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The effects and mechanisms of clinorotation on proliferation and differentiation in bone marrow mesenchymal stem cells

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ABSTRACT

Data from human and rodent studies have demonstrated that microgravity induces observed bone loss in real spaceflight or simulated experiments. The decrease of bone formation and block of maturation may play important roles in bone loss induced by microgravity. The aim of this study was to investigate the changes of proliferation and differentiation in bone marrow mesenchymal stem cells (BMSCs) induced by simulated microgravity and the mechanisms underlying it. We report here that clinorotation, a simulated model of microgravity, decreased proliferation and differentiation in BMSCs after exposure to 48 h simulated microgravity. The inhibited proliferation are related with blocking the cell cycle in G2/M and enhancing the apoptosis. While alterations of the osteoblast differentiation due to the decreased SATB2 expression induced by simulated microgravity in BMSCs.

1. Introduction

Data from real spaceflight or simulated experiments have demonstrated that exposure to microgravity results in osteopenia of approximately 1%–2% per month [1–3], particularly of weight-bearing bone [4,5]. Osteopenia is character as the loss of bone mass and bone mineral, declines in bone mechanical function and negative calcium balance. Furthermore, the progressive bone loss not only was occurred at very early during their spaceflight, but also its recovery required more than 3–4 times longer than periods of mission [6,7]. Thus the deleterious effects induced by microgravity may seriously affect the health and performance of astronaut and seem to be a main biomedical obstacle of long-term manned space missions [8,9]. Even though numerous studies have reported that the effect of microgravity on bone volume, the underlying mechanisms are not well understood , not to mention the countermeasures.

As we all known, process of bone dynamic remodeling depends on an equilibrium between the bone formation and bone absorption in normal bone. It has been shown that microgravity environment break the balance between them, which may count for the bone loss [10–12]. Despite numerous studies elucidating the underlying mechanisms, many aspects still remain unclear. The decrease of bone formation and block of maturation appear to be the main reasons for bone loss induced by microgravity. The effects of microgravity on osteoblast alterations have been widely reported, including the reduced proliferation and activity, decreased osteoblastic function and inhibition of responsiveness to bone related factors, which are considered to the observed reduction in bone formation during spaceflight [13]. However, changes of osteoblast can not fully explain the observed bone loss. Recent works suggest that inhibited differentiation of osteoprogenitor cells into mature osteoblasts may play important roles in bone loss induced by microgravity [14,15].

Bone marrow mesenchymal stem cells (BMSCs) have the capability of self-renewal and differentiation into multifunctional cell including osteoblasts, chondrocytes, adipocytes and other mesodermal cell types under certain inducing conditions. When required, BMSCs can be activated, differentiated into osteoblasts

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and produced active substances for bone remodeling and repair [16]. Therefore, changes of BMSCs function play crucial roles in maintaining bone homeostasis. Given their physiological functions in regulating bone remodeling, BMSCs response to microgravity has become great interest of investigation. However, the effects of microgravity on BMSCs function and the possible mechanisms underlying it remain to be determined. The present study was designed to investigate whether the proliferation and osteogenic potential could be changed as a result of simulated microgravity in BMSCs and further to explore the molecular mechanisms underlying it.

2. Materials and methods

2.1. Cell culture

The C3H10T1/2 cell lines were obtained from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China), and cultured in gelatin-coated 25-cm^2 flasks in MEM medium (Gibco BRL, Shanghai, China) containing 10% fetal bovine serum (FBS), 100 units/mL penicillin and 100 mg/mL streptomycin under normal cell culture conditions (37 °C and 5% CO_2).

2.2. Clinorotation to simulate microgravity

Due to the limitation of real spaceflight, most of experiments for the biological effects of microgravity are conducted in ground-based analogs. The clinostat is one of ground-based tools at cellular responses to simulated microgravity [17,18]. Details and application of the method were as described previously [19,20]. Briefly, C3H10T1/2 were seeded at a total of 1×10^5 cells on 2.5 cm × 3.0 cm coverslips in 6-well plates and cultured at 37 °C in 5% CO_2 for 24 h. Then the coverslips were transferred into the fixture of the chambers which were subsequently filled completely with fresh MEM with 10% FBS and ensured no presence of air bubbles. The chambers were fixed at the clinostat and simulated microgravity (short for SMG) was achieved by rotation around the horizontal axis at 30 rpm for 48 h in an incubator at 37 °C. Meanwhile, cells cultured without rotation were designates a normal 1-G control (short for CON). The differentiation of C3H10T1/2 is induced by administration of 300 ng/ml BMP2.

2.3. RNA extraction and RT-PCR analyses

Reverse transcription-polymerase chain reaction (RT-PCR) was performed with total RNA which was extracted from cultured C3H10T1/2 by Trizol reagent according to the manufacturer's instructions. The first-strand cDNA was synthesized from 500 ng of total RNA using a reverse transcription kit (Takara, Japan). The forward and reverse primer sequences used to amplify the target genes are as follows: ALP, 5′-CAGACCCTCCCCACGAGT-3′, 5′-GGACCTGAGCGTTGGTGTTA-3′ and GAPDH, 5′-GGAAATGAGA-GAGGCCCAGC-3′, 5′-TACGGCCAAATCCGTTCACA-3′. PCR was performed with an initial activation step at 94 °C for 3 min followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 50 s, extension at 72 °C for 7 min. The amplified PCR products were separated by a 2% PAGE and visualized with ethidium bromide under UV light illumination. The intensity of each band was quantified by the Image-J software and relative changes were evaluated by normalized to GAPDH level.

2.4. Construction of SATB2 overexpression vector and transfections

The sense and antisense primers for the full-length coding sequence (CDS) region for SATB2 (GenBank accession no. NM_138673.2) were 5′- atcgGAATTCatggcaaggagcaagcttctcct-3′ and 50- atcgGTCGACggacctcagtgccccaagagggt -30, respectively. The amplified SATB2 product was obtained by RT-PCR from total RNA of C3H10T1/2, then digested and cloned into pEGFP-N2 vector by restriction endonuclease EcoR I, Sal I and T4 Ligase to construct an overexpression plasmid (pEGFP-N2-SATB2). PCR, restriction enzyme, western blot and sequence analysis were used to confirm the recombinant plasmid.

C3H10T1/2 cultured on coverslips at 60–70% confluence, and then were transfected with 3 mg of pEGFP-N2-SATB2 construct by Lipofectamine 2000 Plus (Invitrogen, CA) following the manufacturer's protocol. Cells were exposed to clinorotation for 48 h after 24 h transfection. Vehicle pEGFP-N2 treated cells served as a control.

2.5. Western blot analysis

C3H10T1/2 were washed 3 times with cold PBS and then were lysed with the cell lysis buffer (Cell Signaling Technology, MA), followed by centrifugation at 12,000 g for 10 min at 4 °C. The whole cell protein extract (50 μg/lane) was separated by SDS-PAGE and transferred to a PVDF membrane (Millipore, MA). After blocking with 5% skim milk in TBS containing 0.1% Tween 20 (TBST) for 2 h at the room temperature, the membranes were incubated overnight at 4 °C with the primary antibodies against PCNA (1:1000), Cbfa1 (1:1000), SATB2 (1:1000), Hoxa2 (1:1000), and GAPDH (1:500), respectively. The immunoblots were incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (Santa Cruz Biotech, CA) for 1 h at room temperature and then detected using enhanced ECL detection kit (Amersham, UK) after being washed with TBST. The intensity of each band was quantified by Image-J software and the density ratio represents the relative intensity of each band against those of controls in each experiment.

2.6. Alkaline phosphatase (ALP) assays

ALP can be considered as an early marker of osteoblast differentiation. ALP enzyme activity was evaluated with an ALP Assay Kit according to the manufacturer's protocol. Briefly, the lysed cells were added into Reaction Buffer containing 0.75M p-Nitrophenyl Phosphate, and then mixed well and incubated for 30 min at 37 °C. The absorbance was read at 405 nm.

2.7. Flow cytometry analysis of cell cycle detection and the apoptotic rate

For cell cycle detection and DNA content analysis, C3H10T1/2 were harvested by 0.25% trypsin and fixed in ice-cold 70% ethanol. And then cells were stained with 50 μg/mL propidium iodide (PI) solution containing 10 μg/mL RNaseA (Sigma) and 0.01% ethylenediaminetetraacetic acid. The percentage of cell for each phase including G1, S and G2/M were determined and analyzed by BD FACSDiva™ Software.

For cell apoptosis detection, cells were treated with the same step of above. Cells were stained with the 5% Annexin V-FITC and 5% PI for 15 min at room temperature in the dark. The results were obtained by Flow Cytometry (BD FACSARIA III) and analyzed with BD FACSDiva™ Software.

2.8. Chemicals and reagents

Chemicals and reagents included DMSO, Tris–HCl, PBS, EDTA. Lipofectamine 2000 Plus and Trizol reagent were from Invitrogen Corp. Taq-polymerase for long PCR, restriction enzymes, and the DNA ligation kit were from Takara. GAPDH antibody were from BD

Biosciences (San Jose, CA). PCNA, Cbfa1, SATB2 and Hoxa2 antibody were purchased from ABcom (Cambridge, UK).

2.9. Statistical analysis

The data are shown as mean ± SEM. All the experiments were performed 3 times with similar results for each time. Differences between groups were analyzed by repeated-measures one-way ANOVA and Fisher's PLSD. Statistical significance was accepted at $p < 0.05$.

3. Result

3.1. Clinorotation blocks cell cycle in G2M

In order to investigate the influence of simulated microgravity on the cell cycle, flow cytometry analysis were performed. C3H10T1/2 were cultured in simulated microgravity and 1G conditions, respectively. As shown in Fig. 1, the proportion of cells decreased 0.04-fold in G1 phase, however there were no significant difference between the two groups. While the proportion of cell markedly decreased 0.22-fold in S phase and increased 1.0-fold in G2 phase after 48 h clinorotation in comparison with that in the control. Data present here show that clinorotation for 48 h arrests the cell cycles of in G2/M phases.

We further analyzed the apoptosis of C3H10T1/2 after 48 h clinorotation by flow cytometry analysis. As presented in Fig. 2, compared with the control, early apoptosis increased about 3.35-fold and late death enhanced about 0.44-fold, suggesting that clinorotation for 48 h significantly enhances apoptosis of C3H10T1/2.

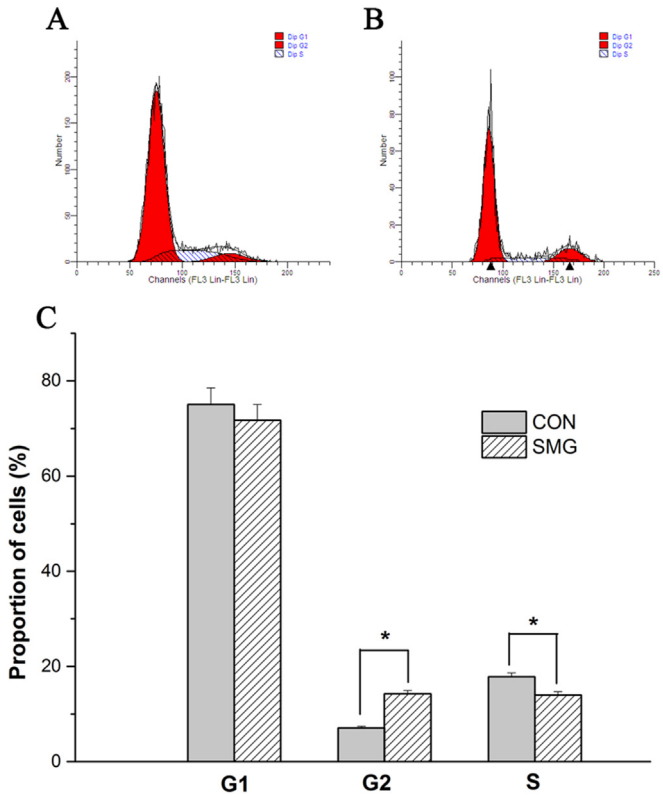

Fig. 1. Clinorotation for 48 h arrested the cell cycle of C3H10T1/2. Cell-cycle histograms in C3H10T1/2 of control (A) and simulated microgravity (B). The lower panel shows the cycle changes of C3H10T1/2 after 48 h clinorotation (C). $^*P < 0.05$, compared with control group, n = 3.

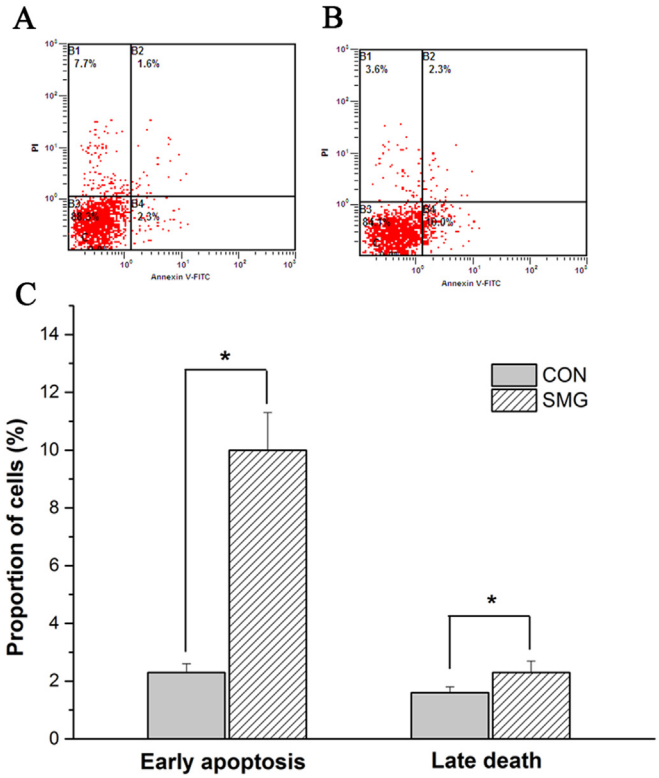

Fig. 2. Clinorotation for 48 h enhanced the apoptosis in C3H10T1/2. Apoptosis of histograms in C3H10T1/2 of control (A) and simulated microgravity (B). The lower panel shows the early apoptosis and late death of C3H10T1/2 after 48 h clinorotation (C). $^*P < 0.05$, compared with control group, n = 3.

3.2. Clinorotation inhibit C3H10T1/2 proliferation and differentiation

Proliferating cell nuclear antigen (PCNA) is a well-accepted marker of proliferation given its role in replication. We next explored the effect of simulated microgravity on the proliferation of C3H10T1/2 by measuring the level of PCNA protein. As presented in Fig. 3A and B, the expression of PCNA protein was evidently attenuated with exposure to clinorotation for 48 h, suggesting that simulated microgravity may cause an inhibition of C3H10T1/2 proliferation.

Alkaline phosphatase (ALP) and Cbfa1 has long been considered as an indicator for osteoblast differentiation. To identify the effects of simulated microgravity on changes of BMSCs differentiation, we next investigated the osteogenic potentials of BMSCs by detecting ALP activity and Cbfa1 levels. C3H10T1/2 were cultured in 300 ng/ml BMP2 medium with or without 48 h clinorotation. RT-PCR analysis revealed that the expressions of ALP mRNA were significantly decreased (Fig. 3C and D). As seen in Fig. 3E, consistent with the results of ALP mRNA expression, ALP activity suppressed significantly in clinorotation group than that in control. The level of Cbfa1 protein also showed a markedly decrease for 48 h simulated microgravity (Fig. 3F and G). Data here indicate that BMSCs differentiation is inhibited during 48 h clinorotation.

3.3. Changes of SATB2/Hoxa2 contribute to the inhibition of BMSCs differentiation

There are many pathways involved in BMSCs differentiation. It has been shown that the levels of SATB2 and its downstream target Hoxa2 are the critical molecules related to osteogenesis. To

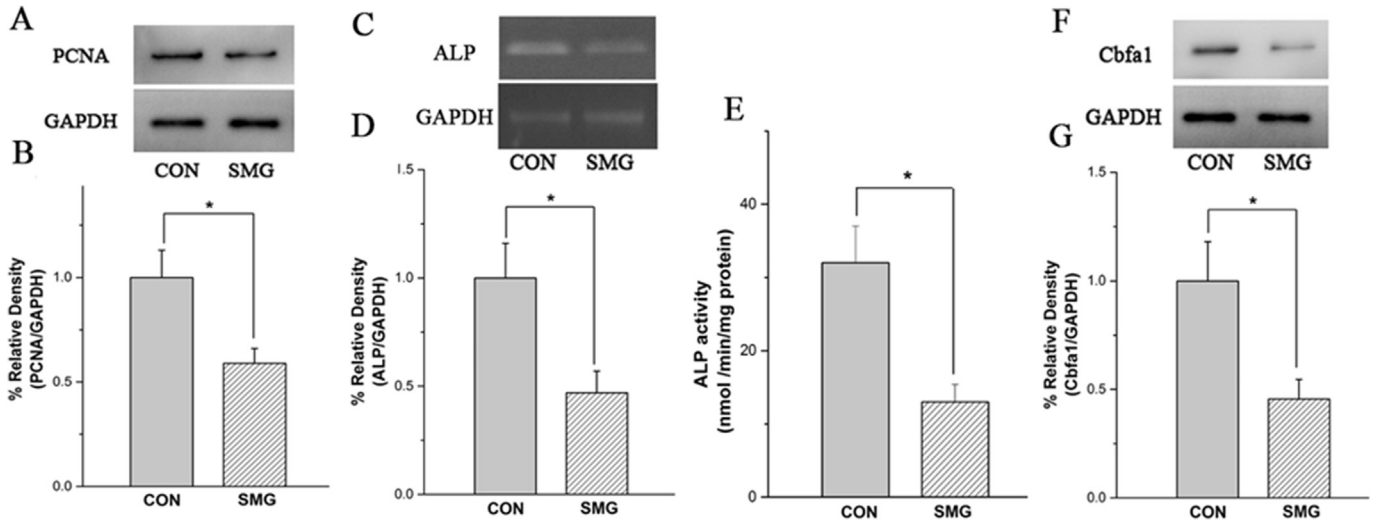

Fig. 3. Clinorotation for 48 h inhibited proliferation and differentiation in C3H10T1/2. A, Effects of 48 h clinorotation on the expression of PCNA protein; B, The relative level of PCNA protein was determined after densitometric scanning of PCNA and GAPDH bands. C, Effects of 48 h clinorotation on the expression of ALP mRNA; D, The relative level of ALP mRNA was determined after densitometric scanning of ALP and GAPDH bands. E, Effects of 48 h clinorotation on the ALP activity. F, Effects of 48 h clinorotation on the expression of Cbfa1 protein; G, The relative level of Cbfa1 protein was determined after densitometric scanning of Cbfa1 and GAPDH bands. The relative level of PCNA, Cbfa1 protein, ALP mRNA and activity normalized as fold over control is shown under each band. $^{*}P < 0.05$, compared with control group, n = 3.

understand the influences of simulated microgravity on the expression of SATB2 and Hoxa2, western bolt were performed with the presence of 300 ng/ml BMP2 for 48 h simulated microgravity. As seen in Fig. 4A and B, the level of SATB2 protein was markedly decreased in clinorotation group than that in control. While higher levels of Hoxa2, which is repressed regulated by SATB2, were detected in C3H10T1/2 after 48 h clinorotation (Fig. 4A and C).

To explore the role of SATB2 in regulating osteoblast differentiation of C3H10T1/2 after exposure to simulated microgravity, we further investigated the effects of excessive SATB2 by transfection with pEGFP-N2-SATB2 on the ALP level. C3H10T1/2 were transfected with pEGFP-N2-SATB2, and then cells were treated with clinorotation condition for 48 h in BMP2 medium. The SATB2 expression (Fig. 4D and E) and ALP activity (Fig. 4F) show that the levels of ALP in the clinorotation cells were significantly enhanced after overexpression of SATB2 in comparison to that without overexpression. Data presented here indicate that the inhibition of osteoblast differentiation induced by simulated microgravity can be eliminated by overexpression of SATB2 in C3H10T1/2.

4. Discussion

In this study, we demonstrated that simulated microgravity could inhibit the proliferation and differentiation in C3H10T1/2. The changes of proliferation are related with the arrested cell cycle in G2M and enhanced apoptosis. While alterations of the osteoblast differentiation due to the decreased SATB2 expression induced by simulated microgravity in C3H10T1/2.

Data from human and rodent studies have revealed that the absence or the reduction of gravity induce almost all physiologic

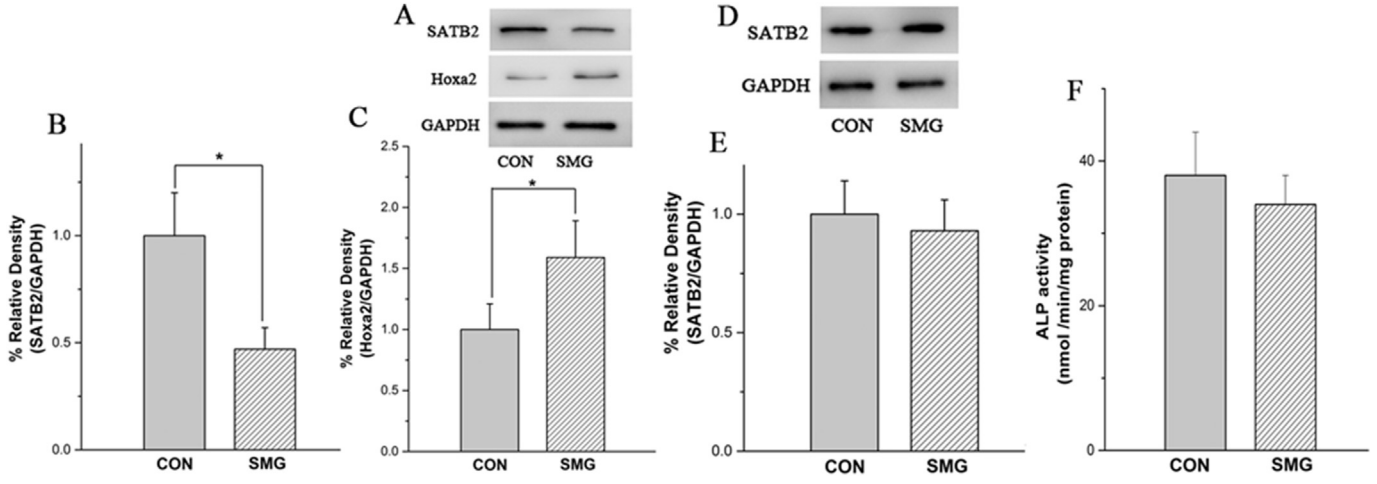

Fig. 4. SATB2 is involved in the inhibition of differentiation in C3H10T1/2 exposed to 48 h clinorotation. A, Effects of 48 h clinorotation on the expression of SATB2 and Hoxa2 protein; B, The relative level of SATB2 protein was determined after densitometric scanning of SATB2 and GAPDH bands. C, The relative level of Hoxa2 protein was determined after densitometric scanning of Hoxa2 and GAPDH bands. D, Effects of 48 h clinorotation on the expression of SATB2 after transfecting with pEGFP-N2-SATB2. E, The relative level of SATB2 protein was determined after densitometric scanning of SATB2 and GAPDH bands. F, Effects of 48 h clinorotation on the ALP activity after transfecting with pEGFP-N2-SATB2. The relative level of SATB2, Hoxa2 and ALP activity normalized as fold over control is shown under each band. $^{*}P < 0.05$, compared with control group, n = 3.

systems changes, such as bone loss, muscle atrophy and some other space adaptation syndromes [21]. These changes are some of the main obstacles to long term expeditions. Although documents have been accumulated for many decades, the bone loss induced by microgravity remains to be solved for astronauts and receives general concern by researchers. Even though a series of in-flight, postflight and ground-based investigations have been made [22], little is known about the basic mechanism underlying it.

Literatures indicated that the unbalanced alterations of osteoblasts, osteocytes, and osteoclasts may contribute to the bone loss induced by microgravity [23]. Furthermore it has been widely accepted that the decreased bone formation appeared to be the main cause of bone loss during spaceflight [14,24]. Therefore reduced activity of functional cells and lack of new osteoblast from progenitors may play important role in the progress [4,25]. BMSCs as a major source of osteoblasts, which can circulate to bone surfaces, commit to proliferation and differentiation into functional cells for bone remodeling and repair [16]. It has confirmed that BMSCs are sensitive to microgravity and undergo several changes such as morphology, proliferation, gene expression, and functional behavior [26,27]. However, to our knowledge, the mechanisms underlying the effects of microgravity on osteogenic function alterations are not clearly understood. Here we confirmed our hypothesis by observing a reduction of proliferation by expression of PCNA in C3H10T1/2 after 48 h clinorotation in respect to controls. The results about the inhibition of proliferation induced by microgravity are in agreement with previous researches [28,29]. To clarify the reason for the reduction of proliferation after exposure to clinorotation, cell cycle and apoptosis analysis were performed. Data reported here showed the arrested cell cycle at G2/M phase and enhanced apoptosis may contribute to the proliferation inhibition.

In addition to the proliferation of BMSCs, the changes of differentiation were also explored after exposure to microgravity. Our current works showed that clinorotation for 48 h inhibited the BMP2 induced differentiation in C3H10T1/2. Our reports on the decreased differentiation were in accordance with those of other studies [15,30,31]. Whereas there were some conflicting literatures about differentiated behavior of MSCs, which should be ascribed to the differences of experimental conditions, i.e., 2D or 3D microgravity simulator used, different duration and the cell lines utilized [32,33]. Moreover, it has been reported that changes of cytoskeleton, extracellular matrix, cytokine/cytokine receptor interactions, MAPK cascade et al. may respond to the decreased differentiation induced by microgravity [26–28,34]. However, the possible mechanisms underlying it are not well understood. Recently, several evidences indicated that transcription factors may be involved in the regulation of osteoblast differentiation. Cbfa1, as an osteoblast-specific transcriptional factor, has been proved to be played critical roles in both the initiation and the regulation of osteoblast differentiation and bone formation [14]. The expression of Cbfa1 is decreased under real and simulated microgravity. Our reports about the inhibition of Cbfa1 in BMSCs are in agreement with them. It has been reported that Cbfa1 can be regulated by SATB2, which represses Hoxa2 expression in the osteoblast lineage. SATB2 is a nuclear matrix-associated transcription factor, which can promote osteoblast differentiation, maturation and bone regeneration [35]. However, there was no report about whether SATB2 participate in the changes of differentiation in BMSCs induced by simulated microgravity. The present study indicated that the level of SATB2 was inhibited with the presence of BMP2 by 48 h simulated microgravity. To clarify the involvement of SATB2 in the regulation of BMSCs differentiation, we next detected the effects of transfection with pEGFP-N2-SATB2 on the ALP level subjects to 48 h clinorotation. Our results showed that SATB2 overexpression can eliminate the inhibition of BMSCs differentiation induced by simulated microgravity. Collectively, these results suggested that the osteoblast differentiation in clinorotation was, at least in part, via the suppression of SATB2 signaling. Furthermore, Hoxa2, which is suppressed by SATB2, acts as a regulator in antagonizing bone formation [36]. Our data in this study showed that clinorotation enhanced the expression of Hoxa2, consistent with the results about inhibition of SATB2 induced by simulated microgravity.

In conclusion, inhibition of SATB2 was involved in the pathway of osteoblast differentiation suppression by clinorotation. To our knowledge, this is the first report demonstrating the relationship of inhibition of SATB2 signaling and osteoblast differentiation suppression under conditions of simulated microgravity in BMSCs in vitro. Interestingly, SATB2, Hoxa2 and Cbfa1 can be interacted with each other and synergizes functionally in osteoblast differentiation and bone formation. The mechanisms accounting for alteration of SATB2, Hoxa2 and Cbfa1 induced by simulated microgravity and a more detailed analysis of the interaction involved in this response are under further investigation.

Conflict of interest

None.

Acknowledgments

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