Simulated Microgravity Regulates Gene transcript Profiles of 2T3 Preosteoblasts : Comparison of the Random Positioning Machine and the Rotating Wall Vessel Bioreactor

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Running Title: Microgravity sensitive genes in osteoblasts

Abstract

Microgravity of spaceflight induces bone loss due in part to decreased bone formation by osteoblasts. We have previously examined the microgravity-induced changes in gene expression profiles in 2T3 preosteoblasts using the Random Positioning Machine (RPM) to simulate microgravity conditions. Here, we hypothesized that exposure of preosteoblasts to an independent microgravity simulator, the Rotating Wall Vessel (RWV), induces similar changes in differentiation and gene transcript profiles, resulting in a more confined list of gravi-sensitive genes that may play a role in bone formation. In comparison to static 1g controls, exposure of 2T3 cells to RWV for 3 days inhibited alkaline phosphatase activity, a marker of differentiation, and downregulated 61 genes and upregulated 45 genes by more than two-fold as shown by microarray analysis. The microarray results were confirmed with real time PCR for downregulated genes *osteomodulin*, *bone morphogenic protein 4 (BMP4)*, *runx2*, and *parathyroid hormone receptor 1*. Western blot analysis validated the expression of three downregulated genes, *BMP4*, *peroxiredoxin IV*, and *osteoglycin*, and one upregulated gene *peroxiredoxin I*. Comparison of the microarrays from the RPM and the RWV studies identified 14 gravi-sensitive genes that changed in the same direction in both systems. Further comparison of our results to a published database showing gene transcript profiles of mechanically loaded mouse tibiae revealed 16 genes upregulated by the loading that were shown to be downregulated by RWV and RPM. These mechanosensitive genes identified by the comparative studies may provide novel insights into understanding the mechanisms regulating bone formation and potential targets of countermeasure against decreased bone formation both in astronauts and in general patients with musculoskeletal disorders.

Key Words: bone formation, alkaline phosphatase, RWV, RPM, microgravity, osteoblasts

Introduction

Exposure to microgravity conditions during spaceflights induces several adaptive and pathophysiological changes to the human body, posing health risks to astronauts during space flights. These pathophysiological changes include reduced cardiovascular performance (1, 2), immune system dysfunction (3), skeletal muscle atrophy (4, 5), bone demineralization (6-9), decreased bone mass (10-13), and increased bone resorption (13, 14). Astronauts lose bone mass as much as 1%-2% per month during spaceflight, and this may be due to either decreasing bone formation by osteoblasts or increasing osteolytic functions of osteoclasts (6, 14). Many of the known space-inflicted pathologies have been the subject of research, and the bone loss has not been adequately counteracted with dietary supplements (12, 15, 16) or rigorous exercise (4). 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, rare, expensive, and impractical to most investigators. As an alternative, and a complementary means in some cases, investigators have developed ground-based systems to simulate microgravity conditions on Earth (17-20). Simulated microgravity is based on the hypothesis that sensing no weight would have similar effects as being weightless (21). The 1D and 3D clinostats such as the Rotating Wall Vessel bioreactor (RWV) and the Random Positioning Machine (RPM) simulate microgravity by continuously moving the gravity vector, a method called gravity-vector averaging, yielding a net-zero sum of gravity vectors averaged over time. The key aspect in gravity-vector averaging is that the movement of the gravity vector must be faster than the cell sensors can detect gravity (19).

For the microgravity simulators to be used widely and accepted as valid model systems to study microgravity effects on human biology, the biological and pathophysiolocial effects of each simulator should be compared to each other and, if possible, to real microgravity conditions. There is one comparison between the RPM and the RWV in which cell proliferation and cytoskeletal organization were evaluated in endothelial cells exposed to both simulators (22). However, detailed experiments assessing biological function and genetic changes were not performed, and there was no discussion relating the observed changes to microgravity-induced pathologies. However, as far as we are aware, no unbiased comparison amongst the various microgravity simulators as related to bone loss studies has been reported.

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 (20). We compiled a list of 140 genes (gravi-sensitive genes) that changed in response to the simulated microgravity conditions with the RPM, and some of them may be involved in decreased bone formation. In contrast, Xing et al, have characterized gene transcript profile changes induced by exposing mouse tibia to mechanical stimulation, an apparent opposite of microgravity conditions, using a four-point bending method (23). Therefore, we hypothesized that simulated microgravity using the RWV inhibits differentiation and alters gene expression profiles of 2T3 pre-osteoblasts, recapitulating the trends produced by the RPM, while inducing the opposite trends produced by mechanical stimulation of bones. To test this hypothesis, we carried out an additional gene transcript profiling study with 2T3 cells exposed to the NASA developed RWV, 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 tibias (23). From these studies, we found 16

genes that were affected in all three studies, suggesting that they may be implicated in microgravity-induced decrease in bone formation.

Results

RWV-simulated microgravity inhibits 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. As shown in Figure 2, exposure of cells to the RWV for 3 days significantly decreased ALP mRNA (Panel A) and its activity (Panel B) by 3-fold in comparison to static 1g controls. This finding, which is consistent to our previously reported data with the RPM (20), 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 alters gene expression profiles of 2T3 cells as determined by microarray studies.

DNA microarray studies were performed on samples obtained from 2T3 cells exposed to the static 1g or RWV-simulated microgravity for 3 days. Among approximately 40,000 genes tested by the Affymetrix array, 61 were downregulated while 43 were upregulated statistically significantly by more than two fold above the static 1g control ($p<0.05$) as shown in a scatter plot of the genes (Figure 3). Table 1 shows a subset of these genes organized by biological process as defined by GoMiner, and each gene is given an associated molecular function where available. Additionally, Table 2 provides a list of genes from the microarray that may be implicated in bone

formation or mineralization as determined by literature survey, and these genes are organized by fold change with molecular function when known. The entire array can be accessed from Gene Expression Omnibus (GEO).

Validation of the microarray data with the quantitative Real Time-PCR and immunoblotting.

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 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 (Figure 4 A-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, 0.62, 0.23, and 1.23. We showed that the fold changes for these genes by Western blot were 0.59, 0.68, 0.32, and 1.58, respectively (Figure 4 F-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 reveals 14 genes that changed in the same way.

There was a subset of genes that demonstrated similar expression changes when exposed to simulated microgravity in both the RPM and RWV experiments. Table 3 displays those genes that changed statistically significantly with $p<0.05$. There were 13 downregulated genes and 1 upregulated gene in this group. With such a small sample size (n=3), we felt it was valuable to evaluate which genes changed commonly in both simulators with less stringent p-value cutoffs. Under this non-stringent condition, there were also 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 3 organizes the significant genes by biological process as defined by GoMiner and associates each gene with a molecular function, if known. Interestingly, many of the genes in both tables have been implicated in skeletal remodeling, including *fibromodulin*, *osteomodulin*, and *osteoglycin* (24-26).

Comparison of microgravity microarrays to a mechanical loading microarray produced a list of 16 genes that changed in opposite directions between microgravity and mechanical loading.

Recently, Xing, et al. published their microarray data on mice that were mechanically loaded (23). Briefly, they loaded mice in a four point bending mechanical device for four days and total RNA was obtained from the region of the mechanically stimulated tibia. The untreatd tibias of the same mice were used as unloaded controls. They used a 22,000 gene Agilent Technologies microarray (23). We compared our RPM and RWV microarrays to their published

data and compiled an interesting list of common genes changing in opposite directions. Sixteen genes that were upregulated by loading were downregulated in the cells exposed to simulated microgravity conditions by the RPM and RWV (Table 4). Table 4 compares the fold changes for the RWV, RPM, and mechanically-loaded mouse tibias. There were many genes that were altered by loading and microgravity that are believed to play roles in bone remodeling, including *pleiotrophin*, *legumain*, and *follistatin* (27-31).

Discussion

We have previously developed an *in vitro* method combining the 3D-clinostat RPM and 2T3 pre-osteoblasts cells cultured in tissue culture disks called Opticells, in which the cells are encapsulated by two polystyrene, gas-permeable membranes to which the cells are adhered (20). The RPM-simulated microgravity significantly inhibited alkaline phosphatase activity over a time course of 9 days, without significantly altering cell morphology or cell proliferation (20). Additionally, using gene microarrays scanning 10,000 mouse genes, we produced a list of 52 upregulated and 88 downregulated genes by more than two-fold above the static 1g control with statistical significance ($p<0.05$).

Here, we used another approach to expose the adherent 2T3 cells to simulated microgravity using the 1-D clinostat RWV bioreactor, which maintains constant suspension of the cells. Unlike the RPM system where the cells were attached to OptiCell 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 RWV-simulated microgravity. To compare the results of the RWV to those published with the RPM, we aimed to control the experimental conditions as much as possible. We used the same seeding density of 10,000

cells/cm^2, and we chose a microcarrier that had the most similarity to the Opticell membrane, which was used in the RPM studies. In our previous RPM studies, 2T3 cells grew well on the OptiCells without requiring any extracellular matrix other than what was available from fetal bovine serum contained in the cell culture medium. Therefore, we used the same culture conditions to grow 2T3 cells on the microcarriers and found no difference in cell growth and morphological characteristics (data not shown). Under this condition, we found that cells adhered to the microcarriers with 80% efficiency (n=9) within a day as determined by cell counting. Most importantly, we independently confirmed the RPM data showing that the RWV produced similar results regarding cell differentiation, as determined by alkaline phosphatase (*ALP*) enzyme activity. These results suggest that the RPM and RWV exposures inhibit cell differentiation of pre-osteoblasts, a finding that seems to be consistent with the microgravity-induced bone loss response.

Previously, it has been controversial whether RWV-simulated microgravity decreases or increases *ALP* activity, but this may be due to a difference of whether the cells were grown as attached vs. suspended cells. Rucci, et. al. found that *ALP* 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 (32). In contrast, Klement, et al. 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(18). It should be noted that in this condition the bone cells were still attached on the extracellular matrix within the embryonic bone tissues. In our current and the previous study, the pre-osteoblasts were grown as adherent cells either on microcarriers or on the OptiCell membranes before and during exposure to the RPM or RWV. These results suggest that the inhibitory effect of the RWV and RPM on the osteoblastic function

requires the bone cells grown as adherent cells during the exposure to the simulated microgravity. Additionally, our finding partially validates 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 way, 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 (33, 34). Additionally, *asporin* (*Aspn*) and *proline arginine-rich end leucine-rich repeat* protein (*PRELP*) were both downregulated by simulated microgravity in both the RWV and RPM by 3.5 and 2.2-fold, respectively. *Asporin* is a cartilage extracellular protein and a member of the well known family of secreted proteoglycans present in many connective tissues called small leucine rich proteoglycans (SLRPs) (35, 36). *Aspn* is known to regulate the activity of transforming growth factor-beta (TGFβ) and forms an association with *calmodulin 1* (*Calm1*), which is an intracellular protein that interacts with a number of proteins involved in signal transduction. The association of *Aspn* and *Calm1* reduces the ability of chondrocytes to make aggrecan and type II collagen (35). Aggrecan and type II collagen are essential constituents of articular cartilage, and collagen abnormalities are associated with skeletal abnormalities (35). *PRELP* is categorized by GoMiner to be involved in skeletal development and has been found to bind collagen type I and type II through its leucine-rich repeat domain (37). 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 (38). This is consistent with the decrease in the *parathyroid hormone receptor 1* levels by simulated microgravity, which was confirmed by RT-PCR. *Osteomodulin* belongs to the SLRP family and may be involved in bone matrix formation (25), and simulated microgravity-induced decrease in *Omd* is consistent with our hypothesis.

Additionally, we confirmed the downregulated expression levels of *bone morphogenic protein 4* (*BMP4*), a member of the bone morphogenic protein (BMP) family, and *BMP4* is involved in skeleton development, including cartilage formation and various joint developments (39, 40). 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 (41). Bone resorption is known to increase beyond normal physiological levels in spaceflight (14), potentially increasing the levels of oxidative stress in the human body. Oxidative stress can lead to breaks in DNA, depletion of cellular energy stores, and cell death, and oxidants play important roles in cell signaling (41). Peroxiredoxins are a family of antioxidants that are often made by cells in response to oxidant production. It has been found that *peroxiredoxin I* is upregulated during bone cell differentiation (41, 42). We found that *PrxI* was upregulated while *peroxiredoxin IV* was downregulated by RWV-simulated microgravity, suggesting that the cells may be responding to oxidative stress agents and trying to compensate for these changes. Moreover, from Table 1, there is a large group of genes involved in cell adhesion that changes upon exposure to simulated microgravity. In general, with the exception of *procollagen XI*, several of these genes that were upregulated are extracellular matrix proteins such as *procollagen IV* and *integrin alpha 6*. There also were several genes involved in cytoskeletal regulation that changed, such as *tubulin*. It also seems that heat shock proteins involved in protein folding change upon

exposure to simulated microgravity. These results indicate possible candidate genes involved in decreased bone formation in astronauts, suggesting that this decrease is related to changes in the expression of genes necessary for osteoblast differentiation, matrix formation, subsequent mineralization, and cartilage maintenance.

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 tibias in a mouse model (23). When we compared their gene chip results to those of the RPM and RWV microarrays, we found 16 genes that were upregulated by mechanical loading and contrastingly dowregulated by simulated microgravity. Moreover, many of these genes are highly involved in bone remodeling. For example, *pleiotrophin* and *osteoglycin* were not only downregulated by simulated microgravity but also upregulated by mechanical loading. *Pleiotrophin* has been found to be involved in early bone development and osteoblast function (27). Overexpression of *pleiotrophin* in mice enhances bone growth early in development, but its effects diminish over time (27). However, the *pleiotrophin* knockout mouse does not have an altered bone remodeling phenotype (28). *Pleiotrophin* is synthesized by osteoblasts at an early stage of differentiation, and the gene is present at new sites of bone formation (29). *Osteoglycin* is a small leucine-rich proteoglycan found in the extracellular matrix of bone, and knockout mice for this gene display collagen fibril diameter abnormalities (24). Additionally, *legumain* has been identified as an inhibitor of osteoclast function and thus bone resorption (30). Simulated microgravity by the RPM and RWV downregulated this inhibitor with fold changes of 0.66 and 0.64, respectively, while loading upregulated *legumain* by 3.3-fold, suggesting that microgravity may induce expression of genes normally involved in bone resorption. Disruption of the tight coupling between bone formation and bone resorption

has been recognized as a general mechanism inducing bone loss (43, 44). Microgravity-induced alteration of genes like *legumain* is consistent with this notion. These comparisons suggest that a subset of these gravity-sensitive genes is also mechanical load-sensitive and may be implicated in microgravity-induced decreased bone formation. 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.

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 by alkaline phosphatase activity that both simulators reproduce a decreased bone formation response as also seen in spaceflight. Furthermore, we have compiled a confined list of genes that change in response to the two different types of simulated microgravity conditions and to mechanical loading, which could provide a limited number of specific targets for interventions to cure or prevent bone loss not only in astronauts but also in patients on Earth with various metabolic musculoskeletal diseases.

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 by us previously (20). For RWV experiments, trypsinized cells were seeded on microcarriers (#P102-1521, Solohill, Ann Arbor, MI), which has a polystyrene core with no extracellular matrix coating. Microbeads, needed to obtain 100cm^2 surface growth area based on the surface area of

each bead, were prepared in sterile PBS (50mg/ml) and autoclaved as suggested by the manufacturer. We plated 1 million cells with 5.6 grams of beads in a low adhesion 100mm plate (Corning, Inc. #3262) so that the cells adhered to the beads and not the plate. The cells were allowed to grow for 3 days to confluency and then transferred to a RWV with approximately 55ml of culture media. Media bubbles were removed with a two-syringe system. For static 1g control, cells were kept in the same incubator in the low adhesion plate with the identical media for the matching experimental duration as the RWV group.

Rotating Wall Vessel (RWV)– Rotation of the RWV (Figure 1) maintains microbeads attached with 2T3 pre-osteoblasts in a continuous free-fall state, simulating a microgravity environment (45). The RWV is rotated in a single mode, and the rate of rotation varies according to cell type, microcarrier density, and number of cell-bead aggregates. For our experiments, we rotated the RWV at 22 RPMs (rotations/minute). Over three days of RWV exposure, the rotation rate did not need to be altered as the cell-bead aggregates maintained suspension. Figure 1-B shows the RPM (20) as a comparison.

Whole cell lysate and alkaline phosphatase enzyme activity—Following the RWV or static 1g exposure, cells and beads were washed with cold PBS 2 times and lysed in 500 µl of a lysis buffer containing 0.1% Triton X-100 in 1mM $MgCl_2$, 20mM Tris-HCl, and 0.1mM $ZnCl_2$. The lysate and bead mixture was centrifuged for 3-5 minutes 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. Protein assay using the Bio-Rad DC protein assay and the alkaline phosphatase activity using a Diagnostics *ALP* assay kit (Sigma) were carried out as we described previously (20).

Immunoblotting—Aliquots of cell lysates (20 μg protein) were resolved on a SDS-PAGE gel and transferred to a polyvinylidene difluoride membrane (Millipore) (46). 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 (46). A polyclonal primary antibody for BMP4 (Santa Cruz Biotechnologies), osteoglycin (R&D Biosciences), and polyclonal antibodies for peroxiredoxin I and peroxiredoxin IV (Lab Frontier, Seoul, Korea) were used for these studies.

RNA isolation—Total RNA was prepared by using the RNeasy Mini kit (Qiagen). Briefly, after two cold PBS washes, 500ul 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 minutes at 1,500 RPMs 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.

Reverse Transcriptase and Real-Time Polymerase Chain Reaction— Total RNA was reverse transcribed by using random primers and a Superscript-II kit (Life Technology) (47). 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 (47). 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 61ºC 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 5 along with Light Cycler conditions. Real-time PCR for the listed genes were carried out in PCR buffer (20mM Tris-Cl, pH 8.4 at 25°C, 4mM $MgCl_2$, 250 μg/ml bovine serum albumin, and 200 μM deoxynucleotides) containing SYBR green (1:84,000 dilution), 0.05unit/μl Taq DNA polymerase, and Taq Start antibody (1:100 dilution) as described previously by us (47).

Affymetrix Gene Microarrays— All RNA samples passed the microarray 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 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 indicates a gene upregulated by simulated microgravity and below 1 indicates a gene downregulated by simulated microgravity. The data were statistically analyzed by DNA chip analyzer (dChip) as described below in Statistical analysis section and filtered for fold change threshold, and the genes that changed in response to simulated microgravity by more than 2-fold above or below the static 1g 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 known functions of each known gene (48).

Statistical analysis—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 (49, 50). 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 t-test built in dChip.

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Figure Legend

Figure 1. 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 incubated in T-75 flasks for static 1g controls for the same period. The alkaline phosphatase activity (A) was determined by a colorimetric assay using cell lysates 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).

Figure 2. The effects of RWV-simulated microgravity on gene transcript profiles of 2T3 cells as determined by Affymetrix microarray analysis. Total RNA was isolated from 2T3 cells exposed to RWV or static 1g 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 66 genes upregulated (red dots) and 45 genes downregulated (green dots) by more than 2-fold compared to static 1g control ($p<0.05$, n=3). Unchanged genes are shown in black in the scatter plot.

Figure 3. Verification of microarray results by real time PCR and Western blot for genes that were upregulated or downregulated by RWV exposure of 2T3 cells. Aliquots of total RNA used for microarray studies (n=3, simulated μg and static 1g) 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 lysates for the Western blot assay using antibodies specific to BMP4 (F), peroxiredoxin IV (Prx IV, G),

osteoglycin (Ogn, H), and Prx I (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 quantitative RT-PCR (E) or Western blot results (J).

Figure 4. **Simulated microgravity using the RWV and the RPM.** A. The Rotating Wall Vessel (RWV) bioreactor is a one-dimensional clinostat that simulates microgravity conditions by maintaining particles in a free-fall state. 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. Both simulators use the gravity vector averaging method to simulate microgravity.

Table 1 A list of selected statistically significant genes sensitive to RWV simulated microgravity in 2T3 cells. Sorted based on typical biological process (p<0.05).

Accession #	*Gene Name*	*Fold Δ	p-value	Molecular Function
Cell Adhesion				
NM_012050	*osteomodulin*	0.10	0.04	aka osteoadherin, may mediate cell attachment
NM_011581	*thrombospondin 2*	0.39	0.03	structural molecule activity; calcium ion binding
BB781435	*nidogen 2*	0.39	0.004	calcium ion binding
NM_007729	*procollagen, type XI, alpha 1*	0.40	0.04	extracellular matrix structural constituent
NM_012043	*immunoglobulin superfamily containing leucine rich repeat (ISLR)*	0.42	0.02	involved in cell attachment
BB250384 L08431	*vascular cell adhesion molecule 1*	0.45	0.02	protein binding
BF225985	*discoidin domain receptor family, member 1*	0.49	0.04	protein kinase activity; protein serine/threonine kinase activity
BC013560	*procollagen, type IV, alpha 2*	2.02	0.03	structural molecule activity; extracellular matrix structural constituent
BG073728	*RGM domain family, member B*	2.24	0.05	protein self binding
BM935811	*integrin alpha 6*	2.47	0.01	receptor activity; protein binding
BF158638	*procollagen, type IV, alpha 1*	2.59	0.002	structural molecule activity; extracellular matrix structural constituent
Cell Cycle				
NM_011817 AK007410	*growth arrest and DNA damage inducible, gamma*	0.40	0.009	structural constituent of ribosome
AK004608	*heat shock protein 8*	2.27	0.04	protein binding; ATP binding
Cell Differentiation				
NM_025711	*asporin*	0.15	0.05	porin activity; cartilage extracellular protein
Development				
NM_031258	*chordin-like 1*	0.16	0.03	unknown
NM_009144	*secreted frizzled-related sequence protein 2*	0.43	0.03	transmembrane receptor and signal transduction activity
BB549310	*olfactomedin 1*	2.66	0.02	unknown
Skeletal Development				
NM_007554	*bone morphogenetic protein 4*	0.40	0.002	cytokine activity
NM_054077	*proline arginine-rich end leucine-rich repeat*	0.43	0.05	extracellular matrix structural constituent
NM_011641	*transformation related protein 63*	0.47	0.04	DNA binding; transcription factor activity
Cell Growth				
NM_010516	*cysteine rich protein 61*	0.24	0.02	protein binding; insulin-like growth factor binding

Accession #	*Gene Name*	*Fold Δ	p-value	Molecular Function
NM_016873	*WNT1 inducible signaling pathway protein 2*	0.39	0.01	phospholipase A2 activity; calcium ion binding
BC020038	*endothelial cell-specific molecule 1*	0.46	0.01	insulin-like growth factor binding
Cytoskeletal Regulation				
NM_008857	*protein kinase C, iota*	0.45	0.04	actin filament organization
AV297945	*myosin X*	0.46	0.046	motor activity; actin binding
AW491660	*tubulin, alpha 4*	2.42	0.007	microtubule based movement; GTPase activity; structural molecule activity
Metabolism				
NM_007934	*glutamyl aminopeptidase*	0.40	0.03	DNA binding; protein binding
AY057913	*brain derived neurotrophic factor*	0.46	0.02	mechanoreceptor differentiation; protein binding; growth factor activity
NM_007436	*aldehyde dehydrogenase family 3, subfamily A1*	2.56	0.04	aldehyde dehydrogenase activity
Proteolysis				
NM_011269	*Rhesus blood group-associated A glycoprotein*	0.36	0.01	cysteine-type endopeptidase activity
AF282844	*matrix metalloproteinase 16*	0.47	0.04	metalloendopeptidase activity
BB658835	*a disintegrin-like and metalloprotease (reprolysin type) with thrombospondin type 1 motif, 5 (aggrecanase-2)*	0.48	0.03	metalloendopeptidase activity; peptidase activity
AK011596	*transferrin receptor*	2.40	0.05	receptor activity; transferrin receptor activity
Signal Transduction				
NM_008046	*follistatin*	0.39	0.02	BMP signaling pathway
Protein Folding				
NM_013560	*heat shock protein 1*	2.03	0.04	unknown
NM_011020	*heat shock 70kDa protein 4 like*	2.06	0.02	ATP binding; unfolded protein binding
Transport				
AK018504	*ras association (RalGDS/AF-6) domain family 2*	0.43	0.27	protein binding
AV344473	*sorting nexin associated golgi protein 1*	0.48	0.008	unknown
BB280137	*RAB guanine nucleotide exchange factor (GEF) 1*	0.48	0.02	DNA binding; zinc ion binding
BB144704	*ATP-binding cassette, sub-family A (ABC1), member 1*	0.48	0.02	ATP binding; ATPase activity
Chromosome Organization				
BB533903	*histone 1, H1c*	0.29	0.03	DNA binding; protein binding
M25487 NM_023422	*histone 1, H2bc*	0.44	0.03	DNA binding

Accession #	*Gene Name*	*Fold Δ	p-value	Molecular Function
Nitric Oxide Synthesis				
BF166000	*high mobility group box 1*	0.41	0.04	nitric-oxide synthase regulator activity
Multidrug Transport				
BB291885	*ATP-binding cassette, sub-family C (CFTR/MRP), member 4*	2.35	0.003	multidrug efflux pump activity
Endocytosis				
AI848122	*low density lipoprotein receptor-related protein 8, apolipoprotein e receptor*	2.30	0.04	receptor activity; calcium ion binding
Stress or Immune Response				
NM_007705	*cold inducible RNA binding protein*	0.21	0.003	
D67017	*heat shock protein 105*	2.54	0.02	protein binding; ATP binding
AW763765	*heat shock protein 1A*	3.18	0.04	ATP binding
Regulation of Cell Growth				
NM_008760	*osteoglycin*	0.23	0.03	growth factor activity
Negative Regulation of Cell Growth				
NM_009517	*wild-type p53-induced gene 1*	2.18	0.03	unknown
Unknown				
BC002065	*serine (or cysteine) proteinase inhibitor, clade A, member 3G*	0.35	0.02	endopeptidase inhibitor activity
NM_133859	*olfactomedin-like 3*	0.40	0.02	unknown
NM_016753	*latexin*	0.44	0.01	enzyme inhibitor activity; metalloendopeptidase inhibitor activity
NM_007984	*fascin homolog 1, actin bundling protein*	0.47	0.04	actin binding; actin filament binding
NM_029632	*protein phosphatase 1, regulatory (inhibitor) subunit 11*	2.17	0.03	unknown
AI266910	*ceroid-lipofuscinosis, neuronal 2*	2.07	0.04	serine-type endopeptidase activity
NM_009266	*selenophosphate synthetase 2*	2.02	0.004	catalytic activity

*** Fold=simulated microgravity expression/static 1g expression**

Table 2 The effect of microgravity on selected genes that may be involved in osteoblast differentiation and matrix mineralization. Sorted based on fold changes.

Accession #	*Gene Name*	*Fold Δ	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
BG229308	*procollagen, type VIII, alpha 2*	0.35	>0.1	present in cartilage
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
BM218630	*protocadherin 18*	0.40	>0.05	calcium ion binding
NM_011693	*vascular cell adhesion molecule 1*	0.41	<0.025	cell adhesion molecule activity
BB396904	*calmodulin 3*	0.41	>0.1	calcium binding; regulates osteoclast differentiation
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
BC002064	*pleiotrophin*	0.43	>0.1	involved in bone mineralization
NM_009821	*runt related transcription factor 1*	0.43	>0.1	mesenchymal cell differentiation
BI111620	*tissue inhibitor of metalloproteinase 3*	0.44	>0.1	may be involved in bone resorption
NM_021355	*fibromodulin*	0.45	>0.05	binds to collagen, may regulate fibril formation, binds to TGF-beta
BB431535	*matrix metalloproteinase 16*	0.47	<0.05	involved in osteoclast function and bone resorption
M93954	*tissue inhibitor of metalloproteinase 2*	0.48	>0.1	involved in bone resorption
AW412729	*procollagen, type XII, alpha 1*	0.48	>0.05	present in collagen; extracellular matrix structural constituent
C76813	*a disintegrin and metalloproteinase domain 17*	0.48	>0.1	involved in matrix protein degradation; prevent osteoclast recruitment
BB371406	*frizzled homolog 2 (Drosophila)*	0.48	>0.1	involved in WNT pathway
NM_009933	*procollagen, type VI, alpha 1*	0.49	<0.1	present in cartilage
AU021035	*syndecan 2*	0.50	>0.1	binds to type 1 collagen, fibronectin, tenascin-C
AA717838	*interleukin 6 signal transducer*	0.50	>0.1	act as stimulators of an early stage of osteoclast formation
BF580235	*cathepsin E*	0.50	>0.1	involved in bone resorption
NM_010511	*interferon gamma receptor 1*	0.52	>0.05	inhibit bone resorption
BE628614	*annexin A4*	0.52	>0.1	expressed in osteoblasts and relocation dependent on calcium
BF537076	*interferon gamma receptor 2*	0.53	>0.1	inhibit bone resorption

Accession #	*Gene Name*	Fold Δ	p-value	Molecular Function
AF153440	*BMP and activin membrane-bound inhibitor, homolog (Xenopus laevis)*	0.53	>0.05	antagonist to BMPs; involved in TGF-beta signaling pathway
NM_011199	*parathyroid hormone receptor1*	0.54	>0.1	transmembrane receptor activity
NM_009627	*adrenomedullin*	0.57	>0.1	neuropeptide hormone activity
AI385532	*thrombospondin 1*	0.59	>0.1	involved in cell attachment
BC014690	*transforming growth factor, beta 3*	0.59	<0.05	growth factor and cytokine activity
NM_011580	*thrombospondin 1*	0.59	>0.1	cell attachment
NM_007743	*procollagen, type I, alpha 2*	0.62	>0.1	the major constituent of bone matrix
BG248060	*bone morphogenetic protein 1*	0.62	>0.1	metalloendopeptidase activity
NM_007737	*procollagen, type V, alpha 2*	0.68	>0.1	present where there is collagen type I
AF053954	*cbfa1/runx2 (osf2)*	0.69	>0.05	essential transcription factor for osteoblast differentiation and bone formation
BB730912	*interleukin 13 receptor, alpha 1*	0.69	>0.1	inhibit bone resorption
NM_020273	*glucocorticoid modulatory element binding protein 1*	0.70	>0.05	transcription factor activity
S80963	*interleukin 13 receptor, alpha 1*	0.72	>0.1	inhibit bone resorption
NM_019444	*RAMP2*	0.73	>0.1	calcitonin signal transducer activity
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
BB082407	*hyaluronan and proteoglycan link protein 4*	1.22	>0.05	present in articular cartilage
BM251152	*chondroitin sulfate proteoglycan 2*	1.23	>0.05	present in cartilage
NM_009758	*bone morphogenetic protein recepto, type 1A*	1.37	>0.1	TGF-β and BMP receptor
BG092290	*insulin-like growth factor 2 receptor*	1.43	<0.025	signal transduction and hormone activity
NM_007802	*cathepsin K*	1.43	>0.1	may regulate elastic fiber formation (calcium ion binding activity)
NM_020275	*tumor necrosis factor receptor superfamily, member 10b*	1.43	<0.05	growth factor and cytokine activity
NM_008965	*prostaglandin E receptor 4*	1.45	>0.1	G-protein coupled receptor_activity
BG069059	*leucine rich repeat (in FLII) interacting protein 1*	1.52	>0.05	leucine rich repeats involved in bone mineralization
NM_010554	*interleukin 1α*	1.58	<0.05	potent stimulators of bone resorption
NM_011361	*serum/glucocorticoid regulated kinase*	1.65	>0.05	transferase activity, transferring phosphorus-containing groups
M94967	*prostaglandin-endoperoxide synthase 2*	2.05	>0.05	prostaglandins important in fluid shear over bone cells
BM935811	*integrin α6*	2.47	<0.05	cell adhesion molecule
AK003744	*cystatin E/M*	3.45	<0.05	antagonist to cathepsin family
NM_011111	*serine (or cysteine) proteinase inhibitor, clade B, member 2*	7.14	>0.05	may be involved in osteoclast function with MMPs and cathepsins
X75557	*proliferin*	14.4	>0.05	involved in cell adhesion; may regulate cathepsin L; involved in cell proliferation

*** Fold=simulated microgravity expression/static expression**

Table 3 A list of statistically significant common genes sensitive to simulated microgravity in 2T3 cells using both RPM and RWV. Sorted based on typical biological process ($p<0.05$).

Accession #	*Gene Name*	*Fold Δ RWV	*Fold Δ RPM	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

*** Fold=simulated microgravity expression/static expression**

Table 4 Comparison of gene expression changes among RWV, RPM, and mechanical load microarrays. Sorted by biological process.

Accession #	*Gene Name*	Fold Δ RWV	p-value RWV	Fold Δ RPM	p-value RPM	Fold Δ *in vivo*	p-value *in vivo*	Molecular Function
Cell Growth & Differentiation								
AK014259	*osteoglycin*	0.22	p<0.05	0.38	p<0.005	2.47	p<0.005	binds to TGF-Beta
NM_008409	*integral membrane protein 2A*	0.31	p>0.1	0.53	p<0.05	2.85	p<0.005	marker gene of osteoblastic cells in bone formation
NM_007792	*cysteine and glycine-rich protein 2*	0.65	p<0.05	0.68	p>0.1	4.12	p<0.0025	zinc ion binding
AK011346	*pleiotrophin*	0.43	p>0.1	0.36	p<0.05	4.29	p<0.0025	involved in bone mineralization; growth factor binding
Cell Adhesion								
NM_015734	*procollagen, type V, alpha 1*	0.56	p>0.05	0.65	p>0.1	1.97	p<0.0025	present in cartilage ECM
NM_007739	*procollagen, type VIII, alpha 1*	0.57	p>0.05	0.68	p>0.1	2.56	p<0.0025	present in cartilage ECM
Cell Death								
NM_008086	*growth arrest specific 1*	0.59	p>0.1	0.53	p<0.01	2.26	p<0.0025	protein binding
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
NM_011175	*legumain*	0.64	p>0.1	0.66	p>0.05	3.25	p<0.005	cysteine-type endopeptidase activity
Signal Transduction								
NM_008809	*platelet derived growth factor receptor, beta polypeptide*	0.74	p>0.1	0.61	p>0.05	1.97	p<0.01	protein serine/threonine kinase activity
NM_009037	*reticulocalbin*	0.69	p<0.05	0.85	p>0.1	2.31	p<0.005	calcium ion binding
Transcription regulation								
NM_010351	*goosecoid*	0.58	p>0.1	0.68	p>0.1	2.3	p<0.005	DNA binding
NM_019791	*melanoma antigen, family D, 1*	0.54	p>0.1	0.55	p<0.005	2.33	p<0.0025	transcription coactivator activity
Cytoskeletal Movement								
NM_007392	*actin, alpha 2, smooth muscle, aorta*	0.64	p>0.1	0.85	p>0.1	1.97	p<0.0025	structural constituent of cytoskeleton
Other								
NM_009128	*stearoyl-coenzyme A desaturase 2*	0.4	p>0.1	0.35	p<0.0025	2.56	p<0.0025	iron ion binding
AK004179	*platelet-derived growth factor receptor-like*	0.45	p<0.005	0.52	p<0.05	2.18	p<0.005	receptor activity

Table 5 Primers and LightCycler conditions used for Real-Time PCR

GeneBank Accession No.	Gene		Primers (5'-3')	bp	LightCycler Conditions	Ref.
NM_009820	*Runx2*	Fw	GACAGAAGCTTGATGACTCTAAACC	171	7 sec; 62ºC	(17)
		Rv	TCTGTAATCTGACTCTGTCCTTGT		9 sec; 72ºC	
NM_007554	*BMP4*	Fw	CTGCGGGACTTCGAGGCGACACTTCT	150	7 sec; 65ºC	(47)
		Rv	TCTTCCTCCTCCTCCTCCCCAGACTG		7 sec; 72ºC	
NM_011199	*PthR1*	Fw	GCACACAGCAGCCAACATAA	531	7 sec at 63ºC	(51)
		Rv	CGCAGCCTAAACGACAGGAA		22 sec at 72ºC	
NM_012050	*Omd*	Fw	GACGGGCTGGTGAATGTGACTATGCTTGA	147	7 sec; 63ºC	(20)
		Rv	CCAAGGGGCATTGATTCTAATCTGTTATT		10 sec; 72ºC	