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Identification of Mechanosensitive Genes in Osteoblasts by Comparative Microarray Studies Using the Rotating Wall Vessel and the Random Positioning Machine

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Abstract Weightlessness or microgravity of spaceflight induces bone loss due in part to decreased bone formation by unknown mechanisms. Due to difficulty in performing experiments in space, several ground-based simulators such as the Rotating Wall Vessel (RWV) and Random Positioning Machine (RPM) have become critical venues to continue studying space biology. However, these simulators have not been systematically compared to each other or to mechanical stimulating models. Here, we hypothesized that exposure to RWV inhibits differentiation and alters gene expression profiles of 2T3 cells, and a subset of these mechanosensitive genes behaves in a manner consistent to the RPM and opposite to the trends incurred by mechanical stimulation of mouse tibiae. Exposure of 2T3 preosteoblast cells to the RWV for 3 days inhibited alkaline phosphatase activity, a marker of differentiation, and downregulated 61 and upregulated 45 genes by more than twofold compared to static 1 g controls, as shown by microarray analysis. The microarray results were confirmed by real-time PCR and/or Western blots for seven separate genes and proteins including *osteomodulin*, *runx2*, and *osteoglycin*. Comparison of the RWV data to the RPM microarray study that we previously published showed 14 mechanosensitive genes that changed in the same direction. Further comparison of the RWV and RPM results to microarray data from mechanically loaded mouse tibiae reported by an independent group revealed that three genes including *osteoglycin* were upregulated by the loading and downregulated by our simulators. These mechanosensitive genes may provide novel insights into understanding the mechanisms regulating bone formation and potential targets for countermeasures against decreased bone formation during space flight and in pathologies associated with lack of bone formation. J. Cell. Biochem. 101: 587–599, 2007.

Key words: bone formation; alkaline phosphatase; RWV; RPM; microgravity; osteoblasts

Exposure to microgravity conditions during spaceflight induces several adaptive and pathological changes to the human body, posing health risks to astronauts during spaceflight. These pathological changes include reduced cardiovascular performance [Arbeille et al., 1995; White and Blomqvist, 1998], immune system dysfunction [Sonnenfeld et al., 2003],

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skeletal muscle atrophy [Katkovsky and Pomyotov, 1976; Miyamoto et al., 1998], and decreased bone mass [Lang et al., 2004; Smith et al., 2005a]. Astronauts lose bone mass as much as 1–2% per month during spaceflight, and this may be due to either decreased bone formation by osteoblasts or increased osteolytic functions of osteoclasts [Carmeliet and Bouillon, 1999; Smith et al., 2005a]. Many of the known space-inflicted pathologies have been the subject of research, and bone loss has not yet been adequately counteracted with nutrition [Smith et al., 2005b] or rigorous exercise [Lang et al., 2004]. This is due in part to the lack of understanding of the mechanisms leading to the effects of microgravity on bone cells.

While it would be ideal to conduct studies to discover the mechanisms in real microgravity conditions, it has become extremely difficult, expensive, and impractical to most investigators. Therefore, it is becoming increasingly important to have several validated in vitro ground-based models, and whenever possible, comparisons should be made to real microgravity, in vivo models of microgravity, and in vivo mechanical loading models. The Rotating Wall Vessel (RWV) bioreactor and the Random Positioning Machine (RPM) are the two most commonly used microgravity simulators. While the RWV models microgravity conditions by maintaining continuous free-fall, the RPM exposes cells and tissues to random speeds and orientation such that the gravity vector is randomly moved relative to the specimen [Schwarz et al., 1992; Hoson, 1997]. The RWV (Fig. 1a), also known as a modeled microgravity device, is designed such that the particles within it are subjected to solid body rotation, which is achieved as the vessel rotates and the liquid accelerates, such that the entire body of fluid is rotating at the same angular velocity as the vessel wall. The RWV generates minimum shear forces since there is no internal mixer. Thus, the cells in the RWV are subjected to solid body rotation and are constantly suspended [Schwarz et al., 1992]. In contrast, the RPM (Fig. 1b) is a three dimensional clinostat that rotates about two orthogonal axes. There are two frames, an inner and an outer frame, which rotate about distinct axes. The platform where the cells rest is the inner frame. The RPM operates in a random mode, where rotation speeds and directions are randomized using software developed by the Dutch Space Agency (Leiden, the Netherlands). The continuous movement of the gravity vector averages the vector to zero over time, a method called gravity-vector averaging [Huijser, 2000].

Both the RWV [Ontiveros et al., 2003; Zayzafoon et al., 2004] and the RPM [Nakamura et al., 2003; Pardo et al., 2005] have been previously used by various groups to assess the effects of microgravity on bone cells. However, it has not been reported whether the various microgravity simulators such as RPM and RWV induce similar changes with regard to bone loss or bone formation. Since the RPM and RWV simulate microgravity by distinct modes, it is imperative to compare the data produced by each simulator.

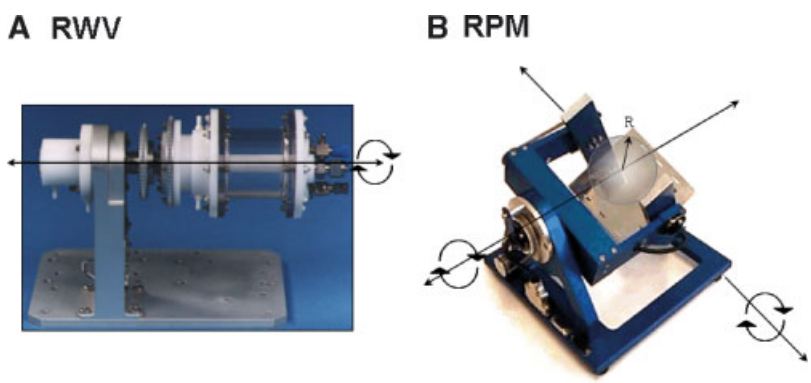


Fig. 1. Simulated microgravity using the RWV and the RPM. **A**: The RWV bioreactor is a clinostat that simulates microgravity conditions by maintaining particles in a free-fall state. The arrow indicates the movement about the axis of rotation. **B**: The Random Positioning Machine (RPM) is a three-dimensional clinostat that simulates microgravity by continuously rotating the cells in a random orientation at random speeds [Pardo et al., 2005]. The RPM moves about two axes of rotation, as shown by the arrows. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Recently, we have characterized gene transcript expression profiles of 10,000 genes using Codelink gene chips in 2T3 murine pre-osteoblasts exposed to RPM-simulated microgravity conditions [Pardo et al., 2005]. We compiled a list of 140 genes (mechanosensitive genes) that changed in response to the simulated microgravity conditions with the RPM, and some of these genes may be involved in decreased bone formation. In contrast, Xing et al. [2005] have characterized gene transcript profile changes induced by exposing mouse tibiae to mechanical stimulation, an apparent opposite of microgravity conditions, using a four-point bending method. Therefore, we hypothesized that exposure to RWV inhibits differentiation and alters gene expression profiles of 2T3 cells, and a subset of these mechanosensitive genes behaves in a manner consistent to the RPM and opposite to the trends incurred by mechanical stimulation of mouse tibiae.

To test this hypothesis, we carried out an additional gene transcript profiling study with 2T3 cells exposed to the NASA-developed RWV bioreactor and compared the results to our previous transcript database obtained with the RPM. In addition, we compared both the RWV and RPM transcript databases to the database obtained from the mechanically loaded mouse tibiae [Xing et al., 2005]. From these studies, we found a subset of genes that was affected in all three studies, suggesting that these genes may be implicated in microgravity-induced decrease in bone formation.

MATERIALS AND METHODS

Cell Culture

2T3 murine osteoblast precursor cells were cultured in MEM (α-minimal essential medium containing 10% fetal bovine serum (Atlanta Biologicals) with 100 U/ml penicillin and 100 μg/ml streptomycin) in a standard humidified incubator (37°C, 5% CO_2) as described previously by us [Pardo et al., 2005]. For RWV experiments, trypsinized cells were seeded on microcarriers (#P102-1521, Solohill, Ann Arbor, MI), which have polystyrene cores with no extracellular matrix coating. The cells were allowed to grow in low adhesion 100 mm plates (Corning, Inc. #3262) for 3 days to confluency and then were exposed to modeled microgravity using the RWV or static 1 g control as described previously by us [Ward et al., 2006].

Rotating Wall Vessel (RWV)

Rotation of the RWV (Fig. 1A) maintains microbeads attached with 2T3 pre-osteoblasts in a continuous free-fall state, simulating a microgravity environment [Schwarz et al., 1992]. The RWV was rotated at 22 rpm to achieve continuous freefall of the cell-bead aggregates. This RWV system has been shown to be an effective simulator of real microgravity conditions as demonstrated in comparative studies using blood mononuclear cells in space flight missions STS-54 and STS-56 [Pellis et al., 1997]. Cells were exposed to the RWV for 3 days so that the data could be directly compared to data obtained with RPM for the same time period [Pardo et al., 2005].

Whole Cell Lysate and Alkaline Phosphatase Enzyme Activity

Following the RWV or static 1 g exposure, cells and beads were washed with cold phosphate buffered saline (PBS) two times and lysed in 500 μl of a lysis buffer containing 0.1% Triton X-100 in 1 mM $MgCl_2$, 20 mM Tris-HCl, and 0.1 mM $ZnCl_2$. The lysate and bead mixture was centrifuged for 3 min at 1,500 rpm to separate the beads from the lysate. The lysate was pipetted out of the mixture and stored at -20°C until needed. A Bio-Rad DC protein assay and alkaline phosphatase activity assay (Sigma) were carried out as we described previously [Pardo et al., 2005].

Immunoblotting

Aliquots of cell lysate were resolved on a SDS–PAGE gel and transferred to a polyvinylidene difluoride membrane (Millipore) [Boo et al., 2002]. The membrane was incubated with a primary antibody overnight at 4°C and then incubated with a secondary antibody conjugated with alkaline phosphatase for 1 h at room temperature. Expression was detected by a chemiluminescence method and the intensities of immunoreactive bands were determined by densitometry [Boo et al., 2002]. Antibodies specific for BMP4 (Santa Cruz Biotechnologies), osteoglycin (R&D Biosciences), and peroxiredoxin I and peroxiredoxin IV (Lab Frontier, Seoul, Korea) were used.

RNA Isolation

Total RNA was prepared by using the RNeasy Mini kit (Qiagen). Briefly, after two cold PBS

washes, 500 μl of RLT lysis buffer containing β-mercaptoethanol was added to the bead and cell mixture to isolate the RNA from the beads and cells. The bead and lysate mixture was centrifuged for 3 min at 1,500 rpm to separate the beads from the RNA, and the RNA was pipetted to the homogenizing column in the RNeasy kit. The RNA was purified with the kit and stored at −80°C until needed.

Affymetrix Gene Microarrays

All RNA samples passed the Affymetrix test for RNA quality and concentration before proceeding to the gene chip study carried out in the Microarray Core Facility at the Baylor College of Medicine. The samples used for these studies were obtained from three independent biological experiments as follows: We pooled cells from three independent RWV experiments carried out at the same time to obtain a single sample (total RNA) that was used for a single microarray. This process minimized inter-experimental variations and provided a sufficient amount of RNA for each microarray. We repeated this process three different times to obtain a final sample size of three microarray chips for static samples and three microarray chips for RWV samples. The array used was Affymetrix GeneChip® Mouse 430 2.0, and the data were background adjusted and normalized to median intensity. The data were transformed so that fold changes were obtained by dividing the averaged normalized intensities of the microgravity samples by the averaged normalized intensities of the static samples. Thus, a fold change above 1.0 indicates a gene upregulated by simulated microgravity and below 1.0 indicates a gene downregulated by simulated microgravity. The data were statistically analyzed by DNA chip analyzer (dChip) as described below in the statistical analysis section and filtered for fold change threshold, and the genes that changed in response to simulated microgravity by more than twofold above or below the static 1 g controls with P values of less than 0.05 were deemed considerable and statistically significant. The number of differentially expressed genes and false discovery rate (FDR) were calculated for each of 500 permutations, and 1,934 genes had P values less than 0.05 at median FDR. Roughly, half of these genes were false positives. GoMiner software (http://www.miblab.gatech.edu/gominer) was used to sort the genes by biological processes and to assign some of the molecular functions of each known gene [Zeeberg et al., 2003].

Reverse Transcriptase and Real-Time Polymerase Chain Reaction

Total RNA was reverse transcribed by using random primers and a Superscript-II kit (Life Technology) [Sorescu et al., 2003]. The synthesized and purified cDNA was amplified using a LightCycler (Roche Applied Science), and the size of each PCR product was verified by agarose gel electrophoresis as described by us [Sorescu et al., 2003]. The mRNA copy numbers were determined based on standard curves generated with the genes of interest and 18S templates. The 18S primers (50 nM at 61°C annealing temperature; Ambion) were used as an internal control for real-time PCR using capillaries (Roche Applied Science), recombinant Taq polymerase (Invitrogen), and Taq start antibody (Clontech). The primer pairs for the quantitative real-time PCR are listed in Table I along with Light Cycler conditions. Real-time PCR for the listed genes were carried out in PCR buffer (20 mM Tris-HCl, pH 8.4 at 25°C, 4 mM $MgCl_2$, 250 μg/ml bovine serum albumin, and 200 μM deoxynucleotides) containing SYBR green (1:84,000 dilution), 0.05 U/μl Taq DNA polymerase, and Taq

TABLE I. Primers and LightCycler Conditions Used for Real-Time PCR

Gene bank accession no.	Gene	Primers (5′–3′)	bp	LightCycler conditions	Ref.
NM_009820	*Runx2*	Fw GACAGAAGCTTGATGACTCTAAACC Rv TCTGTAATCTGACTCTGTCCTTGT	171	7 s at 62°C 9 s at 72°C	Ontiveros et al. [2004]
NM_007554	*BMP4*	Fw CTGCGGGACTTCGAGGCGACACTTCT Rv TCTTCCTCCTCCTCCTCCCCAGACTG	150	7 s at 65°C 7 s at 72°C	Sorescu et al. [2003]
NM_011199	*PthR1*	Fw GCACACAGCAGCCAACATAA Rv CGCAGCCTAAACGACAGGAA	531	7 s at 63°C 22 s at 72°C	Wang et al. [2002]
NM_012050	*Omd*	Fw GACGGGCTGGTGAATGTGACTATGCTTGA Rv CCAAGGGGCATTGATTCTAATCTGTTATT	147	7 s at 63°C 10 s at 72°C	Pardo et al. [2005]

Start antibody (1:100 dilution) as described previously by us [Sorescu et al., 2003].

Statistical Analysis

Data are expressed as mean ± SEM with n numbers representing biological replicates using different cell preparations and RWV experiments. Statistical analysis was performed using the Student's *t*-test for ALP enzyme activity experiments. A significance level of $P < 0.05$ was considered statistically significant. The microarray data was analyzed with the dChip, a program based on the Model-Based Expression Index (MBEI) method [Li and Wong, 2001a,b]. The raw data were normalized using the invariant set normalization, and the average expression values are represented as model-based expression indices. We used the 'PM only model,' and the expression values were expressed in log2 scale. Differential gene expression between two groups of samples was analyzed by the *t*-test built in dChip.

RESULTS

RWV-Simulated Microgravity Inhibited Alkaline Phosphatase Activity

Expression of alkaline phosphatase (ALP) increases as osteoblasts mature and differentiate, and its enzyme activity is often used as a marker for bone formation. Thus, we chose to examine the effects of RWV-simulated microgravity on ALP enzyme activity in pre-osteoblasts. Exposure of cells to the RWV (Fig. 1A) for 3 days significantly decreased ALP activity (Fig. 2A) and mRNA (Fig. 2B) in comparison to static 1 g controls. This finding, which is consistent to our previously reported data with the RPM [Pardo et al., 2005], suggests that the simulated microgravity conditions induced by either the RWV or the RPM results in the similar inhibitory effect on osteoblast differentiation.

RWV-Simulated Microgravity Altered Gene Expression Profiles of 2T3 Cells

DNA microarray studies were performed on samples obtained from 2T3 cells exposed to static 1 g or RWV-simulated microgravity for 3 days. Among approximately 40,000 genes tested by the Affymetrix array, 61 were downregulated while 45 were upregulated statistically significantly ($P < 0.05$) by more than twofold above the static 1 g control as shown in a scatter plot of the genes (Fig. 3). Table II provides a list of genes from the microarray that may be implicated in bone formation or mineralization as determined by literature survey,

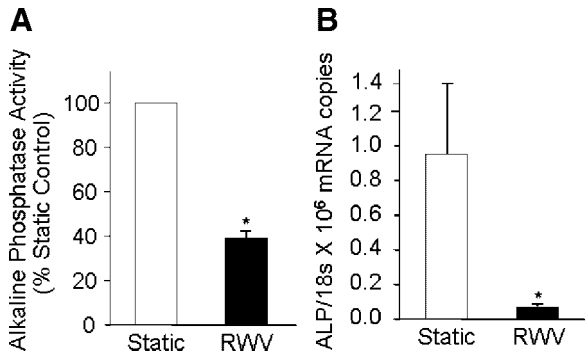


Fig. 2. RWV exposure inhibits alkaline phosphatase activity and mRNA expression in 2T3 cells. Confluent 2T3 cells grown on microcarriers were placed in the RWV for 3 days of rotation or exposed to static 1 g conditions for the same period. The alkaline phosphatase activity (**A**) was determined by a colorimetric assay using cell lysate and *alkaline phosphatase* (*ALP*) mRNA levels (**B**) determined by real time PCR with 18S as an internal control. The graphs show mean ± SEM (*$P < 0.05$, n = 6 for A and n = 3 for B).

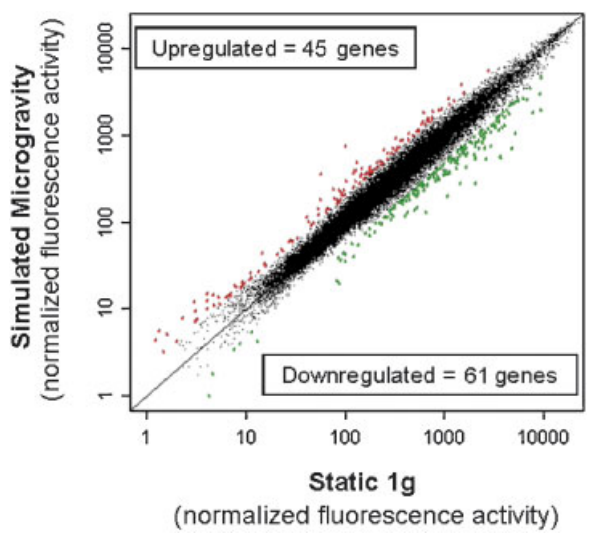


Fig. 3. The effects of RWV-simulated microgravity on gene transcript profiles of 2T3 cells. Total RNA was isolated from 2T3 cells exposed to RWV or static 1 g controls for 3 days. cRNA was then prepared and analyzed by Affymetrix microarrays corresponding to 40,000 mouse genes. The scatter plot shows mean intensities of each gene probe using the data obtained from all microarrays. Statistical analysis identified 45 upregulated genes (red dots, above diagonal line) and 61 downregulated genes (green dots, below diagonal line) by more than twofold compared to static 1 g control ($P < 0.05$, n = 3 each static 1 g and RWV). Unchanged genes are shown in black in the scatter plot. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

TABLE II. The Effect of Microgravity on Selected Genes That May be Involved in Osteoblast Differentiation and Matrix Mineralization

Accession #	Gene name	Fold Δ[a]	*P*-value	Molecular function
NM_012050	*Osteomodulin*	0.10	<0.05	Aka osteoadherin, may mediate cell attachment
NM_008760	*Osteoglycin*	0.23	<0.05	Binds to TGF-beta, no GAG in bone, keratan sulfate in other tissues
NM_025711	*Asporin*	0.28	<0.05	Porin activity; cartilage extracellular protein
BC002065	*Serine (or cysteine) proteinase inhibitor, clade A, member 3G*	0.35	<0.05	May be involved in osteoclast function with MMPs and cathepsins
NM_007729	*Procollagen, type XI, alpha 1*	0.37	<0.05	Present in cartilage
NM_011581	*Thrombospondin 2*	0.39	<0.025	Involved in cell attachment
NM_016873	*WNT1 inducible signaling pathway protein 2*	0.39	<0.05	Involved in WNT pathway, WNT stimulated by BMPs
BB781435	*Nidogen 2*	0.39	<0.005	Calcium binding
NM_007554	*Bone morphogenetic protein 4*	0.40	<0.0025	Growth factor and cytokine activity
NM_011693	*Vascular cell adhesion molecule 1*	0.41	<0.025	Cell adhesion molecule activity
NM_012043	*Immunoglobulin superfamily containing leucine rich repeat (ISLR)*	0.42	<0.05	Involved in cell attachment
NM_009144	*Secreted frizzled-related sequence protein 2*	0.43	<0.05	WNT signaling pathway antagonist
BB431535	*Matrix metalloproteinase 16*	0.47	<0.05	Involved in osteoclast function and bone resorption
BC014690	*Transforming growth factor, beta 3*	0.59	<0.05	Growth factor and cytokine activity
AF053954[b]	*cbfa1/runx2 (osf2)*	0.69	>0.05	Essential transcription factor for osteoblast differentiation and bone formation
NM_008216	*Hyaluronan synthase 2*	0.74	<0.05	With versican-like protein works to captures space destined to become bone
NM_007833	*Decorin*	0.76	<0.025	Binds to collagen and may regulate fibril diameter
NM_007431	*Alkaline phosphatase 2, liver*	0.82	<0.05	Essential for hydroxyapatite formation and matrix mineralization
BG092290	*Insulin-like growth factor 2 receptor*	1.43	<0.025	Signal transduction and hormone activity
NM_020275	*Tumor necrosis factor receptor superfamily, member 10b*	1.43	<0.05	Growth factor and cytokine activity
NM_010554	*Interleukin 1α*	1.58	<0.05	Potent stimulators of bone resorption
BM935811	*Integrin α6*	2.47	<0.05	Cell adhesion molecule
AK003744	*Cystatin E/M*	3.45	<0.05	Antagonist to cathepsin family

Sorted based on fold changes.
[a]Fold = RWV/static 1 g.
[b]Confirmed by RT-PCR with $P < 0.05$.

and these genes are organized by fold change with molecular function as defined by GoMiner, when known. Supplementary Table 1 shows a larger subset of these genes organized by biological process. Supplementary Table 2 list genes that may be involved in osteoblast function with relaxed *P*-value stringency. The entire RWV array data can be accessed from Gene Expression Omnibus (GEO) with accession number GSE4658. The RPM microarray data that we previously published can be accessed with numbers GDS928 or GSE1367 [Pardo et al., 2005].

Quantitative Real Time-PCR and Immunoblotting Validated the Microarray Data

To confirm our microarray data, we performed real time PCR and Western blot analyses for a select subset of genes that may be implicated in bone formation. The same samples that were used for the microarrays were used for the real time-PCR, and additional RWV experiments were performed to obtain protein samples for the Western blots. The 2T3 cells exposed to the RWV had decreases in *runt related transcription factor 2* (*Runx2*), *bone morphogenic protein 4* (*BMP4*), *parathyroid hormone receptor 1* (*PthR1*), and *osteomodulin* (*Omd*) gene expression by a fold change of 0.69, 0.40, 0.54, and 0.10, respectively, as evaluated by the Affymetrix microarray. The gene expression fold changes by real time PCR for *Runx2*, *BMP4*, *PthR1*, and *Omd*, were 0.38, 0.26, 0.59, and 0.10, respectively (Fig. 4A–E). Additionally, we confirmed the expression of downregulated genes *BMP4*, *peroxiredoxin IV* (*PrxIV*), and *osteoglycin* (*Ogn*) and upregulated gene *peroxiredoxin I* (*PrxI*) by immunoblotting. The Affymetrix fold changes for *BMP4*, *PrxIV*, *Ogn*, and *PrxI* were 0.40, 0.66, 0.23, and 1.23. We showed that the fold changes for the proteins by Western blot were 0.59, 0.68, 0.32, and 1.58, respectively (Fig. 4F–J). The different methodologies used, real time PCR, immunoblotting, and the microarray assay, produced highly consistent results, providing a level of assurance regarding the validity of the microarray data.

Comparison of the RPM and RWV Microarrays Revealed 14 Genes That Changed in the Same Direction

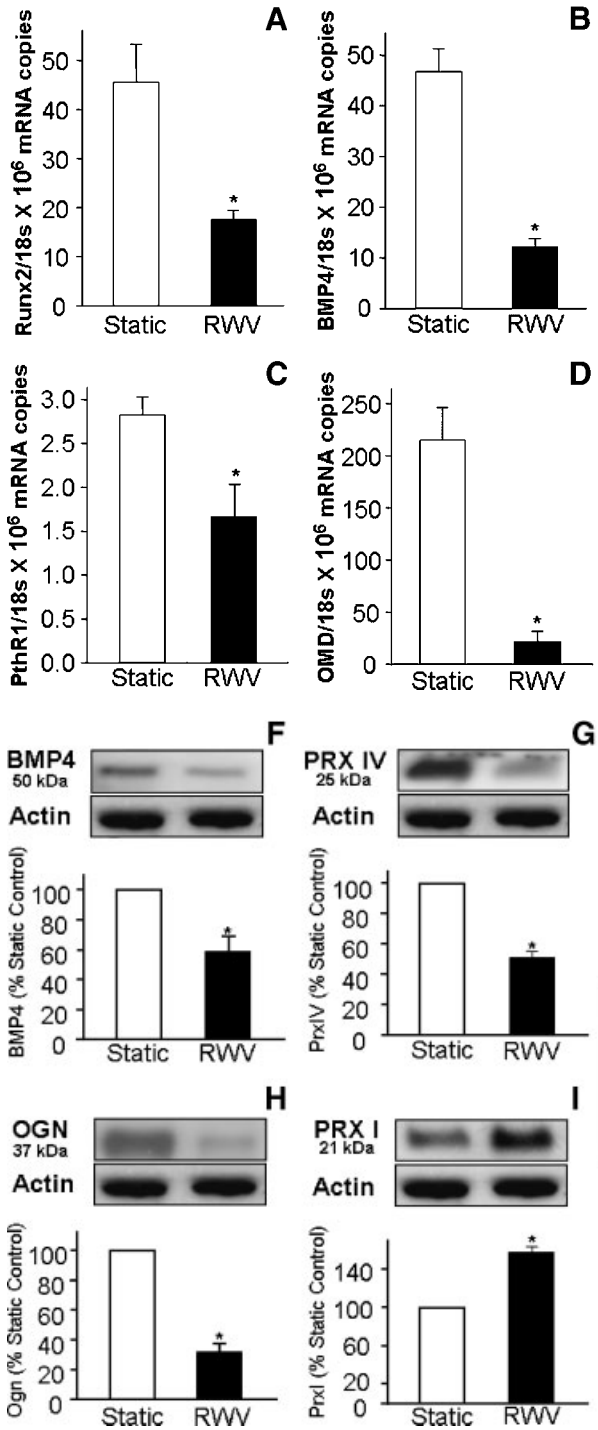
There was a subset of genes that demonstrated similar expression changes when exposed to simulated microgravity in both the RPM and RWV experiments. Table III displays those genes that changed statistically significantly with $P < 0.05$. There were 13 downregulated genes and one upregulated gene in this group. Since our experimental sample sizes ($n = 3$ each) are relatively small, we felt it was important to evaluate which genes changed commonly in both simulators with less stringent *P*-value cutoffs ($P < 0.1$). Under this non-stringent condition, we found additional genes, 11 downregulated and 5 upregulated genes, whose change in expression did not reach statistical significance ($0.05 < P < 0.1$) but changed in the same direction with exposure to both simulators (data not shown). Table III organizes the significant genes by biological process as defined by GoMiner and associates each gene with a molecular function, if known.

Comparison of Microgravity Microarrays to a Mechanical Loading Microarray Showed That Three Genes Changed in Opposite Directions Between Microgravity and Mechanical Loading

Recently, Xing et al. [2005] published microarray data on mouse tibiae that were mechanically loaded. Briefly, they loaded mice in a four point bending mechanical device for 4 days and total RNA was obtained from the region of the mechanically stimulated tibia. The untreated tibiae of the same mice were used as unloaded controls, and they used a 22,000 gene Agilent Technologies microarray. We compared our RPM and RWV microarrays to their published data and found that three genes changed in opposite directions with $P < 0.05$. Table IV lists the three genes that were upregulated by loading and were downregulated in the cells exposed to the RPM or the RWV. However, with less stringent *P* values ($P > 0.05$), there were an additional 13 genes that changed in opposite directions as shown in Supplementary Table 3.

DISCUSSION

We have previously shown that exposure of 2T3 cells grown in Opticell membranes to the 3D-clinostat RPM inhibits alkaline phosphatase activity, while gene transcript studies using 10,000 mouse genes produced a list of 52 upregulated and 88 downregulated genes by more than twofold above the static 1 g control [Pardo et al., 2005]. Here, we used another approach to expose 2T3 cells to simulated



E

Gene	qRT-PCR	Microarray
Runx2	0.38±0.16	0.69±0.02
BMP4	0.26±0.14	0.40±0.02
PthR1	0.59±0.17	0.54±0.06
OMD	0.10±0.27	0.10±0.08

J

Gene	Western	Microarray
BMP4	0.59±0.10	0.40±0.02
PrxIV	0.68±0.06	0.66±0.01
Ogn	0.32±0.15	0.23±0.09
PrxI	1.58±0.04	1.23±0.01

Fig. 4. Verification of microarray results by real time PCR and Western blot. Aliquots of total RNA used for microarray studies ($n = 3$) were used for the quantitative real time PCR assay for *Runx2* (**A**), *BMP4* (**B**), *PthR1* (**C**), and *osteomodulin* (*OMD*, **D**) using 18S rRNA as internal controls. Additional experiments were performed to obtain cell lysate for the Western blot assay using antibodies specific to BMP4 (**F**), peroxiredoxin IV (PrxIV, **G**), osteoglycin (Ogn, **H**), and PrxI (**I**) using β-actin as an internal control. Error bars shown represent mean ± SEM ($n \geq 3$, $^*P < 0.05$). Comparison of the Affymetrix microarray fold changes to the real time-PCR (**E**) or Western blot results (**J**).

TABLE III. A List of Statistically Significant Common Genes Sensitive to Simulated Microgravity in 2T3 Cells Using Both RPM and RWV

Accession #	Gene name	Fold Δ RWV[a]	Fold Δ RPM[b]	Molecular function
Cell adhesion				
NM_012050	*Osteomodulin*	0.10	0.18	Aka osteoadherin, may mediate cell attachment
NM_007729	*Procollagen, type XI, alpha 1*	0.37	0.29	Extracellular matrix structural constituent
NM_012043	*Immunoglobulin superfamily containing leucine rich repeat*	0.18	0.42	Involved in cell attachment
AK004179	*Platelet-derived growth factor receptor-like*	0.45	0.52	Involved in cell attachment and possibly cell proliferation
Cell cycle				
NM_011817	*Growth arrest and DNA damage inducible, gamma*	0.40	0.49	Structural constituent of ribosome
Development				
NM_009144	*Secreted frizzled-related sequence protein 2*	0.43	0.41	Transmembrane receptor and signal transduction activity
Regulation of cell growth				
NM_030127	*Serine protease*	0.74	0.22	Serine-type endopeptidase activity
NM_008760	*Osteoglycin*	0.23	0.38	Growth factor activity
Protein biosynthesis				
NM_026631	*Nucleolar protein family A, member 2*	1.69	2.04	RNA binding; structural constituent of ribosome
Transport				
AK018504	*Ras association (RalGDS/AF-6) domain family 2*	0.27	0.43	Protein binding
Cell differentiation				
NM_025711	*Asporin*	0.15	0.28	Porin activity; cartilage extracellular protein
Metabolism				
NM_007934	*Glutamyl aminopeptidase*	0.40	0.35	Aminopeptidase activity
Skeletal development				
NM_054077	*Proline arginine rich end leucine rich repeat*	0.43	0.45	Extracellular matrix structural constituent
Unknown				
NM_021355	*Fibromodulin*	0.45	0.45	Unknown

Sorted based on typical biological process ($P < 0.05$).
[a]Fold = RWV/static 1 g.
[b]Fold = RPM/static 1 g.

TABLE IV. Comparison of Gene Expression Changes Among RWV, RPM, and Mechanical Load Microarrays

Accession #	Gene name	Fold Δ RWV	*P*-value RWV	Fold Δ RPM[a]	*P*-value RPM	Fold Δ in vivo[b]	*P*-value in vivo	Molecular function
Cell growth and differentiation								
AK014259	*Osteoglycin*	0.22	$P < 0.05$	0.38	$P < 0.005$	2.47	$P < 0.005$	Binds to TGF-Beta
Proteolysis								
NM_008788	*Procollagen C-proteinase enhancer protein*	0.56	$P < 0.01$	0.52	$P < 0.05$	2.15	$P < 0.005$	Nucleic acid binding
Other								
AK004179	*Platelet-derived growth factor receptor-like*	0.45	$P < 0.005$	0.52	$P < 0.05$	2.18	$P < 0.005$	Receptor activity

Sorted by biological process.
[a]RPM data previously published [Pardo et al., 2005].
[b]In vivo refers to mechanical loading data previously published [Xing et al., 2005].

microgravity using the RWV, which maintains the cells in continuous free-fall in culture medium, mimicking a microgravity condition. The RWV is a bioreactor developed by NASA and is most commonly used in the United States [Schwarz et al., 1992; Rucci et al., 2002; Ontiveros et al., 2004]. In contrast, the RPM has been developed by the Japanese and the European Space Agency and is used mostly by scientists outside of the United States, with the exception of our group [Hoson, 1997; Huijser, 2000; Pardo et al., 2005]. Unlike the RPM system where the cells were attached to Opti-Cell membranes and exposed to simulated microgravity, the RWV does not have a similar platform, so the adherent cells had to be grown on microcarriers for exposure to simulated microgravity. Given the need for validated ground-based microgravity simulators, it is imperative that these simulators be compared not only to each other but also to other in vivo mechano-loading and -unloading systems.

To compare the results of the RWV to those published with the RPM, we aimed to match the conditions between the two experiments as much as possible. Most importantly, we found that exposure of 2T3 cells to either the RPM or RWV produced similar inhibitory effect on alkaline phosphatase enzyme activity. These results suggest that the RPM and RWV exposures inhibit cell differentiation of pre-osteoblasts, a finding that is consistent with the expected real microgravity-induced decrease in bone mass.

Previously, it has been controversial whether RWV-simulated microgravity decreases or increases alkaline phosphatase activity, but this may be due to a difference of whether the cells were grown as attached vs. suspended cells. Rucci et al. [2002] found that alkaline phosphatase activity and mRNA expression increased when exposed to the RWV for 2 days using the rat osteoblast-like cell line that was grown as a suspension, which then formed aggregates. In contrast, Klement et al. [2004] showed that exposure to the RWV for up to 14 days blunted ALP activity and bone matrix mineralization of mouse embryonic pre-metatarsal tissue explants. It should be noted that in the Klement et al's study the bone cells were still attached to the extracellular matrix within the embryonic bone tissues. Similarly, in our current RWV and our previous RPM study, the pre-osteoblasts were grown as adherent cells either on microcarriers or on the OptiCell membranes before and during exposure to the simulators. These results suggest that the inhibitory effect of the RWV and RPM on osteoblast function requires the bone cells grown as adherent cells as in vivo during the exposure to the simulated microgravity. Additionally, our finding validates, at least partially, and supports the use of ground-based simulators to study microgravity-induced changes in bone cell biology and pathophysiology.

We also performed gene microarray analysis to determine changes in gene expression profiles of the pre-osteoblasts and compared the results to our published findings with the RPM. We found that 14 genes changed in the same manner, and many of these genes are involved in skeletal remodeling. For example, we confirmed expression levels of *runx2*, which was downregulated by approximately 1.5-fold, and is believed to be a 'master gene' that plays a critical role in the formation of the skeleton. When *Runx2* is genetically knocked out in a mouse model, there is a complete lack of skeleton formation [Komori et al., 1997; Katagiri and Takahashi, 2002]. Additionally, we confirmed the downregulation of *PthR1* levels by RWV-simulated microgravity. A decrease in *parathyroid hormone related protein*, which plays a role in calcium mobilization, has been linked to decreases in bone density and subsequent bone loss in space-flown rats [Torday, 2003]. *Omd* belongs to the small leucine-rich protein (SLRP) family and may be involved in bone matrix formation [Buchaille et al., 2000], and simulated microgravity-induced decrease in *Omd* is consistent with our hypothesis.

Furthermore, we confirmed the downregulated expression levels of *BMP4*, which is involved in skeleton development, including cartilage formation and various joint developments [Tsumaki et al., 2002; Wijgerde et al., 2005]. Moreover, oxidative stress is involved in the etiology of various pathologies, and oxidants are produced under physiological conditions during phagocytosis by macrophages, mitochondrial electron transport, and bone resorption by osteoclasts [Li et al., 2002]. Bone resorption is known to increase beyond normal physiological levels in spaceflight [Smith et al., 2005a], potentially increasing the levels of oxidative stress in the human body.

Peroxiredoxins are a family of antioxidants that are often made by cells in response to oxidant production. It has been found that *PrxI* is upregulated during bone cell differentiation [Kawai et al., 1994; Li et al., 2002]. We found that *PrxI* was upregulated while *PrxIV* was downregulated by RWV-simulated microgravity. These peroxiredoxins may play a differential role in the cells attempting to compensate for the oxidative changes.

To further investigate the functional significance of the microgravity-associated changes in gene expression, we compared our microarray results to independently published data from mechanically loaded tibiae in a mouse model [Xing et al., 2005]. When we compared their gene chip results to those of the RPM and RWV microarrays, we found three genes that were upregulated by mechanical loading and were contrastingly downregulated by simulated microgravity. Also, when less stringent statistical *P* values were applied, we found thirteen additional genes displaying the same trends. For example, *Ogn* was downregulated by the RPM and RWV but upregulated by mechanical loading. *Ogn* is a small leucine-rich proteoglycan found in the extracellular matrix of bone, and knockout mice for this gene display collagen fibril diameter abnormalities [Tasheva, 2002]. These comparisons suggest that a subset of the genes in 2T3 cells are mechanosensitive and may be implicated in microgravity-induced decreased bone mass. Weightlessness or microgravity occurring during spaceflight is characterized as an environment in which the human body is no longer loaded as on Earth. Therefore, it is interesting that many genes that are changed by loading are also changed in the opposite direction by 'unloading' or simulated microgravity.

The widely accepted model of animal-based simulated microgravity is the rodent hindlimb suspension experiment. To date, there has not been a large scale gene expression study performed from bones of animals exposed to hindlimb suspension. However, there have been a few small scale studies investigating specific gene expression. After 4 days of hindlimb suspension of BALB mice, two independent studies found decreases in *collagen type I*, *osteonectin*, *osterix*, and *matrix metalloproteinase 2* [Judex et al., 2005; Zhong et al., 2005]. However, in both the RWV and RPM, the expression levels of these genes did not reach statistical significance but did show a trend towards decreased expression. Additionally, Judex et al. found no change in *cathepsin K* while the RPM increased it and the RWV did not alter it. As well, Judex et al. [2005] showed no change in *runx2*, which was decreased by both the RWV and RPM. Moreover, Zhong et al. [2005] showed that alkaline phosphatase expression decreased, corroborating with the RWV and RPM results. Recently, Hughes-Fulford et al. [2006] examined the effect of real microgravity with or without 1 g field on expression of 24 genes in MC3T3-E1 preosteoblasts. In their study, there was a significant reduction in expression of genes such as *cyclooxygenase 2*, *transforming growth factor beta 1* (*TGFB1*), *fibroblast growth factor 2*, and *osteocalcin* with exposure to real microgravity conditions [Hughes-Fulford et al., 2006]. *TGFB1* expression was downregulated by the RWV, comparable to spaceflight, but it was upregulated by the RPM. Additionally, *prostaglandin EP1* was not altered in spaceflight, corroborating with the RPM data, but the RWV upregulated its expression. Furthermore, spaceflight did not alter *cyclin A*, *cyclin E*, *actin*, or *fibronectin*, which is comparable to both the RWV and the RPM microarray data. Our detailed large scale gene expression studies and comparisons may allow investigators the information needed to select specific genes on which to focus, narrowing the pool of potential therapeutic targets for bone loss.

In conclusion, we have shown that the two different simulators of microgravity produce similar results with regard to bone cell differentiation and osteoblast function. We have shown that both simulators reproduce a decreased bone formation response as also seen in spaceflight. Furthermore, we have compiled a short list of genes that change in response to the two different types of simulated microgravity conditions and to mechanical loading, which may serve as specific targets for interventions to prevent decreased bone mass in spaceflight and for pathologies associated with lack of bone formation.

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