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Gravitational environment produced by a superconducting magnet affects osteoblast morphology and functions

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Abstract

The aims of this study are to investigate the effects of gravitational environment produced by a superconducting magnet on osteoblast morphology, proliferation and adhesion. A superconducting magnet which can produce large gradient high magnetic field (LGHMF) and provide three apparent gravity levels (0g, 1g and 2g) was employed to simulate space gravity environment. The effects of LGHMF on osteoblast morphology, proliferation, adhesion and the gene expression of fibronectin and collagen I were detected by scanning electron microscopy, immunocytochemistry, adhesion assays and real time PCR, respectively, after exposure of osteoblasts to LGHMF for 24 h. Osteoblast morphology was affected by LGHMF (0g, 1g and 2g) and the most evident morphology alteration was observed at 0g condition. Proliferative abilities of MC3T3 and MG-63 cell were affected under LGHMF (0g, 1g and 2g) conditions compared to control condition. The adhesive abilities of MC3T3 and MG-63 cells to extracellular matrix (ECM) proteins (fibronectin, laminin, collagen IV) were also affected by LGHMF (0g, 1g and 2g), moreover, the effects of LGHMF on osteoblast adhesion to different ECM proteins were different. Fibronectin gene expression in MG63 cells under zero gravity condition was increased significantly compared to other conditions. Collagen I gene expression in MG-63 and MC3T3 cells was altered by both magnetic field and altered gravity. The study indicates that the superconducting magnet which can produce LGHMF may be a novel ground-based space gravity simulator and can be used for biological experiment at cellular level.

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Keywords: Large gradient high magnetic field; Simulated weightlessness; Morphology; Proliferation; Adhesion

1. Introduction

Gravity is a universal force and influences everything in nature. Any slight fluctuations and variations in this force can have significant impact on the structure and functions of organisms. The alterations of metabolism

and utilization of various nutrients have been observed during both real and simulated space flight. For astronauts during long term space flight, one of the most important medical risks is bone loss [1,2]. Although human adaptation to the microgravity environment allows astronauts to maintain overall function, the musculoskeletal system rapidly degrades once the force of gravity is disappeared. Bone loss is the most common physiological alteration observed in astronauts performing long-term space flights. Several studies have reported that bone loss approximates 1–2% per month during flights

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Nomenclature

LGHMF	large gradient high magnetic field	PBS	phosphate-buffered saline
NSBRI	National Space Biomedical Research Institute	CPR	critical path roadmap
NASA	National Aeronautics and Space Administration	FN	fibronectin
FCS	fetal calf serum	LN	laminin
FAK	focal adhesion kinase	Collagen IV	Col IV
		ECM	extracellular matrix

lasting 4–6 months [3]. However, some researchers have demonstrated that the range of bone loss in Mir astronauts varies from 0% to 24% per month when measured in cancellous and cortical bone in the tibia (National Space Biomedical Research Institute, NSBRI, Bone Loss Team Strategic Plan). Bone loss of this magnitude has been observed in human bed rest studies and in individuals following spinal cord injury. The bone loss team strategic plan established by the NSBRI aims to study the mechanism of bone loss and to develop effective countermeasures to bone loss. The National Aeronautics and Space Administration (NASA) has also carried out many studies on the mechanism of bone loss and countermeasures.

At present, weightlessness, identical to between 10^{-2} and 10^{-4} times gravity (g), can be obtained in aircraft under freefall conditions [4] and the effects of weightlessness on physical, chemical and biological systems are usually studied. At present, ground-based methods such as clinorotation [5,6] and parabolic aircraft [7] have been employed to simulate space gravity environment or effects. The effects of gravity and mechanical forces at the cellular and molecular level have been studied by utilizing ground-based models. Nevertheless, the cellular and molecular mechanisms are still poorly understood.

Following the development of superconducting magnets, a special designed large gradient high magnet has been developed, which can be used as a new method of simulated weightlessness. The technique produces a simulated hypo-gravity and hyper-4 environment for diamagnetic materials, such as biological macromolecules, cells, tissues and even small model animal [8,9]. Some research results have been reported in this field of large gradient high magnet as a gravitational condition simulator [10]. Valles [10,11] advocates the use of the magnetically adjustable gravity simulator (MAGS). Frogs [12] and frog embryos [13], cell cultures of plants [14] have been levitated in earth's

gravity with this method. Glade et al. [15] reported that weightlessness resulted in microtubule self-organization by using magnetic levitation equipment.

In order to elucidate the biological effects of magnetic-gravitational environment and approve the usefulness of large gradient high magnetic field (LGHMF), in this study, a superconducting magnet (JMTA-16T50MF) which can provide three apparent gravity levels (0g, 1g and 2g) was employed to study the effects of this special gravitational environment on osteoblast MG-63 and MC3T3 cell morphology, proliferation and adhesion. Findings of gravity-induced alterations in these characteristics at a cellular level may provide a mechanistic foundation to improve our understanding of the physiological effects observed during space flight.

2. Materials and methods

2.1. Materials

Tissue culture plate and dish and microlon ELISA strips were purchased from Nunc Inc Roskilde Denmark. Fibronectin, Laminin and Collagen IV were got from BD Biosciences California, America. Anti-Ki-67 and SP immunohistochemistry kit anit-Ki-67 antibody, HRP-IgG and Diaminobenzidine (DAB) were got from Zhongshan Bio-Tech Co., Ltd, Guangzhou, China. Hoechst 33258 was purchased from Invitrogen Corporation. Cell counting kit-8 (CCK-8) was from DojinDo Molecular Technologies, Inc. MEM culture medium, CO₂ independent medium and fetal calf serum (FCS) were purchased from GIBCO™ Invitrogen Corporation and goat anti-rabbit IgG-FITC was from KPL Company, America. RT-PCR reaction kit and TaKaRa SYBR® Premix Ex Taq™ were from TaKaRa, Japan and Trizol was from Invitrogen Corporation. Primers were designed and synthesized by Shanghai Sangon Biological Engineering Technology & Service Co.,

Ltd. Haematoxylin and eosin were purchased from Sino-American Biotechnology Company, Xi'an, China. Permout was from Beyotime Institute of Biotechnology, Haimen, China. Other chemicals used were of reagent grade. The scanning electron microscope (S-3400 N) was purchased from Hitachi High Technologies America Inc. and fluorescence microscope was purchased from Nikon Co. Microplate reader (model 680), Icyler and peltier thermal cyler were purchased from Bio-Rad, California, America FACScalibur flow cytometer was purchased from BD Biosciences.

2.2. Cell culture

The human osteosarcoma cell line MG-63 was purchased from the Cell Collection Center of Shanghai, which obtained the cells originally from American type culture collection (Manassas, VA, USA). Osteoblast MC3T3 cells were purchased from the Cell Bank of the Chinese Academy of Medical Sciences. Cells were grown in complete MEM culture medium supplemented with 2 mM L-glutamine, 1.5 g/L sodium bicarbonate, 0.1 mM non-essential amino acids, 1 mM sodium pyruvate and 10% FCS at 37 °C, 5% CO₂ in a humidified atmosphere. For culturing of the cells in the superconducting magnet, MEM was changed to CO₂ independent medium with 10% FCS and the tissue culture dishes were sealed with parafilm. GIBCO CO₂ independent medium contains a unique phosphate buffering system. Additionally, it has been formulated with components that enhance cellular production and utilization of CO₂ such that an exogenous source of CO₂ is not required for the maintenance of CO₂ dependent cellular functions. A circulating water-bath was used for temperature control during the experiments. The temperature control system mainly includes water-bath pump and related channel system and the temperature range is 37 ± 0.5 °C.

2.3. Superconducting magnet

A superconducting magnet (JMTA-16T50MF) which can provide LGHMF was made by Japan Superconductor Technology Inc. (JASTEC) dependent on the authors' design plan. Maximum magnetic induction intensity (B) is 16.2 tesla (T) and the product of $B \cdot dB/dz$ is 1100 T² m⁻¹. The superconducting magnet can provide three gravity levels (0g, 1g and 2g) and keep stable more than one year (Fig. 1a and b). The magnetic field intensity of three gravity levels (0g, 1g and 2g) was 12, 16 and 12 T, respectively. Four groups were designed in this study, namely, 1g

group (normal gravity, 16 T), control group (normal gravity, geomagnetic field), 2g group (2 gravity, 12 T) and 0g group (zero gravity, 12 T). We have developed many kinds of other experiment equipment matching with the superconducting magnet, which could be used to life sciences including utilizing in structure biology, cell biology, microbiology and development biology. In order to keep the temperature at 37 °C for cell culture in the bore of superconducting magnet, the temperature control system was designed and made by authors. In order to deliver the experimental samples and to accurately detect the gravity levels, an adjustable elevating platform and a gravitational detection system were also developed by the authors (Fig. 1c–g). The gravitational detection system contains pressure sensors.

2.4. Scanning electron microscopy (SEM)

After osteoblast MG-63 and MC3T3 cells were cultured on coverslips in 35 mm petri dishes for 20 h, the petri dishes were, respectively, placed into the bore of LGHMF (0g, 1g and 2g) by an adjustable elevating platform and the gravitational detection system and the cells were continually cultured in LGHMF at 37 °C for 24 h. The coverslips were removed from superconducting magnet, washed twice with phosphate-buffered saline (PBS), pH 7.4 and fixed by 3% glutaraldehyde in 0.1 MPBS, pH 7.4, at 4 °C, overnight. For the SEM, the cells were dehydrated in vacuum for 1 h. The coverslips with the planted cells were mounted with double-sided adhesive tape on aluminium stubs and coated with a 20 nm gold layer. The cells were examined in an S-3400N SEM operated at an accelerating voltage of 10 kV.

2.5. Haematoxylin–eosin (HE) staining

After being cultured for 24 h in room temperature bore of superconducting magnet, the cells were removed from superconducting magnet, washed twice with PBS, pH 7.4 and fixed in 95% ethanol. The coverslips were placed in 20% haematoxylin and 0.5% eosin for 5 min, respectively, and washed by tap water. Then cells were dehydrated by an ethanol gradient and mounted by Permout (Beyotime Institute of Biotechnology, Haimen, China). The slides mounted with Permout were then observed by microscope and photos were taken.

2.6. Hoechst staining

After being cultured for 24 h in the bore of the superconducting magnet, the coverslips planted with cells

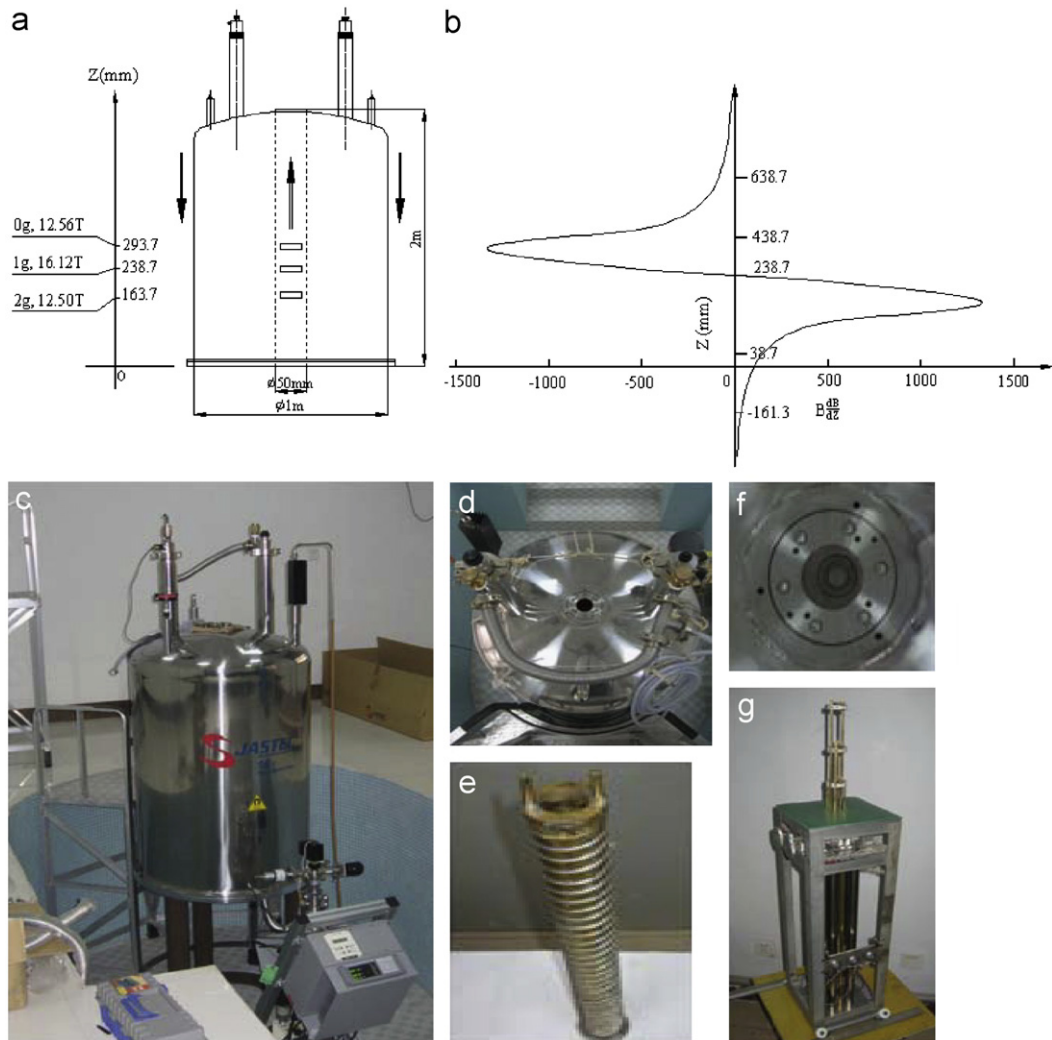


Fig. 1. The gravity level positions (a), magnetic field distribution of large gradient high magnetic field (b) and other equipment matching with biological experiments (c–g). (a) Three different gravity level positions (z axle), apparent gravity (0g, 1g and 2g) and magnetic induction (12, 16 and 12 T) in the bore of superconducting magnet. (b) Magnetic field distribution of the large gradient high magnetic field. (c) Superconducting magnet. (d) The top of superconducting magnet. (e) Water-jacket for temperature control. (f) Top of room temperature bore of superconducting magnet. (g) Adjustable elevating platform.

were washed twice with pre-warmed PBS, pH 7.4 and stained with hoechst 33258 (1 $\mu\text{g}/\text{ml}$) for 5 min and observed by fluorescence microscope.

2.7. Cell proliferation assays

One hundred μl cell suspension ($1 \times 10^5 \text{ ml}^{-1}$) of MG-63 and MC3T3 cells were planted to the microlon ELISA strips and cultured overnight at 37 °C, 5% CO_2 . Then microlon ELISA strips with the planted cells were placed into three gravity levels (0g, 1g and 2g) in the superconducting magnet by an adjustable elevating plat-

form and a gravitational detection system and the cells were continually cultured for 24 h at 37 °C in LGHMF (0g, 1g and 2g). After the microlon ELISA strips were removed from LGHMF, 10 μl cell counting kit was added to the microlon ELISA strips for 2 h and the absorbance was detected by microplate reader at 405 nm. The control group absorbance was subtracted from experimental absorbance and then divided by the control group absorbance, by which the inhibitory rate was calculated. The cell number of every group was also counted by hemocytometer after osteoblasts had been cultured in LGHMF at three gravity levels for 24 h.

Table 1

The primer sequences, annealing temperature, amplification length and GeneBank accession number of collagen I and fibronectin

Gene name	Primer sequences (5' – 3')	Annealing temperature (°C)	Amplification length (bp)	Genebank accession number
<i>Homo sapiens</i>	F: TTC CTG CGC CTG ATG TCC	55	413	NM_000088.2
Collagen I	R: TTT GGG TTG CTT GTC TGT TT			
<i>Homo sapiens</i>	F: AAG GTT CGG GAA GAG GTTGT	59	402	212482.1
Fibronectin	R: TGG CAC CGA GAT ATT CCT TC			
<i>Mus musculus</i>	F: TGTTCTGGTTCTCAGGGTAG	62	253	007743.2
Collagen I	R: TTGTCGTAGCAGGGTCTTTTC			
<i>Mus musculus</i>	F: CCC CAG TGA TGT TAG CAG ACC	61	345	NM_010233.1
Fibronectin	R: GAT GGC AAA AGA AAG CAG AGG			

2.8. Immunocytochemistry staining

After being cultured for 24 h in room temperature bore of superconducting magnet, the cells were fixed by 95% ethanol for 15 min at room temperature. The immunocytochemistry staining assay was according to the supplier's instruction. Briefly, anti-Ki-67 (10 µg/ml) was added at 4 °C, incubated overnight and washed three times with PBS, pH 7.4. Sheep anti-mouse IgG-HRP (1:50) was added to the coverslips for 1.5 h at room temperature. After washing for three times, the cells were stained with DAB for approximately 8–12 min. The cells were washed three times with PBS, stained with haematoxylin and eosin, mounted by Permount and observed by microscope. The percentage of positive cells was calculated by counting the number of positive cells among 100 cells.

2.9. Cell adhesion assays

The cell adhesion assay was performed according to the Cell Adhesion Protocol Manual of BD Biosciences (2001, 1st edition, pp. 24–26). Briefly, after being cultured for 24 h in LGHMF, MG-63 and MC3T3 cells (5×10^4) were detached by 0.25% trypsin, respectively, and transferred into in 96-well micro-plates coated with fibronectin, laminin and collagen IV (3 µg per well). The cells were allowed to adhere for 60 min at 37 °C. The wells were washed three times with PBS and the cells were fixed with 4% formaldehyde. The cells were then stained with 0.5% crystal violet in 20% (v/v) methanol/water and viewed under a microscope. The number of the bound cells was estimated by

solubilizing the dye using 0.1 M sodium citrate and the absorbance was read at 490 nm. Triplicate determinations were done at each data point.

2.10. Real time RT-PCR

After MG-63 cells and MC3T3 cells were cultured in LGHMF (0g, 1g and 2g) for 24 h, total RNA was extracted using the Trizol method as recommended by the manufacturer (Invitrogen, carlsbad, CA). Reverse transcription reactions were run with 1 µg of total RNA. Reagents were included in RT-PCR reaction kit (TaKaRa, Japan). RNA was reverse transcribed and processed for PCR reactions according to the protocol provided by the kits. According to the protocol recommended by TaKaRa SYBR® Premix Ex Taq™ (TaKaRa, Japan), real time relative quantitative RT-PCR analysis was performed with SYBRGreen on an MJ Research by using 18 S rRNA as internal control gene for normalization. The extension steps are as follows: 30 s initial denaturation at 95 °C, followed by 44 cycles for 5 s at 94 °C, 20 s at annealing temperature and 15 s at 72 °C. Forward (F) and reverse (R) primer sequences for *Homo sapiens* and *Mus musculus* Collagen I and fibronectin are shown in Table 1. In order to estimate the relative expression of specific mRNA in the sample, the gene-specific PCR signal was normalized to 18 S RNA amplification product in each sample [16].

2.11. Statistical analysis

Statistically significant differences were determined by Prism statistical software (GraphPad Software Inc).

$P < 0.05$ was considered significant in all cases. All data averages or means are accompanied by standard deviations to indicate the amount of variability in the data. In addition, standard errors of the means were also calculated since they are useful in comparing means of different test groups.

3. Results

3.1. The effects of gravitational environment on cellular morphology

Compared to other conditions, in 0g condition the rugosity of MG-63 cell surface was changed into flat shape and MG-63 cells were obviously enlarged. Moreover, the stereo shape of MG-63 cells in 0g and 1g was not distinct. The secretory vesicles in 0g and 1g conditions were evidently decreased compared to control and 2g conditions (Fig. 2a).

The morphological behaviour of MC3T3 cells was similar to that of MG-63 cells. MC3T3 cultured under control conditions appeared spindle-shaped but in LGHMF (0g, 1g, 2g) exhibited a polygonal shape (Fig. 2). Compared to the other conditions, the MC3T3 cells in 0g condition were not plump (Fig. 2) and the secretory vesicles of MC3T3 cells in LGHMF (0g, 1g, 2g) seemed to be reduced compared to control condition (Fig. 2b).

The results of HE staining showed that the size of MG-63 cell nuclei became larger at 1g condition compared to other conditions (Fig. 3, $P < 0.001$). Compared to the control condition, the size of MC3T3 cells nuclei became larger ($P < 0.001$) and the shape of MC3T3 cells became polygonal in LGHMF (0g, 1g, 2g) (Fig. 3).

The results of Hoechst staining showed that under 0g MG-63 cell nuclei had a rounded appearance with a significant larger nuclear size ($P < 0.05$) (Fig. 4). Compared to other conditions, the size of MC3T3 cell nuclei at 2g conditions became significantly smaller ($P < 0.05$) (Fig. 4). The difference of the size of MC3T3 cell nuclei between 0 and 2g conditions has statistical significance ($P < 0.05$).

3.2. The effects of gravitational environment on cell proliferation

The cell counting results showed that the proliferation of MG-63 cells in LGHMF (0g, 1g, 2g) was accelerated compared to that of control condition. Moreover, the difference of MG-63 cell proliferation between 1g condition and control condition has statistical

significance ($P < 0.001$). However, MC3T3 cell proliferation in LGHMF (0g, 1g, 2g) was significantly inhibited compared to that of control condition. The inhibitory rate of MC3T3 cell proliferation was 16.2%, 54.6% and 51.4%, respectively (Fig. 5). A statistical difference of MC3T3 cell proliferation at 0g condition and 1g condition in LGHMF compared to control condition was found ($P < 0.001$).

The expression of Ki-67 in MG-63 cells under 1g and control conditions was positive but under 0g and 2g conditions it was negative. The positive expression rate of Ki-67 in MG-63 cells at (0g, 1g, 2g) and control conditions was, respectively, 4.28%, 68.29%, 2.8% and 72.6%. However, the expression of Ki-67 in MC3T3 cells at all groups was negative.

3.3. The effects of gravitational environment on cell adhesion to ECM proteins

Compared to the control condition, the adhesive abilities of MG-63 cells to fibronectin and collagen IV in 0g and 2g conditions were significantly decreased ($P < 0.01$) and there was no significance between control condition and 1g condition ($P > 0.05$). However, the adhesive abilities of MG-63 cells to laminin at 2g were significantly increased ($P < 0.05$) compared to that of control condition and the differences were not significant between other conditions and control condition ($P > 0.05$) (Fig. 6).

The adhesive abilities of MC3T3 cells to laminin in 2g condition were dramatically increased ($P < 0.05$) compared to that of control condition. Moreover, the difference of the adhesive abilities of MC3T3 cells to laminin between 2g and 0g conditions had statistical significance ($P < 0.05$). The difference in the adhesive abilities of MC3T3 cells to collagen IV in 1g and control conditions had statistical significance ($P < 0.05$) (Fig. 6).

3.4. The effects of gravitational environment on fibronectin and collagen I gene expression

Analysis of relative gene expression data was according to the $2^{-\Delta\Delta CT}$ and real time quantitative PCR method [16]. The differences of gene expression between control condition and LGHMF conditions (0g, 1g and 2g) were quantified relatively by using 18S rRNA as internal control gene to normalize gene signal of target gene. The results showed that fibronectin gene expression in MG-63 cells at 0g condition was significantly increased compared to other conditions ($P < 0.05$, Fig. 7). The differences of fibronectin gene

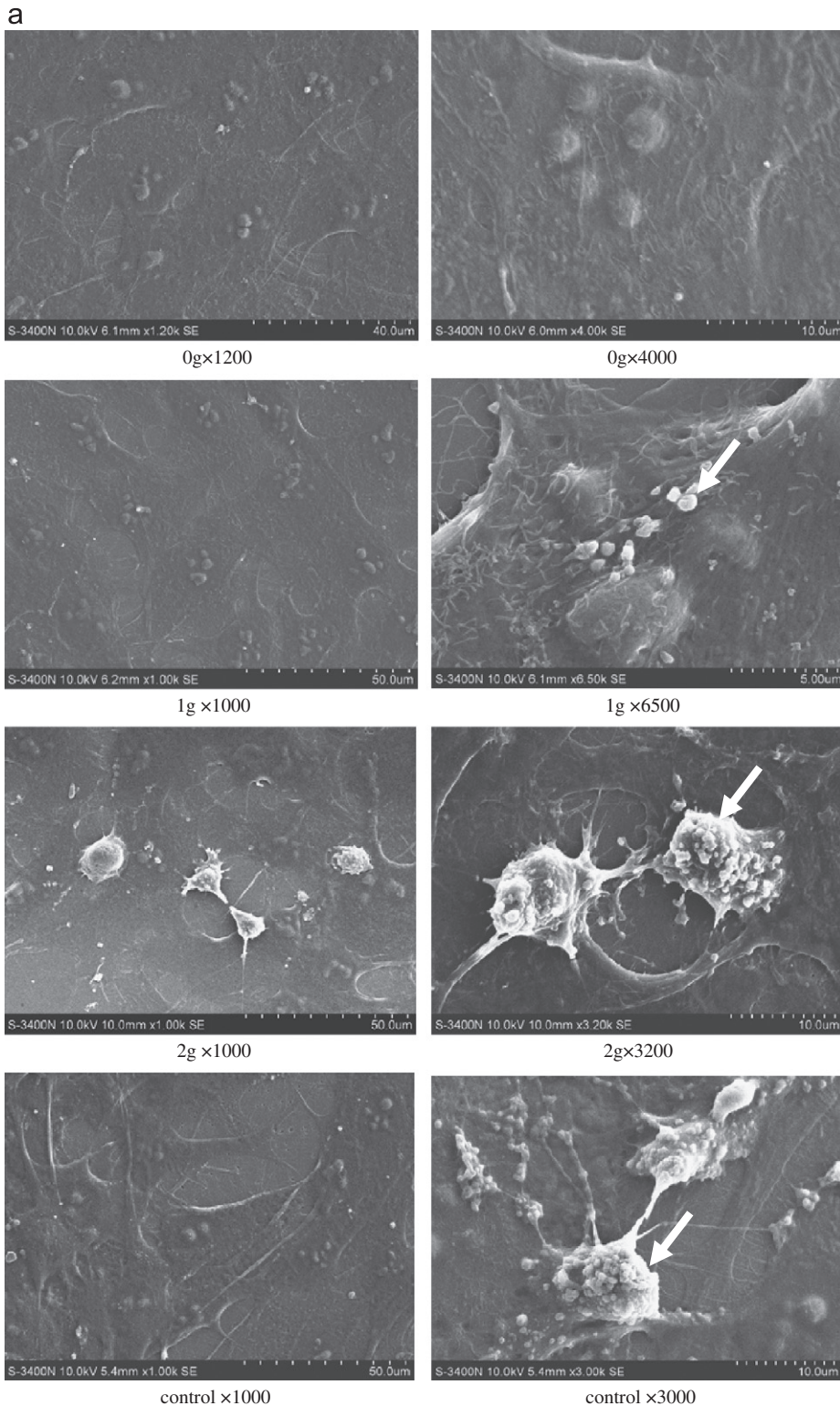


Fig. 2. Detection of the effects of LGHMF on MG-63 (a) and MC3T3 (b) morphology by scanning electron microscopy. After osteoblast MG-63 and MC3T3 cells were cultured in the bore of LGHMF (0g, 1g and 2g) at 37°C for 24 h. The cells were dehydrated in vacuum and mounted with double-sided adhesive tape on aluminium stubs and coated with a 20 nm gold layer. The cells were examined in an S-3400N scanning electron microscope operated at an accelerating voltage of 10 kV. Lower magnification (left) and higher magnification (right) pictures were provided. Arrows indicate secretory vesicles.

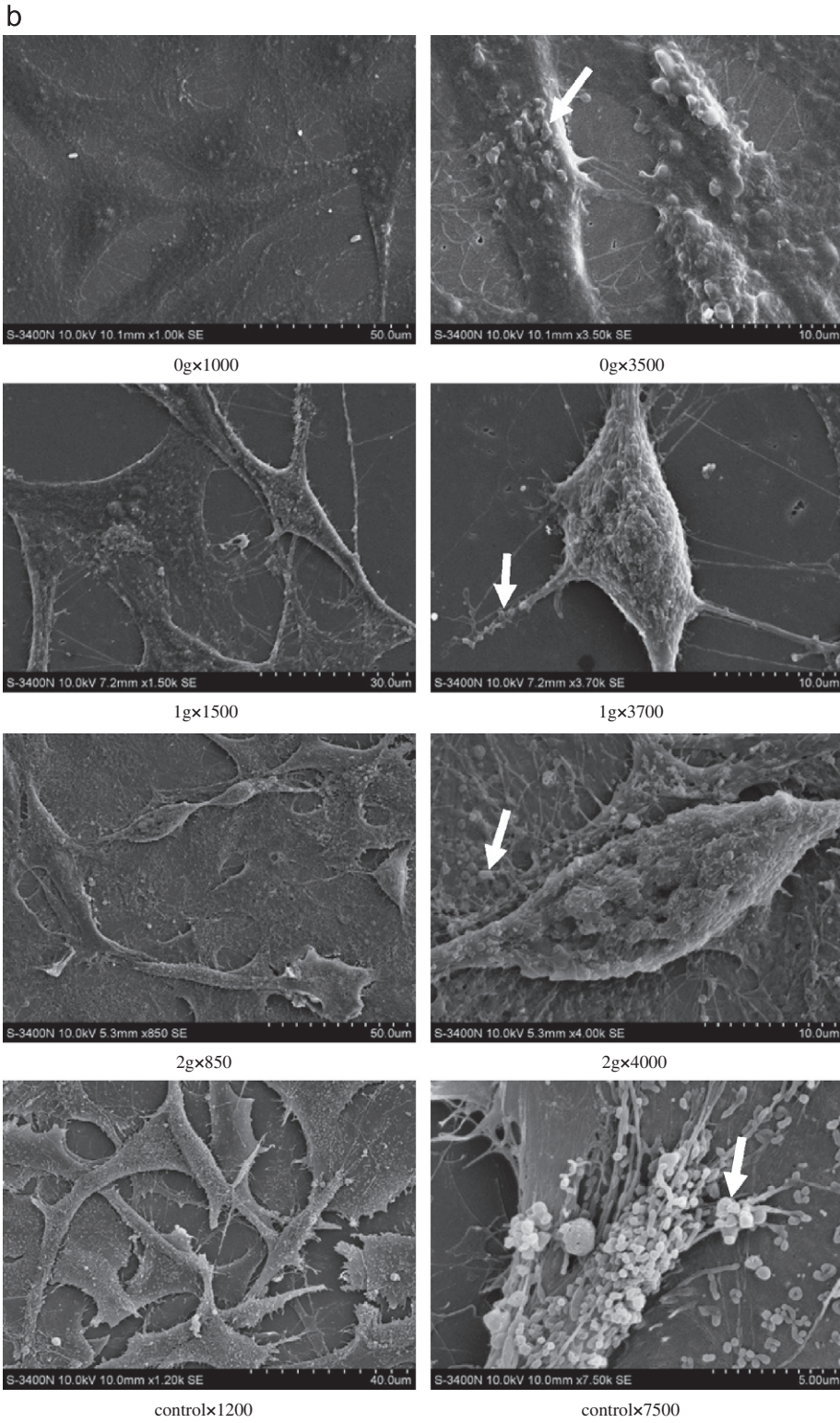


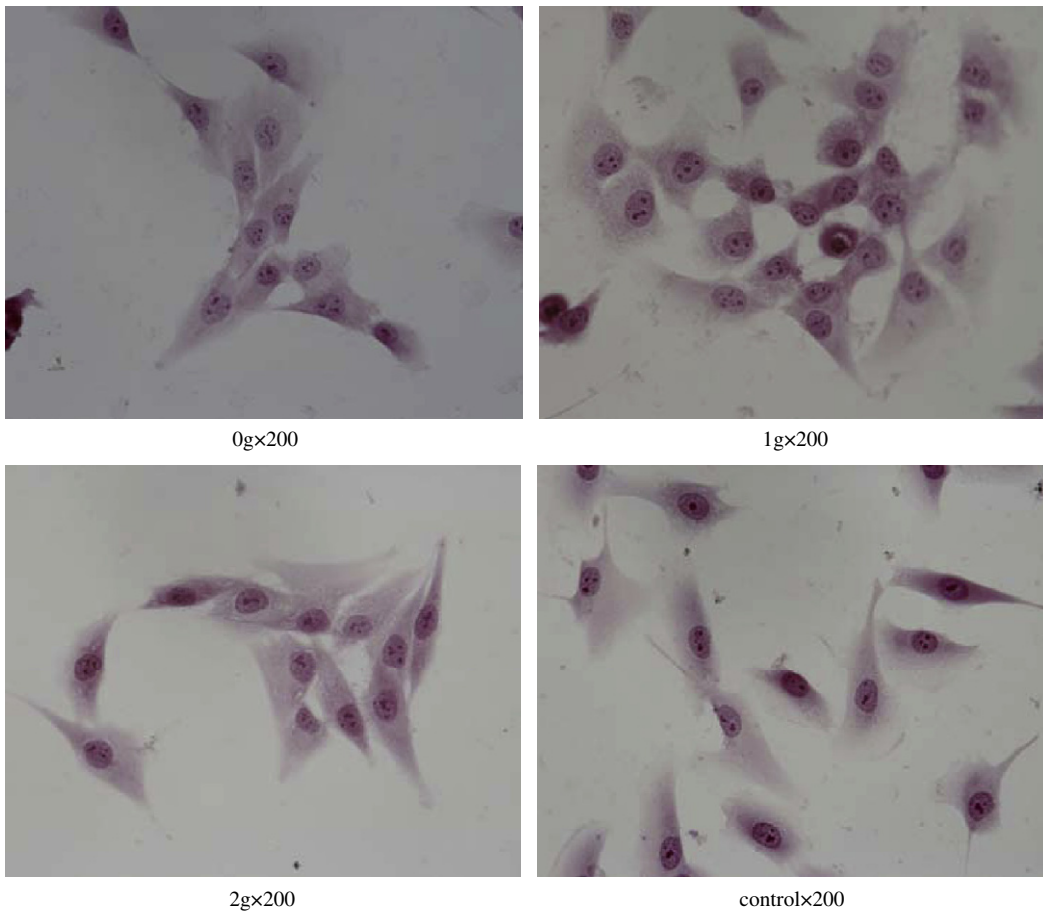
Fig. 2. (continued).

expression in MC3T3 cells among four conditions (0g, 1g and 2g) and control) were not significant.

Collagen I gene expression in MG-63 cells was significantly increased under LGHMF conditions (0, 1 and

2g) compared to control condition ($P < 0.05$, Fig. 8). In MC3T3 cells collagen I gene expression was dramatically increased under 1 and 2g conditions, but under 0g slightly decreased compared to control

a



b

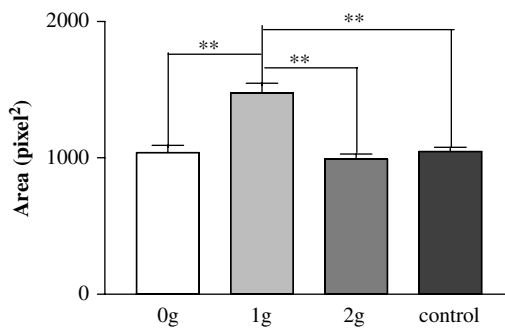
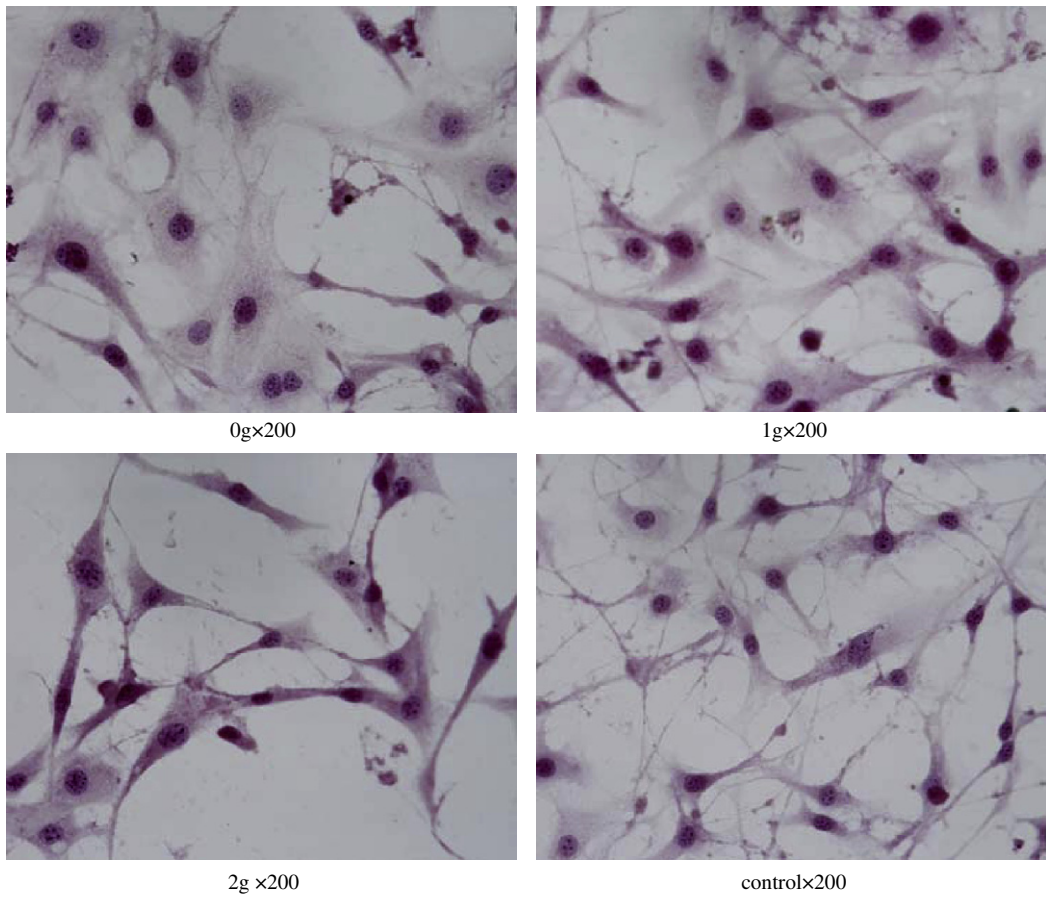


Fig. 3. Detection of the effects of LGHMF on MG-63(a) and MC3T3(c) morphology by HE staining. * $P < 0.05$; ** $P < 0.001$. After osteoblast MG-63 and MC3T3 cells were cultured in the bore of LGHMF (0g, 1g and 2g) at 37 °C for 24 h, the coverslips were removed from superconducting magnet, fixed in 95% ethanol and placed in 20% haematoxylin and 0.5% eosin for 5 min, respectively. Then cells were dehydrated by an ethanol gradient and mounted by Permount. The slides mounted with Permount were then observed by microscope and taken photos ($\times 200$). The size of cell nuclei was quantified by ImageJ software (b) and (d).

C



d

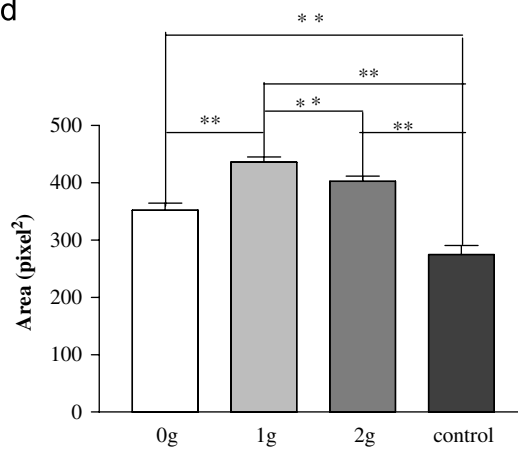


Fig. 3. (continued).

condition. The differences of collagen I gene expression in MC3T3 cells between 0g and 2g conditions, 1g and control conditions had statistical significance ($P < 0.05$, Fig. 8).

4. Discussion

The organism possesses the function to identify mechanics stimulation and response to it. The osteocyte

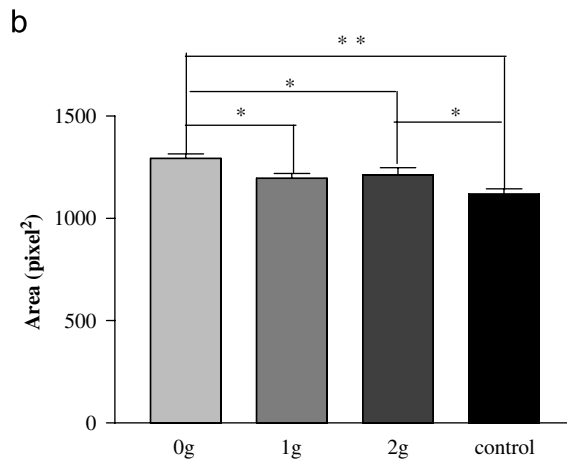
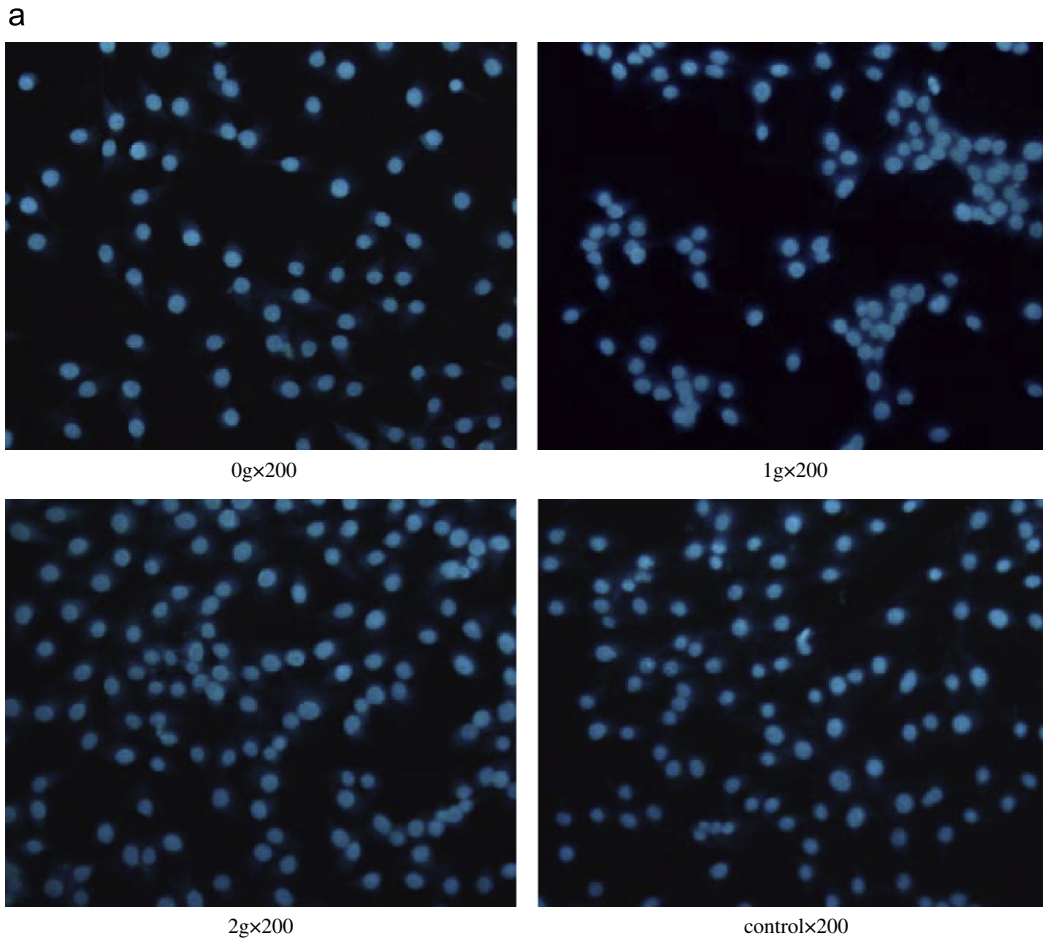
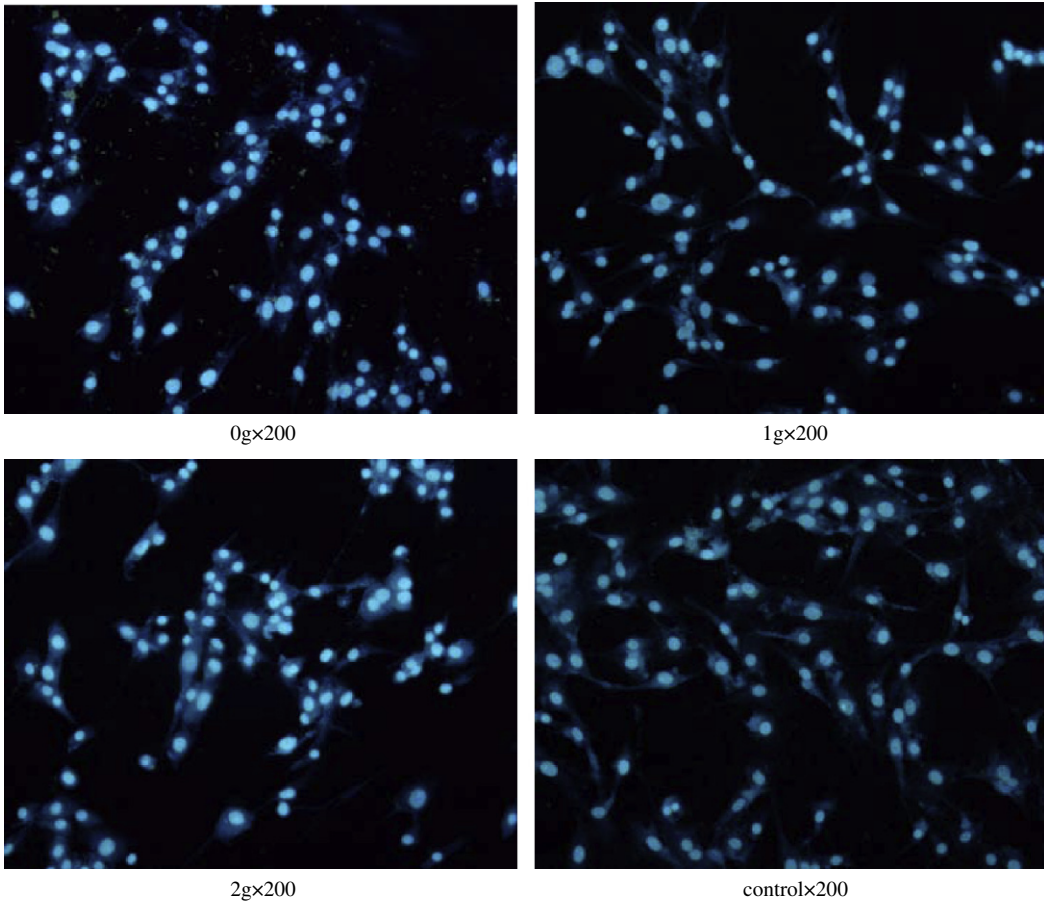


Fig. 4. Detection of the effects of LGHMF on MG-63 cell (a) and MC3T3 cell nucleus morphology (c) by Hoechst 33258 staining. * $P < 0.05$; ** $P < 0.001$. After culturing for 24h in room temperature bore of superconducting magnet, the coverslips coated with cells were washed twice with pre-warmed phosphate-buffered saline (PBS) and stained with Hoechst 33258 (1 $\mu\text{g}/\text{ml}$) for 5 min and observed by fluorescence microscope ($\times 200$). The size of cell nuclei was quantified by ImageJ software (b) and (d).

C



d

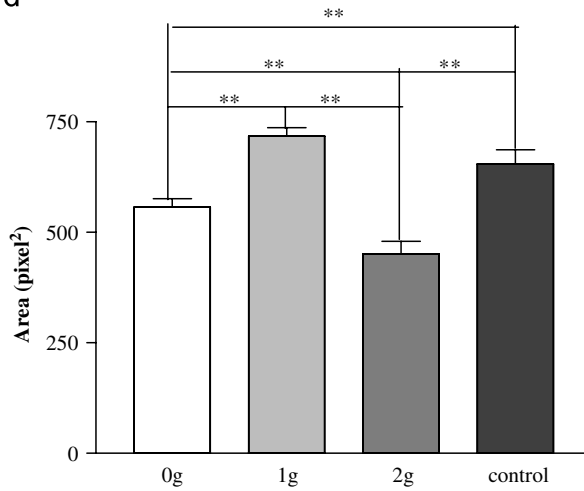


Fig. 4. (continued).

is supposed to sense loading of bone tissue [17]. So far, the mechanisms of how cells sense gravity and how mechanical signals are transmitted and

converted into biochemical signals are still not understood. In addition limited real space flight times and conditions have made the intensive and extensive

research at cellular and molecular level hard to be studied.

In this study, a new special designed superconducting magnet which can provide long-time, steady and

large gradient magnetic field ($B \cdot (dB/dz) = -1500$ to $-1600 \text{ T}^2/\text{m}$) was employed to produce gravitational effects. In the bore superconducting magnet, along with the vertical axis, different positions have different

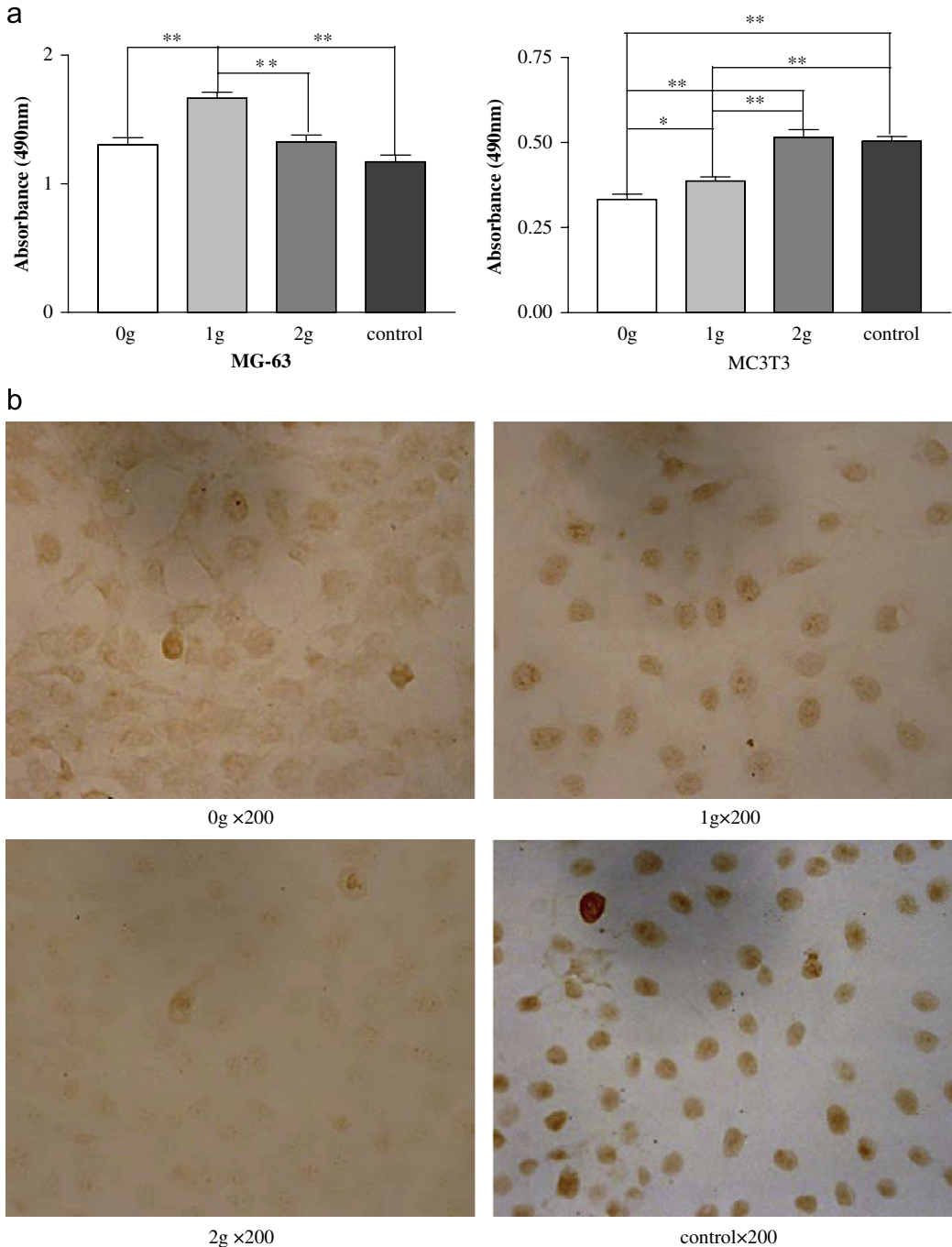
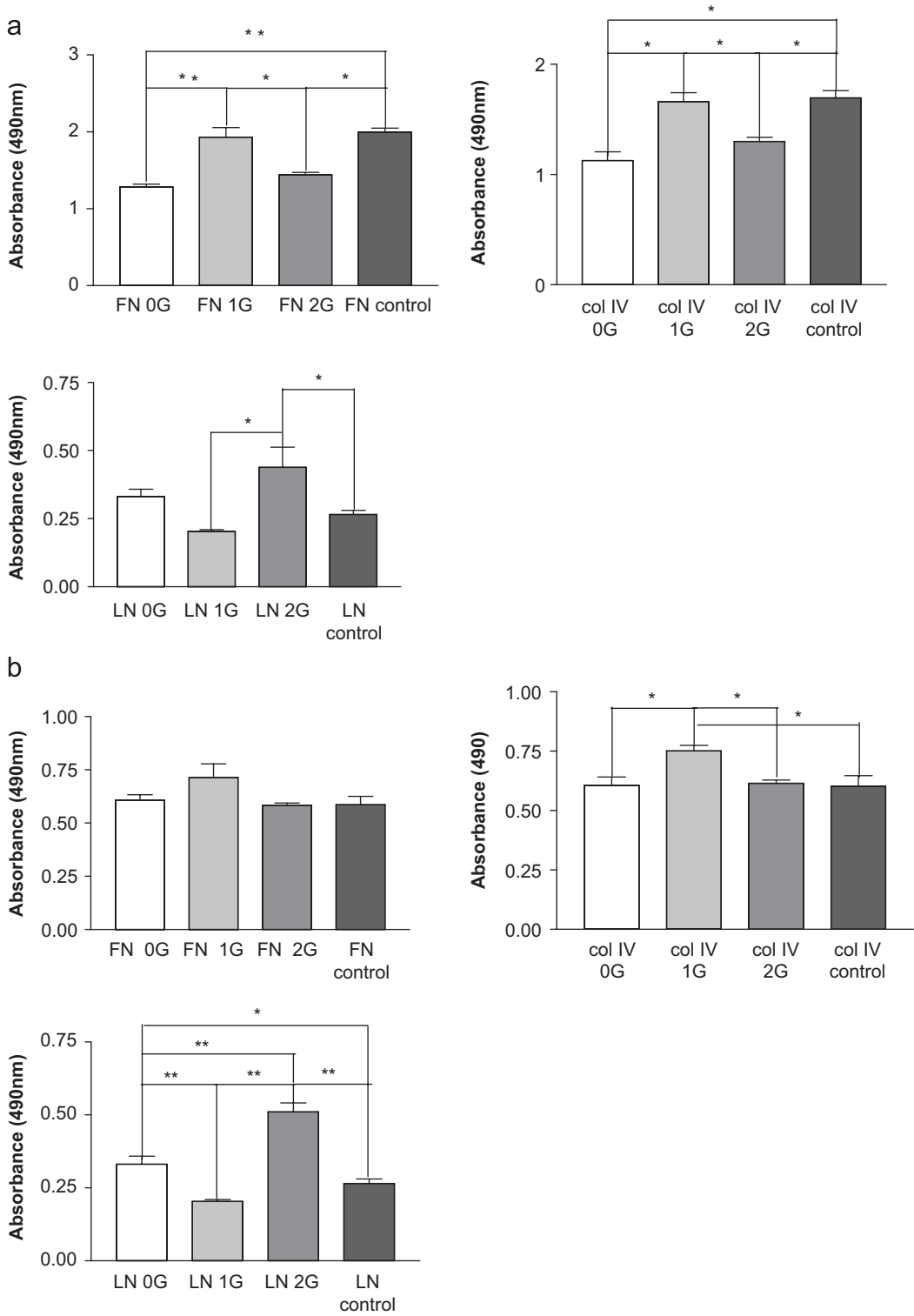


Fig. 5. The effects of LGHMF on MG-63 and MC3T3 proliferation. $*P < 0.05$; $**P < 0.001$. After culturing for 24 h in room temperature bore of superconducting magnet, $10 \mu\text{l}$ cell counting kit was added to the microelon ELISA strips for 2 h and the absorbance was detected by microplate reader at 405 nm (a). The immunocytochemistry staining assay was employed to detect the effects of LGHMF on Ki-67 expression on MG-63 cells (b).



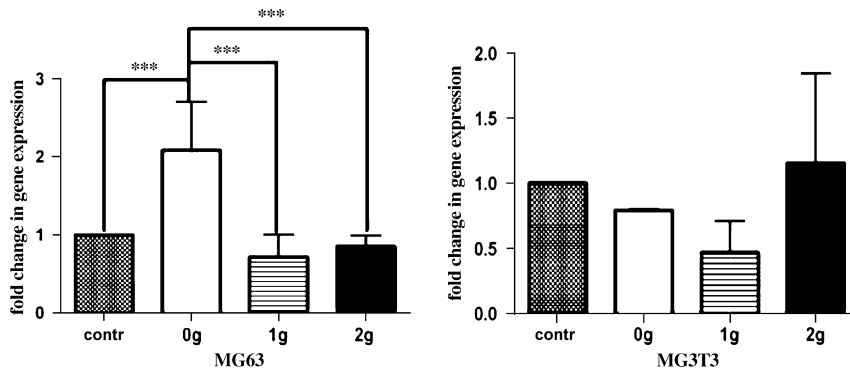


Fig. 7. Detection of the effects of LGHMF on fibronectin gene expression in MG63 cells and MC3T3 cells by real time PCR. * $P < 0.05$; *** $P < 0.001$. After MG-63 cells and MC3T3 cells were cultured in LGHMF (0g, 1g and 2g) for 24h, total RNA was extracted and RNA was reverse transcribed and processed for PCR reactions according to the protocol provided with the kits. Fibronectin gene expression in MG-63 cells under zero gravity increased significantly compared to other conditions ($P < 0.05$). The difference of fibronectin gene expression in MC3T3 cells among four conditions (0, 1, 2g and control) is not significant. The results indicate the effects of LGHMF on fibronectin gene expression in MG63 are the results of altered gravity.

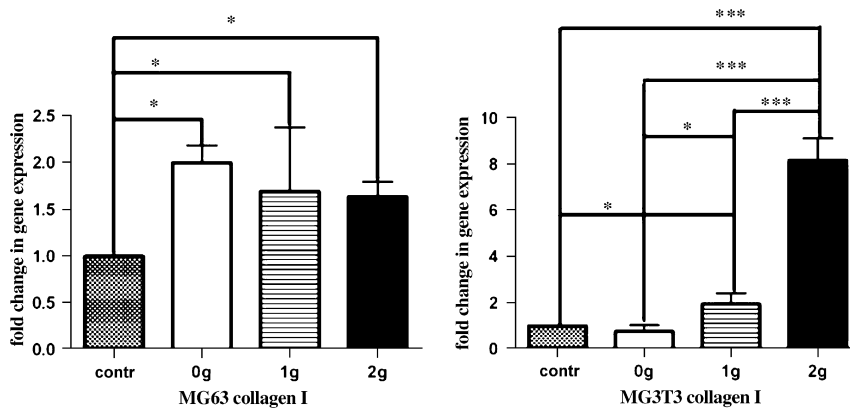


Fig. 8. Detection of the effects of LGHMF on collagen I gene expression in MG63 cells and MC3T3 cells by real time PCR. * $P < 0.05$; *** $P < 0.001$. After MG-63 cells and MC3T3 cells were cultured in LGHMF (0g, 1g and 2g) for 24h, total RNA was extracted and RNA was reverse transcribed and processed for PCR reactions according to the protocol provided with the kits. Collagen I gene expression in MG-63 and MC3T3 cells was significantly increased under 1 and 2g conditions compared to control condition ($P < 0.05$). The differences of collagen I gene expression in MC3T3 cells between zero gravity and 2g conditions, 1g and control conditions had statistical significance ($P < 0.05$). The results indicate that the effects of the magnetic field on collagen I gene expression in MG-63 cells were dominant but in MC3T3 cells both the magnetic field and the altered gravity worked together.

values of $B \cdot (dB/dz)$ and the magnitude and direction of magnetic force acting on specimen are also different, so the apparent gravity of specimen placed in LGHMF

can be attenuated or enhanced in different degree by magnetic force. Here, B in the intensity of the magnetic field, and the dB/dz is the change along with z -axis.

Fig. 6. The effects of LGHMF on MG-63 cell (a) and MC3T3 cell (b) adhesion to ECM proteins. * $P < 0.01$; ** $P < 0.001$. After being cultured for 24h in LGHMF, MG-63 and MC3T3 cells (5×10^4) were detached and added in 96-well micro-plates coated with fibronectin (FN), laminin (LN) and collagen IV (Col IV) ($3 \mu\text{g}$ per well). The bound cells were stained with 0.5% crystal violet. The number of the bound cells was estimated by solubilizing the dye using 0.1 M sodium citrate and reading the absorbance at 490 nm. Triplicate determinations were done at each data point. Compared to control group, the adhesive abilities of MG-63 cells to fibronectin and collagen IV in 0 and 2g groups were significantly decreased ($P < 0.01$) but to laminin in 2g group were significantly increased ($P < 0.05$). The adhesive abilities of MC3T3 cells to laminin in 2g group were dramatically increased ($P < 0.05$) compared to those of control group. Moreover, the difference of the adhesive abilities of MC3T3 cells to laminin in 2g and 0g has statistical significance ($P < 0.05$). The difference of the adhesive abilities of MC3T3 cells to collagen IV in 1g and control has statistical significance ($P < 0.05$).

The product of $B^*(dB/dz)$ means the magnetic force in different position in the magnet. Because the magnetic field intensities are changed along with the z -axis, in different position the production of $B^*(dB/dz)$ is different. So the superconducting magnet can simulate gravitational environment from hypo-gravity ($0g$) to hyper-gravity ($2g$). Like other ground-based simulation methods, LGHMF also has shortcomings. A strong magnetic field coexists with different gravity levels at all time. In order to relatively distinguish the gravitational effects and magnetic field effects, four groups were designed in this study, namely, $1g$ group (normal gravity, $16T$), control group (normal gravity, geomagnetic field), $2g$ group ($2g$ gravity, $12T$) and $0g$ (zero gravity, $12T$). The relatively magnetic effects can be obtained by comparing $1g$ group and control group, and the relatively gravitational effects also can be obtained by comparing ($2g$ and $0g$) group.

Valles from Brown University, Rhode Island in America has been working on magnetic levitation-based Martian and Lunar gravity simulator supported by NASA (Grants: NAG 8-1782, NAG 8-1774 and NNA 04CC57G). Valles has reported that intense $16.7T$ high magnetic fields can alter the geometry of the early cell cleavages of *Xenopus laevis* eggs and the reorientation of cleavage furrows. Moreover, the altered cleavage geometry results from the magnetic field-induced realignment of mitotic structures, which causes a realignment of the centrosome replication and spreading processes [18,19]. Stalcup et al. [14] has reported that the leaves of a transgenic plant produce resonant-type stress response to strong magnetic fields and in magnetic levitation (low gravity) environments ($17T < B < 25T$) but null response in roots. Glade et al. [15] utilized ground-based methods including magnetic levitation and clinorotation to study the effects of weightlessness on physical and biological systems. Glade's findings indicate the effects of weightlessness on microtubule self-organization can be studied using ground-based equipment and the findings closely resemble that of the space flight experiment.

Many human and animal studies have demonstrated that loss of bone formation (osteoblast proliferation) may be the main cause of space bone loss [20]. The problem of decreased osteoblast proliferation in microgravity is a key issue in cell biology that may determine whether we can further explore space [21]. Over the past 10 years evidences of changes in cell biology have been noted in a variety of cells. Specifically, it has been demonstrated that the growth of lymphocytes [22] and osteoblasts [23,24] is inhibited or altered in microgravity environment. The results of the altered

osteoblast proliferation by LGHMF were observed in this study. LGHMF stimulated MG-63 cell proliferation but inhibited MC3T3 cell proliferation. These results indicate the increase of MG-63 cell proliferation is the result of magnetic field, but the effects of LGHMF on MC3T3 cell proliferation are the results of both magnetic field and altered gravity. The effects of LGHMF on the expression of nuclear antigen Ki-67, a proliferation marker, on MG-63 and MC3T3 cells were investigated by immunocytochemistry. The results showed that the expression of Ki-67 in MG-63 cells under 0 and $2g$ conditions was negative but under control and $1g$ conditions was positive. The results indicate that the high magnetic field promotes MG-63 cell proliferation. However, the expression of Ki-67 in MC3T3 cells under $0g$, $1g$, $2g$ and control conditions was negative and maybe Ki-67 antibody does not bind to mouse Ki-67 antigen. The effects of LGHMF on osteoblast proliferation detected by the immunocytochemistry assay were not consistent with those of the CCK-8 assay. The CCK-8 assay depends on the amount of the formazan dye generated by the activity of dehydrogenases in cells and the activity of dehydrogenases is directly proportional to the number of living cells. The expression of the human Ki-67 protein is strictly associated with cell proliferation. During interphase, the antigen can be exclusively detected within the nucleus, whereas in mitosis most of the protein is relocated to the surface of the chromosomes. The fact that the Ki-67 protein is present during all active phases of the cell cycle (G(1), S, G(2) and mitosis), but is absent from resting cells (G(0)), makes it an excellent marker for determining the so-called growth fraction of a given cell population [25]. Hughes-Fulford [26] reported that the flown osteoblasts grew slowly in microgravity with a significantly reduced total cell number and the cytoskeleton of the flight samples had a reduced number of stress fibers and a unique abnormal morphology. However, Chiu et al. [27] studied that cellular proliferation with three-dimensional (3D) tissue-like aggregates of CD34+ mononuclear cells cultured in RWV significantly increased.

Cellular morphology is related to cellular function, so the effects of LGHMF on MG-63 and MC3T3 cell morphology were investigated by HE staining, hoechst 33258 staining and scanning electron microscope. We found that the cellular morphology of MG-63 and MC3T3 cells at $0g$ condition was evidently affected compared to the control condition. So the strong magnetic field and gravitational environment affect osteoblast morphology. No major differences were found in the morphology of MG-63 between flight (Foton-10) and $1g$ or ground samples [28]. Elongated nuclei and

stellar cytoskeleton of MC3T3-E1 (STS-76, 81 and 84) presented in microgravity samples and 1g in-flight centrifugation reversed morphology changes to resemble ground controls [29].

Cell growth and survival are regulated by several environmental signals, including contact with extracellular matrix (ECM), other cells, and soluble growth and survival factors [30]. We hypothesize that gravity is a component of the mechanical environment needed for the efficient transduction of cell growth and survival signals from the ECM. Our findings showed that compared to the control group, the adhesive abilities of MG-63 cells to fibronectin and collagen IV in 0g and 2g conditions were significantly decreased but the adhesive abilities of MG-63 and MC3T3 cells to laminin in 2g condition were significantly increased. Although the fibronectin gene expression in MG-63 cell at 0g condition increased compared to other conditions, the adhesive abilities of MG-63 cells to fibronectin did not increase. These results indicated that the inhibition of cell proliferation in LGHMF may be via interrupting matrix-dependent cell survival pathways and LGHMF altered the ECM-integrin signal transduction pathway. Studies have shown that the ECM component fibronectin strongly supports cell survival in the absence of other soluble growth and survival factors in primary synovial fibroblasts [31,32] and that fibronectin is critical for the survival of bone-forming osteoblasts. Vercoetere [33] showed that mechanical stimulation of primary osteoblasts from hyper-gravity stimulated the activation of focal adhesion kinase (FAK) and promotes matrix-dependent cell survival. Conversely, they observed that the rotating wall vessel microgravity model interrupted matrix-dependent cell survival pathways and increased apoptosis. Hughes-Fulford et al. [34] reported that osteoblast fibronectin mRNA, protein synthesis and matrix were unchanged after exposure to microgravity.

Collagen I as one of the markers of osteoblast function plays an important role in the process of bone formation. Several studies during space flight have shown the decreased serum levels of the C-terminal peptide of pro-collagen type I [3]. Collagen I gene expression was also affected by LGHMF. Collagen I gene expression in MG-63 cells significantly increased but in MC3T3 cells significantly decreased at LGHMF conditions (0, 1 and 2g) compared to control condition ($P < 0.05$). The difference of collagen I gene expression in MC3T3 cells between 0 and 2g conditions, 1g and control conditions has statistical significance ($P < 0.05$). The results indicate the effects of LGHMF on collagen I gene in MC3T3 cells are the results of both magnetic field and

alerted gravity. Studies of osteosarcoma cells in true microgravity have also identified a reduction of collagen type I (Col I) gene expression [35].

In this study, strong magnetic field coexists with different gravity levels at all time, so it is difficult to absolutely distinguish which effects are caused by the strong magnetic field and which by the simulated microgravity. The gravitational effects and magnetic field effects have been relatively discriminated by designing different experimental groups in this study. To sum up, the effects of LGHMF on osteoblast morphology were the results of both magnetic field and altered gravity. The effects of magnetic field were dominant on MG-63 proliferation but on MC3T3 proliferation both magnetic field and altered gravity took important roles. The effects of LGHMF on MG-63 cells adhesion to fibronectin, collagen IV and laminin were the complex results of gravity and magnetic field. The effects of LGHMF on MC3T3 cells adhesion to laminin were the results of altered gravity but to collagen IV were the results of magnetic field. The effects of the magnetic field on collagen I gene expression in MG-63 were dominant but in MC3T3 both the magnetic field and the altered gravity worked together. On fibronectin gene expression, in MG-63 cells the gravitational effects were dominant but in MC3T3 cells the differences were not significant.

In conclusion, the superconducting magnet offers a new ground-based space gravity simulator compared to other simulated method for biological experiments. In this study, the results show that gravitational environment produced by a superconducting magnet significantly affects cell morphology, proliferation, adhesion to ECM components, fibronectin gene and collagen I gene expression. These findings will provide some evidences for studying the mechanism of bone loss. The study indicates that the superconducting magnet is a novel gravity simulator and will be valuable for experimental observations.

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