The isolation and cultivation of bone marrow stem cells and evaluation of differences for neural-like cells differentiation under the induction with neurotrophic factors

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Abstract The bone marrow represents the most common source from which to isolate mesenchymal stem cells (MSCs). They can be obtained directly from patients and successfully induced to form various differentiated cell types. In addition, cell-based transplantation therapies have been proven to be promising strategies for curing disease of the nerve system. Therefore, it was particularly important to establish an easy and feasible method for the isolation, purification, and differentiation of bone marrow stromal cells (BMSCs). The aim of this study was to isolate and characterize putative bone marrow derived MSCs from Sprague–Dawley (SD) rats. Furthermore, differentiation effects were compared between the GDNF-induction group and the BDNF-induction group. Of these, BMSCs were isolated from the SD rats in a traditional manner, and identified based on plastic adherence, morphology, and surface phenotype assays. After induction with GDNF and BDNF, viability of BMSCs was detected by MTT assay and neuronal differentiation of BMSCs was confirmed by using immunofluorescence and Western blotting.

Besides, the number of BMSCs that obviously exhibited neuronal morphology was counted and the results were compared between the GDNF-induction group and BDNF-induction groups. Our results indicate that direct adherence was a simple and convenient method for isolation and cultivation of BMSCs. Furthermore, BMSCs can be induced in vitro to differentiate into neuronal cells by using GDNF, which could achieve a more persistent and stable inducing effect than when using BDNF.

Keywords Bone marrow stem cells · Differentiation · GDNF · BDNF · Neuron

Introduction

A number of investigations have demonstrated that cell-based transplantation therapies have been proven to be promising strategies for relieving symptoms of various neuronal diseases and improving function of damaged neuronal tissues in experimental models (Bae et al. 2007; Dezawa et al. 2005; Lu et al. 2001). As reported by Hu et al., glial restricted precursor cells (GRPs) and Schwann cells (SCs) were co-grafted into normal or injured spinal cord. They found that grafted GRPs differentiated into mature oligodendrocytes and formed new myelin on axons caudal to the injury. Finally, co-grafts of GRPs and SCs promoted recovery

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of function following spinal cord injury (SCI) (Hu et al. 2012). In the experiment of Amemori et al., the human spinal cord cell line (SPC-01) was implanted into the lesion 1 week after SCI. The result of the experiment indicated that transplanted animals displayed significant motor and sensory improvement 2 months after SCI (Amemori et al. 2013). Besides, some researchers also indicated that direct transplantation of neural stem cells (NSCs) into the injured brain enhanced the expression of synaptic protein and regeneration-associated protein, which significantly improved neurological motor function in selected behavioral test compared to saline control rats in a rat model of traumatic brain injury (Ma et al. 2011).

Although the results of the above studies indicated that transplantation of Schwann cells or neural stem cells could be used for curing spinal cord injury or brain injury, another peripheral nerve or brain tissue must be sacrificed for the cultivation of Schwann cells or neural stem cells. Furthermore, technical difficulties existed in the harvesting and expansion of these seed cells to obtain a sufficient amount of cells for cell-based therapy within a reasonable time period. Therefore, it was desirable to harvest the neural-like cells from easily accessible sources other than the Peripheral Nervous System (PNS) or brain tissue to produce the seed cells for cell-based transplantation therapy.

In recent years, marrow stem cells (MSCs) have been proposed as an alternative source for cell therapy and regeneration (Barry and Murphy 2004; Salem and Thiemermann 2010). Previous studies demonstrated that MSCs could replace NSCs and neural precursor cells (NPC) because of their differentiation potential into mesodermal lineages including neural-like differentiation (Rebelatto et al. 2008; Bunnell et al. 2008; Yarak and Okamoto 2010). Some evidences have shown that BMSCs transplantation improved prognosis after stroke, promoted remyelination and contributed to partial recovery of locomotor function in animal models of SCI (Koda et al. 2005; Zurita and Vaquero 2006).

But, it was difficult to control the differentiation of the BMSCs when they were directly transplanted into the central nervous system (CNS), because they tended to differentiate into glial cells when placed in a damaged environment (Hofstetter et al. 2005). However, Cao et al. (2001) found that if the

transplanted cells were pre-cultured, the survival rate of transplanted cells was high and the development of mature neurons was significantly increased. Besides, Joannides et al. (2007) also reported that the induction of stem cells to differentiate into neural-like cells before transplantation would help to control the differentiation of transplanted cells in a damaged environment and would be necessary in the design of therapeutic stem cell-based strategies.

Hence, in order to provide more alternative seed cells for transplantation therapy in future, the primary goal of this study was focused on establishing an efficient method for dividing and expanding MSC from bone marrow. The further target was to explore a novel and feasible method to inducing BMSCs to differentiate into neural-like cells in vitro.

Materials and methods

Separation and cultivation of BMSCs

Sprague-Dawley rats (aged 4 weeks, weighing approximately 100g) were purchased from the Comparative Medicine Center of the Yangzhou University. The local ethics committee approved the animal experimentation protocols and all animal experiments were performed according to the Yangzhou University guidelines for animal care. The rats weighing 100–120 g were euthanized and both bilateral femurs were removed. An 18-gauge sterile needle was inserted into the bone marrow cavity, and marrow was extruded by flushing with 10 ml of Dulbecco's modified Eagle's medium-low glucose (DMEM-LG) (Wisent, Inc., St. Bruno, Quebec, Canada). Then, aspirate was centrifuged and resuspended in DMEM + 20 % certified fetal bovine serum (FBS) (Wisent, Inc.) + 1 % penicillin/streptomycin (Wisent, Inc.). The cell suspension was seeded into 75-mm tissue culture flasks. Half of the volume of the culture medium was changed every 2 days in order to remove non-adherent cells and obtain adherent cells. Adherent cells were incubated at 37 °C in 95 % humidity and 5 % CO₂ and medium was replaced every 2–3 days. Inverted fluorescence microscope (Nikon, Ti-U, Tokyo, Japan) was used to observe the cultivated cells every day. A suspension of the cells was obtained by a 5-min incubation in 0.25 % trypsin/EDTA (Wisent, Inc.) followed by gentle scraping.

Cell surface phenotype

P3 cultivated cells in an optimal growth state that had a higher growth rate and were uniformly spindle-shaped were rinsed in PBS and resuspended in 0.5 ml PBS. Mouse anti-rat CD45-phycoerythrin (PE, Becton Dickinson, Franklin Lakes, NJ, USA), mouse anti-rat CD90-FITC (FITC, Becton Dickinson), mouse anti-rat CD34-PE (PE, Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA) and mouse anti-rat CD44-PE (Santa Cruz) monoclonal antibodies were added separately, followed by 30 min of incubation in the dark at 4 °C. Labeled cells were rinsed in PBS, centrifuged at $200\times g$ for 5 min and resuspended in PBS. Then, labeled cells were analyzed using a flow cytometer.

BMSC induction cultivation

This study was divided into 3 groups: GDNF-induction, BDNF-induction and control group. For each group, cover glasses were put into the 24-well plate before seeding cells. Then, suspensions of the P3 BMSCs were seeded in 24-well plates at 4×10^4 cells/well. After 24 h cultivation, in the GDNF-induction group, medium was replaced by induction medium consisting of 1 ml DMEM-LG containing 10 % FBS and 10 ng GDNF (PeproTech, Rocky Hill, NJ, USA). The induction medium was replaced every three days. In the BDNF-induction group, the medium was replaced by induction medium consisting of 1 ml DMEM-LG containing 10 % FBS and 10 ng BDNF (PeproTech, USA). The induction medium was also replaced every 3 days. In the control group, the culture medium was DMEM-LG containing 10 % FBS. It was changed every 3 days.

Immunofluorescence

Cover glasses were taken out from each group, respectively, after 3 and 9 days of induction. Firstly, the cover glass was rinsed with PBS and fixed in ice-cold 4 % paraformaldehyde for 30 min at room temperature (RT), then washed with PBS three times, 5 min each time. Fixed cells were permeabilized in 0.5 % Triton X-100 (BIOS, Beijing, China) for 20 min and blocked with 1 % (W/V) Bovine Serum Albumin (BSA, dissolved in PBS) (BSA/PBS) (BIOS) for 1 h at RT. Then rabbit anti-rat NSE (abcam, ab53025, Cambridge, MA, USA) monoclonal antibody (1:200 dilution with PBS - 1 % BSA) was added

and cover glasses were kept at 4 °C overnight. The primary antibodies were removed by washing with PBS four times, 5 min each time. Then, the cover glasses were incubated with the secondary antibodies (Green fluorescence, Alexa Flour 488 conjugated F[ab]₂IgG (H+L) (Dawen Biotec, DW-GAR4881, China) for 2 h in the dark at RT. In the dark, cover glasses were washed with PBS and DAPI was added and incubated for 20 min at RT. Stained cells were observed under an inverted fluorescence microscope (Niko, Ti-u, Japan).

After cultivation for 5 and 9 days, detection of MAP-2 from BMSCs was performed as described above. The primary antibodies were rabbit anti-rat MAP-2 (abcam, ab32454) monoclonal antibody (1:800 dilution with PBS - 1 % BSA). After cultivation for 5 days, detection of MAP-2 was performed using the above steps. The primary antibody was rabbit anti-rat MAP-2 (abcam, ab32454) monoclonal antibody (1:800 dilution with PBS - 1 % BSA). The secondary antibody was a goat anti-rabbit (Green fluorescence, Alexa Flour 488 conjugated F[ab]₂IgG (H + L) (Dawen Biotec, DW-GAR4881, China). After cultivation for 9 days, detection of MAP-2 was performed using the above steps. The primary antibody was rabbit anti-rat MAP-2 (abcam, ab32454) monoclonal antibody (1:800 dilution with PBS - 1 % BSA). The secondary antibody was Goat anti-rabbit (Red fluorescence Alexa Flour 488 conjugated F[ab]₂IgG (Cy3) (Dawen Biotec, DW-GAR4881, China).

Western blotting

After 9 days of incubation, cells in the GDNF-induction group and the BDNF-induction group were washed three times in cold PBS. Then protease and phosphatase inhibitors (APPLYGEN, Beijing, China) of the radio immuno precipitation assay (RIPA, APPLYGEN) were added in order to obtain lysate. After incubation on ice for 30 min, the cells were scraped and the samples were centrifuged at $14,000\times g$ for 17 min. The supernatant was collected and the protein concentration was quantified using the Pierce BCA Protein Assay Kit (Thermo, NCI3225CH, Waltham, MA, USA). When the protein concentration had been determined, buffer was added in the supernatant according to the protein concentration. This new protein sample, containing $4\times$ buffer, was boiled for 16 min, and then, the protein sample was frozen at

minus 20 °C. The next day, the frozen sample was thawed at RT. The samples were separated by electrophoresis on 10 % SDS-PAGE and transferred onto a polyvinylidene difluoride membrane. The membranes were blocked with 5 % skimmed milk for 2 h at RT, before the membranes were incubated with anti-rat-NSE (abcam, ab53025) monoclonal antibody (1:800 dilution) overnight at 4 °C. Washing buffer was then used three times for 15 min each time. Horseradish peroxidase-linked goat anti-rabbit secondary antibody (Peroxidase Conjugated IgG (H+L), Dawen Biotec, DW-GAR007, China) was added to the reaction for 2 h at RT and then washed three times with washing buffer. Enhanced chemiluminescence (ECL, Millipore, Billerica, MA, USA) was used to record the results.

Cell viability

For each group, BMSCs suspensions were seeded in five 96-well plates at 1×10^4 cells/well and incubated in 5 % CO₂, at 37 °C for 24 h. For the induction group, medium was then replaced by the corresponding induction medium and induction medium was changed every 2 days. The cells in 96-well plates were incubated, respectively, for 1, 3, 5, 7, and 9 days. At different time-points, 20 µL MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Solarbio, Amresco 0793, Shanghai, China) diluted 1:1,000 with PBS was added to everywell of the 96-well plates. 4 h later after incubation in the dark, in the MTT containing supernatant was removed and 150 µL DMSO (Sigma, D5879, St. Louis, MO, USA) was added. A Bio-

Fig. 1 Identification of cultivated cells. The percentage of stained cells for each antibody was CD34: 0.62 %, CD44: 99.69 %, CD45: 0.28 % and CD90: 95.05 %

Fig. 2 Morphology of BMSCs. **a** 1 day cultivation. The appearance of the cells was polygonal or rod-shaped (green arrows, scale bar 10 μ m). **b** 3 days cultivation. The appearance of the cells was polygon-like or flat-shaped (green arrows, scale bar 10 μ m). **c** 5 days cultivation. The appearance of the cells

was almost uniform and spindle-shaped (green arrows, scale bar 10 μ m). **d** 7 days cultivation. Cells are arranged significantly in a swirling pattern (green circle, scale bar 10 μ m). (Color figure online)

Kinetics reader (PE-1420, Perkin-Elmer, Waltham, MA, USA) was used to measure the absorbance at 492 nm. Three independent duplicate tests were performed. Results of the MTT test were collected and statistical differences among 3 groups were evaluated by the one-way analysis of variance. Significant differences were considered when *p* values were below 0.05.

Results

Identification of cultivated cells

Phenotypic cell surface marker analysis of P3 BMSCs using flow cytometry found that BMSCs highly expressed CD90 (95.05 %), CD44 (99.69 %), and expressed low levels of CD45 (0.28 %) and CD34 (0.62 %). These results revealed that cultivated cells were BMSCs rather than hematopoietic cells or their progenitors (Fig. 1).

Morphology of BMSCs

After 1 day of cultivation, P3 cells adhered to the culture flask wall and the appearance of the cells was polygonal or rod-shaped (green arrows). After 3 days of cultivation, the appearance of the cells was spindle-like or flat-shaped (green arrows), and the cells gradually covered the bottom of the culture flask. After 5 days of cultivation, the morphology of the BMSCs was almost uniform: spindle-shaped (green arrows). After 7 days cultivation, cells reached about 90 % confluence and they were arranged in swirl or radial patterns (green circle) (Fig. 2).

Immunostaining of BMSCs with anti-NSE antibody 3 days after induction

In the BDNF and GDBF induction groups, 3 days after induction, positive staining of the neuronal

Fig. 3 In the GDNF-induction group and BDNF-induction group, the anti-NSE staining is shown as *green* fluorescence. Nuclei are labeled with DAPI, shown as *blue* fluorescence (left panels). The panels show that the cells, strongly stained for NSE, formed tapered triangular and axon-like structures of neuronal

cells (*red arrows*). Some of the cells, weakly stained for anti-NSE, still maintained the flat-shaped or irregular shape (*white arrows*). In the control group, nuclei were also labeled with DAPI, shown as *blue* fluorescence but no staining of anti-NSE was detected (*scale bar 10 µm*). (Color figure online)

marker NSE was detected in the BMSCs. Images showed that some cells were strongly stained with anti-NSE antibody and soma gradually formed a tapered or axon-like structure of neural-like cells (*red arrows*). Whereas some cells were stained very weakly and maintained the flat-shaped or irregular shape (*white arrows*). The images also showed that

the number of cells that were strongly stained with anti-NSE antibody in the GDNF-induction group was higher than that in the BDNF-induction group. In the control group, the expression of NSE was not detected and morphology of BMSCs had no changes compared with the induction groups (*Fig. 3*).

Fig. 4 Immunostaining of BMSCs with anti-MAP-2 antibody 5 days after induction. The expression of MAP-2 was shown as green fluorescence. Red arrows show significant filament structures of cells and white arrows show irregular-shaped or

flat-shaped morphology of cells 5 days after induction (for GDNF-induction group and BDNF-induction group, scale bar 50 μm. For control group, scale bar 100 μm). (Color figure online)

Fig. 5 Quantitation of differentiation into neuron-like cells 7 days after induction: **a** 7 days after induction with GDNF, images show that most of the BMSCs exhibited significant morphological changes including soma retraction, transparency and appearance of filament structures (green arrow), a small number of the cells maintained polygon-like or flat-shaped morphology (red arrow) (scale bar 10 μm) **b** 7 days

after induction with BDNF, images show that most of the BMSCs exhibited filament structures and changes of transparency (green arrow) but most of the cells maintained polygon-like or flat-shaped morphology (red arrow) (scale bar 50 μm). **c** The result of statistical analysis indicated that significant difference existed in the two groups. * $p < 0.05$. (Color figure online)

Immunostaining of BMSCs with anti-MAP-2 antibody 5 days after induction

After 5 days induction, in the GDNF-induction and BDNF-induction group, the marker of mature neuron MAP-2 was detected and shown as green fluorescence (Fig. 4). Red arrows show significant filament structures of neural-like cells and white arrows show spindle-shaped or large flattened morphology. Random fields of vision showed that the morphology of neuronal cells in the GDNF-induction group was more obvious than that in the BDNF-induction group.

Estimates of numbers of neuronal cells derived from BMSCs after 7 days induction

Estimates of neuronal cells were based on counting cells in 10 random visual fields. After 7 days of induction with GDNF, most of the BMSCs exhibited significant morphological changes including soma retraction, transparency and appearance of filament structures (green arrow), but only a small number of cells maintained a polygon-like or flat-shaped morphology (red arrow) (Fig. 5a). After 7 days of induction with BDNF, a small number of BMSCs exhibited filament structures and changes of transparency (green arrow), but most of the cells maintained a polygon-like or flat-shaped morphology (red arrow) (Fig. 5b). The result of statistical analysis indicated that 76.34 ± 0.03 % of the cells obviously exhibited morphology of neuronal cells in the GDNF-induction group but only 45.29 ± 0.02 % of the cells exhibited similar changes in the BDNF-induction group after 7 days of induction (Table 1). Significant differences existed between both groups (Fig. 5c). For the GDNF-induction group, 76.34 ± 0.03 % cells exhibited a significant morphological change including soma retraction and transparency (green arrows), but a small number of cells maintained a polygon-like or flat-shaped morphology (red arrows) after 7 days of induction (Fig. 5b). The result of the statistical

analysis showed that significant differences existed between the BDNF-induction group and the GDNF-induction group (Fig. 5c; Table 1).

Morphological change and Western blot analysis 9 days after induction

Remarkable differences appeared after 9 days of induction. In the GDNF-induction group, cells formed bipolar or multipolar synaptic structures of neural-like cells (Fig. 6a) and expression of NSE was confirmed by Western blots (Fig. 6c, d). However, in the BDNF-induction group, most of cells exhibited obvious cytoplasm lysis (red arrows) and only a small number of cells maintained the bipolar structure of neural-like cells (Fig. 6b). Furthermore, expression of NSE distinctively declined after 9 days of induction (Fig. 6c, d).

Co-expression of NSE and MAP-2 9 days after induction

In the GDNF-induction group, both mature neuron markers and neuron-specific markers were detected and BMSCs still maintained significant bipolar or multipolar synaptic structures of neural-like cells after 9 days of induction (green arrows). The anti-NSE staining is shown as green fluorescence and anti-MAP-2 as red fluorescence (Fig. 7b, c). In the BDNF-induction and the control group, expression of NSE and MAP-2 was not detected at the same time-point (Fig. 7e, f).

Viability of BMSCs during cultivation and induction

The statistical analysis demonstrated that (1) the viability of BMSc in the GDNF-induction group after 5 days of induction was obviously higher than that in the BDNF-induction group and the control group ($p < 0.05$); (2) there were no significant differences among the 3 groups whether BMSCs were cultivated or

Table 1 Estimates of neuronal cells derived from BMSCs 7 days after induction

Count	Group		
	Control group	BDNF-induction group	GDNF-induction group
Percentage of neural-like cells (mean $\pm$ SD, %)	<0.5	45.29 ± 0.02	76.34 ± 0.03

Fig. 6 Morphological change and Western blot analysis 9 days after induction. **a** After 9 days of induction with GDNF, most of the BMSCs formed bipolar or multipolar synaptic structures of neuronal cells (*green arrows*) (*scale bar 50 μ m*). **b** After 9 days of induction with BDNF, most of the BMSCs showed obvious cytoplasm retraction or lysis (*red arrow*) and only a small number of cells exhibited bipolar structures of neuronal cells (*green arrows*) (*scale bar 100 μ m*). **c** and **d**: Expression of NSE among the 3 groups after 3 (**c**) and 9 (**d**) days of induction by using Western blot analysis. (Color figure online)

induced for 1 and 3 days in vitro ($p > 0.05$); (3) the viability of BMSCs in the BDNF-induction group gradually declined and a significant difference was observed after 5 days induction ($p < 0.05$) (Fig. 8).

Discussion

Recently, there has been increasing interest in the study in which injured neural components were replaced by BMSCs that can be easily isolated from small aspirates of bone marrow and expanded in culture (Hofstetter et al. 2002; Shimizu et al. 2007; Keimpema et al. 2009). The results of these studies demonstrated that BMSCs had great prospects in cell replacement therapy and tissue engineering. Therefore, it was particularly important to establish an easy and feasible method for isolation and propagation of BMSCs.

Previous studies indicated that direct adherence was the preferential method for MSC isolation and purification (Rouger et al. 2007; Yang and Yang 2008). Identification of MSC was mainly based on morphology and functional characteristics. Both Cheng et al. (2004) and Reger et al. (2008) demonstrated that CD90 was expressed on BMSCs and absent on hematopoietic stem cells, and CD45 was absent on BMSCs and positive on hematopoietic stem cells. In this experiment, results of flow cytometry identified surface antigens of cultivated cells (Fig. 1). Images of cultivated cells showed that cells had a uniform spindle-shaped or flat-shaped morphology, and characteristics of adherence to a plastic flask (Fig. 2). Immunofluorescent staining further confirmed neuron-like cells differentiation potential of cultivated cells (Fig. 3). Besides, the MTT assay revealed that cells obtained by use of direct adherence had high in vitro viability (Fig. 8). These results support

Fig. 7 Co-expression of NSE and MAP-2 9 days after induction. **a** Image of BMSCs induced by GDNF for 9 days and stained with DAPI (scale bar 50 µm). **b** Image of BMSCs induced by GDNF for 9 days and stained with anti-NSE (green) (scale bar 50 µm). **c** Image of BMSCs induced by GDNF for 9 days and stained with anti-MAP-2 (red) (scale bar 50 µm). **d** The merged images of panels **b** and **c**. **e** Image of BMSCs

induced by BDNF for 9 days and stained with anti-NSE (scale bar 50 µm). **f** Image of BMSCs induced by BDNF for 9 days and stained with anti-MAP-2 (scale bar 50 µm). **g** Image of BMSCs cultured in the control group for 9 days and stained with NSE (scale bar 50 µm). **h** Image of BMSCs cultured in the control group for 9 days and stained with MAP-2 (scale bar 50 µm). (Color figure online)

strong evidence that the cultivated cells were BMSCs rather than hematopoietic stem cells. This also suggested that the direct adherence of whole marrow was a simple and convenient method for isolation and cultivation of BMSCs.

Currently, there are several methods for the induction of differentiation of MSCs into neural cells: (1) exposure to inducing cytokine and neurotrophic factors, such as NGF, GDNF, and EGF (Low et al. 2008; Cohen et al. 2011; Keimpema et al. 2009); (2)

Fig. 8 Comparison of viability of BMSCs among the 3 groups in vitro (* $p < 0.05$, # $p > 0.05$). (Color figure online)

exposure to chemical inducers such as β -mercaptoethanol, DMSO, and glutathione (Woodbury et al. 2000; Kang et al. 2006; Sagara and Makino 2008); (3) a combination of cytokines and chemical inducers; (4) other methods including exposure to traumatic brain homogenate, co-culture in or exposure to culture medium/Chinese medicines (Yong et al. 2008; Wang et al. 2009).

Differentiation of MSC to neuron-like cells was firstly reported by Woodbury et al. (2000), but increasing researches found that chemical inducers had negative effects on viability of MSC during induction in vitro. Neuron-like cells differentiation of MSC was firstly reported by Woodbury et al. in (2000), but increasing number of studies found that chemical inducers had a negative effect on the viability of MSC during the induction in vitro. Besides, although these studies indicated that neuronal differentiation of MSCs could be rapidly achieved by using chemical inducers within 24 h, both the observation for morphological changes of differentiated MSCs and the detection for makers of mature neurons derived from differentiated MSCs were deficient (Woodbury et al. 2000; Kang et al. 2006; Sagara and Makino 2008; Edamura et al. 2012). This could not supply enough evidence to illustrate stability and practicality of differentiated cells for therapeutic stem cell-based strategies. In this experiment, the results of MTT indicated that GDNF significantly improved the viability of BMSCs in the process of induction compared to the control group and the BDNF-induction group (Fig. 8).

After 3 days of induction, images of immunofluorescence showed that some BMSCs changed to the neuron-like morphology with multipolar, rounded cell bodies and long, narrow processes in the GDNF-induction group (Fig. 3). Random field of vision showed that nearly all cells stained positive against NSE and NSE-positive ratios were also higher than in the experiment by Woodbury et al. (2000) and Edamura et al. (2012). They reported that the ratio of NSE-positive cells were $88.5 \pm 8.6\%$ and $78.2 \pm 2.3\%$, respectively. After 5 days of induction with GDNF, the mature neural marker MAP-2 was expressed in BMSCs (Fig. 4), suggesting that BMSCs differentiated into neural-like cells both morphologically and functionally (Sanchez-Ramos et al. 2000). The results of statistical analysis indicated that more than $76.34 \pm 0.03\%$ of BMSCs (after 7 days of induction with GDNF) obviously exhibited a morphology of neuronal cells (Fig. 5). But approximately only $65.7 \pm 19.7\%$ of canine BMSCs exhibited this kind of changes induced by chemical inducers such as DMSO, BHA (butylated hydroxyanisole) and β -mercaptoethanol (Edamura et al. 2012).

In addition, Ye et al. (2011) reported that the cerebrospinal fluid (CSF) could induce BMSCs to differentiate into neural-like cells. Yang et al. discovered that striatal extracts also promoted the dopaminergic differentiation of BMSCs. But both experiments did not indicate what factor or factors in the CSF/striatal extracts were responsible for this effect (Ye et al. 2011; Yang et al. 2012). In this study, after 9 days of induction, MAP-2 and NSE were still successfully detected in the GDNF-induction group (Fig. 8). Furthermore, BMSCs still maintained remarkable morphology of neural-like cells. Therefore, it could be demonstrated that GDNF could successfully induce BMSCs to differentiate into neural-like cells in vitro.

In addition, the results of this study revealed that BMSCs exhibited some different differentiation effects responding to induction with GDNF and BDNF in vitro: (1) after 3 and 5 days of induction with GDNF, the fluorescence intensities of NSE-staining and MAP-2 staining were stronger than those induced by using BDNF (Figs. 3 and 5); (2) after 5 days of induction with GDNF, results of statistical analysis revealed that the viability of the cells was significantly higher than that induced by using BDNF. It was easy to find that the viability of the cells

gradually declined after 5 days of induction with BDNF (Fig. 8); (3) after 7 days of induction with GDNF, BMSCs exhibited more obviously neuronal morphology than those induced by using BDNF (Fig. 5); (4) in the BDNF-induction group, after 9 days of induction, Western blot assay confirmed a significant decrease in NSE expression, however, this phenomenon was not found in the GDNF-induction group (Fig. 6). Based on the above results, GDNF was more appropriate for neuronal differentiation of BMSCs in vitro than BDNF.

Conclusion

Direct adherence of whole bone marrow is a simple and convenient method for isolation and culture of BMSCs. BMSCs could express markers of neurons and change into a significant morphology of neural-like cells by using GDNF, which could achieve a more persistent and stable induction effect than BDNF in vitro.

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