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Upregulation of TrkB by forskolin facilitated survival of MSC and functional recovery of memory deficient model rats

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ABSTRACT

Mesenchymal stem cells (MSCs) are effective vectors in delivering a gene of interest into degenerating brain. In *ex vivo* gene therapy, viability of transplanted MSCs is correlated with the extent of functional recovery. It has been reported that BDNF facilitates survival of MSCs but dividing MSCs do not express the BDNF receptor, TrkB. In this study, we found that the expression of TrkB is upregulated in human MSCs by the addition of forskolin (Fsk), an activator of adenylyl cyclase. To increase survival rate of MSCs and their secretion of tropic factors that enhance regeneration of endogenous cells, we pre-exposed hMSCs with Fsk and transduced with BDNF-adenovirus before transplantation into the brain of memory deficient rats, a degenerating brain disease model induced by ibotenic acid injection. Viability of MSCs and expression of a GABA synthesizing enzyme were increased. The pre-treatment improved learning and memory, as detected by the behavioral tests including Y-maze task and passive avoidance test. These results suggest that TrkB expression of hMSCs elevates the neuronal regeneration and efficiency of BDNF delivery for treating degenerative neurological diseases accompanying memory loss.

1. Introduction

Neurodegenerative diseases are caused by neuronal loss in the nervous system [1]. To facilitate neuronal regeneration in the degenerating brain, mesenchymal stem cells (MSCs) with various developmental potentials [2,3], are particularly considered as therapeutic sources in addition to neural stem cells [4,5]. MSCs derived from human bone marrow or cord blood hardly proliferate but migrate, integrate, and differentiate into cells expressing neuronal markers or glial markers at the micro-environment of the brain when transplanted into the rodent nervous system [6,7]. In the majority of recent studies, MSCs transplanted into injured adult central nervous system (CNS) promote functional improvement by releasing tropic factors without replacement of lost neural cells [3,8–11]. However, there is a correlation between the degree of surviving neurons like MSCs and extent of functional recovery when neurally induced MSCs were transplanted into injured spinal cord [12]. Thus, many groups have attempted to use MSCs as a vehicle to deliver tropic factors to sites of the degenerating nervous system diseases [10,11,13].

The brain-derived neurotrophic factor (BDNF), a member of the neurotrophin family, has been applied for the cell therapy of several neurodegenerative disease models using adult stem cells [14]. When MSCs were transduced by recombinant BDNF-adenovirus (BDNF-Ad) before transplantation into model rats, MSCs secreting BDNF reduced the damage of ischemic lesion following a stroke [11], and enhanced functional recovery after spinal cord injury and corticospinal neuronal survival [15].

Furthermore, BDNF administration allows human MSCs to survive and differentiate into a neural phenotype, suggesting that BDNF may inhibit cell death of MSCs [3,15,16]. In addition, the cell viability of MSCs and the levels of tropic factors released from MSCs were increased after treatment with BDNF, VEGF, and HGF, suggesting autocrine regulation of hMSCs [17]. However, dividing and undifferentiated MSCs seem not to express the BDNF receptor, TrkB [18]. Thus, it has not been elucidated how BDNF promotes viability of MSCs and the expression of neuronal markers.

In this study, we found that the expression of TrkB was promoted in human MSCs by addition of forskolin (Fsk), an activator of adenylyl cyclase. To increase survival rate and differentiation of MSCs secreting tropic factors that enhance regeneration of endogenous cells, we pre-exposed hMSCs with Fsk and then transplanted

Abbreviations: Ad, adenovirus; BDNF, brain-derived neurotrophic factor; cAMP, cyclic adenosine monophosphate; Fsk, forskolin; hMSCs, human mesenchymal stem cells; PKA, protein kinase A.

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hMSCs into the brain of memory deficient rats, a degenerating brain disease model induced by ibotenic acid injection. We demonstrated that transplantation of hMSCs expressing TrkB facilitated neuronal regeneration and the functional recovery by examing behavioral changes on the memory deficient model rats.

2. Materials and methods

2.1. hMSCs culture

The hMSCs were isolated from the bone marrow of a spinal cord injury patient (male, 30 yrs) and provided by Prof. JW. Chang and Prof. DH. Yoon (College of Medicine, Yonsei University, Korea). The cells were maintained in minimum Essential Medium (MEM) – α (Gibco) with 10% fetal bovine serum (FBS, Gibco), 2.2 g/l $NaHCO_3$, 0.6 g/l penicillin, and 0.1 g/l streptomycin (proliferation medium, PM) in 5% CO_2 incubator. To induce differentiation, the cells were changed to 0.5% FBS in α-MEM (differential medium, DM). Addition of forskolin (Fsk, 30 μM) promoted neuron like phenotype.

2.2. Cell labeling with DiI-C18-(3) and transduction with GFP- and BDNF-adenovirus

The hMSCs (passage 4–5) were transduced with recombinant GFP-adenovirus and BDNF-adenovirus [19] at MOI of 50 in 2% FBS media for 2 h, and then labeled with fluorescence dye, DiI-C18-(3) (60 μg/ml, Sigma) for 2 h at room temperature in darkness, following the incubation in PM for overnight. After being changed to DM, the cells were treated with Fsk (30 μM) for 4 h. These cells were then dissociated with 0.05% trypsin, and the number of viable cells was counted in PM containing trypsin inhibitor by trypan blue staining. The cells were washed and resuspended with saline containing 0.7% penicillin/streptomycin, 20 mM HEPES (pH 7.2), and 0.5% glucose and kept on ice until transplantation.

2.3. Animals and transplantation

Male Sprague–Dawley rats (250–270 g) were purchased from the Orient Co., Ltd, (Korea) a branch of Charles River Laboratories (USA). The animals were housed 2 or 3 rats per cage, allowed access to water and food *ad libitum*, and maintained under a constant temperature (23 ± 1 °C), humidity (60 ± 10%) and a 12 h light/dark cycle (light on 07.00–19.00 h). The animal treatment and maintenance were carried out in accordance with the Principle of Laboratory Animal Care (NIH publication No. 85-23, revised 1985) and the Animal Care and Use Guidelines of Kyung Hee University, Korea. All rats were anesthetized with Equithensin for approximately 2 h. Before cell transplantation, ibotenic acid (1.5 μl per animal, 1 mg/ml, Sigma) was injected into the entorhinal cortex as described previously [20]. Rats were received unilateral transplants into the right hippocampus as in the following [21]. Rats were aligned in the stereotaxic device (interaural line 0-alignment), and the cells were placed 1.4 mm lateral to midline, 4.3 mm posterior to bregma, and 2.2 mm below the dura. 2 μl of cell suspension (at a density of 6.0×10^4 cells/μl) in a hamilton syringe were injected using automatic micro-injector over 10 min at a pressure of 10 psi. The syringe was left at the injected position for 10 min and then withdrawn over 5 min to minimize loss of cells from the injection site.

2.4. Immunocytochemical and immunohistochemical staining

Immunostaining analysis of hMSCs and brain slices were carried out as previously described with modifications [20,22]. Shortly, the hMSCs on coverslip (Bellco) at 2×10^4 cells/well was incubated with DM for 1 day and then 30 μM Fsk was added for 2 days. Primary antibodies were incubated with the dilution ratios of 1:500 for antibodies to Pan-neurofilament (Covance) and trkB (SantaCruz). Nucleus was stained with 1 μg/ml DAPI (4,6-diamidino-2-phenylindole) for 5 min. For immunohistochemical analysis, the animals were perfused with 4% paraformaldehyde. All the incubations were carried out on ice to block the diffusion of DiI-C18-(3). Cryosectioned brain slices (35 μm) were permeabilized in 0.5% Triton X-100 for 20 min and blocked in 15% normal serum and 3% BSA for 2 h in a free floating condition. Primary antibodies were incubated for 4 h at 4 °C and then for 1 h at room temperature with the following dilution ratios: ChAT (1:500, Millipore), GAD67 (1:2000, Millipore), Glutamate (1:5000, Sigma), GFP (1:500, SantaCruz), GFP (1:500, Millipore), NeuN (1:500, Millipore). Secondary antibody conjugated with Alexa Fluor 488 (Invitrogen), Cy2 and Cy5 (Jackson Lab) was used for double or triple labeling. Immunostained sections were scanned with a confocal laser microscope (LSM510, LSM5 Pascal. Carl Zeiss).

2.5. Quantification of immunostained cells and statistical analysis

The number of immunostained cells was counted in CA1 field and dentate gyrus region of five hippocampal coronal-sections. Confocal images from every fifth hippocampal cryo-section (AP: Bregma −4.5 to −4.3 mm) were taken for staining analysis under 100× and 400× magnification. Three to four animals per group were processed, and values were expressed as means ± S.E.M. Results of passive avoidance and Y-maze tests, and histological study were analyzed by one-way analysis of variance (ANOVA) followed by the Student–*t* test for multiple comparisons. Statistical significance was set at $p < 0.05$.

3. Results

3.1. TrkB expression of hMSCs upregulated by treatment of Fsk

In an effort to enhance neuroprotection by MSCs releasing BDNF, we searched for factors that upregulated TrkB, the BDNF receptor, as BDNF was reported to increase viability of MSCs by autocrine regulation [16,17]. When we treated hMSCs isolated from human bone marrow with forskolin (Fsk), after neural induction using a differentiation medium (DM), the morphologies were changed to cells with thin and long neurite like structures (Fig. 1A, arrows). Fsk is known to induce expression of TrkB in the cultured cortical neurons [23]. Treatment with forskolin (Fsk) upregulated TrkB in immunoblot assay. Full length, 140 kDa TrkB is internalized and degraded to a smaller size, likely by endogenous BDNF released from MSCs [24]. However, expression of 95 kDa TrkB a newly synthesized form without glycosylation, was largely increased by treatment with fsk in DM for 1 day, compared with hMSCs incubated in DM for 1 and 2 days (Fig. 1B) or hMSCs in a proliferation medium (PM). We confirmed the expression of TrkB in hMSCs expressing neuronal marker, pan-neurofilaments by performing immunocytochemical staining (Fig. 1C).

3.2. Survival rate of transplanted hMSCs were enhanced by pre-treatment with Fsk and transducing with BDNF-adv in the memory deficient model rats

To examine if TrkB expression of hMSCs by Fsk treatment increases the survival rate of MSCs and MSCs releasing BDNF, thereby enhancing neuroprotection, we transplanted hMSCs into the hippocampus of the degenerating brain model rats accompanying memory loss. The chemically lesioned animal model was generated by

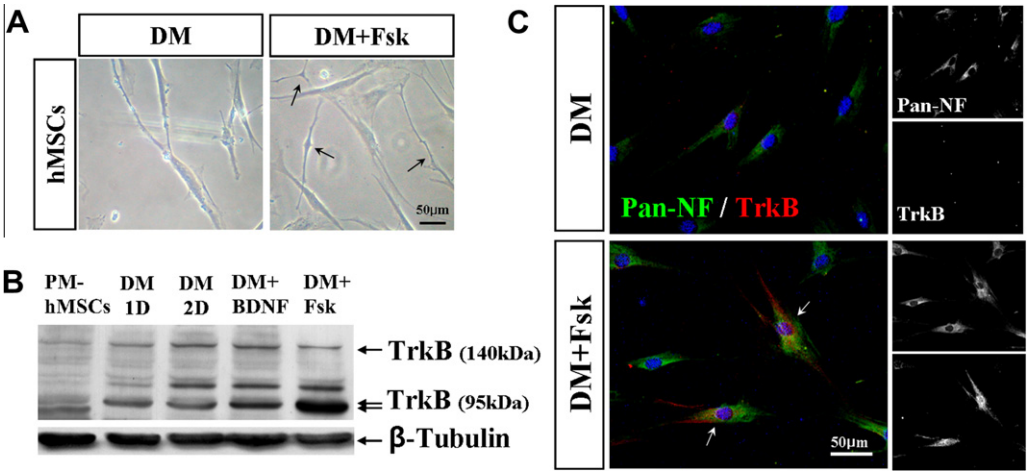

Fig. 1. Forskolin (Fsk) upregulated TrkB expression in human mesenchymal stem cells (hMSCs). (A) Transmit light microscopic images of hMSCs. Fsk treated hMSCs shows morphology containing long neurite-like structures compared with Fsk(−) group. Scale bar = 50 μm. (B) Expression of TrkB (140 kDa and 95 kDa) was increased in Fsk (30 μM) treated hMSCs (Fsk 1D), compared with controls (hMSCs in differential medium (DM)) in immunoblot analysis. (C) Immunocytochemical staining represents Fsk treated hMSCs expressed TrkB more than control (DM 2D). Scale bar = 50 μm.

the injection of ibotenic acid into entorhinal cortex before hMSCs transplantation [20]. Ibotenic acid damages granule cells of dentate gyrus and hippocampal pyramidal cells by destroying neurons in entorhinal cortex which project to hippocampus. To mark the hMSCs to be identified within the host tissue, they were labeled with a fluorescence dye, DiI-C18-(3) and by infecting with recombinant GFP or BDNF adenovirus (GFP-Ad, BDNF-Ad) [19]. GFP-Ad or BDNF-Ad can transduce hMSCs efficiently without any cytotoxic effect and the green fluorescence was detected until 12 weeks after MSC injection. The labeled hMSCs were transplanted into the medial aspect of the dorsal region of hippocampus, above the CA1 subfield. To examine whether hMSCs integrated into the CA1 layer of hippocampus properly, DiI-labeled hMSCs shown in the pyramidal layer and CA1 field, were immunostained with the neuronal marker, NeuN (Fig. 2A). The hMSCs group pre-treated with Fsk and/or BDNF-Ad, showed more NeuN positive cells in the CA1 field (Fig. 2A, Arrows). We confirmed that TrkB was expressed in Fsk treated hMSCs when transplanted into the rat hippocampus, and the TrkB expressing cells were about 80% of pretreated hMSCs, with about 4-fold that of untreated MSCs (Fig. 2B and C). To compare viability of hMSCs, we counted hMSCs that show both the signals of GFP and DiI fluorescence to distinguish them from endogenous cells in the hippocampal area, 12 weeks after transplantation (Fig. 2D). Compared with GFP-Ad transduced control group (hMSCs + GFP-Ad, 3052.00 ± 189.97 cells/ea, Fig. 2(C), hMSCs group treated with Fsk and GFP-Ad (hMSC + Fsk + GFP-Ad) showed enhancement of viability slightly (hMSCs (Fsk+), 4046.25 ± 343.13 cells/ea, Fig. 2(C) but hMSCs group transduced with BDNF-Ad did not increase substantially GFP positive cell counts. However, hMSCs group treated with both Fsk and BDNF-Ad (hMSC + Fsk + BDNF-Ad) showed the highest enhancement of viability (hMSCs (Fsk + BDNF-Ad) 5182.50 ± 293.12 cells/ea, Fig. 2(C). These data indicate that upregulation of TrkB by Fsk enhances viability of hMSCs which are transplanted into the hippocampus of the memory deficient rat brain.

3.3. hMSCs treated with Fsk and BDNF-adv upregulated a GABA synthesizing enzyme

As BDNF is known to promote GABA transmission in neurons [25], we immunostained GFP positive cells with antibodies against GAD67, a GABA synthesizing enzyme, in the brain slices of the memory deficient rats transplanted with hMSCs (Fig. 3). By 12 weeks after transplantation, 15.84 ± 1.53% of GFP + cells in hMSCs group, 13.29 ± 0.87% of hMSCs (F+) group and 27.46 ± 2.63% of hMSCs (F + B) group were stained with GAD67 antibodies in the hippocampal area (Fig. 3). Brain slices were also immunostained with antibodies to glutamate, a neurotransmitter and ChAT, an acethylcholine synthesizing enzyme. The pre-treatment with Fsk and BDNF-Ad did not largely increase the immunoreactive cells. This suggests that the expression of both TrkB and BDNF in MSCs facilitates GAD67 expression in vivo, and this may cause GABA release at the sites of neuronal loss, to improve functional recovery.

3.4. Learning and memory abilities improved by transplantation of hMSCs pre-treated with Fsk and transduced with BDNF-Ad

12 weeks after the transplantation, we conducted two behavioral tests to study the neuronal regeneration. In the Y-maze task, which measured the short-term memory and space perception ability, learning and memory ability was improved in all groups more than in the ibotenic acid injected model group ($p < 0.001$, Fig. 4A). In the passive-avoidance test which measured the long-term learning and memory ability, the latency time was increased in Fsk treated group (hMSCs + Fsk + GFP-Ad) and BDNF transduced group (hMSCs + BDNF-ad), compared with control group (hMSCs + GFP-Ad), and the latency time was increased more in hMSCs + Fsk + BDNF-Ad group compared with other groups, 12 weeks after transplantation (Fig. 4B). These results indicate that transplantation of MSCs expressing TrkB and BDNF is effective in regenerating the injured brain and improving learning and memory abilities.

4. Discussion

We showed here that TrkB is upregulated by Fsk in MSCs and viability of MSCs expressing TrkB and releasing BDNF is increased when transplanted into the hippocampus. Our results of learning and memory tests suggest that enhanced viability of MSCs by pre-treatment of Fsk and BDNF-Ad facilitates to regenerate the injured brain and to recover functionally from memory loss in the model rats.

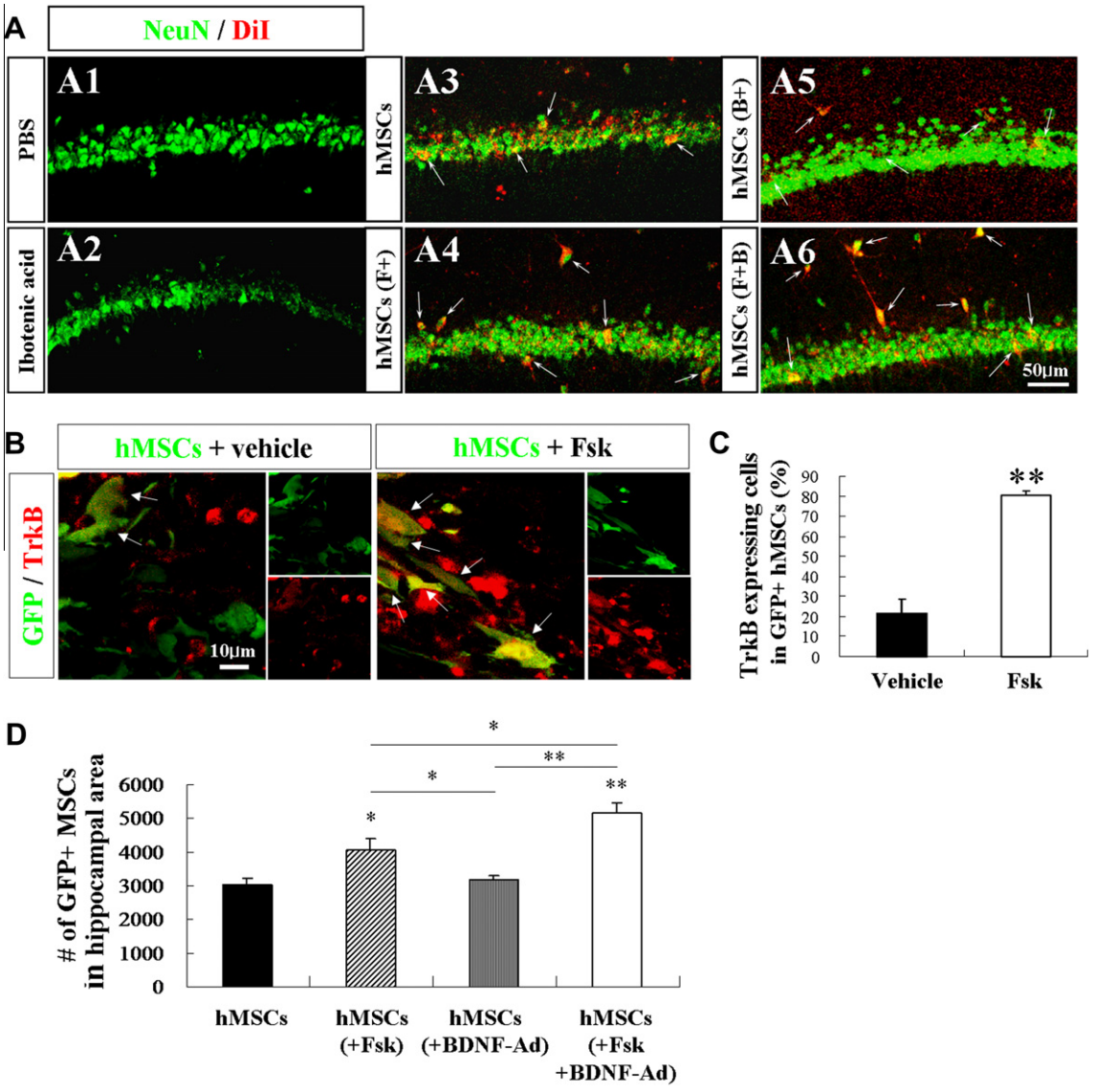

Fig. 2. Pre-treatment of Fsk and BDNF-Ad increased survival rate of hMSCs after transplantation into the memory deficient model rats. (A) Integration of hMSCs into the CA1 field of hippocampus in memory deficiency model rats. In contrast to control group (PBS, A1), memory deficiency model group (using ibotenic acid, A2) showed reduced density of NeuN positive cells. 12 weeks after transplantation of hMSCs, DiI positive hMSCs are integrated into the CA1 field (hMSCs, A3; hMSCs (F+), A4; hMSCs (F + B), A5). Arrows indicate DiI-positive hMSCs merged with NeuN. Scale bars represent 50 μm. (B) 10 days after transplantation, TrkB was immunostained more in Fsk pre-treated hMSCs than vehicle treated hMSCs transplanted into the memory deficiency model rats. Scale bar = 10 μm. (C) TrkB expressing cell numbers are presented as percentages (%) of GFP positive hMSC numbers in the hippocampal slices ($^{**}p < 0.01$, One-way ANOVA). (D) 12 weeks after transplantation, survival rate of hMSCs was measured by counting GFP expressing MSCs in the hippocampal area of brain slices. Pre-treatment with Fsk and BDNF-Ad increased the viability of transplanted hMSCs 1.6 folds more than untreated hMSCs control group ($^{*}p < 0.05$, $^{**}p < 0.01$; One-way ANOVA).

Though the controversy over whether MSCs have the trans-differentiation abilities is unlikely to cease. However, many groups have demonstrated that MSCs derived from adult bone marrows have a great potential as a therapeutic agent by providing tropic factors for the degenerating neural cells, serving guidance for regenerating axons, and releasing neurotransmitters to balance inhibitory and excitatory transmission and improve neuronal functions [4,10,13,15]. Transplantation of MSCs may be more effective when MSCs become differentiated cells or neuron like cells, producing neurotransmitters and neurotrophic factors. Neuronal differentiation from neural stem cells to GABAergic cells are upregulated by BDNF treatments in vitro [26,27]. We have shown that expression of a GABA synthesizing enzyme is facilitated by pre-exposure of MSCs to Fsk and BDNF-Ad before transplantation. Brain endothelial cells forming microvessles are derived from MSCs and release neurotrophic factors such as BDNF, and NT-3 and VEGF [28–30] to promote differentiation of NSCs and regeneration of neurons [31]. The transplanted MSCs may behave as endogenous brain MSCs releasing neurotrophic factors.

Forskolin (Fsk) is an activator of adenylyl cyclase and elevates the intracellular concentration of cyclic adenosine monophosphate (cAMP). Fsk activates the Protein Kinase A (PKA) signaling via elevated cAMP levels which is an important mechanism in neuronal differentiation [32,33]. Recently, several groups reported that Fsk treated MSCs revealed upregulation of neural markers and changed their morphology by extension of neurite-like structures [18,34,35]. Deogracias and colleagues

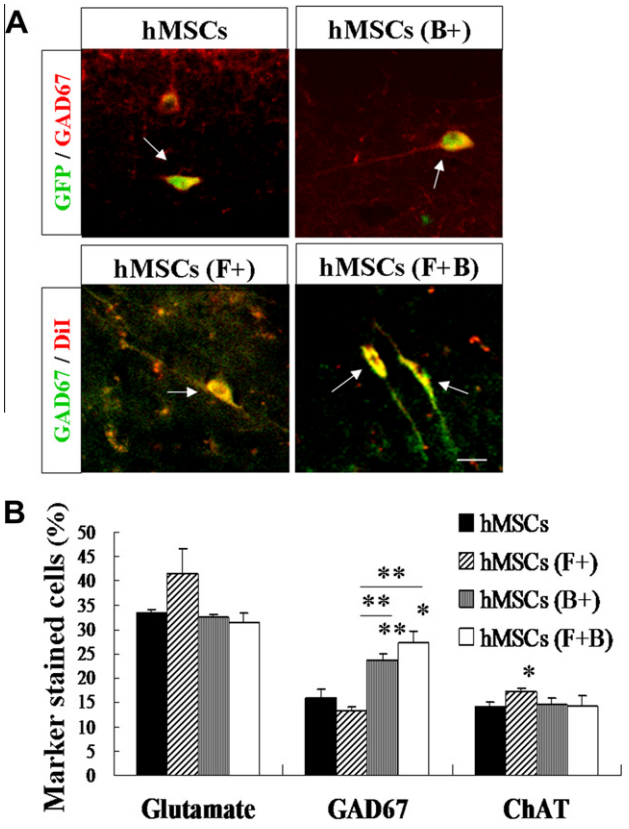

Fig. 3. Production of neurotransmitter and neurotransmitter synthesizing enzymes in MSCs pre-treated with Fsk and BDNF-Ad when transplanted into the memory deficient model rats. (A) MSCs expressing GAD67, a GABA synthesizing enzyme in hippocampus of the memory deficient rat brain. Scale bars represent 20 μm. (B) 12 weeks after transplantation, numbers of GAD67-positive cells, and ChAT, a acethylcholine synthesizing enzyme-stained cells were increased in Fsk (F+) and/or BDNF-Ad (B+) treated hMSCs group (F + B) more than control ($^{**}p < 0.01$, One-way ANOVA) when transplanted into the memory deficient rat brain.

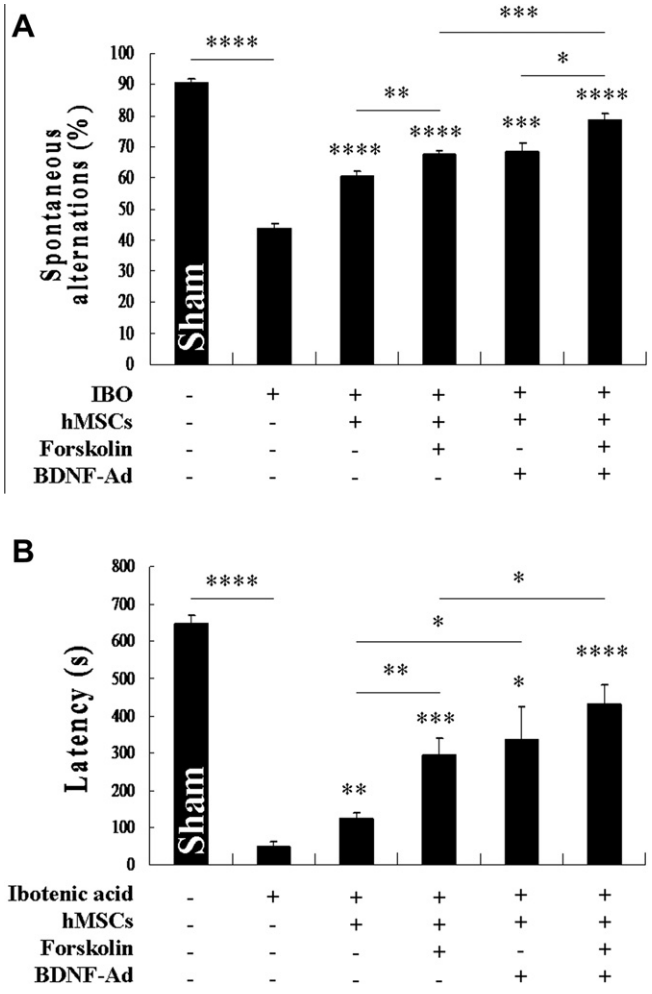

Fig. 4. Ability of learning and memory was improved after pre-exposure of hMSCs to Fsk and/or BDNF-Ad in the memory deficiency rat model. (A) Y-maze task, measuring spatial and short-term memory, shows spontaneous alternation increased in Fsk and BDNF-Ad treated MSC group 12 weeks after transplantation ($^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$, One-way ANOVA). (B) Passive-Avoidance test shows long-term memory was largely improved in Fsk and BDNF-Ad treated MSC group, 12 weeks after transplantation ($^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$, One-way ANOVA).

reported that Fsk rapidly stimulated the expression of TrkB in primary cultured cortical neurons [23]. We observed that Fsk treatment upregulated TrkB (95 kDa) expression in hMSCs to increase cell viability and functional recovery. Neuronal regeneration may depend on the survival rate of MSCs transplanted into the injured sites [12].

BDNF released from MSCs did not increase survival rate of MSCs alone but it was synergistic when TrkB expression was upregulated in the MSCs by Fsk. The secreted BDNF from MSCs also played a role on neuronal protection of the injured endogenous neural cells in the memory deficient model rats when hMSCs were transduced with the BDNF-Ad before transplantation, as consistent with the previous reports on the effects of neurotrophins in neuronal survival and differentiation [36–39].

In conclusion, treatment of MSCs with Fsk increased the survival rate of MSCs transduced with BDNF-Ad by upregulating the expression of TrkB when transplanted into memory deficient model rats. Treatment of MSCs with Fsk also increased expression of GAD, a GABA synthesizing enzyme, suggesting increased release of a neurotransmitter, GABA at sites of nerve injury. The transplantation of MSCs pretreated with Fsk and transduced with BDNF-Ad improved memory function in memory deficient model rats, likely by facilitating neuroregeneration. The data suggest that transplantation of MSCs treated with Fsk and transduced with BDNF-ad may be a useful approach in the treatment of neurodegenerative diseases.

Acknowledgments

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