

## cDNA microarray reveals the alterations of cytoskeleton-related genes in osteoblast under high magneto-gravitational environment

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**The diamagnetic levitation as a novel ground-based model for simulating a reduced gravity environment has been widely applied in many fields. In this study, a special designed superconducting magnet, which can produce three apparent gravity levels (0, 1, and 2 g), namely high magneto-gravitational environment (HMGE), was used to simulate space gravity environment. The effects of HMGE on osteoblast gene expression profile were investigated by microarray. Genes sensitive to diamagnetic levitation environment (0 g), gravity changes, and high magnetic field changes were sorted on the basis of typical cell functions. Cytoskeleton, as an intracellular load-bearing structure, plays an important role in gravity perception. Therefore, 13 cytoskeleton-related genes were chosen according to the results of microarray analysis, and the expressions of these genes were found to be altered under HMGE by real-time PCR. Based on the PCR results, the expressions of WASF2 (WAS protein family, member 2), WIPF1 (WAS/WASL interacting protein family, member 1), paxillin, and talin 1 were further identified by western blot assay. Results indicated that WASF2 and WIPF1 were more sensitive to altered gravity levels, and talin 1 and paxillin were sensitive to both magnetic field and gravity changes. Our findings demonstrated that HMGE can affect osteoblast gene expression profile and cytoskeleton-related genes expression. The identification of mechanosensitive genes may enhance our understandings to the mechanism of bone loss induced by microgravity and may provide some potential targets for preventing and treating bone loss or osteoporosis.**

*Keywords* superconducting magnet; high magneto-gravitational environment; simulated weightlessness; cytoskeleton; microarray

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### Introduction

Bone loss caused by microgravity is one of the most common and serious health problems that the astronauts face in space environment [1,2]. Studies on spaceflights lasting more than 12 months have shown that due to microgravity, astronauts on long missions may lose as much as 20% of their bone mass [3]. However, the mechanism of bone loss induced by microgravity is still not clear. It has been hypothesized that gravity affects cells via three ways, including the roles of cell organelle or molecules as being the cell gravity receptors, adaptation response caused by physical or chemical reaction surrounding cells, and the theory of bifurcations. Most researches are prone to the theory of bifurcations, namely the direct vs. indirect effects at the cellular level [4,5]. Mesland [6] has predicted that cells are a non-linear dynamical system and minute gravity will dramatically affect cell behaviors when particles in cells are in the state of the gravity sensitive-window. Thus, non-linear state transitions (i.e. bifurcations) at the molecular level might function to amplify relatively weak gravity signals at the cellular level and the amplification methods may be through cytoskeleton system and inter-cellular network system.

Cytoskeletons consist of three basic types of filaments and associated protein molecules arranged into chains, maintain cells shape, help cells move, and hold the nucleus in place. The cytoskeleton, as the load-bearing architecture of the cell, plays a role in mechanotransduction [7]. Cytoskeletons have tensegrity to balance compression with tension, and yield to forces without breaking [8]. National Aeronautics and Space Administration (NASA) is interested in cytoskeletons

because cytoskeletons respond to gravity [9]. But what happens when gravity vanishes? How do cells replicate and maintain their genomes, including the regulation of their proliferative capacity and survival? Studies reported that changes in cell shape and function were observed in response to changed gravity [10,11]. Our previous results also showed that simulated weightlessness by means of diamagnetic levitation markedly affected the cytoskeleton alteration of osteoblast (A.R. Qian, unpublished data).

Due to the cost expense and the limited opportunities for spaceflight experiment, it is very necessary to develop ground-based models. Diamagnetic levitation technology is a novel-simulated weightless technique and has recently been applied in life science research. Although at present the studies on the biological effects of high magneto-gravitational environment (HMGE) produced by superconducting magnet with large gradient have just started, the rapid-developing trends have been highlighted. Many studies have utilized this technique, such as those on frogs, frog embryos, and cell cultures of plants. Valles *et al.* [12] have carried out the magnetic levitation-based Martian and Lunar gravity simulator with the support from NASA. Brooks *et al.* [13] have reported that the leaves of transgenic plants produce resonant-type stress response in strong magnetic fields and in magnetic levitation (low gravity) environments ( $17\text{ T} < B < 25\text{ T}$ ) but null response in roots. It has been reported that the magnetic levitation inhibits microtubule self-organization, which is consistent with the results reported in spacecraft [14]. Hammer *et al.* [15] have reported that magnetic levitation of MC3T3 osteoblast cells can be taken as a ground-based simulation of microgravity. These findings indicated that magnetic levitation can be used as a new ground-based model for simulating weightless environment. In this study, a superconducting magnet (JMTA-16 T 50 MF) that can provide large gradient high magnetic field [ $B \cdot (dB/dz) = -1500\text{ T}^2/\text{m} \sim 1100\text{ T}^2/\text{m}$ ] was taken as a model to investigate its effects on gene expression profile of osteoblast. In this inhomogeneous magnetic field, a repulsive force will act on diamagnetic materials (cells) so that different apparent gravities (0, 1, and 2 g) can be obtained, when the field direction is vertical to the ground. Our previous work showed that HMGE affected osteoblast morphology, proliferation, and adhesion [16].

In order to further explore the possible mechanism of cellular morphology and function alterations induced by HMGE, we investigated the effects of HMGE on gene expression profile of osteoblast using Affymetrix

GeneChip HuGene U133 (Affymetrix, Santa Clara, CA, USA). Several mechanosensitive genes were identified by real-time PCR and western blot assays. The identification of specific mechanosensitive genes will enhance our understandings of physiological effects observed during spaceflight and may provide some new clues to further investigate the mechanism of bone loss induced by microgravity.

## Materials and Methods

### Cell cultures

The human osteoblast-like cell line MG-63 was purchased from the Cell Collection Center of Shanghai (China). Cells were seeded in 35-mm tissue-culture plate (Nunc Inc., Roskilde, Denmark) and grown in complete MEM culture medium (Gibco, Carlsbad, CA, USA) 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% fetal calf serum (FCS) (Hyclone, Logan, UT, USA) at 37°C, 5% CO<sub>2</sub> in a humidified atmosphere. For culturing of the cells in HMGE, MEM was changed to CO<sub>2</sub> independent medium (Gibco) with 10% FCS and the tissue culture dishes were sealed with parafilm. The plates seeded with cells were transported into the special positions, namely 0, 1, and 2 g by the object stage. A self-made circulating water-bath was used for temperature control during the experiments. The self-made temperature control system mainly includes water-bath pump and related channel system, and the temperature range is  $37 \pm 0.5^\circ\text{C}$  [16,17].

### High magneto-gravitational environment

A ground-based simulated experimental platform for gravitational biology by the large gradient superconducting magnet was described in detail by Qian *et al.* [17]. Briefly, a superconducting magnet (JMTA-16 T 50 MF, Japan Superconductor Technology Inc., Tokyo, Japan), which can provide an HMGE, was made by Japan Superconductor Technology Inc. (JASTEC) according to our orders. The superconducting magnet generates three different magnetic force fields of -1360, 0, and 1312 T<sup>2</sup>/m in a 50 mm diameter room temperature bore, and three magnetic force fields correspond to three apparent gravity (0, 1, and 2 g) and three magnetic induction intensity (12, 16, and 12 T), respectively. In order to develop a long time and stable ground-based simulated platform for space life science research, some equipments matched with the large magnetic field gradient superconducting magnet for gravitational biology

were designed, including temperature control system, object stage, and observing system.

### Total RNA isolation and microarray preparation

Total RNA was isolated from MG-63 cells exposed to HMGE and controls for 24 h using Trizol method as recommended by the manufacturer's protocol (Invitrogen, Carlsbad, CA, USA). Total RNA was purified by RNeasy Micro Kit (Qiagen, Valencia, CA, USA) and then the purity was analyzed by the 260/280 absorbance ratio as well as denatured gel electrophoresis.

First- and second-strand cDNAs were synthesized using Affymetrix one-cycle cDNA synthesis kit according to the manufacturer's protocol (Affymetrix), respectively. Briefly, a T7 oligo(dT) primer (Affymetrix) was incubated with 5  $\mu$ g total RNA at 70°C for 10 min at room temperature and 2 min on ice. Five times first-strand buffer (4  $\mu$ l), 0.1 M DTT (2  $\mu$ l), and 10 mM dNTP (1  $\mu$ l) were mixed and incubated at 42°C for 2 min. Samples were then incubated with Superscript II RT (200 U/ $\mu$ l, Invitrogen) at 42°C for 1 h to generate first-strand cDNA. The synthesized first-strand cDNA product was placed on ice and incubated with 91  $\mu$ l RNase-free water, 30  $\mu$ l 5 $\times$  second-strand buffer, 3  $\mu$ l dNTP (10 mM), 1  $\mu$ l DNA ligase (10 U/ $\mu$ l), 4  $\mu$ l DNA polymerase (10 U/ $\mu$ l), and 1  $\mu$ l RNase H (10 U/ $\mu$ l, Invitrogen) at 16°C for 2 h to generate second-strand cDNA. Then, 2  $\mu$ l T4 polymerase (Invitrogen) was added, and samples were incubated for another 5 min at 16°C. The reaction was terminated by addition of 10  $\mu$ l EDTA (0.5 M) and the samples were purified using the genechip sample clean-up module (Affymetrix).

cRNA was prepared using GeneChip IVT Labeling Kit (Affymetrix). cRNA was subsequently purified using the genechip sample clean-up module and then was quantified and analyzed by both UV spectrophotometer and denatured gel electrophoresis.

Fragmented cRNA was first pre-hybridized with Genechip hybridization oven 640 (Affymetrix) at 45°C for 10 min, and then, after sample quality evaluation, was hybridized to high-density human genome (HG) U133 Plus 2.0 Arrays (Affymetrix), containing 42,203 genes and 12,397 expressed sequence tags. Hybridization was performed at 45°C for 16 h in a hybridization oven with constant rotation (60 rpm/min). The microarrays were then automatically washed and stained in an Affymetrix Genechip Fluidics Station 450 and fluorescence intensities were scanned with a GeneArray<sup>TM</sup> scanner 3000 (Affymetrix). The scanned images were inspected and analyzed using established

quality control measures. Before comparing two arrays, a normalization method was applied. Prior to performing data comparisons, we scaled the data with 'All Probe Sets' and 'Target Signal' is 100.

### Microarray data analysis

A powerful computer workstation loaded with a GeneChip Operating Software (GCOS) was used to analyze the scanned image and to obtain scaled quantitative information. Comparison analysis was used to analyze the differences in gene expression profile in MG-63 cells exposed to 0, 1, 2 g, and control conditions. In a comparison analysis, four samples, hybridized to four GeneChip<sup>®</sup> probe arrays of the same type, are compared against each other in order to detect and quantify changes in gene expression. During a comparison analysis, each probe set on the experiment array is compared with its counterpart on the baseline array, and a change *P*-value is calculated indicating an increase, decrease, or no change in gene expression. Two sets of algorithms are used to generate change significance and change quantity metrics for every probe set. A change algorithm generates a change *P*-value and an associated change. A second algorithm produces a quantitative estimate of the change in gene expression in the form of signal log ratio (SLR). Thus, an SLR of 1.0 indicates an increase in the transcript level by 2 folds and -1.0 indicates a decrease by 2 folds. An SLR of 0 would indicate no change.

In order to further analyze the microarray data, we used FatiGO Data mining with Gene Ontology (<http://fatigo.bioinfo.cnio.es>) [18]. By inputting gene probe set ID, gene ontology including biological process, cellular component, and molecular functions was obtained. Based on the gene ontology of biological process, the different genes were classified. The selected genes were further analyzed with the NetAffx Analysis Center (<http://www.affymetrix.com/analysis/index.affx>), which is a comprehensive resource of functional annotations and public database information integrated with the probe sets.

### Quantitative real-time PCR analysis

After MG-63 cells were cultured in HGME for 24 h, total RNA was extracted using the Trizol method (Invitrogen). RNA was reverse transcribed and processed for PCRs according to the protocol provided with the kits (TaKaRa, Dalian, China). Primers were designed based on the sequence of the cytoskeleton gene available in GenBank and synthesized (Sangon, Shanghai, China) (**Table 1**).

**Table 1** *Homo* primers of cytoskeleton-related genes in MG-63 cells used for quantitative real-time RT-PCR

Gene name	Primer sequences (5' → 3')	Amplification length (bp)	Annealing temperature (°C)	GenBank accession no.
<i>18S rRNA</i>	AATCAGGGTTCGATTCCGGA CCAAGATCCAACACTACGAGCT	257	60	NR_0032861
<i>WASF2</i>	ATGTTGTGGGCAATGACG TTGGAGGAAGCACTTGGATAT	275	54	NM_006990
<i>Adducin 3</i>	AATGGAAGGTTGGCGAAAT ACGCTGTTGCCTCTGTGC	215	53	NM_013943
<i>CDC42BPB</i>	GTGGAGACCTATGGGAAGAT CCAATGAATGGCAAATGTAA	341	54	NM_006035
<i>Tropomodulin 3</i>	CTCCTTGGAATCTGTCA	193	55	NM_014547
<i>Talin 1</i>	TTTGAAGAGCAGGAGAATGA GAAGGCTTTGGTAGTGGC	253	56	NM_006289
<i>Coactosin-like 1</i>	AGTTGACGGAGGGAGTATTT	256	58	NM_021149
<i>Supervillin</i>	TCACCTACTGCCATAACCC	232	55	NM_003174
<i>Filamin A</i>	AATGGCGTTTACCTGATTGA	186	57	NM_001456
<i>WIPF1</i>	CGATTCAAGAGGCAACTA	276	51	NM_003387
<i>SORBS3</i>	ATCCCTAAGCCCATCAAGC	162	59	NM_005775
<i>Plectin1</i>	GAGGCCATGTCCGGTGAGGA CCAGGACACTCCCGTTGCTC	166	65	NM_000445
<i>Paxillin</i>	ACAGCCCTTGACCGGACGTGGCACC CGTGAAGCATTCCCGGCACACAAAGCA	233	68	NM_002859
<i>SPTBN1</i>	CGACAGTCCTTTGCCCTTGC GGATTGCTGGTATGAACTTATTGC	169	57	NM_003127

Primers of 13 cytoskeleton-related genes and 18S rRNA were designed based on the sequence of each gene available in GenBank (accession no.) and were synthesized.

SYBR Green real-time relative quantitative RT-PCR analysis was performed with SYBR Premix Ex Taq™ according to the manufacturer's protocol (TaKaRa) on an MJ Research (Bio-Rad, Hercules, CA, USA) using 18S rRNA as an internal control gene for normalization. The following peltier thermal cycler experimental run protocol was used as follows: denaturing program (94°C for 5 min), amplification and quantification program with 45 cycles (94°C for 15 s, 57°C for 20 s, 72°C for 15 s, and 80°C for 2 s with a single fluorescence detection), melting curve program (70–95°C, with a heating rate of 0.3°C/s and a continuous fluorescence detection). Forward (F) and reverse (R) primer sequences for *Homo sapiens* cytoskeleton-related genes 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 18S RNA amplification products in each sample.

In this study, the  $2^{-\Delta\Delta Ct}$  method of relative quantification was used to estimate relative changes in

cytoskeleton-associated gene expression in MG-63 cells exposed to HMGE. The  $\Delta\Delta Ct$  calculation for the relative quantification of the cytoskeleton-associated gene was used as follows:  $\Delta\Delta Ct = (Ct_{\text{target gene}} - Ct_{\text{reference gene}})_{\text{exp}} - (Ct_{\text{target gene}} - Ct_{\text{reference gene}})_{\text{cont}}$ , where exp and cont are the experimental and control groups, respectively. Each sample was performed in quadruplex wells, and the Ct of each well was recorded at the end of the reaction. The mean and standard deviation (SD) of the four Ct's were calculated. The changes in cytoskeleton gene expression, normalized to 18S under HMGE conditions, were calculated according to the following equation: amount of target =  $2^{-\Delta\Delta Ct}$  [19].

#### Western blot analysis

After MG-63 cells were cultured in HMGE for 24 h, the cells were collected and re-suspended in lysis buffer. The lysis solution was centrifuged at 16,000 g for 10 min at 4°C. The supernatants were collected, and the protein concentration was detected using Lowery's

method. Equal total protein loads of 100  $\mu$ g were loaded on the lanes of the SDS–polyacrylamide gel and separated by electrophoresis. The proteins were then transferred to a PVDF membrane (Millipore, Billerica, MA, USA). After being blocked with Odyssey blocking buffer (LI-COR Biosciences, Lincoln, NE, USA), the blots were incubated overnight with primary antibodies: chicken monoclonal anti-paxillin (1:100, BD Bioscience, San Diego, CA, USA), goat polyclonal anti-WASF2, rabbit polyclonal anti-WIPF1, and anti-talin (1:100, Santa Cruz, CA, USA) at 4°C. Blots were incubated with IRDye™ 800-labeled secondary antibodies (1:2000, Rockland Immunochemicals, Boyertown, PA, USA) for 1 h with GAPDH (1:50,000, Calbiochem, San Diego, CA, USA) expression as a loading control. The bands were then scanned by Odyssey Infrared Imaging System (LI-COR Biosciences). The expression level of each protein was quantified by Quantity One software v4.6 (Bio-Rad).

### Statistical analysis

Statistically significant differences were determined by Prism statistical software (GraphPad Software Inc., La Jolla, CA, USA). A value of  $P < 0.05$  was considered significant in all cases. All data averages or means are accompanied by SDs to indicate the amount of variability in the data.

## Results

### Effects of HMGE on gene expression profiles

From the scatter graph of expression genes in MG-63 cells exposed to HMGE (**Fig. 1**), the hybridization effect of the whole microarray was very good and came up to the requirements of gene expression analysis. Common genes significantly expressed (red) were in the majority and there were also some indistinctively or uncertainly expressed genes (yellow). Differentially expressed genes (blue) are clearly shown in **Fig. 1**. The eight green diagonals indicated 2-, 3-, 10-, and 30-fold changes of expression, respectively, between two samples.

By performing microarray studies, we were able to analyze the global changes in the gene expression profiles of MG-63 cells exposed to HMGE.  $SLR \geq 1$  or  $SLR \leq -1$  was a significant dividing value to analyze the expression profile, which indicates difference between two groups more than two times. Among 54,613 gene probes examined with the microarray, only 572 genes were down-regulated and 415 genes were up-regulated to statistically significant levels  $>2$  folds in

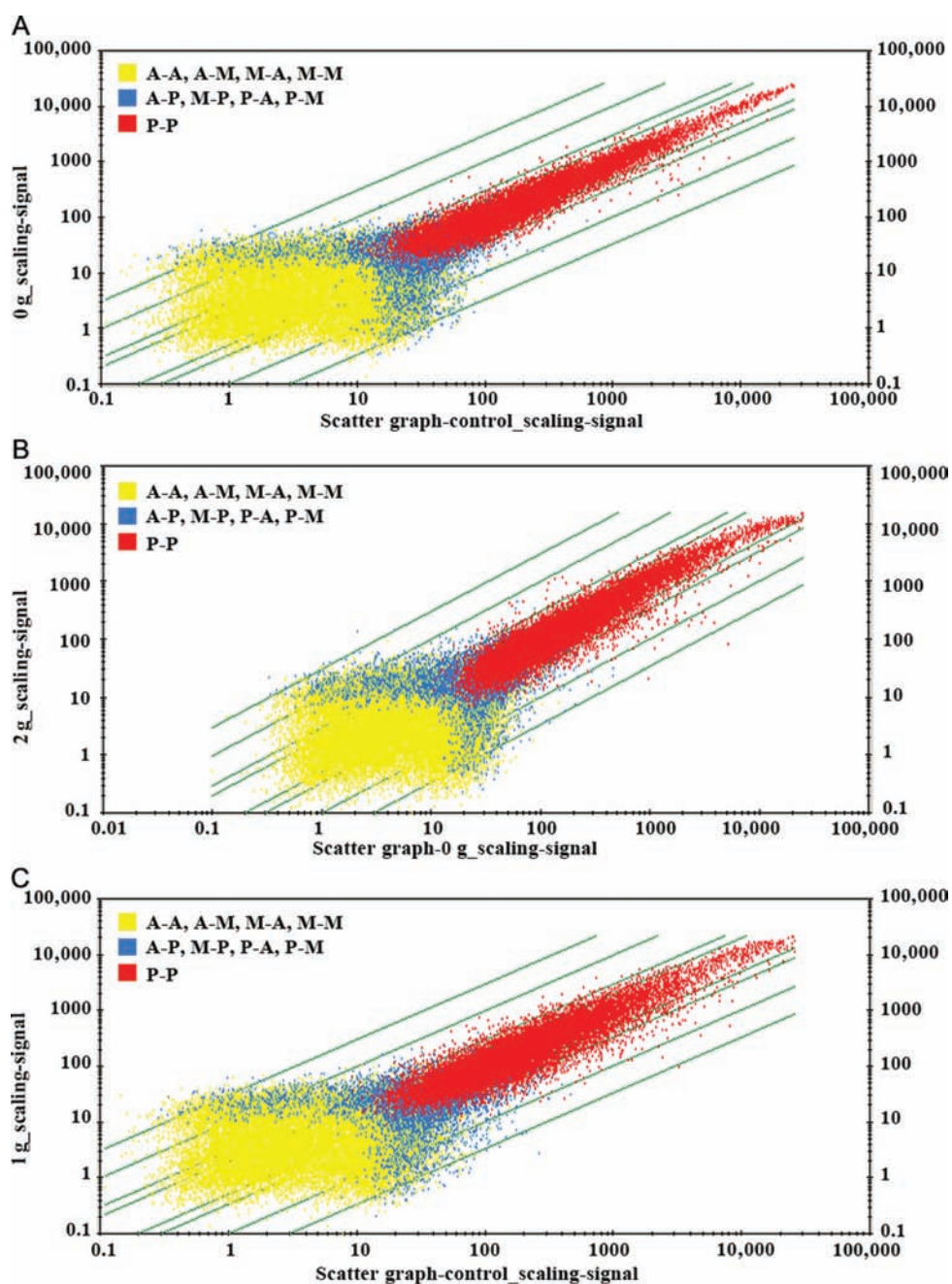
0 g vs. control conditions. In 2 g vs. 0 g conditions, 1048 genes were down-regulated and 367 genes were up-regulated, and in 1 g vs. control conditions, 1579 genes were down-regulated and 1015 genes were up-regulated. **Table 2** lists the up-regulated and down-regulated gene numbers according to SLR from the microarray. It showed that 55 genes were up-regulated ( $SLR \geq 3$ ) and 53 genes were down-regulated ( $SLR \leq -3$ ) by comparing 0 g with control conditions. In addition, 109 genes were up-regulated ( $SLR \geq 3$ ) and 27 genes were down-regulated ( $SLR \leq -3$ ) in MG-63 cells under 0 g vs. 2 g conditions. Sixty-eight genes were up-regulated ( $SLR \geq 3$ ) and 123 genes were down-regulated ( $SLR \leq -3$ ) in MG-63 cells under 1 g vs. control conditions (**Table 2**).

### Bioinformatics analysis of microarray results

FatiGO Data mining with Gene Ontology (<http://fatigo.bioinfo.cnio.es>) was used to analyze the microarray data. The gene numbers up-regulated ( $SLR \geq 1$ ) or down-regulated ( $SLR \leq -1$ ) according to biological process, cellular component, and molecular functions are listed in **Table 3**, respectively. Based on the results of biological process, the NetAffx Analysis Center (<http://www.affymetrix.com/analysis/index.affx>) was used to further analyze the selected genes' function. The alteration of genes involved in 'proliferation', 'cell adhesion', 'apoptosis', 'cell cycle', 'cell communication', 'response to stress', and 'response to external stimulus' was analyzed. The partial genes with  $>2$ -fold change were sorted on the basis of typical cell functions and are shown in **Tables 4–6**. **Table 4** categorizes the genes in MG-63 cells exposed to 0 g environment with known functions from the microarray studies that were up-regulated or down-regulated significantly  $>2$  folds above the control. **Table 5** shows the part of different genes sorted on the basis of typical cell functions in MG-63 cells under 1 g vs. control conditions. **Table 6** shows the part of the different genes sorted on the basis of typical cell functions in MG-63 cells compared with 0 g vs. 2 g conditions. These genes were sorted on the basis of typical cell function using the Gene Ontology, so genes with unknown functions were not included in this analysis.

### Effects of HMGE on cytoskeleton-associated gene expression

The cytoskeleton, as the load-bearing architecture of the cell, is very sensitive to altered gravitational forces and disruption of the cytoskeleton will result in the alteration



**Figure 1 Scatter graph of gene expressions in MG-63 cells exposed to high magnetic-gravitational environment** Scatterplot shows the intensities of each gene probe using data obtained from the microarrays of 0 g vs. control (A), 0 g vs. 2 g (B), and 1 g vs. control (C). Genes changed by HMGE >2 folds were shown as solid dots. The red plots represent significantly expressed common genes and yellow plots mean some indistinctively or uncertainly expressed genes. The blue plots were differentially expressed genes. The eight green diagonals indicated 2-, 3-, 10-, and 30-fold changes of expression, respectively, between two samples.

of cellular structure and function [1,2]. Microarray results showed that the expression of *SPTBN1* (spectrin,  $\beta$ , non-erythrocytic 1) and *supervillin* genes under 0 g condition showed 3.3- and 13.9-fold increase, respectively, compared with that under ground control (Table 7). The expression of 13 cytoskeleton-associated genes (*adducin*

*3*, *coactosin-like 1*, *filamin A*, *SORBS3*, *CDC42BPB*, *tropomodulin 3*, *talín 1*, *SPTBN1*, *supervillin*, *WASF2*, *WIPF1*, *plectin 1*, and *paxillin*) was increased in 0 g vs. 2 g condition, but it was decreased in 1 g vs. control condition (Table 7). The increase in 13 cytoskeleton-associated genes expression except *tropomodulin 3* was

**Table 2** The number of up-regulated and down-regulated genes in MG-63 cells under 0 g vs. control, 0 g vs. 2 g, and 1 g vs. control conditions

Groups	Up-regulation			Down-regulation		
	SLR $\geq 3$	$2 \leq \text{SLR} \leq 3$	$1 \leq \text{SLR} \leq 2$	SLR $\leq -3$	$-3 \leq \text{SLR} \leq -2$	$-2 \leq \text{SLR} \leq -1$
0 g vs. control	55	37	321	53	39	480
0 g vs. 2 g	109	158	1048	27	20	367
1 g vs. control	68	69	879	123	208	1248

RNA from cells sampled at 24 h in HMGE and ground controls was evaluated by cDNA microarray. Comparison analysis was used to analyze the differences in gene expression profile in MG-63 exposed to 0 g, 1 g, 2 g, and control conditions. During a comparison analysis, each probe set on the experiment array is compared with its counterpart on the baseline array, and a change *P*-value is calculated indicating an increase, decrease, or no change in gene expression. One of algorithms produces a quantitative estimate of the change in gene expression in the form of SLR. The number of the up-regulated (SLR  $\geq 1$ , SLR  $\geq 2$ , and SLR  $\geq 3$ ) and down-regulated genes (SLR  $\leq -1$ , SLR  $\leq -2$ , and SLR  $\leq -3$ ) were listed from the microarray.

**Table 3** Classification of differentially expressed genes in MG-63 cells exposed to HMGE

Groups	Biological processes		Cellular components		Molecular functions	
	Up-regulated	Down-regulated	Up-regulated	Down-regulated	Up-regulated	Down-regulated
0 g vs. control	175	211	178	200	185	219
0 g vs. 2 g	416	165	411	171	437	173
1 g vs. control	460	723	493	714	482	764

FatiGO Data mining with Gene Ontology (<http://fatiGO.bioinfo.cnio.es>) was used to analyze microarray data. By inputting gene probe set ID, gene ontology including biological process, cellular component, and molecular functions was obtained in MG-63 cells under 0 g vs. control, 0 g vs. 2 g, and 1 g vs. control condition.

two times more in 0 g than in 2 g condition. However, the expressions of 13 cytoskeleton-associated genes except *WASF2* and *WIPF1* were down-regulated at least two times in 1 g when compared with those under control condition (**Table 7**).

Thirteen cytoskeleton-associated genes sensitive to HMGE were selected from microarray data and SYBR Green-based real-time PCR was used to verify the effects of HMGE on cytoskeleton-associated gene expression at mRNA levels. After being normalized by internal control genes, the relative gene expression levels in experimental groups were obtained comparing with those of control groups. And then, the differences in gene expression between 0 g vs. control, 1 g vs. control, and 0 g vs. 2 g were analyzed (**Table 7** and **Fig. 2**). Most of the selected cytoskeleton-associated genes expression was increased by comparing 0 g with 2 g condition, but it was decreased in 1 g vs. control condition. Two-fold changes of *SPTBN1* and *WASF2* expressions were observed in MG-63 cells exposed to 0 g environment compared with those in control. The

expression of *SPTBN1*, *coactosin-like 1*, *WASF2*, and *supervillin* was up-regulated 5.77, 4.25, 2.38, and 2.08 folds, respectively, in 0 g vs. 2 g. In addition, the expression of *SPTBN1*, *supervillin*, and *paxillin* was down-regulated at least 2 folds in 1 g vs. control (**Table 7** and **Fig. 2**). However, a 6.16-fold increase in *SORBS3* was showed in 1 g vs. control (**Table 7** and **Fig. 2**). In addition, the differences by comparing all pairs of *WASF2* and *talin 1* were significant ( $P < 0.05$ ); however, it was not significant ( $P > 0.05$ ) by comparing all pairs of *add3*, *plectin 1*, and *WIPF1* (**Fig. 2**). The difference in *paxillin* expression compared all pairs except 0 g vs. 2 g was statistically significant ( $P < 0.05$ ).

### Effects of HMGE on cytoskeleton-associated protein expression

In order to screen the cytoskeleton-related genes sensitive to gravity, paxillin, WASF2, WIPF1, and talin 1 were selected on the basis of the results of microarray and real-time PCR and were further verified by western

**Table 4 Selected genes sensitive to diamagnetic levitation environment (0 g) in MG-63 cells sorted on the basis of typical cell functions**

Gene description	GenBank accession no.	Fold change	Molecular functions
<b>Cell adhesion</b>			
Periostin, osteoblast-specific factor	NM_006475	3.48	Protein and heparin binding
Collagen, type XIV, $\alpha 1$	NM_021110	2.46	Extracellular matrix structural constituent
Trophinin	NM_177556	0.33	Protein binding
Protein tyrosine phosphatase, receptor type, F	NM_130440	0.27	Protein tyrosine phosphatase activity
Cysteine-rich, angiogenic inducer,61	NM_001554	0.16	Insulin-like growth factor binding, growth factor binding
Metastasis suppressor 1	NM_014751	0.27	Actin binding, actin monomer binding, receptor binding
<b>Cell cycle</b>			
Cyclin B1	NM_031966	3.25	G2/M transition of mitotic cell cycle
Histone deacetylase 5	NM_005474	0.17	Histone deacetylase activity, protein binding
<b>Proliferation</b>			
Plasminogen-likeA1/B1/B2	NM_002665	8.57	Plasmin activity
Cysteine and glycine-rich protein 2	NM_001321	0.31	Zinc ion binding metal ion binding
<b>Apoptosis</b>			
Prune homolog 2	NM_015225	0.33	Induction of apoptosis G1 phase
<b>Cell communication</b>			
Rho guanine nucleotide exchange factor (GEF) 7	NM_145735	12.13	Regulation of Rho protein signal transduction
Fibroblast growth factor 18	NM_003862	4.59	Signaling pathway
G-protein-coupled receptor 125	NM_145290	18.38	G-protein-coupled receptor protein signaling pathway
RAR-related orphan receptor A	NM_134262	0.072	DNA-dependent signal transduction
Protein tyrosine phosphatase, receptor type, f polypeptide, interacting protein, $\alpha 4$	NM_015053	0.033	Receptor activity, protein binding, cell communication
SMAD family member 2	NM_001003652	0.13	Transforming growth factor $\beta$ receptor signaling pathway
GDNF family receptor $\alpha 2$	NM_001495	0.29	Transmembrane receptor protein tyrosine kinase signaling pathway
<b>Response to stress</b>			
Fanconi anemia, complementation group A	NM_000135	4	Response to DNA damage stimulus
Interleukin 8	NM_000584	3.03	Interleukin-8 receptor binding, inferred from physical interaction
Heat shock 70 kDa protein 2	NM_021979	0.31	Nucleotide binding, protein binding
Hypoxia-inducible protein 2	NM_013332	0.33	Methyltransferase activity

Based on the NetAffx Analysis, we employed FatiGO Data mining with Gene Ontology to classify different genes according to gene molecular functions. The alteration of genes involved in 'proliferation', 'cell adhesion', 'apoptosis', 'cell cycle', 'cell communication', 'response to stress', and 'response to external stimulus' was analyzed. The partial genes were categorized in MG-63 cells exposed to 0 g environment with known functions from the microarray studies that were up-regulated or down-regulated significantly  $>2$ -fold above the control.

blot assay at protein level (**Fig. 3**). The western blot results showed that WASF2 and paxillin expressions were significantly decreased in 0 g condition compared with those in 2 g or control condition ( $P < 0.01$ ). In 1 g

condition, WASF2 expression was clearly increased but paxillin expression was distinctly decreased in comparison with that in control condition ( $P < 0.01$ ). Talin 1 expression in 0 g vs. control condition was not changed

**Table 5 Different genes sorted on the basis of typical cell functions in MG-63 cells under 0 g vs. 2 g conditions**

Gene description	GenBank accession no.	Fold Change	Molecular functions
<b>Cell adhesion</b>			
Pleckstrin homology, Sec7 and coiled-coil domains 3 (PSCD3)	NM_004227	20	ARF guanyl-nucleotide exchange factor activity
Glucocorticoid receptor DNA binding factor 1	NM_004491	7.1	GTPase activator activity
FYN oncogene related to SRC, FGR	NM_153047	6.96	Protein-tyrosine kinase activity
Fibronectin 1	NM_002026	4.4	Extracellular matrix structural constituent, collagen binding
ATPase, Ca <sup>++</sup> transporting, cardiac muscle, slow twitch 2	NM_001681	4	Regulation of the contraction/relaxation cycle
Integrin, $\beta$ 1	AF086249	3.7	Receptor activity
Collagen, type XIV, $\alpha$ 1	NM_021110	3.5	Extracellular matrix structural constituent
Zyxin	NM_001010972	3.2	Mediate adhesion, modulate the cytoskeletal organization
Collagen, type VI, $\alpha$ 1	NM_001848	3.2	Structural component of microfibrils, cell adhesion
Heparan sulfate proteoglycan 2	NM_005529	3	Major component of basement membranes, cell adhesion
Collagen, type XV, $\alpha$ 1	NM_001855	3	Structural molecule activity
<b>Cell cycle</b>			
Calreticulin	NM_004343	4.5	Calcium ion binding
Cyclin-dependent kinase 6	NM_001259	4.3	Protein serine/threonine kinase activity, nucleotide binding
B-cell CLL/lymphoma 3	NM_005178	3.2	Maintenance of protein localization
BCL2-associated X protein	NM_004324	0.35	T cell homeostatic
<b>Cell proliferation</b>			
Growth arrest-specific 1	NM_002048	8	GPI anchor binding
Fibroblast growth factor 9	NM_002010	5.3	Growth factor activity, heparin binding
EGFR, transcript variant 1, mRNA	NM_005228	5	MAP/ERK kinase kinase activity, transmembrane receptor activity
Fibroblast growth factor receptor-like 1	NM_001004358	4.5	Receptor activity, protein binding
EGFR, transcript variant 2, mRNA	NM_201282	0.077	Ossification, cell cycle
<b>Apoptosis</b>			
Baculoviral IAP repeat containing 3	NM_182962	8.3	Protein binding, metal ion binding
Myxovirus (MX1)	NM_002462	5	GTPase activity, GTP binding
Tumor necrosis factor $\alpha$ -induced protein 3	NM_006290	4.5	Negative regulation of I- $\kappa$ B kinase/NF- $\kappa$ B cascade
Fibroblast growth factor receptor 1	NM_015850	3.4	Protein-tyrosine kinase activity
B-cell CLL/lymphoma 6	NM_001706	3.2	DNA binding, protein binding
<b>Cell communication</b>			
Tyrosine kinase, non-receptor, 2	NM_001010938	12.5	Protein-tyrosine kinase activity
Insulin receptor substrate 2	NM_003749	8	Signal transducer activity
Transcription factor AP-2 $\alpha$	NM_001042425	4.4	Regulation of transcription
Rho GDP dissociation inhibitor (GDI) $\alpha$	NM_004309	4	Rho GDP-dissociation inhibitor activity
Rho GTPase activating protein 24	NM_031305	3.2	GTPase activator activity

*Continued*

**Table 5** Continued

Gene description	GenBank accession no.	Fold Change	Molecular functions
Transforming growth factor $\beta$ receptor II	NM_003242	3	Transforming growth factor $\beta$ receptor activity, type II
Mitogen-activated protein kinase activated protein kinase 2	NM_032960	3	Protein serine/threonine kinase activity, signal transducer activity
Wingless-type MMTV integration site family, member 5B	NM_032642	2.9	Signal transducer activity
Rho guanine nucleotide exchange factor (-GEF) 11	NM_014784	2.9	Rho guanyl-nucleotide exchange factor activity
RAS p21 protein activator 3	NM_007368	2.6	GTPase mediated signal transduction
Thyroid hormone receptor-associated protein 3	NM_005119	0.33	Androgen receptor signaling pathway
3-phosphoinositide dependent protein kinase-1	NM_002613	0.13	Protein amino acid phosphorylation,
Response to stress			
Fatty acid desaturase 1	NM_013402	5	Oxidoreductase activity
Junction mediating and regulatory protein	NM_152405	3.7	Transcription coactivator activity, protein binding
Heat shock transcription factor 1	NM_005526	2.4	Transcription factor activity
Response to external stimulus			
Chemokine (C motif) ligand 1	NM_002995	14.3	Chemokine activity
Prostaglandin endoperoxide synthase 2	NM_000963	8	Peroxidase activity, prostaglandin-endoperoxide synthase activity
Interleukin 8	NM_000584	4.3	Interleukin-8 receptor binding
Abhydrolase domain containing 2	NM_152924	4	Hydrolase activity
Protein phosphatase 1, regulatory (inhibitor) subunit 9B	NM_032595	3.4	Actin binding, protein phosphatase inhibitor activity
Thrombospondin 1	NM_003246	3	Endopeptidase inhibitor activity
Peroxiredoxin 5	NM_012094	0.4	Response to oxidative stress

The partial genes were categorized in MG-63 cells in 0 g vs. 2 g with known functions from the microarray studies, which were up-regulated or down-regulated significantly >2 folds.

significantly ( $P > 0.05$ ), but it was evidently altered in 0 g vs. 2 g condition or 1 g vs. control condition ( $P < 0.01$ ). WIPF1 expression in 0 g vs. 2 g condition or 1 g vs. control was obviously changed ( $P < 0.01$ ), but similar between 0 g and control conditions ( $P > 0.05$ ).

## Discussion

The cytoskeleton-related genes in osteoblast that are sensitive to HMGE have been identified by cDNA microarray for the first time in this study. The novel and most significant finding of this study is that exposure of osteoblasts to HMGE (0, 1, and 2 g) distinguishes some genes that are sensitive to low gravity, magnet field, and the

combined environment. Using Affymetrix GeneChip System, the current study obtained a list of genes that were up-regulated, down-regulated, or unchanged by HMGE. The results are helpful to the understanding of the mechanisms that control cell proliferation, cell cycle, apoptosis, response to stress, and so on.

We have developed a ground-based experimental platform which can provide a long-term, persistent, and stable weightless environment for the fundamental study of space life sciences by a superconducting magnet with large gradient high magnetic field [17]. In the bore of the superconducting magnet, along with the vertical axis, different positions have different values of  $B \cdot (dB/dz)$  and the magnitude and direction of magnetic force acting on

**Table 6 Different genes sorted on the basis of typical cell functions in MG-63 cells under 1 g vs. control conditions**

Gene description	GenBank accession no.	Fold change	Molecular functions
<b>Cell adhesion</b>			
ADAM-like, decysin 1	NM_014479	14.9	Metalloendopeptidase activity
Collagen, type II, $\alpha$ 1	NM_033150	6.1	Structural molecule activity
Claudin 11	NM_005602	5.3	Structural molecule activity
Chondroitin sulfate proteoglycan 2 (versican)	NM_004385	3.2	Calcium ion binding, glycosaminoglycan binding
Egf-like module containing, mucin-like, hormone receptor-like 1	NM_001974	3.2	Signal transducer activity, receptor activity
Periostin, osteoblast-specific factor	NM_006475	2.1	Protein binding, heparin binding
Integrin $\beta$ 3-binding protein	NM_014288	2.1	Signal transducer activity
Filamin-binding LIM protein 1	NM_017556	0.27	Protein binding, metal ion binding
Integrin, $\beta$ 3	NM_000212	0.23	Receptor activity, protein binding
Collagen, type I, $\alpha$ 1	NM_000088	0.19	Extracellular matrix structural constituent
Integrin, $\beta$ 5	NM_002213	0.077	Receptor activity
Cysteine-rich, angiogenic inducer 61	NM_001554	0.067	Insulin-like growth factor binding
<b>Cell cycle</b>			
CDC45 cell division cycle 45-like	NM_003504	3.2	Protein binding
Karyopherin $\alpha$ 2 (RAG cohort 1, importin $\alpha$ 1)	NM_002266	3.2	Nuclear localization sequence binding
Cell division cycle 2, G1 to S and G2 to M	NM_033379	3	Cyclin-dependent protein kinase activity, protein binding
Cell division cycle-associated 8	NM_018101	2.6	Protein binding, cell cycle
BCL2-associated X protein	NM_004324	2.5	Protein binding
Cyclin B2	NM_004701	2.3	Translation initiation factor activity
Cyclin-dependent kinase inhibitor 1A (p21, Cip1)	NM_000389	0.5	Cyclin-dependent protein kinase inhibitor activity
Cullin 7	NM_014780	0.05	Protein binding
<b>Proliferation</b>			
Deoxythymidylate kinase	XM_932687	3.2	Thymidylate kinase activity
TTK protein kinase	NM_003318	2.6	Serine/threonine kinase activity
Fibroblast growth factor 18	NM_003862	2.3	Growth factor activity
Glypican 4	NM_001448	0.41	GPI anchor binding
Low-density lipoprotein receptor-related protein 5	NM_002335	0.29	Receptor activity
<b>Cell communication</b>			
Proenkephalin	NM_006211	12.1	Opioid peptide activity
Thyroid hormone receptor-associated protein 3	NM_005119	3	Ligand-dependent nuclear receptor, transcription coactivator
Rho GTPase activating protein 28	NM_030672	2.5	GTPase activator activity
Caspase recruitment domain family, member 8	NM_014959	2.5	Caspase activator activity, protein homodimerization activity
Peroxisome proliferative activated receptor, $\gamma$	NM_138711	0.23	Ligand-dependent nuclear receptor activity
Insulin-like growth factor-binding protein 3	NM_000598	0.27	Insulin-like growth factor binding
Dystrophin myotonic protein kinase	NM_004409	0.25	Magnesium ion binding
ADP-ribosylation factor 4	NM_001660	0.25	GTPase activity, GTP binding

*Continued*

Table 6 Continued

Gene description	GenBank accession no.	Fold change	Molecular functions
Potassium channel tetramerisation domain containing 11	NM_001002914	0.22	Ion channel activity, voltage-gated potassium channel activity
Phosphodiesterase 8B	NM_001029851	0.11	3',5'-cyclic-nucleotide phosphodiesterase activity
Mitogen-activated protein kinase kinase kinase 1 (MAP3K1)	XM_042066	0.02	Protein serine/threonine kinase activity
Response to stress			
RAD9 homolog B	NM_152442	14.9	Protein binding
Transient receptor potential cation channel, subfamily A, member 1	NM_007332	13	Ion channel activity, channel or pore class transporter activity
Proenkephalin	NM_006211	12.1	Opioid peptide activity
PMS1 postmeiotic Segregation increased 1	NM_000534	10.6	DNA binding, GTP binding
Fanconi anemia, complementation group A	NM_000135	4.9	Protein binding
Heat shock 70 kDa protein 2	NM_021979	0.33	Unfolded protein binding
Thioredoxin interacting protein	NM_006472	0.22	Protein binding
Histone deacetylase 5	NM_005474	0.19	Histone deacetylase activity
Response to external stimulus			
Jub, ajuba homolog	NM_032876	22.6	Zinc ion binding, metal ion binding
Serpin peptidase inhibitor, clade	NM_000602	0.33	Serine-type endopeptidase inhibitor activity
Peroxisome proliferators activated receptor $\gamma$	NM_138711	0.23	Ligand-dependent nuclear receptor activity
Thrombospondin 1	NM_003246	0.2	Structural molecule activity

The partial genes with known functions in 1 g vs. 2 g that were up-regulated or down-regulated significantly >2 folds were categorized.

specimen are also different, so the apparent gravity of specimen placed in HMGE can be attenuated or enhanced in different degree by magnetic force. The superconducting magnet therefore can simulate gravitational environment from hypo-gravity (0 g) to hyper-gravity (2 g). Since a high magnetic field coexists with different gravity levels at all time, four groups were designed in this study, namely 1 g group (normal gravity, 16 T), control group (normal gravity, geomagnetic field), 2 g group (2-fold gravity, 12 T), and 0 g group (hypogravity, 12 T). The relative magnetic effects can be obtained by comparing the results of 1 g vs. control group, and the relative gravitational effects also can be obtained by comparing the results of 0 g vs. 2 g group.

Microgravity/weightlessness-induced bone loss in humans and animals has been reported to be mediated at least in part by decreased osteoblast function [20]. In evaluating expressions of >40,000 human genes in MG-63 cells exposed to HMGE at 24 h and corresponding ground controls, we found some differential genes on the basis of typical cell functions, such as

proliferation, cell adhesion, apoptosis, cell cycle, cell communication, response to stress, and response to external stimulus. These results suggest that the characteristics of MG-63 cells may be altered by HMGE and MG-63 cells themselves may be sensitive to altered gravity levels and magnetic field levels. Our previous work showed that HMGE affected osteoblast-like cell MG-63 morphology, adhesion, proliferation, and secretion [16]. Alterations in the morphology, cytoskeleton, and gene expression for growth factors and matrix proteins are observed in osteoblastic cells *in vitro* under microgravity conditions [20]. Pardo *et al.* [21] have reported that simulated microgravity using the random positioning machine inhibits differentiation and alters gene expression profiles of 2T3 preosteoblasts. It has been reported that diamagnetic levitation changes growth, cell cycle, and gene expression of *Saccharomyces cerevisiae* [22,23].

It has also been reported that the cytoskeleton is the sensor of gravity in cells and very sensitive to gravity alteration and that microtubule self-organization is particularly dependent on gravity [24]. Mechanotransduction may be mediated at multiple locations inside the cell

**Table 7** Fold changes of the selected cytoskeleton-related genes in 0 g vs. control, 0 g vs. 2 g, and 1 g vs. control by qPCR and microarray assays

Gene name	0 g vs. control		0 g vs. 2 g		1 g vs. control	
	PCR	Microarray	PCR	Microarray	PCR	Microarray
<i>CDC42BPB</i>	1.94	NC	1.0	3.0	0.66	0.35
<i>Tropomodulin 3</i>	1.15	1.15	0.67	1.4	0.66	0.33
<i>Talin 1</i>	0.74	0.66	1.45	9.1	1.78	0.082
<i>SPTBN1</i>	2.05	3.25	5.77	2.3	0.39	0.29
<i>Supervillin</i>	0.72	13.9	2.08	2.5	0.34	0.38
<i>Adducin 3</i>	0.82	0.76	1.02	2.0	0.72	0.41
<i>Coactosin-like 1</i>	0.67	0.76	4.25	2.0	0.69	0.33
<i>Filamin A</i>	0.84	0.81	1.34	2.6	0.72	0.28
<i>SORBS3</i>	1.69	NC	1.41	2.9	6.16	0.19
<i>WASF2</i>	0.50	0.81	2.38	4.0	0.88	0.54
<i>WIPF1</i>	1.1	NC	1.23	2.1	0.91	0.57
<i>Plectin 1</i>	0.85	0.81	1.61	2.9	0.73	0.38
<i>Paxillin</i>	0.77	NC	1.26	4.8	0.39	0.16

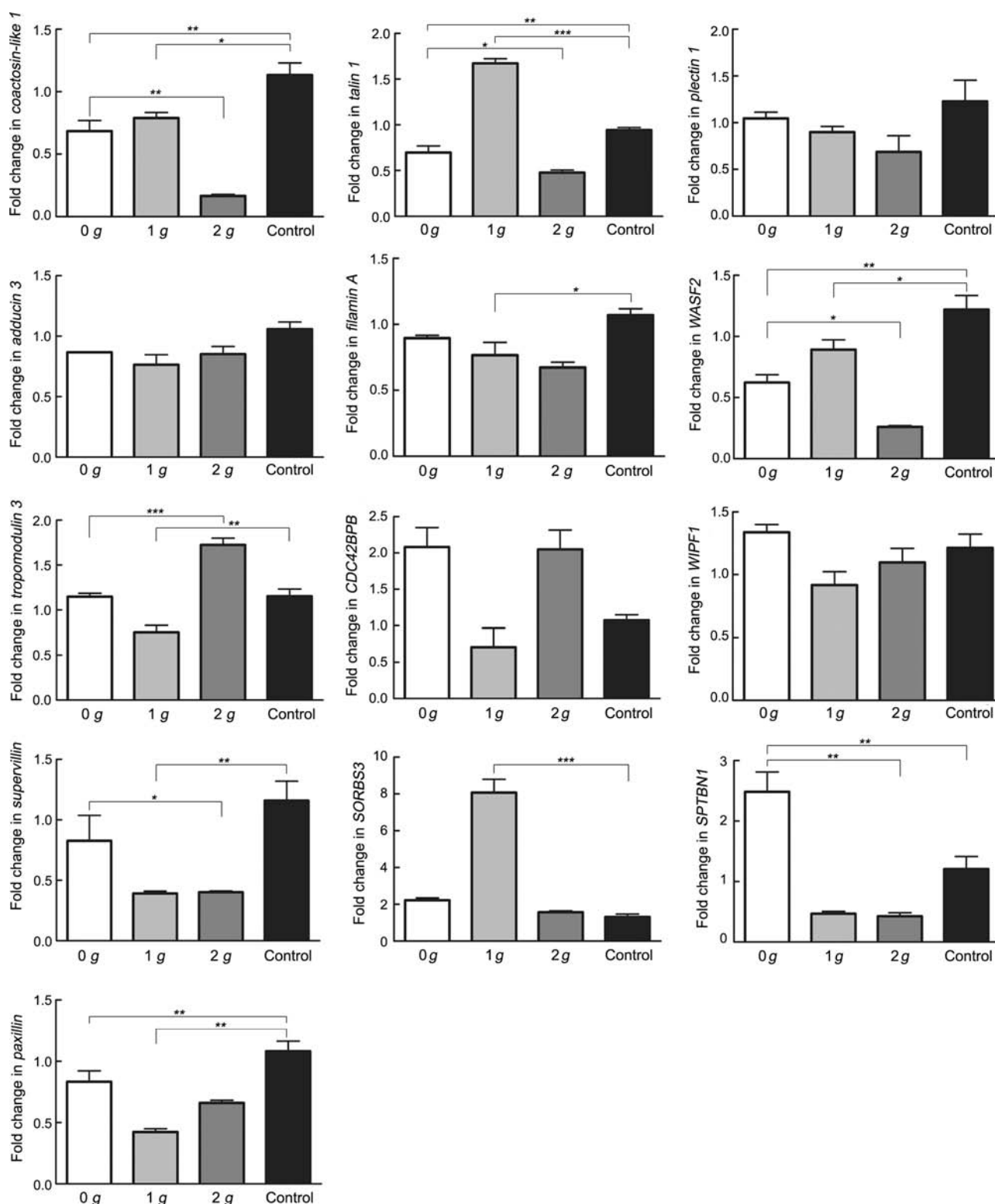
WASF2, WAS protein family, member 2; WIPF1, WAS/WASL interacting protein family, member 1; CDC42BPB, CDC42-binding protein kinase  $\beta$ ; SORBS3, sorbin and SH3 domain containing 3; NC, no change. RNA from cells sampled at 24 h in HMGE and ground controls was evaluated by cDNA microarray and by RT-PCR as described in Materials and Methods. Fold changes of 13 cytoskeleton-related genes in 0 g vs. control, 0 g vs. 2 g, and 1 g vs. control by QPCR and microarray analysis were listed.

through force-induced rearrangements within a tensionally integrated cytoskeleton [7]. The effects of weightlessness on microtubule self-organization can be studied using ground-based equipment and the findings closely resemble that of the spaceflight experiment [25]. Alenghat and Ingber [26] have reported that all signals point to cytoskeleton, matrix, and integrins. We therefore wonder what will happen when gravity disappears or diminishes and what changes in gene expression profile will occur when the cytoskeleton relaxes.

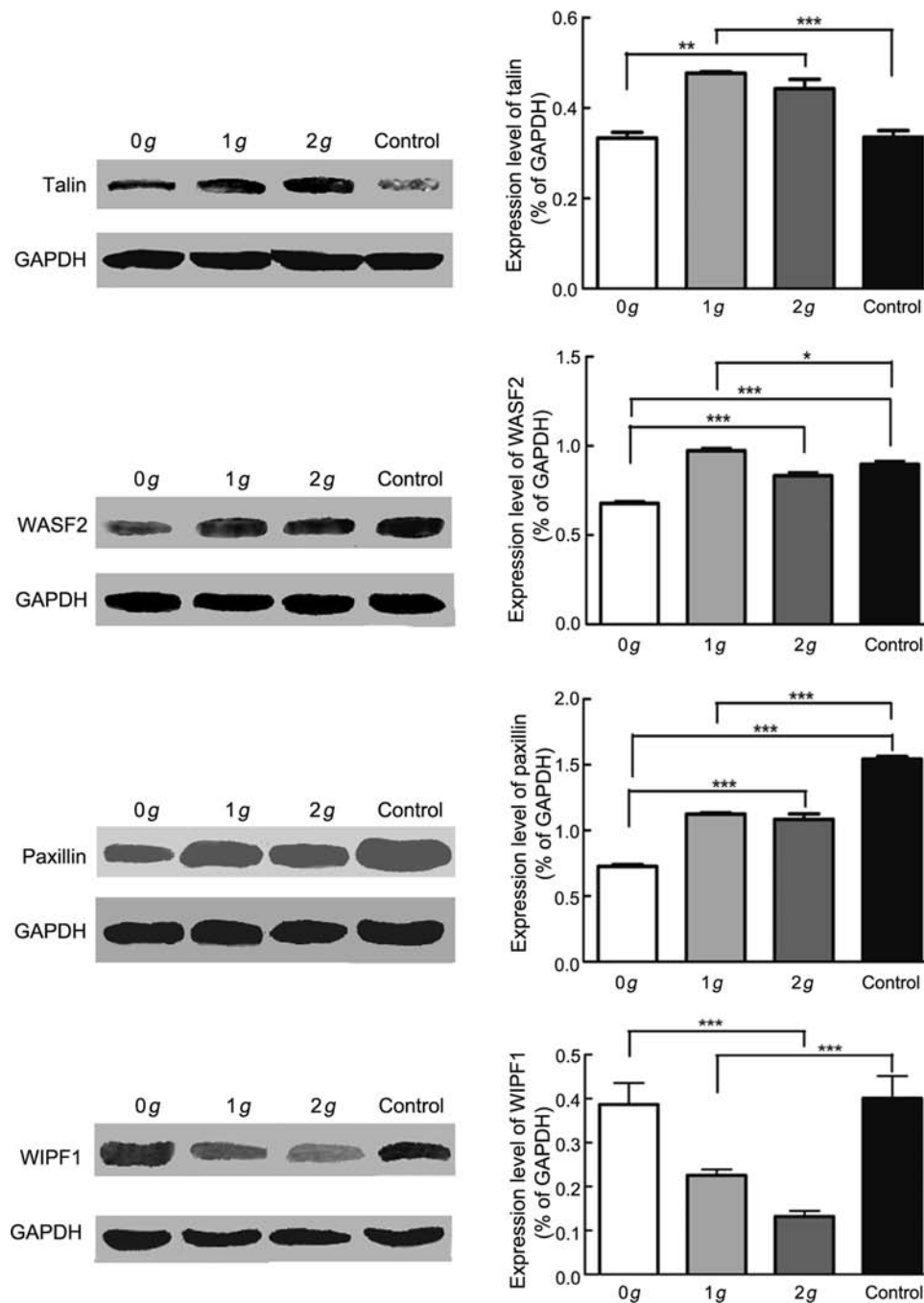
cDNA microarray revealed that the expression of 13 cytoskeleton-related genes (*adducin 3*, *coactosin-like 1*, *filamin A*, *SORBS3*, *CDC42BPB*, *tropomodulin 3*, *talin 1*, *SPTBN1*, *supervillin*, *WASF2*, *WIPF1*, *plectin 1*, and *paxillin*) was significantly altered by HMGE. The expression of 13 cytoskeleton-related genes was all up-regulated in diamagnetic levitation (0 g) vs. 2 g condition, but it was down-regulated in 1 g vs. ground control. The changes in cytoskeleton-related genes induced by HMGE may imply that cytoskeletal elements are not properly reassociated or there are some anomalies in translation downstream. It has been reported recently that modeled microgravity by clinorotation differentially inhibits T-lymphocyte transcription factor activation [27]. Our finding that MG-63 cell exposure to diamagnetic levitation (0 g) vs. 2 g condition increased *plectin 1*

expression is consistent with the spaceflight data obtained with Jurkat cells [28]. We also found that diamagnetic levitation (0 g) significantly raised *SPTBN1* and *supervillin* expression in comparison with control. Lewis *et al.* [28] reported that filamin and *SPTBN1* were not up-regulated in space-flown compared with ground control in leukemic T lymphocytes by cDNA microarray. Spectrin, a major cytoskeletal protein in a wide-range of cells, plays an important role in maintenance of plasma membrane integrity and cytoskeletal structure [29,30]. We therefore hypothesize that cytoskeleton-related genes may be via readjusting themselves expression or distribution to further influence cytoskeleton arrangement to respond to external environment alteration.

Both of PCR and microarray analysis showed that *WASF2* and *WIPF1* gene expressions were up-regulated in 0 g vs. 2 g condition. It suggests that *WASF2* and *WIPF1* genes are more sensitive to altered gravity than to altered magnetic field. *WASF2* is a downstream effector of Cdc42 that is implicated in actin polymerization and cytoskeletal organization as well as it appears to be involved in mediating actin reorganization by inducing the formation of actin filament clusters in response to GTP binding and activating Rac [31]. *WIPF1* encodes a protein that plays an important role in the organization of the actin cytoskeleton. Alteration of *WASF2* and



**Figure 2** Microarray results were verified using real-time PCR for genes that were up-regulated or down-regulated in response to HMGE. Total RNA was extracted and qPCR assay was used to further identify for 13 selected genes. The method of relative quantification was used to estimate the relative expression changes of cytoskeleton-associated gene expression in MG-63 cells exposed to HMGE. The changes in cytoskeleton gene expression, normalized to 18S or GAPDH under HMGE were calculated. Fold changes in cytoskeleton gene expression relative to control were shown. The difference between 0g vs. control, 0g vs. 2g, and 1g vs. control was statistically analyzed by one-way ANOVA. \*\*\* $P < 0.001$ ; \*\* $P < 0.01$ ; \* $P < 0.05$ .



**Figure 3** Detection of the effects of HMGE on paxillin, talin 1, WASF2, and WIPF1 expressions in MG-63 cells by western blot analysis After MG-63 cells were cultured in HMGE for 24 h, the cells were collected and re-suspended in lysis buffer. Equal total protein loads were loaded on the lanes of the SDS–polyacrylamide gel and separated by electrophoresis. The proteins were then transferred to a PVDF membrane. After blocking with Odyssey blocking buffer, the blots were incubated overnight with antibodies against paxillin, WASF2, WIP, and talin 1. Blots were incubated with IRDye 800-labeled secondary antibodies for 1 h with GAPDH expression as a loading control. The bands were then scanned by Odyssey Infrared Imaging System. The expression level of each protein was quantified by Image J software and the difference between 0 g vs. control, 0 g vs. 2 g, and 1 g vs. control was statistically analyzed by one-way ANOVA. \*\*\* $P < 0.001$ ; \*\* $P < 0.01$ ; \* $P < 0.05$ .

WIPF1 message in MG-63 cells exposed to 0 g environment for 24 h may imply that cytoskeletal-related proteins readjust to respond to gravity environment

alteration. Our previous studies reported that HMGE also affects the expression of cytoskeletal linker protein, microtubule actin cross-linking factor (MACF1), and the

association of MACF1 with actin and microtubule cytoskeleton [32]. Tropomodulin 3 is a ubiquitous TMOD isoform present in non-erythroid cells, where it regulates dynamic actin processes [33]. Our results showed that *tropomodulin 3* expression was decreased under high magnetic field 1 g vs. control condition by PCR and microarray analysis. It suggests that tropomodulin 3 may be involved in cytoskeleton rearrangement induced by high magnetic field. Paxillin and talin are focal adhesion proteins, which directly or indirectly connect microfilaments or microtubules to cell membrane [34]. Our results indicated that HMGE also changed *paxillin* and *talin 1* expressions. Infanger *et al.* [35] reported that long-term conditions of mimicked weightlessness increased talin expression in thyroid cells.

PCR analysis of *talin 1* and *SORBS3* under high magnetic field 1 g vs. control condition was not in accord with microarray analysis. The expression of *tropomodulin 3* in 0 g vs. 2 g by PCR analysis was also inconsistent with microarray analysis. The discordance of real-time PCR vs. microarray may be explained by the decreased sensitivity of the microarray *per se*, or by the relatively low expression levels of these genes. It is reported that both quantitative real-time PCR (qPCR) and microarray analysis have inherent pitfalls; thus, the transcriptional levels quantified with each method are different [36].

Based on the data of the microarray and real-time PCR, we performed western blot assay to examine the expression levels of four selected proteins (talin 1, paxillin, WASF2, and WIPF1) with specific antibodies. Accordant with PCR and microarray analysis, western blot analysis showed that WIPF1 expression were significantly increased in 0 g condition compared with that in 2 g or control condition, whereas WASF2 and paxillin expressions were both significantly decreased in 0 g condition compared with that in control condition. However, PCR analysis of *WASF2*, *paxillin*, and *talin 1* expressions in 0 g vs. 2 g or 1 g vs. control disagreed with western blot analysis. The disagreement of mRNA and protein levels suggests that the effects of HMGE on *WASF2* and *paxillin* genes on transcription and translation stage are different. With the results of microarray, real-time PCR, and western blot, we confirmed that *WASF2* and *WIPF1* are more sensitive to altered gravity levels than to altered magnetic field, and *talin 1* and *paxillin* were sensitive to both magnetic field and gravity changes.

In summary, the present study used DNA microarray analysis to provide a new and comprehensive cognition to the effects of HMGE on gene expression profiles in

osteoblast-like cells, and has selected the genes that may be sensitive to altered gravity or magnetic field. The study shows that *WASF2* and *WIPF1* genes are mechanosensitive cytoskeleton-related genes. The identification of mechanosensitive cytoskeleton-related genes will help us to understand the mechanism of bone loss to open a new route for the therapeutic control of bone mass and provide new potential countermeasures.

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