Basic fibroblast growth factor increases the transplantation-mediated therapeutic effect of bone mesenchymal stem cells following traumatic brain injury

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Abstract. Basic fibroblast growth factor (bFGF) has proven useful for neural stem and progenitor cells during the transplantation-mediated therapeutic effect of bone mesenchymal stem cells (BMSCs). Endogenous bFGF expression levels increase during brain development and gradually diminish with aging. To date, few studies have been conducted on exogenous bFGF promoting BMSC transplantation-mediated functional recovery in adult rats following traumatic brain injury (TBI). The results of the present study showed that BMSCs in the TBI cortex and dentate gyrus showed differentiation along the glial and neuronal lines, which are possibly enhanced by bFGF. The neuronal differentiation rate was not consistent with neurological functional recovery rate over time. bFGF may promote the transplantation-mediated therapeutic effect of BMSCs more significantly and rapidly in rats following TBI, with a small proportion of differentiated neurons. In conclusion, exogenous bFGF functions as a booster of the transplantation-mediated therapeutic effect of BMSCs following TBI.

Introduction

Bone mesenchymal stem cells (BMSCs) have been shown to exhibit the ability to improve the neurological functional outcome of central nervous system (CNS) disorders, including stroke and traumatic brain injury (TBI) (1,2). Moreover, BMSCs are capable of proliferating and differentiating into neurons and glial cells *in vivo* and *in vitro* (3,4). These studies affirm BMSCs as a potential candidate for use in cellular therapy for CNS disorders. However, this type of therapy presents difficulties in the treatment of brain injury due to the limited plasticity of the nervous system and the complicated pathological processes involved. The viability of transplanted stem cells may be reduced in *in vivo* conditions, including a low-oxygen environment or during an immunoreaction. Moreover, neurotrophic factors are imperative for tissue regeneration as an insufficient level of neurotrophins weakens the proliferation and differentiation of transplanted stem cells (5). Thus, increasing the viability of grafts through the utilization of neurotrophic factors is an important strategy in promoting the efficacy of cell therapy for the treatment of CNS diseases. Among a large number of neurotrophic factors, the basic fibroblast growth factor (bFGF) has been shown as a potent mitogen for neural stem and progenitor cells *in vitro* and *in vivo*. Evidently, bFGF fulfills a mediative function in inducing neurogenesis (6) and enhancing neural survival and outgrowth (7). Furthermore, bFGF alone has been demonstrated to induce BMSC neuronal differentiation effectively *in vitro* (8). However, endogenous bFGF expression levels diminish with aging but increase during brain development (9,10). Thus, BMSC-derived neural functional recovery may be promoted through the administration of exogenous bFGF following various brain injuries in the adult mammal. To date, few studies have been conducted on exogenous bFGF promotion of BMSC transplantation-mediated functional recovery in adult rats following TBI. The current study was performed to investigate the therapeutic potential of exogenous bFGF to promote BMSC transplantation for adult brain repair, as well as to examine the effects of bFGF on BMSC transplantation in adult rats following TBI.

Materials and methods

Animals. A total of 48 male Sprague-Dawley rats (age, 3 months; weight, ~300 g) were obtained from the Experimental Animal Center, Fourth Military Medical University (Xi'an, China) to be used in the present study. This study was conducted

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in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (1996). The animal use instructions were reviewed and approved by the Institutional Animal Care and Use Committee of the Fourth Military Medical University. Rats were divided into four groups: A, (n=6) rats without TBI; B, (n=6), rats with TBI that underwent no treatment; C, (n=18) TBI group treated with BMSC transplantation and D, (n=18) TBI group treated with BMSC transplantation and bFGF.

Cultivation of BMSCs. BMSCs were isolated and cultured according to a typical method (11) with modifications. Briefly, bone marrow was harvested from the femurs and tibias of one month-old male Sprague-Dawley rats through suction using a 20 ml sterile syringe. For anticoagulation, 5 ml heparin (100 IU/ml) was used. The recovery of the nucleated cells was accomplished by centrifugation at 900 x g. BMSCs ($<4x10^5$) were loaded into 3 ml Percoll (Sigma, St. Louis, MO, USA) with a density of 1.089 g/ml, in a 15 ml conical tube. The cells were separated following centrifugation at 1,100 x g for 20 min at 20°C. The primary BMSCs were cultured in flasks at $2x10^5$ cells/ml with Dulbecco's modified Eagle's medium (DMEM) F12 (Gibco-BRL, Carlsbad, CA, USA) containing 15% fetal bovine serum (Hyclone, Logan, UT, USA) in a carbon dioxide incubator (37°C, 5% CO_2). The medium was replaced every three days. When adherent cells reached ~80% confluency, the cells were propagated to the next passage. Following three passages of culture and identification by flow cytometry (Biomedika Instruments Inc., Dollard-des-Ormeaux, QC, Canada), BMSCs were ready for analysis and transplantation by trypsinization using 0.25% trypsin and 0.02% EDTA.

Labeling of BMSCs with bromodeoxyuridine (BrdU). When the cells had reached 60% confluency, rat BMSCs were labeled with BrdU (Sigma) at 3 μg/ml concentration by the addition of BrdU to the culture and the mixture was incubated for two days. Following washing three times with sterile phosphate-buffered saline (PBS), the BMSCs were trypsinized and used in subsequent *in vivo* experiments.

TBI model. The TBI model was developed as described in a previous study (12) with modifications. Following administration of chloral hydrate (Kangjie Technology and Development Co., Ltd., Wuhan, China) anesthesia [300 mg/kg, intraperitoneally (i.p.)], the scalps were incised and a bone window was developed over the right forelimb motor cortex. Specifically, the rats were placed into a stereotactic frame (Wandong Instruments, Ltd., Wuhan, China). The center of the bone window was 2.5 mm lateral to the midline and 1.5 mm posterior to the bregma. The diameter of the bone window was 5.0 mm. The bone was thinned over the right forelimb motor cortex using a high-speed microdrill (Ruijing Medical Equipment Co., Ltd., Wuhu, China) and the last layer of the bone was removed with forceps. A 40 g weight was inserted into the top of the tube (25 cm) and was allowed to slide through the tube to deliver a blow onto the motor cortex to induce TBI. The bone flap was placed *in situ* following TBI, without fixing.

Surgical procedures. One day following TBI modeling, the rats were anesthetized using chloral hydrate (300 mg/kg, i.p.) and were placed into a stereotactic frame (Wandong Instruments, Ltd.) for the microinjection procedure. Prior to transplantation, BMSCs were digested with trypsin, washed twice with DMEM and centrifuged. The BMSCs were diluted in PBS at a density of $1x10^5$ cells/μl. A microinjector was slowly inserted 4 mm vertically through a hole drilled on the skull into the right lateral ventricle of each animal. The microinjections were conducted at a rate of 1 μl/min. BMSCs ($1x10^6$ cells/μl) were unilaterally implanted into the left lateral ventricle of the rats. Groups C and D received BMSC infusions, whereas groups A and B received a normal saline infusion. The needle was left in place for an additional 10 min prior to being slowly drawn back. Following TBI, this intralateroventricular injection of bFGF solution (PeproTech, Rocky Hill, NJ, USA) was performed for seven consecutive days (400 ng/day). Group D received a bFGF infusion, whereas groups A, B and C received a normal saline infusion. The needle was kept in the targets for a further 5 min prior to being slowly withdrawn. Six rats from groups C and D at days 10 and 20 post-TBI, respectively and six from groups A and B, in addition to the remainder of groups C and D at day 30 post-TBI, were euthanized by intraperitoneal injection of pentobarbital natrium and perfused with 4% paraformaldehyde.

Immunohistochemical staining. Immunohistochemical staining was performed by BrdU labeling to identify the transplanted BMSCs. Formalin-fixed and paraffin-embedded brain tissue blocks were sliced into 4-μm thick sections. The sections were deparaffinized in xylene, followed by rehydration in a decreasing concentration gradient of ethanol solution. The sections were subjected to microwave heat-induced epitope retrieval in boiled EDTA for 2 min. Following washing with 0.1 M PBS, these sections were pretreated with 0.3% H_2O_2 in methanol for 30 min at room temperature to prevent endogenous peroxidase activity. The sections were then blocked with PBS, 3% skimmed milk and 3% normal donkey serum for 2 h at room temperature and anti-BrdU (sheep, 1:200; Abcam, Cambridge, MA, USA) was applied onto the sections, which were subsequently incubated for 24 h at 4°C in a humidified chamber. Following rinsing with PBS with Tween-20, the samples were reacted with biotinylated secondary antibody (Donkey anti-sheep IgG, 1:500; Abcam Inc., Cambridge, MA, USA) diluted in 0.1 M PBS (1:500) for 2 h at room temperature. Subsequently, these sections were incubated with an avidin-biotin peroxidase complex (Sigma) diluted in 0.1 M PBS (1:500) for 2 h at room temperature. The samples were developed in a staining solution (DAB). All steps were performed according to the manufacturer's instructions. The sections were examined under a light microscope, (Carl Zeiss, Oberkochen, Germany). To confirm whether the staining satisfactorily developed, cells were counterstained with hematoxylin to facilitate the visualization of the immunostained product. In addition, non-immune serum was used in the controls for the primary antibodies or omission of the primary antibodies.

Immunofluorescent double-labeling. Double-immunostaining with immunofluorescence was performed. The parallel sections were processed for immunofluorescent double labeling with antibodies against BrdU and markers for mature neurons (NeuN) and astrocytes (glial fibrillary acidic protein, GFAP). The staining procedure used was similar to the BrdU staining

procedure described in the previous section with modifications. The primary antibodies used were mouse anti-NeuN (1:500, Millipore, Billerica, MA, USA), mouse anti-GFAP (1:500, Millipore) and sheep anti-BrdU (1:500, Abcam). Secondary antibodies used were Alexa Fluor 488 anti-mouse IgG (1:500, Millipore) or Cy3-conjugated anti-goat IgG (1:500, Chemicon, Temecula, CA, USA). Sections in EDTA buffer were boiled in a microwave for 2 min to retrieve the antigen. Following DNA denaturation, endogenous peroxidase and serum blocking, sections were incubated with primary antibodies for 48 h at 4°C with constant shaking. Following washing three times, sections were incubated with secondary antibodies for 4 h at room temperature. Vectashield cover slips (Kangjie Technology and Development Co., Ltd.) were placed on the slides, which were examined using an Olympus BX51 fluorescent microscope (Olympus, Tokyo, Japan).

Quantification of cells. Sections were examined using an Olympus Image System CAST program (Olympus BX-51) to quantify the number of BrdU-positive cells in the dentate gyrus (DG) and cortex. Results are expressed as the average number of cells per specimen in the target areas that consist of the DG and cortex. Eight sections per sample at the level of DG spanning between -2.56 and -5 mm of bregma and eight sections per sample at the level of TBI cortex spanning between -1 and -2 mm of bregma were assessed by a blinded pathologist. All BrdU-positive cells in the DG and TBI cortex were counted regardless of size or shape under magnification x200. These immunofluorescent double-labeling slides were examined using an Olympus fluorescent microscope to quantify the number of BrdU-positive cells that had differentiated to the varying cell types. In counting the double-stained sections, all BrdU-labeled cells, which were located in the DG and cortex, were examined for colocalization with NeuN or GFAP in sections. Only those cells for which the BrdU-positive cells were unambiguously associated with a specific cell type-specific marker were considered to be double-labeled. Immunofluorescent double-labeled images were imported into Photoshop under magnification x200 (Olympus BX-51) for merging and the number of BrdU-positive cells (red fluorescence image) which colocalized with NeuN or GFAP (green fluorescence image) was counted on a computer monitor.

Neurological functional evaluation. All tests began at 5:00 pm, following the nocturnal habits of rats. Beginning on the day following TBI modeling, continuing every 10th day thereafter for one month and prior to euthanasia, animals were examined using two standard tests to assess the sensorimotor function in the limbs, as well as vestibulomotor function (13). Specifically, the forelimb placing test measures sensorimotor function in each forelimb as the animal places the limb on a tabletop in response to visual, tactile and proprioceptive stimuli (total score=0-10; 10=maximally impaired) (13). The modified beam balance test examines vestibulomotor activity as the animal balances on a narrow beam (30x1.3 cm) for 60 sec (score range=1-7; 7=maximally impaired) (13).

Statistical analyses. All statistical analyses were performed using SPSS® version 16.0 software (SPSS Inc., Chicago, IL, USA). One-way analysis of variance and the Bonferroni test were used to compare the differences between three or four groups and groups in pairs, respectively. The t-test was used to compare the differences between those in the contralateral hemisphere and those in the ipsilateral hemisphere. $P<0.05$ was considered to indicate a statistically significant difference.

Results

The majority of BrdU-labeled cells were identified in the injured hemisphere concentrated around the contusion cortex and the DG, whereas the minority were located in the contralateral hemisphere (Fig. 1). In group D, immunofluorescent double-labeling showed the highest expression of astrocytes and neuron markers, with a small differentiation proportion ($P<0.05$; Fig. 2A-I; Fig. 3E-H). The majority of BMSCs in the TBI cortex and DG showed differentiation along the glial line, which was possibly enhanced by bFGF (Fig. 2J-R; Fig. 3G-H). Statistical significance was observed in the number of BrdU-positive cells in the ipsilateral DG and cortex between groups C and D at 10, 20 and 30 days post-injury ($P<0.05$; Fig. 3C-D). The trend in the ipsilateral DG in groups C and D was towards the significant increase in the number of mitotically BrdU-positive cells immediately following injury, which decreased marginally 20 days post-injury (F_C, 26.182; F_D, 26.524; $P<0.05$; Fig. 3C). The same results were observed for groups C and D in the ipsilateral cortex (F_C, 22.962; F_D, 12.180; $P<0.05$; Fig. 3D).

The peak increase in the neuronal differentiation rate in the DG occurred 20 days post-injury in groups C and D (F_C, 5.91; F_D, 26.882; $P<0.05$; Fig. 3E). In the ipsilateral cortex, the same results were observed for groups C and D (F_C, 7.562; F_D, 35.192; $P<0.05$; Fig. 3F).

A statistically significant improvement was observed in the forelimb placing test scores (F_C, 303.881; F_D, 456.813; $P<0.001$; Fig. 3A), as well as in the modified beam balance test scores (F_C, 111.825; F_D, 281.817; $P<0.001$; Fig. 3B). The recovery tempos of groups B and C were more rapid in the first 20 days ($P<0.001$), gradually decreasing for the remainder of the study period, as evidenced in the forelimb placing (P_B, 0.426; P_C, 0.512; Fig. 3A) and modified beam balance test scores (P_B, 0.623; P_C, 0.474; Fig. 3B). At a different time point, group D constantly showed the most significant improvement, which is inconsistent with the neuronal differentiation rate, as demonstrated in the recovery of the forelimb placing (F_{10}, 224.626; F_{20}, 67.216; F_{30}, 93.925; $P<0.001$; Fig. 3A) and modified beam balance tests (F_{10}, 110.009; F_{20}, 48.110; F_{30}, 41.961; $P<0.001$; Fig. 3B).

Discussion

Currently available engrafted stem cells are primarily classified into two types: Neural and non-neural stem cells. As non-neural stem cells, BMSCs have an advantage over neural and embryonic stem cells, which are more difficult to prepare and culture and are limited in availability. Notably, the utilization of BMSCs does not create ethical issues. In the current study, the lower survival rate of the grafted cells is attributed to the shortage of neurotrophic support due to the occurrence of numerous newly engrafted cells and/or to the less conducive environment for cell survival due to injury. However, the current results indicate that following transplantation, cells survive *in vivo* for at a minimum of one month even without bFGF treatment.
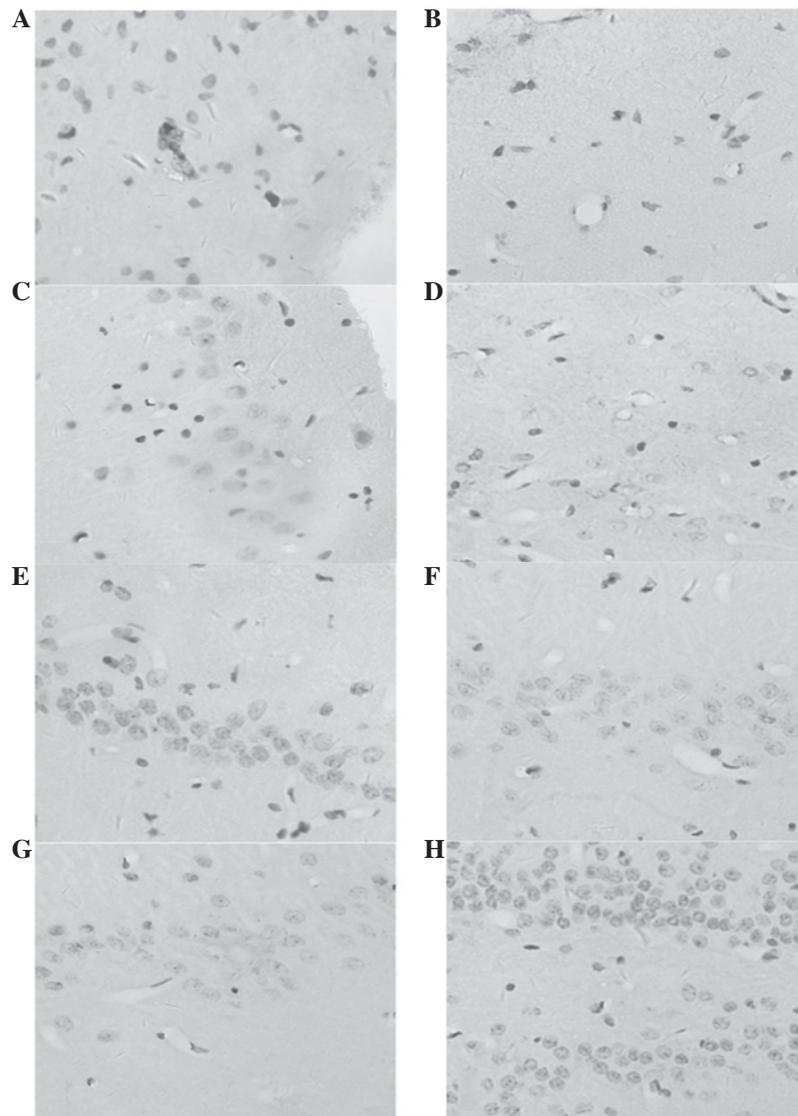
Figure 1. BMSC distribution in the DG and cortex following TBI. Immunohistochemistric images at four weeks following TBI show BrdU$^+$ cells in the DG and cortex. (A) BrdU$^+$ cells in the contralateral cortex of the BMSC group; (B) BrdU$^+$ cells in the ipsilateral cortex of the BMSC group; (C) BrdU$^+$ cells in the contralateral cortex of the BMSC group with bFGF treatment; (D) BrdU$^+$ cells in the ipsilateral cortex of the BMSC group with bFGF treatment; (E) BrdU$^+$ cells in the contralateral DG of the BMSC group; (F) BrdU$^+$ cells in the ipsilateral DG of the BMSC group; (G) BrdU$^+$ cells in the contralateral DG of the BMSC group with bFGF treatment and (H) BrdU$^+$ cells in the ipsilateral DG of the BMSC group with bFGF treatment. BMSCs, Bone mesenchymal stem cells; DG, dentate gyrus; TBI, traumatic brain injury; BrdU, bromodeoxyuridine; bFGF, basic fibroblast growth factor.

bFGF is a mitogen for neuronal and non-neuronal stem cells, showing multifunctional and pleiotropic activities. Throughout the developmental stages and even in mature animals, bFGF provides important extracellular signals for regulating neurogenesis (14,15). Furthermore, the beneficial effect of bFGF on functional recovery may be due to the increased connectivity (16). These results demonstrate that the survival, proliferation and differentiation of cells within the CNS are crucially dependent on signals provided by bFGF. In the present study, one of the essential contributions of bFGF in BMSCs transplantation therapy for TBI is the enhancement of the viability of transplanted cells. An increased viability of transplanted stem cells is indispensable for tissue regeneration. The therapeutic effect of BMSCs on the brain was evident in the current experiments. Moreover, the therapeutic effect may be further augmented with exogenous bFGF following TBI. Specifically, an intraventricular injection of bFGF immediately following TBI significantly increased the permeation of implanted cells into damaged areas, that is, the subventricular zone and the DG. Moreover, by determining the cell fate of these newly engrafted cells, the growth factor treatment generated more neurons and astrocytes than that identified in the group with injury alone or that which was induced with a vehicle infusion.

Based on previous studies, an extremely low percentage of differentiated neurons from transplanted cells were observed following transplanting stem cells into TBI models (17-19). Analogous results have been demonstrated in the present study. The proportion of differentiated neurons or astrocytes in this study was not significantly larger than those of previous studies. The question of whether a majority of BMSCs differentiate into neurons and astrocytes over time was also addressed. The possibility that, as time elapsed, an increased number of donor cells may present phenotypic markers of neurons and astrocytes, exists. The present data indicated that the number
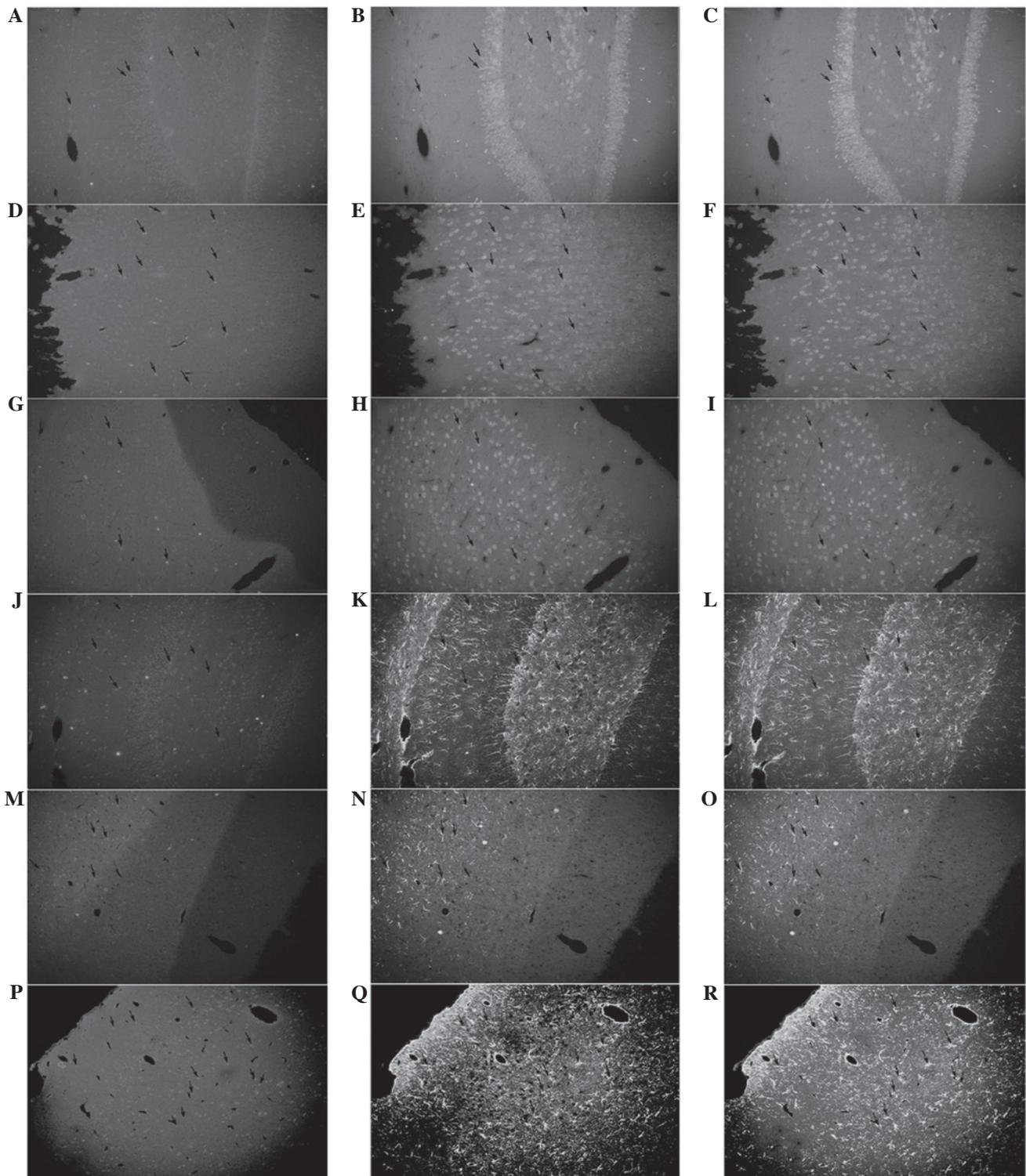
Figure 2. BMSCs in the DG and cortex differentiate into neurons and astrocytes following TBI. Immunofluorescent double-labeled images of the DG and cortex at four weeks following TBI showing double labeling of BrdU⁺ cells with the neuronal marker NeuN and the astrocytic marker GFAP (A-R). Arrows indicate BrdU-positive cells (red), neuronal marker NeuN or astrocytic marker GFAP (green) and merged (yellow). (A-C) BrdU⁺-NeuN⁺ in ipsilateral DG; (D-F) BrdU⁺-NeuN⁺ in ipsilateral cortex; (G-I) BrdU⁺-NeuN⁺ in contralateral cortex; (J-L) BrdU⁺-GFAP⁺ in ipsilateral DG; (M-O) BrdU⁺-GFAP⁺ in contralateral cortex and (P-R) BrdU⁺-GFAP⁺ in ipsilateral cortex. BMSCs, Bone mesenchymal stem cells; DG, dentate gyrus; TBI, traumatic brain injury; BrdU, bromodeoxyuridine; GFAP, glial fibillary acidic protein.

of donor cells expressing neuronal, astrocytic phenotypes was lesser at later time points compared with earlier time points. Rats under the effect of bFGF following TBI recovered most quickly. However, the neuronal differentiation rate was not consistent with the neurological functional recovery rate over time. The functional improvement in the present experiments may have resulted from the paracrine effect of the transplanted BMSCs or of bFGF itself, rather than from the neuronal differentiation of the transplanted stem cells. When the brain is damaged, reactions of neurotrophic factors may lead to
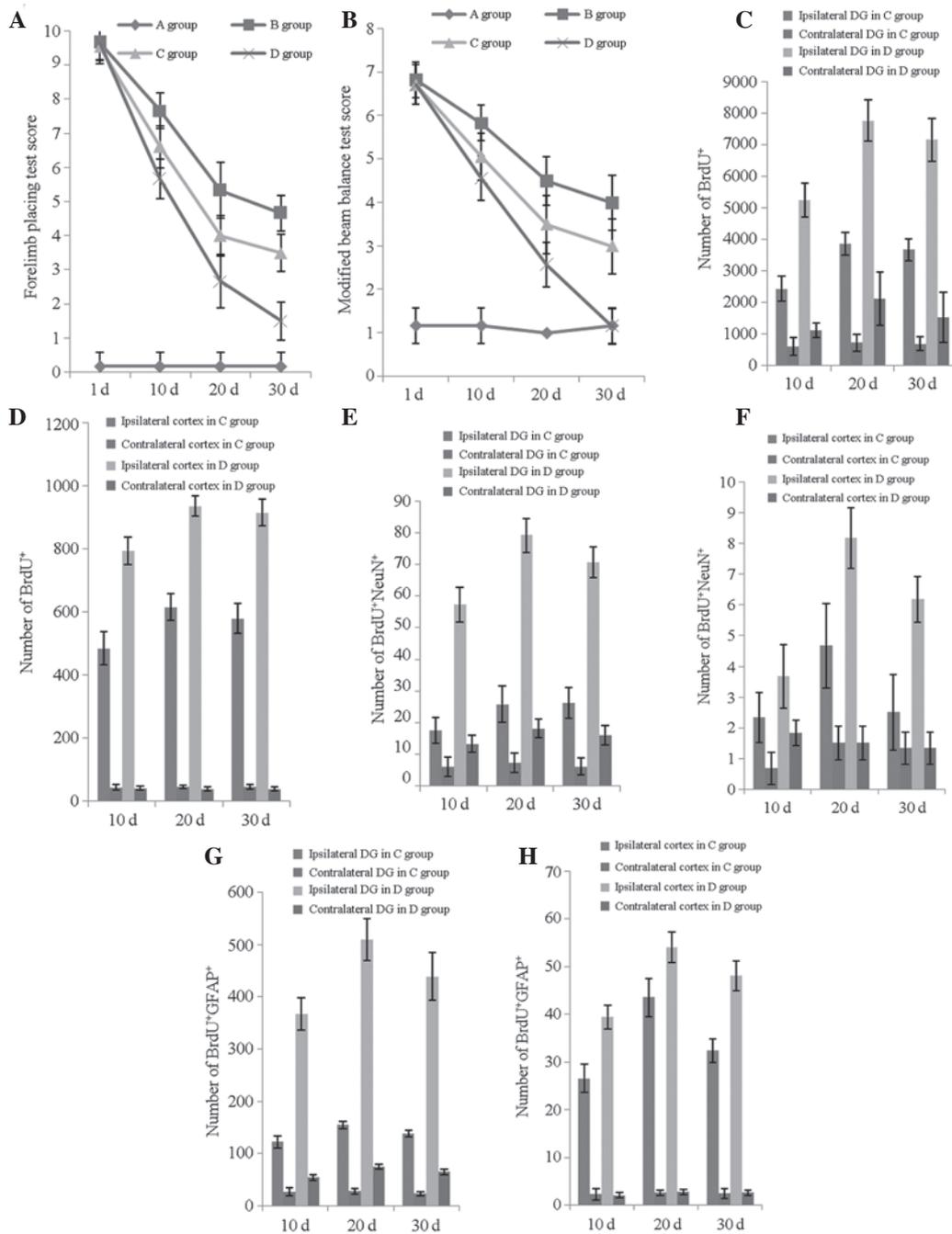
Figure 3. Neurological functional evaluation and number of BrdU⁺/NeuN⁺/GFAP⁺ cells at various time points. (A) Forelimb placing test scores of the four groups at 1, 10, 20 and 30 days post-TBI; (B) modified beam balance test scores of the four groups at 1, 10, 20 and 30 days post-TBI; (C) number of BrdU⁺ cells in the ipsilateral and contralateral DG in groups C and D at 10, 20 and 30 days post-TBI; (D) number of BrdU⁺ cells in the ipsilateral and contralateral cortices in groups C and D at 10, 20 and 30 days post-TBI; (E) number of BrdU⁺/NeuN⁺ cells in the ipsilateral and contralateral DG in groups C and D at 10, 20 and 30 days post-TBI; (F) number of BrdU⁺/NeuN⁺ cells in the ipsilateral and contralateral cortices in groups C and D at 10, 20 and 30 days post-TBI; (G) number of BrdU⁺/GFAP⁺ cells in the ipsilateral and contralateral DG in groups C and D at 10, 20 and 30 days post-TBI and (H) number of BrdU⁺/GFAP⁺ cells in the ipsilateral and contralateral cortices in groups C and D at 10, 20 and 30 days post-TBI. Groups: A, rats without TBI; B, rats with TBI that underwent no treatment; C, TBI treated with transplantation of BMSCs; and D, TBI treated with transplantation of BMSCs and basic fibroblast growth factor. DG, dentate gyrus; TBI, traumatic brain injury; BrdU, bromodeoxyuridineBMSCs, bone mesenchymal stem cells.

changes in the surrounding brain tissue (20,21). Specific gene expression becomes altered and a large number of potentially damaging and restorative neurochemical factors are released and upregulated. Such factors lead to delayed cellular dysfunction and death or tissue regeneration following brain injuries, including TBI (22). These neurotrophic factors may be the cause of the beneficial effect and may protect host neurons and facilitate the host regeneration observed in other stem cells,

including neural stem cells (23,24). Therefore, the beneficial effect of bFGF treatment on neural function may not be due to its protective function on the cyto-architecture, but rather due to other mechanisms. In the current experiments, the exogenous injection that maintains bFGF for an appropriate period enhanced bFGF efficacy for nerve regeneration, as the endogenous bFGF content is low in normal physical conditions, thus, following TBI, imbalance occurs.

Although the present results provide certain reference values for the clinical utilization of BMSCs, further studies on this preliminary research are required. Following TBI, performing an immediate clinical administration is extremely difficult due to the short window of opportunity. One-day postinjury treatment, although clinically possible, may be difficult due to a significant portion of patients with severe head injury being transferred from smaller hospitals. For the majority of patients, moving from the smaller hospitals to the central hospitals would take more than a week. Hence, confirming the efficacy of BMSCs when administered at more than one time point following TBI, for example for one week or more, is essential. Furthermore, the treatment dosage (BMSCs and bFGF) at various time points following TBI also has a key function in affecting the outcome of BMSC transplantation. Previous results showed that a larger dose, specifically, $4x10^6$ BMSCs, was required to be efficacious compared with the $2x10^6$ BMSC dose, which was effective when administered one day following injury (17-19). These observations are in agreement with those of other studies concerning neural injury. Methylprednisolone treatment administered within 3 h of spinal cord injury requires a shorter treatment (smaller total dose) to be efficacious than when initiated 3 h post-injury (25,26). An intervention early in the injury phase requires less tissue repair than at later stages as damage from a neural injury does not simply occur at the time of injury, but is progressive and continues over time (27). To date, few studies regarding the correlation between the dosage, time windows, intervention and therapeutic effect of BMSCs have been conducted.

In conclusion, exogenous bFGF has become a booster for BMSC transplantation-mediated functional recovery following TBI, which has generated great interest in the neuroscience community. The results of the present study add to the understanding of the biological characteristics of cells with neurotrophic factors, and augments their potential for clinical utilization.

Acknowledgements

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