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Modeled Microgravity Inhibits Osteogenic Differentiation of Human Mesenchymal Stem Cells and Increases Adipogenesis

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Space flight-induced bone loss has been attributed to a decrease in osteoblast function, without a significant change in bone resorption. To determine the effect of microgravity (MG) on bone, we used the Rotary Cell Culture System [developed by the National Aeronautics and Space Administration (NASA)] to model MG. Cultured mouse calvariae demonstrated a 3-fold decrease in alkaline phosphatase (ALP) activity and failed to mineralize after 7 d of MG. ALP and osteocalcin gene expression were also decreased. To determine the effects of MG on osteoblastogenesis, we cultured human mesenchymal stem cells (hMSC) on plastic microcarriers, and osteogenic differentiation was induced immediately before the initiation of modeled MG. A marked suppression of hMSC differentiation into osteoblasts was observed because the cells failed to express ALP, collagen 1, and osteonectin. The expression of runt-related transcription factor 2 was also inhibited. Interestingly, we found that peroxisome proliferator-activated receptor γ (PPARγ2), which is known to be important for adipocyte differentiation, adipsin, leptin, and glucose transporter-4 are highly expressed in response to MG. These changes were not corrected after 35 d of readaptation to normal gravity. In addition, MG decreased ERK- and increased p38-phosphorylation. These pathways are known to regulate the activity of runt-related transcription factor 2 and PPARγ2, respectively. Taken together, our findings indicate that modeled MG inhibits the osteoblastic differentiation of hMSC and induces the development of an adipocytic lineage phenotype. This work will increase understanding and aid in the prevention of bone loss, not only in MG but also potentially in age-and disuse-related osteoporosis. (*Endocrinology* 145: 2421–2432, 2004)

OSTEOPOROSIS IS A SERIOUS age-related condition that is largely preventable but frequently undiagnosed until a major life-altering fracture occurs. In addition, bone loss is the major skeletal problem that has been reported in humans after prolonged space flight and weightlessness. In the most severe forms of microgravity (MG)-induced bone loss, there is an approximately 2% decrease in bone mineral density in only 1 month, equal to that of a postmenopausal woman in 1 yr (1). Both preventing and treating bone loss are important goals, to create a healthier life for the elderly and astronauts. That MG-induced bone loss occurs is not surprising, because gravity and mechanical loading are known to be essential for the maintenance of skeletal integrity. Skeletal abnormalities due to MG are not exclusive to humans (2, 3). Laboratory animals, such as rats (4, 5) and mice (6), have also been shown to be affected. Interestingly, morphological changes observed in the bones of elderly osteoporotic persons and in patients after disuse dramatically resemble those observed in astronauts after space flights (2).

The mechanisms involved in producing MG-induced bone loss remain unclear. One factor many studies agree on is that altered osteoblast function and development play an important role in MG-induced bone loss. Tetracycline labeling of rat bones before and after space flight demonstrates decreased bone formation in both cortical and cancellous bone (7–11). Development of procedures to culture cells *in vitro* while modeling MG has expanded the possibilities of identifying important factors that regulate cellular proliferation, differentiation, and function.

Ground-based studies on the effects of MG on bone are performed using different models. Some of the early studies were performed using the rat hindlimb elevation model. This model produced fluid shifts similar to these that occur in space travel and is well tolerated by animals, with minimal evidence of stress, as indicated by continued weight gain (12) and normal levels and circadian rhythms of corticosterone (13). Unfortunately, ground-based models for simulating the effects of MG on bone must overcome enormous technical problems to enable us to have confidence in the obtained data. Although there is disagreement about whether ground-based studies truly simulate MG (14), certain cell culture systems provide a model system for investigating altered gravity effects. The system we used in this study, the Rotary Cell Culture System (RCCS), is equipped with High Aspect Ratio Vessels (HARVs; Synthecon, Inc., Houston, TX). The vessel rotates around a horizontal axis and allows for diffusion of oxygen and carbon dioxide across a semipermeable membrane. The HARV demonstrates very low shear stress

Abbreviations: ALP, Alkaline phosphatase; COL, collagen; Glut4, glucose transporter-4; hMSC, human mesenchymal stem cells; JNK, c-Jun N-terminal kinase; MG, microgravity; MG-E, MG-experienced; OC, osteocalcin; PPAR, proliferator-activated receptor; RCCS, Rotary Cell Culture System; Runx2, runt-related transcription factor 2; RWV, rotary wall vessel; SDS, sodium dodecyl sulfate.

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(0.5 dyn/cm^2) for 1- or 2-mm cellular aggregates (14). During simulated MG, the vessel wall and medium containing cells bound to microcarrier beads rotate at the same speed, producing a vector-averaged gravity comparable with that of near-earth free-fall orbit (15–17). This system was developed by NASA at the Johnson Space Center and has been previously used to simulate the effects of a MG environment on numerous cell culture systems (18, 19). These properties make the HARV a useful tool for studying cellular physiology in a ground-based cell culture system that demonstrates both low shear stress and a gravity-averaged free-fall paradigm (20).

Studies of the effects of MG on bone have demonstrated that the development of osteoblasts is markedly affected when exposed to real and simulated MG conditions. It has been reported that exposing osteoblast-like cells to real MG for 4 d influenced their actin distribution and decreased growth in response to serum stimulation (21). Furthermore, it has also been shown that real MG for 9 d causes a decrease in alkaline phosphatase (ALP) gene expression in cultured human osteoblasts (22). In addition, it has been reported that modeled and real MG effects on osteoblast differentiation are comparable. It has been shown that 7 d of modeled MG reduces the expression of osteoblastic markers, such as ALP and osteocalcin (OC) (23).

Human mesenchymal stem cells (hMSC) are multipotent cells present in adult bone marrow, can replicate as undifferentiated cells, and have the potential to differentiate into different lineages of mesenchymal tissues, including bone, cartilage, fat, muscle, and marrow stroma (24). The osteogenic differentiation of cultured hMSC has been previously characterized (24). Under special *in vitro* conditions, the isolated hMSC form osteoblasts capable of matrix mineralization. The development of the osteoblastic lineage was shown to be dependent on the activation of runt-related transcription factor 2 (Runx2) and ERK MAPK (25, 26). In addition, the activation of p38 MAPK and peroxisome proliferator-activated receptor (PPAR)γ2 accompanied by suppression of Runx2 expression were shown to be important in inducing the differentiation of hMSC to adipocytes (27).

Runx2, also known as Cbfa1, is essential for the differentiation of osteoblasts from mesenchymal precursors (26, 28, 29), because homozygous Runx2 mice show a complete lack of functional osteoblasts (28, 29). Moreover, this factor is required for bone matrix synthesis by differentiated osteoblasts (30), indicating that it regulates osteoblast gene expression at multiple levels. Runx2 expression and activity are modulated during mesenchymal stem cell proliferation, differentiation, and mechanical loading and in response to a variety of extracellular ligands, including TGF-β, bone morphogenetic protein, vitamin D, IGF, estrogen, PTH, and others. The central role of Runx2 in osteogenesis suggests a potentially critical function in the regulation of the hMSC response to MG. Similarly, PPARγ2, a member of the nuclear receptor superfamily of ligand-activated transcription factors, has been shown to be expressed early in the adipocyte differentiation program (31). It acts synergistically with CCAATT enhancer-binding protein α (32) to coordinate the adipocyte differentiation cascade (33, 34). These findings led us to hypothesize that MG-induced bone loss could be due to decreased osteoblast formation through inhibition of Runx2 expression concurrently with increased expression of PPARγ2. Our results support this hypothesis and clearly demonstrate that modeled MG suppresses the osteogenic differentiation of mesenchymal stem cells. In addition, these findings provide evidence that modeled MG creates an artificial environment in which the osteogenic differentiation of hMSC is suppressed, possibly mimicking the effects of aging on the human bone. This will provide us with an appropriate tool to target specific mechanisms involved in decreasing osteoblastogenesis in the aging population, as well as in astronauts.

Materials and Methods

Isolation of hMSC

hMSC were isolated from surgical waste ribs or femoral heads. Bone marrow compartments were gently flushed with α-MEM containing heparin (10 U/ml) and deoxyribonuclease I (1 μg/ml). Marrow cells were pelleted by centrifugation at 500 $\times$ *g* at room temperature for 10 min, and the fat-containing supernatant was decanted. The cell pellet was resuspended in 20 ml α-MEM containing 10% heat-inactivated fetal bovine serum and further purified by gradient density centrifugation using 15 ml Histopaque-1077 (Sigma, St. Louis, MO). Cells present at the interface were harvested, washed, and seeded into T-175 flasks, without disturbance, for 7 d. The attached stromal cells were fed every 3–4 d thereafter. The isolated human stromal cells are able to form mineralized matrix under osteogenic conditions (35), or to differentiate into adipocytes after glucocorticoid induction (36). All the cells used in this study were between passages 2 and 5. We used in our studies hMSC obtained from University of Alabama at Birmingham's hMSC generation facility with an approval from the Institutional Review Board at the University of Alabama at Birmingham. The cells originate from different sources regarding age, race, and gender. In addition, we performed studies on cells obtained from Cambrex Bio Science, Inc. (Walkersville, MD), which were donated by a 19-yr-old Caucasian female. Our results were always consistent regardless of the source of the cells.

Rotary wall vessel (RWV) bioreactor

The RWV bioreactor (Model HARV, size 10 ml) was purchased from Synthecon. It consists of a cylindrical growth chamber that contains a flat silicone rubber gas transfer membrane for oxygenation. The basic principles of this system are: 1) solid body rotation around a horizontal axis, resulting in randomizing the gravitational vector, low fluid shear stress, and three-dimensional spatial freedom; and 2) oxygenation by diffusion of dissolved gasses from the reactor chamber, yielding a vessel devoid of gas bubbles (37). The fluid dynamic principles of the RWV bioreactor allow oxygenation without turbulence, colocalization of particles with different sedimentation rates, and reduction of fluid shear forces. The culture technique commonly employed with these RWVs is the coinoculation of cells and microcarrier beads, into the rotary vessel, followed by an extended culture period. The numerical model used to predict the motion of multiple beads in the rotary vessel is based on the equations of motion for each bead in the rotating reference frame. Details of these equations of motion can be found in previous reports (38, 39). Significant forces included in these equations include the drag force, net buoyancy force from the difference in particle and fluid density, the centrifugal force, and the coriolis force from the rotation of the bioreactor (40).

Cell culture and differentiation

hMSC were maintained in DMEM with 10% fetal bovine serum. Upon reaching subconfluency, hMSC were detached using 0.05% trypsin/0.53 mM EDTA (Invitrogen Life Technologies, Carlsbad, CA), and 10^6 cells were pooled and seeded on 90- to 150-μm plastic microcarriers (SOLOHILL Engineering Labs, Ann Arbor, MI). Cells were cultured on plastic microcarriers in low adhesion plates in normal gravity for 7 d in DMEM with 10% FBS. Unattached cells were counted 24 h after plating on beads. This revealed that 92 $\pm$ 1% of cells successfully attached on

beads within 24 h. Media were changed twice during this period. Cells were then transferred to: 1) RWV to model MG; and 2) 100-mm plates as a gravity control. Osteogenic differentiation was induced immediately before initiation of modeled MG by supplementing maintenance medium with 10 nM dexamethasone, 10 mM β-glycerophosphate, and 50 μM ascorbic acid-2-phosphate. HARVs were rotated to reach a state of solid body rotation where the cells-beads aggregates are suspended within the vessel with little shear force. Medium was changed every 3 d, and cells were harvested 7 d after the initiation of modeled MG and processed for mRNA and protein extraction as described below.

To control for the effect of cells growing in a three-dimensional normal gravity environment or without osteogenic induction, control cells were grown on microcarrier beads in tissue culture dishes with or without osteogenic supplements.

Cell proliferation assay

For [^{3}H]-thymidine incorporation, hMSC cells were cultured on plastic microcarriers, and osteogenesis was induced under normal and modeled MG condition as described above. At the end of the treatment, [^{3}H] thymidine was added to the culture medium at a concentration of 5 μCi/ml. Cells were then incubated for 30 min at 37 C. Culture medium was removed by aspiration, and cells were washed twice with cold PBS. Samples were kept on ice, and 10% cold trichloroacetic acid was added for 5 min. Cells were then solubilized at room temperature with 10% sodium dodecyl sulfate (SDS) for 2 min, after the removal of trichloroacetic acid. Finally, samples were transferred to scintillation vials, and [^{3}H] was counted.

RNA extraction and RT-PCR

Total RNA was extracted using the TRIzol method as recommended by the manufacturer (Invitrogen Life Technologies). The yield and purity of RNA was estimated spectrophotometrically using the A_{260}/A_{280} ratio. The quality of RNA was examined by gel electrophoresis. One microgram of RNA was reverse transcribed using M-MLV reverse transcriptase, and the equivalent of 10 ng was used for the PCRs. These were carried out in a final vol of 25 μl containing 0.2 mM deoxynucleotide triphosphates, 120 nM of each primer, and 1 U Taq-DNA-polymerase. TaqMan real-time quantitative RT-PCR analysis was performed using the relative-standard curve method with SYBRGreen (TaqMan PCR detector 5700; PerkinElmer Applied Biosystems) (35). The expression of 18S rRNA was used as control. The sequences for the specific primers used in this study are listed in Table 1. Regular PCR was performed using recombinant TaqDNA polymerase and a Bio-Rad (Hercules, CA) Thermal Cycler; 30 cycles; melting, 15 sec at 95 C; annealing and extension, 60 sec at 60 C. Amplified products were then loaded on a 2% agarose gel and subjected to electrophoresis. Digital pictures were taken and analyzed using Kodak Digital Science 1D image analysis software (Eastman Kodak Co., Rochester, NY).

Whole-cell protein extraction

At the end of the study, cells were washed with chilled PBS and centrifuged at 800 $\times$ *g* for 5 min at 4 C, then resuspended in lysis buffer (50 mM Tris, pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, and 10% glycerol). A mixture of protease and phosphatase inhibitors consisting of 2 mM phenylmethylsulfonylfluoride, 5 μg/ml aprotinin, 1 mM EGTA, 10 mM NaF, 1 mM sodium pyrophosphate, 1 mM sodium orthovanadate, and 0.1 mM β-glycerophosphate was added to the lysis buffer. Samples were then centrifuged at 14,000 rpm for 30 min at 4 C, and the protein concentration of the supernatant was measured using the Bio-Rad DC protein assay (41).

Western blot analysis

Whole-cell protein extracts were loaded (30 μg/lane) onto an SDS mini-PAGE system. After electrophoresis, proteins were transferred to a polyvinylidene difluoride membrane, Immobilon-P, (Millipore Co., Billerica, MA) using a Bio-Rad wet transfer system. Protein transfer efficiency and size determination were verified using prestained protein markers. Membranes were then blocked with Blotto B (Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h at room temperature and subsequently incubated overnight with antibodies directed against MAPK proteins as well as their phosphorylated forms (Santa Cruz Biotechnology). Signals were detected using a horseradish peroxidase-conjugated secondary antibody and an enhanced chemiluminescence detection kit (ECL, Amersham Bioscience, Buckinghamshire, UK) (35).

Animal studies

Balb/c mice, 5-d-old littermates, were killed; and the calvarial bone was dissected (0.5 $\times$ 0.5 mm). Tissue was cut symmetrically so that it was comparable in shape to cell-bead aggregate. These studies were approved by the Institutional Animal Care and Use Committee at the University of Alabama at Birmingham. Study of the calvarial response to extracellular stimuli has been previously used successfully to examine osteoblast response *ex vivo* to hormones, such as TGF-β (42), and glucose (41). Tissues were washed three times with PBS and initially cultured in α-MEM supplemented with 10% FBS under normal gravity for 2 d to allow for equilibration with the new environment. Calvariae were then divided into two groups, one of which continued to be cultured under normal gravity while the other was subjected to modeled MG by culture in the RWV bioreactor for 7 d (one calvaria in each vessel). Culture medium was supplemented with 10 mM β-glycerophosphate and 50 μM ascorbic acid-2-phosphate. At the end of the study, frozen and plastic sections were taken of the tissue, or it was processed for RNA extraction, as described above. Modified Goldner Trichrome, ALP, and von Kossa staining were used to evaluate bone thickness, osteoblast number, and mineralization by histomorphometric measurements using BioQuant image analysis (BioQuant Image Analysis Corporation, Nashville, TN). To establish baseline data as control, freshly dissected calvarial bone tissue from 5-d-old mice was examined as described above.

Statistical analysis

All statistical analyses were performed using the Microsoft Excel data analysis program for Student's *t* test analysis or using the SPSS (Chicago, IL) statistical analysis program for ANOVA with the Bonferroni test. Experiments were repeated at least three times, unless otherwise stated. Values were expressed as mean $\pm$ SE. To make the variance independent of the mean, statistical analysis of real-time PCR data was performed after logarithmic transformation (43).

TABLE 1. The sequence of primers used

Primer	Forward (5′-3′)	Reverse (5′-3′)
ALP	CCATTCCCACGTCTTCACATT	AAGGGCTTCTTGTCTGTGTCACT
Cbfa1	GATGACACTGCCACCTCTGACTT	AAAAAGGGCCCAGTTCTGAAG
COL IA1	GAGACTGTTCTGTTCCTTGTGTAACTGT	TGCCCCGGTGACACATC
Osteonectin	CCTCCTTTACCTTTCAGTGCAGTT	CAGTCCGTGCTCCCAAAAGT
Osteocalcin	CGCAGCCACCGAGACACCAT	GGGCAAGGGCAAGGGGAAGA
PPARγ2	AAACTCTGGGAGATTCTCCT	TCTTGTGAATGGAATGTCTT
Adipsin	CAAGCAACAAAGTCCCGAGC	CCTGCGTTCAAGTCATCCTC
Glut 4	CGCAGAGAGCCACCCCAGGAAA	GGGTGACGGGGTGGACGGAGAG
Leptin	GTGCGGATTCTTGTGGCTTT	GGAATGAAGTCCAAACCGGTG
18S	CGCCGCTAGAGGTGAAATTCT	CGAACCTCCGACTTTCGTTCT

Results

The effect of modeled MG on bone formation and mineralization was determined using calvariae from 5-d-old mice. The calvarial bone tissue was harvested and then processed to establish a baseline or cultured under normal gravity for 2 d to allow for equilibration to the new environment. Cultured samples were then divided into two groups. One group was cultured in normal gravity while the other was subjected to modeled MG by culture in the RWV bioreactor. Average calvarial bone thickness was measured using BioQuant image analysis and was shown to be increased 3-fold after 7 d of culture in normal gravity (Fig. 1B). In contrast, modeled MG completely inhibited this increase. In addition, there was an increase in the density and number of cells lining the bone surface compared with normal gravity (Fig. 1A). We also performed von Kossa staining, which detects calcium in the bone matrix. Similar to bone thickness, mineralized bone/total bone area was also increased after 7 d of culture in normal gravity, whereas no increase in mineralization was observed in calvariae that was cultured under modeled MG conditions (data not shown). This inhibition of mineralization was not due to an increase in bone resorption as a result

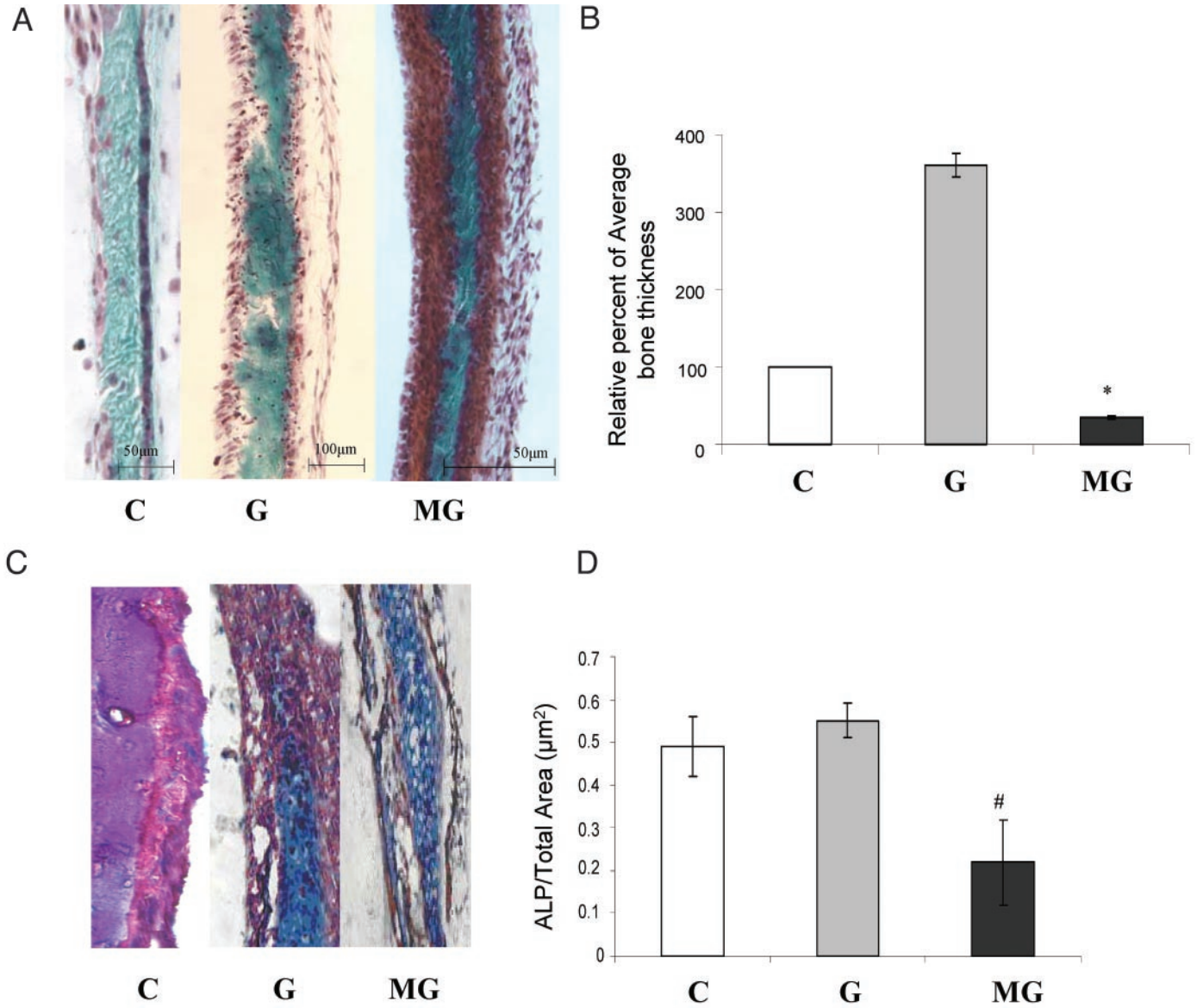

FIG. 1. Modeled MG inhibits bone formation and ALP activity in cultured mouse calvariae. Calvariae from 5-d-old mice were harvested and processed as baseline control (C) or cultured under normal (G) and modeled MG for 7 d. A, Modified Goldner's Trichrome staining was performed to distinguish the mineralized bone (*green*) from the bone lining cells (*red*) in the calvarial sections. The *white horizontal bar* indicates the thickness of the bone. Representative pictures are shown from three different experiments. Panel C, ALP staining was performed to visualize cells exhibiting activity (*red*). Representative pictures are shown of two different experiments. Each experiment was performed on three separate mice littermates. Histomorphometric measurements of calvariae were performed using BioQuant image analysis to measure (B) bone thickness and (D) area of ALP activity, which was then normalized to the total bone area. Values were obtained from the same number of experiments as above and represent mean ± SE. The values from MG-cultured calvariae were significantly different when compared with gravity; *, $P \leq 0.01$; #, $P \leq 0.03$.

of an increase in osteoclast function. Staining for the activity of tartrate-resistant acid phosphatase, a marker for osteoclasts, showed no detectable activity in tissue grown in both gravity and MG conditions (data not shown). These results suggest that, in response to modeled MG, bone formation is decreased, possibly due to decreased osteoblast formation, increased osteoblast apoptosis, decrease in the function of the mature osteoblast, or a combination of all of these. We also attempted to measure the width and area of osteoid (newly formed, unmineralized bone), which proved to be problematic and the data impossible to interpret. Osteoid was only visible at small and irregular parts of the calvarial sections. We felt that the measurement of these areas would be subjective and thus biased.

ALP staining (Fig. 1, C and D) confirms that MG decreases osteoblast number, by demonstrating a 3-fold decrease in ALP-expressing cells in calvariae cultured under MG conditions. The decrease in mineralization of the calvariae and ALP-expressing osteoblasts was not due to an increase in cell death and apoptosis. Terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling staining showed no difference in apoptosis between normal and MG conditions (data not shown).

To confirm the histological observations, we examined ALP and OC gene expression in calvariae harvested from 5-d-old mice before and after culture in both gravity and modeled MG conditions. As expected, MG caused 5- and 2.5-fold decreases in the expression of ALP and OC, respectively (Fig. 2). This decrease in gene expression could reflect a decline in mature osteoblast function. Alternatively, it could be attributed to a lack of osteoblast formation in response to modeled MG, as well as a decline in the function of the osteoblasts that were already present in the calvarial tissue, before the start of the gravity alteration.

To specifically examine the effect of modeled MG on osteoblast formation, we examined the response of isolated hMSC to modeled MG. To accomplish this, we cultured mesenchymal stem cells in osteogenic and nonosteogenic medium (24). Cells were cultured on plastic microcarriers for 7 d to allow them to adhere and aggregate. Osteoblastogenesis was then induced for 7 d. Figure 3A demonstrates that the hMSC cultured on plastic microcarrier beads can be successfully induced to differentiate into osteoblasts. The expression of early osteoblast gene markers has been induced in the newly differentiated osteoblasts but not in the hMSC. Actin and 18S expression was also examined and remained unchanged in the hMSC compared with osteoblasts. In addition, there was no difference in the osteogenic differentiation of hMSC cultured on normal culture plates compared with those cultured on microcarrier beads (data not shown).

We then examined the response of isolated hMSC, cultured under osteogenic conditions, to modeled MG. HMSC were cultured for 7 d on plastic microcarriers under nonosteogenic conditions in normal gravity. Cell aggregates were then divided into two groups before inducing osteoblastogenesis. One continued to be cultured under normal gravity conditions for another 7 d, in the presence of osteogenic medium, while exposing the second group to modeled MG by culture of the cells in an RWV bioreactor under osteogenic conditions. Figure 3B demonstrates that 7 d of modeled MG is sufficient to suppress the osteogenic differentiation of hMSC as demonstrated by the total inhibition of the expression of ALP, COL 1A1, and osteonectin. The expression of actin and 18S remained constant. Osteogenic induction in this study was for 7 d. Therefore, we chose to examine those genes that are expressed early in the development of the osteoblast. We also examined the expression of OC despite the fact that it is a late marker of differentiation; but as expected, its expression was undetectable (data not shown). These results are consistent with our data from the cultured mouse calvariae studies and suggest that the suppression of osteoblast formation by modeled MG plays a role in the decrease in bone formation and mineralization.

The transcription factor, Runx2, is essential for the development of osteoblasts from mesenchymal precursors, osteoblast maturation, and bone formation. Here we analyzed mRNA expression of Runx2 in hMSC, in response to osteogenic induction, in normal and modeled MG. Figure 4A clearly demonstrates our success in differentiating hMSC into osteoblasts that express Runx2, whereas Fig. 4B demonstrates that modeled MG suppresses this Runx2 expression. The decrease in expression of the osteoblastic gene markers was not due to a decrease in hMSC proliferation in response to MG. Figure 5 demonstrates that the proliferation

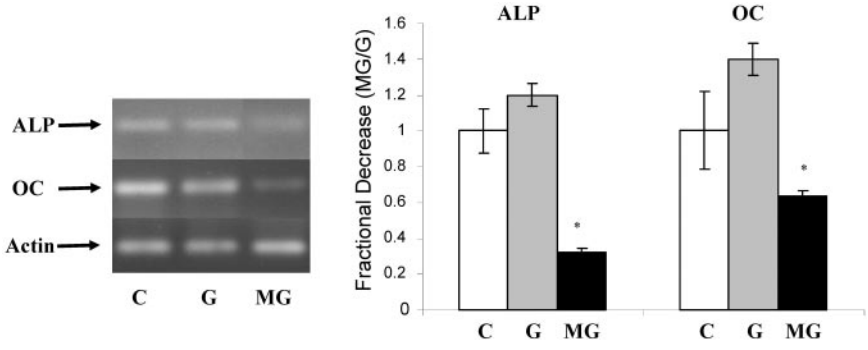

FIG. 2. Modeled MG decreases ALP and OC gene expression in cultured mouse calvariae. Calvariae from 5-d-old mice were harvested and processed as baseline control (C) or cultured under normal (G) and modeled MG for 7 d. RNA was extracted from the tissue at the end of the study. RNA was reverse transcribed and used for conventional and semiquantitative RT-PCRs using primers for ALP, OC, and actin. Representative pictures of three different experiments of conventional RT-PCR are shown. Values were obtained by semiquantitative RT-PCR from the same number of experiments as above and represent mean fold decrease ± SE in gene mRNA expression relative to 18S expression. The values for MG-cultured calvariae were significantly different when compared with gravity controls; *, $P \leq 0.01$.

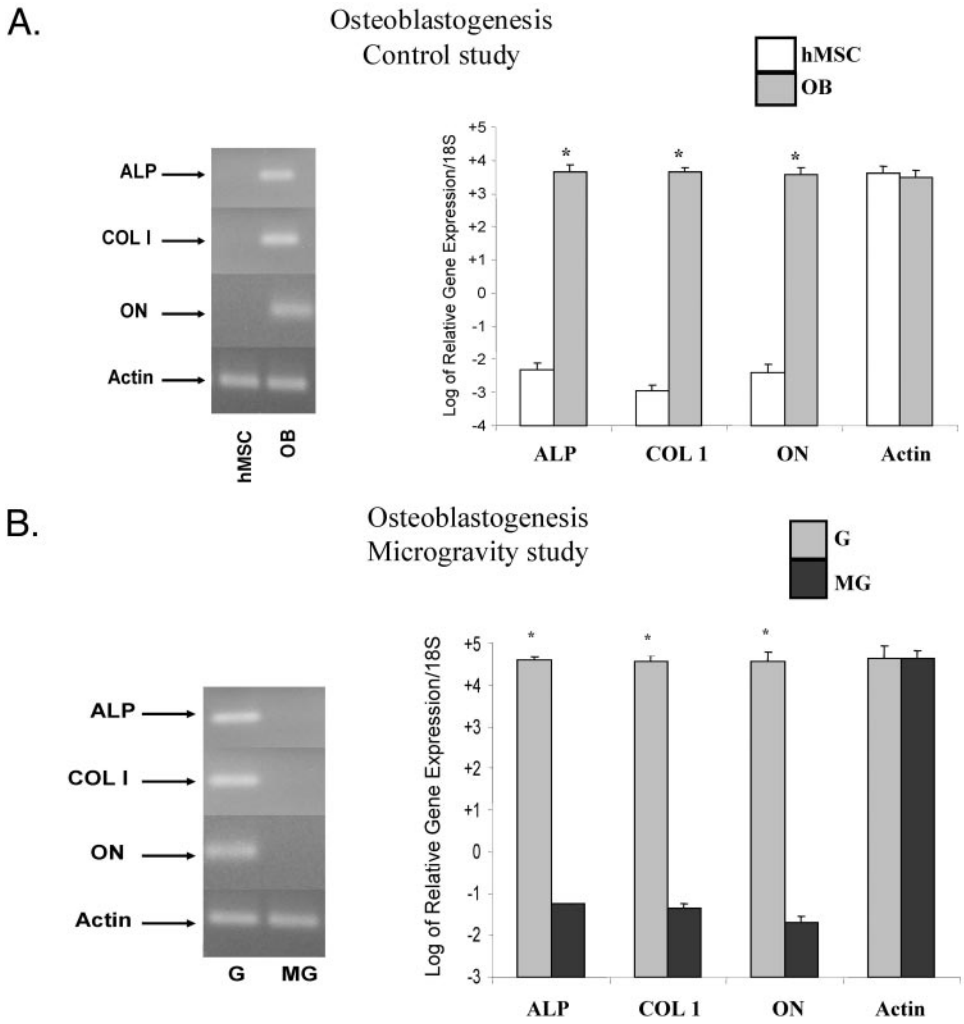

FIG. 3. Modeled MG suppresses osteoblastogenesis. HMSC were cultured on plastic microcarriers for 5 d. Control studies: A, Cells were cultured in osteogenic medium to induce osteoblastogenesis (OB) while leaving others without any induction (hMSC). MG studies: B, Cells cultured under normal gravity in the presence of osteogenic medium (G) while exposing the second group to modeled MG by culturing the cells in an RWV bioreactor (MG) under osteogenic conditions. RNA was extracted at the end of the study. Conventional and semiquantitative RT-PCRs were performed using primers for ALP, COL 1A1, osteonectin (ON), and actin. Representative pictures from four different experiments of conventional RT-PCR are shown. Relative gene expression (mean ± SEM) was obtained from semiquantitative RT-PCR. Tabulated concentrations were normalized to 18S expression and plotted on a log scale. The values for MG-cultured cells were significantly different when compared with normal gravity controls; *, $P \leq 0.01$.

of hMSC reaches a plateau and then significantly decreases within the first 7 d of culture and before osteogenic induction. In addition, Fig. 5 demonstrates that modeled MG does not alter incorporation of [^{3}H] thymidine.

To determine whether hMSC are capable of recovering normal function as mature osteoblasts after a short period of modeled MG, we exposed hMSC cultured in osteogenic medium to 7 d of modeled MG and then continued their culture at normal gravity, under osteogenic induction, for another 30 d. In a parallel experiment, we maintained hMSC in osteogenic medium for the whole duration of the study, as a control (Fig. 6). Interestingly, we have found that the expression of ALP increased 3-fold in MG-experienced (MG-E) cells when compared with control. This suggests that the pattern of gene expression in the MG-E cells has been altered and the cells could be either trapped at an early stage of osteoblastic development, despite the fact that they have been cultured for 30 d in normal gravity, or they have differentiated into another mesenchymal lineage incapable of expressing osteoblastic markers. In addition, the return to normal gravity did not induce the expression of COL 1A1, osteonectin, OC, and Runx2, which was 4- to 5-fold lower than their expression in gravity-cultured hMSC/osteoblasts. This decrease in gene expression was not due to a decrease in the viability of the hMSC after returning them to normal gravity, because expression of actin and 18S remained constant.

It is known that osteoblasts and adipocytes develop from the same mesenchymal stem cells by the activation of the transcription factors, Runx2 and PPARγ2, respectively. Here we demonstrate that exposing hMSC to 7 d of modeled MG not only decreases Runx2 expression but also increases that of PPARγ2, making these cells potentially committed to become adipocytic (Fig. 7A). In addition, Fig. 7B shows that the expression of adipsin, leptin, and glucose transporter-4 (Glut4) (adipocyte gene markers) is also increased after 7 d of modeled MG. This expression remained up-regulated even after the return to normal gravity (Fig. 7B, MG-E).

Given the important role of MAPK in the regulation of hMSC development and lineage commitment, we examined the effects of modeled MG on MAPK activation. Figure 8 shows that total levels of c-Jun N-terminal kinase (JNK), ERK, and p38 were unchanged in hMSC after 7 d of modeled MG, under osteogenic conditions. However, examination of active phosphorylated-MAPK forms identified a decrease in phosphorylated-ERK and an increase in phosphorylated-p38. Changes in the activation of the JNK pathway were not evident. Together, our data indicate that 7 d of modeled MG

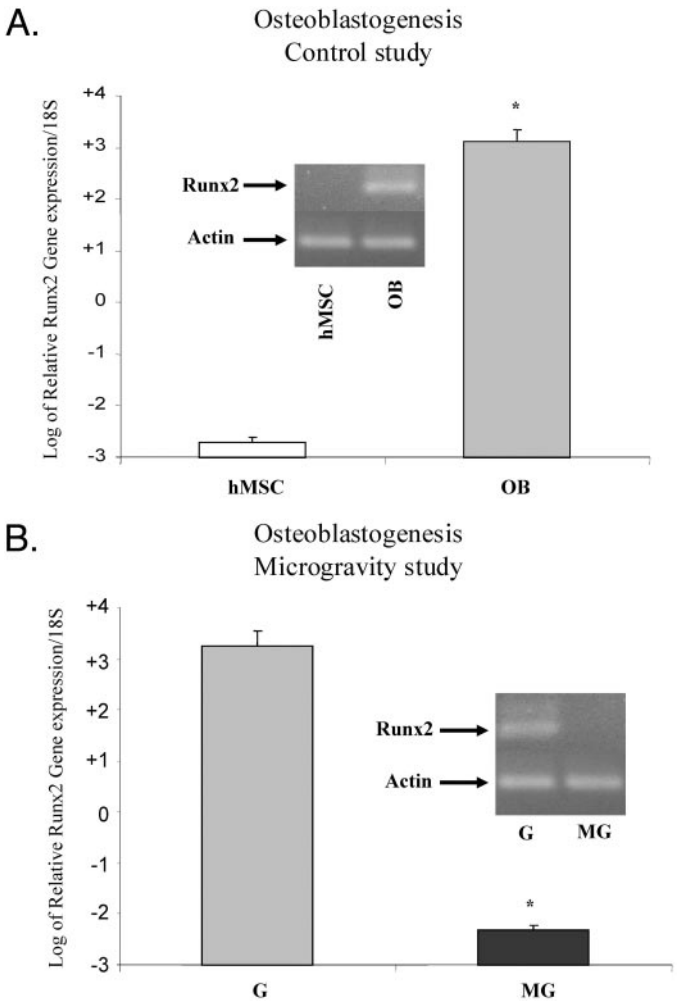

FIG. 4. Modeled MG suppresses Runx2 expression. HMSC were cultured on plastic microcarriers in different conditions. Control studies: A, Cells were cultured in osteogenic medium to induce OB while leaving others without any induction (hMSC). MG studies: B, Cells cultured under normal gravity in the presence of osteogenic medium (G) while exposing the second group to modeled MG by culturing the cells in an RWV bioreactor (MG) under osteogenic conditions. RNA was extracted at the end of the study, and conventional and semiquantitative RT-PCRs were performed using primers for Runx2. Representative pictures of PCRs are shown for four different experiments. Relative gene expression (mean ± SEM) was obtained from semiquantitative RT-PCR. Tabulated concentrations were normalized to 18S expression and plotted on a log scale. The values for MG-cultured cells were significantly different when compared with normal gravity controls; *, $P \leq 0.01$.

using the RWV bioreactor inhibits the osteoblastic differentiation of hMSC coupled with a decrease in the activation of ERK and the complete suppression of Runx2 and osteoblast gene marker expression. Concomitantly, modeled MG increased the activation of p38 and the expression of PPARγ2 and other adipogenesis gene markers, which could contribute to the overall decrease in bone formation and mineralization in response to weightlessness.

Discussion

Skeletal abnormalities, including osteopenia (7, 44–49), decreased bone formation (7, 48, 50–52), decreased mineralization (44), and reduced bone strength (53, 54), have been identified in astronauts and animals after space flight, raising the concern that long-duration missions may have a negative impact on astronauts' health. Similarly, these abnormalities are increasingly evident in elderly men and women and in patients after extended period of disuse. The effects of long-duration space flight on bone mineral density show considerable individual and site variation.

To address the issue of bone loss in MG, investigators have used several techniques and tools to model MG in earth-based studies. Some of the early studies to model the effects of MG on bone were performed using the rat hindlimb elevation model. Unfortunately, ground-based models for simulating MG effects on bone must overcome enormous technical problems to enable us to have confidence in the obtained data. Although there is disagreement about whether ground-based studies truly simulate MG (14), certain cell culture systems provide a model for investigating altered gravity effects. The RCCS we used for our studies has been widely used and validated by numerous reports. Similar to the hindlimb elevation model, this system has its unique limitations, as demonstrated by several conflicting reports (22, 55). The RCCS simulates MG by randomizing the gravitational vector in response to the rotation of the culture vessel around a horizontal axis. This may or may not be identical with true MG. The constant change in direction of the gravitational field may result in alteration of different signaling pathways, which may be different from those altered in response to a near-complete lack of any gravitational field. Nonetheless, the design of the RCCS is based on the same principles that cause reduced gravity as a result of orbital space flights (38, 39). Despite all limitations and contradictory reports, until the experiments are conducted under true MG in space, validated earth-bound models will have to suffice.

We used cultured mouse calvariae as a model to examine the effects of modeled MG on osteoblast formation and differentiation. Calvariae from newly born mice are mineralized postnataly, providing a perfect opportunity to examine the role of different treatments on osteoblast formation and differentiation. This method has been previously used successfully to examine osteoblast responses to different conditions, such as treatment with TGF-β (42) and glucose (41).

Our results are consistent with other earth-based studies (47). We demonstrate that short-term modeled MG (7 d) causes a decrease in bone growth and mineralization. Our results are also consistent with data generated from space-based studies on rhesus monkeys (56). Interestingly, Rodionova *et al.* (56) reported that, in addition to the decline in osteoid mineralization, there was a significant increase in the fibrotic zones in the bone, suggesting that osteoblasts were transformed to fibroblast-like cells. Similarly, we show an increase in the cellularity of layers surrounding the bone, as if the osteoblast progenitors are unable to differentiate into osteoblasts or dedifferentiate into fibroblastic stromal cells. Osteoblasts are distinguished by their ability to express ALP. Here, we demonstrate that the number of cells capable of expressing ALP declined 3-fold in modeled MG compared with control.

In many tissues, changes in gene expression have been

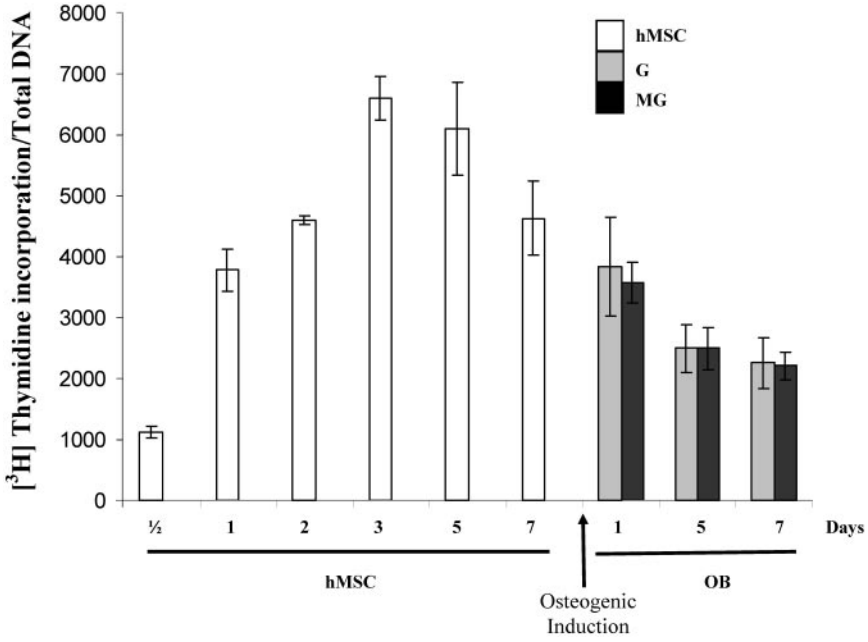

FIG. 5. Modeled MG does not alter hMSC proliferation during osteoblastogenesis. HMSC cells were cultured on microcarrier plastic beads in normal gravity without osteogenic induction (hMSC) for 12 h, 1, 2, 3, 5, 7 d. Osteogenic induction was then performed to induce OB as indicated, and cells were cultured either in normal (G) or modeled MG (MG) for 1, 5, and 7 d. At the end of the culture period, 5 μCi/ml [^{3}H] thymidine was added for 30 min, and thymidine incorporation was measured. Results from the incorporation of [^{3}H] thymidine (mean ± SEM) are combined from two experiments (duplicate determination for each condition).

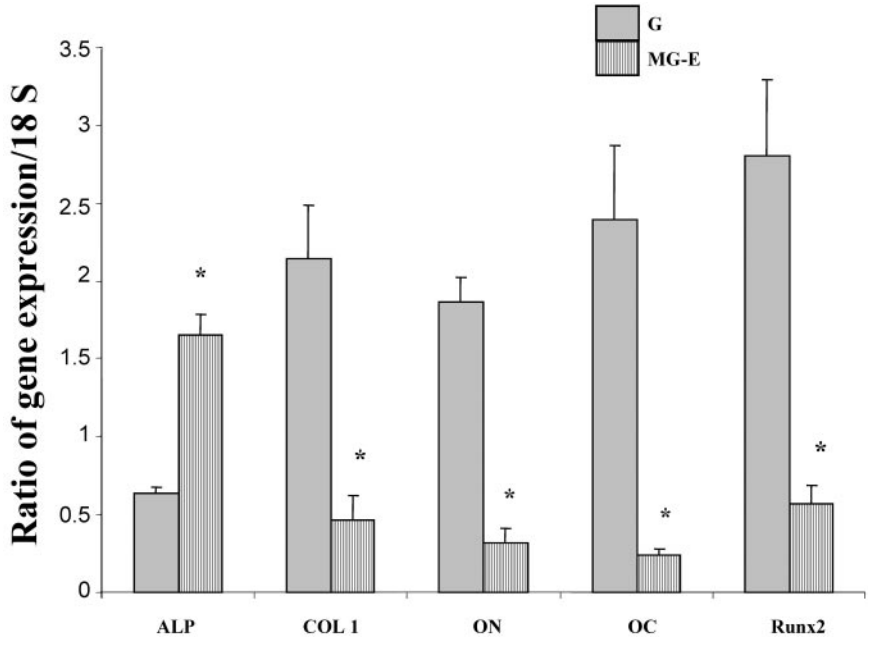

FIG. 6. Modeled MG effects are not reversible. HMSC cells were cultured under normal gravity in the presence of osteogenic medium (G) while a second group was exposed to modeled MG by culture of the cells in an RWV bioreactor for 7 d under osteogenic conditions, then returned to normal gravity for a period of 30 d readaptation (MG-E), while leaving the G cells in normal gravity for all 37 d. RNA was extracted at the end of the study, and semiquantitative RT-PCRs were performed using primers for ALP, COL 1A1, ON, OC, and Cbfa1/Rnux2. Values were obtained from three experiments and represent mean values ± SE in gene mRNA expression relative to 18S expression. The values for osteoblast gene expression were significantly different when compared with hMSC controls. The values for MG-cultured cells were similarly significantly different when compared with normal gravity controls; *, $P \leq 0.05$.

reported to be associated with the phenotypic changes in response to MG. In mouse muscle, the expression of the transcription factor, myoD, and the myogen gene are significantly reduced after space flights (57). Similarly, analysis of rat bone after space flight demonstrated a decrease in COL 1 and OC expression (58). We show that the expression of both ALP and OC was significantly reduced in cultured mouse calvariae in response to 7 d of modeled MG. These observations could be explained by: 1) a decrease in the osteogenic differentiation of bone mesenchymal stem cells; 2) a decrease in the differentiation and function of osteoblasts; and 3) an increase in osteoblast apoptosis. Interestingly, Rucci *et al.* (55) reported a decrease in the viability and proliferation of cells cultured in the RWV bioreactor under modeled MG conditions. They attributed this observation to an increase in apoptosis. Contrary to their findings, we found no increase in apoptosis in cultured calvariae under MG when compared with the gravity control. This may be attributed to the different type of cells used in their study (rat osteosarcoma cells, stably transfected with mouse estrogen receptor, ROS.SMER#14). In addition, the above study showed an increase in ALP and OC gene expression in response to modeled MG. This is in contrast to data from several labs, including ours, where a decline in osteoblast gene marker expression has been demonstrated (22, 23, 59).

Here we show that hMSC cultured on microcarriers can successfully differentiate into functional osteoblasts capable of expressing gene markers that are totally absent in undif-

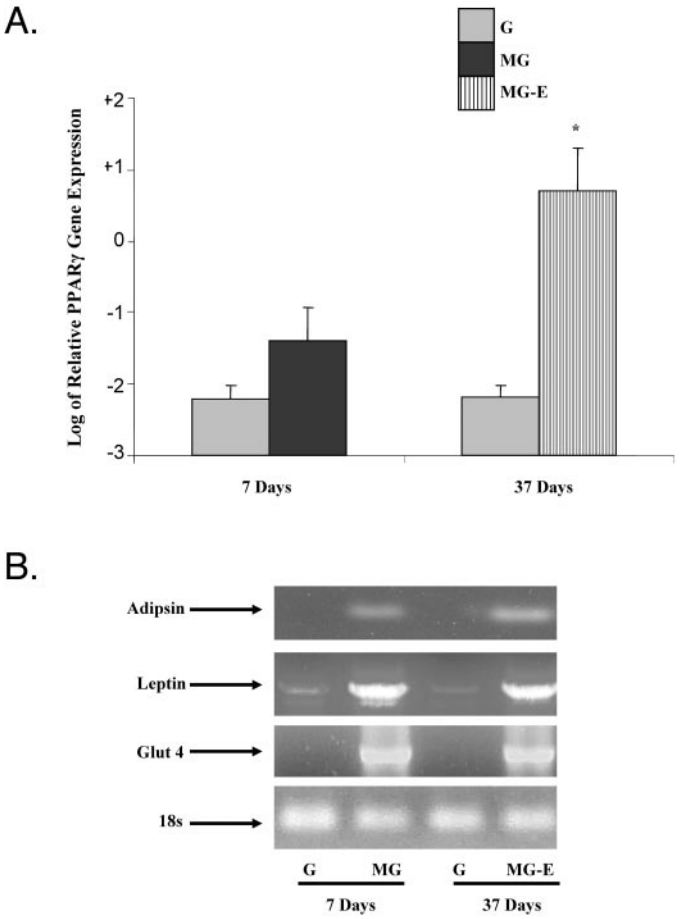

FIG. 7. Modeled MG induces the expression of PPARγ2 and several adipogenic markers. HMSC cells were cultured in normal gravity conditions in the presence of osteogenic medium (G) while exposing a second group to modeled MG by culturing the cells in an RWV bioreactor (MG) for 7 d under osteogenic conditions. MG cells were then returned to gravity for a period of 30 d (MG-E) while leaving the G cells without any gravity alteration for the whole 37 d. RNA was extracted on d 7 and at the end of the study, and semiquantitative and regular RT-PCRs were performed using primers for PPARγ2, adipsin, leptin, and Glut-4. A, Relative PPARγ2 gene expression (mean ± SEM) was obtained by semiquantitative RT-PCR. Tabulated concentrations were normalized to 18S expression and plotted on a log scale. The values for MG-E cells were significantly different when compared with normal gravity controls; $P \leq 0.05$. B, Representative pictures of standard PCRs for adipsin, leptin, and Glut-4 are shown for three different experiments.

ferentiated hMSC. Although our results are in contrast to those reported by some, where the undifferentiated hMSC express low levels of ALP, collagen (COL) 1, and Runx2, our results are consistent with various other reports demonstrating that levels of these genes are undetectable before osteogenic induction (60, 61). This variation could be due to the stage of differentiation and the purity of the hMSC at the time of isolation, clone variation, donor age, and number of passages *in vitro* (62). This system provided us with a strong tool to study the effects of modeled MG on the osteoblastic differentiation of hMSC. The expression of these osteogenic markers was not detected in hMSC, cultured under modeled MG conditions, whereas actin and 18S expression remained constant. This led us to conclude that 7 d of modeled MG is sufficient to totally suppress osteoblast formation. We have also exposed hMSC to longer periods of modeled MG (14 and 21 d). This gave similar results, because hMSC did not differentiate into osteoblasts, regardless of the period of exposure (data not shown). Based on this, we performed all remaining studies after 7 d of modeled MG. This earth-based demonstration of the ability of modeled MG to inhibit cellular differentiation is consistent with space-flight studies both *in vivo* from mouse skeletal muscle (57) and *in vitro* culture of MG-63 osteoblasts (23).

Runx2 is an essential transcription factor for the osteogenic differentiation of hMSC (28, 63). We demonstrate here that modeled MG completely inhibits the expression of Runx2. These results are similar to those derived from an available animal model where the Runx2 gene is knocked-out and there is a complete lack of osteoblast development (28, 29). Interestingly, rats exposed to skeletal unloading induced by hindlimb suspension also demonstrate a decrease in the expression of Runx2, as well as COL 1 and OC. In addition, the same study demonstrated an increase in adipocyte differentiation (64). Similarly, our results demonstrate that modeled MG increases the expression of PPARγ2, a critical transcription factor involved in adipogenic differentiation (65). The accompanied increase in the expression of adipsin, leptin, and Glut4 indicates that modeled MG is leading to the adipogenic rather than the osteogenic differentiation of hMSC. Our present findings suggest that modeled MG acts on the common precursor cell in the bone marrow to promote its commitment to the adipocyte lineage, at the expense of the osteoblastic lineage. These changes in gene expression were not reversible. Thirty days after readaptation to normal gravity, the expression of osteoblast gene markers (with the exception of ALP) remained lower than in the gravity control. Our data are in contrast to an *in vivo* study on rats exposed to MG in space (66), where bone formation was fully compensated within 14 d of readaptation. In support of our data, cosmonauts (n = 15) who suffer from serious bone loss in space have been reported to continue to have significant tibial bone loss 6 months after readaptation (67). It has been previously suggested that MG resets the pattern of gene expression in osteoblasts (68). Our data clearly demonstrate that, during 7 d of MG, the gene expression in mesenchymal stem cells shifts toward the adipogenic instead of the osteoblastogenic. Therefore, even after the return to gravity, hMSC that are now preadipocytic cannot successfully differentiate into osteoblasts, thus explaining their inability to significantly express osteoblastic markers except ALP. Interestingly, it has also been shown that the activation of PPARγ in mouse bone marrow cells by thiazolidinediones induces adipogenesis instead of osteoblastogenesis, as detected by an increase in the gene expression of adipsin and PPARγ, without affecting ALP activity (69). This suggests that MG alters the pattern of phenotypic gene expression in hMSC supporting adipogenesis, without affecting ALP.

Modulation of Runx2 and PPARγ2 expression by MG can occur through a variety of pathways, including those that regulate transcription and/or those that result in posttranslational activity. It is known that the protein kinase A and ERK pathways activate and phosphorylate Runx2 (70–72). It has also been shown that the inhibition of ERK in hMSC does

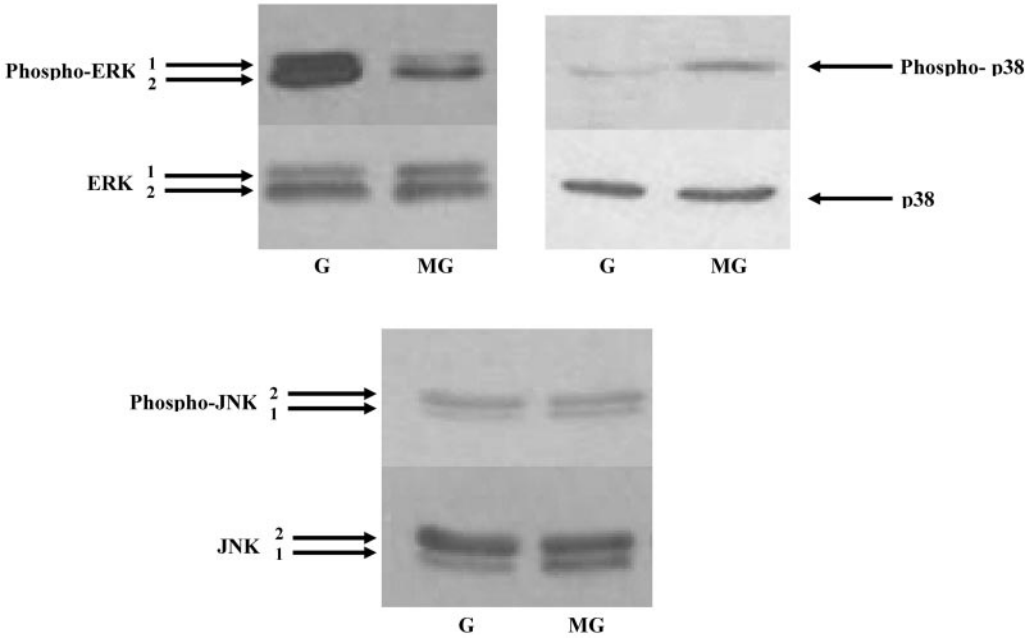

FIG. 8. Modeled MG decreases ERK activation and enhances p38 phosphorylation. HMSC were cultured on plastic microcarriers for 5 d. One group of cells were then cultured under normal gravity in the presence of osteogenic medium (G) while exposing another to modeled MG by culturing the cells in an RWV bioreactor (MG) under osteogenic conditions. Cells were harvested at the end of the study for whole-cell protein extraction. Extracts (30 μg/lane) were separated by 10% SDS-PAGE. Immunoblots were developed using specific antibodies directed against phosphorylated and total ERK, JNK, and p38. The autoradiograph shown is representative of three separate experiments.

not only lead to inhibition of osteoblastogenesis but also to the induction of adipogenesis (73). We show here that modeled MG decreases the phosphorylation of ERK, which ultimately could lead to a decrease in the activity and expression of Runx2 and its target genes, COL 1A1 and OC. Interestingly, hypergravity influences the differentiation of osteoblast-like cells by modulating the same pathways but in a different direction. Gebken *et al.* (74) have shown that exposing human osteoblast cells to hypergravity results in a 40% increase in COL 1 synthesis, a response mediated by increasing ERK phosphorylation. The inhibition of this pathway suppressed the hypergravity-induced stimulation of COL synthesis. On the other hand, it has been shown in other systems that the activation of p38 MAPK is a cellular response to mechanical stress (41) and potentially leads to PPARγ2 activation (75). Our results suggest that the effects of modeled MG on hMSC differentiation could be due, in part, to a decrease in ERK and an increase in p38 MAPK activity. The JNK phosphorylation was not altered by modeled MG exposure.

This work provides new insight into the effects of MG on bone formation by specifically investigating the differentiation of hMSC. We show that modeled MG, using the RCCS, inhibits osteoblastic differentiation of hMSC and induces markers of adipocytic lineage development. This is accompanied by the modulation of MAPK activity, ERK and p38, and the expression of the transcription factors, Runx2 and PPARγ2. This *in vitro* system provides an excellent model for investigation of molecular mechanisms of osteoblastogenesis and mesenchymal stem cell signaling. As such, it presents a unique opportunity to investigate molecular mechanisms for reversal of decreased osteoblastogenesis that may be translated to mechanisms highly relevant to human diseases such as osteoporosis.

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