JNS

LABORATORY INVESTIGATION

J Neurosurg 122:856–867, 2015

Effect of exosomes derived from multipluripotent mesenchymal stromal cells on functional recovery and neurovascular plasticity in rats after traumatic brain injury

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OBJECT Transplanted multipotent mesenchymal stromal cells (MSCs) improve functional recovery in rats after traumatic brain injury (TBI). In this study the authors tested a novel hypothesis that systemic administration of cell-free exosomes generated from MSCs promotes functional recovery and neurovascular remodeling in rats after TBI.

METHODS Two groups of 8 Wistar rats were subjected to TBI, followed 24 hours later by tail vein injection of 100 μg protein of exosomes derived from MSCs or an equal volume of vehicle (phosphate-buffered saline). A third group of 8 rats was used as sham-injured, sham-treated controls. To evaluate cognitive and sensorimotor functional recovery, the modified Morris water maze, modified Neurological Severity Score, and foot-fault tests were performed. Animals were killed at 35 days after TBI. Histopathological and immunohistochemical analyses were performed for measurements of lesion volume, neurovascular remodeling (angiogenesis and neurogenesis), and neuroinflammation.

RESULTS Compared with the saline-treated group, exosome-treated rats with TBI showed significant improvement in spatial learning at 34–35 days as measured by the modified Morris water maze test ($p < 0.05$), and sensorimotor functional recovery (i.e., reduced neurological deficits and foot-fault frequency) was observed at 14–35 days postinjury ($p < 0.05$). Exosome treatment significantly increased the number of newly generated endothelial cells in the lesion boundary zone and dentate gyrus and significantly increased the number of newly formed immature and mature neurons in the dentate gyrus as well as reducing neuroinflammation.

CONCLUSIONS The authors demonstrate for the first time that MSC-generated exosomes effectively improve functional recovery, at least in part, by promoting endogenous angiogenesis and neurogenesis and by reducing inflammation in rats after TBI. Thus, MSC-generated exosomes may provide a novel cell-free therapy for TBI and possibly for other neurological diseases.

http://thejns.org/doi/abs/10.3171/2014.11.JNS14770

KEY WORDS angiogenesis; exosomes; functional recovery; mesenchymal stromal cell; neurogenesis; traumatic brain injury

Traumatic brain injury (TBI) is a major cause of death and long-term disability worldwide.[41] Although many neuroprotective therapeutic trials for TBI have been undertaken in the past, no broadly applicable, safe, and efficacious treatment has been identified.[94] There is a compelling need to develop therapeutic approaches designed to improve functional recovery after TBI. Multipotent mesenchymal stromal cells (MSCs) are a heterogeneous subpopulation consisting of mesenchymal stem and progenitor cells that can be harvested from bone marrow, adipose tissue, skin, umbilical cord blood, and peripheral blood as well as other organs.[28] Extensive research conducted during the last decade has shown great promise for MSCs as an effective therapy for brain injuries, including TBI in experimental models,[14,46,56,66] and potentially in clinical settings.[18,105] Previous studies from our

ABBREVIATIONS BrdU = 5-bromo-2′-deoxyuridine; DCX = doublecortin; DG = dentate gyrus; EBA = endothelial barrier antigen; FBS = fetal bovine serum; GFAP = glial fibrillary acidic protein; GFP = green fluorescent protein; miRNA = microRNA; mNSS = modified Neurological Severity Score; MSC = mesenchymal stromal cell; MWM = Morris Water Maze; NeuN = neuron-specific nuclear protein; PBS = phosphate-buffered saline; TBI = traumatic brain injury.

SUBMITTED April 4, 2014. **ACCEPTED** November 11, 2014.

INCLUDE WHEN CITING Published online January 16, 2015; DOI: 10.3171/2014.11.JNS14770.

DISCLOSURE This work was partially supported by NIH Grant No. R01NS081189 (Dr. Xin). The authors report no conflict of interest concerning the materials or methods used in this study or the findings specified in this paper.

group and others have shown that only a small proportion of transplanted MSCs actually survive and that few MSCs differentiate into neural cells in injured brain tissues.[45,54] The predominant mechanisms by which MSCs participate in brain remodeling and functional recovery are related to their secretion-based paracrine effect rather than a cell replacement effect.[14,46] Whereas the predominant role of MSC paracrine activity in brain tissue remodeling after injury has already been established,[9,15,46] whether MSC-generated exosomes promote brain remodeling and functional recovery after TBI remains unknown.

Exosomes are small-membrane vesicles of endosomal origin with a size of 30–120 nm in diameter.[86] They are generated by many cell types and contain not only proteins and lipids but also mRNAs and microRNAs (miRNAs).[4] Exosomes are well suited for small functional molecule delivery, and increasing evidence indicates that they have a pivotal role in cell-to-cell communication.[70] In contrast to transplanted exogenous MSCs, the MSC-derived exosomes do not proliferate, are less immunogenic, and are easier to store and deliver than MSCs.[36] Recent studies indicate that exosomes and microvesicles derived from multipotent MSCs have therapeutic promise in cardiovascular, liver, and kidney diseases.[7,17,60] We have previously demonstrated that exosomes generated from MSCs promote neurite remodeling and functional recovery in rats after stroke.[91] As a proof-of-principle study of MSC exosomes as a novel cell-free alternative therapeutic approach for TBI, we tested the hypothesis that systemically administered exosomes generated from MSCs improve functional outcome in animals with TBI, with therapeutic benefits reflecting those observed with systemically administered MSCs. In the present study, we intravenously administered exosomes generated by MSCs to rats subjected to TBI induced by controlled cortical impact injury, and we investigated cognitive and sensorimotor functional recovery as well as the potential mechanisms underlying therapeutic effects.

Methods

All experimental procedures were approved by the Henry Ford Health System Institutional Animal Care and Use Committee. To prevent potential biases of performance and detection, the persons who performed the experiments, collected data, and assessed outcome were blinded throughout the course of the experiments and were unaware of the treatment allocation.

Exosome Generation and Collection From MSCs

The MSC expansion was performed according to previously described methods.[91] Briefly, bone marrow from adult male rats was mechanically harvested by flushing the cavity of the femurs with phosphate-buffered saline (PBS), and the cells were washed and suspended in culture medium. Three days later, cells that tightly adhered to the plastic flasks were considered to be P0 MSCs. The MSCs were conventionally cultured with a modified minimum essential medium (Hyclone) containing 20% fetal bovine serum ([FBS], Gibson Laboratory) and penicillin-streptomycin on 75-cm^2 tissue culture flasks (Corning). For the exosome isolation, conventional culture medium was replaced with an exosome-depleted FBS medium (EXO-FBS-250 A-1, System Biosciences) when the cells reached 60%–80% confluence, and the MSCs were cultured for an additional 48 hours. The media were then collected and exosomes were isolated by the ExoQuick exosome isolation method according to the manufacturer's instructions. Briefly, ExoQuick-TC (2.5 ml) was added to 10 ml of media, incubated 12 hours at 4°C, and then centrifuged at 1500*g* for 30 minutes to obtain pelleted exosomes. The supernatant (nonexosomal fraction) of the samples was removed without disturbing the exosome pellets, and the pellets were resuspended in 200 μl of PBS. We quantitated the exosomes by measuring the total protein concentration with the micro-bicinchoninic acid protocol (Pierce), and we analyzed particle size by using a qNano nanopore-based exosome detection system according to the manufacturer's instructions (Izon).

Animal Model and Experimental Groups

A well-established controlled cortical impact rat model of TBI was used for the present study.[23] Adult male Wistar rats (2–3 months old) weighing 325 ± 11 g were anesthetized with intraperitoneally administered chloral hydrate (350 mg/kg body weight). Rectal temperature was maintained at 37°C ± 5°C by using a feedback-regulated water-heating pad. Rats were placed in a stereotactic frame. Two 10-mm-diameter craniotomies were performed adjacent to the central suture, midway between the lambda and bregma. The second craniotomy allowed for lateral movement of cortical tissue. The dura mater was kept intact over the cortex. Cortical injury was delivered by impacting the left (ipsilateral) cortex with a pneumatic piston containing a 6-mm-diameter tip at a rate of 4 m/sec and 2.5 mm of compression. Velocity was measured with a linear velocity displacement transducer.

The study animals were randomly divided into 3 groups of 8 rats: Group 1, TBI + exosomes; Group 2, TBI + vehicle (PBS); and Group 3, sham injury and sham treatment. Exosomes generated from MSCs (100 μg total protein of exosome precipitate in 0.5 ml PBS per rat) or an equal volume of PBS (0.5 ml) were administered intravenously over 5 minutes via the tail vein, starting 1 day after injury; this protocol was chosen based on our recent stroke study with exosomes in rats.[91] Animals with TBI that were treated with PBS were used as a control group. Sham-injured, sham-treated animals underwent surgery without injury or treatment. For labeling of proliferating cells, 100 mg/kg 5-bromo-2′-deoxyuridine (BrdU) was injected intraperitoneally into rats daily for 10 days, starting 1 day after TBI. The dose and time for BrdU injection were based on our previous TBI studies in rats.[96] All animals were allowed to survive for 35 days after TBI.

Evaluation of Neurological Outcome

Modified Neurological Severity Score Test

Neurological functional measurement was performed using the modified Neurological Severity Score (mNSS) test.[10] The test was conducted in all rats preinjury and at 1, 4, 7, 14, 21, 28, and 35 days after TBI. The mNSS is a composite of the motor (muscle status, abnormal movement), sensory (visual, tactile, and proprioceptive), and

reflex tests and has been used in previous studies.[53] Neurological function was graded on a scale of 0–18 (normal score 0; maximal deficit score 18). In the severity scores of injury, 1 point is awarded for each abnormal behavior or for lack of a tested reflex; thus, the higher the score the more severe the injury.

Foot-Fault Test

To evaluate sensorimotor function, the foot-fault test was performed before TBI and at 1, 4, 7, 14, 21, 28, and 35 days after TBI. The rats were allowed to walk on a grid. With each weight-bearing step, a paw might fall or slip between the wires and, if this occurred, it was recorded as a foot fault.[5] A total of 50 steps were recorded for the right forelimb.

Morris Water Maze Test

To measure spatial learning impairments, an updated version of the Morris Water Maze (MWM) test was used.[13] The procedure was modified from previous versions[64] and has been used for spatial memory assessment in rodents with brain injury.[13] The MWM test was performed 1 month postinjury. At each testing interval, animals were tested with 4 trials per day for 5 consecutive days (on Days 31–35 after TBI). A blue swimming pool (1.8 m in diameter) was located in a large room, where there were many clues external to the maze (e.g., pictures on the walls, lamps, and a camera on the ceiling); these were visible from the pool and presumably were used by the rats for spatial orientation. The position of the cues remained unchanged throughout the experiment. Data collection was automated using the HVS Image 2020 Plus Tracking System (US HVS Image), as described previously.[57]

For data collection, the swimming pool was subdivided into 4 equal quadrants formed by imaging lines. At the start of each trial the rat was placed at 1 of 4 fixed starting points, randomly facing toward a wall (designated North, South, East, and West), and allowed to swim for 90 seconds or until it found the platform, which was transparent and invisible to the animals. If the animal found the platform by spatial navigation, it was allowed to remain on it for 10 seconds. If the animal failed to find the platform within 90 seconds, it was placed on the platform for 10 seconds. Throughout the test period, the platform was located in the northeast quadrant 2 cm below the water in a randomly changing position, including locations against the wall, toward the middle of the pool, or off-center but always within the target quadrant. If the animal was unable to locate the platform within 90 seconds, the trial was terminated and a maximum score of 90 seconds was assigned. If the animal reached the platform within 90 seconds, the percentage of time traveled within the northeast (correct) quadrant was calculated relative to the total amount of time spent swimming before reaching the platform, and this percentage was used for statistical analysis. The latency to find the hidden escape platform was also recorded and analyzed.

The advantage of this version of the water maze is that each trial takes on the key characteristics of a probe trial, because the platform is not in a fixed location within the target quadrant. In the traditional version of the MWM test, the position of the hidden platform is always fixed and is relatively easy for rodents to find. With the modified MWM test we used in this study, the platform is relocated randomly within the correct quadrant with each training trial. The rodents must spend more time searching within the target quadrant; therefore each trial effectively acts as a probe trial. The advantage of this protocol is that rodents should find the platform purely and extensively by reference to the extramaze spatial cues, which improves the accuracy of spatial performance assessment in the MWM.[13]

Tissue Preparation

Rats were anesthetized with an overdose of chloral hydrate administered intraperitoneally and then were perfused transcardially with saline solution, followed by 4% paraformaldehyde in 0.1 M PBS (pH 7.4). Rat brains were removed and immersed in 4% paraformaldehyde for 2–4 days. Using a rat brain matrix (Activational Systems, Inc.), each forebrain was cut into 2-mm-thick coronal blocks for a total of 7 blocks (from bregma 5.2 mm to bregma −8.8 mm) per animal.[71] The tissues were embedded in paraffin and a series of 6-μm-thick slides were cut. For lesion volume measurement, one 6-μm-thick section from each of 7 coronal blocks was traced by a microcomputer imaging device (MCID, Imaging Research), as described previously.[11] The volumes of the ipsilateral and contralateral cortices were computed by integrating the area of each cortex measured at each coronal level and the distance between 2 sections. The cortical lesion volume was expressed as a percentage calculated by the following formula: [(contralateral cortical volume − ipsilateral cortical volume)/(contralateral cortical volume)] × 100%.[79]

Immunohistochemical Studies

Antigen retrieval was performed by boiling sections in 10 mM citrate buffer (pH 6) for 10 minutes. After washing with PBS, sections were incubated with 0.3% H_2O_2 in PBS for 10 minutes, blocked with 1% bovine serum albumin containing 0.3% Triton X-100 at room temperature for 1 hour, and incubated with either mouse anti-doublecortin ([DCX], 1:200; Santa Cruz Biotechnology), anti–endothelial barrier antigen ([EBA], 1:1000; Covance), anti-CD68 (1:200; Serotec), or anti–glial fibrillary acidic protein ([GFAP], 1:1000; Dako) at 4°C overnight. For negative controls, primary antibodies were omitted. After washing, sections were incubated with biotinylated anti–mouse or anti–rabbit antibodies (1:200; Vector Laboratories, Inc.) at room temperature for 30 minutes. After an additional washing, sections were incubated with an avidin-biotin-peroxidase system (ABC kit; Vector Laboratories, Inc.), visualized with diaminobenzidine (Sigma), and counterstained with hematoxylin.

Immunofluorescent Staining

We performed double immunostaining to identify newly generated endothelial cells (BrdU/EBA+) and newly formed mature neurons (BrdU/NeuN+) in the lesion boundary zone and dentate gyrus (DG) 35 days after TBI. Briefly, after being deparaffinized and rehydrated, brain sections were boiled in 10 mM citric acid buffer (pH 6) for 10 minutes. After washing with PBS, sections were incubated in 2.4 N HCl at 37°C for 20 minutes. Sections

were incubated with 1% bovine serum albumin containing 0.3% Triton X-100 in PBS. Sections were then incubated with mouse anti-NeuN antibody (1:200; Chemicon) or anti-EBA at 4°C overnight. For negative controls, primary antibodies were omitted. Fluorescein isothiocyanate–conjugated anti–mouse antibody (1:400; Jackson ImmunoResearch) was added to sections at room temperature for 2 hours. Sections were then incubated with rat anti-BrdU antibody (1:200; Dako) at 4°C overnight. Sections were then incubated with Cy3-conjugated goat anti–rat antibody (1:400; Jackson ImmunoResearch) at room temperature for 2 hours. Each of the steps was followed by three 5-minute rinses in PBS. Tissue sections were mounted with Vectashield mounting medium (Vector Laboratories).

Cell Counting and Quantitation

For analysis of angiogenesis, we focused on the lesion boundary zone and DG. The EBA+ endothelial cells, CD68+ microglia/macrophages, GFAP+ astrocytes, BrdU+ cells, and EBA/BrdU-colabeled cells were counted in the lesion boundary zone and the DG. For analysis of neuroblasts, DCX+ cells were examined within the subgranular zone and the granule cell layer of the DG of the hippocampus. For analysis of neurogenesis, we counted BrdU+ cells and NeuN/BrdU-colabeled cells in the DG and its subregions, including the subgranular zone, the granular cell layer, and the molecular layer. The fields of interest were digitized under the light microscope (Eclipse 80i, Nikon) at a magnification of either 200 or 400, using a CoolSNAP color camera (Photometrics) interfaced with the MetaMorph image analysis system (Molecular Devices), as described in detail previously.[104] In brief, 5 fields of view in the lesion boundary zone from the epicenter of the injury cavity (bregma –3.3 mm) and 9 fields of view in the ipsilateral DG were counted in each section. From our previous experience, our interrater reliability was greater than 95% when the cell counts were compared between 2 independent trained blinded observers scoring the same sections of an animal. In the present study, 1 blinded observer performed the cell counting in all brain sections.

Statistical Analysis

Data are presented as the mean ± SD. The ANOVA test was used for repeated measurements of spatial performance and sensorimotor function. For cell counting, a 1-way ANOVA followed by post hoc Tukey tests was used to compare the differences between the exosome-treated, PBS-treated, and sham-injured, sham-treated groups. Pearson correlation coefficients were calculated to examine relationships between cognitive functional recovery and immunostaining. Differences were considered significant if the p value was < 0.05.

Results

Isolation of Exosomes From MSC Culture Medium

Exosomes accumulate as intraluminal vesicles inside multivesicular bodies,[21,25,76,85] while microvesicles (size 100 to approximately 1000 nm) are small, plasma-membrane–derived particles that are released into the extracellular environment by the outward budding and fission of the plasma membrane.[2,16,65] A precise and clear distinction between the endosomal origin exosomes (30–120 nm) and microvesicles is lacking, and it is technically difficult to definitively separate microvesicles from the culture media by currently available methods like ultracentrifugation, density gradient separation, chromatography, and immunoaffinity capture techniques.[81] In the present study we used the ExoQuick-TC kit with centrifugation; this is one of the standard methods for isolating exosomes.[82] Due to the relatively low centrifugal force used in the ExoQuick isolation process, the precipitation of nonexosomal proteins and nucleotides is minimized, whereas nonexosomal protein contamination can occur in prolonged ultracentrifugation methods.[19] Using a qNano nanopore-based exosome detection system, we demonstrated that MSCs generate exosome-enriched particles with a peak diameter at 116 ± 49 nm (Fig. 1), which is consistent with MSC exosomes that we identified with transmission electron microscopy, using the ExoQuick-TC kit for exosome isolation.[32] Exosomes were the primary component in our isolated precipitant, which was verified by transmission electron microscopy for size, and by Western blot and confocal fluorescence microscopy for characteristic markers.[32,90,92]

Administration of MSC Exosomes Significantly Enhances Spatial Learning in Rats After TBI

Spatial learning measurements were performed during the last 5 days (31–35 days postinjury) prior to planned death by using a modified MWM test, which is very sensitive to evidence of hippocampal injury.[13] The greater the percentage of time the animals spend in the correct quadrant (i.e., northeast, where the hidden platform was located) of the water maze, the better their spatial learning function. The percentage of time spent by sham-injured rats in the correct quadrant increased significantly between 32 and 35 days after sham operation, compared with time spent in the correct quadrant on the 1st day of testing (Day 31) (Fig. 2A; $F_{4, 35} = 29.58$, $p < 0.01$). In the testing of spatial memory among the 3 groups, no significant between-group effect on the time spent in the cor-

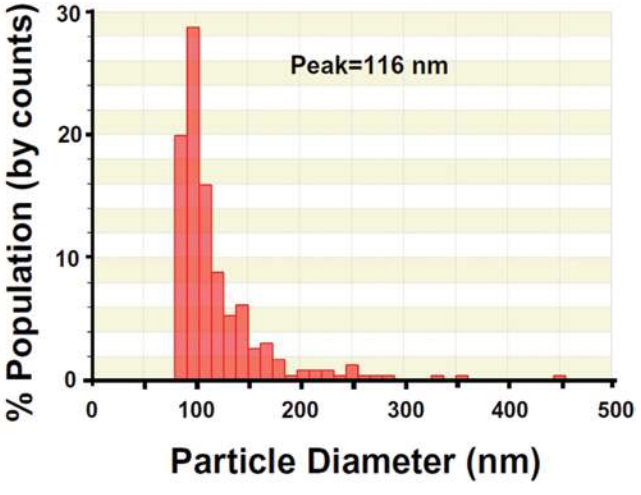

FIG. 1. Bar graph showing results of measurement by a qNano nanopore-based exosome detection system; the percentage population of MSC-generated exosome-enriched particles by counts is shown, with a peak diameter of 116 ± 49 nm. Figure is available in color online only.

rect quadrant was detected on the 1st day of testing in the MWM (Day 31 postinjury, $F_{2,21} = 0.32$, $p = 0.73$); however, a statistically significant between-group effect on the time spent in the correct quadrant was noted in the MWM test on succeeding days (Day 32: $F_{2,21} = 17.14$, $p < 0.01$; Day 33: $F_{2,21} = 29.19$, $p < 0.01$; Day 34: $F_{2,21} = 43.49$, $p < 0.01$; and Day 35: $F_{2,21} = 38.39$, $p < 0.01$). Relative to the PBS group, post hoc Tukey testing demonstrated significantly increased time spent in the correct quadrant in the exosome group at Days 33–35 ($p < 0.01$).

Administration of MSC Exosomes Significantly Promotes Sensorimotor Functional Recovery in Rats After TBI

Neurological functional measurement was performed in rats using the mNSS test. The mNSS is a composite of the motor, sensory, and reflex tests and has been used in our previous TBI studies.[55] Neurological function is graded on a scale of 0–18; the higher the score, the more severe the injury. The mNSS was close to 12 in rats with TBI (both the PBS and exosome groups) on Day 1 post-TBI, indicating that neurological functional deficits were comparable in all rats with TBI before treatment (Fig. 2B; $t(14) = 0.48$, $p = 0.64$). Significant reduction in the mNSS was found over time in the PBS-treated animals from Day 4–35 compared with Day 1 postinjury ($F_{6,49} = 127.82$, $p < 0.01$), suggesting that a significant spontaneous sensorimotor functional recovery occurred after TBI. However, compared with the PBS treatment, functional recovery was significantly increased in the exosome-treated group on Days 14–35 after TBI (Day 14: $F_{2,21} = 579.91$, $p < 0.01$; Day 21: $F_{2,21} = 479.04$, $p < 0.01$; Day 28: $F_{2,21} = 670.23$, $p < 0.01$; and Day 35: $F_{2,21} = 594.58$, $p < 0.01$ [with ANOVA followed by post hoc Tukey tests]). Exosome treatment also significantly reduced the frequency of forelimb foot-fault occurrence compared with PBS-treated controls (Fig. 2C—Day 14: $F_{2,21} = 196.31$, $p < 0.01$; Day 21: $F_{2,21} = 163.40$, $p < 0.01$; Day 28: $F_{2,21} = 91.84$, $p < 0.01$; and Day 35: $F_{2,21} = 91.28$, $p < 0.01$ [with ANOVA followed by post hoc Tukey tests]).

Administration of MSC Exosomes Significantly Increases Vascular Density and Angiogenesis in Rats After TBI

Endothelial barrier antigen staining was performed to identify mature vasculature in the brain after TBI.[44] Traumatic brain injury alone (without addition of exosomes) significantly increased the density of vessels in the lesion boundary zone ($F_{2,21} = 215.68$, $p < 0.01$) and DG ($F_{2,21} = 129.00$, $p < 0.01$) of the ipsilateral hemisphere compared with sham-injured controls (Fig. 3). Exosome treatment significantly increased the vascular density in the injured cortex and DG compared with the PBS treatment (Fig. 3, $p < 0.01$, with ANOVA followed by post hoc Tukey tests). Exosome treatment significantly increased angiogenesis identified by EBA/BrdU+ double labeling for newly generated endothelial cells in the lesion boundary zone and DG compared with the PBS treatment (Fig. 3, $p < 0.01$). The Pearson correlation analyses further showed the following: 1) spatial learning was positively correlated to EBA+ vascular density in the DG region ($R^2 = 0.81$, $p < 0.01$); and 2) sensorimotor functional recovery was positively correlated to EBA+ vascular density in the lesion boundary zone ($R^2 = 0.64$, $p < 0.01$).

Administration of MSC Exosomes Significantly Increases Neurogenesis in the DG in Rats After TBI

To investigate effects of exosome treatment on cell proliferation in the DG, we injected BrdU intraperitoneally into rats once daily for 10 days, starting 24 hours postinjury. Animals were killed 35 days after TBI, and immunostaining was performed on paraffin-embedded brain coronal sections.[62] Although TBI alone (without exosomes) significantly increased cell proliferation compared with the sham-injured group, exosome therapy significantly increased the number of BrdU+ cells compared with the PBS treatment (Fig. 4A–D; $F_{2,21} = 503.38$, $p < 0.01$). Immature neurons (neuroblasts) were identified with DCX staining. Traumatic brain injury reduced the number of DCX+ immature neurons compared with the sham-injured group (Fig. 4E and F; $F_{2,21} = 17.39$, $p < 0.01$). Compared with the PBS group, exosome therapy significantly increased the number of DCX+ cells (Fig. 4F–H; $p < 0.01$), with their long dendrites projecting into the granule cell layer. We further demonstrated that exosome therapy increased the number of DCX/BrdU+ newly generated, immature neu-

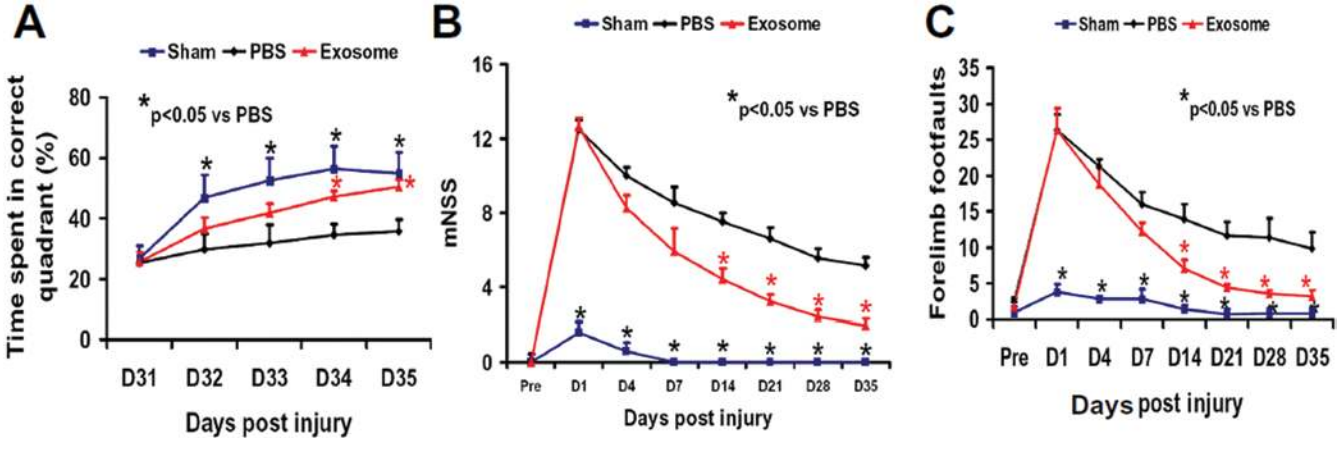

FIG. 2. Graphs showing that treatment with exosomes derived from MSCs significantly improves spatial learning in the MWM test **(A)**, sensorimotor function measured by mNSS **(B)**, and right forelimb foot-fault test scores **(C)** in rats after TBI (8 rats per group). Data represent the mean ± SD. Figure is available in color online only.

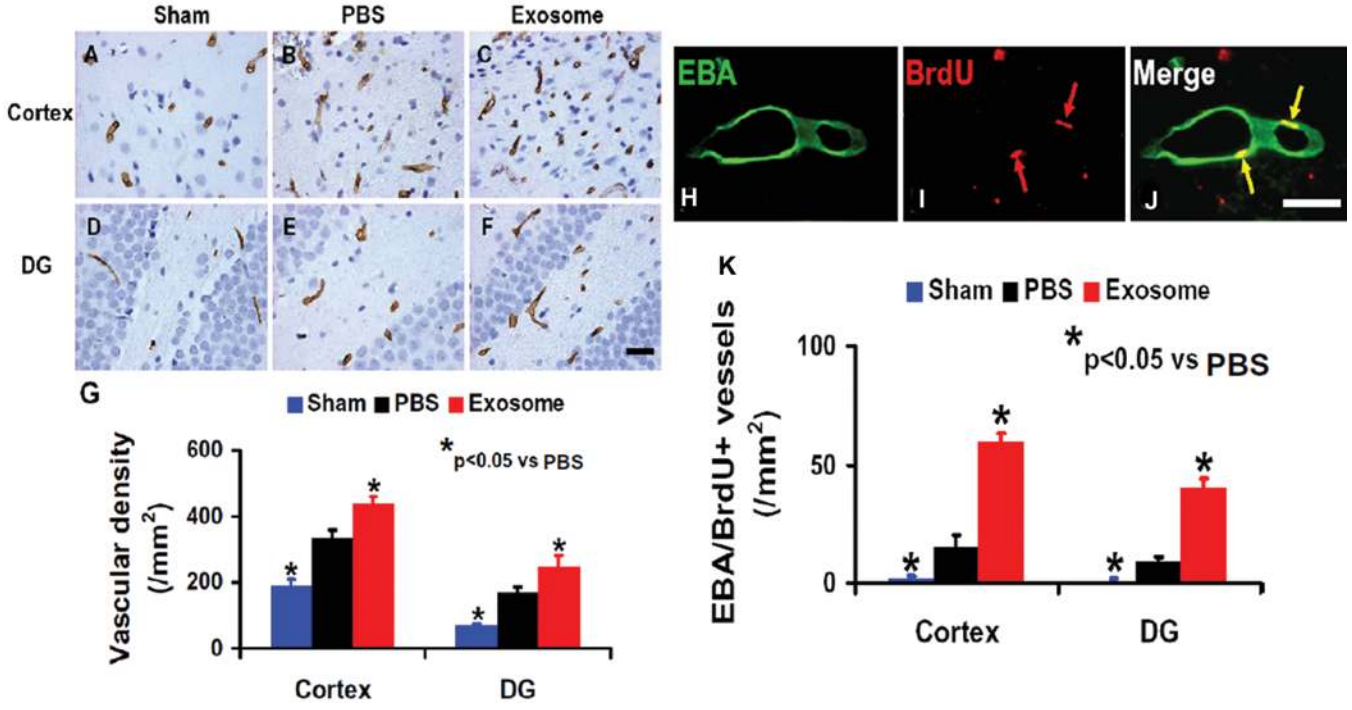

FIG. 3. Treatment with exosomes derived from MSCs significantly increases brain vascular density and angiogenesis in rats after TBI (8 rats per group). EBA staining was performed for detection of mature vasculature at Day 35 after TBI in the lesion boundary zone and DG of the sham-injured group **(A and D)**, PBS-treated group **(B and E)**, and exosome-treated group **(C and F)**. Double staining for EBA (*green*, **H**) and BrdU (**I**, *red arrows*) was performed to identify newly formed mature vessels (**J**, *yellow arrows*) in the brain at Day 35 after TBI. Bar = 25 μm **(F and J)**. Data in the bar graphs **(G and K)** represent the mean ± SD. Figure is available in color online only.

rons, some of which projected long dendrites into the granule cell layer (Fig. 4I–L; $F_{2,21} = 42.65$, $p < 0.01$). To identify newly generated neurons in the DG, double labeling for BrdU (proliferating marker) and NeuN (mature neuronal marker) was performed. Exosome treatment significantly increased the number of newly generated neurons detected in the granule layer of the DG compared with the PBS-treated controls (Fig. 4M–P; $F_{2,21} = 207.16$, $p < 0.01$). Our data also show a significant positive correlation between spatial learning tested by the MWM and the number of newly formed mature neurons ($R^2 = 0.92$, $p < 0.01$).

Administration of MSC Exosomes Significantly Reduces Brain Inflammation in Rats After TBI

We performed CD68 staining to identify macrophages/microglia in the brain after TBI.[42] As shown in Fig. 5, TBI alone (without exosomes) significantly increased the density of CD68+ cells in the lesion boundary zone ($F_{2,21} = 150.23$, $p < 0.01$) and DG ($F_{2,21} = 792.51$, $p < 0.01$) of the ipsilateral hemisphere compared with sham-injured controls. Exosome treatment significantly reduced the CD68+ cell density in the injured cortex and DG compared with the PBS treatment ($p < 0.01$, with ANOVA followed by post hoc Tukey tests). The Pearson correlation analyses showed that 1) spatial learning was inversely correlated to CD68+ cell density in the DG region ($R^2 = 0.45$, $p < 0.01$); and 2) sensorimotor functional recovery was inversely correlated with CD68+ cell density in the lesion boundary zone ($R^2 = 0.41$, $p < 0.01$). The GFAP staining was performed to identify reactive astrocytes in the brain after TBI.[73] Traumatic brain injury alone (without exosomes) significantly increased the density of GFAP+ cells in the lesion boundary zone ($F_{2,21} = 221.49$, $p < 0.01$) and DG ($F_{2,21} = 103.94$, $p < 0.01$) of the ipsilateral hemisphere compared with sham-injured controls. Exosome treatment significantly reduced the GFAP+ astrocyte density in the injured cortex and DG compared with the PBS treatment ($p < 0.01$, with ANOVA followed by post hoc Tukey tests). The Pearson correlation analyses showed that 1) spatial learning was inversely correlated to GFAP+ astrocyte density in the DG region ($R^2 = 0.76$, $p < 0.01$); and 2) sensorimotor functional recovery was inversely correlated to GFAP+ astrocyte density in the lesion boundary zone ($R^2 = 0.64$, $p < 0.01$).

Administration of MSC Exosomes Does Not Alter Cortical Lesion Volume in Rats After TBI

Cortical lesion volume was measured 35 days post-TBI, as described previously.[97] No differences in lesion volume were observed between the PBS group and the exosome group (14.9% ± 0.9% for PBS group vs 14.2% ± 2.9% for exosome group, $t(14) = -0.68$, $p = 0.51$).

Identification of MSC-Generated Exosomes

Exosomes generated from MSCs were identified with Alix, the exosomal marker protein, by using Western blot analysis and were observed to be within a size range of 40–120 nm by the transmission electron microscopy im-

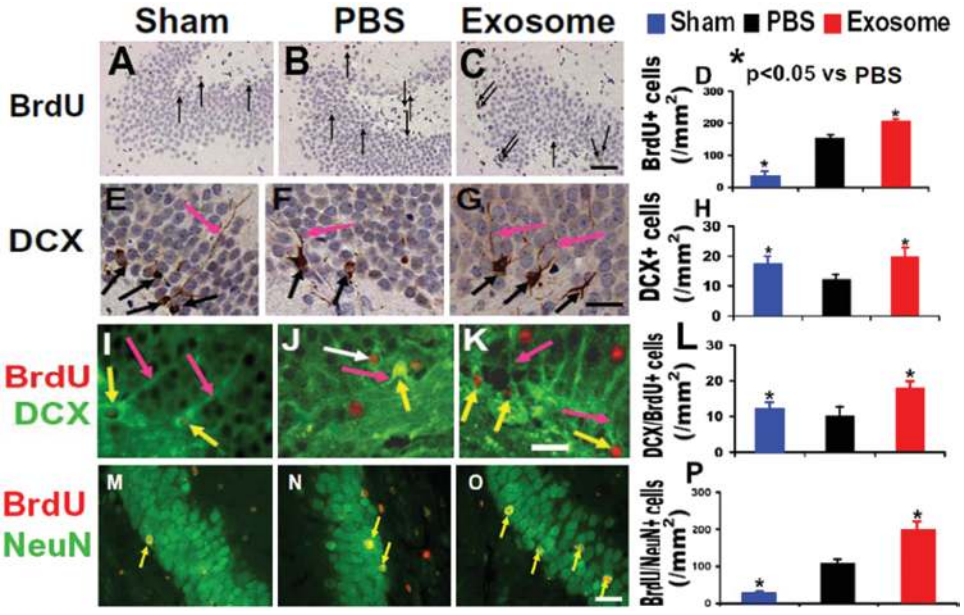

FIG. 4. Treatment with exosomes derived from MSCs significantly increases cell proliferation and neurogenesis in the DG of rats killed on Day 35 after TBI (8 rats per group). BrdU staining for cell proliferation (**A–D**, *black arrows*). DCX staining for immature neurons (**E–H**, *black arrows* for DCX+ cells and *pink arrows* for dendrites). Double staining with BrdU *(red)*/DCX *(green)* for newly generated immature neurons indicated by *yellow arrows* (**I–L**; *pink arrows* for dendrites). BrdU *(red)*/NeuN *(green)* for newly formed mature neurons (**M–P**, *yellow arrows*). Bar = 50 μm (A–C) and 25 μm (E–G, I–K, M–O). Data in the bar graphs (D, H, L, and P) represent the mean ± SD. Figure is available in color online only.

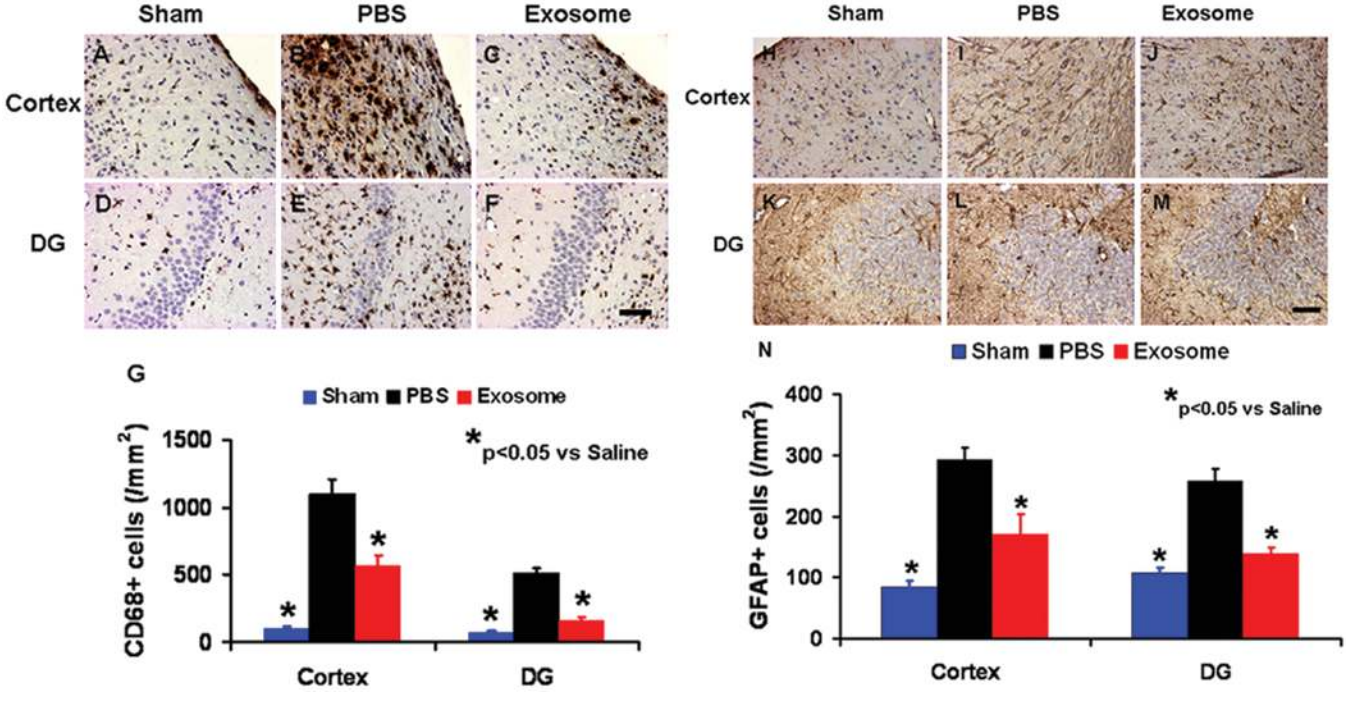

FIG. 5. Treatment with exosomes derived from MSCs significantly reduces the number of activated GFAP+ astrocytes and CD68+ microglia/macrophages in the brains of rats killed on Day 35 after TBI (8 rats per group). CD68 staining for activated microglia/macrophages **(A–F)**. GFAP staining for reactive astrocytes **(H–M)**. Bar = 50 μm **(F and M)**. Data in the bar graphs **(G and N)** represent the mean ± SD. Figure is available in color online only.

age, as described in our previous study.[90–92] In the present study, using a qNano nanopore-based exosome detection system, we demonstrated that MSCs generate exosome-enriched particles, with a peak diameter at 116 ± 49 nm.

Discussion

In this study we demonstrate for the first time that systemic administration of cell-free exosomes generated by MSCs, with treatment initiated 24 hours postinjury in rats with TBI, does not alter cortical lesion volume compared with PBS treatment. However, this treatment has the following significant effects: 1) improves cognitive and sensorimotor functional recovery; 2) increases the number of newly formed neuroblasts and mature neurons in the DG; 3) increases the number of newly formed endothelial cells in the lesion boundary zone and DG; and 4) reduces brain inflammation. Improved functional recovery after treatment of TBI with exosomes generated from MSCs is significantly associated with increased brain angiogenesis and neurogenesis as well as with reduced neuroinflammation. Our results suggest that intravenous administration of exosomes generated from MSCs may represent a novel therapeutic approach for treatment of TBI.

The mechanisms of action of exosomes on functional recovery after TBI are not clear. Our recent study demonstrates that intravenous administration of cell-free MSC-generated exosomes improves functional recovery and enhances neurite remodeling, neurogenesis, and angiogenesis in rats after stroke.[91] The beneficial effects of systemic administration of MSCs and cell-free exosomes generated by MSCs appear to be similar in animal models of stroke and TBI, in terms of improved functional recovery and increased neuroplasticity, including angiogenesis and neurogenesis.[14,43,91] These data support the premise that the beneficial effects of MSC treatment are at least partly mediated by exosomes from MSCs. Cells produce exosomes with components and functions that mirror those of their parent cells.[33] Therefore, exosomes function in a highly cell origin–dependent manner. Exosomes contain proteins, lipids, mRNAs, and miRNAs, which can be transferred to recipient cells and modify their characteristics.[99] Further studies are warranted to identify the molecular constituents of the exosomes, including specific miRNAs and growth factors that promote angiogenesis and neurogenesis after TBI. When the specific molecules necessary for a therapeutic effect are known, selective manipulation of expression of those molecules in the parent MSCs may lead to an enhancement of the therapeutic efficiency of isolated exosomes.

The 100 μg total protein of exosomes injected into each rat in this study was collected from approximately 2×10^6 MSCs, a number of MSCs equivalent to the effective amount that we previously used in the MSC-based treatment for TBI (2×10^6 MSCs per rat).[54] Our previous study suggests that human MSCs cultured with cerebral tissue extract from rats with TBI demonstrated a time-dependent increase of various growth factors, including brain-derived neurotrophic factor, nerve growth factor, and vascular endothelial growth factor.[12] Because cellular stress increases exosome release from cell lines,[39] MSCs within the injured brain tissue may release more exosomes to the brain. However, our previous studies indicate that only a small percentage (< 1%) of MSCs transplanted via tail vein injection can be detected in the injured brain.[59]

Although our recent study in which exosomes tagged with green fluorescent protein (GFP) were used demonstrated that exosome-enriched extracellular particles were released from MSCs that were intravenously administered to rats with stroke and transferred to adjacent astrocytes and neurons,[92] it is unclear what amount of exosomes generated by transplanted MSCs is formed in the brain after intravenous MSC administration. Whether a higher dose of exosomes provides a better functional recovery in rats after TBI is unclear. Further studies are warranted to determine a dose-response efficacy for this novel mode of exosome treatment for TBI. In addition, we cannot exclude the possibility that exosomes may act, as cell-based therapies possibly do, on extracerebral tissues to indirectly promote neurovascular remodeling and functional recovery post-TBI. Mesenchymal stromal cells used as cell therapy after TBI may act as remote "bioreactors" via stimulation of lung macrophages and spleen T regulatory cell production (probably because many intravenously injected MSCs are trapped by these organs), leading to systemic remote effects on the CNS.[87] Further investigation is warranted to determine whether these nano-sized exosomes are trapped in those organs and have remote effects on brain.

Many molecules that have been individually tested in preclinical TBI models have not shown efficacy in a clinical setting,[61] suggesting that combination therapies with these molecules may be required to target complex multiple secondary injury mechanisms involved in the TBI. Exosomes contain very complex molecular cargo.[37,100] The benefit and potential strength of exosome treatment, as with stem cell therapy, is that we are seeking multiple targets. We have demonstrated in rats with stroke that treatment with MSCs transfers miRNAs via exosomes to recipient parenchymal cells.[92] MicroRNAs also regulate a myriad of genes.[38] It is this multitargeted approach, rather than the traditional single molecular pathway approach, that elicits the therapeutic potency of exosome or cell-based therapy. Treatment with MSC-generated exosomes is an alternative approach for targeting the complex TBI.

Endothelial barrier antigen–positive cells are endothelial cells that constitute the vessels.[48] Increased newly formed vessels (angiogenesis) may contribute to functional recovery after TBI, as demonstrated by us and others.[51,63,93] Exosome treatment–induced angiogenesis may contribute motor functional recovery by promoting neurite growth and synaptogenesis in the brain after stroke.[91] In the DG, angiogenesis is well coupled with neurogenesis, which may play an important role in improving learning and memory after brain injury.[3,49,69,98] Neurogenesis (i.e., a process by which new neurons are generated from neural stem/progenitor cells) occurs in mammals during adulthood and is involved in the pathology of different neurological disorders, and thus neurogenesis may be a potential target area for treatments.[80] Neurogenesis is stimulated by TBI in rodents and humans.[34,72,107] Accumulating evidence shows a strong correlation between certain types of memory functions and adult neurogenesis in the hippocampus;

for example, blocking neurogenesis pharmaceutically[103] or genetically[6] impairs spatial learning and memory after TBI, whereas enhancing neurogenesis through various treatments promotes learning and memory.[35,52,77] Immature DG cells that undergo maturation are also implicated in modulating learning and memory.[20,106] There is evidence for an increase in newly formed neurons around the lesion area.[29,95] Subventricular zone cells generate neuroblasts and can migrate from their normal route along the rostral migratory stream to the injured area after TBI.[30,78] To date, there is no evidence for migration of subgranular zone–derived cells beyond the hippocampus after brain injury.

We have previously used different routes (intraarterial, intravenous, and intracerebral) to administer MSCs to rodents with TBI.[50,56,58] Although these routes exhibit promising therapeutic effects,[50,55,56,58] there are some disadvantages for each one. For example, relatively few MSCs can be injected intracranially, intraarterial injection of MSCs can cause brain ischemia, and intravenous injection results in body-wide distribution of MSCs.[53] In fact, the efficacy of MSC transplantation in treating TBI in animal models seems independent of cell replacement.[15,31] In the present study, exosomes promote neurovascular remodeling and improve functional recovery after TBI, and they reflect the beneficial effects of their parent cells. Considering the nano size of exosomes and their many advantages, exosomes present a new weapon for the treatment of TBI in terms of easy administration and the potential to act as drug delivery vehicles across the blood-brain barrier.[1,8] In the present preliminary study, we focused on the efficacy of exosome therapy for TBI and did not perform this treatment on animals without TBI, considering that exosome therapy would be used only for treatment of patients with TBI rather than healthy people. Although cell-free exosome-based therapy offers several advantages over MSCs, including easier storage and reduced safety risks, it is necessary to determine the safety of exosomes in animals before initiating a clinical trial. Of note, a clinical trial using umbilical cord blood–derived MSC microvesicles and exosomes in Type I diabetes mellitus is ongoing (ClinicalTrials.gov, NCT02138331).

In the current study, activation of GFAP+ astrocytes and CD68+ microglia/macrophages was significantly suppressed in the exosome-treated rats compared with the PBS-treated group. This antiinflammatory effect is similar to that of MSC therapy in animal models of stroke[83,89] and TBI.[101] Astrocytes and microglia are distributed throughout the brain, and one of their main functions is to monitor and sustain neuronal health.[88] Activated astrocytes and microglia release pro- and antiinflammatory cytokines, free radicals, antioxidants, and neurotrophic factors, which contribute to neuronal death as well as survival mechanisms during neurodegeneration[74] and after TBI.[42,103] It remains a subject of debate whether activated microglia/macrophages promote neuronal survival or exacerbate neuronal damage.[27] Here, we demonstrate that suppression of activated microglia/macrophages by exosomes may, at least in part, contribute to increased angiogenesis and neurogenesis, and subsequent improvement in functional recovery after TBI.

In the present 35-day study, functional recovery was significantly accelerated (as evidenced by reduced mNSS and foot-fault scores) after TBI in the exosome-treated rats compared with the PBS-treated animals. Our previous long-term (3-month) studies indicate that the PBS-treated animals with TBI continue to recover slowly after the 35-day time point.[55,67] Traumatic brain injury produces behavioral deficits, with different recovery rates over time, dependent on injury type, severity, and size and sex, age, and different tasks performed.[22,67,68,75] Whether exosome treatment yields a long-term permanent reduction in chronic deficits must be determined in a future study. It is important to quantify the amount of exosomes in the injured brain. Correlation between exosome levels in the brain and functional recovery may provide additional insight into the mechanism of action and help determine the dose-response efficacy of exosomes for treatment of TBI. The present study is not final proof of the superiority of exosomes. Investigators still need to optimize exosome production and quality control, to determine dosing, timing, toxicity, and mechanisms of MSC exosome treatment.

Some additional limitations in the present study should be noted. Extracellular particles and soluble factors are important mechanisms underlying MSC therapy.[37,40,47,100] Extracellular particles include released smaller, more homogeneous exosomes of endocytic origin and rather heterogeneous microvesicles formed by the outward budding and fission of the plasma membrane.[26,40] In our study, we focused on exosomes and did not compare them with the nonexosomal fraction of the media. Our first step was to investigate whether treatment of TBI solely with exosomes derived from MSCs provides significant functional benefit compared with PBS treatment, which is an unexplored area in the TBI field. Although the supernatant of the samples was carefully removed after centrifugation, we do not exclude the possibility of microvesicle components in the content of our injected precipitate, and we will not exclude a contribution of microvesicles to mediating TBI recovery. In addition, we generated exosomes from MSCs rather than from other cells or stem-like cells. We do not exclude the possibility that other cells (e.g., embryonic stem cells) may also generate exosomes that may be effective as a treatment for TBI.

Another caveat of the present study is that although we demonstrated a significant therapeutic and neuroplasticity effect of systemic exosome administration, we did not investigate the presence of the exosomes within the brain after TBI. However, exosomes are nanovesicles and probably enter into the brain.[84,102] By expressing a neuron-targeting protein on the surface of exosomes, filling them with short interfering RNA and injecting them intravenously into mice, Alvarez-Erviti et al. have achieved specific gene knockdown in the brain.[1] In our recent stroke study, by tagging exosomes with CD68-GFP, we demonstrated that exosome-enriched particles were released into brain and CSF from intravenously administered CD68-GFP MSCs and transferred to adjacent astrocytes and neurons in the rat brain.[92] Thus, systemic exosome administration may be a means by which to deliver the active components of cell-based therapy to the CNS. A study is warranted to inject tagged exosomes, either isolated from CD63-GFP MSCs[92] or labeled with other fluorescent dyes such as PKH,[24] into the animals and to track the distribu-

tion of these exosomes in the brain (the DG, injury site, and target cells) as well as other organs.

Conclusions

In the present study, we demonstrate for the first time that intravenous administration of exosomes generated from MSCs improves functional recovery, promotes neurovascular remodeling (angiogenesis and neurogenesis), and also reduces neuroinflammation in rats after TBI. This discovery opens a novel avenue for treatment of TBI and possibly other neurological diseases.

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