

# Immunoregulatory effect of neuronal-like cells in inducing differentiation of bone marrow mesenchymal stem cells

J. XU, H. LU, Z.-N. MIAO, W.-J. WU, Y.-Z. JIANG, F. GE, W.-F. FANG, A.-H. ZHU, G. CHEN, J.-H. ZHOU, Y.-Z. LU, Z.-F. TANG, Y. WANG

Wuxi Third People's Hospital, Liangxi, Wuxi, Jiangsu, China

**Abstract.** – **OBJECTIVE:** To evaluate the immunoreactivity of bone marrow mesenchymal stem cells (BMSCs), and explore the biological characteristics and capabilities of BMSCs and the potential to be differentiated into neuronal cells *in vitro*.

**MATERIALS AND METHODS:** The BMSCs were isolated and proliferated *in vitro* to generate the xenogeneic mixed lymphocyte reaction. Moreover, peripheral BMSCs (pBMSCs) were added according to different ratios, which methods were stated as follows: 1: Dulbecco's Modified Eagle Medium (DMEM) + 10% Fetal Bovine Serum (FBS) + 1 µmol/L all-trans-retinoic acid (ATRA) + 20 µg/L basic fibroblast growth factor (bFGF) + 20 µg/L epidermal growth factor (EGF); 2: DMEM + 2% dimethyl sulfoxide (DMSO) + 100 µmol/L butylated hydroxyanisole (BHA). The immunofluorescence and immunohistochemical staining were finally used to evaluate the differentiation capabilities of human BMSCs (hBMSCs) induced in neuronal cells.

**RESULTS:** hBMSCs inhibited the lymphocyte proliferation in the mixed lymphocyte reaction (MLR) system at a proportional inhibition rate with additional numbers of stem cells. At hour 2 after culture with method 1, the plasma of hBMSCs shrank to nuclei and perinuclear bodies and was visualized under the light microscope. At hours 3-5, most of the hBMSCs formed neuron-like cells with total cell number unchanged. Afterward, the hBMSCs turned into bipolar or multipolar shaped cells and interconnected into a large network at Day 3. With immunofluorescence and immunohistochemical staining, 60-70% of the hBMSCs showed neurospecific enolase (NSE) positive and 45-50% glial fibrillary acidic protein (GFAP) positive while the Nestin-positive cells decreased to 3.4%. However, when cultured 2 hours with method 2, the most of the hBMSCs formed bipolar or multipolar shaped cells, then died after 48 hours. 40-50% NSE and 35-40% GFAP were positively expressed. Significantly, the rate of Nestin-positive cells decreased from 63% to 1.6% from hour 2 after culture to hour 48.

**CONCLUSIONS:** hBMSCs may be effective for cell therapy and tissue engineering for the capability of differentiating into neuronal-like cells, as well as the capability of inhibiting lymphocyte proliferation in MLR system.

## Key Words

Immunomodulatory effect, Neuronal-like cell, Bone marrow mesenchymal stem cells.

## Introduction

The human bone marrow mesenchymal stem cells (hBMSCs) with the ability of self-replication and multiple potential differentiation, can significantly promote the recovery of spinal cord injury. Since the hBMSCs are phenotypically immaturity, the recipients of which have no graft vs. host disease (GVHD) or reduced GVHD after allogeneic cell transplantation. Unfortunately, there are various factors participating to the chemical and biological induction of hBMSCs into the neurons *in vitro*. The differentiation of hBMSCs into neurons and neuroglia is a complex mechanism.

Recent researches suggested that, except for supporting hematopoiesis *in vitro* and *in vivo*, the mesenchymal stem cells (MSCs) are also capable of inhibiting allogeneic immune response and reducing graft vs. host (GVH) reaction. Bartholomew et al<sup>1</sup> had discovered that the baboon MSCs not only inhibited a proliferative response to potent T-cell *in vitro* when added into a mixed lymphocyte reaction culturing system, but also prolonged skin graft survival when administered intravenously to donor itself or the recipients. These immunoregulatory features are equivalent to the immunosuppressants such as Cyclosporine A. Fukuda et al<sup>2</sup> also showed in their research on

mice that the donors' bone marrow mesenchymal stem cells (hBMSCs) had greatly affected acute and chronic GVHD after the hematopoietic stem cell transplantation. If the recipients had both hematopoietic stem cells (HSCs) and MSCs, the post-transplantation GVHD occurrence rate was significantly lower than those which had only HSCs. Although the immunoregulatory mechanism of the MSCs has not been elucidated, it is the most popular transplantation research hotspot nowadays. The animal researches have already been proved for its usefulness in future applications of transplantation.

Currently, the stem cell transplantation therapy comprises three major research hotspots: (1) providing MSCs directly to repair the damaged tissue; (2) inducing the stem cells *in vitro*, differentiating into specific tissue cells; genetically modifying the stem cells then differentiating into healthy specific tissue cells to treat genetic disorders; (3) building material to "construct" tissues or organs *in vitro* for transplantation. Most researches were conducted according to the second hotspot, especially in the differentiation and clinical application of genetically modified stem cells.

Some researches<sup>3,4</sup> proved that the hBMSCs can differentiate into at least eight types of tissue-specific cells such as osteoblast, chondroblast, fibroblast, cardiac muscle cell, skeletal muscle cell, hepatocyte, epithelium and neuron. In neurology, the researches were mainly focusing on the correlation between the MSCs and neurological tissue cells. Some researches<sup>5,6</sup> suggested that, the marked neural stem cells were administered into the body of a lethally irradiated animal, and differentiated into blood cells, which affected the bone marrow, spleen, and circulating blood system of the recipients. Whereas, MSCs also were capable of differentiating into neuron and neuroglia *in vivo*<sup>7,8</sup>. However, the researches on the differentiation of MSCs into neuron and neuroglia *in vitro* relatively lagged behind. Woodbury et al<sup>9</sup> set-up a series of experiments of induction, which resulted in the hBMSCs differentiating into neurons *in vitro*. It also indicated that various factors may contribute to the differentiation of hBMSCs into neuron and neuroglia.

This study demonstrated that the hBMSCs had immunoregulatory capabilities by cultured in Mixed Lymphocyte Reaction (MLR) System, and it also proved that the hBMSCs were capable of differentiation into neuronal cells, which provided a theoretical background for hBMSCs transplantation *in vivo*.

## Materials and Methods

### Reagents and Equipment

The reagents and equipment are stated as follows:

- DMEM-LG (Gibco BRL, Grand Island, NY, USA)
- All-Trans-retinoic acid (Sigma-Aldrich, Gaithersburg, MD, USA),
- Butylated hydroxyanisole (Sigma-Aldrich, Gaithersburg, MD, USA),
- Penicillin-Streptomycin (Gibco BRL, Grand Island, NY, USA),
- DMSO (Sigma-Aldrich, Gaithersburg, MD, USA),
- sABC kit (Wuhan BOSTER, Wuhan, Hubei, China),
- DAB brown detection kit (Wuhan BOSTER, Wuhan, Hubei, China),
- Anti-Mouse IgG-FITC antibody Mouse in goat (Sigma-Aldrich, Gaithersburg, MD, USA),
- Fetal Bovine Serum (FBS) (Hyclone, Logan, UT, USA),
- $\beta$ -FGF (CYTOLAB, Mogi das Cruzes, SP, Brasil),
- EGF (Sigma-Aldrich, Gaithersburg, MD, USA),
- Human Neuron-Specific Enolase (NSE) (Wuhan BOSTER, Wuhan, Hubei, China),
- Rabbit anti-human antibody (Wuhan BOSTER, Wuhan, Hubei, China),
- Anti-Human Glial Fibrillary Acid Protein (GFAP) (Wuhan BOSTER, Wuhan, Hubei, China),
- Mouse Anti-Human CD44 Monoclonal antibody (Exalpha Biological Inc., Shirley, MA, USA),
- CD45 (IMMUNOTECH, Marlborough, MA, USA),
- CD29 CD106 and CD105 (Ansell, Stillwater, MN, USA),
- CD34 (Caltag Laboratories, Buckingham, MK, UK),
- Mitomycin (Kunming Pharmaceutical Co., Kunming, Yunnan, China),
- Liquid Scintillation Counter (Pharmacia, Shanghai, China),
- CK40 Inverted Phase Contrast Microscope (Olympus, Tokyo, Japan),
- BX51 Fluorescence Microscope (Olympus, Tokyo, Japan),
- Imaging Digital DP50 (Olympus, Tokyo, Japan),
- Flow Cytometry (Beckman-Coulter, Indianapolis, IN, USA),
- Carbon Dioxide Incubator (EVERCO, Cornelius, OR, USA).

Adult human bone marrow was harvested by surgical procedures (unsalvageable fragments of cancellous bones during the hip joint operations or cancellous bones recovered during the autogenous iliac bone grafting procedures). All participants have signed informed consent and this study was in accordance with the term of the Ethics Committee of our Hospital (Wuxi Third People's Hospital, Wuxi, Jiangsu, China).

## Methods

### *Isolation and Enrichment of hBMSCs*

Under sterile condition, the human bone marrow suspension solution, which was anti-coagulated by adding heparin, was diluted by D-Hank's solution of 10 ml (Beijing Suolaibao Tech Co. Ltd. Beijing, China) at volume ratio 1:1. Then the solution mixture was added slowly into 5 ml Ficoll-Paque solution (Beijing Suolaibao Tech Co. Ltd. Beijing, China) in a 15 ml conical centrifuge tube. Then, the mixture was centrifuged for 15 minutes at 1800 rpm, and the mononuclear cell fraction at the interface between plasma and Ficoll-Paque solution (Pharmacia Fine Chemicals, Stockholm, Sweden) was collected. Then the cell suspension was rinsed by D-Hank's solution once and diluted to concentration  $1 \times 10^8/l$ . Then, it was and planted in  $25 \text{ cm}^2$  culture flasks on the medium containing Dulbecco's Modified Eagle Medium-Low Glucose (DMEM-LG) (Gibco BRL, Grand Island, NY, USA) + 1% Penicillin (Gibco BRL, Grand Island, NY, USA) and Streptomycin (Gibco BRL, Grand Island, NY, USA) + 5 mg/L basic Fibroblast Growth Factor (bFGF) (CYTOLAB, Mogi das Cruzes, SP, Brasil) + 10% (v/v) fetal bovine serum (FBS), in humidified  $37^\circ\text{C}$ , 5%  $\text{CO}_2$  incubator. After 72 hours, all mediums had to be changed every 2 days until the cell colonies were visible and grew up to 70% of the confluence. After that, all mediums were removed and the culture flasks were rinsed once by D-Hank's solution, and added 2.5 g/l trypsin into the culture flasks for 3-5 minutes, until the cells were retracted and the retraction was increased through the inverted phase contrast microscope technique. Then neutralized trypsin, pipetted the cell suspension vigorously with a 10 ml pipet to wash the cells off the bottom of the flask, transferred the cell suspension to another tube, adjusted the cell concentration of the suspension to (v/v) 1:3 and replanted them onto the culture medium. After 2 days, the mediums had to be changed again, and repeated above steps to replant and re-culture.

### *Mixed Lymphocyte Reaction System by BMSCs and T Lymphocytes*

#### *Reaction Cells*

The human peripheral blood mononuclear cells (hPBMCs) of adult donors had been isolated by Ficoll-Paque Density Gradient Centrifugation. The hPBMCs were cultured in 6-well plates, incubated for 2 hours at  $37^\circ\text{C}$ . All non-adherent cells were aspirated and mixed with sheep red blood cells, and then incubated for 15 minutes at  $37^\circ\text{C}$ . After that, the cell suspension was centrifuged at 14000 rpm for 5 minutes, the supernatant was discarded, and the red cell lysis buffer containing Ammonium chloride was added. Finally, the T lymphocyte with 92.5% purity was prepared.

#### *Stimulating Cells*

We adjusted the 3<sup>rd</sup> generation hBSCs suspension concentration to  $1 \times 10^6/ml$ , and added Mitomycin (Kunming Pharmaceutical Co., Kunming, Yunnan, China) into the suspension until the Mitomycin concentration reached  $25 \mu\text{g/ml}$ , then incubated 45 minutes at  $37^\circ\text{C}$ . Finally, we rinsed five times by Roswell Park Memorial Institute-1640 (RPMI-1640) medium (Thermo Fisher Scientific, Waltham, MS, USA) supplemented with 10% FBS.

#### *Grouping*

During the "two-way" MLR test, the hPBMCs of two donors were added at the same concentration ( $1 \times 10^5$ ) to each well on the 96-well plate. According to different hBMSCs/hPBMC ratio, mitomycin-treated-hBMSCs at different concentration levels were also added to the well. There were positive control wells, which no hBMSCs were added. When incubated at the 6<sup>th</sup> day,  $1.85 \times 10^4 \text{ Bq } ^3\text{H-Thymidine}$  ( $^3\text{H-TdR}$ ) was added to each well, and cultured for another 16-18 hours. Then we collected all cells on 49-fiberglass paper. After drying, the paper was measured by a liquid scintillation counter (Pharmacia, Shanghai, China) and expressed as cpm.

### *Differentiation of hBMSCs into neuron-like cells*

#### *Induction Method 1*

We planted the 3<sup>rd</sup> generation hBSCs cell suspension into 6-well culture plates. When the colonies reached 60% of confluence, we changed the culture mediums to induction mediums according to following the formula: DMEM + (v/v) 10% FBS +  $1 \mu\text{mol/L ATRA}$  +  $20 \mu\text{g/L bFGF}$  +  $20 \mu\text{g/L EGF}$ , in humidified  $37^\circ\text{C}$ , (v/v) 5%  $\text{CO}_2$  incubator.

### Induction Method 2

The procedures were as same as induction method 1, and we changed the culture mediums to induction mediums according to following the formula: DMEM + (v/v) 2% DMSO + 100  $\mu$ mol/L BHA, in humidified 37°C, (v/v) 5% CO<sub>2</sub> incubator.

### Fixation, Immunofluorescence and Immunohistochemical stain

When the differentiation of the hBMSCs at the 2<sup>nd</sup>, 6<sup>th</sup>, 24<sup>th</sup>, 48<sup>th</sup> hour and 7<sup>th</sup> day, the differentiated cells were then stained by immunofluorescence and immunochemical stains. The procedures were stated as follows:

1. Discarded all culture mediums, rinsed the cells by phosphate buffered saline (PBS) three times;
2. Added 40 g/L Paraformaldehyde (PFA) (Shanghai Xiangsheng Biotech Co. Ltd. Shanghai, China) to cell suspension for cell fixation for 20 minutes, then rinsed with PBS three times again;
3. Added 0.3% TritonX-100 (Shanghai Xiangsheng Biotech Co. Ltd. Shanghai, China) for 20 minutes and rinsed with PBS three times;
4. 10% sheep serum was added to the cell suspension and sealed up at room temperature for 20 minutes;
5. Aspirated all sheep serum and added anti-Human Neuron-Specific Enolase (NSE) (Wuhan BOSTER, Wuhan, Hubei, China), Rabbit anti-human antibody (v/v 1:500) (Wuhan BOSTER, Wuhan, Hubei, China), Anti-Human Glial Fibrillary Acid Protein (GFAP) Glial Fibrillary, antibody Human in rabbit (v/v 1:1000) (Wuhan BOSTER, Wuhan, Hubei, China) antibody Human and incubated at 37°C for 2 hours;
6. Rinsed with PBS three times, added immuno-stain Anti-Mouse IgG-FITC antibody Mouse in goat (v/v 1:500) (Sigma, Gaithersburg, MD, USA) and incubated 37°C for 30 minutes;
7. After the staining process was completed, all samples were observed under Fluorescence Microscope;
8. OR cell suspension was immune-histochemical stained by adding DAB brown detection solution (Wuhan BOSTER, Wuhan, Hubei, China) and incubated at 37°C for 5-10 minutes;
8. After the staining process was completed, all samples were observed under an ordinary microscope.

### Statistical Analysis

The results were statistically analyzed by SPSS software (Inc. Chicago, IL, USA). The one-way ANOVA with S-N-K tests, and post-hoc multiple

comparisons Student's *t*-test and F-test for paired data were employed to test the probability of significant differences between samples with  $p < 0.05$

## Results

### MLR System by hBMSCs and T Lymphocytes

During the 5-day co-culture *in vivo* of hBMSCs and T lymphocytes, the <sup>3</sup>H-TdR incorporation showed that in the hBMSCs/hPBMC group with ratio 1:5, and with the cpm value of 11506.98±2483.65, there were significant statistical differences compared to other groups ( $p < 0.05$ ). There was a tendency when the hBMSCs/hPBMC ratio decreased, which led to the hBMSCs number decreased as well with the cpm value increased accordingly. However, the cpm values were lower than the positive control group (hMLR group). Therefore, the hBMSCs' capability of inhibiting T Lymphocytes in MLR was proportionate to their cell concentration level. The more hBMSCs, the more strength of inhibition, as shown in Figure 1.

### Differentiation, Immunofluorescence and Immunochemical stain of hBMSCs

#### Induction Method 1

After differentiation for 2 hours, hBMSCs became visible under the light microscope that the cell plasma was contracted to the nuclei, and the perinuclear bodies began to be visible. After differentiation for 3-5 hours, most of the hBMSCs showed neuronal morphology. The bodies of hBMSCs became round and had neurite extensions of which with some branches at the tip. Some neurite extensions were inter-connected with each other to form a network structure. However, no cell number changed significantly. After differentiation for 3 days, most of the hBMSCs transformed

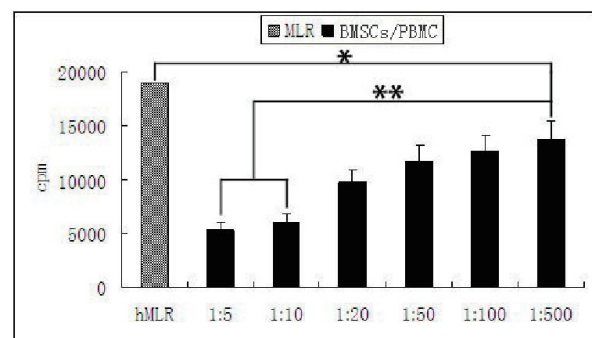
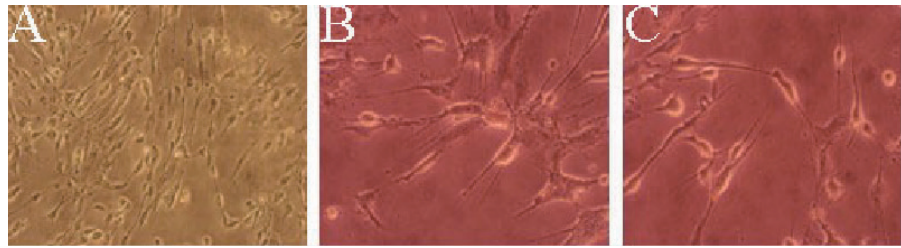


Figure 1. "Two way" mixed lymphocyte reaction test at different hBMSCs/hPBMC ratio.



**Figure 2.** Morphological changes of hBMSCs differentiated into neuron-like cells induced by method 1 at (A) 2h (B) 48h (C) 72h. (Phase Contrast Microscope,  $\times 100$ ).



into bi-polar or multipolar shape, and also had an axon-like or dendrite-like process. When stained, 60-70% of hBMSCs showed NSE positive and 45-50% GFAP positive. The Nestin-positive cells decreased to 3.4%, as shown in Figures 2 and 3.

#### Induction Method 2

After differentiation for 2 hours, hBMSCs became a smaller size than usual. The cell plasma was contracted to their nuclei and had neurite extensions and changed into bipolar and multiple polar shaped cells. After 48 hours, most of the differentiated hBMSCs floated above the mediums, then died (Figure 4). When stained, 40-50% of the cells showed NSE positive and 35-40% showed GFAP positive. The Nestin-positive cells elevated to 63% after 2 hours of differentiation, then decreased to 1.6% after 48 hours (Figure 5A and 5B). It is possible to demonstrate that the hBMSCs may differentiate into neuron progenitor cells at specified time spot and specified concentration level of inducer, then they may develop into full grown neuron cells.

#### Without induction

There were very rare hBMSCs which expressed no GFAP, showed 0.1-0.2% NSE positive and 10% Nestin-positive (Figure 5C).

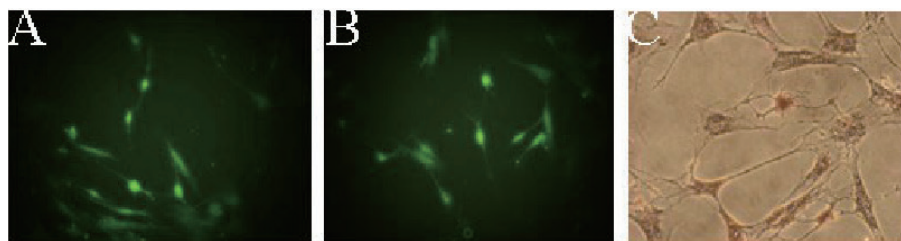
## Discussion

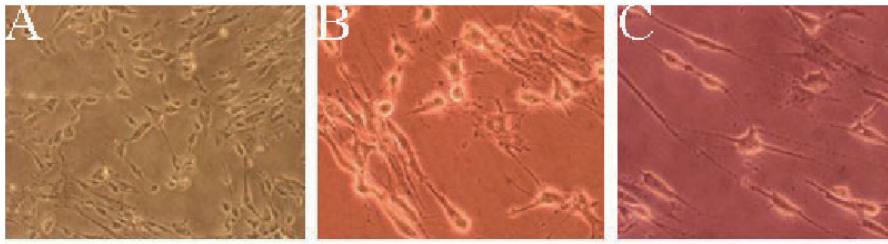
Recent researches showed the capability of the MSCs to inhibit the allogeneic immune response and alleviate GVHD. The immunoregulatory

features of MSCs are the most popular research hotspots of transplantation<sup>10,11</sup>. The mechanism of MSCs' immunoregulation is a complex and related to various factors. The immunogenicity of MSCs is relatively low and there are not MHC-II type of immunogens, and CD 80 (B7-1), CD 86 (B7-2) and CD 40 as co-stimulators to the immune response. Therefore, T Lymphocyte is unable to attack MSCs since the messengers are missing<sup>12,13</sup>. The immunoregulation features of MSCs bring the widespread attention of both domestic and overseas researchers.

However, none of the MHC-II antigens, Fas ligand, or co-stimulation T Lymphocyte activation factors such as B7-1, B7-2 or CD 40L is expressed by MSCs. It was considered that the MSCs are not the main cause for the proliferation of allogeneic T Lymphocytes<sup>14</sup>. Some researchers had BMSCs co-incubated with IFN- $\gamma$ . The BMSCs can highly express most of MHC-I antigens and even some MHC-II antigens by induction. They added CD 28 during the T Lymphocyte mixed reaction. However, all above procedures failed to lead to the proliferation of allogeneic T Lymphocytes *in vitro*<sup>15</sup>. Other researches<sup>16-18</sup> demonstrated that despite the stimulators and effectors derived from the same or unrelated donors, the MSCs always inhibit lymphocytes, and the strength of inhibition is proportionate with the number of MSCs, which meant that the MSCs can regulate the proliferation of T Lymphocytes without MHC. Dinicla et al<sup>19</sup> added autologous and allogeneic BMSC into MLR after peripheral lymphocytes stimulation *in vitro*, resulted in inhibition of T Lym-

**Figure 3.** Neuron-specific enolase was expressed after induction by method 1 at (A) 2h (B) 72h (Immunofluorescent staining $\times 100$ ) (C) 48h. GFAP was expressed at 48 hours after induction by method 1 (SABC method  $\times 100$ ).





**Figure 4.** Morphological changes of bone marrow mesenchymal stem cells differentiated into neuron-like cells induced by method 2 at (A) 2h (B) 24h (C) 48h (Phase Contrast Microscope,  $\times 100$ ).

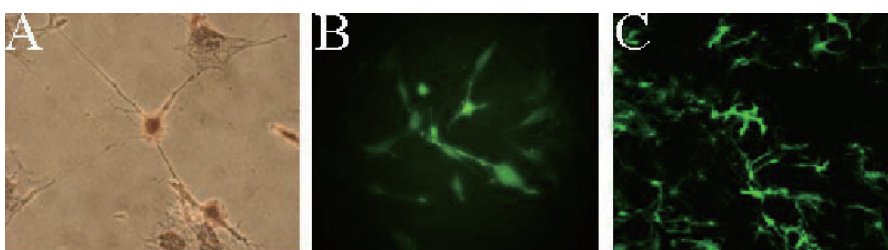
phocytes. Klyushnenkova et al<sup>20</sup> showed that the MSC can express the MHC-II antigens and costimulatory molecules such as B7-1. If the autologous or allogeneic MSCs were introduced into the MLR system of the stimulators and effectors, the inhibition of the primary and secondary MLR immediately occurred. In overall, MSC has a negative regulatory capability to elicit T lymphocytes proliferative responses due to active suppressive mechanisms<sup>20</sup>.

In the experiment, hBMSCs/hPBMCs=1:5 group also showed that the stimulated T lymphocytes were significantly inhibited by hBMSCs after co-cultured with hBMSCs, the results accorded with Kramperal's research. Kramperal et al<sup>19</sup> believed that the strength of T lymphocyte inhibition was proportionated with the number of MSCs. They also proved that soluble factors produced during the culture of hBMSCs with T Lymphocytes can inhibit the T Lymphocyte proliferation. Moreover, such inhibition was transmissible, and these factors prevented antigen-presenting cells from contacting with T lymphocytes. Further researches discovered that such inhibition was only functioning to autologous antigen memory cells and specific T lymphocytes, antigen response T lymphocytes were excluded from the process. By using neutralizing monoclonal antibodies, such as transforming growth factor  $\beta 1$  and hepatocyte growth factor, hBMSCs would effectively abrogate synergic effects by both factors and T lymphocytes proliferation was revitalized<sup>20,21</sup>.

Researches on hBMSCs cell cycle showed that about 90% of BMSCs were at resting  $G_0/G_1$  phase.

After completing the genetic analysis of BMSCs, Silva et al<sup>22</sup> discovered that BMSCs not only encoded the genes of mesenchymal tissues, but also the genes of endothelium and epithelium tissues. Theoretically, it proved that the BMSCs had multiple differentiation potentials and were capable of differentiating into multiple system/organs derived from different germ layers. Such plasticity and potentiality would provide the cell strong basis for tissue repair.

Neural induction from BMSCs, which derived from mesoderm, is at the initial stage. Such differentiation is called "Trans-differentiation" because the mesoderm stem cells are differentiating into ectoderm differentiated cells. All-trans retinoic acid (ATRA), which is the acid derivative of Vitamin A, is able to enhance the quantity of neural cells *in vitro* by the dose-dependant effect. It regulates the differentiation of neural stem cells through Epidermal Growth Factor (EGF) modulation, and it also increases the neuron and astrocyte population *in vitro*. With Nerve Growth Factor (NGF), it effectively induces the embryonic stem cell to differentiate into neural stem cell<sup>23</sup>. Basic Fibroblast Growth Factor (bFGF) is relatively more widespread and complicated in differentiation. It keeps EGF-induced neural stem cells survive and proliferate *in vitro*, and it induces the neural stem cell to differentiate into neuron. It is able to keep neuron and neuroglia survives *in vitro*, and it induces the neuronal cell to synthesize neuron-specific gene which products SCG-100 and acetylcholine transferase. Also, it stimulates the growth of axons in sympathetic



**Figure 5.** A, GFAP was expressed at 48 hours after induction by method 2 (SABC method,  $\times 100$ ). B, Neuron-specific enolase was expressed at 48 hours after induction by method 2 (Immunofluorescent staining,  $\times 100$ ). C, hBMSCs expressed Nestin (Immunofluorescent staining,  $\times 100$ ).

and parasympathetic nerve cells, which shows the mitogen-like features<sup>24,25</sup>. It was reported that, when both bFGF and ATRA were introduced, 16% BMSCs had neurofilament (NF) in the cytoplasm<sup>26</sup>. Epidermal Growth Factor (EGF) is a mitogenic molecule, which promotes neural stem cell division without species specificity, and it also induces the neural stem cell to differentiate into astrocyte and stimulates axon extension which keeps neuron survives<sup>9</sup>.

NSE and GFAP are two types of cytoplasm protein; when NSE is presenting in neurons, GFAP is mostly expressed in astrocyte. In the experiment, after hBMSCs have been successfully induced, immunofluorescence stain was used to show the existence of NSE and GFAP. ATRA and BHA were capable of inducing the hBMSC to differentiate into astrocyte and neuron. The un-induced hBMSCs had Nestin expression. The experiment showed that at the initial stage of induction, the Nestin-positive hBMSC increased and the Nestin-positive hBMSC decreased. Overall, it proved that after induction, hBMSCs transformed from nascent to adult<sup>27</sup>.

### Conclusions

This research showed that hBMSCs were able to be isolated and proliferated *in vitro*. hBMSCs are easily obtained, quickly proliferated *in vitro* and are sufficient for transplantation without the requirement of immortalization of cell lineage. Besides, hBMSCs have multiple differentiation potentials and foreign genes are easily transferred and expressed. Also, they are able to be autologous transplanted, which ultimately avoids GVHD. Therefore, hBMSCs are the potential reliable sources for autologous neuron cell transplantation. The next stage of research has to emphasize the purification of hBMSCs. Whether or not the induced hBMSCs have similar physiological features as the normal neural cells is also left for future study. For the fully functioning neural cells, they are able to synthesize the neurotransmitters, set-up neural network by dendrite-like connection with other neural cells or even other tissues, receive and transmit nerve impulses and control all physiological activities of the human bodies.

### Conflict of Interest

The Authors declare that they have no conflict of interests.

### References

- 1) BARTHOLOMEW A, STURGEON C, SIATSKAS M, FERRER K, MCINTOSH K, PATIL S, HARDY W, DEVINE S, UCKER D, DEANS R, MOSELEY A, HOFFMAN R. Mesenchymal stem cells suppress lymphocyte proliferation in vitro and prolong skin graft survival in vivo. *Exp Hematol* 2002; 30: 42-48
- 2) FUKUDA K, FUJITA J. Mesenchymal, but not hematopoietic, stem cells can be mobilized and differentiate into cardiomyocytes after myocardial infarction in mice. *Kidney Int* 2005; 68: 1940-1943.
- 3) ZHANG T, SHAO H, XU KQ, KUANG LT, CHEN RF, XIU HH. Midazolam suppresses osteogenic differentiation of human bone marrow-derived mesenchymal stem cells. *Eur Rev Med Pharmacol Sci* 2014; 18: 1411-1418.
- 4) SAFFORD KM, RICE HE. Stem cell therapy for neurologic disorders: therapeutic potential of adipose-derived stem cells. *Curr Drug Targets* 2005; 6: 57-62.
- 5) GROVE JE, BRUSCIA E, KRAUSE DS. Plasticity of bone marrow-derived stem cells. *Stem Cells* 2004; 22: 487-500.
- 6) SOTIROPOULOU PA, PEREZ SA, GRITZAPIS AD BAXEVANIS CN, PAPAMICHAIL M. Interactions between human mesenchymal stem cells and natural killer cells. *Stem Cells* 2006; 24: 74-85.
- 7) WANG G, BUNNELL BA, PAINTER RG, QUINIONES BC, TOM S, LANSON NA JR, SPEES JL, BERTUCCI D, PEISTER A, WEISS DJ, VALENTINE VG, PROCKOP DJ, KOLLS JK. Adult stem cells from bone marrow stroma differentiate into airway epithelial cells: potential therapy for cystic fibrosis. *Proc Natl Acad Sci U S A* 2005; 102: 186-191.
- 8) YANO S, KURODA S, SHICHINOHE H, HIDA K, IWASAKI Y. Do bone marrow stromal cells proliferate after transplantation into mice cerebral infarct? A double labeling study. *Brain Res* 2005; 1065: 60-67.
- 9) WOODBURY D, SCHWARZ EJ, PROCKOP DJ, BLACK IB. Adult rat and human bone marrow stromal cell differentiate into neurons. *J Neurosci Res* 2000; 61: 364-370.
- 10) BATTULA VL, BAREISS PM, TREML S, CONRAD S, ALBERT I, HOJAK S, ABELE H, SCHEWE B, JUST L, SKUTELLA T, BÜHRING HJ. Human placenta and bone marrow derived MSC cultured in serum-free, b-FGF-containing medium express cell surface frizzled-9 and SSEA-4 and give rise to multilineage differentiation. *Differentiation* 2007; 75: 279-291.
- 11) ZHANG X, SODA Y, TAKAHASHI K, BAI Y, MITSURU A, IGURA K, SATOH H, YAMAGUCHI S, TANI K, TOJO A, TAKAHASHI TA. Successful immortalization of mesenchymal progenitor cells derived from human placenta and the differentiation abilities of immortalized cells. *Biochem Biophys Res Commun* 2006; 351: 853-859.
- 12) PASQUINELLI G, TAZZARI P, RICCI F, VASELLI C, BUZZI M, CONTE R, ORRICO C, FORONI L, STELLA A, ALVIANO F, BAGNARA GP, LUCARELLI E. Ultrastructural characteristics of human mesenchymal stromal (stem) cells derived from bone marrow and term placenta. *Ultrastruct Pathol* 2007; 31: 23-31.
- 13) DELORME B, CHATEAUVIEUX S, CHARBORD P. The concept of mesenchymal stem cells. *Regen Med* 2006; 1: 497-509.
- 14) VAANANEN HK. Mesenchymal stem cells. *Ann Med* 2005; 37: 469-479.



- 15) ALMEIDA-PORADA G, SHABRAWY D, PORADA C, ZANJANI ED. Differentiative potential of human meta-nephric mesenchymal cells. *Exp Hematol* 2002; 30: 1454-1462.
- 16) DOMINICI M, LE BLANC K, MUELLER I, SLAPER-CORTENBACH I, MARINI F, KRAUSE D, DEANS R, KEATING A, PROCKOP DJ, HORWITZ E. Minimal criteria for defining multipotent mesenchymal stromal cells. The international society for cellular therapy position statement. *Cytotherapy* 2006; 8: 315-317.
- 17) PAROLINI O, ALVIANO F, BAGNARA GP, BILIC G, BÜHRING HJ, EVANGELISTA M, HENNERBICHLER S, LIU B, MAGATTI M, MAO N, MIKI T, MARONGIU F, NAKAJIMA H, NIKAIKO T, PORTMANN-LANZ CB, SANKAR V, SONCINI M, STADLER G, SURBEK D, TAKAHASHI TA, REDL H, SAKURAGAWA N, WOLBANK S, ZEISBERGER S, ZISCH A, STROM SC. Concise review: isolation and characterization of cells from human term placenta: outcome of the first international workshop on placenta derived stem cells. *Stem Cells* 2008; 26: 300-311.
- 18) MIAO Z, JIN J, CHEN L, ZHU J, HUANG W, ZHAO J, QIAN H, ZHANG X. Isolation of mesenchymal stem cells from human placenta: comparison with human bone marrow mesenchymal stem cells. *Cell Biol Int* 2006; 30: 681-687.
- 19) KRAMPFERAL M, GLENNIE S, DYSON J, SCOTT D, LAYLOR R, SIMPSON E, DAZZI F. Bone marrow mesenchymal stem cells inhibit the response of naïve and memory antigen-specific T cells to their cognate peptide. *Blood* 2003; 101: 3722-3729.
- 20) KLYUSHNENKOVA E, MOSCA JD, ZERNETKINA V, MAJUMDAR MK, BEGGS KJ, SIMONETTI DW, DEANS RJ, MCINTOSH KR. T cell responses to allogeneic human mesenchymal stem cells: immunogenicity, tolerance, and suppression. *J Biomed Sci* 2005; 12: 47-57.
- 21) BEYTH S, BOROVSKY Z, MEVORACH D, LIEBERGALL M, GAZIT Z, ASLAN H, GALUN E, RACHMILEWITZ J. Human mesenchymal stem cells alter antigen-presenting cell maturation and induce T-cell unresponsiveness. *Blood* 2005; 105: 2214-2219.
- 22) SILVA GV, LITOVSKY S, ASSAD JA, SOUSA AL, MARTIN BJ, VELA D, COULTER SC, LIN J, OBER J, VAUGHN WK, BRANCO RV, OLIVEIRA EM, HE R, GENG YJ, WILLERSON JT, PERIN EC. Mesenchymal stem cells differentiate into an endothelial phenotype, enhance vascular density, and improve heart function in a canine chronic ischemia model. *Circulation* 2005; 111: 150-156.
- 23) LI M, PEVNY L, LOVELL-BADGE R, SMITH A. Generation of purified neural precursors from embryonic stem cells by lineage selection. *Curr Biol* 1998; 8: 971-974.
- 24) SIEBER-BLUM M, ZHANG JM. Growth factor action in neural crest cell diversification. *J Anat* 1997; 191: 493-499.
- 25) PINCUS DW, KEYOUNG HM, HARRISON-RESTELLI C, GOODMAN RR, FRASER RA, EDGAR M, SAKAKIBARA S, OKANO H, NEDERGAARD M, GOLDMAN SA. Fibroblast growth factor-2/brain-derived neurotrophic factor-associated maturation of new neurons generated from adult human subependymal cells. *Ann Neurol* 1998; 43: 576-585.
- 26) KIM BJ, SEO JH, BUBIEN JK, OH YS. Differentiation of adult bone marrow stem cells into neuron progenitor cells in vitro. *Neuroreport* 2002; 13: 1185-1188.
- 27) YANG YJ, LI XL, XUE Y, ZHANG CX, WANG Y, HU X, DAI Q. Bone marrow cells differentiation into organ cells using stem cell therapy. *Eur Rev Med Pharmacol Sci* 2016; 20: 2899-2907.