The simulated microgravity enhances multipotential differentiation capacity of bone marrow mesenchymal stem cells

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Abstract Multi-differentiation capability is an essential characteristic of bone marrow mesenchymal stem cells (BMSCs). Method on obtaining higher-quality stem cells with an improved differentiation potential has gained significant attention for the treatment of clinical diseases and developmental biology. In our study, we investigated the multipotential differentiation capacity of BMSCs under simulated microgravity (SMG) condition. F-actin staining found that cytoskeleton took on a time-dependent change under SMG condition, which caused spindle to round morphological change of the cultured cells. Quantitative PCR and Western Blotting showed the pluripotency marker OCT4 was up-regulated in the SMG

condition especially after SMG of 72 h, which we observed would be the most appropriate SMG duration for enhancing pluripotency of BMSCs. After dividing BMSCs into normal gravity (NG) group and SMG group, we induced them respectively in endothelium oriented, adipogenic and neuronal induction media. Immunostaining and Western Blotting found that endothelium oriented differentiated BMSCs expressed higher VWF and CD31 in the SMG group than in the NG group. The neuron-like cells derived from BMSCs in the SMG group also expressed higher level of MAP2 and NF-H. Furthermore, the quantity of induced adipocytes increased in the SMG group compared to the NG group shown by Oil Red O staining. The expression of PPAR γ 2 increased significantly under SMG condition. Therefore, we demonstrated that SMG could promote BMSCs to differentiate into many kinds of cells and predicted that enhanced multi-potential differentiation capacity response in BMSCs following

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SMG might be relevant to the changes of cytoskeleton and the stem cell marker OCT4.

Keywords Simulated microgravity · Bone marrow mesenchymal stem cells · Pluripotency · Differentiation · OCT4

Abbreviations

SMG	Simulated microgravity
NG	Normal gravity
MSC	Mesenchymal stem cell
BMSC	Bone marrow mesenchymal stem cell
VWF	Von Willebrand factor
MAP2	Microtubule-associated protein 2
NF-H	Neurofilament heavy chain
OCT4	Octamer-binding transcription factor 4

Introduction

As an important member of the stem cell family, the mesenchymal stem cells (MSCs) have recently become a widespread focus of study for more and more researchers. It can be obtained from various tissues and the main source of isolated MSCs comes from bone marrow. Relatively easy to separate and culture is one of the main advantages of bone marrow mesenchymal stem cell (BMSC). In some specific conditions, BMSCs can differentiate into several distinct cell types, including chondrocytes, osteoblasts, smooth muscle cells, adipocytes, cardiac myocytes, endothelial cells and neurons (Pittenger et al. 1999; Oswald et al. 2004; Kodama et al. 2005, 2006; Tamama et al. 2006, 2008; Owen and Friedenstien 1988). Due to BMSC's capabilities in regenerating tissue, self-renewal and multipotential differentiation, it is highly desirable to use them in therapeutic renewal methods following aging, disease or trauma. They can also play an important role in wound healing, organ donation and chronic immune-suppressive therapies (Pittenger et al. 1999; Barry and Murphy 2004; Phinney and Prockop 2007; Prockop 1997). However, the problems of the low induction efficiency and high risk of the stem cells in clinical application have not been addressed yet (Colter et al. 2000; Crisostomo et al. 2006). Therefore, the development of a method that can produce higher-quality stem cells with improved differentiation potential is highly significant.

A strong relationship between the morphology and function of stem cells has been demonstrated (Graziano et al. 2007; McBride et al. 2008). Geometric shape cues have shown to have great effect on the differentiation of human BMSCs (Kilian et al. 2010). McBeath and his coworkers recently confirmed that cell shape and size had a large effect on the fate of MSCs (McBeath et al. 2004). It has been reported that mechanical boundary conditions can change the gene expressing pattern and consequently the functional behavior of MSCs. For example, MSCs regulated angiogenesis according to their mechanical environment. Osteogenic proliferation and differentiation appeared when MSCs were stimulated by mechanical loading (Koike et al. 2005; Yoshikawa et al. 1997; Kasper et al. 2007; Mauney et al. 2004).

Microgravity, as one of the most essential elements of space flight, can induce extensive physiological changes in human (Basso et al. 2005). Our work concurred with previous observation that the shape of spindle stem cells derived from rat BMSCs turned round after simulated microgravity (SMG) (Chen et al. 2011). However, the significance of the shape change for the stem cell itself has not been explained sufficiently yet. Many studies have demonstrated that the change in geometric shape could bring cytoskeletal reorganization (Li et al. 2009), which could alter the multipotential differentiation fates of BMSCs by affecting the relative signaling pathways such as RhoA or the stem cell surface markers (Hosu et al. 2008; Yuge et al. 2011).

In this study, we report data obtained from SMG experiments performed with BMSCs. The objectives of this study were to investigate changes in morphology, cytoskeleton, the pluripotency marker and multipotential differentiation capacity of BMSCs under SMG condition and to examine the underlying relationships of these changes.

Materials and methods

Isolation and cell culture of BMSCs

Bone marrow mesenchymal stem cells were isolated by their adherence to plastic as described in our previously published paper (Chen et al. 2011), with slight modification. Both tibias and femurs were isolated from 2-week-old Sprague–Dawley (SD) rats according to standard outlined by the Chinese Ethics

Committee. The bone marrow containing MSCs was washed out by ice-cold DMEM (low glucose, Gibco; Carlsbad, CA, USA) and supplemented with 15 % fetal bovine serum (FBS, Gibco). We then gathered and cultured the cells in 25 cm² plastic flasks at 37 °C with 5 % CO₂, removing the refreshing medium every 3–4 days. When the cells reached 70–80 % confluence, they were treated with 0.25 % trypsin and separated at a ratio of 1:3. Our study chose the 3rd to 6th passage cells for testing their surface makers CD44, CD45, CD90 and CD34 with Flow Cytometry. Every independent experiment began from cell culture of BMSCs. If $n = 3$, we needed to choose three SD rats to isolate and culture the BMSCs, respectively, and then perform the corresponding experiments as follows.

Culture under simulated microgravity condition

Simulated microgravity (SMG) was operated according to a previously published paper (Li et al. 2009). A rotating vessel was used to create the weightless environment which is often referred to as “SMG”. This equipment with high aspect ratio vessels was designed to provide a vector-averaged gravity environment through the medium that contained cells rotating at the same speed with the vessel wall (Fig. 1D). Clinorotation technology (30 rpm) that models microgravity actually prevented the cell from feeling the effects of gravity, the gravity vector thus escaped its detection machinery. In our previous study, we found cell configuration, the cytoskeleton, apoptotic rate and the expression of OCT4 of the BMSCs have no change in different NG durations (0, 48, 72, 120 h) (Supplementary material 1). So in our study, we have chosen to culture the BMSCs in the NG condition for 72 h to represent the NG group. We divided the vessels randomly. Normal gravity group vessels were kept in the same room as the machine only without clinorotation. SMG group vessels were rotated with different durations (48, 72, 120 h). Flow Cytometry tested the surface markers CD44, CD45, CD90 and CD34 of SMG 72 h group.

Flow Cytometry analysis of the apoptotic rate

The effect of SMG on BMSCs apoptosis was investigated in this step. After the cells were cultured under NG and SMG conditions of different rotation durations, they were treated with 0.25 % trypsin. Their

density was adjusted to 10⁶/mL, 100 µL was transferred to 5 mL centrifuge tubes and then two groups cells were stained with the Annexin V-FITC and PI (BD Pharmingen, San Diego, CA, USA) for 15 min without light. The results were analyzed by fluorescence activated cell sorter (FACScan) cytometer (Elite ESP, Miami, FL, USA) and WinList software (Verity Software House, Topsham, ME, USA).

F-actin staining

The changes of cytoskeleton were observed after microgravity stimulations. Briefly, the cells were fixed in 4 % paraformaldehyde for 30 min and then washed in PBS 3 times. After being immersed in 0.01 % Triton X-100 for 30 min, they were covered with 1 % BSA at room temperature for 1 h to block the non-specific bindings. They were subsequently incubated with FITC-Phalloidin (5 µg/mL, Sigma, USA) for 2 h at 37 °C. BMSCs were then stained with DAPI (1:500, Sigma) for 7 min for nucleolus staining. Cells were imaged using IPLab software (Scanalytics, Fairfax, VA, USA).

Quantitative PCR

The expression of the pluripotency marker OCT4 at gene level was detected after SMG simulations. Total RNAs were isolated from SMG groups of different durations (0, 48, 72 and 120 h) using Trizol reagent (Invitrogen), and then reverse-transcribed into cDNA. Real-time PCR technique quantified the cDNAs using a SYBR green PCR mix, directed by the ABI Prism 7900HT. The sequences of the specific primers were: forward, 5'-GAACAGTTTGCCAAGCTGCTG-3', and reverse, 5'-CCGTTACAGAACCATACCTCG-3'. Real-time PCR were performed using a thermal cycler consisting of an initial holding at 42 °C for 2 min, then 37 °C for 15 min, 85 °C 5 s. This was then followed by a two-step PCR program: 95 °C for 5 s and 60 °C for 34 s for 45 cycles. Quantitative analyses of the data were carried out using an ABI PRISM 7900 sequence detection system (Applied Biosystems). The values were expressed as fold change relative to the expression of GAPDH.

Endothelium oriented differentiation of BMSCs

As described previously (Oswald et al. 2004), the BMSCs of NG group and SMG 72 h group were cultivated in DMEM, supplemented with 10 % FBS and

Fig. 1 **A(a)** Flow Cytometry analysis of CD44, CD90, CD45 and CD34 in normal BMSCs; **(b)** flow Cytometry analysis of CD44, CD90, CD45 and CD34 in BMSCs which were cultured under SMG for 72 h. **B** Phase-contrast microscopic analysis of the effect of SMG on the morphology of BMSCs. The scale bar

is 40 μ m. **C** The changes of the ratio of the width/length of BMSCs in the different groups. * Denotes $P < 0.05$, versus the NG group. # Denotes $P < 0.05$, versus the SMG 48 h group, $n = 10$. **D** The clinostat system modeled microgravity

100 ng/mL vascular endothelial growth factor (VEGF, Invitrogen), 50 ng/mL epidermal growth factor (Invitrogen) and 1 μ g/mL hydrocortisone (Sigma-Aldrich)

for 14 days, aiming to induce endothelial differentiation. An inverted phase-contrast microscope (Eclipse TE 300; Nikon Co., Tokyo, Japan) was subsequently

used to examine the morphological changes. The expression level of VWF and CD31 were analyzed by Immunofluorescent and Western Blotting.

Adipogenic differentiation of BMSCs

Both of the NG group cells and the SMG 72 h group cells were treated with adipogenic medium composed of 1 $\mu\text{mol/L}$ dexamethasone (Sigma), 0.5 mmol/L isobutylmethylxanthine (IBMX, Sigma), 10 $\mu\text{mol/L}$ bovine insulin (Sigma), 200 $\mu\text{mol/L}$ indomethacin (Sigma) and DMEM + 10 % FBS. After 7 days, a phase-contrast microscope showed a change in shape. Here, Oil Red O staining was applied to examine the efficiency of adipogenic induction as described previously (Xiang et al. 2007). Western Blotting was used to analyze the expression level of PPAR γ 2.

Neuron oriented differentiation of BMSCs

After the stimulation of SMG 72 h, the two groups BMSCs were induced to differentiate into neuron-like cells as previously described (Chen et al. 2011). Briefly, the cells were cultured in neuronal induction medium consisting of DMEM + 10 % FBS, 10 $\mu\text{g/L}$ basic fibroblast growth factor (b-FGF, R&D Systems, Inc., Minneapolis, MN, USA), 10 $\mu\text{g/L}$ human epidermal growth factor (hEGF, R&D Systems, Inc.), 1 mmol dibutyl cyclic AMP (dbcAMP, Sigma, St. Louis, MO, USA) and 0.5 mmol isobutylmethylxanthine (IBMX, Sigma, St. Louis, MO, USA) for 14 days. Morphological changes were observed by phase-contrast microscope. Immunofluorescent and Western Blotting were used to analyze the expression level of Microtubule-associated protein 2 (MAP2) and Neurofilament heavy chain (NF-H).

Immunofluorescent staining

After cultured in the induced intermediary for 14 days, the cells were analyzed by fluorescence staining. The neuron oriented induced cells were incubated with primary antibodies to MAP2 (dilution 1:200; Santa Cruz, CA, USA) and to NF-H (dilution 1:200; Santa Cruz, CA, USA), respectively. The endothelium oriented induced cells were incubated with an anti-VWF antibody (dilution 1:200; Santa Cruz, CA, USA) and an anti-CD31 antibody (dilution 1:200; Santa Cruz, CA, USA), respectively, at 4 °C overnight. After we washed

out the primary antibodies, fluorescein isothiocyanate (FITC) or TRITC-conjugated fluorescent secondary antibodies (dilution 1:100; Santa Cruz, CA, USA) were incubated, respectively, for 1 h at 37 °C in the dark. At last the nucleoli were stained by DAPI for 7 min. The number of positive cells was divided by the total number of cells to calculate the rate of positivity.

Western Blotting

The cells were washed three times in phosphate-buffered saline (PBS) and then lysed in RIPA buffer containing protease and phosphatase inhibitors (Sigma-Aldrich). Protein concentrations were quantified, and subsequently, separated on a sodium dodecyl sulfate (SDS)—12 % polyacrylamide gels, then electrophoretically transferred onto nitrocellulose membranes. Membranes were blocked and probed. The concentrations of the primary antibodies used were for the anti-MAP2 antibody (dilution 1:500), NF-H (dilution 1:500), for the anti-VWF antibody (dilution 1:500), for the anti-CD31 antibody (dilution 1:500), for the anti-PPAR γ 2 antibody (dilution 1:500) and for the anti-GAPDH antibody (1:2000). BMSCs cultured in normal media after rotated for 0 h, 48 h, 72 h and 120 h were incubated with an anti-OCT4 antibody as the primary antibody (dilution 1:500; Santa Cruz, CA, USA), the primary antibodies overnight at 4 °C. The next day, they were made to hybridized horseradish peroxidase-labeled the goat anti-rabbit secondary antibodies fluorescein isothiocyanate (FITC) or TRITC (dilution 1:100; Santa Cruz, CA, USA) respectively. Membranes were exposed to X-ray film for chemiluminescence detection. The bands were analyzed using the Quantity One software. Data were normalized by GAPDH protein level and the corresponding relative expression ratio was calculated as protein of interest/GAPDH.

Statistical analysis

Data were expressed as mean $\pm$ SD and analyzed using SPSS 13.0 (SPSS, Chicago, IL, USA). Statistical analyses were performed using the two-tailed Student's *t* test for two groups. The one-way ANOVA was used to analyze the results of more than two groups. When one-way ANOVA showed a significant difference, the significance between the groups was calculated with the Bonferroni test for multiple comparisons. Different levels represented significance at *P* values <0.05.

Results

Cell culture and cell identification

The 3rd to 6th passage cells which were prepared for this research displayed spindle-shaped or broadened flattened morphology. Flow Cytometry results showed that these cells were CD44 and CD90 positive (the ratios were 94.74 and 93.57 %, respectively) and CD45 and CD34 negative [Fig. 1A(a)]. After the cells have been cultured in SMG condition for 72 h, they were also CD44 and CD90 positive (the ratios were 93.04 and 92.83 %, respectively) and CD45 and CD34 negative [Fig. 1A(b)].

Morphological change of BMSCs

After SMG stimulation, the shape of the cells shifted to roughly round, which obviously contrasted with the spindle cells cultured in NG (Fig. 1B). We measured the width and length of the cells and calculated the ratio to describe the morphology by the MetaMorph 7.1 software (Universal Imaging, Downingtown, USA). The statistical data indicated that the most significant change of the shape occurred after SMG 72 h and differences were found rarely between SMG 72 and 120 h (Fig. 1C).

Apoptotic rate analysis

Flow Cytometry results showed that there was little difference of the quantity of apoptotic BMSCs among different groups. This indicated that SMG did not induce apoptosis of BMSCs (Fig. 2a).

Time-dependent change of cytoskeleton

The cytoskeleton of BMSC was sensitive to SMG. This was especially true for the change in microfilaments that are composed of F-actin. It was observed that F-actin organized clearly into bundles as thick filaments in the NG group. The filaments bundles were oriented along the long axis of the cells, displaying a typical radial array. However, SMG induced transient changes of the cytoskeleton, made microfilaments disturbed and induced the modification of some bundles. The most obvious change was found in SMG 72 h. The bundles were thin, dispersed and lost their long-range orientation that appeared under the NG condition. Further more, some were even observed

to be perpendicular to the substratum. However partial restoration of F-actin occurred in SMG 120 h. The bundles showed signs of polymerization and restored the preferential orientation toward the cell periphery (Fig. 2b).

Effect of simulated microgravity on pluripotency marker

Analysis by Quantitative PCR detected that cells maintained in SMG could up-regulate OCT4 expression. While the expression of OCT4 was changed slightly after 48 h of SMG simulation, more obvious changes were found after SMG 72 h (Fig. 3a). However, this up-regulation had no significance in the SMG 120 h group compared with the 72 h group analyzed by the one-way ANOVA. Furthermore, the results of Western Blotting were very similar to those obtained with Quantitative PCR (Fig. 3b). Therefore, we deduced that 72 h might be the most appropriate SMG duration for the highest pluripotency of BMSCs.

Endothelial differentiation of BMSCs

After the cells were induced with endothelium oriented revulsive, we found cell morphology exhibited endothelium-like changes and phase-contrast microscope showed that there were more endothelium-like cells in the SMG group than in the NG group (Fig. 4a). Immunostaining showed that the positivity rate of VWF and CD31 were higher in SMG than in the NG group cells (Fig. 4b). Western Blotting analysis confirmed the above immunostaining observations. As shown in Fig. 4c, the induced cells in the SMG group expressed higher VWF and CD31 levels than that seen in the NG group ($P < 0.05$). This showed that SMG could enhance the endothelium oriented differentiation of BMSCs.

Adipogenic differentiation of BMSCs

After we induced the cells for 7 days, the number of the adipogenic-like cells was higher in the SMG group as observed by phase-contrast microscope (Fig. 5a). Oil Red O staining indicated that the numbers of derived adipocytes in the SMG group were higher than those in the NG group (Fig. 5b). Western Blotting showed that the expression of PPAR γ 2 increased significantly under SMG condition (Fig. 5c). This indicated that SMG could promote adipogenic differentiation of BMSCs.

Fig. 2 **a** FACS analysis of the apoptotic rate of BMSCs in four groups. **b** Time-dependent change of cytoskeleton. The scale bar is 20 μ m. The changes of filaments bundles orientation are shown in the red rectangles. (Color figure online)

Neuron oriented differentiation of BMSCs

When these cells were placed in neuronal induction medium for 14 days, many of them took on long neuron-like cell processes and these cells developed long branching that contacted with neighboring cells. Phase-contrast microscope showed that there were more neuron-like cells in the SMG group than in the

NG group (Fig. 6a). MAP2 and NF-H were shown to have lower expression in the NG group than that in the SMG group, as observed by immunostaining (Fig. 6b). Western Blotting concurred with the immunostaining results either at 7 or 14 days after induction (Fig. 6c). This evidence suggested that SMG was helpful for the neuron oriented differentiation of BMSCs.

Fig. 3 **a** Quantitative PCR analysis of the expression of OCT4. OCT4 expression was normalized to endogenous GAPDH in the same samples. The results represent three independent experiments, where each experiment was repeated three times. **b** Western Blotting showed the changes of OCT4 expression

for different SMG durations. The results represent 4 independent experiments, where each experiment was repeated three times. * Denotes $P < 0.05$, versus the NG group. # Denotes $P < 0.05$, versus the SMG 48 h group

Discussion

Microgravity, as one of the most important elements of space flight, has been reported to cause extensive physiological changes in MSCs, including the maintenance of the undifferentiated state of BMSCs and potentiating the proliferation and differentiation of the cells to a single direction (Yuge et al. 2006, 2011). In this study, we further demonstrated that SMG could enhance the multipotential differentiation capacity of BMSCs which enriches the previous studies.

We used a clinostat for simulating microgravity, which could generate a multidirectional G force that placed the cells in a constant direction changing the gravity environment (Yuge et al. 2003). As a result, the cells had no time to respond to normal gravity. While it is impossible for this device to entirely model a weightless environment, it is a comprehensive and feasible research method for use in the space life science field (Klaus 2001).

This study illustrated that the morphology of BMSCs changed from spindle to round shaped in the SMG condition which was caused by a change of the cytoskeleton. But the BMSCs did not lose their immunophenotype. The idea that the cytoskeletal tension can affect the stem cell fate via RhoA pathway is widely accepted (McBeath et al. 2004). This study found that the cytoskeleton present time-dependent change. The most obvious changes were found after SMG 72 h and partial restoration occurred after SMG 120 h. Consequently, it

can be deduced that the duration of rotation for SMG was fundamental in directing the differentiation fate of BMSCs. RhoA is a protein associated with actin cytoskeleton has also been reported to be affected by SMG. Our previous study found that the activation of RhoA in the SMG 72 h group decreased significantly but increased in SMG 10 days. In addition, our study also found that the expression of osteoblastic gene ALP was lower in SMG 72 h group but higher in SMG 10 day group (Zhang et al. 2013). We can therefore speculate that 72 h stimulation might down-regulate the RhoA pathway associated genes to encourage the differentiation to endothelium, adipocyte or neuron. On the other hand, a long duration for SMG might activate the RhoA pathway and reorganized the cytoskeleton to advance BMSCs in differentiating to other cells such as osteogenic progenitor cells. Therefore, SMG can enhance the pluripotency of BMSCs through adjustments in the duration. But still more experiments are needed to confirm this hypothesis.

It is clearly evident that the differentiation potential and functional activities of MSCs were decided by multilevel nature. In addition to the cytoskeleton, the surface markers of BMSCs were also very important. It was interesting to find that not only the cytoskeleton, but also the surface markers of the stem cells had been modified. OCT4 is a key transcription factor that can regulate the pluripotency and self-renewal of stem cells. It has been established that levels of OCT4 expression were crucial for the maintenance of pluripotency and for governing the fate of the stem cell.

Fig. 4 Effect of SMG on the endothelium oriented differentiation of BMSCs. **a** Phase-contrast microscopic image of the differentiated cells from both groups. The scale bar is 100 μ m. **b** Immunostained images of VWF and CD31. The scale bar is

40 μ m. **c** Western Blotting analysis of the expression of VWF and CD31. The results represent three independent experiments, where each experiment was repeated three times.* Denotes $P < 0.05$, compared with the NG group

Both the up-regulation and the down-regulation of the level of OCT4 could affect the differentiation potential of stem cells (Niwa et al. 2000). We found that SMG can lead to up-regulation of the OCT4 gene, especially after 72 h stimulation which was the same duration causing the most obvious changes of the shape and the cytoskeleton of BMSCs. Though the cytoskeleton was re-established and the preferential orientation toward the cell periphery was restored at SMG 120 h, the shape change of BMSCs in the SMG 120 h group was not significant compared with the SMG 72 h group. However, the expression level of OCT4 no longer increased after SMG 72 h. Some experts found that the more complex change of BMSCs occurred after SMG 120 h (Gershkovich et al. 2011). We also agree with that the change of the multipotential differentiation

capacity of stem cell may be relevant to the telomerase activity. But, results reported by Yuge et al. (2006) showed that telomere length for the NG group or the SMG group did not change during culturing, and telomerase activity was undetectable in both groups in their study. The specific effect of the telomerase activity on the multipotential differentiation capacity of stem cell is unclear. In addition, there is no evidence that OCT4 expression can be affected by the telomerase activity. So, when we designed this experiment, we did not consider that the influence of telomerase activity could impact the results.

A number of studies show that CD31 and VWF play important roles in endothelium maturation and angiogenesis. CD31 is also known as platelet-endothelial cell adhesion molecule-1 (PECAM-1) which is the

Fig. 5 Effect of SMG on the adipogenic differentiation of BMSCs. **a** Phase-contrast microscopic images showed that there were more adipocyte-like cells in the SMG group. The scale bar is 100 μ m. **b** Images of Oil Red O staining of the cells from both groups cells. The scale bar is 100 μ m. **c** Western Blotting

analysis of the expression of PPARγ2. The results represent three independent experiments, where each experiment was repeated three times.* Denotes $P < 0.05$, compared with the NG group

only known member of the CAM family on platelets. CD31 proteins which are expressed by endothelial cells, mediate homotypic or heterotypic cell adhesion and thus play a role in cardiovascular development (Jin et al. 2012). Vascular endothelial cells synthesize and secrete VWF. VWF is only expressed in normal endothelial cells (Ruggeri 2003). Thus, they are regarded as endothelial-specific markers. MAP2 and NF-H are involved in the formation of the cytoskeleton of nerve cells. MAP2 can participate in composing the structure of different parts of nerve cells and can be found mostly in dendrites and axon. Neurofilaments usually contain three intermediate filament proteins: L, M, and H which are involved in the maintenance of neuronal caliber. NF-H has an important function in mature axons. So according to the previous article by Yuge et al. (2011) we have chosen MAP2 and NF-H as the neural differentiation markers. Peroxisome proliferator-activated receptor gamma 2 (PPARγ2) is an adipocyte-specific nuclear hormone receptor that has recently been identified as a critical transcription factor involved in adipogenic differentiation in MSCs.

Previous research suggested that the physiologic role of PPARγ2 is to regulate development of the adipose lineage in response to endogenous lipid activators and that this factor may serve to link the process of adipocyte differentiation to systemic lipid metabolism (Muruganandan et al. 2011). So we have chosen the PPARγ2 as an adipogenic differentiation marker of BMSCs. After induction, we found that the positivity expression rate of VWF, CD31, MAP2, NF-H and Oil Red O staining increased indeed in the SMG 72 h group, which demonstrated that 72 h SMG could promote BMSCs to differentiate into many kinds of cells. As we all known that endothelium, adipocytes and neuron cells were all force-insensitive cells, and previous report showed that the force-insensitive cells were easily affected by short time SMG. However, the osteoblasts are force-sensitive cells and they were easily affected by long term SMG. So we have chosen BMSCs differentiation into endothelium, adipocytes and neuron-like cells to research the multipotential differentiation capacity of BMSCs in short time SMG condition and we indeed demonstrated that SMG could

Fig. 6 Effect of SMG on the neuron oriented differentiation of BMSCs. **a** Phase-contrast microscopic images of the cells from two groups. The scale bar is 100 μ m. **b** Immunostained images of MAP2 and NF-H. The scale bar is 40 μ m. **c** Western Blotting analysis of the expression of MAP2 and NF-H after MSCs

cultured in the neuronal differentiation media for 7 and 14 days. The results represent three independent experiments, where each experiment was repeated three times. * Denotes $P < 0.05$, compared with the NG group

promote BMSCs to differentiate into these kinds of cells.

A previous study reported that SMG boosted their survival rate by maintaining BMSCs in an undifferentiated state, thus enhancing the efficiency of differentiation (Yuge et al. 2011). In accordance with our observation, we predicted that the up-regulated OCT4 gene and cytoskeleton were relevant to the possible mechanisms of SMG affecting BMSCs. But in our study, we only little discussed the modifications of other capabilities of BMSCs under the SMG condition. We will further study further effects of SMG on BMSCs such as the proliferation and migration capacities in our future work.

In this study, we used as base for induction condition normal gravity (for unification). The cells

were first treated in the “micro-gravity” conditions and then treated with various differentiation cocktails under normal gravity conditions. In the previous study, we found that most of the BMSCs did not differentiate into the corresponding cells when they were induced in the SMG condition, but the stem cell surface markers were up-regulated (Yuge et al. 2011). We suspected that SMG condition was helpful for maintaining the undifferentiated state of BMSCs and consequently can improve the induction efficiency of BMSCs in the NG condition. This may be another reason of the SMG enhancing multipotential differentiation capacity of BMSCs. However, more experimental studies are needed to confirm this in our future work.

We demonstrated that the morphology of BMSCs was changed under the SMG condition and thus had larger capability to differentiate to other cell types. This result further demonstrated that there was some interplay between the geometric cues of the cells and their functions (Kilian et al. 2010). While previous report illustrated that SMG was helpful to expand stem cell populations in vitro (Yuge et al. 2006), our work further demonstrated that SMG enabled cells to maintain pluripotency and therefore enhance multipotential differentiation capacity.

Conclusion

In this study, we demonstrated that BMSCs exposed to microgravity became morphologically modified from a spindle to a round shape. This subsequently increased the differentiation potential of BMSCs through cytoskeletal changes and up-regulation in the multipotential mark OCT4. We also highlighted 72 h as the most appropriate SMG duration for enhancing the pluripotency of BMSCs. Therefore, in the light of not inducing apoptosis, SMG can be expected to provide a novel method for obtaining higher-quality stem cells with an improved capacity for differentiation and self-renewal. This suggests great potential for use in clinical applications and developmental biology.

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