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ARTICLE

Simulated Microgravity Suppresses Osteoblast Phenotype, Runx2 Levels and AP-1 Transactivation

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Abstract Conditions of disuse such as bed rest, space flight, and immobilization result in decreased mechanical loading of bone, which is associated with reduced bone mineral density and increased fracture risk. Mechanisms involved in this process are not well understood but involve the suppression of osteoblast function. To elucidate the influence of mechanical unloading on osteoblasts, a rotating wall vessel (RWV) was employed as a ground based model of simulated microgravity. Mouse MC3T3-E1 osteoblasts were grown on microcarrier beads for 14 days and then placed in the RWV for 24 h. Consistent with decreased bone formation during actual spaceflight conditions, alkaline phosphatase and osteocalcin expression were decreased by 80 and 50%, respectively. In addition, runx2 expression and AP-1 transactivation, key regulators of osteoblast differentiation and bone formation, were reduced by more than 60%. This finding suggests that simulated microgravity could promote dedifferentiation and/or transdifferentiation to alternative cell types; however, markers of adipocyte, chondrocyte, and myocyte lineages were not induced by RWV exposure. Taken together, our results indicate that simulated microgravity may suppress osteoblast differentiation through decreased runx2 and AP-1 activities. J. Cell. Biochem. 88: 427–437, 2003.

Key words: bone; osteoblast; cbfa1; clinostat; RWV

Increased mechanical loading associated with weight bearing exercise causes bone compression, tension, and shear stress ultimately leading to enhanced bone formation [Sessions et al., 1989; McDonald et al., 1994; Forwood et al., 1996; Haapasalo et al., 1996; Turner and Pavalko, 1998; Daly et al., 1999; Cullen et al., 2001]. Given the important role for mechanical loading in the maintenance of skeletal mineralization and strength, it is not surprising that conditions of disuse result in bone loss [Sessions et al., 1989; Kannus et al., 1996; Jadvar, 2000; Uebelhart et al., 2000]. Decreased mechanical loading and aging are associated with decreased bone mineral density and increased fracture risk [Vernikos, 1996; Collet et al., 1997; Uebelhart et al., 2000]. This phenomenon is clearly seen under space flight conditions where bone is exposed to minimal mechanical loading resulting in decreased bone mineral density in humans [Vernikos, 1996; Collet et al., 1997; Vico et al., 2000], monkeys [Zerath et al., 1996b], rats [Kitajima et al., 1996; Zerath et al., 1996a; Lafage-Proust et al., 1998] and mice [Van Loon et al., 1995; Bateman et al., 2002]. The cellular and molecular mechanisms that regulate bone mineral density in response to loading or unloading remain unclear. However, suppression of bone formation under decreased loading suggests a role for osteoblasts [Morey and Baylink, 1978; Jee et al., 1983; Wronski et al., 1987; Vico et al., 1988; Turner et al., 1995; Vico et al., 1998]. Osteoblasts are derived from mesenchymal stem cells that advance to the osteoblast lineage and progressively differentiate through the upregulation of runx2, AP-1, and other transcription factors. Under normal conditions this leads to upregulation of alkaline phosphatase followed by osteocalcin expression and bone formation. However, if factors involved in this progression are altered, bone loss can occur.

Two possible scenarios leading to decreased osteoblast function under unloading conditions are: (1) a decrease in pre- and/or

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committed-osteoblast proliferation resulting in fewer cells to form bone or (2) a reduced ability to differentiate and produce bone (either by dedifferentiation, transdifferentiation, or direct effects on osteoblast function). When osteoblast histology is examined after spaceflight, an increase in less-differentiated and a decrease in more-differentiated osteoblasts can be seen [Garetto et al., 1990]. This suggests that microgravity causes a block in the differentiation pathway of osteoblasts. Studies examining osteoblasts in vitro under microgravity conditions indicate that reduced gravity can directly alter osteoblast function, apart from changes in systemic factors or interactions with other cells such as osteoclasts [Hughes-Fulford and Lewis, 1996; Carmeliet et al., 1997; Carmeliet et al., 1998]. For example, 4 days of space flight directly influences mouse osteoblast actin distribution and decreases cell growth in response to serum [Hughes-Fulford and Lewis, 1996]. Space flight also suppresses human and embryonic chick osteoblast differentiation as marked by reduced alkaline phosphatase and osteocalcin activity, secretion and/or expression [Carmeliet et al., 1997; Carmeliet et al., 1998; Landis et al., 2000]. In addition, space flight conditions suppress TGF-beta and vitamin D induction of alkaline phosphatase and osteocalcin expression [Carmeliet et al., 1997]. In vivo models of unloading, such as hind limb suspension, also result in decreased bone mass [Globus et al., 1986; Wronski and Morey-Holton, 1987] and suppressed osteoblast function [Kostenuik et al., 1997; Dehority et al., 1999; Ahdjoudj et al., 2002]. Taken together, these data implicate space flight induced decreases in mechanical force as a regulator of osteoblast function.

To address the role of microgravity on cells on earth, ground based models of space flight associated microgravity conditions have been developed [Klement and Spooner, 1993]. These units include the clinostat and rotating wall vessel (RWV), which utilize solid phase rotation to maintain cells in suspension, with or without microcarrier beads as an attachment substrate (see Fig. 1). Under these conditions cells experience randomized g-vectors and minimal shear stress [Goodwin et al., 1993; Unsworth and Lelkes, 1998; Hammond and Hammond, 2001]. This environment has been demonstrated to alter the differentiation, phenotype and gene expression patterns of several cell types including rat adrenal medullary cells [Lelkes et al., 1998], tracheal epithelial cells [Felix et al., 2000], skeletal muscle [Slentz et al., 2001], peripheral blood lymphocytes [Risin and Pellis, 2001], and human prostate carcinoma cells [Clejan et al., 2001]. Findings from osteoblast cultured in the RWV indicate altered cytokine expression [Rucci et al., 2002], apoptosis [Sarkar et al., 2000; Rucci et al., 2002], and variable effects on differentiation [Kunisada et al., 1997; Granet et al., 1998; Rucci et al., 2002].

Progression to a mature osteoblast phenotype is associated with three stages: growth, extracellular matrix maturation, and extracellular mineralization. Each stage is marked by the temporal expression and repression of genes [Owen et al., 1990; Lian and Stein, 1992]. Nuclear run-on analyses demonstrate a significant transcriptional component to the regulated pattern of gene expression [Stein et al., 1996]. Many transcription factors are involved in regulating osteoblast gene expression and subsequent differentiation, including runx2 and AP-1. Runx2 is a homeodomain protein critical in regulating osteoblast lineage selection and differentiation [Lian et al., 1998; Ducy, 2000; Karsenty, 2001]. Runx2 binds to and regulates gene expression through the osteoblast-specific cis-acting element 2 (OSE2) [Ducy and Karsenty, 1995; Banerjee et al., 1997] which is found in the promoter region of many major osteoblast-specific genes including alkaline phosphatase, bone sialoprotein, osteocalcin, and matrix metalloproteinase-13 [Ducy et al., 1997; Lian et al., 1998; Harada et al., 1999; Jimenez et al., 1999]. In mice, inactivation of runx2 through deletion or mutation blocks bone formation [Komori et al., 1997; Otto et al., 1997]. In humans, a heterozygous mutation in runx2 results in cleidocranial dysplasia [Lee et al., 1997; Mundlos et al., 1997], marked by delayed suture formation and the absence of clavicle formation.

AP-1 members have been shown to be developmentally regulated [McCabe et al., 1995] and also play a role in regulating expression of genes which promote osteoblast differentiation including collagen I, alkaline phosphatase, and osteocalcin [McCabe et al., 1996; Lian et al., 1998; Palcy et al., 2000; Zayzafoon et al., 2000]. The AP-1 family of transcription factors is composed of seven Fos and Jun members which dimerize with each other (Fos–Jun, Jun–Jun) prior to binding DNA. Overexpression of specific

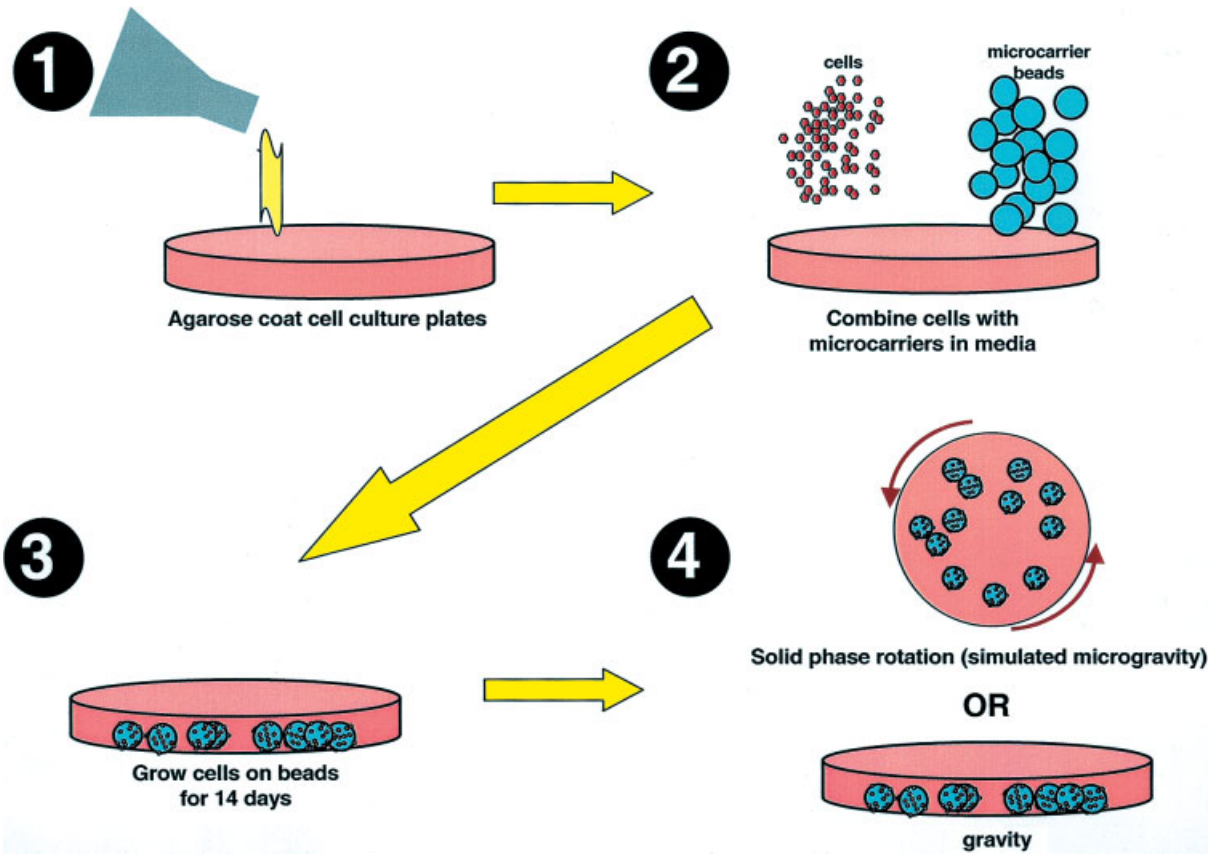

Fig. 1. Experimental design. Bacterial culture plates are coated with 1% agarose/1 × PBS to prevent cells from attaching to plates (**1**). Cells and microcarrier beads are combined to allow attachment of cells to beads (**2**). Cells are cultured on beads for 14 days (**3**). After 14 days, half of the cells on beads are put into the RWV to simulate microgravity conditions for 24 h, while the other half of the cells are maintained on cell culture plates to serve as unit gravity controls (**4**).

family members such as delta Fos B [Jochum et al., 2000b; Sabatakos et al., 2000] or Fra-1 [Jochum et al., 2000a] results in increased mouse bone formation, while suppression of Fra-2 expression results in decreased bone nodule formation in tissue culture [McCabe et al., 1996]. Because many of the genes regulated by AP-1 are also regulated by runx2, it is conceivable that cooperative regulation of these factors occurs at some, if not all of the sites. Work by D'Alonzo et al. [2002] shows that c-Fos and c-Jun physically interact with runx2 to regulate parathyroid hormone dependent MMP13 expression in osteoblasts. This suggests that runx2 and AP-1 may directly interact to activate transcription.

Based on the above findings, we determined the involvement of runx2 and AP-1 in osteoblast phenotype modulation under simulated microgravity conditions in the RWV. While other studies have demonstrated long-term influences of simulated microgravity on osteoblast phenotype, we focused on immediate events associated with a 24 h exposure to simulated microgravity. Our results demonstrate that this amount of time is sufficient to downregulate markers of osteoblast differentiation and suppress runx2 expression and AP-1 transactivation. The coordinate decrease in runx2 and AP-1 implicates them in the transcriptional regulation of genes responsible for decreased osteoblast differentiation under conditions of unloading.

METHODS

Cell Culture System

MC3T3-E1 cells [Sudo et al., 1983], subcloned for maximal alkaline phosphatase staining and mineralization, were used for all studies.

Osteoblasts were seeded at 1,000 cells/mg gelatin-coated microcarrier beads, which are 90–150 μm in diameter (Solohill, Ann Arbor, MI). Osteoblasts were cultured for 14 days and fed every two days with α-MEM containing 10% fetal calf serum (Atlanta Biologicals Norcross, GA) and supplemented to a final concentration of 2 mM β-glycerophosphate (Sigma, St. Louis, MO) and 25 μg/ml ascorbic acid (Sigma). On day 14, half of the cells on microcarriers were placed into the RWV to simulate microgravity, while the other half were left at unit gravity.

Rotating Wall-Vessel (RWV)

The RWV cell culture system (models 55 ml STLV and 10 ml HARV) was purchased from Synthecon, Inc. (Houston, Texas) and used for studies of simulated microgravity. The RWV bioreactor is a cylindrical vessel containing an inner oxygenator core (surrounded by a membrane) and an outer core filled with culture media into which cells grown on microcarrier beads are placed (Fig. 1). The vessel is connected to a rotator base and rotated along its longitudinal axis at a speed where cells establish a near solid phase rotation with the culture medium. The fluid dynamics in the RWV allow for co-localization of cells and aggregates of different sedimentation rates, three-dimensional spatial freedom, reduced fluid shear forces, and oxygenation without turbulence by diffusion [Hammond and Hammond, 2001].

RNA Analysis

Total RNA was extracted using the TRI Reagent RNA isolation reagent (Molecular Research Center, Inc., Cincinnati, OH) and integrity was verified by formaldehyde-agarose gel electrophoresis (Fig. 3). First strand cDNA synthesis was performed by reverse transcription with 2 μg of total RNA using the Superscript II kit with oligo d($T_{12\text{-}18}$) primers as described by the manufacturer (Invitrogen, Carlsbad, CA). cDNA (1 μl) was amplified by PCR in a final volume of 25 μl using the SYBR Green PCR Core Reagent kit (Applied Biosystems, Warrington, United Kingdom) with 10 pmol of each primer (Integrated DNA Technologies, Coralville, IA). TBP primers were used as internal controls along with the primers of genes being analyzed, including those associated with stages of osteoblast differentiation: collagen I (Col I), alkaline phosphatase (AP), and osteocalcin (OC) (Table I). Real time PCR was carried out for 40 cycles using the iCycler (Bio-Rad, Hercules, CA) and data were evaluated using the iCycler software. RNA-free samples, a negative control, did not produce amplicons. Melting curve and gel analyses were used to verify single products of the appropriate base pair size.

Transient Transfections

MC3T3-E1 cells were seeded onto microcarriers as described above. Twenty-four hours later, cells on microcarriers were transferred to 1% agarose/1X PBS coated 6-well tissue culture plates and cotransfected using lipofectamine (Gibco, Rockville, MD) with 900 ng of a 6XAP-1 luciferase reporter (Gibco) and 100 ng of a pSV40 β-galactosidase reporter to normalize transfection efficiency in a total volume of 1 ml serum-free Opti-MEM (Gibco). Five hours after transfection, 1 ml of α-MEM containing 20% FBS was added to the transfectants. Cells were cultured for 14 days and then transferred into the RWV or unit gravity control plates for 24 h. Cells were harvested, washed with 1 × PBS, lysed for analysis of luciferase and β-galactosidase activities using the protocol provided by the manufacturers (Promega, Madison, WI; Clontech, Palo Alto, CA, respectively), and quantitated on a Turner TD 20E luminometer (Turner Designs, Sunnyvale, CA).

Statistical Analysis

All statistical analyses were performed using Microsoft excel data analysis program

TABLE I. PCR Primers

mRNA	Left primer (5′-3′)	Right primer (5′-3′)
AP	CAG TAT GAA TTG AAT CGG AAC AAC C	CAG CAA GAA GAA GCC TTT GAG G
OC	ACG GTA TCA CTA TTT AGG ACC TGT G	ACT TTA TTT TGG AGC TGC TGT GAC
COL1	CCC TCA ACC CCG TCT ACT TC	GGA GAT GCC AGA TGG TTA GG
PPAR	AGA AAT TAC CAT GGT TGA CAC AGA G	GTG AAT GGA ATG TCT TCA TAG TGT G
SOX9	AAG CTG GCA AAG TTG ATC TGA AG	CAA GTA TTG GTC AAA CTC ATT GAC G
MEF2A	ATT CAA CTC CAA TTC CTC TTC CTC	AAA TAC TGG TGC AAA TAG TTT AGG C
RUNX2	GAC AGA AGC TTG ATG ACT CTA AAC C	TCT GTA ATC TGA CTC TGT CCT TGT G
TBP	CCA TTC TCA AAC TCT GAC CAC TGC	CAG AAG CTG GTG TGG CAG

for *t*-test analysis. Experiments were repeated at least three times. Values are expressed as a mean ± SE.

RESULTS

To address the influence of acute exposure to unloading, differentiating osteoblasts were transferred to a rotating wall vessel (RWV) to simulate microgravity. Figure 1 illustrates the experimental design. Osteoblasts were seeded onto microcarrier beads in agarose-coated plates to prevent attachment of cells to the plate surface. After 14 days, at which point markers of osteoblast differentiation were detectable, beads were either retained at unit gravity or placed into the RWV. The RWV was rotated at 20–25*g* to approximate solid phase rotation.

Measurement of alkaline phosphatase and osteocalcin mRNA levels revealed that 24 h of RWV exposure dramatically suppressed these markers of osteoblast maturation compared to control cultures (Fig. 2). Specifically, alkaline phosphatase and osteocalcin expression were decreased to less than 20 and 50% of control levels, respectively. This supports the hypothesis that osteoblast differentiation is suppressed under acute RWV conditions. In contrast, TATA binding protein (TBP) mRNA levels were unchanged by RWV exposure (Fig. 3) demonstrating that decreases in alkaline phosphatase and osteocalcin expression are not due to changes in general cellular transcription. In addition, RNA was found to be similarly intact under both unit gravity and microgravity conditions (Fig. 3).

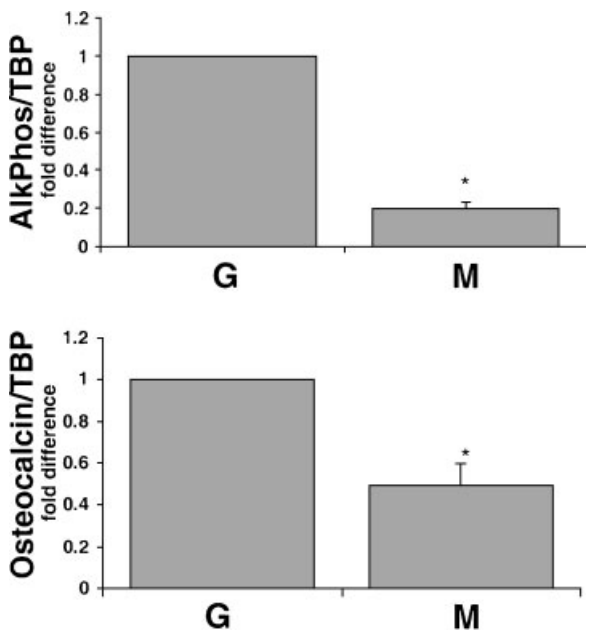

Fig. 2. Acute RWV exposure decreases alkaline phosphatase and osteocalcin expression. MC3T3 E1 osteoblasts were cultured for 14 days and left at unit gravity (G) or simulated microgravity (M) for 24 h. Whole cell RNA was extracted and 2 μg of RNA subjected to reverse transcription followed by real time PCR to quantitate alterations in alkaline phosphatase (AlkPhos) and osteocalcin expression. Values are expressed relative to TBP expression, averaged and then graphed as a fold difference compared to unit gravity controls (n = 4, $*P < 0.02$).

Given that runx2 is a major transcription factor involved in the regulation of osteoblast lineage commitment, osteoblast gene expression, and osteoblast differentiation [Lian et al., 1998; Karsenty, 2001], we measured the influence of RWV exposure on runx2 expression in MC3T3-E1 osteoblasts. Figure 4 demonstrates that 24 h in the RWV is sufficient to suppress runx2 expression by more than 50%. This finding is consistent with the suppression of markers of the mature osteoblast phenotype.

To determine if suppression of runx2 correlates with altered expression of genes associated with early stages of osteoblast growth and differentiation, collagen I mRNA levels were measured. Figure 5 demonstrates that collagen I expression is not decreased by RWV exposure and its associated suppression of runx2 expression. In fact, a slight but significant increase in collagen I was observed. This could indicate that the RWV selectively influences genes associated with specific stages of osteoblast differentiation or that 24 h of RWV treatment initiates regression to an early differentiation phenotype.

Alternatively, suppression of runx2 could signify a lineage shift. Pluripotent mesenchymal cells can give rise to multiple cell types, including osteoblasts, adipocytes, myocytes, and chondrocytes. Therefore, we examined the expression of early gene markers of these lineages. We measured levels of Sox9 to examine chondrogenesis [Lefebvre et al., 2001], myocyte enhancer factor 2A (MEF2A) for myogenesis [Naya and Olson, 1999], and peroxisome proliferator-activated receptor (PPAR) gamma expression for adipogenesis [Chawla and Lazar, 1994]. While MEF2A was not detectable in our samples (but was present in positive control myocytes), our findings of more than three separate experiments demonstrate no significant enhancement in the expression of Sox9 or PPAR gamma (0.9 ± 0.2, 0.96 ± 0.5, respectively;

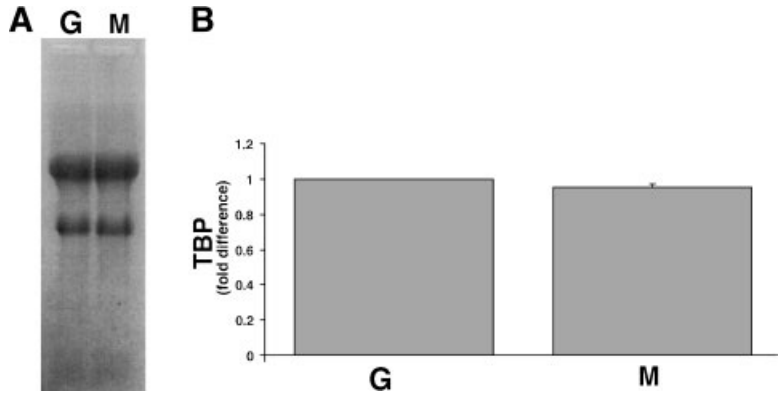

Fig. 3. Acute RWV exposure does not alter RNA integrity or total cellular transcription. MC3T3 E1 osteoblasts were cultured for 14 days and left at unit gravity (G) or simulated microgravity (M) for 24 h. **A**: Whole cell RNA was extracted and analyzed by agarose gel electrophoresis to verify RNA integrity under gravity and simulated microgravity conditions. **B**: Two micrograms of RNA were subjected to reverse transcription followed by real time PCR to quantitate alterations in TBP expression. Averaged values from five separate experiments are expressed as fold difference relative to controls.

expressed as an average fold increase relative to unit gravity controls ± SE). This finding suggests that culturing MC3T3-E1 mouse osteoblasts for 24 h in the RWV does not lead to a detectable lineage shift.

The regulation of osteoblast phenotype and gene expression involves a significant transcriptional component. To address the influence of the RWV on general transcription factor activities, we measured expression of an SV40 promoter driven reporter plasmid in MC3T3-E1 cells. As shown in Figure 6, the RWV had no effect on SV40 promoter activity. In contrast, activity of an AP-1 dependent promoter reporter was suppressed by more than 50%, suggesting selective influences on AP-1 transactivation.

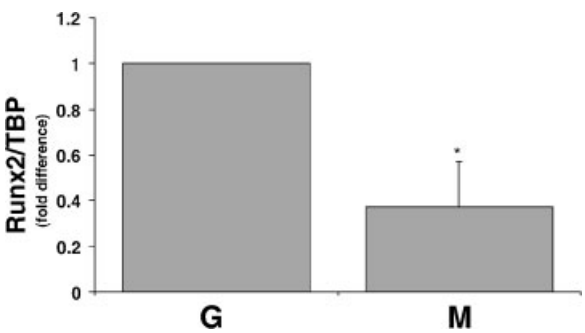

Fig. 4. Acute RWV exposure results in decreased runx2 expression. MC3T3 E1 osteoblasts were cultured for 14 days and left at unit gravity (G) or simulated microgravity (M) for 24 h. Whole cell RNA was extracted and 2 μg of RNA were subjected to reverse transcription followed by real time PCR to quantitate alterations in runx2 expression. Values are expressed relative to TBP expression, averaged and then graphed as a fold difference compared to unit gravity controls (n = 3, $*P < 0.05$).

DISCUSSION

Decreased mechanical loading on the skeleton can result in suppression of osteoblast phenotype and bone formation. This has been demonstrated in humans [Vico et al., 1987; Collet et al., 1997; Vico et al., 1998; Uebelhart et al., 2000; Vico et al., 2000], rats [Morey and Baylink, 1978; Jee et al., 1983; Wronski et al., 1987; Vico et al., 1988; Turner et al., 1995; Vico et al., 1998], and mice [Van Loon et al., 1995; Bateman et al., 2002] under conditions of bed rest, disuse, and spaceflight. To address the role of mechanical unloading on osteoblast

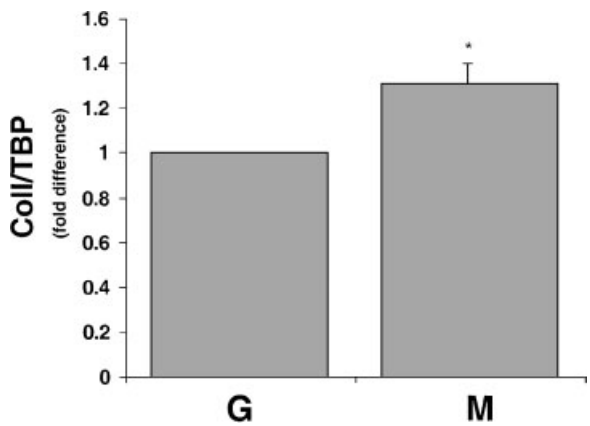

Fig. 5. Acute RWV exposure results in a modest increase in collagen I expression. MC3T3 E1 osteoblasts were cultured for 14 days and left at unit gravity (G) or simulated microgravity (M) for 24 h. Whole cell RNA was extracted and 2 μg of RNA were subjected to reverse transcription followed by real time PCR to quantitate alterations in collagen I (Col I) expression. Values are expressed relative to TBP expression, averaged and then graphed as a fold difference compared to unit gravity controls (n = 3, $*P < 0.05$).

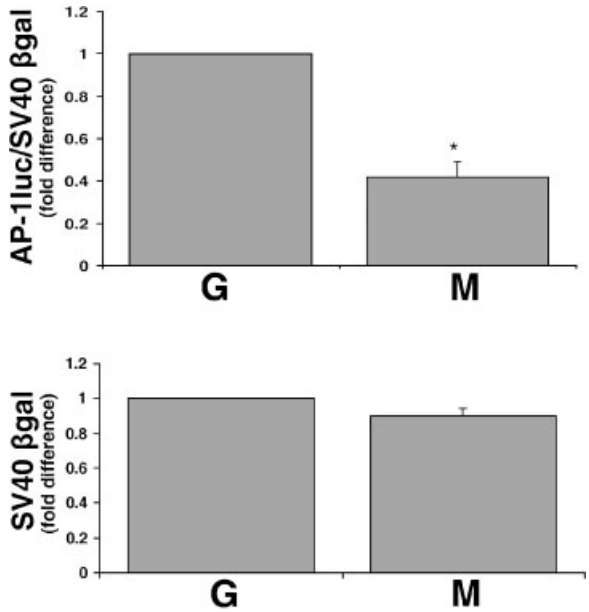

Fig. 6. Acute RWV exposure results in decreased AP-1 transactivation but does not alter general cellular transcription. MC3T3 E1 osteoblasts were seeded onto microcarrier beads and transfected with a 6X AP-1 luciferase reporter along with a constitutively active SV40 β-gal expression vector to normalize for transfection efficiency. Transfected cells were grown on microcarrier beads for 14 days then left at unit gravity (G) or simulated microgravity (M) for 24 h. Cell lysates were analyzed for luciferase activity indicative of AP-1 activity, and normalized to SV40 β-gal expression. Values represent the average fold difference, relative to unit gravity controls, of three separate experiments ($P < 0.01$).

phenotype we used the NASA approved RWV clinostat. After 24 h in the RWV, markers of osteoblast differentiation, alkaline phosphatase and osteocalcin, are significantly suppressed in mouse osteoblasts. In addition, we find that mRNA levels of runx2 and AP-1 transactivation, regulators of osteoblast differentiation and bone formation [Komori et al., 1997; Otto et al., 1997; Lian et al., 1998; Ducy, 2000; Jochum et al., 2000a,b; Sabatakos et al., 2000; Karsenty, 2001], are significantly suppressed compared to unit gravity controls. This finding is consistent with the suppression of markers of osteoblast differentiation.

While there is some variability in results obtained from space flight, many studies demonstrate that the decrease in bone formation seen in spaceflight correlates with a suppression in osteoblast phenotype marked by decreased osteocalcin expression and secretion [Bikle et al., 1994; Collet et al., 1997; Bateman et al., 2002]. For example, space flight suppresses osteocalcin and collagen I expression in committed embryonic chick osteoblasts [Landis et al., 2000]. In addition, space flight and hindlimb suspension decreases osteocalcin and increases alkaline phosphatase and IGF-1 expression in rats [Bikle et al., 1994], supporting a model where microgravity exposure suppresses the mature osteoblast phenotype and enhances an immature phenotype. Our data is consistent with this model; however, we find that alkaline phosphatase expression is also decreased. Similar to our findings, cultures of human osteoblastic MG-63 cells grown for 9 days aboard the unmanned Foton 10 spaceflight exhibit a 51% and 19% decrease in alkaline phosphatase and osteocalcin, respectively [Carmeliet et al., 1998]. Clinorotation of human osteoblast-like cells, HuO9, also leads to decreased alkaline phosphatase and osteocalcin expression [Kunisada et al., 1997]. Taken together human, rat, chick, and mouse osteoblasts respond to models of unloading such as spaceflight, hindlimb suspension, and clinorotation by suppressing gene expression associated with differentiation.

In contrast to the above mentioned studies by ourselves and others, there are reports suggesting that osteoblast phenotype is not modified or enhanced under conditions of spaceflight [Harris et al., 2000] or RWV culturing [Granet et al., 1998; Sarkar et al., 2000]. Differences may lie in within a variety of issues including species, acute versus chronic exposure, and normal versus transformed phenotypes. For example, several studies have cultured rat osteosarcoma (ROS) cells in the RWV and demonstrated an upregulation in the expression of markers of osteoblast differentiation [Granet et al., 1998; Sarkar et al., 200]. Osteosarcoma cells exhibit a proliferative phenotype and at the same time have robust expression of genes associated with differentiation. The expression of genes involved with differentiation during proliferation indicates that these genes are already able to override normal stage dependent regulation and therefore may not be affected by developmental stage regulation induced by simulated microgravity conditions. Thus the linkage between growth and differentiation observed in normal cells is lost, so the consequences of microgravity on transformed cells may not fully reflect in vivo responses. In addition, these studies involved 8–10 days exposure to the RWV; adaptation to the environment over chronic periods of exposure could also result in different findings.

Few studies have examined models of unloading on mice or mouse cells. Understanding mouse responses provides both fundamental knowledge and the opportunity to incorporate transgenic, knockout and mutant mice to address mechanisms of microgravity or disuse phenotypes. In vitro studies demonstrate that MC3T3-E1 osteoblasts have acute increases in fibronectin [Hughes-Fulford and Gilbertson, 1999], exhibit cytoskeletal changes, and reduced cycloxygenase-2 expression [Hughes-Fulford et al., 1998] and prostaglandin synthesis [Hughes-Fulford and Lewis, 1996]. These studies focused on responses of osteoblasts that have not reached later stages of differentiation, therefore expression of markers of maturation were not examined. Ex vivo studies carried out by Van Loon et al [Van Loon et al., 1995] demonstrate that space flight decreases mineralization of isolated fetal mouse bones, but did not address the expression of genes associated with differentiation. More recently, in vivo analysis of mice in space flight for 12 days demonstrated significant bone loss [Bateman et al., 2002]. Consistent with our data, they also find a decrease in alkaline phosphatase and osteocalcin mRNA levels.

In addition to suppression of osteoblast phenotypic markers, we also demonstrate that runx2 mRNA levels are suppressed. Runx2 is associated with enhanced expression of osteocalcin [Banerjee et al., 1997; Ducy et al., 1997; Xiao et al., 1999] and is essential for the progression of osteoblast differentiation and mouse bone development [Komori et al., 1997; Otto et al., 1997]. Runx2 expression increases with the onset of MC3T3-E1 differentiation [Seth et al., 2000] and can be activated through post-translational modification [Xiao et al., 2000]. Here we show that an acute 24 hour exposure in the RWV is enough to suppress runx2 expression. Consistent with this finding, skeletal unloading has also been demonstrated to rapidly suppress osteocalcin as well as runx2 expression [Ahdjoudj et al., 2002] while mechanical stretching has been demonstrated to enhance runx2 expression [Ziros et al., 2002].

The suppression of runx2 expression could signal the transition of osteoblasts to a different phenotype. Differences in culture conditions, such as high density culturing, can influence chondrogenesis [Ahrens et al., 1977; Duke et al., 1993]. However, we did not find an increase in Sox9 expression, a marker of the chondrocytic lineage. This suggests that osteoblasts are not transdifferentiating to chondrocytes and is consistent with the role of runx2 (which is suppressed in these cells) in hypertrophic chondrocytic differentiation [de Crombrugghe et al., 2001]. Given that bone loss associated with osteoporosis and immobilization is also associated with an increase in marrow adipose tissue [Minaire et al., 1974; Burkhardt et al., 1987; Ahdjoudj et al., 2002] we also examined expression of PPAR gamma, an early marker of adipogenesis. A 24 h exposure to the RWV did not induce PPAR gamma, suggesting that adipogenesis or transdifferentiation to adipoctyes did not occur within the time frame we examined. Finally, we also examined MEF2A levels in our cells. Expression was undetectable, although detectable in control muscle cells. Taken together, these findings suggest that 24 hours of RWV exposure does not lead to a lineage switch. Because MC3T3-E1 cells are less pluripotent than some other cell systems such as C2C12 cells or marrow cells, transdifferentiation to other cell types may not be possible. In addition, a longer time course may be required to detect lineage changes [Ahdjoudj et al., 2002].

The importance of regulating transcription factor activities during the development of bone has been extensively demonstrated, although the role during spaceflight related bone loss is less well understood. Though critical for normal bone development and maintenance, limited studies have examined AP-1 transcription factors in response to unloading. These studies utilized different models of unloading, but demonstrate the same phenomenon. Using A431 epidermal cells, basal c-fos expression was unchanged, while EGF induced c-fos expression was decreased in the RWV [de Groot et al., 1991]. Sato et al. [1999] demonstrate that MC3T3-E1 cells exposed to microgravity, on the sounding rocket TR-1A6, also exhibited depressed c-fos induction by EGF. In addition, bone marrow stromal cells isolated from 5 days unloaded rats express 50% less c-fos [Kostenuik et al., 1997]. These findings support our results indicating a selective decrease in AP-1 transactivation as a result of RWV exposure.

To our knowledge, this is the first report demonstrating the coordinate suppression of mouse osteoblast phenotype, runx2 expression, and AP-1 transactivation under conditions of decreased mechanical loading. This finding is

consistent with the role of runx2 and AP-1 in the regulation of osteoblast differentiation and bone formation. The mechanisms accounting for this rapid effect on Runx2 expression and a more detailed analysis of specific AP-1 family members involved in this response are under investigation.

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