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Direct Intrathecal Implantation of Mesenchymal Stromal Cells Leads to Enhanced Neuroprotection via an NFκB-Mediated Increase in Interleukin-6 Production

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Mesenchymal stromal cell (MSC) therapy has shown promise for the treatment of traumatic brain injury (TBI). Although the mechanism(s) by which MSCs offer protection is unclear, initial in vivo work has suggested that modulation of the locoregional inflammatory response could explain the observed benefit. We hypothesize that the direct implantation of MSCs into the injured brain activates resident neuronal stem cell (NSC) niches altering the intracerebral milieu. To test our hypothesis, we conducted initial in vivo studies, followed by a sequence of in vitro studies. In vivo: Sprague-Dawley rats received a controlled cortical impact (CCI) injury with implantation of 1 million MSCs 6 h after injury. Brain tissue supernatant was harvested for analysis of the proinflammatory cytokine profile. In vitro: NSCs were transfected with a firefly luciferase reporter for NFκB and placed in contact culture and transwell culture. Additionally, multiplex, quantitative PCR, caspase 3, and EDU assays were completed to evaluate NSC cytokine production, apoptosis, and proliferation, respectively. In vivo: Brain supernatant analysis showed an increase in the proinflammatory cytokines IL-1α, IL-1β, and IL-6. In vitro: NSC NFκB activity increased only when in contact culture with MSCs. When in contact with MSCs, NSCs show an increase in IL-6 production as well as a decrease in apoptosis. Direct implantation of MSCs enhances neuroprotection via activation of resident NSC NFκB activity (independent of PI3 kinase/AKT pathway) leading to an increase in IL-6 production and decrease in apoptosis. In addition, the observed NFκB activity depends on direct cell contact.

Introduction

Approximately 1.5 million people suffer traumatic brain injury (TBI) yearly in the United States. The annual mortality approaches 50,000 with the remaining patients suffering from varying levels of long-term sequelae [1]. Overall, 6.5 million patients are burdened by the physical, cognitive, and psychosocial deficits associated with TBI [2] leading to a total economic impact of ~60 billion dollars [3,4].

Bone marrow and umbilical cord blood-derived mesenchymal stromal cells (MSCs) have shown potential as a novel therapy for a number of central nervous system disorders including TBI [5], ischemic stroke [6], and spinal cord injury [7]. Initial in vivo research has shown MSC engraftment at the site of injury with possible differentiation into neuronal cells [8,9]; however, despite the promising results of initial studies, doubt remains about the frequency of transdifferentiation and the clinical significance of cellular engraftment [4,10,11].

While the mechanism of action remains unknown, modulation of inflammatory cytokine production could represent a mechanism for neuroprotection. In vitro, human MSCs cocultured with subpopulations of immune cells showed an increase in production of the anti-inflammatory cytokines interleukins (IL)-4 and IL-10 with a concomitant decrease in the proinflammatory cytokines tumor necrosis factor (TNF)-α and interferon (IFN)-γ [12]. Additionally, intravenous infusion of MSCs was associated with an increase in serum IL-10 in a murine sepsis model [13]; however, the effect of MSCs on the intracerebral microenvironment after neural injury remains controversial. Recently, MSCs directly implanted into the normal rat hippocampus were found to stimulate an inflammatory response as evidenced

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by infiltration of ED1-positive microglia/macrophages and astrogliosis with failure of engraftment at 14 days [14].

After direct intracerebral implantation in a TBI model, MSCs are in contact with neurons, astrocytes, glial cells, and neural stem cells (NSCs). Endogenous NSC proliferation is thought to be critical to the neuroreparative response to injury. The focus of this study was to investigate the effect of MSC implantation on cytokine production in vivo and to evaluate the potential role of NF-kappa B (NFκB) activation in NSCs in vitro. Previous work has shown that late NFκB activation can provide endogenous neuroprotection and up-regulation of antiapoptotic molecules in a hypoxia–ischemia model [15]. To test our hypothesis, a series of in vivo and in vitro experiments were performed to evaluate potential MSC modulation of inflammatory cytokine production focusing on an NFκB-dependent pathway.

Experimental Design

In vivo

Three groups ($n = 6$) of Sprague-Dawley rats underwent controlled cortical impact (CCI) injury (2 groups) or sham injury (1 group). Previously isolated MSCs were stereotactically injected into the injury cavity and penumbra 6 h after injury as previously described [16]. Next, 6 h after MSC injection, the brain tissue was homogenized and the supernatant collected. Using a Bio-Plex cytokine assay system (Bio-Rad Laboratories, Hercules, CA), the intracerebral inflammatory cytokine levels were measured.

The additional 2 groups ($n = 6$) of Sprague-Dawley rats underwent CCI. Six hours after injury 1 group received stereotactic injection of 1 million QDOT-labeled MSCs with the second group serving as a control. The animals were perfused with paraformaldehyde and the brain tissue was fixed. Subsequently, the tissue was sectioned and the brains were viewed using fluorescent microscopy to observe MSC engraftment.

In vitro

To evaluate the change in cytokine production observed in vivo, NSCs were transfected with a firefly luciferase reporter for NFκB. The transfected cells were placed in direct contact co-culture with MSCs (1 group stimulated with TNFα) to investigate the effect of increasing MSC concentration on NFκB activation. Furthermore, NSCs were placed in transwell (non-contact) co-culture to evaluate the need for direct cell contact and transfected with dominant negative vectors for IKKβ, AKT (K179M), and the PI3 kinase inhibitor LY294002 to analyze the potential mechanism of NFκB activation. Of note, all NFκB transfection groups contained 4 samples ($n = 4$).

In order to investigate inflammatory cytokine production in vitro, 2 groups of NSCs and MSCs were placed in direct contact co-culture (1 group stimulated with TNFα; $n = 6$). After 6 h of incubation, cytokine levels were measured in the media using the Bio-Plex cytokine assay system (Bio-Rad Laboratories, Hercules, CA). To further investigate the source of IL-6 production, a quantitative real-time PCR was completed on separate groups of direct contact co-cultures after flow cytometric sorting of MSCs based upon CD29 and Stro-1 positivity.

Next, 2 groups ($n = 3$) of NSCs and MSCs were placed in direct contact co-culture (1 group stimulated with TNFα for 6 h). Using an EdU proliferation assay kit (Invitrogen, Carlsbad, CA), the fraction of actively dividing NSCs was calculated using flow cytometry in order to investigate the effect of MSCs on NSC proliferation in vitro.

Additionally, separate NSC/MSC co-cultures ($n = 3$) were placed in glucose-free media and incubated in an oxygen-free environment for 6 h to stress the cells. Using a caspase 3 antibody (Cell Signaling Inc., Danvers, MA), the fraction of apoptotic NSCs was measured using flow cytometry.

Methods

All protocols involving the use of animals were in compliance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* and were approved by the University of Texas Institutional Animal Care and Use Committee (protocol HSC-AWC-07-055).

Data Analysis

Unless otherwise indicated, all values are represented as mean ± SEM. Values were compared using analysis of variance (ANOVA) with a post-hoc Tukey analysis. A P value of ≤ 0.05 was used to denote statistical significance.

In vivo

Controlled cortical impact injury. A CCI device (eCCI Model 6.3; VCU, Richmond, VA) was used to administer a unilateral brain injury as described previously [17]. The 225–250 g male rats were anesthetized with 4% isoflurane and a 1:1 mixture of N_2O/O_2 and the head was mounted in a stereotactic frame. With the head held in a horizontal plane, a midline incision and subsequent 7- to 8-mm craniectomy was performed on the right cranial vault. The center of the craniectomy was placed at the midpoint between bregma and lambda, 3 mm lateral to the midline, overlying the tempoparietal cortex. Animals received a single impact of 3.1-mm depth of deformation with an impact velocity of 5.8 m/s and a dwell time of 150 ms (moderate-to-severe injury) at an angle of 10° from the vertical plane using a 6-mm diameter impactor tip, making the impact orthogonal to the surface of the cortex. The impact was delivered onto the parietal association cortex. Sham injuries were performed by anesthetizing the animals, making the midline incision, and separating the skin, connective tissue, and aponeurosis from the cranium. The incision was then closed. The body temperature was maintained at 37°C by the use of a heating pad. Previously obtained serial arterial PaO_2 and $PaCO_2$ measurements have shown that animals do not become hypoxic or hypercarbic during this procedure [16].

Preparation and intracerebral placement of MSCs. After expansion per protocol, MSCs were collected and suspended in the phosphate-buffered saline (PBS) vehicle. Cells were checked for viability via Trypan blue exclusion and counted using a hemocytometer. MSCs were then suspended at a concentration of 10,000 c/μL. Immediately prior to intracerebral cell placement, the cells were titrated gently 8–10 times to ensure a homogeneous mixture of cells.

Six hours after CCI, rats were again anesthetized as described earlier and the head was mounted in a stereotactic

frame. The midline incision was opened and the injured brain was exposed. A 25-gauge microinjection needle was used to stereotactically guide the placement of the cells in the area of injury and the penumbral area. At all 12 injection sites, 8.3 μL PBS containing 83,000 MSCs was injected over 10 s at 2–3 mm below the cortex, for a total placement of 1×10^6 MSCs per treatment animal. Sham treatment animals underwent identical procedures, with the injections only consisting of the PBS vehicle [18]. The incisions were closed and the animals allowed to recover.

Brain homogenate supernatant fluid collection. Three groups of 6 rats underwent a CCI injury (2 groups) or sham injury (1 group). The groups were sacrificed at 6 h after CCI or sham injury. Their brains were extracted and 4 regions (consisting of coronal sections of entire hemisphere), relative to the injury, were isolated: the site of direct injury, penumbral region, ipsilateral frontal region, and contralateral region as we have previously described [17]. The sections were weighed to ensure each section was 120 mg, gently minced with a pellet pestle, diluted in low-glucose DMEM (Gibco, Carlsbad, CA), vortexed for 30 s, and centrifuged for 6 min at 1,000*g*. The supernatant, containing intracerebral, interstitial fluid, was collected [16].

Cytokine analysis. Cytokines were detected in the brain homogenate supernatant fluid and NSC/MSC co-culture supernatant using the Bio-Plex cytokine assay system (Bio-Rad Laboratories, Hercules, CA). Concentrations of IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-10, IFN-γ, granulocyte macrophage colony-stimulating factor, and TNFα were simultaneously evaluated using a commercially available multiplex bead-based immunoassay (Rat 9-Plex; Bio-Rad Laboratories). The assay was performed per the manufacturer's instructions and the details have been previously published by our group and others [19,20]. High standard curves (low RP1 target value) for each soluble cytokine were used, ranging from 2 to 32,000 pg/mL. A minimum of 100 beads per cytokine region were evaluated and recorded. Values with a coefficient of variation beyond 10% were not included in final data analysis. All samples were run in duplicate [21].

Immunohistochemistry. Brains from sacrificed rats were placed in paraformaldehyde for at least 24 h. After tissue processing, the tissues were embedded in formalin and cut into ~50 μm sections and placed on slides. After deparafinizing, the tissue sections were stained with 4′,6-diamidino-2-phenylindole (DAPI) (for nuclear visualization) (Invitrogen, Carlsbad, CA) and visualized with fluorescent microscopy.

In vitro

Isolation, characterization, and labeling of rat mesenchymal stem cells. MSCs were isolated from the bone marrow of Sprague-Dawley rats and expanded in MAPC media as previously described [22]. Flow cytometric immunophenotyping was used to ensure that the MSCs were CD11b−, CD45−, CD29+, CD49e+, CD73+, CD90+, CD105+, and Stro-1+. MSCs were labeled with the Qtracker 655 cell-labeling kit (Invitrogen, Carlsbad, CA) per the manufacturer's protocol. Cell-labeling efficiency was greater than 90%. MSCs between passages 3 and 5 were utilized for all experiments.

Isolation and characterization of rat neural stem cells. NSCs were purchased from Alphagenix (Alphagenix, Inc., Sioux Falls, SD). Flow cytometric immunophenotyping was used to ensure the NSCs were nestin+ and GFAP− as we have previously described [18]. The manufacturer's protocol was followed to culture and expand the NSCs as neurospheres. Cells between passages 2 and 4 were utilized for all experiments.

Neural Stem Cell (NSC) transfection. NSCs were allowed to expand in culture using NSC growth media (Invitrogen, Carlsbad, CA). After 48 h in culture, the cells were separated from the plates using 0.25% trypsin and placed into 24-well plates at a density of 2.5×10^4 cells per well. After 24 h of incubation, the cells were transfected with a firefly luciferase NFκB vector and Renilla luciferase standard using Lipofectamine 2000 per the manufacturer's protocol (Invitrogen). In brief, 4 μg of an NFκB vector was placed in 50 μL of OPTIMEM buffer solution and 2 μL of Lipofectamine was added to 48 μL of OPTIMEM. Both were allowed to incubate at room temperature for 5 min. At this point, the 2 solutions were combined and allowed to incubate for an additional 20 min. One hundred microliters of the combined Lipofectamine/NFκB vector was added to each well. After 4 h of incubation, the solution was replaced with warmed media. The samples were allowed to incubate for 24 h.

In addition to the NFκB vector, NSC samples were transfected with and without dominant negative vectors for IKKβ, AKT (K179M), and the PI3 kinase inhibitor LY294002. This was completed by adding 0.25 μg of IKKβ and AKT (K179M) or 20 mM of LY294002 with the NFκB vector at the time of transfection.

Twenty-four hours after transfection, MSCs were placed in direct (contact) co-culture and transwell (no contact) culture with the NSC samples at varying densities to investigate a potential dose response. Next, the samples were stimulated for 6 h with 10 ng/mL of TNFα. The samples were then washed with phosphate-buffered saline and subsequently placed at −80°C for lysis.

Measurement of luciferase activity. Measurement of the NFκB firefly and Renilla luciferase activity was completed with the Dual Luciferase Reporter Assay System (Promega, Madison, WI). In brief, 100 μL of passive lysis buffer solution was added to each sample well. The plates were placed on shaker for 15 min to allow complete cell lysis. For each sample, 10 μL of lysed cell solution was added to 100 μL of primary NFκB luciferase reporter and placed in luminometer (Berthold Inc., Oak Ridge, TN). Of note, the luminometer was set for 2 s lag time with 10 s read time. Once value was recorded, 100 μL of secondary Renilla luciferase reporter was added and sample was replaced in luminometer for measurement.

IL-6 immunohistochemistry. NSCs were allowed to expand in culture using NSC growth media (Invitrogen, Carlsbad, CA). After 48 h in culture, the cells were separated from the plates using 0.25% trypsin and placed into 24-well plates at a density of 2.5×10^4 cells per well. After 24 h incubation, 2.5×10^4 MSCs were placed in direct co-culture with the NSC population. The co-culture was allowed to incubate overnight followed by 6 h of stimulation with 10 ng/mL of TNFα. A conjugated CD29-PE antibody (BD Biosciences, San Jose, CA) was added to differentiate the different cell types (1 h incubation). Next, the co-cultures were fixed with 4% paraformaldehyde (30 min), permeabilized in 0.5% Triton X-100 (30 min), blocked in 4% FBS–PBS (30 min), and incubated with IL-6 antibody (Abcam Inc., Cambridge, MA) overnight. Next, a FITC secondary antibody (Zymed Inc., San Francisco, CA) was added. Finally, the co-cultures were viewed with fluorescent microscopy.

RNA extraction. RNA was isolated from MSCs alone, NSCs alone, MSCs grown in co-culture with NSCs and then separated, and NSCs grown in co-culture with MSCs and then separated using RNA-Bee (Tel-Test, Inc., Friendswood, TX) and following the manufacturer's suggested protocol. All cell separations were performed using a BD Influx™ cell sorter (BD Biosciences, San Jose, CA) utilizing CD29 and Stro-1 as MSC identifying markers. After isolation of RNA, samples were immediately treated with DNase (to remove genomic DNA contamination) and an RNAse inhibitor and stored at −80°C until time of use.

Real-time quantitative PCR. Specific quantitative assays for IL-6 and 36b4 were developed using Beacon Designer, AlleleID (Premier Biosoft, Palo Alto, CA), or RealTimeDesign (Biosearch Technologies, Novato, CA) based on the NCBI refseq sequences. Assay-specific information is provided in Table 1.

cDNA was synthesized in 10 µL total volume by reverse transcription of 1 µL of the RNA stock solution using 300 nM specific reverse primer, 500 µM dNTPs Superscript II buffer and Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA). Each sample was assayed in triplicate plus a control without reverse transcriptase to access for DNA contamination. Each plate also contained an assay-specific sDNA (synthetic amplicon oligo) standard spanning a 5-log template concentration range and a no template PCR control. Each plate was covered and incubated for 30 min at 50°C followed by 75°C for 10 min.

The total volume of reaction (10 µL) was added to 40 µL of PCR mix containing 1× PCR buffer, 300 nM forward and reverse primers, 3 mM $MgCl_2$, Taq Polymerase, and 100 nM fluorogenic probe. Amplification and quantitation based on real-time monitoring of amplification was carried out using an ABI Prism 7700 (Applied Biosystems, Foster City, CA) using the following cycling conditions: 1 min at 95°C followed by 40 cycles of 95°C for 12 s and 60°C for 30 seconds. Values of transcripts in unknown samples was obtained by interpolating their Ct (PCR cycles to threshold) values on a standard curve derived from known amounts of cognate, specific amplicons. Transcript levels were normalized to the level of 36b4 RNA. All determinations were performed in triplicate and with a no RT control.

Proliferation assay. The percentage of NSCs actively proliferating (S phase) when in co-culture with MSCs was calculated using Click-iT™ EdU Flow Cytometry Assay Kit (Invitrogen, Carlsbad, CA). The manufacturer's protocol was followed. In brief, NSCs were placed in co-culture with MSCs as described earlier in this manuscript. One group was stimulated for 6 h with 10 ng/mL of TNFα. At this point 15 mM of EdU was added and allowed to incubate for 1 h. Next, we washed the cells with 4% bovine serum in DMEM (4% FBS) and then added an antibody to CD29 to differentiate the NSCs and MSCs using the flow cytometer. After 30 min of incubation, the cells were washed and fixed with paraformaldehyde. This step was repeated twice to permeabilize the cells and then the anti-EdU antibody "cocktail" provided by the manufacturer was added. Finally, the cells were washed followed by the addition of Ribonuclease and CellCycle488-Red stain to analyze the cell cycle distribution using flow cytometry.

Caspase 3 assay. NSCs were allowed to expand in culture using NSC growth media (Invitrogen, Carlsbad, CA). After 48 h in culture, the cells were separated from the plates using 0.25% trypsin and placed into 6-well plates at a density of 1.0×10^5 cells per well. After 24 h incubation, 1.0×10^5 MSCs were placed in direct co-culture with the NSC population. The co-cultures were washed and glucose-free media was added to each well. Next, the co-cultures were placed in an oxygen-deprived environment for 6 h. The co-cultures were then fixed with 4% paraformaldehyde (30 min), permeabilized in 0.5% Triton X-100 (30 min), blocked in 4% FBS–PBS (30 min) and incubated with FITC-conjugated caspase 3 antibody (Cell Signaling Inc., Danvers, MA) and APC-conjugated nestin (BD Biosciences, San Jose, CA) for 1 h. Finally, using flow cytometry, the caspase positivity was measured.

Results

In vivo

Cytokine analysis of brain tissue lysate 6 h after direct implantation of MSCs in a rat TBI model showed an increase in the cytokines IL-1α (penumbral region), IL-1β (penumbral region), IL-6 (direct injury area and penumbral region), and TNFα (penumbral region) compared to vehicle control (Table 2).

Immunohistochemistry after MSC implantation showed 3%–5% of the implanted MSCs to be located in the brain. Of the MSCs located in the brain, 73% were located on the side ipsilateral to the cortical injury, 58% were found in the ventricles (Fig. 1A), and 26% were found in the direct injury and penumbral areas. Figure 1B shows MSCs located around a needle tract 6 h after direct implantation.

In vitro

NSC/MSC co-culture without TNFα stimulation showed a statistically significant increase in NFκB activity ($P = 0.0004$).

Table 1. Quantitative Assays for IL-6 and 36b4 Developed for Quantitative Real-Time Polymerase Chain Reaction (qPCR)

Transcript	IL-6	36B4
Accn. #	NM_012289	NM_022402
Forward primer	AGCCAGTTGCCTTCTTGG	AGAGGTGCTGGACATCACAG
Reverse primer	CCTCTCCGGACTTGTGAAG	CATTGCGGACACCCTCTAG
Fluorogenic probe	FAM-ACTGATGTTGTTGACAGCCACTGCC-BHQ1	FAM-CAGGCCCTGCACACTCGCTT-BHQ1
Amplicon length	74	62
Lowest limit of detection	200	230
PCR efficiency	~100%	99%

Sequences of forward primer, reverse primer, and probe.

Table 2. Cytokine Concentrations (pg/mL) Measured in Brain Tissue Supernatant From the Direct Injury and Penumbral Areas

	IL-1α	*IL-1β*	*IL-6*	*TNFα*
Direct injury area Sham injury	5 ± 1*	28 ± 4*	34 ± 3*	25 ± 1*
Direct injury area CCI + vehicle control	129 ± 32	3,292 ± 720	323 ± 59	77 ± 9
Direct injury area CCI + MSC implantation	183 ± 28	3,344 ± 517	603 ± 96*	58 ± 8
Penumbral area Sham injury	4 ± 0.2	25 ± 2*	29 ± 3	29 ± 2*
Penumbral area CCI + vehicle control	37 ± 10	1,004 ± 328	131 ± 17	45 ± 7
Penumbral area CCI + MSC implantation	121 ± 19*	2,059 ± 268*	291 ± 45*	52 ± 3

Data analysis shows an increase in interleukin 6 (IL-6) found in the direct injury area after mesenchymal stem cell (MSC) implantation as well as an increase in IL-1α, IL-1β, and IL-6 found in the penumbral area.

Abbreviation: CCI, controlled cortical impact.

*Indicates significant difference ($P < 0.05$) in cytokine production when compared to NSC culture alone based upon ANOVA with Tukey Kramer post hoc.

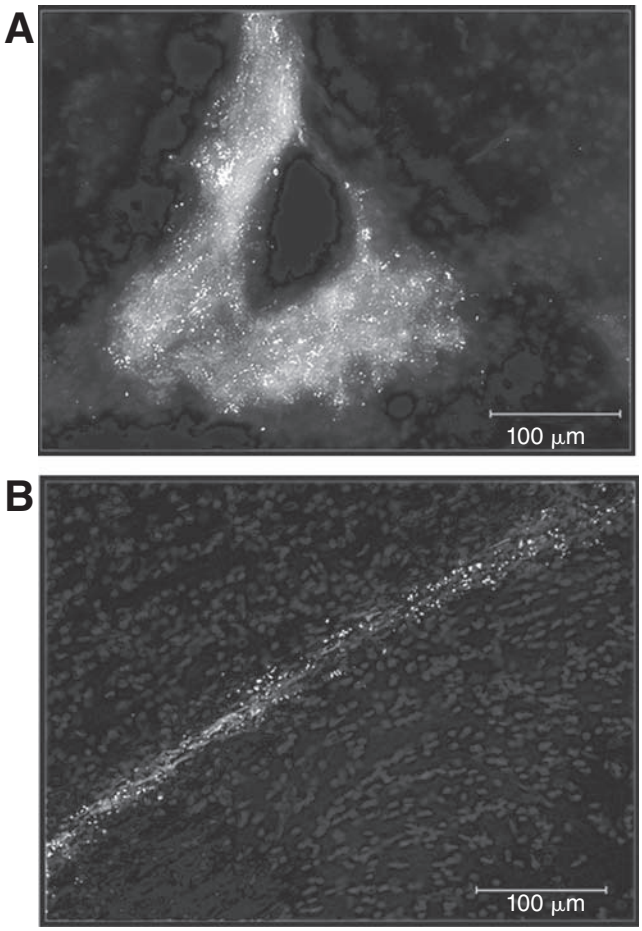

FIG. 1. Mesenchymal stromal cells (MSCs) double labeled: red with quantum dots, green with green fluorescent protein. Nuclei stained with 4′,6-diamidino-2-phenylindole (DAPI). (**A**) Fifty-eight percent of the MSCs were located in the ventricles. (**B**) MSCs located around the needle track transversing the cerebral cortex in the penumbra (10× magnification).

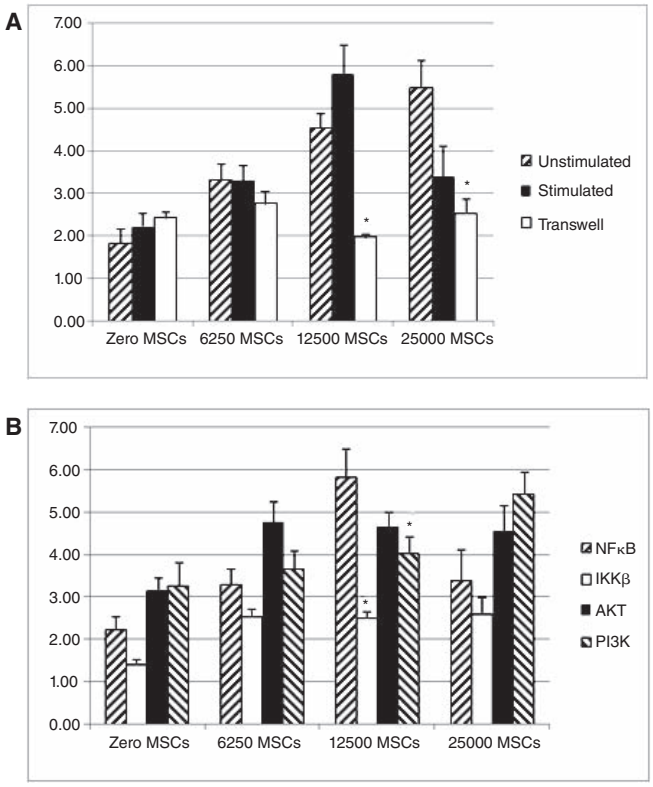

FIG. 2. Nuclear factor kappa B (NFκB) firefly luciferase activity divided by Renilla luciferase activity measured in neuronal stem cell (NSC)/mesenchymal stromal cell (MSC) co-cultures. (**A**) Difference is found between unstimulated contact cultures and transwell cultures. (**B**) Difference is found between neuronal stem cells (NSCs) transfected with NFκB alone and NSCs transfected with IKKβ.

Table 3. Cytokine Production Measured in Media from Neuronal Stem Cell (NSC)/Mesenchymal Stromal Cell (MSC) Co-Cultures Stimulated with Tumor Necrosis Factor Alpha (TNFα) for 6 h

	IL-1α	*IL-1β*	*IL-6*
NSCs alone	3.1 ± 0.66	5.9 ± 0.79	0 ± 0
MSCs alone	2.4 ± 0.31	3.1 ± 0.32	0 ± 0
6,250 MSCs	6.5 ± 0.98*	4.3 ± 0.39	$2.4 \times 10^4 \pm 2.3 \times 10^4$
12,500 MSCs	6.4 ± 0.48*	3.7 ± 0.59	$1.4 \times 10^{10} \pm 1.0 \times 10^{10}$
25,000 MSCs	5.9 ± 0.54*	4.0 ± 0.42	$3.8 \times 10^{18} \pm 1.6 \times 10^{18}$*

An increase in interleukin (IL) 1-α with co-culture that is likely due to an additive effect. Significant increase in IL-6 production is observed with increasing MSC dosage.

*Indicates significant difference ($P < 0.05$) in cytokine production when compared to NSC culture alone based upon ANOVA with Tukey Kramer post hoc.

Additionally, similar co-cultures that were stimulated with TNFα showed a potential dose response as NFκB activity initially increased ($P = 0.005$ calculated with 12,500 MSCs in co-culture) but returned to control levels with increasing amounts of MSCs. Transwell (non-contact) NSC/MSC co-cultures showed no difference in NFκB activity for any MSC concentration. Figure 2A compares NFκB activity between direct NSC/MSC co-cultures (stimulated and unstimulated) and transwell co-cultures. No difference was found between stimulated and unstimulated direct co-cultures at any MSC concentration; however, difference between unstimulated controls and transwell cultures was noted with increasing MSC concentration.

Figure 2B compares NSC/MSC direct contact co-cultures after stimulation with TNFα. The NSC populations were transfected with dominant vectors for IKKβ, AKT, and a PI3 kinase inhibitor (PI3). A difference between NSCs transfected with the dominant negative vector for IKKβ and the NFκB vector alone was observed at an MSC concentration of 12,500 ($P = 0.002$). No difference was noted between the AKT and NFκB vectors for any MSC concentration.

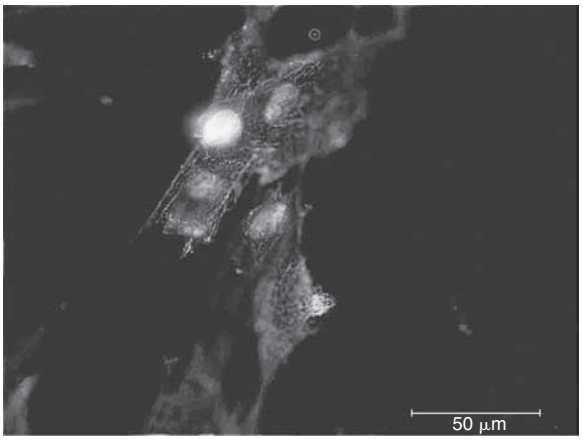

FIG. 3. Neuronal stem cells (NSCs) in direct contact culture with mesenchymal stromal cells (MSCs; stained red with conjugated CD29-PE antibody). Interleukin (IL)-6 secretion is localized to the NSC (stained green with IL-6 antibody with FITC secondary). Nuclei stained with 4′,6-diamidino-2-phenylindole (DAPI) blue (40× magnification).

In order to evaluate the production of proinflammatory cytokines in vitro, Bio-Plex cytokine analysis was completed analyzing culture media from NSC/MSC co-cultures that had been stimulated for 6 h with TNFα. A significant increase in IL 1-α production is observed with co-culture; however, this observation is likely due to an additive effect. Additionally, while NSCs and MSCs alone in culture do not produce IL-6, an increase in IL-6 production is observed with increasing MSC dosage in co-culture ($P = 0.002$). Table 3 shows inflammatory cytokine production measured in NSC/MSC co-cultures.

While the in vitro cytokine data clearly show an increase in IL-6, it is not possible to clearly identify NSCs or MSCs as the source of production. To evaluate the potential source, an IL-6 antibody was incubated in the co-cultures after stimulation for 6 h with TNFα. Figure 3 shows IL-6 production (represented by FITC green stain) from the NSCs only when in direct contact with the underlying MSCs (stained red). Therefore, it appears that MSCs are potentially stimulating the NSCs to produce and secrete IL-6 via direct contact. In order to quantify the source of IL-6 production, NSC/MSC co-cultures were stimulated for 6 h with TNFα followed by flow cytometric cell sorting based upon MSC CD29 and Stro-1 positivity. Next, a real-time quantitative PCR was completed to measure the amount of IL-6 production from both MSCs and NSCs. Figure 4 shows that MSCs and NSCs placed alone in monoculture had minimal IL-6 production; however, when placed in direct contact culture, a significant increase in IL-6 production was found generated primarily from the MSC population. It is also important to note that a significant increase ($P = 0.037$) in NSC IL-6 production was noted when placed in direct contact culture with MSCs.

Figure 5A shows that stimulation of NSCs with TNFα caused a trend toward increased proliferation as indicated by percentage of cells found in the synthesis (S) phase of the cell cycle. The trend reaches significance ($P = 0.01$) at high MSC co-culture concentrations; however, no dose-related difference between MSC concentration was found.

NSC/MSC co-cultures stressed with oxygen/glucose deprivation for 6 h showed a significant decrease in the fraction of apoptotic NSCs (0.197) when compared to NSC cultured alone (0.382; $P = 0.001$). Figure 5B–5C shows the flow cytometry overlays displaying the decreased NSC apoptotic fraction.

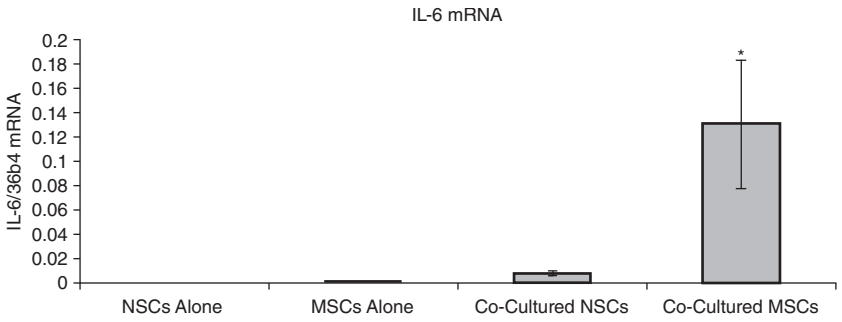

FIG. 4. Real-time quantitative polymerase chain reaction (qPCR) for interleukin 6 (IL-6) production from mesenchymal stromal cell (MSC) and neuronal stem cell (NSC) monocultures as well as NSC/MSC direct contact co-cultures after flow cytometric sorting based upon MSC CD29 and Stro-1 positivity. Minimal IL-6 production is observed with MSC and NSC monocultures; however, NSC/MSC co-cultures show a significant increase (*$P = 0.002$) in IL-6 production.

Discussion

In vivo

While initial in vivo research investigating the intravenous infusion of MSCs has shown potential neuroprotection via up-regulation of the anti-inflammatory response [13], our data show an increase in the proinflammatory cytokines IL-1α, IL-1β, and IL-6 after direct implantation of MSCs in a rat TBI model. Immunohistochemistry showed that very few (3%–5%) of the implanted MSCs remained within the brain with the majority of cells (58%) found in the ventricles (Fig. 1). The small proportion of MSCs that engrafted in the parenchymal tissue was located adjacent to the needle tracts (Fig. 1). The distribution of cells could indicate that only a few engrafted MSCs are capable of secreting significant amounts of proinflammatory cytokines; however, the observed increase in cytokine production could also be due to further parenchymal injury secondary to the cellular injection. A third potential explanation could be the activation of resident neuronal cells by the implanted MSCs to up-regulate cytokine production.

In vitro

The observed increase in proinflammatory cytokine levels could be produced by resident neuronal cells that come into contact with the implanted MSCs. Therefore, to investigate the possible activation of resident cell niches, NSCs (representing neuronal cell lineages) were transfected with the aforementioned NFκB vectors. NSC NFκB activity increased with rising MSC concentration in direct co-culture regardless of stimulation by TNFα; however, no increase in

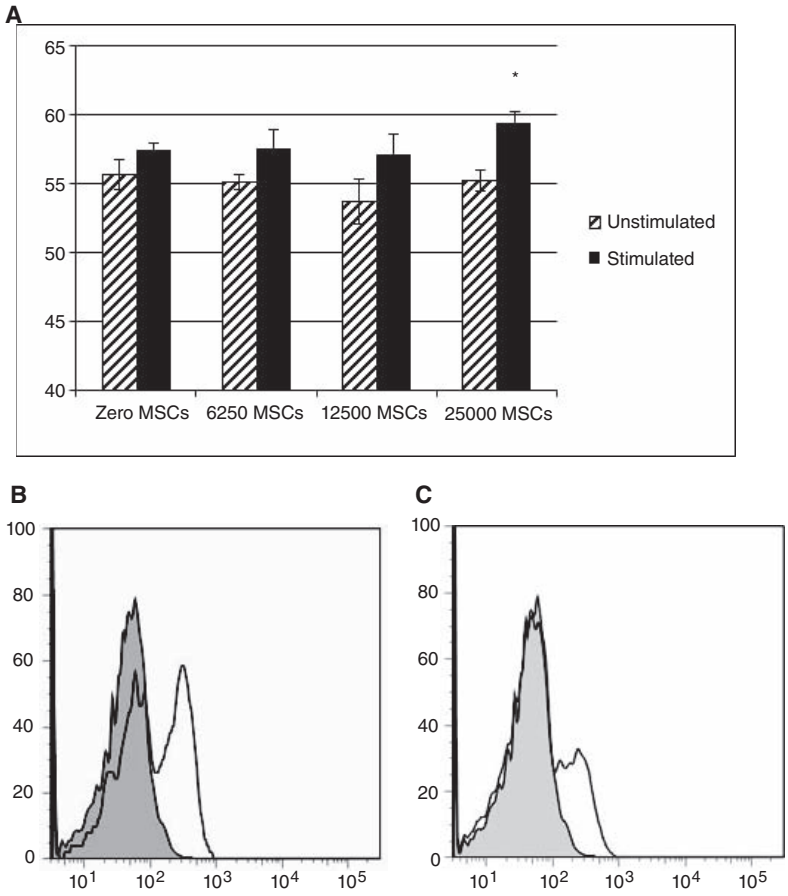

FIG. 5. (**A**) EDU assay shows an increase in neuronal stem cell (NSC) proliferation when stimulated with tumor necrosis factor alpha (TNFα); however, mesenchymal stem cell (MSC) co-culture has no effect. (**B** and **C**) Flow cytometric histogram overlays for NSC caspase 3 expression. Gray area (isotype control). Transparent overlay (apoptotic fraction). (**B**) NSCs cultured alone. (**C**) NSCs/MSC contact culture. *$P < 0.05$.

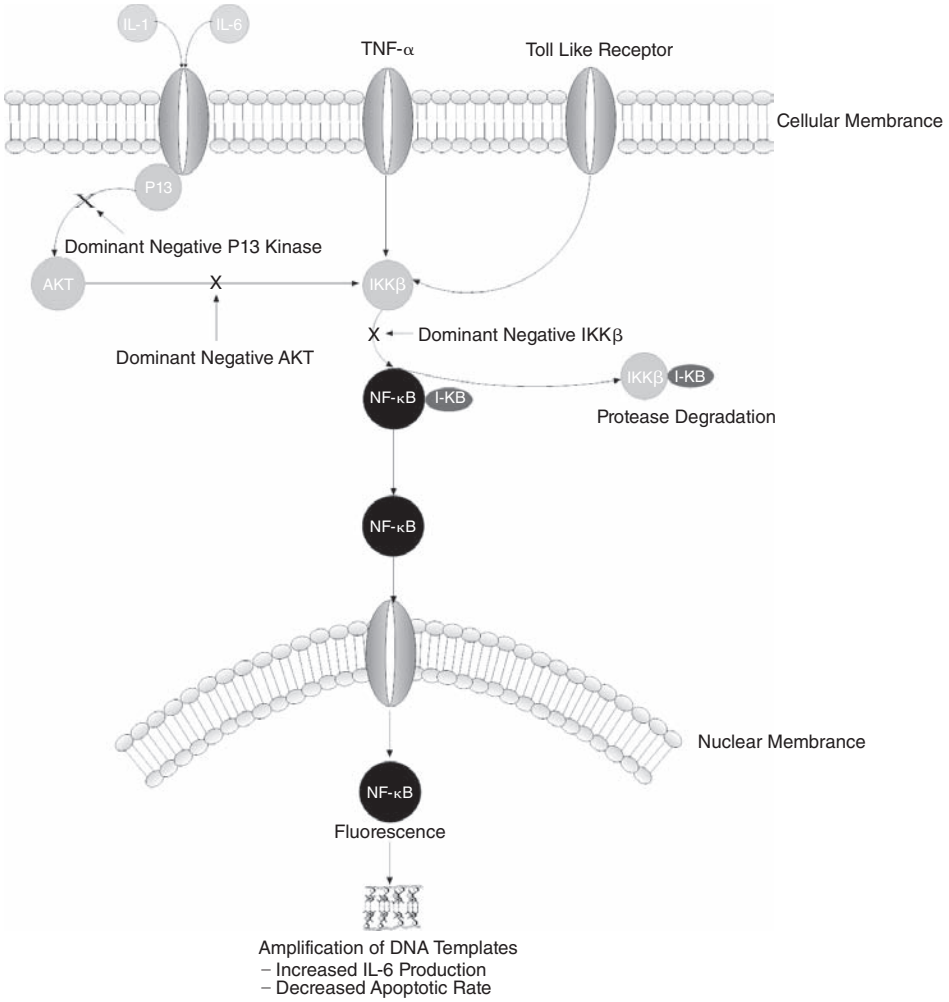

FIG. 6. Nuclear factor kappa B (NFκB) pathway detailing the point of action for the dominant negatives vectors IKKβ, AKT, and the PI3 kinase inhibitor. Activation of the neuronal stem cell (NSC) NFκB pathway leads to increased IL-6 production and decreased apoptosis thereby enhancing neuroprotection.

activity was noted when the cells were placed in transwell (non-contact) culture (Fig. 2A). Our data show that the presence of MSCs alone cause NSC NFκB activation and that direct cell-to-cell contact is required for MSC therapy to have an effect.

Furthermore, attenuation of NFκB activity was seen with the dominant negative IKKβ proving that the observed luminescence was due to activation of the NFκB firefly luciferase. No difference was observed between NSCs transfected with dominant negative vectors for PI3 kinase and AKT showing that the observed NFκB activation is independent of the AKT/PI3 kinase pathway (Fig. 2B). Figure 6 outlines the NFκB pathway.

While NSC NFκB activation could lead to increased cytokine production, it is a transcription factor that is common to several pathways (cellular proliferation and apoptosis) and not specific for an inflammatory response. In order to evaluate the functional effect of NFκB activation, a Bio-Plex cytokine assay was completed. Supernatant collected from NSCs or MSCs alone in culture showed no significant cytokine production; however, NSC/MSC co-cultures showed a significant increase in IL-6 production (Table 2; $P = 0.002$). Quantitative PCR has shown that the implanted MSCs are the primary source of IL-6 production; however, a significant increase in NSC IL-6 production when compared to monocultures was observed. Immunohistochemistry confirms NSC activation and IL-6 production (Fig. 3).

To further investigate the potential functional effect of NSC NFκB activation, both EdU proliferation assay and caspase 3 apoptosis assay were completed. Figure 5A shows that co-culture with MSCs does not affect the proliferative rate. Conversely, Figure 5C shows a significant decrease in the NSC apoptotic rate ($P = 0.001$) when compared to NSCs cultured alone. Therefore, the observed increase in NSC NFκB activity appears to stimulate production of the inflammatory cytokine IL-6 and promote neuroprotection by decreasing apoptosis.

Conclusions

Initial in vivo investigation into the direct implantation of bone marrow-derived MSCs has shown mixed results [14,23] and remains highly controversial. Our data have shown that MSCs potentially activate NFκB in resident NSC niches via

direct contact. In addition, the in vitro data show that the increase in NFκB activity leads to an increase in NSC-derived IL-6 with a concurrent reduction in apoptosis.

Previous research using a cryolesion model has shown that IL-6 production has been associated with improved wound healing, decreased apoptosis, and a decrease in oxidative species for acute neuropathological conditions like TBI [24]. Furthermore, similar experiments using an IL-6 knockout mouse have shown decreased monocyte recruitment and glial cell activation leading to decreased neuronal cell survival and wound healing with increased oxidative stress [25]. Our results show that the direct implantation of MSCs leads to resident neuronal cell activation and IL-6 secretion leading to enhanced neuroprotection.

Optimization of progenitor cell delivery is a field requiring further investigation. Intravenous infusion offers a minimally invasive vehicle with the potential of widespread distribution [26]; however, a clinically limited number of cells reach the arterial system due to a significant first pass pulmonary effect [27]. Intra-arterial infusion could bypass the lungs allowing for more focused delivery and enhanced cellular engraftment [28]; however, high cerebral engraftment rates are associated with impedance of cerebral microvascular blood flow [29]. Direct intracerebral implantation via stereotactic injection would allow for focused delivery with the potential for high levels of engraftment.

Due to the observed decrease in apoptosis and neuroprotective effects of IL-6 after acute neuropathologic insult, the direct implantation of progenitor cells is an attractive vehicle of delivery. Furthermore, the observed effect required only limited progenitor cell engraftment. Additional research into the implantation of progenitor cell-seeded nanofiber scaffolds could enhance engraftment and potentiate neuroprotection. Overall, direct implantation of bone marrow-derived MSCs appears to enhance neuroprotection via activation of resident NSC NFκB leading to decreased apoptosis and improved cell viability.

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References

1. Thurman DJ, C Alverson, KA Dunn, J Guerrero and JE Sniezek. (1999). Traumatic brain injury in the United States: A public health perspective. J Head Trauma Rehabil 14:602–615.
2. (1999). Consensus conference. Rehabilitation of persons with traumatic brain injury. NIH consensus development panel on rehabilitation of persons with traumatic brain injury. JAMA 282:974–983.
3. Faul M, MM Wald, W Rutland-Brown, EE Sullivent and RW Sattin. (2007). Using a cost-benefit analysis to estimate outcomes of a clinical treatment guideline: testing the Brain Trauma Foundation guidelines for the treatment of severe traumatic brain injury. J Trauma 63:1271–1278.
4. Walker PA, SK Shah, MT Harting and CS Cox. (2009). Progenitor cell therapies for traumatic brain injury: barriers and opportunities in translation. Dis Model Mech 2:23–38.
5. Qu C, A Mahmood, D Lu, A Goussev, Y Xiong and M Chopp. (2008). Treatment of traumatic brain injury in mice with marrow stromal cells. Brain Res 1208:234–239.
6. Liao W, J Xie, J Zhong, Y Liu, L Du, B Zhou, J Xu, P Liu, S Yang, J Wang, Z Han and ZC Han. (2009). Therapeutic effect of human umbilical cord multipotent mesenchymal stromal cells in a rat model of stroke. Transplantation 87:350–359.
7. Lim JH, YE Byeon, HH Ryu, YH Jeong, YW Lee, WH Kim, KS Kang and OK Kweon. (2007). Transplantation of canine umbilical cord blood-derived mesenchymal stem cells in experimentally induced spinal cord injured dogs. J Vet Sci 8:275–282.
8. Zhao LR, WM Duan, M Reyes, CD Keene, CM Verfaillie and WC Low. (2002). Human bone marrow stem cells exhibit neural phenotypes and ameliorate neurological deficits after grafting into the ischemic brain of rats. Exp Neurol 174:11–20.
9. Deng J, BE Petersen, DA Steindler, ML Jorgensen and ED Laywell. (2006). Mesenchymal stem cells spontaneously express neural proteins in culture and are neurogenic after transplantation. Stem Cells 24:1054–1064.
10. English D, SK Klasko and PR Sanberg. (2006). Elusive mechanisms of "stem cell"-mediated repair of cerebral damage. Exp Neurol 199:10–15.
11. Castro RF, KA Jackson, MA Goodell, CS Robertson, H Liu and HD Shine. (2002). Failure of bone marrow cells to transdifferentiate into neural cells in vivo. Science 297:1299.
12. Aggarwal S and MF Pittenger. (2005). Human mesenchymal stem cells modulate allogeneic immune cell responses. Blood 105:1815–1822.
13. Nemeth K, A Leelahavanichkul, PS Yuen, B Mayer, A Parmelee, K Doi, PG Robey, K Leelahavanichkul, BH Koller, JM Brown, X Hu, I Jelinek, RA Star and E Mezey. (2009). Bone marrow stromal cells attenuate sepsis via prostaglandin E(2)-dependent reprogramming of host macrophages to increase their interleukin-10 production. Nat Med 15:42–49.
14. Coyne TM, AJ Marcus, D Woodbury and IB Black. (2006). Marrow stromal cells transplanted to the adult brain are rejected by an inflammatory response and transfer donor labels to host neurons and glia. Stem Cells 24:2483–2492.
15. Nijboer CH, CJ Heijnen, F Groenendaal, MJ May, F van Bel and A Kavelaars. (2008). A dual role of the NF-kappaB pathway in neonatal hypoxic-ischemic brain damage. Stroke 39:2578–2586.
16. Harting MT, F Jimenez, SD Adams, DW Mercer and CS Cox, Jr. (2008). Acute, regional inflammatory response after traumatic brain injury: Implications for cellular therapy. Surgery 144:803–813.
17. Lighthall JW. (1988). Controlled cortical impact: a new experimental brain injury model. J Neurotrauma 5:1–15.
18. Harting MT, LE Sloan, F Jimenez, J Baumgartner and CS Cox, Jr. (2009). Subacute neural stem cell therapy for traumatic brain injury. J Surg Res 153:188–194.
19. Penolazzi L, E Lambertini, E Tavanti, E Torreggiani, F Vesce, R Gambari and R Piva. (2008). Evaluation of chemokine and cytokine profiles in osteoblast progenitors from umbilical cord blood stem cells by BIO-PLEX technology. Cell Biol Int 32:320–325.
20. Adams SD, RS Radhakrishnan, KS Helmer and DW Mercer. (2008). Effects of anesthesia on lipopolysaccharide-induced changes in serum cytokines. J Trauma 65:170–174.
21. Harting MT, LE Sloan, F Jimenez, J Baumgartner and CS Cox, Jr. (2009). Subacute neural stem cell therapy for traumatic brain injury. J Surg Res 153:188–194.

22. Harting M, F Jimenez, S Pati, J Baumgartner and C Cox, Jr. (2008). Immunophenotype characterization of rat mesenchymal stromal cells. Cytotherapy 10:243–253.
23. Mahmood A, D Lu and M Chopp. (2004). Marrow stromal cell transplantation after traumatic brain injury promotes cellular proliferation within the brain. Neurosurgery 55:1185–1193.
24. Penkowa M, M Giralt, N Lago, J Camats, J Carrasco, J Hernandez, A Molinero, IL Campbell and J Hidalgo. (2003). Astrocyte-targeted expression of IL-6 protects the CNS against a focal brain injury. Exp Neurol 181:130–148.
25. Penkowa M, T Moos, J Carrasco, H Hadberg, A Molinero, H Bluethmann and J Hidalgo. (1999). Strongly compromised inflammatory response to brain injury in interleukin-6-deficient mice. Glia 25:343–357.
26. Gao J, JE Dennis, RF Muzic, M Lundberg and AI Caplan. (2001). The dynamic in vivo distribution of bone marrow-derived mesenchymal stem cells after infusion. Cells Tissues Organs 169:12–20.
27. Fischer UM, MT Harting, F Jimenez, WO Monzon-Posadas, H Xue, SI Savitz, GA Laine and CS Cox. (2009). Pulmonary passage is a major obstacle for intravenous stem cell delivery: The pulmonary first pass effect. Stem Cells Dev 18:10.
28. Li Y, J Chen, L Wang, M Lu and M Chopp. (2001). Treatment of stroke in rat with intracarotid administration of marrow stromal cells. Neurology 56:1666–1672.
29. Walczak P, J Zhang, AA Gilad, DA Kedziorek, J Ruiz-Cabello, RG Young, MF Pittenger, PC van Zijl, J Huang and JW Bulte. (2008). Dual-modality monitoring of targeted intraarterial delivery of mesenchymal stem cells after transient ischemia. Stroke 39:1569–1574.

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