

Intracerebroventricular Transplantation of Human Mesenchymal Stem Cells Induced to Secrete Neurotrophic Factors Attenuates Clinical Symptoms in a Mouse Model of Multiple Sclerosis

Yael Barhum · Sharon Gai-Castro ·
Merav Bahat-Stromza · Ran Barzilay ·
Eldad Melamed · Daniel Offen

Received: 30 June 2009 / Accepted: 8 October 2009 / Published online: 10 November 2009
© Humana Press 2009

Abstract Stem cell-based therapy holds great potential for future treatment of multiple sclerosis (MS). Bone marrow mesenchymal stem cells (MSCs) were previously reported to ameliorate symptoms in mouse MS models (experimental autoimmune encephalomyelitis, EAE). In this study, we induced MSCs to differentiate in vitro into neurotrophic factor-producing cells (NTFCs). Our main goal was to examine the clinical use of NTFCs on EAE symptoms. The NTFCs and MSCs were transplanted intracerebroventricularly (ICV) to EAE mice. We found that NTFCs transplantations resulted in a delay of symptom onset and increased animal survival. Transplantation of MSCs also exerted a positive effect but to a lesser extent. In vitro analysis demonstrated the NTFCs' capacity to suppress mice immune cells and protect neuronal cells from oxidative insult. Our results indicate that NTFCs-transplanted ICV delay disease symptoms of EAE mice, possibly via neuroprotection and immunomodulation, and may serve as a possible treatment to MS.

Keywords Multiple sclerosis (MS) · Mesenchymal stem cells (MSCs) · Experimental autoimmune encephalomyelitis (EAE)

Introduction

Multiple sclerosis (MS) is an inflammatory, demyelinating, and neurodegenerative disease of the central nervous system. MS is chronic, remitting–relapsing disease characterized by patchy perivenular inflammatory infiltrates in areas of demyelination and axonal loss (Noseworthy et al. 2000). This is the most common cause of neurological disability affecting young adults. The common treatment for MS is based on immunomodulation, mostly interferon- β or glatiramer acetate (Hemmer et al. 2006). Though effective in symptomatic alleviation, the current immunomodulating drugs do not stop the ongoing neurodegeneration.

Experimental autoimmune encephalomyelitis (EAE), the animal model for MS, is a T cell-mediated inflammatory disease of the central nervous system (CNS) with variable degrees of demyelination and axonal damage. The disease is elicited by immunization with myelin antigens—such as myelin-oligodendrocyte glycoprotein (MOG) and myelin basic protein—and adjuvant, resulting in a CD4⁺ T helper-1 cell response that attacks the myelinated areas of the CNS. The EAE mice demonstrate inflammation, demyelination, and neurodegeneration (Whitham et al. 1991).

Stem cell-based regenerative medicine raises great hope for the treatment of MS. Previous reports have shown the potential of cell-based therapy for EAE through transplantation of oligodendrocytes or neural stem cells (Archer et al. 1997; Pluchino et al. 2003, 2005; Einstein et al. 2007). A different approach for the utilization of stem cells in EAE treatment involves their use as a vehicle to deliver therapeutic molecules to the lesioned CNS (Makar et al. 2008a, b).

Y. Barhum · S. Gai-Castro · M. Bahat-Stromza · R. Barzilay ·
E. Melamed · D. Offen (✉)
Felsenstein Medical Research Center,
Tel Aviv University, Sackler School of Medicine,
Petach Tikva 49100, Israel
e-mail: doffen@post.tau.ac.il

Bone marrow mesenchymal stem cells (MSCs) are multipotent cells that can be induced to differentiate into various cell types under specific conditions (Pittenger et al. 1999). Some reports indicate that MSCs are also capable of undergoing neuroectodermal differentiation (Herzog et al. 2003). MSCs are also known for their capacity to induce immunomodulation (Aggarwal and Pittenger 2005; Le Blanc and Ringden 2007). Recent studies revealed that MSCs could improve neurological deficits of various damages or diseases of the central nervous system such as Parkinson's disease, brain trauma, spinal cord injury, and multiple sclerosis (Barzilay et al. 2006). Reports were also made indicating that intravenous and intraperitoneal administration of MSCs in the EAE mice improved the disease symptoms (Zhang et al. 2005; Kassis et al. 2008; Lianhua et al. 2009; Gordon et al. 2008). However, the optimal therapeutic approach for cell therapy to repair mature brain and spinal cord in MS has not yet established (Pluchino and Martino 2005; Payne et al. 2008).

MSCs have also been described for their capacity to produce and secrete neurotrophic factors both in vitro and in vivo (Chen et al. 2002; Pisati et al. 2007). Our previous studies have demonstrated that bone marrow MSCs can be induced in vitro to produce increased levels of neurotrophic factors (neurotrophic factor-producing cells, NTFCs). Intra-striatal injection of NTFCs into rat models of Parkinson's and Huntington's disease demonstrated impressive migration toward the sight of the lesion (Sadan et al. 2008) and significant clinical improvement (Bahat-Stroomza et al. 2009; Sadan et al. 2009). In the current study, we hoped to exploit the NTFCs' migratory properties and ability to secrete neurotrophic factors in the lesioned EAE mouse CNS.

Methods

Isolation and Culture of Human MSCs Human MSCs were isolated from bone marrow aspirates collected from the iliac crest of healthy donors following informed consent. The whole bone marrow sample was fractionated on a FICOL density gradient (FICOL-PAGUE) after which the cells were plated in polystyrene plastic tissue culture dishes in growth medium consisting Dulbecco's modified Eagle's medium (DMEM) supplemented with 15% fetal calf serum (FCS), 2 mM L-glutamine, 100 µg/ml streptomycin, 100 U/ml penicillin, and 12.5 U/ml nystatin (SPN; all from Biological Industries, Beit Haemek, Israel). Growth medium was changed twice a week, and cells were maintained at 37°C in a humidified 5% CO₂ incubator.

NTFC Differentiation Protocol MSCs (passages 3–6) were used for differentiation experiments. Growth medium was

replaced for 72 h with step 1 induction medium consisting of DMEM (SPN, L-glutamine), supplemented with 20 ng/ml human epidermal growth factor (PeproTech Inc, London, UK), 20 ng/ml human basic fibroblast growth factor (R&D Systems, Minneapolis, MN, USA), and N2 supplement (5 µg/ml insulin, 20 nM progesterone, 100 µM putrescine, 30 nM selenium, and 100 µg/ml transferrin, all from Sigma, St. Louis, MO, USA). The medium was then replaced with step 2 for 72 hours differentiation medium consisting of DMEM (SPN, L-glutamine) supplemented with 1 mM dibutyryl cyclic AMP (Sigma), 0.5 mM isobutylmethylxanthine (Sigma), 5 ng/ml human platelet-derived growth factor (PeproTech Inc.), and 50 ng/ml human neuregulin (1-β1 NRG1-β1-GGF-2; R&D Systems).

Immunocytochemistry Cells were fixed with 4% paraformaldehyde then stained with the following primary antibodies: rabbit antigial cell line-derived neurotrophic factor (anti-GDNF; 1:100, Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit anti-nerve growth factor (NGF; 1:200, Santa Cruz), rabbit anti-brain-derived neurotrophic factor (anti-BDNF; 1:100, R&D Systems), and rabbit anti-ciliary neurotrophic factor (anti-CNTF; 1:100, R&D Systems) followed by the secondary antibodies goat anti-rabbit Alexa-488 (1:200, Molecular Probes, Invitrogen, Carlsbad, CA, USA) and goat anti-mouse Alexa-568 (1:200, Molecular Probes). Goat anti-rabbit biotinylated (1:200; Jackson laboratories, West Grove, PA, USA) and streptavidin-Alexa-488 (1:200, Molecular Probes) antibodies were used for GDNF staining. Nuclear DNA was stained by 4,6-diamidino-2-phenylindole dihydrochloride (DAPI; 1:1,000, Sigma). Slides were visualized with a fluorescence Olympus IX70-S8F2 microscope. Images were processed using Image-Pro Plus software.

RNA Isolation and Real-Time PCR Total RNA was isolated with TRIzol (Invitrogen) according to the manufacturer's instructions. Reverse transcription (RT) was carried out on 0.5 µg RNA samples using the 10 U enzyme RT-superscript II (Invitrogen) in a mixture containing 2 µM Random primer (Invitrogen), 1× buffer supplied by the manufacturer, 10 mM dithiothreitol, 20 µM deoxyribonucleotide triphosphates, and RNase inhibitor (RNaguard, Amersham Pharmacia Biotech, UK). RT was performed in a thermocycler with the following program: 25°C for 10 min, 42°C for 2 h, 70°C for 15 min, and 95°C for 15 min. Real-time polymerase chain reaction (PCR) was performed in an ABI Prism 7700 sequence detection system (Applied Biosystems, Foster City, CA, USA) using Syber green PCR master mix and specific primers (see Table 1) at final concentrations of 500 nmol/l. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control. The amplification protocol consisted of

Table 1 Primers sequence for RT-PCR

Gene	Primer	Sequence
GAPDH	F	5'-CGACAGTCAGCCGCATCTT-3'
	R	5'-CCAATACGACCAAATCCGTTG-3'
GDNF	F	5'-TCAAATATGCCAGAGGATTATCCTG-3'
	R	5'-GCCATTTGTTTATC TGGTGACCTT-3'
BDNF	F	5'-AGCTCCGGGTTGGTATACTGG-3'
	R	5'-CCTGGTGGAACCTCTTTGCG-3'
NGF	F	5'-CATGCTGGACCCAAGCTCA-3'
	R	5'-GACATTACGCTATGCACCTCAGTG-3'
CNTF	F	5'-CCTGACTGCTCTTACGGAATCCTAT-3'
	R	5'CCCATCCGCAGAGTCCAG-3'
VEGF	F	5'-TGTGCCCACTGAGGAGTCC-3'
	R	5'-GGTTTGATCCGCATAATCTGC-3'

40 cycles at 94°C for 15 s followed by 60°C for 1 min each. Quantitative calculations of the gene of interest versus GAPDH was done using the $\Delta\Delta CT$ method, as instructed in the user bulletin 2 ABI prism 7700 sequence detection system (updated October 2001).

Measurement of Secreted Neurotrophic Factors The medium was collected after the 2 step differentiation protocol (described above). The protein level of GDNF, NGF, and BDNF was measured using the specific Emax ImmunoAssay (Promega, Madison, WI, USA) according to the manufacturer's protocol. The absorbance at 450 nm was recorded on a Microplate Reader (Model 550, BioRad, Hercules, CA, USA). The results were calculated for one million cells.

Cell Survival Assay The neuroblastoma cell line SH-SY5Y cells (ATCC, Manassas, VA, USA) were grown in basal media consisting of DMEM with 10% FCS, 2 mM L-glutamine, 100 μ g/ml streptomycin, 100 U/ml penicillin, and 12.5 U/ml nystatin. The SH-SY5Y cells were plated in 96-well plates and treated with 10 μ M butyric acid (Sigma) daily for 2 days to induce neuronal differentiation. After visual confirmation of neuronal differentiation, cells were applied either with human MSCs' or NTFCs' conditioned media (CM) and immediately exposed to oxidative insult of hydrogen peroxide (Sigma) for 24 h. Cell viability after treatments was analyzed by adding (3-(4, 5-dimethylthiazole-2-yl)-2, 5-diphenyltetrazolium bromide) (MTT) solution to each well followed by incubation at 37°C for 3 h. The medium was then removed, and the formazan crystals were dissolved in dimethyl sulfoxide. Absorbance was determined at 564 nm in the enzyme-linked immunosorbent assay (ELISA) reader. Cell viability was evaluated in triplicates for each treatment.

Animals Six- to 8-week-old C3H.SW female mice weighing 20 g were obtained from Jackson Laboratories. The animals were housed in standard conditions (constant temperature (22 \pm 1°C), humidity (relative, 30%), and a 12-h light/dark cycle) and were allowed free access to food and water. The animals and protocol procedures were approved and supervised by the Tel Aviv University and the Israel Ministry of Health ethical committee.

Induction of Chronic EAE and Cell Transplantation Chronic EAE was induced according to known protocols (Whitham et al. 1991; Offen et al. 2004). Briefly, female C3H.SW mice (6–8 weeks old) were immunized twice, at days 1 and 8, by subcutaneous injection with an emulsion containing myelin-oligodendrocyte glycoproteins (pMOG35–55, 3.75 mg/kg in C3H.SW) in incomplete Freund's adjuvant (CFA, Difco, Detroit, MI, USA) containing 200- μ g heat-activated *Mycobacterium tuberculosis* in a total volume of 0.2 ml. Six days post-EAE induction, mice received bilateral intracerebroventricular (ICV) injections of 2.5×10^5 cells (MSCs or NTFCs) or phosphate buffered saline (PBS) in a volume of 2 μ l using a Hamilton 10- μ l syringe with a 26-gauge needle. The coordinates of the injections were as follows: AP -0.35 mm, ML ± 0.72 mm, and V -2.3 mm, from bregma based on the mouse stereotaxic atlas (Paxinos & Watson). The animals developed acute EAE clinical signs, which appeared 12 days after immunization. Mice were scored for clinical signs using a score scale: 0, no paralysis; 1, loss of tail tonicity; 2, mild hind limb weakness; 3, complete hind limb paralysis; 4, paralysis of four limbs; 5, total paralysis; and 6, death.

T Cell Proliferation Assay The proliferation response of spleen cells was tested 14 days after the first MOG injection. Four animals were killed by cervical dislocation; the spleens were removed and mechanically dissociated. Splenocytes were washed twice with PBS and placed for 1 h in RPMI 1640 medium supplemented with 2 mM glutamine, 50 μ M 2-mercaptoethanol, antibiotics (100 U/ml penicillin G, 100 μ g/ml streptomycin), and 10% heat-inactivated fetal calf serum (all from Biological Industries) in 37°C. Splenocytes were then counted and plated at a concentration of 3×10^5 cells/well in MSCs' or NTFCs' CM (DMEM incubated for 24 h with the cells) or in DMEM containing 1 ng/ml BDNF, 1 ng/ml NGF, and 0.1 ng/ml GDNF (Peptotech). Subsequently, MOG peptide (2 and 10 μ g/well) or concanavalin A (2 μ g/well, Sigma) was added in triplicate wells. The cells were incubated for 72 h at 37°C in humidified air containing 5% CO₂. ³H thymidine (1 μ Ci/well) was added for the last 16 h of incubation, and the cultures were then harvested and counted using a Matrix 96 Direct beta counter (Packard Instr., Meriden, CT, USA). The proliferative response was

measured using ^3H thymidine incorporation expressed as mean counts per minute recorded in triplicate wells.

Immunohistochemistry At the endpoint of each experiment, animals were perfused with 4% paraformaldehyde, and brains and spinal cords were removed and preserved at -70°C . Serial frozen sections (8 μM thick) of the brains and spinal cords were performed on a microtome cryostat. Sections were rinsed with PBS then placed in citric acid buffer solution, pH 6.0, microwaved until boiling, and allowed to cool slowly to room temperature. Tissues were blocked with 5% normal goat serum in PBS for 1 h. Sections were incubated for 24 h with mouse anti-human nuclear antigen (hNu; 1:50; Chemicon, Millipore, Temecula, CA, USA) and rabbit anti-BDNF 1:100 (Santa Cruz). After washes, the sections were incubated with Alexa Fluor 568-conjugated goat anti-rabbit (1:200; Molecular Probes). For hNu staining, biotin goat anti-mouse (Zymed, Invitrogen) followed by streptavidin-conjugated Alexa Fluor 488 (1:200; Molecular Probes, Invitrogen). Nuclear DNA was stained with DAPI (1:1,000, Sigma). Sections were covered with fluorescence mounting medium (DAKO, Glostrup, Denmark). Slides were visualized with a fluorescence Olympus IX70-S8F2 microscope. Images were processed using Image-Pro Plus software.

Statistical Analysis Statistical analysis of data sets was carried out with the aid of SPSS for windows (version 13.0). In Fig. 2a, data were analyzed by one-way analysis of variance (ANOVA) with repeated measures within subjects' factor time and with repeated measures within subjects' factor group, followed by post hoc Tukey's test. In Fig. 2b, survival analysis is displayed as a Kaplan–Meier curve. In Figs. 3c and 4a, b, data were analyzed by ANOVA followed by multiple paired comparisons (Scheffe's test). Significance was considered for $p < 0.05$.

Results

Isolation and Characterization of Human Bone Marrow MSCs MSCs were produced from freshly harvested adult human bone marrow aspirates. After two to five passages, cells were characterized according to the position paper from Dominici et al. (2006). Cell surface phenotype was determined using FACS caliber. Human MSCs were found to ubiquitously express CD29, CD44, CD73, and CD105 (>95%), whereas hematopoietic markers such as CD14, CD34, and CD45 were absent (<1%). Mesenchymal differentiation capacity of MSCs was confirmed by successful adipogenic and osteogenic differentiation as described in our previous work (Blondheim et al. 2006).

Figure 1 Differentiated human NTFCs produce enhanced levels of neurotrophic factors. **a–h** Immunocytochemistry analysis of neurotrophic factors expression by NTFCs before and after induction of differentiation. Untreated MSCs expressed baseline levels of BDNF, NGF, and CNTF and did not express GDNF, while the neurotrophic factors expression was much higher in the differentiated NTFCs. Photographs were taken at $\times 200$. **i** Real-time PCR analysis shows higher levels of gene expression of the neurotrophic factors BDNF, NGF, CNTF, EGF, and GDNF in differentiated NTFCs compared with MSCs ($p < 0.05$ for all levels of gene expression in NTFCs compared to parental MSCs). **j** ELISA analysis of NTFCs' conditioned media shows secretion levels of BDNF, NGF, and GDNF (amounts are normalized for 10^6 cells)

NTFCs Express Neurotrophic Factors In vitro analysis of the NTFCs revealed their neurotrophic factor expression profile. The NTFCs highly expressed the mRNA and proteins of GDNF, BDNF, NGF, and CNTF (Fig. 1a–i). Furthermore, ELISA analysis revealed that the differentiated NTFCs are also capable of secreting the neurotrophic factors BDNF, GDNF, and NGF (Fig. 1j).

NTFCs and MSC Transplantation Improves Motor Function and Suppresses MOG-Induced Chronic EAE Mice In vivo experiments were performed to test the efficacy of MSCs and NTFCs intracerebroventricular injection to suppress EAE. Mice were stereotactically injected with NTFCs, MSCs, or PBS bilaterally to the cerebral ventricles. The mice that received PBS developed clinical symptoms on day 12, while MSCs- and NTFCs-treated mice showed a significant reduction in the severity of the clinical EAE signs. Though not statistically significant, we found a trend that NTFCs were superior in suppressing the disease as compared to MSCs (Fig. 2a). We observed a similar picture evaluating the effect of cell injection on survival. While PBS-injected mice showed 30% survival rate at 30 days from MOG injection, both groups injected with cells showed increased survival at that time point. But while the MSCs-treated group showed 60% survival, the NTFCs-treated group showed a higher survival rate of 80% (Fig. 2b).

NTFCs Promote Neuronal Survival In Vitro Neuroblastoma cell line SH-SY5Y cells die from oxidative stress when exposed to hydrogen peroxide at different concentration (0–500 nM). We found that both MSCs' and NTFCs' CM have a neuroprotective effect on the neuroblastoma cell line at different H_2O_2 concentrations (Fig. 3). However, the NTFCs' CM protected almost all of the insulted cells, while the MSCs exerted only partial neuroprotection (at 50 nM H_2O_2 , $74.19 \pm 3.36\%$ versus $100 \pm 6\%$ survival in MSCs' or NTFCs' conditioned medium, respectively; at 100 nM H_2O_2 , $57.98 \pm 4.45\%$ versus $92 \pm 15.79\%$ survival in MSCs' or in NTFCs' CM, respectively, $p < 0.05$).

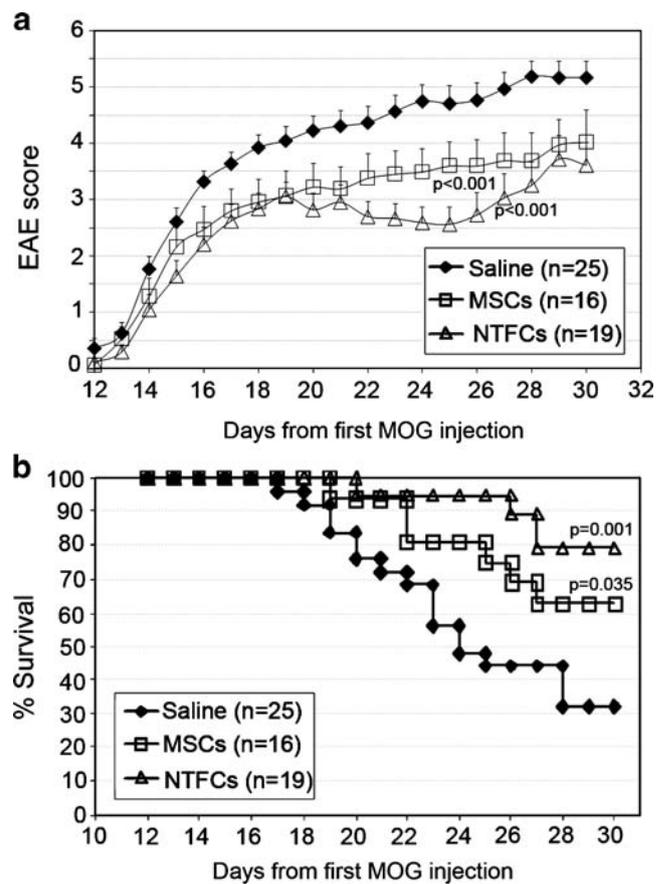
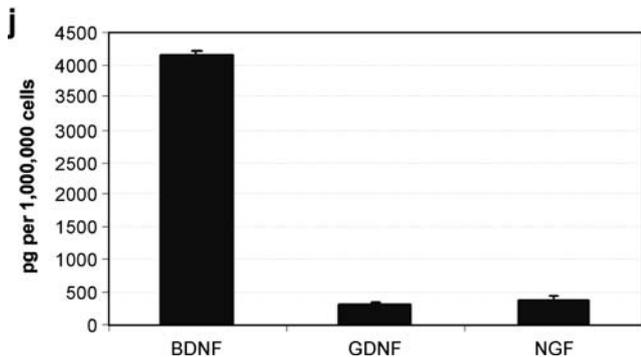
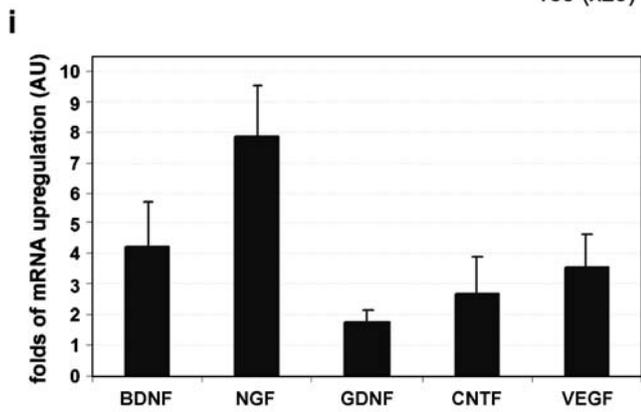
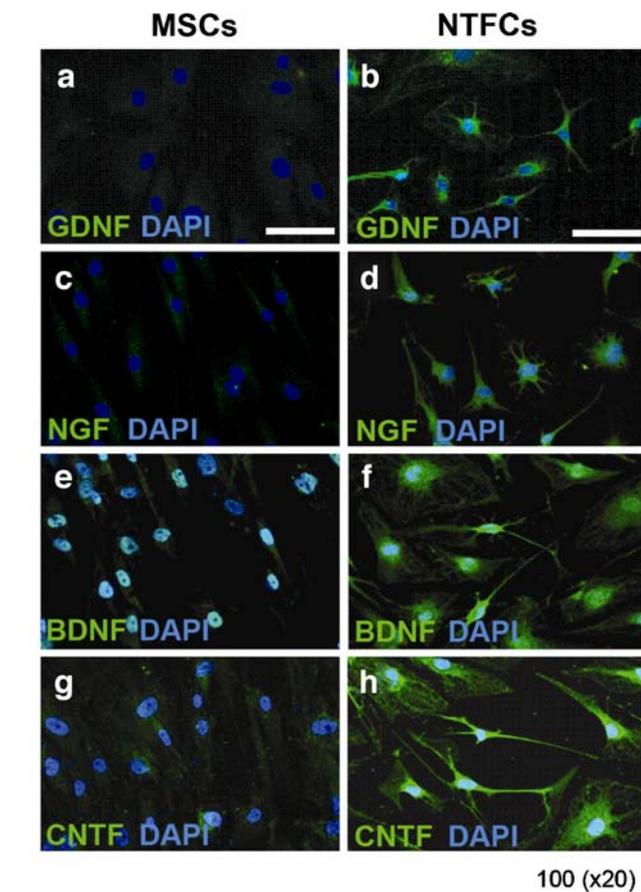


Figure 2 EAE clinical score and survival of EAE mice following bilateral ICV transplantation of human mesenchymal stem cells or differentiated human neurotrophic factors secreting cells (*NTFCs*). MOG-EAE-induced mice were injected with human MSCs, human *NTFCs*, or with PBS as control 6 days after MOG injection. **a** Clinical score and **b** survival rate were monitored through a month from first MOG injection (*p* values are presented in relation to saline-injected group)

NTFCs' Conditioned Medium Reduces MOG-Induced Immune Cells' Proliferation Spleen cells were isolated from EAE-induced mice 14 days after the first MOG injection. The proliferation response of spleen cells to MOG was tested when the cells were grown in freshly prepared serum media (considered as 100% baseline proliferation) compared to CM collected from *NTFCs* or *MSCs*. The cells were exposed to MOG at different concentration (2 and 10 $\mu\text{g/ml}$), and cell proliferation was analyzed by ^3H thymidine incorporation (Fig. 4a). Both *MSCs'* and *NTFCs'* CM decreased the proliferation response of the spleen cells following exposure to 0.2 $\mu\text{g/ml}$ MOG insult, with no significant difference between *MSCs'* and *NTFCs'* CM (29.26 \pm 6.22% proliferation in *MSCs'* CM, $p<0.05$, and 37.07 \pm 4.4% in *NTFCs'* CM, $p<0.05$). However, when insulted with higher MOG concentrations (10 $\mu\text{g/ml}$), only *NTFCs'* CM, but not *MSCs'* CM, significantly reduced splenocyte proliferation (72.87 \pm 11.82% proliferation in *MSCs'* CM and 44.28 \pm 3.15% in

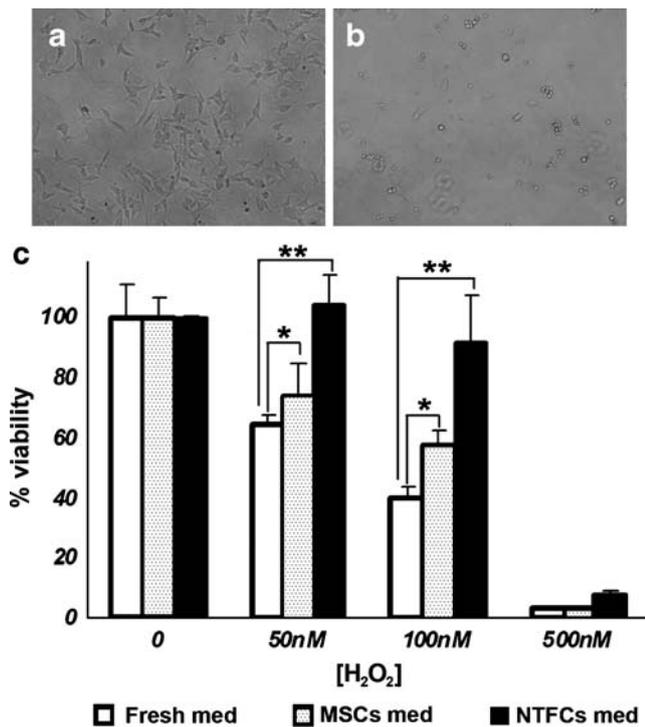


Figure 3 Differentiated NTFC-conditioned medium protect neuroblastoma cell line SH-SY5Y from oxidative stress: **c** SH-SY5Y cells were incubated in freshly prepared medium or conditioned media collected from MSCs or differentiated NTFCs. The cells were exposed to hydrogen peroxide at different concentration (0–500 nM), and cell viability was analyzed using the MTT method (* $p < 0.05$, ** $p < 0.005$). **a** SHSY-5Y cells exposed to H₂O₂ while cultured in conditioned medium collected from differentiated NTFCs. **b** SHSY-5Y cells exposed to H₂O₂ while cultured in fresh medium

NTFCs' CM, $p < 0.05$). Notably, spleen cells exposed to concanavalin A, which stimulates nonspecific T cell proliferation, also showed suppressed proliferation following incubation in MSCs' or NTFCs' CM.

In order to evaluate the effect of secreted neurotrophic factors on spleen cells obtained from EAE mice, splenocytes were incubated either in freshly prepared serum-free medium supplemented with human recombinant GDNF, BDNF, and NGF or in NTFCs' CM (Fig. 4b). The results showed that the supplementation of human recombinant neurotrophic factors indeed suppressed the splenocyte proliferation, though to a lesser extent than the NTFCs' conditioned medium in 10 $\mu\text{g}/\text{ml}$ MOG concentration ($38 \pm 10\%$ proliferation in NTFCs' conditioned medium and $53 \pm 13\%$ proliferation in serum-free medium supplemented with recombinant factors, $p < 0.05$).

NTFCs Survive in EAE Mice Brain for 30 Days Six days after the first MOG injection, we injected differentiated NTFCs, untreated MSCs, or PBS ICV. The animals were analyzed for EAE scoring during a month for disease symptoms (as described above). After a month, sections of

EAE mice brains were stained with specific anti-human nucleus antigen antibodies to identify human cells. hNu-positive cells were found around the lateral ventricles of the NTFC-transplanted animals that survived (Fig. 5). Moreover, some of the human cells were positively stained for BDNF.

Discussion

In this manuscript, we describe the therapeutic advantage of ICV transplantation of human MSCs induced to secrete neurotrophic factors in a chronic EAE mouse model. Various mechanisms can potentially underlie this stem cell transplantation therapeutic effect in MS/EAE: (1) neuroprotection of the host's CNS mediated by the transplanted

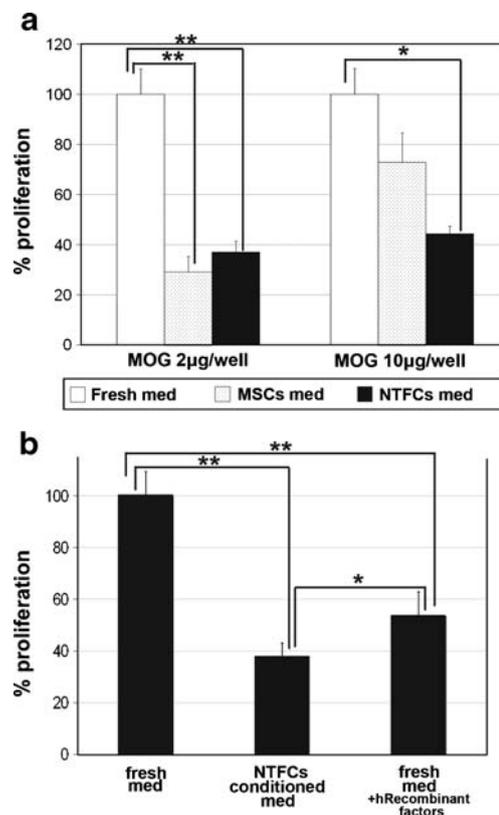
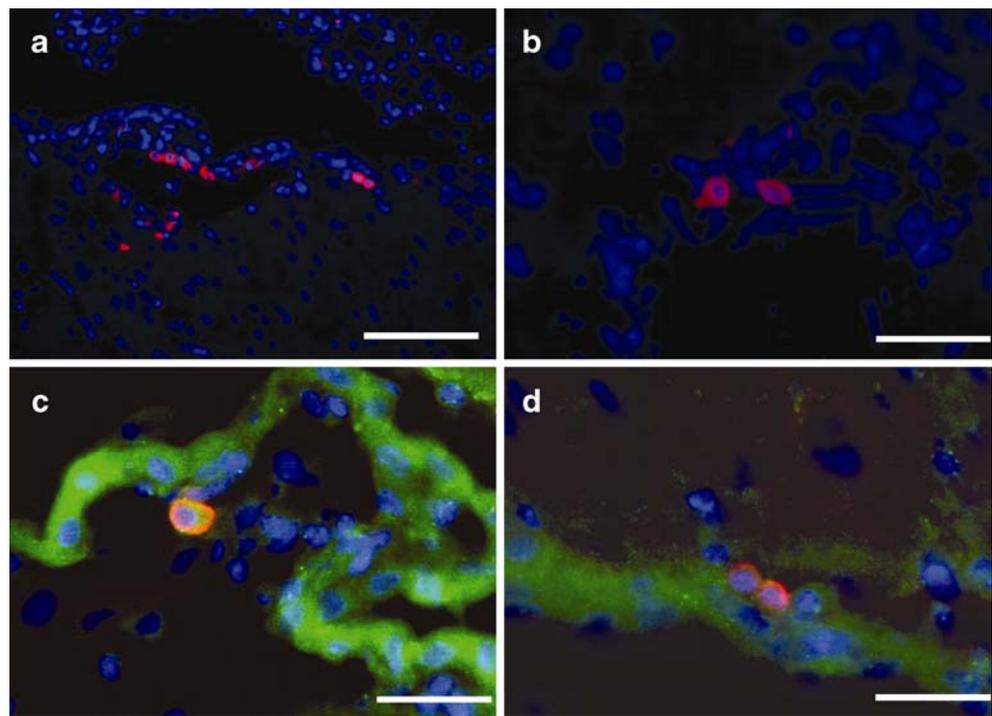


Figure 4 Differentiated human NTFC-conditioned medium reduces EAE mice splenocyte proliferation in response to MOG: **a** splenocytes were incubated with conditioned media collected from MSCs or differentiated NTFCs. The cells were exposed to MOG at different concentration (2 and 10 $\mu\text{g}/\text{ml}$), and cell proliferation was analyzed by ³H thymidine incorporation. **b** Splenocyte cells were grown either in freshly prepared serum-free DMEM, in conditioned media collected from differentiated NTFCs, or in freshly prepared serum-free DMEM supplemented with human recombinant factors, 1 ng/ml BDNF, 1 ng/ml NGF, and 0.1 ng/ml GDNF. Cell proliferation was analyzed by ³H thymidine incorporation (* $p < 0.05$, ** $p < 0.005$)

Figure 5 Immunohistochemistry staining of brain section: **a–d** sections of EAE mice brains 30 days posttransplantation were stained with anti-hNu (*red*), anti-BDNF (*green*), and with DAPI (*blue*). Cells positive to hNu and BDNF were found around the lateral ventricles (scale bars in **a**=100 μ m, in **b** and **d**=50 μ m, and in **c**=25 μ m)



stem cells' interaction with the host cells through cell–cell contact or secretion of factors (Pisati et al. 2007), (2) immunomodulation of host cells induced systemically or locally by the transplanted stem cells (Uccelli et al. 2006; Fibbe et al. 2007), and (3) remyelination of the affected CNS lesions executed by functional transplanted cells (Keirstead 2005).

MSCs have not yet been reported to undergo convincing functional oligodendrocyte differentiation *in vitro*. Previous works describing MSC transplantation in EAE models have shown little evidence of oligodendrocyte transdifferentiation (Zhang et al. 2005). Therefore, it would be fair to assume that, in our work, the transplanted cells did not remyelinate the affected areas in the CNS. However, functional *in vitro* characterization of our NTFCs strongly imply that the therapeutic advantage of NTFCs could be attributed to their neuroprotective properties, as shown in the NTFCs' supernatant capacity to protect the neuronal cell line SH-SY5Y cells from oxidative stress, and to their immunomodulating ability, as shown in the NTFCs' supernatant capacity to suppress the EAE mice spleen cell reaction to MOG.

The observed capacity of the MSCs and NTFCs to suppress MOG-induced spleen cells proliferation fall in place with a previous report indicating that murine MSCs exert their therapeutic effect through inhibiting the specific T cell response against MOG in a similar EAE model (Zappia et al. 2005). In that study, cells were injected intravenously and were found to rapidly migrate to the spleen and draining lymph nodes. We cannot, however,

conclude that this suppression was MOG specific, as we observed significant suppression of proliferation of cells exposed to concanavalin A.

Another recent study involving MSC transplantation to a similar MOG-induced mice EAE model compared the administration of mouse MSC intravenously (IV) or ICV (Kassis et al. 2008). Though the clinical outcome was positive in both administration methods, the authors conclude that ICV injection is superior to IV since it results in a more local immunomodulating outcome in the lesioned sites as manifested in a significant decrease in lymphocytic infiltrates observed in ICV injected mice compared with IV injected animals.

The therapeutic benefit of MSC transplantation in EAE animal models has already been demonstrated (Zhang et al. 2005, 2006; Kassis et al. 2008; Zappia et al. 2005; Gerdoni et al. 2007). Nonetheless, our study demonstrates the added value of inducing the cells to differentiate to NTFCs prior to transplantation as the animals treated with NTFCs displayed a better clinical outcome than the MSC-transplanted mice. *In vitro* analysis of the human NTFCs revealed that they express and secrete high levels of factors that are known for their crucial role in neuroprotection and neuroregeneration in multiple sclerosis (Loeb 2007). Among the secreted factors, the NTFCs were specifically found to express and secrete high levels of BDNF, which was reported to play a key role in the neuroprotective aspects of the two leading drugs prescribed for MS patients, glatiramer acetate, and interferon- β (Ziemssen et al. 2002; Caggiula et al. 2006). Moreover, a recent report described

the positive effect of BDNF administration (through transplantation of genetically engineered bone marrow stem cells) to EAE mice (Makar et al. 2008b). Another factor found to be secreted in the NTFCs is NGF, which was reported to be crucial for the growth and differentiation of brain precursor cells in the subventricular zone of EAE rats (Triaca et al. 2005). Furthermore, in a report describing the beneficial effect of human MSC transplantation to EAE mice, it was demonstrated that the mechanism underlying the clinical improvement is an increase in NGF expression in the transplanted mice brains (Zhang et al. 2006).

The capacity to home to damaged sites in the CNS is a crucial aspect when attempting to employ cell therapy in MS due to the multifocal nature of the disease. MSCs are known for their migratory properties owing to their eclectic expression of chemokine receptors and ligands (Ponte et al. 2007; Ringe et al. 2007). In our lab, we have shown the impressive migration capacities of transplanted MSCs and NTFCs in the damaged brain (Sadan et al. 2008, 2009; Bahat-Stroomza et al. 2009; Hellman et al. 2006). In addition to their capacity to migrate, we have demonstrated that the NTFCs are capable of suppressing immune cell reaction to MOG. Exposure of these immune cells to a medium containing human recombinant neurotrophic factors demonstrated that these factors, at least to some extent, are involved in the immune suppression. The NTFCs were also efficient in protecting neuronal cell line from oxidative stress, demonstrating neuroprotective superiority over untreated MSCs. Taken together, the NTFCs' traits (migration and neurotrophic factor secretion capacity) imply that the paracrine function of the cells mediates the clinical improvement observed in the mice described in this work, either through immunomodulation, neuroprotection, or possibly other cell–cell interactions. Hopefully our results will lay the foundation for possible autologous stem cell regenerative therapy for MS patients in the future.

Acknowledgment The work presented in this manuscript was supported, in part, by the Norma and Alan Aufzein Chair for Parkinson's Disease Research.

References

- Aggarwal S, Pittenger MF (2005) Human mesenchymal stem cells modulate allogeneic immune cell responses. *Blood* 105:1815–1822
- Archer D, Cuddon P, Lipsitz D, Duncan I (1997) Myelination of the canine central nervous system by glial cell transplantation: a model for repair of human myelin disease. *Nat Med* 3:54–59
- Bahat-Stroomza M, Barhum Y, Levy YS, Karpov O, Bulvik S, Melamed E, Offen D (2009) Induction of adult human bone marrow mesenchymal stromal cells into functional astrocyte-like cells: potential for restorative treatment in Parkinson's disease. *J Mol Neurosci* 39:199–210
- Barzilay R, Levy SY, Melamed E, Offen D (2006) Adult stem cells for neuronal repair. *Isr Med Assoc J* 8:61–66
- Blondheim NR, Levy YS, Ben-Zur T, Burshtein A, Cherlow T, Kan I, Barzilay R, Bahat-Stroomza M, Barhum Y, Bulvik S, Melamed E, Offen D (2006) Human mesenchymal stem cells express neural genes, suggesting a neural predisposition. *Stem Cells Dev* 15:141–164
- Caggiula M, Batocchi AP, Frisullo G, Angelucci F, Patanella AK, Sancricca C, Nociti V, Tonali PA, Mirabella M (2006) Neurotrophic factors in relapsing remitting and secondary progressive multiple sclerosis patients during interferon beta therapy. *Clin Immunol* 118:77–82
- 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* 59:687–691
- Dominici M, Le-Blanc K, Mueller I, Slaper-Conterbach I, Marini FC, Krause DS, Deans RJ, Keating A, Prockop DJ, Horwitz EM (2006) Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. *Cytotherapy* 8:315–317
- Einstein O, Fainstein N, Vaknin I, Mizrahi-Kol R, Reihartz E, Grigoriadis N, Lavon I, Baniyash M, Lassman H, Ben-Hur T (2007) Neural precursors attenuate autoimmune encephalomyelitis by peripheral immunosuppression. *Ann Neurol* 61:209–218
- Fibbe WE, Nauta AJ, Roelofs H (2007) Modulation of immune responses by mesenchymal stem cells. *Ann N Y Acad Sci* 1106:278
- Gerdoni E, Gallo B, Casazza S, Musio S, Bonanni I, Pedemonte E, Mantegazza R, Frassoni F, Mancardi G, Pedotti R, Uccelli A (2007) Mesenchymal stem cells effectively modulate pathogenic immune response in experimental autoimmune encephalomyelitis. *Ann Neurol* 61:219–227
- Gordon D, Pavlovska G, Glover CP, Uney JB, Wraith D, Scolding NJ (2008) Human mesenchymal stem cells abrogate experimental allergic encephalomyelitis after intraperitoneal injection, and with sparse CNS infiltration. *Neurosci Lett* 448:71–73
- Hellman MA, Panet H, Barhum Y, Melamed E, Offen D (2006) Increased survival and migration of engrafted mesenchymal bone marrow stem cells in 6-hydroxydopamine-lesioned rodents. *Neurosci Lett* 395:124–128
- Hemmer B, Nessler S, Zhou D, Kieseier B, Hartung H (2006) Immunopathogenesis and immunotherapy of multiple sclerosis. *Nature Clinical Practice Neurology* 2:201–211
- Herzog EL, Chai L, Krause DS (2003) Plasticity of marrow-derived stem cells. *Blood* 102:3483–3493
- Kassir I, Grigoriadis N, Gowda-Kurkalli B, Mizrahi-Kol R, Ben-Hur T, Slavin S, Abramsky O, Karussis D (2008) Neuroprotection and immunomodulation with mesenchymal stem cells in chronic experimental autoimmune encephalomyelitis. *Arch Neurol* 65:753–761
- Keirstead HS (2005) Stem cells for the treatment of myelin loss. *Trends Neurosci* 28:677–683
- Le Blanc K, Ringden O (2007) Immunomodulation by mesenchymal stem cells and clinical experience. *J Intern Med* 262:509–525
- Lianhua B, Lennon DP, Eaton V, Maier K, Caplan AI, Miller SD, Miller RH (2009) Human bone marrow-derived mesenchymal stem cells induce Th2-polarized immune response and promote endogenous repair in animal models of multiple sclerosis. *Glia* 57:1192–1203
- Loeb JA (2007) Neuroprotection and repair by neurotrophic and gliotrophic factors in multiple sclerosis. *Neurology* 68:S38–S42
- Makar TK, Trisler D, Bever CT, Goolsby JE, Sura KT, Balasubramanian S, Sultana S, Patel N, Ford D, Singh IS, Gupta A, Valenzuela RM, Dhib-Jalbut S (2008a) Stem cell based delivery of IFN- β reduces relapses in experimental autoimmune encephalomyelitis. *J Neuroimmunol* 196:67–81

- Makar TK, Trisler D, Sura KT, Sultana S, Patel N, Bever CT (2008b) Brain derived neurotrophic factor treatment reduces inflammation and apoptosis in experimental allergic encephalomyelitis. *J Neurol Sci* 270:70–76
- Noseworthy JH, Lucchinetti C, Rodriguez M, Weinshenker BG (2000) Multiple sclerosis. *N Engl J Med* 343:938–952
- Offen D, Gilgun-Sherki Y, Barhum Y, Benhar M, Grinberg L, Reich R, Melamed E, Atlas D (2004) A novel low molecular weight antioxidant crosses the blood brain barrier and attenuates experimental autoimmune encephalomyelitis. *J Neurochem* 89:1241–1251
- Payne N, Siatskas C, Bernard CCA (2008) The promise of stem cell and regenerative therapies for multiple sclerosis. *J Autoimmun* 31:288–294
- Pisati F, Bossolasco P, Meregalli M, Cova L, Belicchi M, Gavina M, Marchesi C, Calzarossa C, Soligo D, Lambertenghi-Delilieri G, Bresolin N, Silani V, Torrente Y, Polli E (2007) Induction of neurotrophin expression via human adult mesenchymal stem cells: implication for cell therapy in neurodegenerative diseases. *Cell Transplant* 16:41–55
- Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, Moorman MA, Simonetti DW, Craig S, Marshak DR (1999) Multilineage potential of adult human mesenchymal stem cells. *Science* 284:143–147
- Pluchino S, Martino G (2005) The therapeutic use of stem cells for myelin repair in autoimmune demyelinating disorders. *J Neurol Sci* 233:117–119
- Pluchino S, Quattrini A, Brambilla E, Gritti A, Salani G, Dina G, Galli R, Del Carro U, Amadio S, Bergami A, Furlan R, Comi G, Vescovi AL, Martino G (2003) Injection of adult neurospheres induces recovery in a chronic model of multiple sclerosis. *Nature* 422:688–694
- Pluchino S, Zanotti L, Rossi B, Brambilla E, Ottoboni L, Salani G, Martinello M, Cattalini A, Bergami A, Furlan R, Comi G, Constantin G, Martino G (2005) Neurosphere-derived multipotent precursors promote neuroprotection by an immunomodulatory mechanism. *Nature* 436:266–271
- Ponte AL, Marais E, Galloway N, Langonne A, Delorme B, Hérault O, Charbord P, Domenech J (2007) The in vitro migration capacity of human bone marrow mesenchymal stem cells: comparison of chemokine and growth factor chemotactic activities. *Stem Cells* 25:1737–1745
- Ringe J, Strassburg S, Neuman K, Endres M, Notter M, Gerd-Rudiger B, Kaps C, Sittlinger M (2007) Towards in situ tissue repair: human mesenchymal stem cells express chemokine receptors CXCR1, CXCR2 and CCR2, and migrate upon stimulation with CXCL8 but not CCL2. *J Cell Biochem* 101:135–146
- Sadan O, Shemesh N, Barzilay R, Bahat-Stromza M, Melamed E, Cohen Y, Offen D (2008) Migration of neurotrophic factor-secreting mesenchymal stem cells towards a quinolinic acid lesion as viewed by MRI. *Stem Cells* 26:2542–2551
- Sadan O, Bahat-Stroomza M, Barhum Y, Levy YS, Pisneysky A, Peretz H, Bar Ilan A, Bulvik S, Shemesh N, Krepel D, Cohen Y, Melamed E, Offen E (2009) Protective effects of neurotrophic factor-secreting cells in a 6-OHDA rat model of Parkinson disease. *Stem Cells Dev* 18(8):1179–1190
- Triaca V, Tirassa P, Aloe L (2005) Presence of nerve growth factor and TrkA expression in the SVZ of EAE rats: evidence for a possible functional significance. *Exp Neurol* 191:53–64
- Uccelli A, Moretta L, Pistoia V (2006) Immunoregulatory function of mesenchymal stem cells. *Eur J Immunol* 36:2566–2573
- Whitham RH, Bourdette DN, Hashim GA, Herndon RM, Ilg RC, Vandenbark AA, Offner H (1991) Lymphocytes from SJL/J mice immunized with spinal cord respond selectively to a peptide of proteolipid protein and transfer relapsing demyelinating experimental autoimmune encephalomyelitis. *J Immunol* 146:101–107
- Zappia E, Casazza S, Pedemonte E, Benvenuto F, Bonanni I, Gerdoni E, Giunti D, Ceravolo A, Cazzanti F, Frassoni F, Mancardi G, Uccelli A (2005) Mesenchymal stem cells ameliorate experimental autoimmune encephalomyelitis inducing T-cell anergy. *Blood* 106:1755–1761
- Zhang J, Li Y, Chen J, Cui Y, Lu M, Elias SB, Mitchell JB, Hammill L, Vanguri P, Chopp M (2005) Human bone marrow stromal cell treatment improves neurological functional recovery in EAE mice. *Exp Neurol* 195:16–26
- Zhang J, Li Y, Lu M, Cui Y, Chen J, Noffsinger L, Elias SB, Chopp M (2006) Bone marrow stromal cells reduce axonal loss in experimental autoimmune encephalomyelitis mice. *J Neurosci Res* 84:587–595
- Ziemssen T, Kumpfel T, Klinkert WEF, Neuhaus O, Hohlfeld R (2002) Glatiramer acetate-specific T-helper 1- and 2-type cell lines produce BDNF: implications for multiple sclerosis therapy. *Brain* 125:2381–2391