NEURAL REGENERATION RESEARCH
January 2014, Volume 9, Issue 1
www.nrronline.org

Transfection of the glial cell line-derived neurotrophic factor gene promotes neuronal differentiation

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doi:10.4103/1673-5374.125327
http://www.nrronline.org/

Accepted: 2013-11-30

Abstract

Glial cell line-derived neurotrophic factor recombinant adenovirus vector-transfected bone marrow mesenchymal stem cells were induced to differentiate into neuron-like cells using inductive medium containing retinoic acid and epidermal growth factor. Cell viability, microtubule-associated protein 2-positive cell ratio, and the expression levels of glial cell line-derived neurotrophic factor, nerve growth factor and growth-associated protein-43 protein in the supernatant were significantly higher in glial cell line-derived neurotrophic factor/bone marrow mesenchymal stem cells compared with empty virus plasmid-transfected bone marrow mesenchymal stem cells. Furthermore, microtubule-associated protein 2, glial cell line-derived neurotrophic factor, nerve growth factor and growth-associated protein-43 mRNA levels in cell pellets were statistically higher in glial cell line-derived neurotrophic factor/bone marrow mesenchymal stem cells compared with empty virus plasmid-transfected bone marrow mesenchymal stem cells. These results suggest that glial cell line-derived neurotrophic factor/bone marrow mesenchymal stem cells have a higher rate of induction into neuron-like cells, and this enhanced differentiation into neuron-like cells may be associated with up-regulated expression of glial cell line-derived neurotrophic factor, nerve growth factor and growth-associated protein-43.

Key Words: nerve regeneration; bone marrow mesenchymal stem cells; cell differentiation; neuron-like cells; glial cell line-derived neurotrophic factor; recombinant adenovirus vector; transfection; retinoic acid; epidermal growth factor; nerve growth factor; growth-associated protein-43; neural regeneration

Du J, Gao XQ, Deng L, Chang NB, Xiong HL, Zheng Y. Transfection of the glial cell line-derived neurotrophic factor gene promotes neuronal differentiation. Neural Regen Res. 2014;9(1):33-40.

Introduction

In certain diseases of the central nervous system, such as stroke and Parkinson's disease, many neurons degenerate and die, and neural functioning is impaired[1-2]. There is still no effective treatment that can improve the reconstruction of neuronal networks in damaged neural tissue or enhance neural functional recovery. Bone marrow mesenchymal stem cells (BMSCs) are adult stem cells in bone marrow, and studies have shown that cultured bone marrow mesenchymal stem cells *in vitro* have the ability to secrete various neurotrophic factors[3-5] and can be induced into a neuronal phenotype under specific experimental conditions[6-8]. Transplanted bone marrow mesenchymal stem cells can differentiate into neuron-like cells in the brain and compensate for neurological deficits following brain injury[9-10]. These studies suggest that transplantation of bone marrobone marrow mesenchymal stem cellsw mesenchymal stem cells has a therapeutic effect and the potential for clinical application. In addition, the use of bone marrow mesenchymal stem cells possesses many benefits, such as ease of harvesting, the possibility of autotransplantation, ability to express exogenous genes, and minimal host immune rejection[11-12]. Therefore, bone marrow mesenchymal stem cells have been heralded as an ideal cell type for transplantation to treat neurological disorders. However, studies have shown that the differentiation rate of grafted bone marrow mesenchymal stem cells into mature neuron-like cells is very low[13-14]. Therefore, it is very important to establish an efficient and stable induction protocol to promote the differentiation of bone marrow mesenchymal stem cells into neuron-like cells *in vitro* and elucidate the mechanisms underlying differentiation for the treatment of central nervous system diseases.

Neurotrophic factors have been demonstrated to exert potent effects on neurons, such as promotion of survival, neurite branching, synaptogenesis, modulation of electrophysiological properties and synaptic plasticity. Glial cell line-derived neurotrophic factor (GDNF), a distantly related member of the transforming growth factor-beta superfamily and a potent neurotrophic factor, can affect neuronal differentiation, development, growth and survival in the central nervous system and have neuroprotective effects against a variety of neuronal insults[15-19]. However, the effects of glial cell line-derived neurotrophic factor are transient, and its repeated administration into brain parenchyma or the

intraventricular space is needed[20]. In addition, as a large protein, glial cell line-derived neurotrophic factor has difficulty passing through the blood-brain barrier[21]. Thus, glial cell line-derived neurotrophic factor is limited in its clinical applicability[21]. Direct intravenous administration of human mesenchymal stem cells transfected with the glial cell line-derived neurotrophic factor gene in rats subjected to middle cerebral artery occlusion results in an increase in glial cell line-derived neurotrophic factor levels and a reduction in infarct volume in the affected hemisphere and an improvement in behavioral performance compared with injection of human mesenchymal stem cells alone[22]. Moreover, transplantation of glial cell line-derived neurotrophic factor gene-modified bone marrow mesenchymal stem cells promote differentiation into neurofilament-positive cells and have better therapeutic effects in intracerebral hemorrhage models in rats than transplantation of empty virus-transfected bone marrow mesenchymal stem cells[23]. Therefore, bone marrow mesenchymal stem cells secreting glial cell line-derived neurotrophic factor may hold therapeutic potential for central nervous system diseases.

Retinoic acid, also known as all-trans retinoic acid, is a vitamin A derivative that plays an essential role during the development of the nervous system, and is a potent regulator of morphogenesis, cell growth, proliferation and differentiation[24]. It was reported that a cocktail of induction factors containing basic fibroblast growth factor and retinoic acid induces bone marrow mesenchymal stem cells to differentiate into neurons[25]. Epidermal growth factor, a mitotic growth factor, can promote the maturation, proliferation and survival of nerve cells in the central nervous system *in vitro*[26], and it can stimulate the proliferation and maturation of neural stem cells as well[27]. Moreover, bone marrow mesenchymal stem cells, which are of non-neuronal origin, can also be induced by epidermal growth factor to adopt neuronal phenotypes[28]. These observations indicate that retinoic acid and epidermal growth factor have a potent ability to induce neuronal differentiation.

In this study, we sought to develop an efficient and reliable protocol for inducing the differentiation of bone marrow mesenchymal stem cells into neuron-like cells. We used all-trans retinoic acid plus epidermal growth factor to induce the differentiation of bone marrow mesenchymal stem cells transfected with recombinant glial cell line-derived neurotrophic factor adenovirus, and we examined their phenotypic differentiation into neuron-like cells. We sought to provide experimental support for the therapeutic use of glial cell line-derived neurotrophic factor gene-modified bone marrow mesenchymal stem cells in transplantation strategies for central nervous system diseases.

Results

Morphological changes during the differentiation of recombinant adenovirus vector-transfected bone marrow mesenchymal stem cells into neuron-like cells

Morphological changes in bone marrow mesenchymal stem cells were monitored using phase-contrast inverted microscopy. Untreated bone marrow mesenchymal stem cells displayed a flat shape with round, triangular or spindle-shaped bodies and short processes (Figure 1A). Two days after transfection of bone marrow mesenchymal stem cells with empty virus (empty virus-transfected bone marrow mesenchymal stem cells (EV/BMSCs)), the cells remained flat in shape, similar to the untreated bone marrow mesenchymal stem cells (Figure 1B). Two days after transfection of bone marrow mesenchymal stem cells with recombinant glial cell line-derived neurotrophic factor adenovirus vector (GDNF/BMSCs), the cells lost their flat appearance and displayed a gradual shrinkage of the cell body, and extended long tapering processes (Figure 1C). At 4 days, induced EV/BMSCs and GDNF/BMSCs displayed striking morphological changes, taking on a spherical or spindle shape and extending processes (Figure 1D, E).

Increase in viability after differentiation of recombinant glial cell line-derived neurotrophic factor adenovirus vector-transfected bone marrow mesenchymal stem cells into neuron-like cells

The viability of GDNF/BMSCs, EV/BMSCs and uninfected bone marrow mesenchymal stem cells gradually increased with time in culture ($P < 0.05$). Cell viability in the GDNF/BMSCs induction group was significantly higher than that in the EV/BMSCs induction or uninfected bone marrow mesenchymal stem cells group ($P < 0.05$). Moreover, no significant difference in cell viability was detected between the EV/BMSCs induction and uninfected bone marrow mesenchymal stem cells groups ($P > 0.05$). These results suggest that the recombinant adenovirus vector did not lower bone marrow mesenchymal stem cells viability, and that glial cell line-derived neurotrophic factor promoted bone marrow mesenchymal stem cells proliferation (Table 1).

Expression of microtubule-associated protein 2 after the differentiation of recombinant glial cell line-derived neurotrophic factor adenovirus vector-transfected bone marrow mesenchymal stem cells into neuron-like cells

Immunofluorescence staining and reverse transcription (RT)-PCR detection revealed that GDNF/BMSCs and EV/BMSCs induced by all-trans retinoic acid and epidermal growth factor expressed microtubule-associated protein 2 protein and mRNA, but the ratio of microtubule-associated protein 2-positive cells and mRNA in the GDNF/BMSCs induction group was higher than that in the EV/BMSCs induction group ($P < 0.05$). In the control group, no microtubule-associated protein 2 immunolabeling or mRNA expression was detectable (Figures 2, 3, Table 2).

Expression of glial cell line-derived neurotrophic factor, nerve growth factor and growth-associated protein-43 after the differentiation of recombinant glial cell line-derived neurotrophic factor adenovirus vector-transfected bone marrow mesenchymal stem cells into neuron-like cells

Glial cell line-derived neurotrophic factor, nerve growth factor and growth-associated protein-43 protein expression levels in supernatant using the enzyme-linked immunosorbent assay (ELISA) were consistent with mRNA expression levels in cell pellets determined with RT-PCR detection. Expression was highest in the GDNF/BMSCs induction group, followed

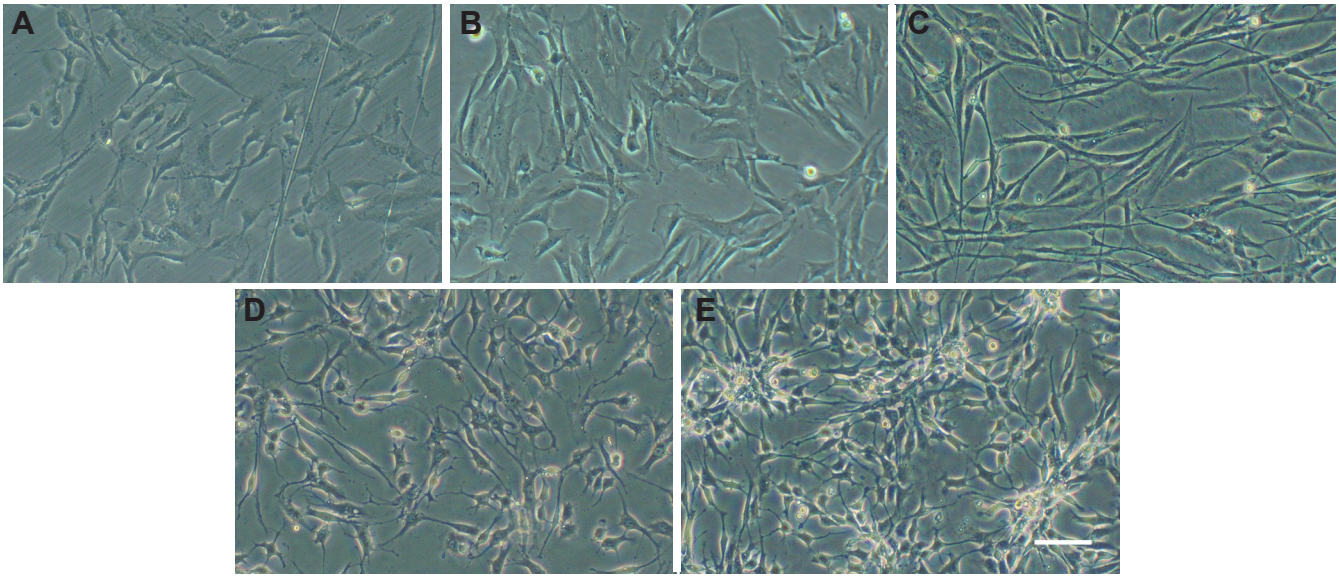

Figure 1 Morphology of BMSCs (phase-contrast inverted microscopy, × 100).
(A) Untreated BMSCs showed a flat morphology.
(B) At 2 days after transfection, BMSCs transfected with empty virus (EV/BMSCs) remained flat in shape.
(C) At 2 days after transfection, BMSCs transfected with recombinant GDNF adenovirus vector (GDNF/BMSCs) showed retracted bodies and extended processes.
(D, E) At 4 days, EV/BMSCs and GDNF/BMSCs treated with the induction agent had noticeable morphological changes, taking on a spherical or spindle shape and extending processes.
BMSCs: Bone marrow mesenchymal stem cells; EV: empty virus plasmid; GDNF: glial cell line-derived neurotrophic factor.

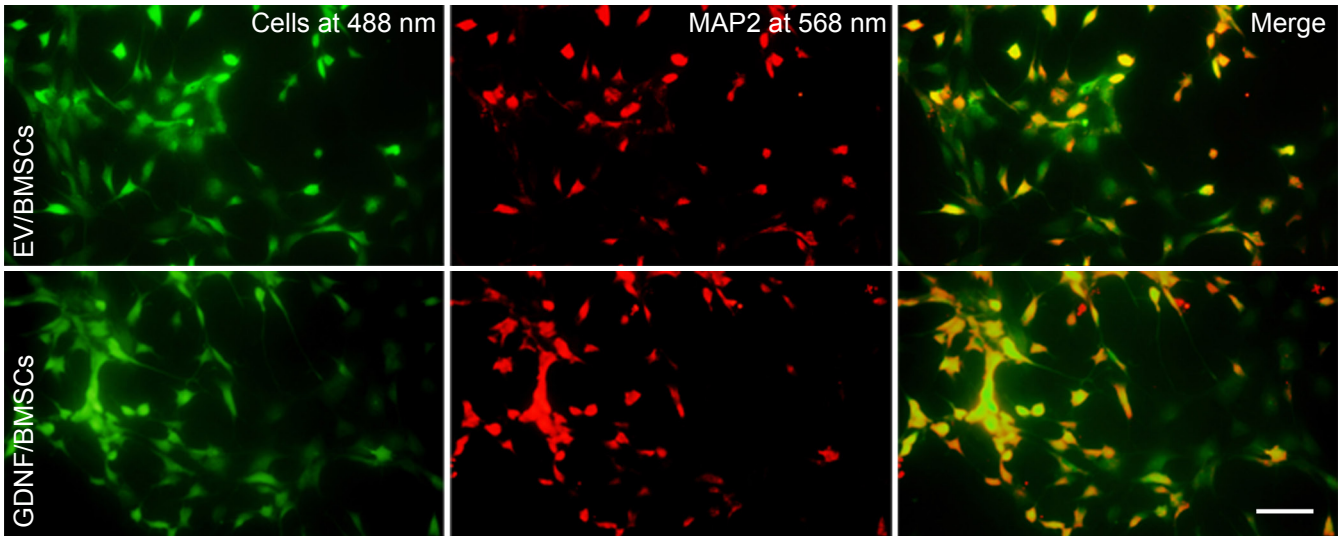

Figure 2 MAP2-positive cells after differentiation of recombinant GDNF adenovirus vector-transfected BMSCs into neuron-like cells (immunofluorescence staining, × 100).
Green fluorescence showing cells in the EV/BMSCs and GDNF/BMSCs induction groups. MAP2-positive cells were stained red by Cy3. Yellow fluorescence showed colocalization of green and red fluorescence. BMSCs: Bone marrow mesenchymal stem cells; EV: empty virus; GDNF: glial cell line-derived neurotrophic factor; MAP2: microtubule-associated protein 2.

by the EV/BMSCs induction group. Expression was lowest in the control group. Comparisons of the protein and mRNA levels of these three proteins among the three different groups revealed significant differences in expression ($P < 0.05$; Figure 4, Table 3).

Discussion

Bone marrow mesenchymal stem cells can differentiate into cells expressing neuronal markers[6-8, 25, 28]. This study revealed that GDNF/BMSCs and EV/BMSCs induced by all-trans retinoic acid and epidermal growth factor express the neuronal marker microtubule-associated protein 2, which confirms that these cells can differentiate into neuron-like cells. Furthermore, we showed that following induction, GDNF/BMSCs have stronger expression of microtubule-associated protein 2 protein and mRNA compared with EV/BMSCs.

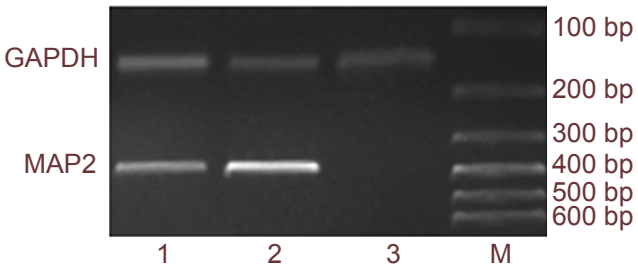

Figure 3 MAP2 mRNA expression after differentiation of GDNF/BMSCs into neuron-like cells (reverse transcription-PCR).
GAPDH: 141 bp; MAP2: 375 bp. 1: EV/BMSCs induction group; 2: GDNF/BMSCs induction group; 3: control group; M: marker; BMSCs: bone marrow mesenchymal stem cells; EV: empty virus plasmid; GDNF: glial cell line-derived neurotrophic factor; MAP2: microtubule-associated protein 2.

Table 1 Viability (absorbance) of BMSCs after infection by empty virus and recombinant GDNF adenovirus vector

Group	Time after infection (hour)		
	24	48	72
Uninfected BMSCs	0.20±0.02	0.31±0.02[a]	0.49±0.05[ab]
EV/BMSCs	0.20±0.03	0.31±0.01[a]	0.50±0.02[ab]
GDNF/BMSCs	0.25±0.03[c]	0.38±0.02[ac]	0.62±0.07[abc]

MTT assay data are presented as mean ± SD, and the experiment was repeated five times. Cell viability was validated using one-way analysis of variance. [a]$P < 0.05$, *vs.* 24 hours; [b]$P < 0.05$, *vs.* 48 hours; [c]$P < 0.05$, *vs.* uninfected BMSCs and EV/BMSCs groups. BMSCs: Bone marrow mesenchymal stem cells; EV: empty virus plasmid; GDNF: glial cell line-derived neurotrophic factor.

Table 2 Changes in MAP2-positive cell percentage and MAP2 mRNA relative amount after recombinant GDNF adenovirus vector-transfected BMSCs differentiated into neuron-like cells

Group	MAP2-positive cells	MAP2 mRNA
EV/BMSCs induction	52.79±4.74	1.25±0.23
GDNF/BMSCs induction	83.06±7.34[a]	1.70±0.52[a]

MAP2-positive cell ratio was measured using immunofluorescence staining, and mRNA was measured using reverse transcription-PCR. Data are presented as mean ± SD, and the experiment was repeated five times. Positive cell percentage was compared using one-way analysis of variance. [a]$P < 0.05$, *vs.* EV/BMSCs induction group. BMSCs: Bone marrow mesenchymal stem cells; EV: empty virus plasmid; GDNF: glial cell line-derived neurotrophic factor; MAP2: microtubule-associated protein 2.

In our previous report, primary rat cortical neurons were subjected to hypoxia and then treated with GDNF/BMSCs or EV/BMSCs for 12 hours or 1, 2, 3 or 5 days. The GDNF/BMSCs induction group had significantly lowered apoptosis compared with the bone marrow mesenchymal stem cells group at any given time. GDNF/BMSCs induction group had higher expression of the neuronal marker neurofilament-200 compared with the EV/BMSCs induction group when the two types of cells were induced by all-trans retinoic acid *in vitro*.

Moreover, when transplanted into the hemorrhagic striatum, the GDNF/BMSCs induction group had a significant

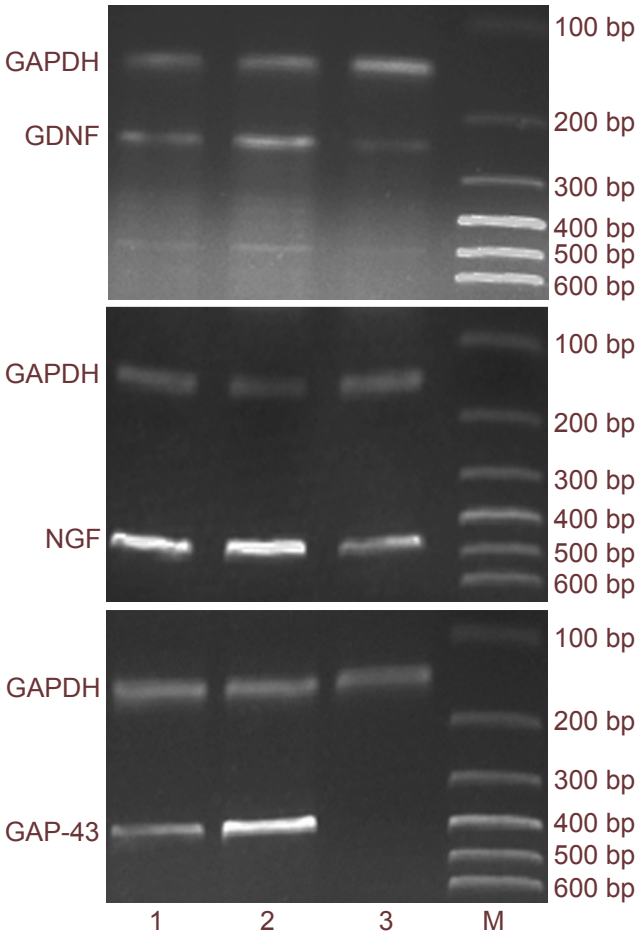

Figure 4 GDNF, NGF and GAP-43 mRNA expression after GDNF/BMSCs differentiation into neuron-like cells (reverse transcription-PCR).
GDNF: 242 bp; NGF: 456 bp; GAP-43: 381 bp; GAPDH: 141 bp. 1: EV/BMSCs induction group; 2: GDNF/BMSCs induction group; 3: control group; M: marker; BMSCs: bone marrow mesenchymal stem cells; EV: empty virus plasmid; GDNF: glial cell line-derived neurotrophic factor; NGF: nerve growth factor; GAP-43: growth-associated protein-43.

amelioration of functional deficits, reduced lesion volume, a lower number of apoptotic cells, and more neurofilament-positive grafted cells compared with the bone marrow mesenchymal stem cells group. The greater therapeutic efficiency of GDNF/BMSCs may result from a higher level of glial cell line-derived neurotrophic factor protein expression in the brain[23]. Guo et al.[29] observed that bone marrow mesenchymal stem cells cultured in induction media supplemented with glial cell line-derived neurotrophic factor, interleukin-1 beta, mesencephalic glial-cell-conditioned medium and flash-frozen mesencephalic membrane fragments could be induced to differentiate into dopaminergic neuron-like cells expressing neural-specific enolase, microtubule-associated protein 2a and b, and tyrosine hydroxylase. Gao et al.[30] reported that bone marrow mesenchymal stem cells induced with glial cell line-derived neurotrophic factor-containing fetal gut conditioned medium for 10 days expressed more Glial cell line-derived neurotrophic factor mRNA than non-induced bone marrow mesenchymal stem cells, and the differentiated cells expressed the neuronal markers neural-specific

Table 3 Changes in GDNF, NGF and GAP-43 protein levels (pg/mL) in supernatant and GDNF, NGF and GAP-43 mRNA (relative absorbance) after differentiation of recombinant GDNF adenovirus vector-transfected BMSCs into neuron-like cells

	GDNF		NGF		GAP-43	
Group	Protein	mRNA	Protein	mRNA	Protein	mRNA
Control	39.84±3.73	0.48±0.12	63.16±4.62	0.97±0.38	2.23±1.64	0
EV/BMSCs induction	109.16±2.72[a]	0.82±0.13[a]	183.54±7.17[a]	1.98±0.74[a]	80.15±7.77[a]	1.66±0.42[a]
GDNF/BMSCs induction	190.43±6.58[ab]	1.22±0.19[ab]	232.85±13.33[ab]	2.44±0.78[ab]	105.19±8.51[ab]	2.15±0.49[ab]

Protein content was detected using enzyme-linked immunosorbent assay, and mRNA was detected using reverse transcription-PCR. Data are presented as mean ± SD, and the experiment was repeated five times. Protein levels were compared using one-way analysis of variance, and mRNA relative amount was analyzed using two-way analysis of variance. [a]$P < 0.05$, *vs.* control group; [b]$P < 0.05$, *vs.* EV/BMSCs induction group. BMSCs: Bone marrow mesenchymal stem cells; EV: empty virus plasmid; GDNF: glial cell line-derived neurotrophic factor; NGF: nerve growth factor; GAP-43: growth-associated protein-43.

enolase and neurofilament protein, and the glial cell marker glial fibrillary acidic protein. Our ELISA and RT-PCR results showed that glial cell line-derived neurotrophic factor/bone marrow mesenchymal stem cells and EV/BMSCs induced by all-trans retinoic acid and epidermal growth factor expressed more glial cell line-derived neurotrophic factor and nerve growth factor than non-induced bone marrow mesenchymal stem cells. Moreover, expression levels were greater in the GDNF/BMSCs induction group. These results suggest that glial cell line-derived neurotrophic factor can induce the differentiation of bone marrow mesenchymal stem cells into neuron-like cells.

Our ELISA and RT-PCR results showed that the expression of another neurotrophic factor, nerve growth factor, in the GDNF/BMSCs induction group was higher than in the EV/BMSCs induction group. Gao et al.[30] reported that bone marrow mesenchymal stem cells cultured in fetal gut condition medium with glial cell line-derived neurotrophic factor could produce more glial cell line-derived neurotrophic factor and nerve growth factor. Nerve growth factor is a critical neurotrophic factor that promotes neuronal survival and neurite growth. Nerve growth factor has significant regulatory effects on neuronal development, differentiation, growth, regeneration and functional properties[31-32]. Thus, recombinant glial cell line-derived neurotrophic factor adenovirus vector-transfected bone marrow mesenchymal stem cells might increase expression of glial cell line-derived neurotrophic factor and nerve growth factor to promote the neuronal differentiation of bone marrow mesenchymal stem cells. In a previous report from our group, glial cell line-derived neurotrophic factor adenovirus vector-transfected neural stem cells and native neural stem cells were used to treat rats subjected to cerebral ischemia[21]. The glial cell line-derived neurotrophic factor adenovirus vector-transfected neural stem cells group had higher survival rate of implanted cells and significantly improved functional deficits than the neural stem cells group, which might be correlated with higher expression of synaptophysin, postsynaptic density-95, brain-derived neurotrophic factor and neurotrophin-3 in the glial cell line-derived neurotrophic factor adenovirus vector-transfected neural stem cells group[21]. Previous studies demonstrated that cultured bone marrow mesenchymal stem cells *in vitro* express not only glial cell line-derived neurotrophic factor and nerve growth factor, but also other neurotrophic factors, such as brain-derived neurotrophic factor, ciliary neurotrophic factor and basic fibroblast growth factor[33-34]. Therefore, we speculate that the GDNF/BMSCs induced by neuronal induction agents might increase expression of various factors that promote neuronal growth, which might create a beneficial environment for the neuronal differentiation of bone marrow mesenchymal stem cells.

Growth-associated protein-43 is a membrane phosphoprotein that is highly concentrated in the growth cone membrane and is closely correlated with axonal outgrowth. It can modulate the response of neurons to axonal guidance signals, it can induce neurons to grow new termini in the absence of other trophic factors[35], and it is a critical factor for neuronal growth[36]. Growth-associated protein-43 may modulate growth cone motility, synaptic plasticity and neurotransmitter release[37]. The protein kinase C-mediated phosphorylation of growth-associated protein-43 may influence cell surface behavior by modulating the action of actin in growing axons and developing nerve termini[38]. Studies have shown that, in transgenic mice, overexpression of growth-associated protein-43 leads to the spontaneous formation of new synapses and enhances sprouting after injury. Regulation of growth-associated protein-43 expression likewise has profound effects on neurite outgrowth in culture[39]. The neuron-like cells induced by basic fibroblast growth factor treatment of bone marrow mesenchymal stem cells exhibit characteristic electrophysiological properties, and the expression of growth-associated protein-43 steadily increases with time[40]. When bone marrow mesenchymal stem cells are transplanted into rats with ischemic stroke, growth-associated protein-43 expression in these transplanted cells increases[41]. In the present study, the induced GDNF/BMSCs expressed more growth-associated protein-43 than EV/BMSCs, which suggests that growth-associated protein-43 is involved in the differentiation of bone marrow mesenchymal stem cells into neuron-like cells and neurite outgrowth, and that recombinant glial cell line-derived neurotrophic factor adenovirus vector transfection promotes the differentiation of bone marrow mesenchymal stem cells into neuron-like cells.

In conclusion, induced GDNF/BMSCs express higher levels of microtubule-associated protein 2, glial cell line-derived

neurotrophic factor, nerve growth factor and growth-associated protein-43 protein and mRNA than induced EV/BMSCs. Although the mechanisms are not clear at present, these results suggest that recombinant glial cell line-derived neurotrophic factor adenovirus vector transfection might promote the differentiation of bone marrow mesenchymal stem cells into neuron-like cells, and the mechanism may be correlated with up-regulation of expression of glial cell line-derived neurotrophic factor, nerve growth factor and growth-associated protein-43. Thus, our study offers a more effective *in vitro* protocol for promoting the differentiation of bone marrow mesenchymal stem cells into neuron-like cells. Our findings provide experimental support for the use of recombinant glial cell line-derived neurotrophic factor adenovirus vector transfected bone marrow mesenchymal stem cells in transplantation procedures for the treatment of central nervous system diseases.

Materials and Methods

Design

A controlled, *in vitro* cytology experiment.

Time and setting

This study was performed in the Research Center for Preclinical Medicine, Luzhou Medical College, China from February to December 2011.

Materials

Passage 2 bone marrow mesenchymal stem cells from Sprague-Dawley rats (RASMX-01001) were purchased from Cyagen Biosciences Inc., Guangzhou, Guangdong Province, China. Recombinant glial cell line-derived neurotrophic factor adenovirus vector (pAdEasy-1-pAdTrack CMV-glial cell line-derived neurotrophic factor) and EV (pAdEasy-1-pAdTrack CMV) were provided by the Department of Neurobiology, Luzhou Medical College, Luzhou, Sichuan Province, China. Adeasy-1 plasmid contains the gene for green fluorescent protein. The viral titers were 1×10^9 PFU/mL and the rate of transfection was 100%[21, 23].

Methods

Culture and transfection of bone marrow mesenchymal stem cells

Passage 2 bone marrow mesenchymal stem cells were plated in Dulbecco's modified Eagle's medium (DMEM; Sigma, St. Louis, MO, USA) containing 10% fetal bovine serum (Hyclone, Logan City, UT, USA) in a humidified environment with 5% CO_2 at 37°C. The bone marrow mesenchymal stem cells were passaged at 1:2 using 0.25% trypsin solution (Sigma) at full confluence. After 5 passages, the bone marrow mesenchymal stem cells were plated at a density of 1×10^5 cells/mL in a 50-mL culture flask. When the bone marrow mesenchymal stem cells reached 80% confluence, 120 μL glial cell line-derived neurotrophic factor virus (recombinant glial cell line-derived neurotrophic factor adenovirus vector) solution and 3 mL DMEM were added to each flask to incubate for 2 days at 37°C to obtain glial cell line-derived neurotrophic factor/bone marrow mesenchymal stem cells, or 120 μL BPV virus solution and 3 mL DMEM to obtain EV/BMSCs.

3-(4,5-Dimethylthiazol-2-yl) 2, 5-diphenyl tetrazolium bromide (MTT) assay for the viability of bone marrow mesenchymal stem cells

GDNF/BMSCs, EV/BMSCs and uninfected bone marrow mesenchymal stem cells were plated at a density of 1×10^4 cells/well in 150 μL DMEM in 96-well plates at 37°C for 24, 48 or 72 hours. After washing with PBS, cells were incubated at 37°C for 4 hours with 150 μL medium and 20 μL 0.5% MTT solution (Sigma) in the dark. The absorbance was determined using a microplate reader (BioTeK, ELX800, Winooski, VT, USA) at 490 nm wavelength.

Induction of bone marrow mesenchymal stem cells

The GDNF/BMSCs and EV/BMSCs were plated in 50-mL culture flasks and coverslips precoated with poly-L-lysine in six-well plates. At 80% confluence, the cells were washed twice with PBS, then serum-free DMEM-F12 (Sigma) with 1 μmol/L all-trans retinoic acid (Sigma), 20 μg/L epidermal growth factor (Invitrogen, Carlsbad, CA, USA) and 1% N-2 supplement was added to culture flasks and wells, followed by incubation for 4 days for the induction groups (GDNF/BMSCs induction group and EV/BMSCs induction group). The EV/BMSCs were cultured in serum-free DMEM/F12 medium without any induction agent for 4 days as control group. All coverslips were fixed with 4% paraformaldehyde for 30 minutes, followed by three 10-minute washes in PBS for microtubule-associated protein 2 immunofluorescence detection. The supernatants in the culture flask were collected and stored at −80 °C for glial cell line-derived neurotrophic factor, nerve growth factor and growth-associated protein-43 protein analysis by ELISA. Cell pellets in the culture flask were collected for microtubule-associated protein 2, glial cell line-derived neurotrophic factor, nerve growth factor and growth-associated protein-43 mRNA measurement by RT-PCR.

Immunofluorescence for detecting microtubule-associated protein 2-positive cells

The cells on coverslips were incubated with rabbit anti-microtubule-associated protein 2 antibody (1:100; Abcam, Cambridge, UK) at 4°C overnight. Cells were then incubated with biotinylated goat anti-rabbit IgG (1:100; Wuhan Boster Biological Technology, Wuhan, China) secondary antibody at 37°C for 1 hour, and the cells were then incubated with streptavidin biotin complex-Cy3 (1:100; Wuhan Boster Biological Technology) at 37°C for 30 minutes. A negative control was carried out using the same procedures without primary antibody. The cells on coverslips were visualized with green fluorescence at 488 nm, and microtubule-associated protein 2-positive cells were visualized with red fluorescence at 568 nm under a fluorescence microscope (Olympus, Sunnyvale, CA, USA). Confocal images (double-fluorescent cells) were yellow fluorescent and were obtained with a laser

scanning confocal microscope imaging system (Olympus). Values of microtubule-associated protein 2-positive cell ratio were calculated by the number of cells with yellow fluorescence/the number of cells with green fluorescence × 100%.

ELISA for detecting glial cell line-derived neurotrophic factor, nerve growth factor and growth-associated protein-43 protein expression

Glial cell line-derived neurotrophic factor, nerve growth factor and growth-associated protein-43 protein in the supernatants were detected using the glial cell line-derived neurotrophic factor, nerve growth factor and growth-associated protein-43 ELISA kit (R&D, Emeryville, CA, USA). Absorbance values were determined using a microplate reader (BioTeK, ELX800, VT, USA) at 450 nm. According to the absorbance of the standard sample and the corresponding concentration value, we drew a standard curve and calculated the concentrations of glial cell line-derived neurotrophic factor, nerve growth factor and growth-associated protein-43 proteins.

RT-PCR for detecting microtubule-associated protein 2, glial cell line-derived neurotrophic factor, nerve growth factor and growth-associated protein-43 mRNA expression

Total RNA was extracted from cell pellets using the TRIZOL reagent (Invitrogen). 2 μg of RNA was reverse-transcribed into cDNA using Takara RNA PCR kit (avian myeloblastosis virus) Ver. 3.0 (Takara Biotechnology, Dalian, Liaoning Province, China), and the reaction conditions were 30°C for 10 minutes, 42°C for 30 minutes, 99°C for 5 minutes, 5°C for 5 minutes. cDNA was subsequently amplified by PCR with specific primers. Sequences of the primers, sizes of the products and the annealing temperatures were as follows:

The PCR reaction consisted of 35 cycles of denaturing at 94°C for 30 seconds, annealing for 30 seconds, and extension at 72°C for 4 minutes. The PCR products were separated on a 2% (w/v) agarose gel with ethidium bromide, and visualized and photographed using a gel documentation system (Bio-Rad Laboratories, Segrate, Milan, Italy). GAPDH was included in the PCR as an internal control. The relative absorbance amounts of mRNA were normalized by comparison with GAPDH levels.

Statistical analysis

All data were presented as mean ± SD. Cell viability, positive cell percentage and protein levels were compared using one-way analysis of variance, and mRNA relative amounts were assessed using two-way analysis of variance. A P value < 0.05 was considered statistically significant.

Author contributions: *All authors contributed to study design and evaluation and conducted the experiments. All authors had read and approved the final version of the manuscript submitted.*
Conflicts of interest: *None declared.*
Peer review: *The article investigated the differentiation of recombinant glial cell line-derived neurotrophic factor adenovirus vector-transfected mesenchymal stem cells into neuron-like cells and the possible underlying mechanisms.*

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Copyedited by Patel B, Li HH, Zhu WJ, Yu J, Qiu Y, Li CH, Song LP, Liu WJ, Zhao M