCs can (i) improve functional recovery, (ii) home directly into the site of injury, (iii) differentiate into neural cells (neurons, glial cells), (iv) stimulate endogenous regeneration (axonal growth), (iv) reveal hypoimmunogenic nature and could be tolerated by rats for 3 weeks after transplantation without immunosuppression.

MATERIAL AND METHODS

Cell Culture and Labeling

hMSCs were obtained from bone marrow taken from the iliac crest of three adult human donors. Two females (25 years, 30 years) and one male donor (55 years) were used in present study. Each aspirate was diluted 1:1 with

Hanks' balanced salt solution (HBSS; GIBCO) and layered over 10 mL of Ficoll (Ficoll-Paque; Amersham Pharmacia). After centrifugation at $1500 \times g$ for 30 min, mononuclear cells were collected from the interface. Cells were suspended in Dulbecco's modified Eagle medium (DMEM), centrifuged at $1000 \times g$ for 10 min and resuspended in DMEM supplemented with 10% FBS and 1% of antibiotics (penicillin, streptomycin, amphotericin B). Number and viability of cells was estimated by Trypan blue staining. Cells were seeded in tissue culture flasks at a density of 40,000 viable cells/cm^2 and cultured at 37°C in humidified atmosphere with 5% of CO_2. On the day 4, the non-adherent cells were removed from the flasks and the adherent cells fed with fresh, complete medium with supplements. The adherent fibroblastoid-shaped cells were cultured and subsequently passaged four times until the final use. Seeding density for subculture of hMSC at all subsequent passages was approximately 5000 cells/cm^2. Cells were grown to confluency before each passage (3–4 days). Medium was exchanged every second day. Twenty-four hours prior to transplantation hMSC (undifferentiated passage 4) were incubated with DMEM containing 5 μM BrdU (5-bromo-2 deoxyuridine), which is an analogue of thymidine that incorporates into DNA during S phase of cell division (Sigma-Aldrich, St. Louis, MO). The following day, cells were washed several times with DMEM and re-suspended at 2×10^6 in 0.5 mL DMEM. Prepared cells were maintained at room temperature and gently re-suspended prior to transplantation. After surgery, a sample of remaining cells were-plated overnight to verify viability.

Spinal Cord Trauma

All experimental procedures were approved by the Institutional ethical committee for animal research and were in correlation with the Slovak Law for Animal protection (No. 115/1995). The spinal cord injury (SCI) was induced by modified balloon-compression technique in adult male Wistar rats ($n = 30$) weighting between 300 and 320 g, according to our previous study (Vanicky *et al.*, 2001). Briefly, animals were anesthetized with 2% halothane in room air in an induction box and were maintained with 1–1.5% halothane delivered by an inhalation mask. For a core temperature measurements, a thermal probe was inserted into the rectum. Afterwards, the shaved skin was cut and the paravertebral muscle overlying the L1-Th8 vertebrae were freed from the attachments and the soft tissue and corresponding spinous processes were removed. Using a dental drill a small hole was performed in vertebral arch of Th10 and a 2-French Fogarty catheter was inserted into the dorsal epidural space through this hole, aiming cranially to Th8-9 of the spinal level. After stabilization of the body temperature at 37°C, the balloon was inflated with 12.5 μL of saline for 5 min. Then, catheter was deflated, removed from epidural space, the soft tissue and skin were sutured in anatomical layers and finally animals were disconnected from anesthesia. All experimental rats were hydrated with cc 5 mL of Ringer's solution and were allowed to recover in warmed cages and kept in pairs during survival. Manual bladder expression was required for 7–14 days after injury, until bladder reflex was established. No antibiotic treatment was performed.

Transplantation of hMSCs

All SCI rats were randomly dividend into (1) transplantation group ($n = 15$). Approximately, 1×10^6 hMSCs in 0.5 mL DMEM were slowly injected during 5 min into a right femoral vein, in anesthetized rats at 7 days after SCI. The (2) control group ($n = 15$) received i.v. injection of DMEM similarly as transplanted group, but without hMSC. Immunosuppressive therapy was not used in any animals. All animals were intracardialy perfused at 28 days after SCI (21 days after transplantation).

Behavioral Testing of Motor Function (BBB Scoring)

Animals were evaluated before surgery and once a week after surgery for 3 weeks. Each rat was tested for 4 min by two blinded examiners. The motor performance was tested by using the Basso–Beattie–Bresnahan (BBB) 21-point open field locomotor scale. BBB scores, which categorize combinations of rat hindlimb movements, trunk position and stability, stepping, coordination, paw placement, toe clearance, and tail position, where 0 represents no locomotion and 21 normal motor function was analysed.

Immunohistochemistry

At the end of the survival (28 days after SCI), all animals were deeply anesthetized with a ketamine–xylozine cocktail (6 mg/kg of xylozine, 60 mg/kg of ketamine) and transcardially perfused with saline, followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PB). Spinal cords were removed, post-fixed in the same fixative overnight at 4°C and cryoprotected in PB containing 30% sucrose. Frozen spinal cords sections (10–40 μm thick) were taken from a 1.2-cm long spinal cord centered on the injury epicenter, embedded in tissue teck, dissected into three blocks (rostral, epicenter, caudal, each 0.4 cm thick) and stored at −20°C. Cryostat spinal cord sections cut from rostral and caudal regions were immersed in PB solutions (floating staining method); the sections cut from epicenter were mounted directly onto slides (Color Frost/Plus; Fisher, Pittsburgh, PA). Representative slides (sections were taken at 200 μm intervals, 20 sections/per each block), 10 sections were stained with hematoxylin-eosin to assess tissue morphology and determine the injury epicenter, 25 sections were used for quantitative analysis (NUMA/Dapi and BrdU/APC, described later), 10 sections performed for neuronal, glial markers and GAP-43 immunocytochemistry. Survival of transplanted hMSC was established either by BrdU or by human nuclear antibody (NUMA) that was followed by additional secondary labeling with primary antibodies determining neuronal or glial phenotype. For immunocytochemical detection of BrdU, DNA was denaturated to expose the antigen. Free floating spinal cord sections were incubated in 1 N HCl at 37°C for 30 min. Sections were subsequently rinsed for 10 min at 25°C in 0.1 M boric acid (pH 8.5), followed by appropriate rinsing in PBS, and incubation with primary antibody, rat anti-BrdU (1:300, Chemicon) for 24 h at 4°C. All blocking steps were performed in 1 × PBS/0.2% Triton-X (TX-100) 5% normal goat serum. Following 3 h, blocking sections were incubated with one of the following primary

antibodies: rat anti-BrdU (1:300, Chemicon), human specific nuclear antibody, the mitotic spindle protein NUMA (1:50; Chemicon), mouse anti-NeuN, rabbit anti-MAP2 (a markers for neurons; 1:1000; Calbiochem), rabbit anti-GFAP (a marker for astrocytes; 1:1000; Sigma), mouse anti-APC expressing adenomatus polyposis coli (a marker for oligodendroglial cells; 1:200; Calbiochem), rabbit anti-GAP-43 (a marker for axonal growth; 1:500; Chemicon) for 24–48 h at 4°C. This was followed by PBS/TX wash (3×5 min) and incubation either with goat anti-mouse–rabbit biotinylated secondary antibodies, followed by ABC elite Vector kit, and developed by DAB, or with secondary goat anti-mouse, anti-rabbit antibody conjugated to a fluorescent marker (Alexa 488 or 594, 1:300, Molecular Probes) for 1 h at room temperature (RT) and for general nuclear staining Dapi (1:400) was added to the final secondary antibody solutions. Control sections were processed to identical staining procedure, but the primary antibodies were omitted. After staining, sections were dried at RT and covered by Prolong—antifade mounting medium. Stained slides were analyzed using Olympus Light or Fluorescence microscope (Olympus BX 40) and captured by Olympus digital camera. Overlaid images were prepared with Photoshop 7.0 and digitally stored for further processing procedure. For quantification analysis, 15 NUMA immunostained and Dapi counterstained sections (percent of grafted cells), 10 sections identified by BrdU positivity and APC coexpression (percent of graft-differentiating oligodenroglia) were analyzed from each transplanted animal. Two identical fields of view from the ventral and lateral white matter taken at each section under an $\times 40$ objective were captured using digital color camera attached to the fluorescence microscope. The number of Dapi/NUMA positive nuclei and BrdU/APC cells were counted in the same field on each section, confirmed by overlaid images NUMA/Dapi and BrdU/APC with Photoshop software, afterwards processed for Image tool analyzing system (UTHSCSA, Image Tool, 3.0), to count positive nuclei. Data are presented as percentage of NUMA immunoreactive nuclei within the total Dapi positive cells or percentage of APC expressing cells within the total BrdU positive nuclei in the ventrolateral white matter.

Statistical Analysis

Behavioral data determined by BBB scores were averaged across hind limbs and differences between experimental groups were analyzed by using the Kruskall–Wallis and Mann–Whitney *U*-tests or one-way ANOVA tests. All data are presented as mean values $\pm$ SEM. Differences between groups were considered statistically significant if $P < 0.05$.

RESULTS

Cell Culture

Primary culture of hMSCs contained extra fraction of non-adherent small hematopoetic CD34+ cells (Fig. 1A and C), which were gradually depleted in further passages (Fig. 1D). Moreover, almost pure population of slightly elongated flat

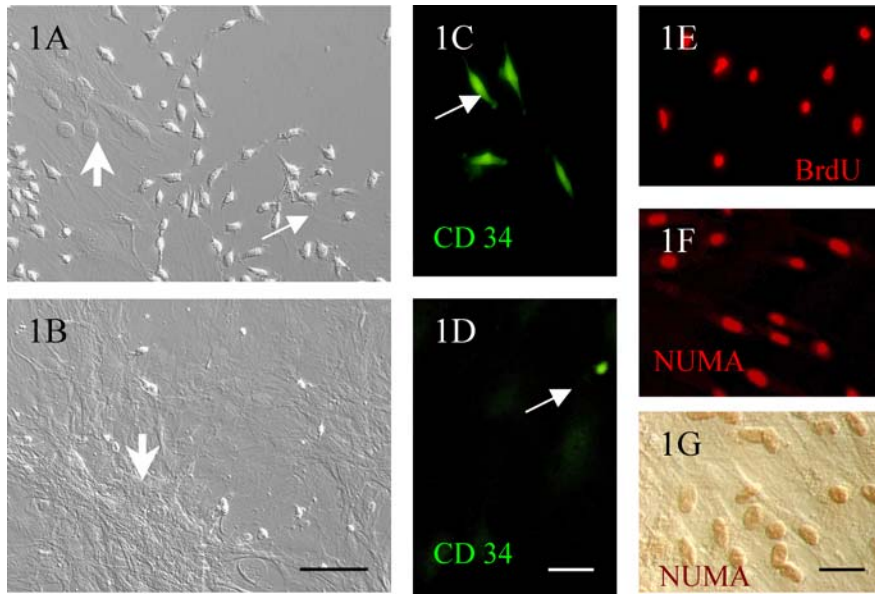

Fig. 1. hMSCs in the primary culture (A, C) and after cultivation at fourth passage (B, D–G). Primary culture contained heterogeneous population of flat adherent (thick arrow) (A) and small hematopoetic CD34+ cells (thin arrow) (C). Note, that almost pure population of slightly elongated flat fibroblastoid hMSCs appeared after fourth passage (thick arrow) with few residual CD34+ cells (thin arrow) (B, D). hMSCs immunoassayed with BrdU antibody indicating good quality of in vitro prelabeling and outlasting proliferation activity even at fourth passage (E). The hMSCs showed specific nuclei staining with human NUMA antibody by means of fluorescence and light immunocytochemical analysis (F, G). Nomarski objective (A, B, G). Scale bars: 100 μm, A, B; 50 μm, C–G.

fibroblastoid hMSCs appeared after four passage in DMEM with additional supplements (Fig. 1B and D). The multipotency and mesenchymal origin of these cells were determined by their adipogenic, chondrogenic, and osteogenic differentiations (data not shown). Prelabeling of hMSC with BrdU during 24 h showed $64 \pm 3\%$ of BrdU immunopositive cells, which indicate to their continuous proliferation activity at fourth passage (Fig. 1E). Immunocytochemical staining of hMSC with NUMA antibody confirmed specific nuclear staining using either secondary antibody for fluorescence or light microscopy (Fig. 1F and G).

Behavioral Testing

BBB Score 0–7 Days After SCI

Animals tested prior to surgery did not show any functional deficits, BBB score = 21. However, spinal cord injury caused in all rats flaccid hindlimb paralysis with BBB score = 0 at 24 h. At 1 week after injury, rats started to recover, showing slight isolate movements of one or two hindlimb joints with small variations among

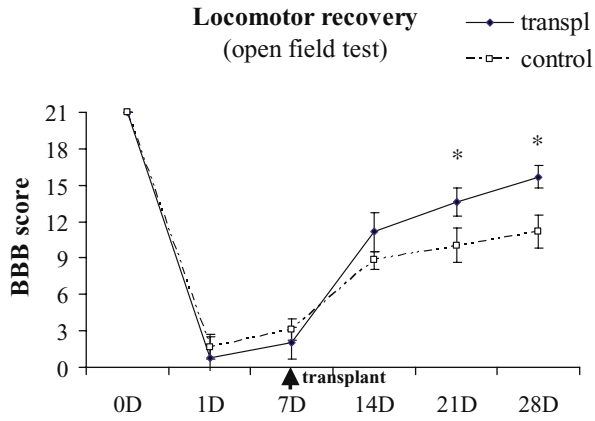

Fig. 2. Locomotor recovery of hindlimb function after SCI and hMSCs transplantation at 7 days evaluated in controls ($n = 15$) and transplants ($n = 15$) during 28 survival period. There were no significant differences of BBB scores between the groups at 14 days after injury ($P > 0.05$). Transplants reached significantly ($P < 0.05$) greater locomotor recovery at 21 and 28 days after SCI when compared with control group. The arrowhead on the abscissa indicates the time of transplantation. Data are presented as means ± SEM. Asterisk (*) denotes statistical differences ($P < 0.05$).

rats. At this stage, when forward movement was entirely associated with forelimb stepping, the rats were processed for transplantation (Fig. 2).

BBB Score 14–28 Days After SCI

A gradual recovery of hindlimb locomotion was seen over the following 2–3 weeks in all rats; however, the transplanted group ($n = 15$) reached significantly ($P < 0.05$) greater locomotor recovery at 21 and 28 days after SCI (BBB = 11.1 ± 1.5/14D, BBB = 13.6 ± 2.1/21D, BBB = 15.7 ± 1.5/28D) when compared with control group ($n = 15$) (BBB = 8.7 ± 0.7/14D, BBB = 10.1 ± 1.4/21D, BBB = 11.0 ± 1.3/28D) (Fig. 2). In transplants, mild behavioral differences (not significant, $P > 0.05$) could be seen at 14 days after SCI (Fig. 2). Rats begin to support their weight on the plantar surface of the paw during the stance and during the forward movement. After 21 days, they showed almost normal stepping when the frequency of plantar stepping took precedence over dorsal stepping. As the survival extended (28 days), the rats continued to improve also the fine details of locomotion such as paw rotation (during stance and lift) and toe clearance during stepping. However, some abnormalities in trunk stability and lack of the normal tail-up position persisted in all rats. On the contrary, the controls showed lower degree of locomotor recovery, characterized by extensive movement of all three hindlimb joints followed by sweeping movement with prominent dorsal stepping at 14 days. Afterwards, rats begin to support their weight and showed occasional steps associated

with no coordination at 21 days. This locomotor pattern remained uniform and only slight movement improvement showing frequent-consistent steps with no coordination occurred at 28 days (Fig. 2).

Immunocytochemistry

Distribution, Survival, and Quantification of Transplanted hMSCs

Transverse spinal cord sections cut from each spinal cord block (rostral, epicenter, caudal) contained donor cells expressing NUMA antibodies, that were infiltrating the injury site and migrating up to 3 mm rostrocaudally to the injury epicenter. The majority of transplanted NUMA positive nuclei could be found in the spared ventral and lateral white matter funiculi and around the newly formed cavities (Fig. 3A–C). Quantitative analyses revealed $3.95 \pm 0.7\%$ of NUMA positive cells calculated from total Dapi counterstained cells taken from identical fields of ventrolateral white matter (Fig. 3D–F).

Neural Differentiation of hMSCs

Spinal cord sections double labeled with BrdU and NeuN antibody showed that hMSCs cells found in white and gray matter did not express NeuN antibody (Fig. 4A and B) and rare NUMA/MAP2 immunoreactive cells in the ventral horn or in the lamina VII could be seen (Fig. 4D and F). Double labeling of the adjacent sections revealed absence of colocalization with NUMA/GFAP antibody (Fig. 5A), suggesting that astrocytes detected in the grafted spinal cord were host-derived. However, in the ventrolateral white matter tracts, $2 \pm 0.4\%$ of BrdU positive cells expressing APC immunoreactivity were observed, indicating that fraction of transplanted cells differentiated into oligodendrocytes (Fig. 5B–D). Furthermore, increased GAP-43 immunoreactivity outlining regrowing axons within damaged dorsal and lateral white matter tracts after transplantation occurred (Fig. 6D and C). Dense network of GAP-43 immunoreactive axons of different thickness oriented in various directions were present in both cranial and caudal segment close (2 mm) to the lesion epicenter (Fig. 6D). In contrast, the sections taken from control rats revealed only moderate GAP-43 immunoreactivity, particularly in the gray matter regions (Fig. 6A and B).

DISCUSSION

Homing of hMSCs

Previous studies have shown migration of MSCs to the site of injury following various pathological events as bone fracture, myocardial infarction, or ischemic cerebral injury (Shake *et al.*, 2002; Wang *et al.*, 2002). Similarly, in the present study, intravenously infused hMSCs were migrating to the site of spinal cord lesion. Although, the mechanisms that guide homing of implanted cells following SCI remain

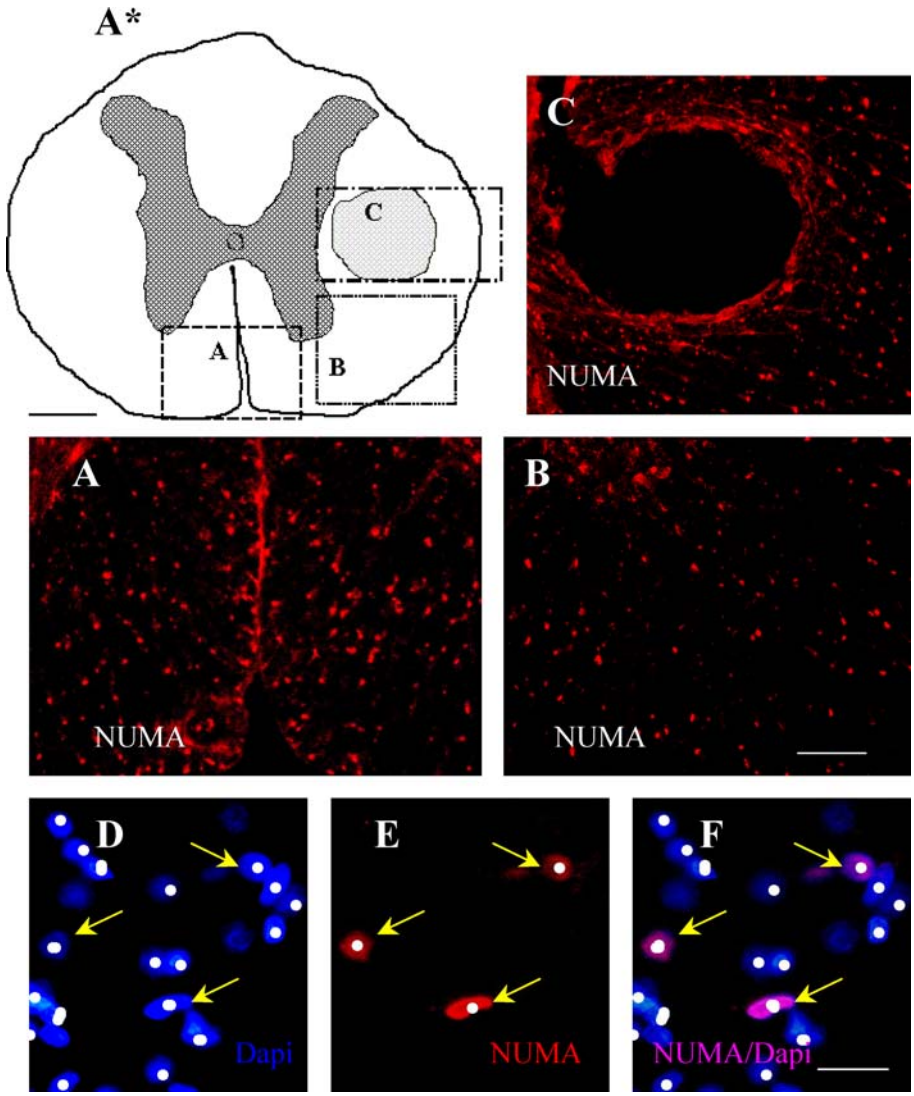

Fig. 3. Expression of NUMA antibody in the coronal section of thoracic spinal cord (Th8) transplanted with hMSC. The majority of NUMA cells occurred in the ventral (A), lateral (B) white matter tracts, or occupied newly formed cavities (C). Boxed areas are taken from schematic spinal cord transversal section diagram A*. The percent of NUMA (E) positive cells were calculated from total Dapi counterstained cells (D) taken from identical fields of ventro-lateral white matter, and coloclized with NUMA positivity (F). Scale bars: 500 μm, A*; 100 μm, A–C; 25 μm, D–F.

unclear, the release of inflammatory chemotactic agents by damaged tissue, or expression of specific integrins by MSCs may play a crucial role in these processes (Liechty *et al.*, 2000). In addition, there is growing evidence that factors as monocyte chemoattractant protein-1 (MCP-1) (Wang *et al.*, 2002) and SDF-1α (Askari *et al.*, 2003) synthesized after cerebral or myocardial ischemia promote homing of

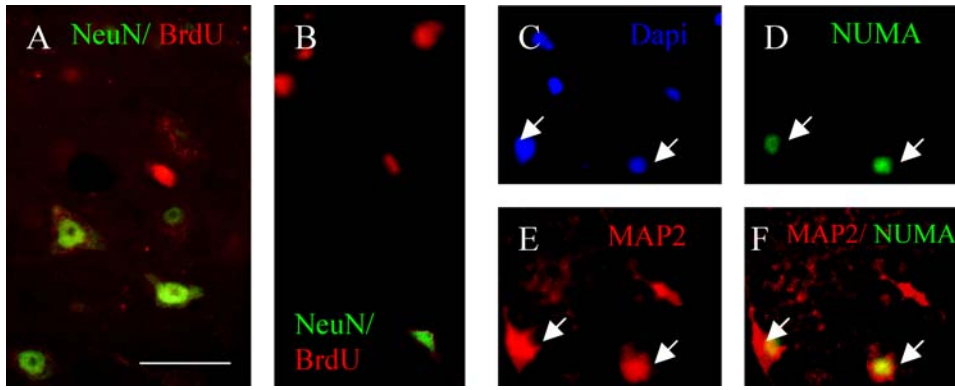

Fig. 4. NeuN (A, B) and MAP2 (E, F) staining of coronal thoracic spinal cord section taken from transplanted rats. Note, BrdU prelabeled hMSCs (*red*) distributed in the ventral (A) and dorsal horn (B) did not reveal NeuN expression (*green*). NUMA immunoreactive transplants (*green*, D) colocalized with MAP2 positivity in the lamina VII (F). Dapi counterstained nuclei in the identical sections (C). Scale bars 50 μm, A–F.

MSCs to the injury site. The present findings correlate with suggested mechanisms of MSCs migration following SCI (Liu *et al.*, 2005; Perrin *et al.*, 2005), but further studies defining intrinsic signals should be proposed.

Neural Differentiation and Functional Recovery

To investigate whether differentiation of transplanted hMSCs in vivo correlate with functional recovery, we have evaluated expression of specific neural markers depicting donor cell phenotype lineage development. Transplanted hMSCs were found to differentiate into oligodendrocytes (APC), but not into cells expressing

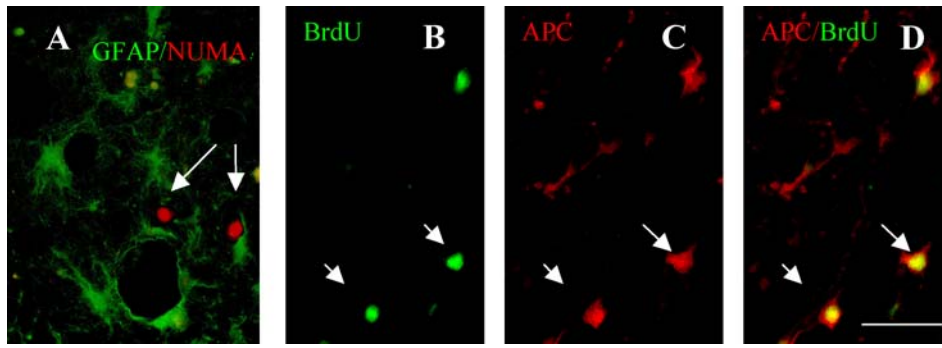

Fig. 5. Expression of glial markers identified by GFAP (A) and APC (C, D) immunoreactivity in transplants. NUMA expressing hMSCs (*arrows pointing to red nuclei*) did not colocalized with GFAP (*green*) positivity (A). Double labeling of the sections with BrdU (B, *green*) and APC antibody (C, *red*) revealed that some grafted cells become oligodendrocytes (D). Scale bars 50 μm, A–D.

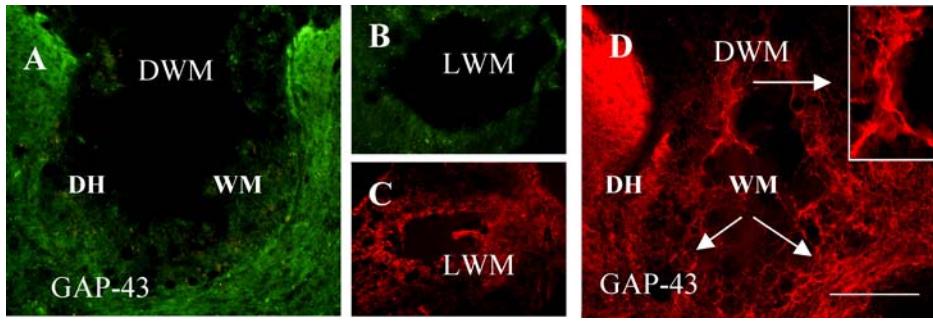

Fig. 6. Distribution of GAP-43 positivity in coronal sections of controls (A, B) and transplants (C, D). Note, increased GAP-43 immunorectivity outlining regrowing axons within damaged dorsal (D) and lateral (C) white matter tracts after transplantation, but only moderate GAP-43 positivity in control rats subjected to trauma (A, B). Scale bars 100 μm, A, D; 50 μm, B, C.

neuronal markers (NeuN). This indicates that hMSCs may facilitate recovery from spinal cord injury by remyelinating spared white matter tracts (Akiyama *et al.*, 2002) and not by neuronal differentiation as documented by other studies (Chopp *et al.*, 2000). It should be noted that only 2 $\pm$ 0.4% of grafted hMSCs distinguished by BrdU/APC immunoreactivity were oligodendrocytes, while many of them remained undifferentiated. Thus, the functional recovery observed in present study may be mediated by other processes such as neurotropic factors, chemokines, and cytokines released by MSCs (Chen *et al.*, 2002; Chopp and Li, 2002). Particularly, production of neurotrophic factors (NGF, BDNF), vascular endothelial growth factor (VEGF) and in part chemokines (Chen *et al.*, 2003) create a permissive environment not only for axonal growth but also for appropriate axonal guidance within damaged spinal cord (Li *et al.*, 2002). Exogenous delivery of bone marrow derived endothelial progenitors (EPCs) or their endogenous stimulation by VEGF or granulocyte-macrophage colony-stimulating factor (GM-CSF) has been shown to enhance angiogenesis and promote functional recovery in stroke (Willing *et al.*, 2003). In addition, MSC may facilitate recovery from spinal cord lesion by releasing brain natriuretic peptide (BNP) and other vasoactive factors that reduce edema and decrease vascular pressure (Song *et al.*, 2004; Mechirova and Domorakova, 2002). Furthermore, up-regulation of neuroprotective factors that are probably driven by transplanted MSCs may support greater axonal outgrowth, known to be associated with host plasticity. Considerable enhancement of GAP-43 positive axons distribution at the lesion site due to MSCs transplantation was seen in present study. The correlation between increased GAP-43 expression and axonal growth and sprouting (Cizkova *et al.*, 1997) has made GAP-43 a commonly used indicator of neuronal growth propensity. However, whether activated endogenous outgrowth is associated with regeneration of functionally relevant tracts (e.g. rubrospinal, corticospinal) depends on the appropriate orientation of GAP-43 axons that should be directed towards the correct target (Neuhuber *et al.*, 2005). Therefore, the majority of outgrowing axons that could acquire relevant synaptic connections

should be oriented in longitudinally directed manner to that of spinal cord (Ankeny *et al.*, 2004), which should be considered in our further studies.

Immunogenic Activity

In present study, we were able to detect transplanted hMSC at the end of survival period, even though we have eliminated immunosuppressive treatment. This may correlate with suggested hypoimmunologic nature of MSCs, which is explained by the absence of HLA Class II expression, low expression of co-stimulatory molecules, leading to inhibition of naïve and memory T-cell responses as well as B lyphocytes attenuation for antibody production (Di Nicola *et al.*, 2002; Tse *et al.*, 2003). These findings imply for unique MSCs immunomodulatory approaches, that could be used for immunosuppression to induce allotransplantation tolerance or even to attenuate autoimmune, inflammatory responses (Le Blanc and Ringden, 2005). Furthermore, immunomodulatory effects of MSC (fetal, adult) were documented in many in vitro and in vivo animal studies (Irons *et al.*, 2004; Mansilla *et al.*, 2005), but the interspecies differences should be considered as well. Since, we did not evaluate the immunogenic activity of hMSCs, it is unknown from present results which processes could account for the survival of the transplanted cells.

Although some experimental studies in animals or pre-clinical human studies demonstrate the effectiveness and safety of MSCs therapy there are still many questions to be answered regarding the mechanisms of engraftment, homing, inter-cellular interactions, immunological profiles in vivo differentiation as well as long-term safety. Together, these studies support further investigation of MSCs as a potential SCI repair strategy.

ACKNOWLEDGMENTS

This work was supported by APVV 51-002105, VEGA 2/5136/25, STAA 51-011-604. We express our thanks to Marika Špontáková for her great assistance in the immunocytochemical analysis as well as to Monika Ragáliová and Ivana Kupsáková for their helpful advice in cell culture preparation.

REFERENCES

Aggarwal, S., and Pittenger, M. F. (2005). Human mesenchymal stem cells modulate allogeneic immune cell responses. *Blood* **105:**1815–1822.

Akiyama, Y., Radtke, C., Honmou, O., and Kocsis, J. D. (2002). Remyelination of the spinal cord following intravenous delivery of bone marrow cells. *Glia* **39:**229–236.

Ankeny, D. P., McTigue, D. M., and Jakeman, L. B. (2004). Bone marrow transplants provide tissue protection and directional guidance for axons after contusive spinal cord injury in rats. *Exp. Neurol.* **190:**17–31.

Askari, A. T., Unzek, S., Popovic, Z. B., Goldman, C. K., Forudi, F., Kiedrowski, M., Rovner, A., Ellis, S. G., Thomas, J. D., DiCorleto, P. E., Topol, E. J., and Penn, M. S. (2003). Effect of stromal-cell-derived factor 1 on stem-cell homing and tissue regeneration in ischaemic cardiomyopathy. *Lancet* **362:**697–703.

Cizkova, D., Racekova, E., and Vanicky, I. (1997). The expression of B-50/GAP-43 and GFAP after bilateral olfactory bulbectomy in rats. *Physiol. Res.* **46:**487–495.

Di Nicola, M., Carlo-Stella, C., Magni, M., Milanesi, M., Longoni, P. D., Matteucci, P., Grisanti, S., and Gianni, A. M. (2002). Human bone marrow stromal cells suppress T-lymphocyte proliferation induced by cellular or nonspecific mitogenic stimuli. *Blood* **99:**3838–3843.

Gussoni, E., Soneoka, Y., Strickland, C. D., Buzney, E. A., Khan, M. K., Flint, A. F., Kunkel, L. M., and Mulligan, R. C. (1999). Dystrophin expression in the mdx mouse restored by stem cell transplantation. *Nature* **401:**390–394.

Hamano, K., Li, T. S., Kobayashi, T., Kobayashi, S., Matsuzaki, M., and Esato, K. (2000). Angiogenesis induced by the implantation of self-bone marrow cells: A new material for therapeutic angiogenesis. *Cell Transplant.* **9:**439–443.

Hofstetter, C. P., Schwarz, E. J., Hess, D., Widenfalk, J., El Manira, A., Prockop, D. J., and Olson, L. (2002). Marrow stromal cells form guiding strands in the injured spinal cord and promote recovery. *Proc. Natl. Acad. Sci. U.S.A.* **99:**2199–2204.

Chen, J., Zhang, Z. G., Li, Y., Wang, L., Xu, Y. X., Gautam, S. C., Lu, M., Zhu, Z., and Chopp, M. (2003). Intravenous administration of human bone marrow stromal cells induces angiogenesis in the ischemic boundary zone after stroke in rats. *Circ. Res.* **92:**692–699.

Chen, X., Li, Y., Wang, L., Katakowski, M., Zhang, L., Chen, J., Xu, Y., Gautam, S. C., and Chopp, M. (2002). Ischemic rat brain extracts induce human marrow stromal cell growth factor production. *Neuropathology* **22:**275–279.

Chopp, M., and Li, Y. (2002). Treatment of neural injury with marrow stromal cells. *Lancet Neurol.* **1:**92–100.

Chopp, M., Zhang, X. H., Li, Y., Wang, L., Chen, J., Lu, D., Lu, M., and Rosenblum, M. (2000). Spinal cord injury in rat: treatment with bone marrow stromal cell transplantation. *Neuroreport* **11:**3001–3005.

Irons, H., Lind, J. G., Wakade, C. G., Yu, G., Hadman, M., Carroll, J., Hess, D. C., and Borlongan, C. V. (2004). Intracerebral xenotransplantation of GFP mouse bone marrow stromal cells in intact and stroke rat brain: Graft survival and immunologic response. *Cell Transplant.* **13:**283–294.

Jackson, K. A., Majka, S. M., Wang, H., Pocius, J., Hartley, C. J., Majesky, M. W., Entman, M. L., Michael, L. H., Hirschi, K. K., and Goodell, M. A. (2001). Regeneration of ischemic cardiac muscle and vascular endothelium by adult stem cells. *J. Clin. Invest.* **107:**1395–1402.

Jendelova, P., Herynek, V., Urdzikova, L., Glogarova, K., Kroupova, J., Andersson, B., Bryja, V., Burian, M., Hajek, M., and Sykova, E. (2004). Magnetic resonance tracking of transplanted bone marrow and embryonic stem cells labeled by iron oxide nanoparticles in rat brain and spinal cord. *J. Neurosci. Res.* **76:**232–243.

Jiang, Y., Jahagirdar, B. N., Reinhardt, R. L., Schwartz, R. E., Keene, C. D., Ortiz-Gonzalez, X. R., Reyes, M., Lenvik, T., Lund, T., Blackstad, M., Du, J., Aldrich, S., Lisberg, A., Low, W. C., Largaespada, D. A., and Verfaillie, C. M. (2002). Pluripotency of mesenchymal stem cells derived from adult marrow. *Nature* **418:**41–49.

Kim, B. J., Seo, J. H., Bubien, J. K., and Oh, Y. S. (2002). Differentiation of adult bone marrow stem cells into neuroprogenitor cells *in vitro*. *Neuroreport* **13:**1185–1188.

Kim, S., Yoon, Y. S., Kim, J. W., Jung, M., Kim, S. U., Lee, Y. D., and Suh-Kim, H. (2004). Neurogenin1 is sufficient to induce neuronal differentiation of embryonal carcinoma P19 cells in the absence of retinoic acid. *Cell Mol Neurobiol.* **24:**343–356.

Krause, D. S., Theise, N. D., Collector, M. I., Henegariu, O., Hwang, S., Gardner, R., Neutzel, S., and Sharkis, S. J. (2001). Multi-organ, multi-lineage engraftment by a single bone marrow-derived stem cell. *Cell* **105:**369–377.

Le Blanc, K., and Ringden, O. (2005). Immunobiology of human mesenchymal stem cells and future use in hematopoietic stem cell transplantation. *Biol. Blood Marrow Transplant.* **11:**321–334.

Li, Y., Chen, J., Chen, X. G., Wang, L., Gautam, S. C., Xu, Y. X., Katakowski, M., Zhang, L. J., Lu, M., Janakiraman, N., and Chopp, M. (2002). Human marrow stromal cell therapy for stroke in rat: Neurotrophins and functional recovery. *Neurology* **59:**514–523.

Liechty, K. W., MacKenzie, T. C., Shaaban, A. F., Radu, A., Moseley, A. M., Deans, R., Marshak, D. R., and Flake, A. W. (2000). Human mesenchymal stem cells engraft and demonstrate site-specific differentiation after in utero transplantation in sheep. *Nat. Med.* **6:**1282–1286.

Liu, S. Q., Ma, Y. G., Peng, H., and Fan, L. (2005). Monocyte chemoattractant protein-1 level in serum of patients with acute spinal cord injury. *Chin. J. Traumatol.* **8:**216–219.

Mansilla, E., Marin, G. H., Sturla, F., Drago, H. E., Gil, M. A., Salas, E., Gardiner, M. C., Piccinelli, G., Bossi, S., Petrelli, L., Iorio, G., Ramos, C. A., and Soratti, C. (2005). Human mesenchymal stem cells are tolerized by mice and improve skin and spinal cord injuries. *Transplant. Proc.* **37:**292–294.

Mechirova, E., and Domorakova, I. (2002). NADPH-diaphorase activity in the spinal cord after ischemic injury and the effects of pretreatment with Ginkgo biloba extract (EGb 761). *Acta Histochem.* **104:**427–430.

Mezey, E., Key, S., Vogelsang, G., Szalayova, I., Lange, G. D., and Crain, B. (2003). Transplanted bone marrow generates new neurons in human brains. *Proc. Natl. Acad. Sci. U.S.A.* **100:**1364–1369.

Munoz-Elias, G., Marcus, A. J., Coyne, T. M., Woodbury, D., and Black, I. B. (2004). Adult bone marrow stromal cells in the embryonic brain: engraftment, migration, differentiation, and long-term survival. *J. Neurosci.* **24:**4585–4595.

Neuhuber, B., Timothy Himes, B., Shumsky, J. S., Gallo, G., and Fischer, I. (2005). Axon growth and recovery of function supported by human bone marrow stromal cells in the injured spinal cord exhibit donor variations. *Brain Res.* **1035:**73–85.

Orlic, D. (2003). Adult bone marrow stem cells regenerate myocardium in ischemic heart disease. *Ann. N. Y. Acad. Sci.* **996:**152–157.

Perrin, F. E., Lacroix, S., Aviles-Trigueros, M., and David, S. (2005). Involvement of monocyte chemoattractant protein-1, macrophage inflammatory protein-1alpha and interleukin-1beta in Wallerian degeneration. *Brain* **128:**854–866.

Pittenger, M. F., Mackay, A. M., Beck, S. C., Jaiswal, R. K., Douglas, R., Mosca, J. D., Moorman, M. A., Simonetti, D. W., Craig, S., and Marshak, D. R. (1999). Multilineage potential of adult human mesenchymal stem cells. *Science* **284:**143–147.

Racekova, E., Fercakova, A., and Orendacova, J. (2000). Neural stem cells: Possibilities of regeneration in the adult CNS. *Bratisl. Lek. Listy.* **101:**450–454.

Racekova, E., Orendacova, J., Martoncikova, M., and Vanicky, I. (2003). NADPH-diaphorase positivity in the rostral migratory stream of the developing rat. *Brain Res. Dev. Brain Res.* **146:**131–134.

Sanchez-Ramos, J., Song, S., Cardozo-Pelaez, F., Hazzi, C., Stedeford, T., Willing, A., Freeman, T. B., Saporta, S., Janssen, W., Patel, N., Cooper, D. R., and Sanberg, P. R. (2000). Adult bone marrow stromal cells differentiate into neural cells in vitro. *Exp. Neurol.* **164:**247–256.

Schwartz, R. E., Reyes, M., Koodie, L., Jiang, Y., Blackstad, M., Lund, T., Lenvik, T., Johnson, S., Hu, W. S., and Verfaillie, C. M. (2002). Multipotent adult progenitor cells from bone marrow differentiate into functional hepatocyte-like cells. *J. Clin. Invest.* **109:**1291–1302.

Shake, J. G., Gruber, P. J., Baumgartner, W. A., Senechal, G., Meyers, J., Redmond, J. M., Pittenger, M. F., and Martin, B. J. (2002). Mesenchymal stem cell implantation in a swine myocardial infarct model: Engraftment and functional effects. *Ann. Thorac. Surg.* **73:**1919–1926.

Song, S., Kamath, S., Mosquera, D., Zigova, T., Sanberg, P., Vesely, D. L., and Sanchez-Ramos, J. (2004). Expression of brain natriuretic peptide by human bone marrow stromal cells. *Exp. Neurol.* **185:**191–197.

Tse, W. T., Pendleton, J. D., Beyer, W. M., Egalka, M. C., and Guinan, E. C. (2003). Suppression of allogeneic T-cell proliferation by human marrow stromal cells: Implications in transplantation. *Transplantation* **75:**389–397.

Vanicky, I., Urdzikova, L., Saganova, K., Cizkova, D., and Galik, J. (2001). A simple and reproducible model of spinal cord injury induced by epidural balloon inflation in the rat. *J. Neurotrauma* **18:**1399–1407.

Wang, L., Li, Y., Chen, X., Chen, J., Gautam, S. C., Xu, Y., and Chopp, M. (2002). MCP-1, MIP-1, IL-8 and ischemic cerebral tissue enhance human bone marrow stromal cell migration in interface culture. *Hematology* **7:**113–117.

Willing, A. E., Vendrame, M., Mallery, J., Cassady, C. J., Davis, C. D., Sanchez-Ramos, J., and Sanberg, P. R. (2003). Mobilized peripheral blood cells administered intravenously produce functional recovery in stroke. *Cell Transplant.* **12:**449–454.

Wu, S., Suzuki, Y., Ejiri, Y., Noda, T., Bai, H., Kitada, M., Kataoka, K., Ohta, M., Chou, H., and Ide, C. (2003). Bone marrow stromal cells enhance differentiation of cocultured neurosphere cells and promote regeneration of injured spinal cord. *J. Neurosci. Res.* **72:**343–351.

Yang, M., Donaldson, A. E., Jiang, Y., and Iacovitti, L. (2003). Factors influencing the differentiation of dopaminergic traits in transplanted neural stem cells. *Cell. Mol. Neurobiol.* **23:**851–864.

Zhong, C., Qin, Z., Zhong, C. J., Wang, Y., and Shen, X. Y. (2003). Neuroprotective effects of bone marrow stromal cells on rat organotypic hippocampal slice culture model of cerebral ischemia. *Neurosci. Lett.* **342:**93–96.

Zurita, M., and Vaquero, J. (2004). Functional recovery in chronic paraplegia after bone marrow stromal cells transplantation. *Neuroreport* **15:**1105–1108.