Protective effects of bone marrow stromal cell transplantation in injured rodent brain: Synthesis of neurotrophic factors

ARTICLE *in* JOURNAL OF NEUROSCIENCE RESEARCH · JUNE 2005

Impact Factor: 2.59 · DOI: 10.1002/jnr.20494 · Source: PubMed

8 AUTHORS, INCLUDING:

Qin Chen
Baylor College of Medicine
7 PUBLICATIONS **247** CITATIONS

SEE PROFILE

Regino Perez-Polo
University of Texas Medical Branch at Galve…
86 PUBLICATIONS **1,952** CITATIONS

SEE PROFILE

Journal of Neuroscience Research 80:611–619 (2005)

Protective Effects of Bone Marrow Stromal Cell Transplantation in Injured Rodent Brain: Synthesis of Neurotrophic Factors

Qin Chen,[1,2,6]★† Yan Long,[1,2†] Xiaoqing Yuan,[1,2] LingLong Zou,[1,2] Jiao Sun,[1,2] Shengdi Chen,[6] J. Regino Perez-Polo,[5] and Keyi Yang[1–4]

[1]Center for Cell and Gene Therapy, Baylor College of Medicine, Houston, Texas
[2]Department of Neurosurgery, Baylor College of Medicine, Houston, Texas
[3]Department of Neurology, Baylor College of Medicine, Houston, Texas
[4]PADRECC, Neurology Care Line, VA Medical Center, Houston, Texas
[5]Department of Human Biological Chemistry and Genetics, University of Texas Medical Branch, Galveston, Texas
[6]Department of Neurology, Ruijin Hospital, Shanghai Second Medical University, Shanghai, P.R. China

Several groups have suggested that transplantation of marrow stromal cells (MSCs) promotes functional recovery in animal models of brain trauma. Recent studies indicate that tissue replacement by this method may not be the main source of therapeutic benefit, as transplanted MSCs have only limited ability to replace injured central nervous system (CNS) tissue. To gain insight into the mechanisms responsible for such effects, we systematically investigated the therapeutic potential of MSCs for treatment of brain injury. Using in vitro studies, we detected the synthesis of various growth factors, including nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), glial cell line-derived neurotrophic factor (GDNF), and neurotrophin-3 (NT-3). Enzyme-linked immunosorbent assay (ELISA) demonstrated that MSCs cultured in Dulbecco's modified Eagle medium (DMEM) produced substantial amounts of NGF for at least 7 weeks, whereas the levels of BDNF, GDNF and NT-3 remained unchanged. In studies in mice, after intraventricular injection of MSCs, NGF levels were increased significantly in cerebrospinal fluid by ELISA, confirming our cell culture results. Further studies showed that treatment of traumatic brain injury with MSCs could attenuate the loss of cholinergic neuronal immunostaining in the medial septum of mice. These studies demonstrate for the first time that by increasing the brain concentration of NGF, intraventricularly transplanted MSCs might play an important role in the treatment of traumatic brain injury.

Key words: marrow stromal cells; traumatic brain injury; nerve growth factor; transplantation; neurotrophic factor

Transplantation of bone marrow (BM) stromal cells has the potential to improve therapeutic strategies for neurologic injury and degenerative diseases of the brain. The number of donor cells engrafting in the brain, as well as functional recovery, is greater after marrow stromal cell (MSC) transplantation compared to that with whole BM transplantation (Mahmood et al., 2002). Many investigators have reported that grafting MSCs into the central nervous system (CNS) significantly promotes neuronal recovery in animal models of trauma (Lu et al., 2001a; Mahmood et al., 2001a), ischemia (Chen et al., 2000; Hess et al., 2002), and other neurologic disorders (Ader et al., 2001). The mechanisms by which transplanted MSCs provide therapeutic benefit, however, remain unknown. Several independent groups have suggested that engrafted MSCs can differentiate into neurons (Jin et al., 2003) and macroglia in vitro (Sanchez-Ramos et al., 2000) and in vivo (Lu et al., 2001b; Mezey et al., 2003; Weimann et al., 2003) and can significantly reduce motor and neurologic deficits (Lu et al., 2001a). Nonetheless, engrafted MSCs alone do not seem sufficient to replenish damaged neurons and repair brain injury through transdifferentiation. More recently, the physiologic relevance of "bone-to-brain" transdifferentiation has come under close scrutiny (Wagers et al., 2002). In rodents, after BM cell transplantation, thousands of donor-derived cells were detected throughout the brains of recipients, but none displayed neuronal, macroglial, or endothelial characteristics, even after injury (Vallieres and Sawchenko, 2003). Adult BM cells were incapable of transdifferentiation

†Qin Chen and Yan Long contributed equally to this article.

Contract grant sponsor: NIH; Contract grant number: RO1-NS35502-05; Contract grant sponsor: Texas Higher Education Coordinating Board; Contract grant number: ATP 004949-0074, ATP 004949-049.

★Correspondence to: Qin Chen, Center for Cell and Gene Therapy and Department of Neurosurgery, Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030. E-mail: qchen1@bcm.tmc.edu

Received 25 January 2005; Accepted 21 February 2005

Published online 4 May 2005 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/jnr.20494

into neural cells and were lineage restricted (Castro et al., 2002). Several independent groups demonstrated that cell fusion of MSCs with neurons, hepatocytes, and cardiac muscle cardiomyocytes, rather than MSC transdifferentiation, may contribute to the development or maintenance of these key cell types (Alvarez-Dolado et al., 2003; Vassilopoulos et al., 2003). Tissue replacement therefore does not seem to be the main avenue by which cell transplantation restores brain function. There is thus a need to consider other mechanisms that might account for the therapeutic benefit of MSC transplantation.

Neurotrophic factors have well-established roles in survival, differentiation, and function of CNS neurons. Exogenous nerve growth factor (NGF), for example, plays a critical role in neuronal plasticity and regenerative potential as well as the inhibition of neural apoptosis after traumatic brain injury (TBI; DeKosky et al., 1994; Dixon et al., 1997; Goss et al., 1997, 1998). Several studies found that brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3) support the survival of injured CNS neurons in vitro and in vivo, induce neurite outgrowth, and increase the expression of key enzymes for neurotransmitter synthesis (Mocchetti and Wrathall, 1995). Glial cell line-derived neurotrophic factor (GDNF) is capable of protecting against hippocampal neuronal death (Kim et al., 2001), attenuating brain swelling, and reducing the lesion volume (Hermann et al., 2001) after TBI.

In an effort to provide additional insight into how MSCs might enhance functional recovery after TBI, we tested both in vitro and in vivo the possibility that MSC transplantation into the CNS stimulates the production of neurotrophic factors, which in turn account for the MSC-associated recovery from neuronal injury. The results show a striking increase in the NGF concentration in MSC cultures, and the cerebrospinal fluid (CSF) of MSC-transplanted mice contained markedly higher NGF levels than did CSF from untreated controls. Comparable changes were not observed for the other neurotrophic factors examined in this study. Enhanced NGF synthesis thus provides a plausible mechanism for the attenuation of cholinergic deficits seen after TBI and MSC transplantation in our model system.

MATERIALS AND METHODS

Animals

Adult C57BL/6 (Harlan) mice weighing 25 ± 2 g were studied. They were fed and watered ad lib and maintained on a 12-hr light/dark cycle. All procedures were in accordance with the Guide for the Care and Use of Laboratory Animals (NIH publication no.85-23, revised 1985) and were approved by the Animal Care Committee of the Baylor College of Medicine.

TBI Model

TBI was induced with a controlled cortical impact (CCI) device (Dixon et al., 1991). Briefly, mice ($n = 24$) were anesthetized with 0.05-ml intramuscular injections of a combination of anesthetics, containing 42.8 mg of ketamine, 8.6 mg of xylazine, and 1.4 mg of acepromazine per milliliter. Their heads were then secured on a stereotaxic frame. A craniotomy, 4 mm in diameter, was then carried out over the right parietal cortex between the bregma and lambda. A moderate-grade CCI injury was induced with a pneumatically driven 3-mm piston at a velocity of 3 m/sec, 1.5-mm deformation. The bone flap was then repositioned, and the skin was closed with wound clips. Sham-operated mice ($n = 24$) underwent craniectomy without CCI. Sham-operated uninjured animals served as controls.

Isolation of Mouse Bone Marrow Stromal Cells

Fresh BM cells were harvested aseptically from the femurs and tibias of 4-week-old C57BL/6 mice by inserting a 27-gauge needle connected to a 1-ml syringe into the shaft of the bone and removing BM with 2 ml of phosphate-buffered saline (PBS). The cells were disaggregated by gentle pipetting several times and filtered through a 70-μm nylon mesh to remove any remaining clumps of tissue. After 5 min of centrifugation at 200 × *g*, the cell pellet was resuspended in Dulbecco's modified Eagle medium (DMEM) containing 20% (vol/vol) fetal bovine serum (FBS; Invitrogen) supplemented with 1% penicillin and streptomycin, and seeded into 25-cm^2 culture flasks. After 72 hr of incubation, the nonadherent cells were removed. The adherent cells (MSCs) were collected and resuspended in fresh flasks and transfected with an adenoviral vector encoding the enhanced green fluorescent protein (eGFP). After 24 hr of incubation, the cells were resuspended in Hank's balanced salt solution (HBSS) buffer containing propidium iodide (10 μl/1 ml HBSS); only GFP-positive cells, selected by fluorescent-activated sorting, were used in the transplantation experiments.

Intraventricular MSC Transplantation

At 5 hr after TBI or sham-operation, the heads of both the TBI and sham-operated mice were secured on a Kopf stereotaxic frame. Using an aseptic procedure, we slowly injected MSCs transduced with GFP (2×10^5 cells in 5 μl of PBS) with a 10-μl Hamilton syringe into the ipsilateral ventricles of TBI ($n = 12$) and sham-operated ($n = 12$) mice over 10 min. The corresponding site of the right ventricles was 0.7 mm posterior to the bregma, 1.3 mm lateral to the sagittal suture, and 2.0 mm below the dura. The needle was kept in place for an additional 5 min to avoid donor cell reflux. The sham-operated/vehicle ($n = 12$) and TBI/vehicle ($n = 12$) groups received the same volume of PBS as a brain injection. Immunosuppressants were not used after the injection.

CSF Withdrawal

At 13 and 45 days after MSC transplantation, CSF was collected by a modified method originated by Fleming et al. (1983). Each group ($n = 6$) and six naive mice were anesthetized by intramuscular injection of a ketamine-based drug combination (as described above) and placed on a platform in a prone position. The posterior neck skin was cleared, razed, and sterilized with alcohol. A No. 27 butterfly needle attached to a 1-ml syringe was used to puncture the skin at the central point between the base of the skull and the first vertebra. The needle was inserted carefully through the nuchal muscles and

the arachnoid membrane into the cisterna magna between the cerebellum and the medulla oblongata. Approximately 10 μl of clean CSF was gently aspirated from each mouse.

Immunocytofluorescence of NGF

Cultures were fixed with 4% paraformaldehyde in PBS (pH 7.4) for 30 min at room temperature and then blocked with 20% normal horse serum in PBS for 1 hr, followed by incubation with rabbit polyclonal anti-NGF (1:500; Chemicon, Temecula, CA), as primary antibody, at 4°C overnight. After washing with PBS, cultures were incubated with Texas red-conjugated goat anti-rabbit IgG (1:200; Vector, Burlingame, CA) at room temperature for 1 hr. Fluorescence signals were detected with a Nikon E300 microscope. Controls included omission of the primary antibody.

Immunohistochemical Assessment

After withdrawal of CSF, the brains were fixed by transcardial perfusion with PBS containing 4% paraformaldehyde. The brains were removed and postfixed in the same fixative for 1 day at 4°C, followed by 2 or 3 days of cryprotection in 30% sucrose in PBS until the tissues completely sank. Cryosections (30 μm) were cut and incubated overnight at 4°C with NGF (1:500; Chemicon) as primary antibody. After washing with PBS, the sections were incubated with second antibody conjugated to Texas red (1:200; Vector). A laser fluorescence microscope was used to examine tissues for immunofluorescence. To control for possible nonspecific reactions of the secondary antibody in each independent experiment, we substituted PBS for the primary antibodies.

Enzyme-Linked Immunosorbent Assay of NGF Levels in CSF

For in vivo studies, NGF levels in CSF were measured with a commercially available enzyme-linked immunosorbent assay (ELISA) system (Promega, Madison, Wisconsin) according to procedures recommended by the manufacturer. Briefly, 96-well plates were coated with anti-NGF antibody, which binds soluble NGF, and then the plates were incubated overnight at 4°C. A standard NGF dilution series was prepared. Standard NGF and sample dilutions were added to the antibody-coated wells, followed by 6 hr of incubation at room temperature. After washing, a second specific monoclonal antibody was added to each well and the mixture was incubated overnight at 4°C. After washing again with buffer, the amount of specifically bound monoclonal antibody was detected with a species-specific antibody conjugated to horseradish peroxidase as a tertiary reactant. The unbound conjugate was removed by washing, and after incubation with a chromogenic substrate, the color change was assessed. The optical density of each well was measured within 30 min with a microplate reader set at 450 nm. The NGF concentrations were determined from a standard concentration curve by a computer-assisted software program (Bio-Rad, Hercules, CA).

Detection of Neurotrophic Factors Secreted by MSCs

For in vitro studies, cell culture media were collected at 1, 2, 3, 4, 5, 6, and 7 weeks after the instigation of MSC cultures. Levels of NGF, BDNF, GDNF and NT-3 were measured with a commercially available ELISA system (Promega) according to the procedures recommended by the manufacturer.

ChAT Immunohistochemistry and Cell Count

Choline acetyltransferase (ChAT) immunohistochemical investigations were carried out as described previously (Zou et al., 1999). Briefly, the cryosections were washed in PBS and incubated with 0.5% H_2O_2 in PBS for 10 min to remove endogenous peroxide activity. After three washes in PBS, the sections were blocked with 3.0% normal rabbit serum at room temperature for 1 hr then incubated overnight at 4°C with polyclonal goat anti-ChAT antibody (1:200; Chemicon). After three washes in PBS, the sections were incubated with rabbit anti-goat (1:200) and avidin-biotin complex (ABC) reagents (Vector) according to the procedures recommended by the manufacturer. The ChAT-positive cells developed a dark-brown reaction product after 3–5 min of incubation in freshly prepared diaminobenzidine solution containing 0.03% hydrogen peroxide. The sections were subsequently dried on Fisher glass slides, dehydrated in graded ethanol, cleared in xylene, and coverslipped.

Sections approximately 860 μm rostral to the bregma were examined for ChAT-positive cells. These sections represent a region densely populated with ChAT-positive neurons that can serve as a surrogate for the medial septal neuron population (Hagg and Varon, 1993). ChAT-positive neurons were defined as immunolabeled cell bodies, regardless of intensity of staining (Gage et al., 1988). ChAT-positive cells were quantified bilaterally in eight consecutive sections using National Institutes of Health (NIH) Scion Image for Windows software. Briefly, photomicrographs of the brain sections, taken with a digital camera (AxioCam; Carl Zeiss, Jena, Germany) at 100× magnification, were converted to a binary image by setting a threshold for pixel intensity. Thresholding value was chosen according to the maximized contrast between reaction production and background. The same threshold was used for every individual image. Each ChAT-positive cell body detectable above the background level was counted. Values were compared for injury alone versus injury with MSC injection groups as well as the ipsilateral versus contralateral to the side of injury group. Findings in the injured mice were compared to those in intact mice.

Statistical Analyses

All data are reported as group means ± standard deviations (SD). A one-way analysis of variance (ANOVA) test, followed by the post Student-Newman test, was used to assess variances. P values less than 0.05 were accepted as significant.

RESULTS

Transplanted MSCs Migrate to Injured Areas

GFP-positive cells were not present in the brain sections from PBS-treated mice on Day 13 or 45 postinjection. Many such cells, however, were found near the engraftment site or in both sides of the ventricle in Day-13 sections from mice transplanted with MSCs. By Day 45 posttransplantation, most MSCs had migrated from

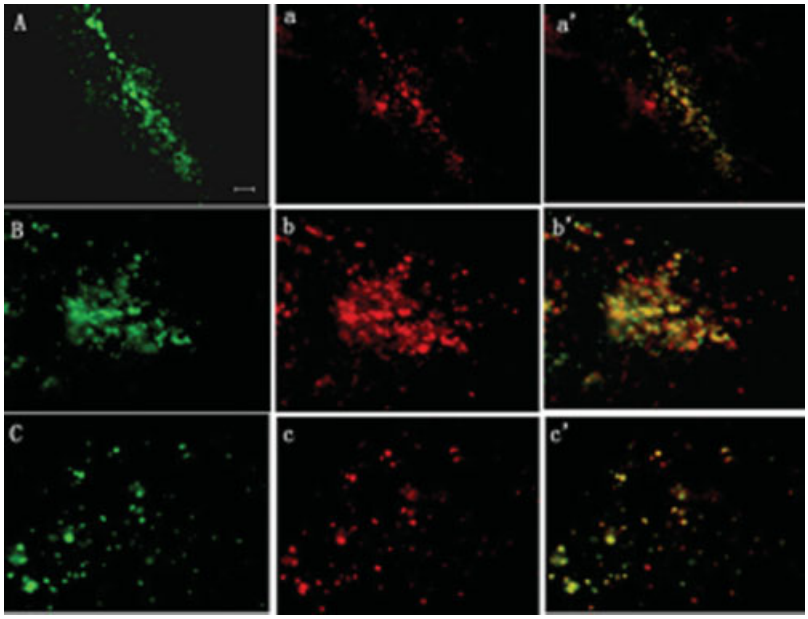

Fig. 1. Expression of nerve growth factor (NGF) increased after marrow stromal cell (MSC) transplantation in mice with traumatic brain injury (TBI). Coronal sections were processed for immunofluorescent staining. MSCs were stained positive by green fluorescent protein (GFP; green). At 13 days after transplantation, MSCs were observed in boundary zone of injection track (**A**) and cortex (**B**). Most MSCs showed NGF immunoreactivity, as evidenced by red immunofluorescence, at 13 days after transplantation at the localized around injection track (**a**) and cortex (**b**). At 45 days after transplantation, most MSCs had migrated from the engraftment site toward the area of injury (**C**) and expressed NGF(**c**). Overlays of the corresponding GFP (green) and NGF (red) staining (**a′–c′**), show that most of the transplanted MSCs expressed NGF (yellow). Several marrow-derived, GFP-positive cells not expressing NGF are also present. Scale bar = 50 μm. Figure can be viewed in color online via www.interscience.wiley.com.

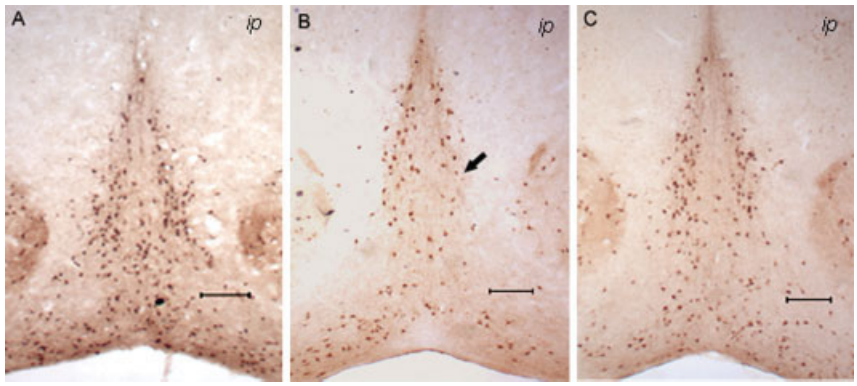

Fig. 2. ChAT immunostaining in coronal sections of adult mouse medial septum after traumatic brain injury (TBI) and ipsilateral intraventricular infusion of vehicle or marrow stromal cells (MSCs). Transplantation of MSCs reduced the loss of ChAT-immunopositive neurons at 13 and 45 days post-TBI in the mouse medial septal regions as shown by ChAT immunohistochemical staining. **A:** ChAT-immunopositive neurons in the medial septal area of naive mouse. **B:** Loss of ChAT-immunopositive neurons in the medial septal area (arrow) at 13 days after controlled cortical impact and vehicle intraventricular injection. **C:** The reduced number of ChAT immunopositive neurons in the medial septal area attenuated after 13 day MSCs treatment; ip, side ipsilateral to injury. Scale bar = 150 μm. Figure can be viewed in color online via www.interscience.wiley.com.

the engraftment site toward the area of injury, and some were found in multiple areas distant from the site of injection (Fig. 1A–C). Fewer GFP-positive cells were present at the injury site on subsequent examinations and had disappeared altogether by Day 90 posttransplantation (data not shown). This result may have been due to cell fusion that could have inactivated or eliminated donor gene expression overtime (Alvarez-Dolado et al., 2003).

Transplanted MSCs Attenuate Cholinergic Neuronal Injury After TBI

To determine the effect of the transplanted MSCs on the cholinergic neurons in the traumatically injured brain, we quantified the number of ChAT-positive cells in the medial septum at 13 and 45 days postinjection. The unilateral traumatic injury significantly and bilaterally reduced the number of ChAT-positive neurons at both time points. In the absence of MSC transplantation, the numbers of ChAT-positive neuronal cells in the ipsilateral medial septum after CCI decreased to 55.1 and 59.3% of the sham/vehicle and to 54.7and 54.08% of the naive mouse values at 13 and 45 days, respectively ($P < 0.05$ for both comparisons). When transplanted MSCs were present, however, the number of ChAT-positive neuronal cells had increased significantly ($P < 0.05$) to 83.7% of the sham/vehicle and 83.1% of the naive mouse values, by day 13 (Fig. 2 and 3A).

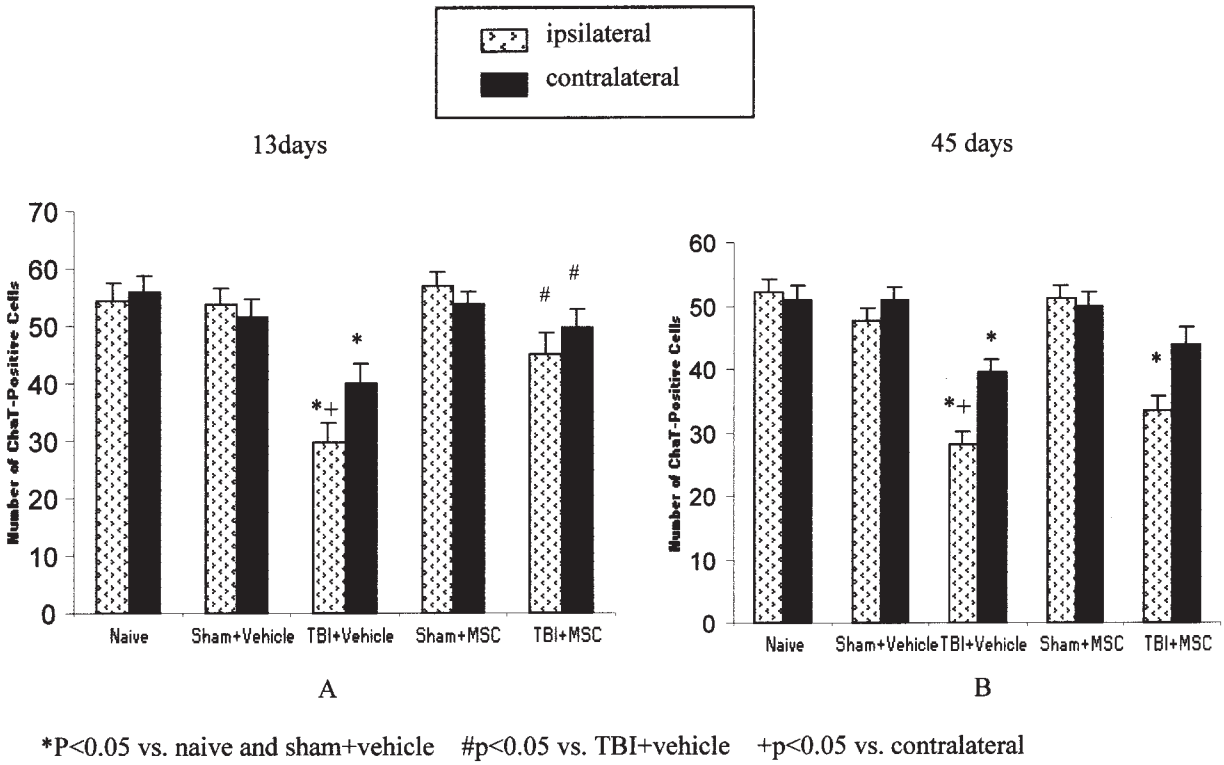

Fig. 3. The effects of marrow stromal cell (MSC) treatment on the number of ChAT-positive neurons in the medial septum. **A, B:** MSC transplantation significantly attenuates the ChAT-positive neuronal loss in mouse septal regions. The data are presented as means ± SEM, $n = 6$ per group. Injured mice treated with vehicle alone showed significant ipsilateral and contralateral reduction in the number of ChAT-positive neuronal cell ($P < 0.05$) at 13 and 45 days after traumatic brain injury (TBI; cortical impact) compared to that in naive and sham mice. In the MSC-transplanted group, however, the number of ChAT-positive neuronal cells increased significantly ($P < 0.05$) up to normal levels on both sides compared to in mice that received vehicle alone at 13 day posttransplantation. This indicated that MSC treatment reversed the loss of ChAT-positive in mouse septal regions. There was a significant decrease in the number of ChAT-positive cells at the ipsilateral vs. the contralateral medial septal areas in injured mice treated with vehicle alone ($P < 0.05$). *$P < 0.05$ vs. naive and sham + vehicle; #$P < 0.05$ vs. TBI + vehicle; +$P < 0.05$ vs. contralateral side.

Although the number of ChAT-positive cells in MSC-treated mice with brain injury did not significantly exceed that in vehicle-treated injured mice at 45 days postinjection, it rose to 70.4% of the sham/vehicle and 64.2% of the naive mouse results (Fig. 3B). The number of neurons was also increased significantly ($P < 0.05$) in sham-operated animals undergoing MSC transplantation. These findings indicate a marked beneficial effect of transplanted MSCs on the number of ChAT-positive neurons in CCI-injured mice.

Increased Concentration of NGF But Not Other Neurotrophic Factors in MSC Cultures

Because tissue replacement by MSCs cannot account for brain functional recovery after TBI and cell transplantation, we asked whether MSCs might produce NGF and other neurotrophic factors after being injected into mouse brain. Figure 4 establishes the expression of NGF by MSCs in vitro. The morphologic characteristics of the cultured cells (Fig. 4A) were consistent with preferential attachment of MSCs to culture dishes (Azizi et al., 1998; Colter et al., 2001). Immunocytochemical staining of the MSCs at 10 days of culture demonstrated NGF (red staining) in a substantial fraction of the cultured cells, suggesting NGF protein synthesis by these MSCs. Using ELISA techniques, we next sought to determine the relative secretion of NGF compared to other neurotrophin factors at 7, 14, 21, 28, 35, 42, and 49 days after instigation of MSC cultures. The results (Fig. 5) showed a significantly higher concentration of NGF at each of seven time points compared to that for the medium-only control value ($P < 0.05$), in contrast to BDNF, GDNF, and NT-3 levels, which remained unchanged over the entire culture period (data not shown). Together, these data suggest preferential and constitutive secretion of NGF by MSCs.

Transplanted MSCs Produce NGF in Recipient Mouse Brain

The above findings led us to test the expression of NGF by MSCs in vivo, using GFP-labeled MSCs and Texas red-conjugated secondary antibodies to visualize

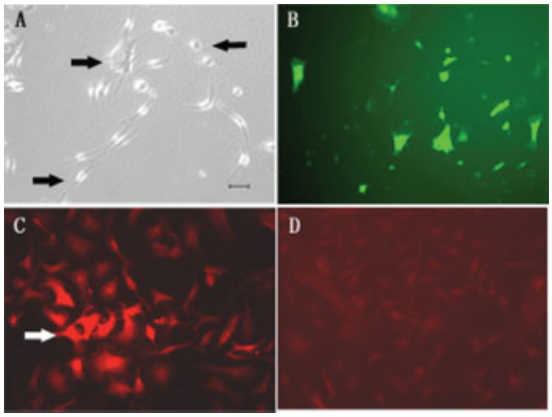

Fig. 4. Immunostaining for nerve growth factor (NGF) shows that NGF protein was expressed in cultured marrow stromal cells (MSCs). **A:** MSCs from mice were cultured in medium. Bright-field photomicrograph showing small round, spindle-shaped and large flat cells (arrows), consistent with MSC. **B:** After 1 day transfected with an adenoviral vector encoding the enhanced green fluorescent protein (eGFP), most MSCs expressed green fluorescent protein (GFP; green). **C:** Some MSCs contained NGF protein, as evidenced by red immunofluorescence (arrow). **D:** Omission of the primary antisera resulted in loss of specific staining. Images are representative of at least three independent experiments per panel. Scale bar = 20 μm. Figure can be viewed in color online via www.interscience.wiley.com.

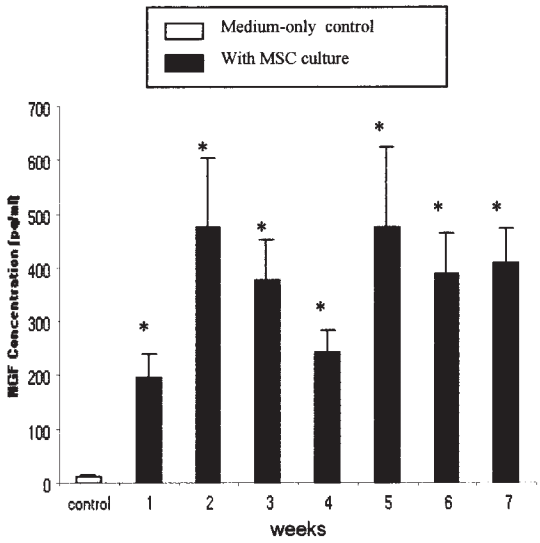

Fig. 5. Enzyme-linked immunosorbent assay (ELISA) of nerve growth factor (NGF) protein in media from marrow stromal cell (MSC) cultures. Values are mean ± SD from four different wells. NGF protein increased from the first week and persisted for at least 7 weeks. There was a statistically significant difference between MSC cultures and controls in all the time points studied ($P < 0.05$; $n = 4$).

NGF expression in coronal cryostat sections (30 μm thick). As shown in Figure 1, immunofluorescence was observed predominantly in GFP-positive cells consistent with the production of NGF by BM-derived stromal

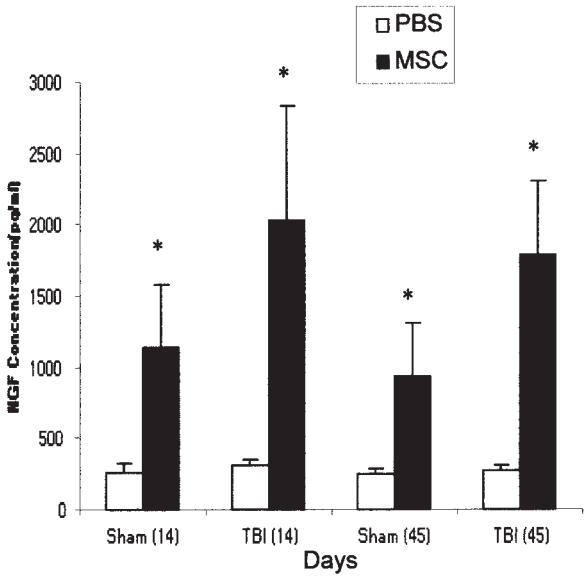

Fig. 6. Enzyme-linked immunosorbent assay (ELISA) of nerve growth factor (NGF) protein in cerebrospinal fluid (CSF) from mice after intraventricular injection of marrow stromal cells (MSCs). MSC transplantation significantly enhanced the levels of NGF in CSF from both the traumatic brain injury mice and sham-operated mice when compared to that from phosphate-buffered saline-injected controls. All data were presented as mean ± SD from six mice. *$P < 0.05$ compared to control.

cells. Analysis of CSF samples, taken at 13 and 45 days posttransplantation by ELISA methods, clearly indicated that MSC transplantation had enhanced the levels of NGF in CSF from both the TBI and sham-operated mice by comparison with PBS-injected controls (Fig. 6). After TBI, the mean concentrations of NGF were 2,029 ± 801 in MSC-injected mice versus 305 ± 39 pg/ml in PBS controls at 13 days posttransplantation (6.7-fold increase), and 1,786 ± 516 versus 276 ± 32 pg/ml (6.5-fold increase) at 45 days. Although the mean NGF concentration in sham-injured mice injected with MSCs was only about half that in TBI animals, it showed a 4.5-fold increase over the PBS control at 13 days and a 3.8-fold increase at 45 days posttransplantation. All statistical comparisons were significant at the $P < 0.05$ level.

DISCUSSION

Effective therapeutic strategies to ameliorate or prevent the neurodegenerative effects of TBI are needed urgently. Transplantation of BM stromal cells recently has received much attention as a novel approach to treatment that can improve neuronal function (Mahmood et al., 2001b). We show here for the first time that intraventricularly transplanted MSCs preferentially migrate toward the site of brain injury and significantly reduce ChAT-positive neuronal cell loss in the mouse medial septal regions, apparently by secreting NGF.

MSCs have been transplanted into rats and mice by several different routes, each having limitations and

potential complications. Intravenous or intraarterial infusion of MSCs can lead to cell migration into organs other than the brain, and the fate of these migrating cells remains unclear (Lu et al., 2001b; Mahmood et al., 2003). Intracerebral injection of cells is highly invasive and requires craniotomy (Azizi et al., 1998). Although ventricular administration of limits the number of cells that can be injected, the advantage of this route is that it avoids systemic dissemination. An intraventricular injection route is clinically relevant because ventriculostomies are often carried out to monitor intracranial pressure in patients with severe traumatic brain injuries. Injection of MSCs into the lateral ventricle may enable cells to migrate to various regions of the brain, including the forebrain, cerebellum, striatum, the molecular layer of the hippocampus, and the subependyma of olfactory bulb (Kopen et al., 1999). Moreover, the intraventricular route of administration does not produce any pathologic changes in cellular morphology, as we have reported previously (Zou et al., 1999). In present study, intraventricularly injected MSCs migrated from the injection site and distributed over the boundary zone of injury, ensuring adequate numbers of NGF-secreting cells to promote recovery from TBI.

Numerous studies have demonstrated that TBI can produce spatial memory deficits, and these disturbances may be related to a loss of cholinergic immunoreactivity in the medial septal area and a decreased capacity of cholinergic neurons to release acetylcholine (Ach) (Schmidt and Grady, 1995; Dixon et al., 1997). The present results show that CCI reduces the number of ChAT-immunopositive neurons ipsilaterally and contralaterally within the medial septal area at 13 and 45 days postinjury. This bilateral reduction in the number of ChAT-immunopositive neurons could be reversed at both times by MSC treatment via the intraventricular route.

What are the mechanisms responsible for attenuating cholinergic deficits after MSC transplantation in mice with TBI? One possibility is that the transplanted marrow cells integrate into the brain, replacing damaged cells and reconstructing neuronal circuits. As yet, however, there is no compelling published data to indicate that MSCs function in this manner. Although many authors have concluded that transplanted MSCs can transdifferentiate into cells with neural characteristics (Mezey et al., 2000; Mahmood et al., 2002), the proportion of MSCs undergoing differentiation in the brain seems small. In three recent studies, only 1–8% of intrastriatally grafted stromal cells expressed markers typical of astrocytes and neurons (Li et al., 2000), and 0.2–2.3% of the total number of neurons originated from donor-derived cells (Brazelton et al., 2000; Mezey et al., 2000). It is doubtful that such a low fraction of engrafted cells with neural phenotypes could effectively repair cortical damage. Moreover, expression of neural markers does not necessarily mean that these cells are capable of the complex functions of mature neurons. Several recent publications suggest that evidence for the adoption of other cellular phenotypes by MSCs may simply reflect fusion of the engrafted cells with host cells and not radical changes in their program of differentiation (Terada et al., 2002). The ability of transplanted MSCs to replace injured CNS cells thus seems limited, raising the possibility that mechanisms other than tissue replacement contribute to the beneficial CNS effects of this form of cell transplantation therapy.

Experiments with rodent models of TBI indicate that neurotrophic factors can prevent neuronal death and dysfunction (Dixon et al., 1997). Recovery from TBI thus may be facilitated by the activity of different neurotrophic factors, such as NGF, BDNF, GDNF, and NT-3. Increased levels of neurotrophic factors after TBI have been shown to support neuronal survival, stimulate neuronal plasticity, induce neuronal repair, and reestablish functional connections in the brain (Sinson et al., 1997; Philips et al., 2001). A number of lines of evidence indicates that NGF can improve posttraumatic spatial memory due to an increase in ACh transmission (Dixon et al., 1997) and enhanced production of ChAT, both induced by exogenous NGF (Tirassa et al., 2003). We have observed previously significant increases in ChAT mRNA after transfection of cells with the NGF gene (Zou et al., 1999). It is therefore reasonable to propose that one or more of these polypeptides contribute to the therapeutic effects of MSCs in mice with TBI. This hypothesis was supported by the detection of NGF protein synthesis by a substantial fraction of MSCs over at least 7 weeks, whereas concentrations of BDNF, NT-3 and GDNF in the culture medium remained slight or negligible over the entire culture period. Our in vitro results expand a recent report of a time-dependent increase of NGF production by human MSCs within 7 days of culture (Chen et al., 2002). In that study, the cultured MSCs (three to five passages) were assessed for their secretion of growth factors in various medium. The authors also found that BDNF levels were increased at 1, 4, and 7 days in normal brain extract medium, and at 7 days in TBI-conditioned medium. The discrepancy between their finding and ours can be attributed to differences in the time points studied (1, 4, and 7 days vs. 1, 2, 3, 4, 5, 6, and 7 weeks) and in the types of MSCs (human vs. mouse), as well as in the culture conditions (knockout medium conditioned with TBI or normal brain extract vs. nonconditioned complete DMEM). Nonetheless, the available data suggest that BDNF expression may be transient, at best. The lack of evidence for appreciable secretion of NT-3 in our study agrees with the profile of NT-3 expression by BM cells reported by Laurenzi et al. (1998), who failed to detect expression of mRNA encoding NT-3. Importantly, CSF samples from MSC-transplanted mice, with or without TBI, showed significantly increased levels of NGF relative to those in CSF from sham controls, confirming the outcome of studies with cultured MSCs. We conclude that enhanced expression of NGF in the brain due to the infiltration of transplanted MSC, contributes significantly to the recovery from TBI often seen after transplantation of bone marrow stromal cells. Measurements

of neurotrophic factors in this study restricted to NGF, BDNF, GDNF, and NT-3. Because MSCs may secrete other growth and trophic factors (e.g., VEGF, HGF; Chen et al., 2002), we cannot exclude the possibility that increased activity by such molecules after MSC transplantation further enhanced recovery from TBI. Finally, NGF levels did not differ significantly between TBI and sham injured mice posttransplantation, indicating that tissue injury is not a prerequisite for enhanced production of NGF by MSCs.

Various insults to the brain, such as trauma, ischemia, or degenerative diseases, have been reported to induce upregulated expression of neurotrophic factors and their respective receptors, although it remains unclear how long this stimulation persists. Most studies suggest that injury-induced increases of NGF expression are transient (Oyesiku et al., 1999). We showed previously that NGF mRNA expression in ipsilateral hippocampus increased 1 hr after TBI and remained increased at 3 and 5 hr postinjury (Yang et al, 1995). DeKosky et al. (1994) reported that the increased cortical levels of NGF protein persisted for up to 7 days after TBI in rat, apparently the longest time that endogenous NGF levels have been measured after injury to the brain. Results of the present study suggest that elevations of endogenous NGF last no longer than 13 days postinjury, an insufficient time to compensate for TBI-induced neurologic deficits. Clearly, a major challenge is to devise strategies that will boost NGF activity in injured or diseased brain for prolonged times.

Our studies indicate the therapeutic potential of intraventricular administration of MSCs for restoring brain function after TBI. Although the activity and differentiation of MSCs after transplantation remains in question, there is the exciting prospect that their secretion of neurotrophic factors stimulate endogenous repair mechanisms to promote brain repair and functional recovery, apparently without the requirement for complete neural differentiation and introgression with the host's nervous system. Identification for the specific type(s) of MSCs responsible for increased production of NGF after transplantation is therefore imperative and should allow refinement of current strategies of MSC transplantation, leading in turn to improved results in the treatment of brain damage and neurologic diseases.

ACKNOWLEDGMENT

We thank Dr. J. Gilbert for editing the article.

REFERENCES

Ader M, Schachner M, Bartsch U. 2001. Transplantation of neural precursor cells into the dysmyelinated CNS of mutant mice deficient in the myelin-associated glycoprotein and Fyn tyrosine kinase. Eur J Neurosci 14:561–566.

Alvarez-Dolado M, Pardal R, Garcia-Verdugo JM, Fike JR, Lee HO, Pfeffer K, Lois C, Morrison SJ, Alvarez-Buylla A. 2003. Fusion of bone-marrow-derived cells with Purkinje neurons, cardiomyocytes and hepatocytes. Nature 425:968–973.

Azizi SA, Stokes D, Augelli BJ, DiGirolamo C, Prockop DJ. 1998. Engraftment and migration of human bone marrow stromal cells implanted in the brains of albino rats—similarities to astrocyte grafts. Proc Natl Acad Sci USA 95:3908–3913.

Brazelton TR, Rossi FM, Keshet GI, Blau HM. 2000. From marrow to brain: expression of neuronal phenotypes in adult mice. Science 290:1775–1779.

Castro RF, Jackson KA, Goodell MA, Robertson CS, Liu H, Shine HD. 2002. Failure of bone marrow cells to transdifferentiate into neural cells in vivo. Science 297:1299.

Chen X, Katakowski M, Li Y, Lu D, Wang L, Zhang L, Chen J, Xu Y, Gautam S, Mahmood A, Chopp M. 2002. Human bone marrow stromal cell cultures conditioned by traumatic brain tissue extracts: growth factor production. J Neurosci Res 69:687–691.

Chen J, Li Y, Chopp M. 2000. Intracerebral transplantation of bone marrow with BDNF after MCAo in rat. Neuropharmacology 39: 711–716.

Colter DC, Sekiya I, Prockop DJ. 2001. Identification of a subpopulation of rapidly self-renewing and multipotential adult stem cells in colonies of human marrow stromal cells. Proc Natl Acad Sci USA 98: 7841–7845.

DeKosky ST, Goss JR, Miller PD, Styren SD, Kochanek PM, Marion D. 1994. Upregulation of nerve growth factor following cortical trauma. Exp Neurol 130:173–177.

Dixon CE, Clifton GL, Lighthall JW, Yaghmai AA, Hayes RL. 1991. A controlled cortical impact model of traumatic brain injury in the rat. J Neurosci Methods 39:253–262.

Dixon CE, Flinn P, Bao J, Venya R, Hayes RL. 1997. Nerve growth factor attenuates cholinergic deficits following traumatic brain injury in rats. Exp Neurol 146:479–490.

Fleming JO, Ting JY, Stohlman SA, Weiner LP. 1983. Improvements in obtaining and characterizing mouse cerebrospinal fluid. Application to mouse hepatitis virus-induced encephalomyelitis. J Neuroimmunol 4:129–140.

Gage FH, Armstrong DM, Williams LR, Varon S., Armstrong DM, Williams LR, Varon S. 1988. Morphological response of axotomized septal neurons to nerve growth factor. J Comp Neurol 269:147–155.

Goss JR, Taffe KM, Kochanek PM, DeKosky ST. 1997. The antioxidant enzymes glutathione peroxidase and catalase increase following traumatic brain injury in the rat. Exp Neurol 146:291–294.

Goss JR, O'Malley ME, Zou L, Styren SD, Kochanek PM, DeKosky ST. 1998. Astrocytes are the major source of nerve growth factor upregulation following traumatic brain injury in the rat. Exp Neurol 149: 301–309.

Hagg T, Varon S. 1993. Neurotropism of nerve growth factor for adult rat septal cholinergic axons in vivo. Exp Neurol 119:37–45.

Hermann DM, Kilic E, Kugler S, Isenmann S, Bahr M. 2001. Adenovirus-mediated glial cell line-derived neurotrophic factor (GDNF) expression protects against subsequent cortical cold injury in rats. Neurobiol Dis 8:964–973.

Hess DC, Hill WD, Martin-Studdard A, Carroll J, Brailer J, Carothers J. 2002. Bone marrow as a source of endothelial cells and NeuN-expressing cells after stroke. Stroke 33:1362–1368.

Jin K, Mao XO, Batteur S, Sun Y, Greenberg DA. 2003. Induction of neuronal markers in bone marrow cells: differential effects of growth factors and patterns of intracellular expression. Exp Neurol 184: 78–89.

Kim BT, Rao VL, Sailor KA, Bowen KK, Dempsey RJ. 2001. Protective effects of glial cell line-derived neurotrophic factor on hippocampal neurons after traumatic brain injury in rats. J Neurosurg 95:674–679.

Kopen GC, Prockop DJ, Phinney DG. 1999. Marrow stromal cells migrate throughout forebrain and cerebellum, and they differentiate into astrocytes after injection into neonatal mouse brains. Proc Natl Acad Sci USA 96:10711–10716.

Laurenzi MA, Beccari T, Stenke L, Sjolinder M, Stinchi S, Lindgren JA. 1998. Expression of mRNA encoding neurotrophins and neurotrophin receptors in human granulocytes and bone marrow cells—enhanced neurotrophin-4 expression induced by LTB4. J Leukoc Biol 64: 228–234.

Li Y, Chopp M, Chen J, Wang L, Gautam SC, Xu YX, Zhang Z. 2000. Intrastriatal transplantation of bone marrow nonhematopoietic cells improves functional recovery after stroke in adult mice. J Cereb Blood Flow Metab 20:1311–1319.

Lu D, Mahmood A, Wang L, Li Y, Lu M, Chopp M. 2001a. Adult bone marrow stromal cells administered intravenously to rats after traumatic brain injury migrate into brain and improve neurological outcome. Neuroreport 12:559–563.

Lu D, Li Y, Wang L, Chen J, Mahmood A, Chopp M. 2001b. Intraarterial administration of marrow stromal cells in a rat model of traumatic brain injury. J Neurotrauma 18:813–819.

Mahmood A, Lu D, Yi L, Chen JL, Chopp M. 2001a. Intracranial bone marrow transplantation after traumatic brain injury improving functional outcome in adult rats. J Neurosurg 94:589–595.

Mahmood A, Lu D, Wang L, Li Y, Lu M, Chopp M. 2001b. Treatment of traumatic brain injury in female rats with intravenous administration of bone marrow stromal cells. Neurosurgery 49:1196–1203.

Mahmood A, Lu D, Wang L, Chopp M. 2002. Intracerebral transplantation of marrow stromal cells cultured with neurotrophic factors promotes functional recovery in adult rats subjected to traumatic brain injury. J Neurotrauma 19:1609–1617.

Mahmood A, Lu D, Lu M, Chopp M. 2003. Treatment of traumatic brain injury in adult rats with intravenous administration of human bone marrow stromal cells. Neurosurgery 53:697–702.

Mezey E, Chandross KJ, Harta G, Maki RA, McKercher SR. 2000. Turning blood into brain: cells bearing neuronal antigens generated in vivo from bone marrow. Science 290:1779–1782.

Mezey E, Key S, Vogelsang G, Szalayova I, Lange GD, Crain B. 2003. Transplanted bone marrow generates new neurons in human brains. Proc Natl Acad Sci USA 100:1364–1369.

Mocchetti I, Wrathall JR. 1995. Neurotrophic factors in central nervous system trauma. J Neurotrauma 12:853–870.

Oyesiku NM, Evans CO, Houston S, Darrell RS, Smith JS, Fulop ZL, Dixon CE, Stein DG. 1999. Regional changes in the expression of neurotrophic factors and their receptors following acute traumatic brain injury in the adult rat brain. Brain Res 833:161–172.

Philips MF, Mattiasson G, Wieloch T, Bjorklund A, Johansson BB, Tomasevic G, Martinez-Serrano A, Lenzlinger PM, Sinson G, Grady MS, McIntosh TK. 2001. Neuroprotective and behavioral efficacy of nerve growth factor-transfected hippocampal progenitor cell transplants after experimental traumatic brain injury. J Neurosurg 94:765–774.

Sanchez-Ramos J, Song S, Cardozo-Pelaez F, Hazzi C, Stedeford T, Willing A, Freeman TB, Saporta S, Janssen W, Patel N, Cooper DR, Sanberg PR. 2000. Adult bone marrow stromal cells differentiate into neural cells in vitro. Exp Neurol 164:247–256.

Schmidt RH, Grady MS. 1995. Loss of forebrain cholinergic neurons following fluid-percussion injury: implications for cognitive impairment in closed head injury. J Neurosurg 83:496–502.

Sinson G, Perri BR, Trojanowski JQ, Flamm ES, McIntosh TK. 1997. Improvement of cognitive deficits and decreased cholinergic neuronal cell loss and apoptotic cell death following neurotrophin infusion after experimental traumatic brain injury. J Neurosurg 86:511–518.

Terada N, Hamazaki T, Oka M, Hoki M, Mastalerz DM, Nakano Y, Meyer EM, Morel L, Petersen BE, Scott EW. 2002. Bone marrow cells adopt the phenotype of other cells by spontaneous cell fusion. Nature 416:542–545.

Tirassa P, Triaca V, Amendola T, Fiore M, Aloe L. 2003. EGF and NGF injected into the brain of old mice enhance BDNF and ChAT in proliferating subventricular zone. J Neurosci Res 72:557–564.

Vallieres L, Sawchenko PE. 2003. Bone marrow-derived cells that populate the adult mouse brain preserve their hematopoietic identity. J Neurosci 23:5197–5207.

Vassilopoulos G, Wang PR, Russell DW. 2003. Transplanted bone marrow regenerates liver by cell fusion. Nature 422:901–904.

Weimann JM, Charlton CA, Brazelton TR, Hackman RC, Blau HM. 2003. Contribution of transplanted bone marrow cells to Purkinje neurons in human adult brains. Proc Natl Acad Sci USA 100:2088–2093.

Wagers AJ, Sherwood RI, Christensen JL, Weissman IL. 2002. Little evidence for developmental plasticity of adult hematopoietic stem cells. Science 297:2256–2259.

Yang K, Mu XS, Xue JJ, Perez-Polo JR, Hayes RL. 1995. Regional and temporal profiles of c-fos and nerve growth factor mRNA expression in rat brain after lateral cortical impact injury. J Neurosci Res 42:571–578.

Zou LL, Huang L, Hayes RL, Black C, Qiu YH, Perez-Polo JR, Le W, Clifton GL, Yang K. 1999. Liposome-mediated NGF gene transfection following neuronal injury: potential therapeutic applications. Gene Ther 6:994–1005.