Effects of GDNF-Transfected Marrow Stromal Cells on Rats with Intracerebral Hemorrhage

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Objective: The present study aimed to investigate the effects of Mesenchymal stem cells/glial cell line derived neurotrophic factor (MSCs/GDNF) transplantation on nerve reconstruction in rats with intracerebral hemorrhage. *Methods*: GDNF transduction to MSCs was using adenovirus vector pAdEasy-1-pAdTrack-CMV prepared. Intracerebral hemorrhage (ICH) was induced by injection of collagenase and heparin into the caudate putamen. At the third day after a collagenase-induced ICH, adult male SD rats were randomly divided into saline group, MSCs group and MSCs/GDNF group. Immunofluorescence and RT-PCR were performed to detect the differentiation of MSCs or MSCs with an adenovirus vector encoding GDNF gene in vivo and in vitro. *Result*: After 6 hours of induction, both MSCs and MSCs/GDNF expressed neuro or glial specific markers and synaptic-associated proteins (SYN, GAP-43, PSD-95); additionally, they secreted bioactive compounds (BDNF, NGF-β). MSCs/GDNF transplantation, compared to MSCs and saline solution injection, significantly improved neurological functions after ICH. The grafted MSCs or MSCs/GDNF survived in the striatum after 2 weeks of transplantation and expressed the neural cell-specific biomarkers NSE, MAP2, and GFAP. *Conclusion*: These findings demonstrate that MSCs/GDNF transplantation contributes to improved neurological function in experimental ICH rats. The mechanisms are possibly due to neuronal replacement and enhanced neurotrophic factor secretion.

Key Words: Mesenchymal stem cells—intracerebral hemorrhage—transplantation—synaptophysin—growth associated protein—glial cell line derived neurotrophic factor

Introduction

Stroke is one of the most serious diseases affecting health and quality of life. Of all strokes, 20% are intracerebral hemorrhage (ICH) strokes.[1] ICH is a common acute cerebral vascular disease with high mortality and morbidity. ICH induces inflammation, blood-brain barrier dysfunction, and oxidative stress,[2-6] resulting in the interruption of integrity of the neural network and synaptic connections, ultimately leading to the emergence of various clinical neurological dysfunctions.[7,8] All of these pathophysiological changes are the main reasons for nerve dysfunction and disabilities. Thus, understanding the pathophysiological mechanisms of ICH and identification of new therapeutic targets plays significant roles in ICH.

To date, there are no effective treatments available for ICH. However, cell-based therapy has been considered to be the most effective to replace lost or dysfunctional neurons and glial cells after ICH. Grafted cells can secrete various growth factors or some therapeutic gene products, which has become a hot research field.[9-12] Mesenchymal stem cells (MSCs) present the advantages of convenience, being easily separated, cultured, amplified, and purified, in contrast to embryonic and neural stem cells. MSCs with multidirectional differentiation potential can be differentiated into neuron-like cells under modulated microenvironments.[13,14]

Recent studies have indicated that glial cell line derived neurotrophic factor (GDNF) plays an important role in the growth, development, differentiation, maintenance, and damage repair of several types of neurons in the

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central nervous system.[15-19] In this study, we investigated the effects of GDNF gene modification on differentiation and synapse-related protein expression in MSCs and whether GDNF gene modification can improve the therapeutic efficiency of MSCs transplantation in rats with collagenase-induced intracerebral hemorrhage.

Methods

Culture and Infection of MSCs with Adenovirus

Rat MSCs (Cyagen Biosciences Co., China) were resuscitated and cultured in DMEM supplemented with 10% fetal bovine serum (FBS) (Hyclone, USA) in a humidified incubator (37°C, 5% CO_2). When the MSCs became confluent, they were resuspended in 0.25% trypsin solution (Beyotime, China) and then subcultured. Fourth passage MSCs were infected with GDNF virus or control virus to establish MSCs/GDNF or MSCs (GFP labeling).

Neural Induction of MSCs/GDNF and MSCs

MSCs/GDNF and MSCs were seeded on sterile glass coverslips in 24-well culture plates at a seeding density of 1×10^5 cells per well. Two days later, the medium was replaced with preinduction medium composed of DMEM with 10% FBS and 1×10^{-3} mol/L β-mercaptoethanol (β-ME) for 24 hours, followed by incubation with neuronal induction medium, which consisted of DMEM with 1×10^{-3} mol/L β-ME, 1×10^{-6} mol/L all-trans retinoic acid (RA) (Sigma, USA) and 2% dimethyl sulfoxide (DMSO) for 6 hours.

Immunocytochemistry

After induction, cells were fixed in 95% alcohol for 15 minutes and washed 3 times with 0.01 M phosphate buffer solution (PBS), then treated with 0.2% TritonX-100 at room temperature for 20 minutes and 5% normal goat serum at 37°C for 40 minutes, and then incubated overnight with primary antibody (rabbit anti-MAP2, GFAP, SYN, GAP-43, and PSD-95 at the concentration of 1:100) at 4°C. After washing with PBS, the cells were incubated with secondary antibody (1:100) at 37°C for 2 hours, washed with PBS 3 times, and treated with CY3 fluorescent dye, followed by observation under a microscope.

Reverse transcription (RT)-PCR

Total RNA was extracted from infected MSCs and MSCs/GDNF with TRIzol Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. cDNA was prepared by using oligo(dT), total RNA was reverse transcribed at 42°C using Invitrogen products, and amplification was initially performed at a low annealing temperature. Quantitative RT-PCR was performed using the SYBR Green RT-PCR system on an ABI 7000 PCR instrument (Applied Biosystems, Foster City, CA) with a 3-stage amplification program under the following conditions: 2 μL of cDNA was used in each subsequent PCR amplification, with 1.5 U per reaction of platinum Taq DNA polymerase, 2 μL of SYBR Green (10 mmol/L), and 1 μL (10 mmol/L) of primers. PCR was performed with the following settings: 94°C for 2 minutes, and 45 cycles of the standard thermal amplification cycling with denaturation at 94°C for 20 seconds, annealing at 52°C for 20 seconds (for MAP2) or 54°C for 20 seconds (for βACT), or 56°C for 20 seconds (for BDNF, NGF-β), then extension at 72°C for 30 seconds. Relative quantification of each gene was calculated after normalization to β-ACT by using the $2^{-\Delta\Delta CT}$ method. The primers used for amplification are listed in Table 1.

Preparation of the ICH Rat Model

SD rats (weight between 250 and 300 g; males only). All animals were purchased from the Experimental Animals Center for Third Military Medical University, China), were anesthetized by intraperitoneal (IP) 2% sodium pentobarbital (40 mg/kg), and then fixed in the stereotaxic frame (myNeurolab, USA). A burr hole (1 mm) created with a triangle-edged needle was inserted into the striatum (ML = 3.0 mm, P = 0.2 mm, V = −6.5 mm), and saline solution (3 μL) containing 1 μL of 2.0 U/μL heparin and 2 μL of collagenase (0.125 U/μL) was injected into the caudate putamen at 0.3 μL/min over a period of 10 minutes. The needle was slowly withdrawn after another

Table 1. *Primer sequences*

Gene	Primer sequence (5′-3′)	Product length (bp)	Tm
MAP2	F:GCAAGGATAGTTCAAGTAGT	204	52˚C
MAP2	R:CATCTTCGAGGCTGTAAGT		
BDNF	F:GGTGTCGTAAAGTTCCACCA	131	56˚C
BDNF	R:GCCAAGTTGCCTTGTCCGT		
NGF-β	F:CATGCTGGACCCAAGCTCA	103	56˚C
NGF-β	R:TGGACATTACGCTATGCACCT		
β-ACT	F:GAAGATCAAGATCATTGCTCCT	111	54˚C
β-ACT	R:TACTCCTGCTTGCTGATCCACA		

5 minutes. A modified neurological severity score (mNSS) above 8 points indicated success of the model.

Cell Transplantation and Materials

Rats with neurological deficits were randomly divided into 3 groups after collagenase-induced ICH: MSCs/GDNF group, MSCs group and saline group; the groups were stereotaxically grafted with MSCs/GDNF, MSCs (20 μL cell suspension at a concentration of 2.5×10^7 cells/mL) and or 20 μL of saline, respectively, on third day after operation. Each group was divided into 2 subgroups according to the feeding time (1 w, 2 w). Rats (n = 5 in each of the groups) were again anesthetized and perfused through the heart with 200 mL of cold saline and 200 mL of 4% paraformaldehyde. Sections (10 μm thickness) of brains were stained for immunofluorescence.

Immunofluorescence Staining

Frozen sections were treated with 0.2% TritonX-100 at room temperature (RT) for 20 minutes, then with 5% normal goat serum at 37°C for 40 minutes, and then incubated with primary antibody (rabbit anti-MAP2, NSE, GFAP) overnight at 4°C under humidified conditions. After washing with PBS, the sections were incubated with secondary antibody (1:100) at 37°C for 2 hours, washed with PBS 3 times and treated with CY3 fluorescent dye. Fluorescent images were captured using a fluorescence microscope (Olympus, Japan).

Statistical Analysis

Experimental data are reported as the mean ± SEM. Statistical analysis was performed using 1-way ANOVA, followed by post hoc tests. P values of less than .05 were considered statistically significant.

Results

Culture, Infection, and Induction of MSCs

Cultured MSCs presented mainly spindle, triangle, or polygon shapes as viewed under a phase-contrast microscope (Fig 1A). When MSCs were infected with pAdEasy-1-pAdTrack-CMV-GDNF or empty pAdEasy-1-pAdTrack-CMV vector for 2 days, the cells showed green fluorescence under a fluorescence microscope (Fig 1B). After being induced for 6 hours, most cells became neuron-like in shape, with cell body shrinkage, process eruption, and extension into reticulation (Fig 1C).

Differentiation of MSCs and MSCs/GDNF Treated with Induction Medium In Vitro

After MSCs and MSCs/GDNF were cultured with induction medium, the cells differentiated into neurons and astrocytes (Fig 2). Rates of MAP2 and GFAP positive cells in the MSCs/GDNF group were higher than those in the MSCs group; the differences were significant, with $P < .05$ (Fig 3A).

Expression of BDNF, NGF-β, and MAP2 mRNAs in MSCs and MSCs/GDNF after Neuronal Induction

By quantitative real-time RT-PCR, NGF-β and MAP2 mRNA levels increased, while BDNF mRNA level decreased significantly in the MSCs/GDNF group as compared with the MSCs group (Fig 3B).

Expression of GAP-43 and SYN and PSD-95 Proteins in MSCs and MSCs/GDNF after Neuronal Induction

We investigated the neurons formed from MSCs and MSCs/GDNF in terms of synaptic structure formation. Both MSCs and MSCs/GDNF were treated with neuronal induction medium for 6 hours, and the cells were studied for GAP-43, SYN, and PSD-95 production by immunofluorescence, because they are considered crucial components of the axon and synapse. The results showed that GAP-43 and SYN and PSD-95 protein levels in MSCs/GDNF were significantly higher than those in MSCs, with $P < .05$ (Figs 3C and 4).

Differentiation of Grafted MSCs and MSCs/GDNF In Vivo

Following transplantation into ICH rat hemorrhage lesion site, some grafted cells were MAP2, NSE, and GFAP positive, suggesting that grafted cells could differentiate into neurons and astrocytes at 7 and 14 days after transplantation (Fig 5). Compared with the MSCs group, the percentage of MAP2 positive grafted cells at 1 week and the percentages of NSE positive grafted cells at 1 and 2 weeks in the MSCs/GDNF group were significantly increased after grafting, with $P < .05$. In contrast, no significant differences in the percentage of MAP2 positive grafted cells at 2 weeks and the percentages of GFAP positive grafted cells at 1 and 2 weeks were observed between the MSCs and MSCs/GDNF groups (Fig 3D).

The Expression of MAP2, NSE, and GFAP in Peripheral Hemorrhage Areas

Expression levels of MAP2, NSE, and GFAP were evaluated to determine the effects of MSCS and MSCs/GDNF transplantation on neurons and astrocytes in peripheral hemorrhage area at 1 and 2 weeks. Immunofluorescence staining revealed that cell transplantation increased the intensity of MAP2 and NSE proteins and decreased the intensity of GFAP proteins in comparison with that in the NS group at 1 and 2 weeks, with $P < .05$ (except the intensity of GFAP in the MSCs group at 1 week). Moreover, in comparison with the MSCs group, the intensities of MAP2 and NSE proteins were significantly higher, and the intensity of GFAP proteins were lower at 1 and 2

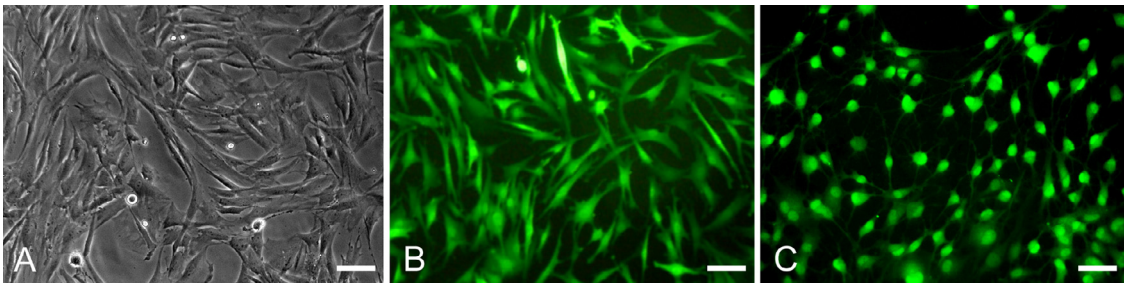

Figure 1. *Culture, infection, and induction of MSCs. (A) Cultured MSCs of SD rat at 3 days after resuscitation. (B) MSCs infected with adenovirus for 2 days lighted green fluorescence. (C) MSCs/GDNF exposed to neuronal induction medium for 6 hours showed neuron-like shape. Scale bar: 50 μm. (Color version of figure is available online.)*

weeks, with $P < .05$ (except the intensity of NSE in the MSCs/GDNF group at 2 weeks; Figs 3E and 6).

Discussion

Stroke is a leading cause of long-term disability worldwide. Currently, neurotrophic factors combined with cell replacement therapies is the most effective treatment strategy for stroke. In the present study, the GDNF gene was transfected into MSCs, and then cells were induced for differentiation using β-ME, RA, and DMSO. Differentiated MSCs/GDNF and MSCs had the morphological characteristics of neuronal cells and expressed the neuronal cell-specific markers MAP2 and GFAP. In addition, the rates of MAP2 and GFAP positive cells in the MSCs/GDNF group were higher than those in the MSCs group. Although the potential mechanism of the GDNF-mediated enhancement of differentiation remains unclear, it has been widely reported that GDNF is currently the most powerful of the dopaminergic neurotrophic factor, which is a kind of motor neuron trophic factor.[20] Recently, evidences have indicated that encapsulated GDNF-secreting cells improve graft survival[21] and that GDNF exhibits a distinct antioxidant effect.[22] GDNF encounters difficulty in crossing the blood-brain barrier due to its high molecular weight. MSCs offer great potential in carrying therapeutic proteins to damaged areas.[23] GDNF delivery by ex vivo gene therapy using MSCs may be more beneficial than direct brain infusion of neurotrophin.[24] In our experiment, the GDNF gene was introduced into MSCs using a recombinant replication-deficient adenoviral vector encoding GDNF with GFP. The differentiated neuronal phenotype of induced MSCs depends on administered GDNF.

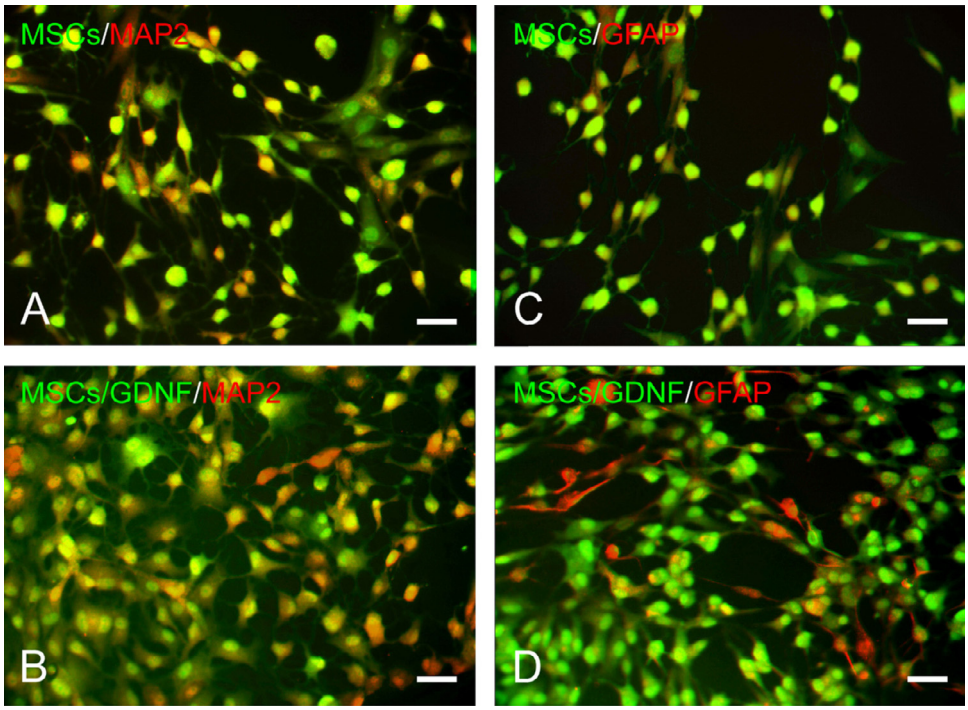

Figure 2. *Differentiation of MSCs and MSCs/GDNF in vitro. Green fluorescence showed MSCs or MSCs/GDNF in vitro. Neurons (MAP2 positive) and astrocytes (GFAP positive) were stained in red fluorescence. Yellow fluorescence showed colocalization of green and red. MSCs and MSCs/GDNF were differentiated into neurons (A and B, respectively) and astrocytes (C and D, respectively). Scale bar: 50 μm. (Color version of figure is available online.)*

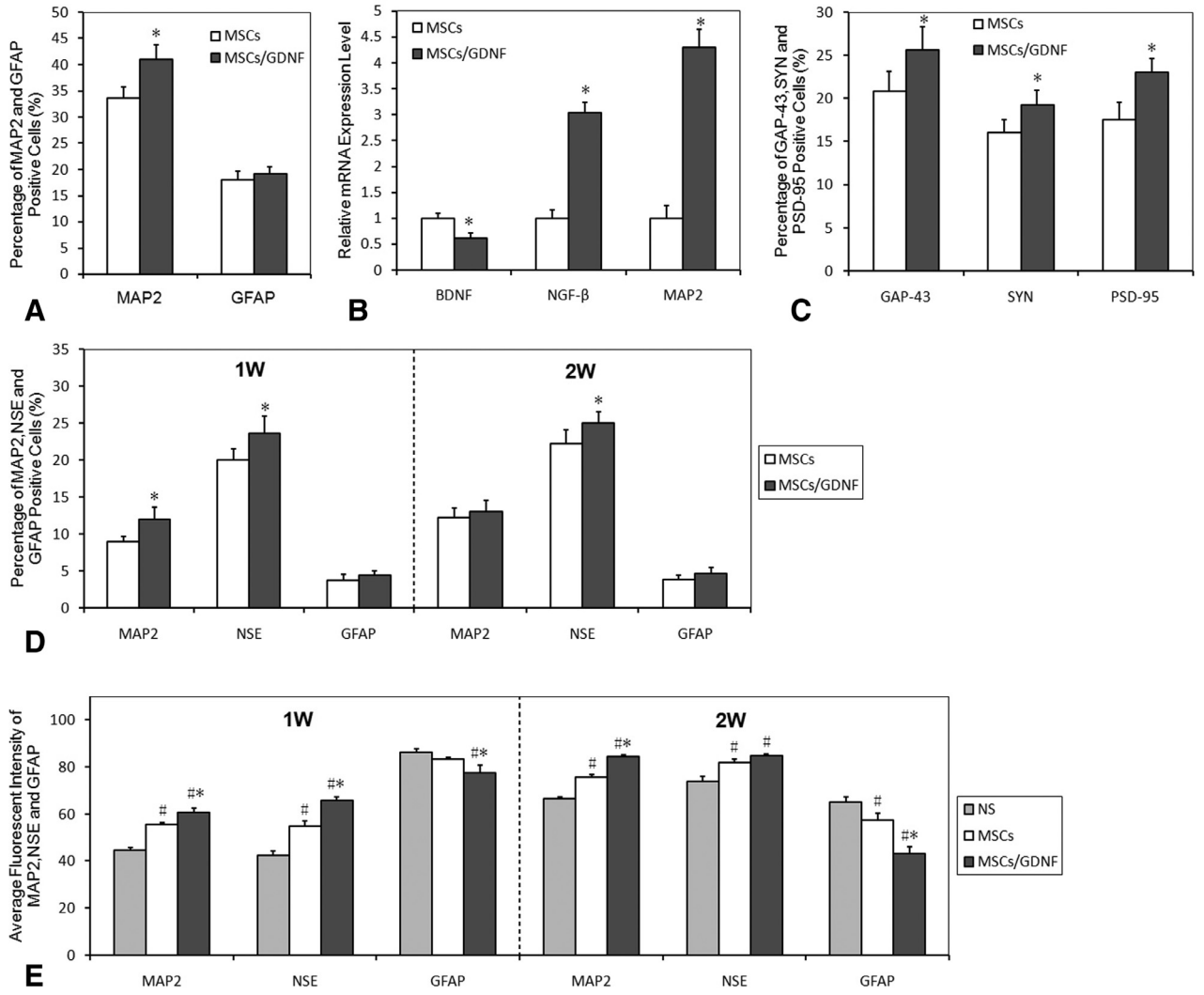

Figure 3. *All statistical graphs on the differentiation of MSCs and MSCs/GDNF in vivo and in vitro. (A) Differentiation of MSCs and MSCs/GDNF in vitro. Values were calculated as follows: green and red double fluorescent cells/green fluorescent cells ×100%. (B) Real time-PCR analysis of the expression of BDNF, NGF-β, and MAP2 mRNAs in MSCs and MSCs/GDNF treated with neuronal induction medium for 6 hours. GAPDH was used as an internal control. (C) The percentage of GAP-43/SYN/PSD-95 positive cells was calculated and analyzed when MSCs and MSCs/GDNF treated with neuronal induction medium for 6 hours. (D) Differentiation of grafted MSCs and MSCs/GDNF in the brains of ICH rats at 1 and 2 weeks after cell transplantation. (E) Immunofluorescence assay of MAP2, NSE, and GFAP in ICH rats at 1 and 2 weeks after saline injection or cell transplantation. The fluorescence intensity was quantified by Image Pro software. Data are shown as mean ± SEM. *P < .05, versus MSCs group; #P < .05, versus NS group. (Color version of figure is available online.)*

MSCs/GDNF and MSCs secrete a variety of bioactive compounds such as neurotrophic factors that can affect the neuron viability through autocrine or paracrine modes of action.[25,26] In our experiment, the mRNA expression levels of MAP2, BDNF, and NGF-β in MSCs/GDNF were higher than those in MSCs. The secretion capacities of MSCs/GDNF or MSCs have been suggested as the primary mechanism contributing to the protection and rehabilitation of injured brain tissue. It was reported that BDNF can induce BMSCs to differentiate into nerve cells in vitro.[27,28] The results showed that GAP-43, SYN, and PSD-95 protein levels in the MSCs/GDNF group were significantly higher than those in the MSCs group. Plasticity and functional remodeling are important characteristics of the nervous system. Synapses are the junctions between nerve cells, but SYN and GAP43 are used as closely related sensitive indicators of synaptic reconstruction,[29] as SYN is a kind of membrane protein related to the synaptic structure and function.[30] It is widely known that GAP-43 can be used as a regulated protein of neuronal development, axonal growth, synaptic plasticity, and reconstruction.[31] PSD-95 is key to synapse formation and maturation.[32] Accordingly, we detected the expression levels of SYN, GAP-43, and PSD-95. Other researchers have shown that edaravone can induce hUMSCs to differentiate into neuron-like cells that express synaptic markers such as SYN, PSD95, and GAP-43.[33]

The present study demonstrated that intrastriatally transplanted MSCs/GDNF or MSCs integrated with the host brain and saved the dying cells surrounding

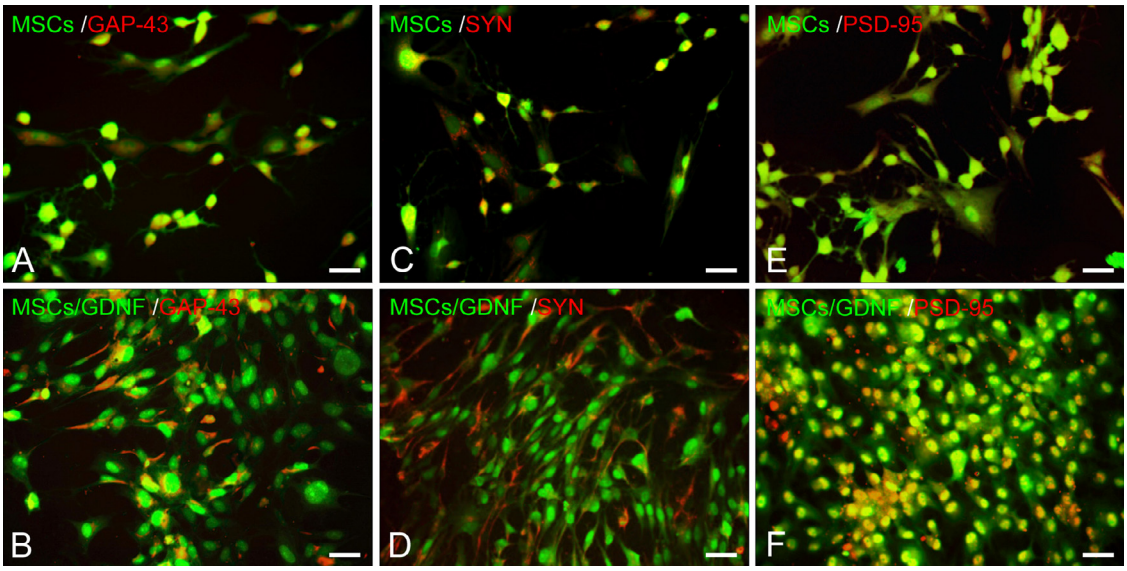

Figure 4. *Effect of MSCs and MSCs/GDNF on the expression of GAP-43, SYN, PSD-95. GAP-43 (A and B), SYN (C and D), and PSD-95 (E and F) protein expression of the induced cells. MSCs and MSCs/GDNF were treated with conditioned medium, and red fluorescence labeled for GAP-43/SYN/PSD-95. Scale bar: 50 μm. (Color version of figure is available online.)*

intracerebral hemorrhage, inhibited glial scar formation, and promoted the restoration of neurological function, which might be correlated with enhanced intensity of MAP2 and NSE proteins and decreased intensity of GFAP proteins. Both MSCs/GDNF and MSCs are used for regeneration purposes for transdifferentiation into neuron-like cells to replace those that are missing. Marrow stromal cells enhance axonal plasticity, which may underlie neurological functional recovery. Several groups of investigators have illustrated that in ICH, MSCs can increase the expression of neurotrophic factors, decrease neuronal apoptosis, enhance synaptic plasticity, strengthen endogenous neurogenesis, and alleviate brain water content.[34-36] Recent evidences indicated that the capacity of MSCs is related to paracrine expression of neurotrophic factors and cytokines,[37] reducing the volume of the infarct size, exerting anti-inflammatory and angiogenic properties,[38] and forming a bio-bridge.[39]

MAP2 is a neuron-specific cytoskeletal protein that is used as a marker of neuronal phenotype,[40] NSE is widely used and accepted as a neuronal marker,[41] and GFAP is known as a marker of astroglial cells. The group with

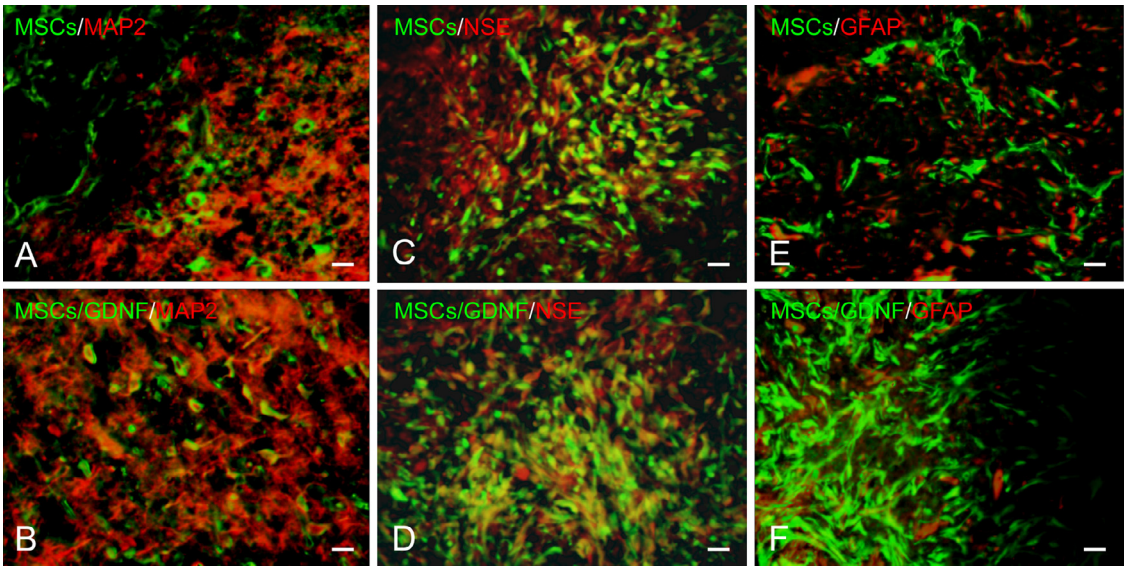

Figure 5. *Differentiation of grafted MSCs and MSCs/GDNF in the brains of ICH rats. Green fluorescence showed MSCs or MSCs/GDNF in vivo. Neurons (MAP2 and NSE positive) and astrocytes (GFAP positive) were stained in red fluorescence. Yellow fluorescence showed colocalization of green and red. MSCs and MSCs/GDNF were differentiated into neurons (A and C, and B and D, respectively) and astrocytes (E and F, respectively). Scale bar: 50 μm. (Color version of figure is available online.)*

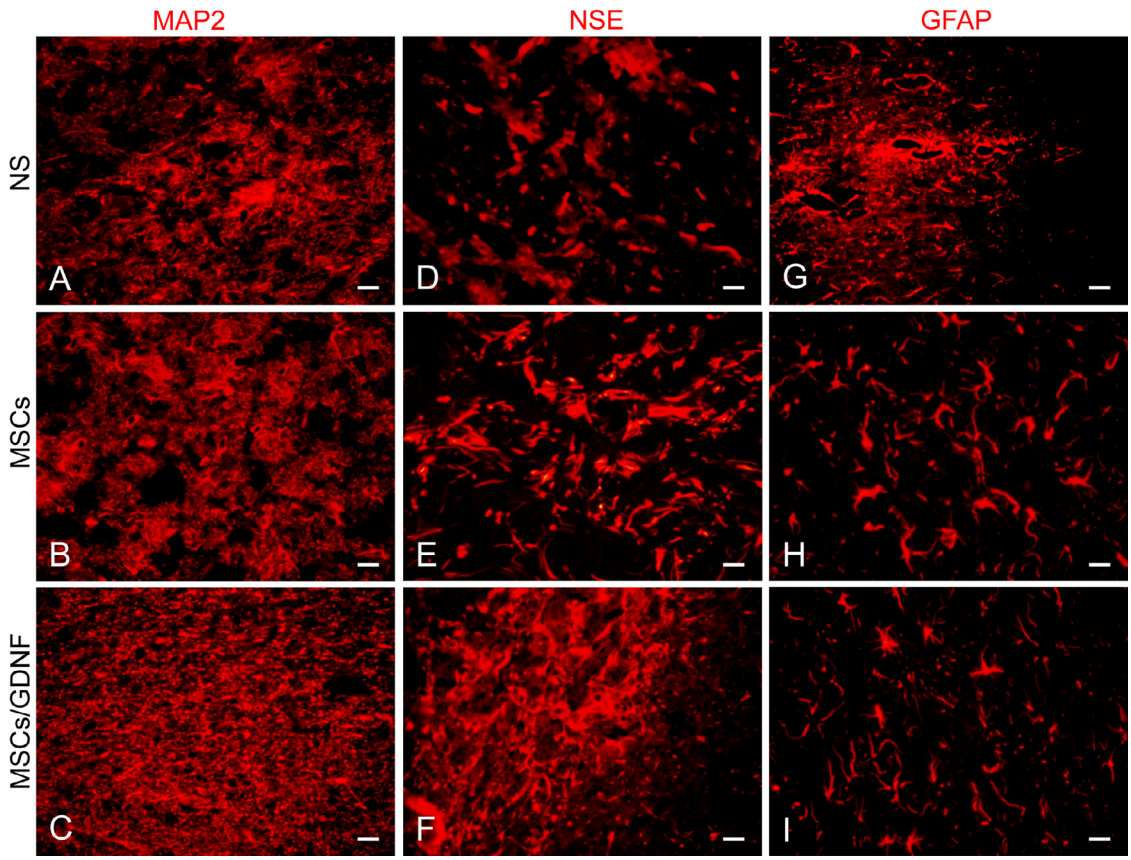

Figure 6. *The expression of MAP2, NSE, and GFAP in peripheral hemorrhage areas. Cell transplantation increased the intensity of MAP2 and NSE proteins and decreased the intensity of GFAP proteins in comparison with the NS group. MAP2, NSE, and GFAP proteins were stained in red fluorescence. Scale bar: 50 μm. (Color version of figure is available online.)*

transplantation of MSCs/GDNF exhibited differentiation into the highest number of MAP2+ and NSE+ cells in the striatum at 1 week and 2 weeks after ICH, compared with the controls transplanted only with MSCs or saline. These data suggest that the posthemorrhage microenvironment is constantly evolving and promoting differentiation of transplanted cells. MSCs transplanted into ICH rats enhance the survival and differentiation of neural cells.[42] Transplantation of induced pluripotent stem cells in experimental ICH in rats shows potential for neuronal replacement.[43] The effects of stem cell therapy can also influence the microenvironment of the central nervous system.[44] Transplantation of GDNF-modified MSCs could be used to improve the therapeutic efficiency of cell-based transplantation and reveal the function of GDNF in promoting neurogenesis, thereby participating in the repair of nerve tissue. We think that the potential for transplantation is more promising for the replacement of lost neurons.

Conclusion

MSCs show strong potential as appropriate scaffold material by delivering therapeutic genes to brain injury regions. The molecular and cellular mechanisms by which MSCs exert their biological activities are not fully understood, although it has been speculated that MSCs replace lost cells and secrete of essential growth factors.

Competing Interests

The authors declare no competing interests.

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