OSTEOBLAST ONCOSTATIN M SIGNALING IN MODELED SPACEFLIGHT

by

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ABSTRACT

Bone deterioration is a challenge in long-term spaceflight with significant connections to terrestrial disuse bone loss. Prolonged unloading and radiation exposure, defining characteristics of space travel, have both been associated with changes in inflammatory signaling via IL-6 class cytokines in bone. While there is also some evidence for perturbed IL-6 class signaling in spaceflight, there has been scant examination of the connections between free fall, radiation, and inflammatory stimuli in bone. Our lab and others have shown that the IL-6 class cytokine oncostatin M (OSM) is an important regulator of bone remodeling. We hypothesize that spaceflight alters osteoblast OSM signaling, contributing to the decoupling of osteolysis and osteogenesis. To test this hypothesis, we induced OSM signaling in murine MC3T3 E1 pre-osteoblast cells cultured in modeled free fall, using a rotating wall vessel bioreactor, and with exposure to radiation typical of a solar particle event. We measured effects on inflammatory signaling, osteoblast maturation and activity, osteoclast recruitment, and mineralization. There were time dependent interactions among all conditions in the regulation of IL-6 production. OSM induced transcription of the OSM receptor β and IL-6 receptor α subunits, collagen α1(I), osteocalcin, sclerostin, RANKL, and osteoprotegerin. Measurements of osteoid mineralization suggest that the spatial organization of the osteoblast environment is an important consideration in understanding bone formation. Taken together, these results support a role for altered OSM signaling in spaceflight bone loss.

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LIST OF ABBREVIATIONS

ANOVA	analysis of variance
BSA	bovine serum albumin
ECM	extracellular matrix
ELISA	enzyme-linked immunosorbent assay
EVM	extra-vehicular mineralization
FBS	fetal bovine serum
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GP130	glycoprotein 130
HARV	high aspect ratio vessel
IL-6	interleukin-6
IL-6Rα	interleukin-6 receptor α subunit
ISS	International Space Station
LEO	Low Earth Orbit
LET	linear energy transfer
LIF	leukemia inhibitory factor
LIFRα	leukemia inhibitory factor receptor α subunit

MC3T3	MC3T3 E1 murine pre-osteoblast cell line
MCSF	macrophage colony stimulating factor
MSC	mesenchymal stem cell
OSM	oncostatin M
OSMRβ	oncostatin M receptor β subunit
PBS	phosphate buffered saline
RANKL	receptor activator of nuclear factor κB ligand
RCCS	Rotary Cell Culture System
RT-PCR	reverse transcription polymerase chain reaction
RWV	rotating wall vessel
SEM	standard error of the mean
SOCS	suppressor of cytokine signaling
SPE	solar particle event

INTRODUCTION

Deteriorating bone health is a substantial barrier to human exploration deeper into the solar system[1]. Each month in space, astronauts lose approximately 1% of the mineral density in their weight-bearing bones[2–5]. Some of this loss may never be fully recovered after return to Earth. The deterioration is likely to worsen during travel to Mars or near-Earth asteroids, when a fractured vertebra or femur could cripple the mission. These missions could last two years or more, greatly extending the astronauts' exposure to the features of spaceflight most relevant to bone health: free fall and ionizing radiation. While it is conventional to use the term "microgravity," and somewhat less frequently "weightlessness" and "zero gravity," astronauts in the International Space Station (ISS) experience 90% of the gravitational force at Earth's surface. Astronauts in free fall, and their skeletons, are mostly experiencing unloading, the freedom from being compressed against the ground[3,6,7]. While unloading is as characteristic of Earth orbit as it is for interplanetary spaceflight, radiation exposure increases beyond low Earth orbit (LEO)[8,9]. While ISS astronauts are exposed to little more radiation than they are on Earth, as one moves beyond the shield of Earth's magnetosphere, radiation exposure increases. Galactic cosmic rays and solar particle events (SPE) become important sources of radiation. Unpredictable SPE can rapidly deliver up to 2 Gy of radiation, comparable to doses used in cancer radiotherapy to kill tumor cells[8,10]. There are also recognized clinical parallels between spaceflight bone loss and disuse osteoporosis, a common complication of inactivity from bed rest, immobilization, or sedentary lifestyle[11–13]. As the analogies to

clinical radiation exposure and disuse osteoporosis suggest, addressing threats to bone health from spaceflight may have significant benefits for the earthbound as well. To date, efforts to counter deteriorating skeletal health in space have focused on resistive exercise and nutrition, with limited success[2–4,14]. Space and weight constraints would likely further limit exercise as an intervention beyond LEO. Taken together, these facts imply that pharmacological countermeasures to bone loss will likely be required for exploration deeper into the solar system. In turn, pharmacological interventions require understanding the molecular pathways that induce bone loss in spaceflight, but these pathways are poorly understood.

Bone is constantly broken down and replaced by a cyclical multicellular process called the bone remodeling cycle[15–19]. Bone is calcified extracellular matrix (ECM) produced by osteoblasts, specialized fibroblasts whose differentiation from mesenchymal stem cells (MSC) is determined primarily by the transcription factor RUNX2[20]. After proliferation and additional maturation, marked by the transcription factor osterix[21], the osteoblast will produce osteoid, the organic component of the bone ECM. Osteoid is primarily type I collagen. Additional components such as osteocalcin regulate the mechanical and chemical properties of the bone[22], including promoting the crystallization of hydroxyapatite, the mineral component of bone. As the ECM is produced and matures, some osteoblasts will become encased and terminally differentiate to osteocytes[23]. An important function of the osteocyte is to limit the excessive formation of bone, largely through the production of sclerostin[24,25], a Wnt-signaling antagonist that potently inhibits the maturation and activity of osteoblasts. When age or increased mechanical strain damages bone, the osteoblast lineage cells initiate the bone remodeling cycle by

recruiting osteoclasts[26,27]. Osteoclasts are multinucleate phagocytic cells specialized to degrade and remove the damaged bone. They differentiate from myeloid precursors, primarily under the influence of macrophage colony stimulating factor (MCSF) and receptor activator of nuclear factor κB ligand (RANKL) produced by the osteoblast lineage[28]. The osteoclasts are, in turn, closely followed by osteoblast bone replacement. This is triggered by coupling factors that are not yet well defined[29,30], but may involve specialized osteal macrophages. This cycle normally carefully balances the extent and location of removal and replacement to adapt to changing mechanical demands on the bone. This mechanical sensitivity originates in the osteoblast lineage[23,32].

Spaceflight leads to a decoupling of removal and replacement resulting in bone loss. The available data suggest that this is characterized by a transient initial increase in osteoclast activity followed by sustained decrease in osteoblast activity, but provide little into the molecular mechanism involved[3,5]. The osteoblast lineage is likely central because it is permanently present throughout bone and confers mechanical sensitivity. Somewhat more detail is available on the osteoblast response to free fall and space radiation, largely from ground-based models of spaceflight, although most mechanistic questions remain unanswered[3,32-34]. The vast majority of these publications examine one factor in isolation; very little is known about the combined effects of free fall and radiation. The disruption of inflammatory cytokine signaling is an emerging feature of spaceflight with potential relevance to these questions[35–38]. Ground-based experiments are showing changes in inflammatory pathways[39–41], as may have been expected from the alterations in inflammatory signals seen in disuse osteoporosis[11–13] and radiation exposure[42–44].

The bone remodeling cycle parallels wound healing, most obviously in the case of a gross fracture, but also on the microscale[45]. Like in wound healing, inflammatory cytokines are important signals in bone remodeling. Inflammatory cytokines are secreted factors that coordinate cell behavior during inflammation. The IL-6-type cytokines are an important group with broad effects in human health and disease, including in bone[46]. They take their name from their prototypical member, interleukin-6 (IL-6). They are also called the GP130 cytokines, after glycoprotein 130 (GP130), the receptor subunit shared by all members of the class. GP130 is expressed by a wide array of cell types. Specificity is achieved by the more limited expression of ligand-specific receptor subunits that work in conjunction with GP130. All of the major cell types in bone express some combination of GP130 cytokines and their receptors[46], as do many of the immune cells produced in the contiguous marrow space and involved in bone remodeling[47,48]. The cytokines with the most demonstrated importance in bone are IL-6, leukemia inhibitory factor (LIF), and oncostatin M (OSM). IL-6 signals through a complex comprising the IL-6 receptor α subunit (IL-6Rα) and a homodimer of GP-130. LIF and most GP130 cytokines signal through a complex of GP130 and LIF receptor α subunit (LIFRα), often with an additional ligand-specific subunit[46]. OSM signals through either the LIFRα – GP130 dimer or a dimer of GP130 and the OSM receptor β subunit (OSMRβ). OSM is the focus of research in our laboratory.

Some important discoveries have been made about the function of OSM in bone, but the picture of its function in bone is far from complete. For some time, it has been clear that OSM induces osteoblasts to secrete IL-6[49]. The primary action of IL-6 in bone is to amplify the generation and activation of osteoclasts[50,51], and perception of OSM has

been colored by the role of its better known relative. Indeed, OSM has been shown in cell culture to promote osteoclastogenesis by inducing osteoblast RANKL expression and reducing expression of osteoprotegerin[52–55]. However, it has also been shown that OSM promotes differentiation and activation of osteoblasts, mineral formation, and represses the expression of sclerostin[49,56–59]. *In vivo* data are complicated and suggest that some effects are species specific[60]. Even in cell culture, OSM appears to have different actions at different points in the osteoblast lineage[59]. Clearly many complications in the action of OSM in bone remain to be illuminated.

The work presented here addresses two areas of uncertainty: the mechanisms of bone loss in spaceflight and the function of OSM in bone. We hoped to shed light on both questions by looking for interactions between them. Because OSM is known to act on both the osteoblast lineage and through it the osteoclast, changes in its action could account for many of the effects of spaceflight on bone. Defining a connection to osteoblast mechanotransduction and radiation response would also contribute to our understanding of OSM in bone. To this end, we hypothesize that spaceflight alters osteoblast OSM signaling such that the decoupling of osteolysis and osteogenesis is exacerbated.

To test this hypothesis, we examined the actions of OSM on GP130 signaling, osteoclast recruitment, and osteoblast activity in a model of spaceflight. Our model system used the MC3T3 E1 mouse pre-osteoblast cell line[61] (MC3T3), which has been shown to recapitulate important actions of OSM seen in primary cells[57,62,63]. To induce OSM signaling, culture medium was supplemented with recombinant mouse OSM at 25 ng/ml. In one set of experiments, spaceflight was modeled by culturing cells on

Cytopore-2 cellulose microcarriers in the NASA-developed Rotary Cell Culture System (RCCS). This rotating wall vessel (RWV) type bioreactor models free fall through gravitational vector averaging and low shear stress[6,64]. In deference to convention, this condition is hereafter described as modeled microgravity (MMG). In an additional set of experiments modeling exposure to a SPE, cell cultures were irradiated prior to culture in MMG. The effect of these conditions on GP130 cytokines and receptors, osteoclast recruitment, and osteoblast activity were then examined. Our results support the hypothesis that spaceflight conditions alter the action of OSM signaling in osteoblasts.

MATERIALS AND METHODS

Cell Culture

The MC3T3 E1 subclone 4 mouse pre-osteoblast cell line[61] and the UMR-106 rat osteosarcoma cell line were obtained directly from the American Type Culture Collection (Rockville, MD). MC3T3 cells were maintained in MEM (Life Technologies, Grand Island, NY) supplemented to make α-MEM without ascorbic acid. All supplement components were from Sigma-Aldrich (St. Louis, MO). UMR-106 were maintained in DMEM (Hyclone, Logan, UT). All culture media were supplemented with 10% fetal bovine serum and 100 U/mL each of penicillin and streptomycin. These supplements were obtained from Hyclone (Logan, UT). Cells were maintained at 37 °C, 5% carbon dioxide, and 95% humidity.

Microcarrier Culture

Cytopore 2 macroporous cellulose microcarriers were purchased from GE Healthcare (Pittsburgh, PA). Microcarriers were hydrated in phosphate buffered saline (PBS) at 20 mg/ml and autoclaved at 20 minutes at 121 °C. Microcarriers were rinsed twice in sterile PBS then transferred to culture medium for at least 16 hours prior to seeding cells.

To seed the microcarriers, MC3T3 E1 cells were grown to confluence, trypsinized, and suspended in culture medium with microcarriers in a tissue culture flask. Concentration of cells during seeding was 50,000 cells / ml and 5 mg microcarriers / ml.

The mixture was agitated by gentle pipetting every 20 minutes for 3 hours and then adjusted to 2 mg / ml. After seeding microcarriers were incubated as above, microcarriers were agitated each morning and afternoon. Approximately one-half the medium was replaced every three to four days. Experiments were initiated on the seventh day after seeding.

Osteogenic Differentiation and Cytokine Stimulation

To induce osteogenic differentiation, MC3T3 cells were transferred to standard α-MEM (Life Technologies) supplemented with an additional 50 μg/ml ascorbic acid and 5 mM phosphate buffer at pH 7.4. Mineralization medium was also supplemented as above. Cells cultured on microcarriers had supernatant medium removed and replaced with differentiation medium with mixing 3 times. To induce OSM signaling, culture medium was supplemented with 25 ng/ml recombinant mouse OSM (R&D Systems).

Modeled Microgravity

The Rotary Cell Culture System (RCCS) and 10-ml high aspect ratio vessels (HARVs) were purchased from Synthecon (Houston, TX). The RCCS is designed to model free fall[6,64]. The HARV rotates around a horizontal axis so that medium and microcarriers undergo solid body rotation, which averages the gravitational vector experienced by cells to near zero. For Cytopore 2 microcarriers, a rotational speed of 18 rpm was found to be optimal by visual inspection. Oxygenation occurs through a gas-permeable membrane, preventing the formation of bubbles and ensuring smooth rotation for the microcarriers.

Modeled microgravity experiments lasted 7 days. At 12 h, 48 h, 96 h, and 7 days, samples were collected for analysis. The culture medium was removed from the HARV. After ensuring uniform suspension of the microcarriers, a portion of the medium was retained so that the microcarriers would be evenly divided among the samples. The remaining microcarriers were returned to the HARV along with fresh medium matching the experimental condition. As controls at normal gravity, microcarriers were cultured in tissue culture flasks at identical concentrations and volumes to the RCCS. Samples were collected by the same procedure.

Radiation

MC3T3 cells were prepared on Cytopore 2 microcarriers as above. Approximately 18 h prior to irradiation, cells were transferred to a sterile 50 ml conical tube at a concentration of 2 mg Cytopore per ml. This concentration provides excess culture medium. The tubes were then packed in an insulated, pre-warmed box with a 2 L bottle of water at 37° C and shipped overnight to Dr. Jeffrey Willey, Radiation Biology Section, Wake Forest University Medical Center. Tubes were exposed to 1 Gy at 364 rad/s from a ^{137}Cs source. Tubes were repacked and return shipped overnight. Controls were subjected to a sham irradiation procedure. Upon return, cells were immediately transferred to mineralization medium and modeled microgravity or control conditions. Samples were collected as described above at 12 h, 48 h, 96 h, and 7 days.

Semi-Quantitative Reverse Transcription Polymerase Chain Reaction

Microcarriers were allowed to settle in a conical tube and the supernatant medium was aspirated. RNA was extracted using 1 ml RNA-STAT 60 (Tel-test Friendswood,

TX) for each 1-2 mg microcarriers. After a 20 min. incubation, chloroform (200 µl/1ml of RNA-STAT 60) was added followed by vortexing for 10 seconds, and centrifugation at 12,000rpm. The upper aqueous layer was transferred to a new tube containing isopropanol (0.5ml/1ml of RNA STAT60), followed by vortexing for 10 seconds and incubation on ice for 15 minutes. The mixture was centrifuged at 12,000rpm and the supernatant discarded. The pellet was washed by adding 1ml of 75% ethanol, mixed and centrifuged at 12,000rpm and the supernatant discarded. The RNA was allowed to air-dry in a sterile environment and resuspended with nuclease free water.

cDNA was generated from this RNA using a commercially available reverse transcriptase kit (Applied Biosystems) per manufacturer instructions. The cDNA generated from the reverse transcription reaction was used in a 25 µl PCR reaction containing 2.5 µl of 10x PCR buffer, 2.5mM dNTPs, 10mM primers, 5U GoTaq polymerase (Promega, Madison, WI) and 2 µl cDNA. Amplifications were carried out as follows: initial denaturation at 95°C for 2 minutes, followed by the indicated number of cycles of 95°C for 1 min, annealing temperature for 1 min, 72°C for 1 min, then a final extension of 72°C for 10 minutes. Primer pairs and reaction conditions for each target are provided in Table 1. The PCR products were electrophoresed on a 1% Tris-Agarose gel containing 0.5 µg/ml of ethidium bromide at 80 volts for 45 minutes. The gels were imaged using a Kodak Image station and exposed for 10 seconds. Band densities were calculated using the ImageJ software (NIH) and normalized to GAPDH. For RT-PCR analyses, the sample size was 4.

Enzyme-Linked Immunosorbent Assay

To measure secreted IL-6, conditioned medium was collected from each sample collected above. IL-6 was quantified with the mouse IL-6 DuoSet kit from R&D Systems according to the manufacturer's instructions. Plates were washed with PBS at pH 7.4 containing 0.05% Tween-20 (PBS-T) and blocked using PBS containing 1% IgG-free BSA (Jackson Immunological West Grove, PA). The substrate used was Thermo Pierce (Rockford, IL) 1-Step Ultra TMB. A seven point standard curve was prepared by serial dilution of the included 1000 pg/ml standard. Sample concentrations were interpolated from a 4 parameter logistic fit of the standards. All samples and standards were assayed in duplicate. All ELISA analyses have a sample size of 3.

Alizarin Red Staining

For monolayer experiments, MC3T3 cells were cultured in mineralization medium for 6 or 14 days in 12-well plate. For microcarrier experiments, a 2 mg sample was retained from each MMG and MMG with radiation experiment and cultured for an additional week in experimental conditions. Medium was aspirated and the cells were washed once with PBS, then fixed in 10% formalin for 15 minutes. Formalin was aspirated and the cells were washed three times with deionized water. Four-hundred µl of 40 mM alizarin red (Millipore, Billerica, MA) was added to each sample. After a 20 minute incubation at room temperature, the stain was aspirated and the cells were washed four times with deionized water.

For extraction and spectrophotometric quantification, 400 µl 10% acetic acid was added to each well. The matrix was disrupted with a pipette tip in monolayer or trituration in microcarrier experiments. The sample was then transferred to (or retained

in) a micro-centrifuge tube and incubated for 30 min at 85°C. Tubes were then transferred to ice for 5 minutes, then centrifuged 20 minutes at 16,000xg. Ten standards were prepared by serial dilution from 4 mM alizarin red. Samples and standards were adjusted to pH 4.2 with 10% ammonium hydroxide. One hundred µl of each sample and standard was transferred in duplicate to a 96-well plate and the absorbance read at 405 nm. Sample concentration was calculated by comparison to a linear least-squares best fit of the standards.

For quantification by confocal microscopy and densitometry, a microscope slide of the sample was prepared after washing but before the extraction procedure. The sample was imaged on a Zeiss LSM 510 Meta system combined with the Zeiss Axiovert Observer Z1 inverted microscope and ZEN 2009 imaging software (Carl Zeiss, Inc., Thornwood, NY). Excitation was at 540 nm and emission was measured at 580 nm. A sample of at least 20 microcarriers was imaged under identical settings with intensity at 580 nm saved as 8-bit grayscale. Using the ImageJ software (NIH), thresholding was applied at 30/255 to eliminate background, then the integrated intensity of each image was calculated. Integrated intensity per bead was calculated and used to represent alizarin red staining.

Statistics

MMG experiments were analyzed using three-way analysis of variance (ANOVA) with repeated measures of each combination of MMG and OSM induction (corresponding to a culture vessel) at each level of time. MMG with radiation experiments were analyzed using four-way ANOVA with repeated measures of each combination of radiation, MMG, and OSM induction (corresponding to a culture vessel)

at each level of time. Multiple comparisons were conducted with Tukey's HSD test *post hoc*. Each response variable was treated separately. For all comparisons, $\alpha = 0.05$. In figures, bars and asterisks (*) indicate $p < 0.05$ for the indicated main effect. Any interactions identified in the test had a $p < 0.05$. Calculations were performed in the R statistical environment (R Project).

RESULTS

OSM and MMG Synergistically Induce IL-6 Secretion

To assess the action of osteoblast OSM signaling in MMG, we first examined the transcription of the cytokines and receptors most closely associated with OSM by semi-quantitative reverse transcription polymerase chain reaction (RT-PCR) (Fig. 1A). OSM supplementation was observed to induce the transcription of OSMRβ (Fig. 1B) and IL-6Rα (Fig. 1C). Induction of the receptors reached its peak at 48 h and remained stable afterward. These effects of OSM have not been previously described in osteoblasts. MMG had no statistically significant effect on these targets.

IL-6 was the most substantially affected of the examined transcripts (Fig. 1D). Both OSM supplementation and MMG independently induced IL-6 transcription, which is consistent with prior results[49,65] . Additionally, the combination of OSM supplementation and MMG (hereafter OSM+MMG) increased IL-6 transcription by more than twice what would be expected from even multiplicative combination of their individual effects, reaching 70-fold by 7 d. This synergistic effect has not previously been described. OSM's effect was seen as early as 12 h after treatment and increased throughout the 7 d. The independent effect of MMG was not observed until 48 h and remained stable afterward. The synergistic effect from OSM+MMG was detected from 48 h, along with the effect from MMG alone. The amplification of IL-6 induction demonstrates for the first time that MMG does alter the effect of OSM signaling.

To ensure that the effects of OSM signaling induction and MMG extended to the secretion of the IL-6 protein, we tested the cell culture conditioned medium using ELISA (Fig. 2). Again, both OSM supplementation and MMG were shown to independently and synergistically induce osteoblast IL-6 production, with the effect of MMG lagging OSM in both time and scale. OSM alone increased secretion by as much as 200-fold over control conditions, while the effects of MMG on mRNA and secreted protein were proportional at 48 h. The fold increase in secreted protein was much larger than the transcriptional change. This may reflect the accumulation of protein in the culture medium as IL-6 was produced at increasing rates. It is also possible the at post-transcriptional regulatory effects account for difference. Regardless, the interaction of MMG and OSM is confirmed at the protein level. This supports the hypothesis that spaceflight alters OSM signaling in the osteoblast.

Radiation Limits the Effect of OSM Induction on IL-6 Secretion but Enhances the Effect of MMG

The action of OSM signaling on osteoblast inflammatory factors was next examined in the context of a more complete model of spaceflight including both MMG and radiation typical of a solar particle event (SPE). Statistically significant interactions ($p < 0.01$) between OSM signaling and these spaceflight conditions were again observed in the regulation of IL-6 production. Radiation had no significant additional effect on any other targets assessed by RT-PCR (Table 2). The changes in IL-6 mRNA measured by RT-PCR were entirely reflected in the measurements of secreted IL-6. A general increase in the concentration of IL-6 compared to the experiments without radiation is attributed to the increase in Cytopore microcarrier concentration from 4 mg/ml to 4.5 mg/ml,

increasing the number of cells, and the fewer time points at which samples were collected, decreasing the dilution of the conditioned medium with fresh medium. Both changes were made to provide sufficient cell numbers for the additional experimental factor. Sham irradiation control recapitulated the effects of the RCCS-only model with its substantial synergistic increase in secretion (Fig. 3A, left column), validating the radiation procedure. As in the experiments without radiation, OSM increased IL-6 secretion by at least 10-fold under all conditions. Consequently, to facilitate comparison of other conditions, the results of the experiments with radiation are separated into panels showing results without-OSM (Fig. 3B) and with-OSM (Fig. 3C).

Intriguingly, the effects of radiation in combination with either OSM supplementation were opposite of the effects of radiation alone or radiation with MMG. Radiation alone increased IL-6 secretion relative to the control at 48 h and 7 d by approximately 5-fold, confirming a response recently reported for the first time in osteoblasts43 (Fig 3B). Radiation in combination with MMG increased the secretion of IL-6 relative to either factor alone. The scale of this increase compared to multiplicative combination of the factors was approximately two-fold, comparable to the synergistic effect seen from OSM+MMG in absence of radiation. Contrary to these increases in IL-6 secretion, irradiation decreased the effect of OSM supplementation at all time points by a substantial margin, approximately 50% (Fig 3C). Finally, radiation did not change the induction of IL-6 by OSM+MMG except at 12 h, when all conditions respond as if MMG were not present. Considered together, these complicated interactions again support the action of spaceflight conditions on OSM signaling.

OSM Counteracts the Effect of MMG on the RANKL:Osteoprotegerin Ratio

It has been observed that IL-6 functions primarily in bone to magnify osteoclast recruitment and activity[50,51]. OSM is also known to enhance RANKL expression and osteoclast activation[52,54,55]. To determine if spaceflight conditions also interact with these actions of OSM, we measured the transcription of the osteoblast-produced factors most important for osteoclastogenesis: MCSF, RANKL, and osteoprotegerin (Fig. 4A). We found that RANKL (Fig. 4B) was upregulated by MMG. MMG alone also decreased the transcription of osteoprotegerin, so that the ratio of RANKL to its decoy receptor would be increased, which favors increased osteoclastogenesis. In these experiments, OSM did not exert a statistically significant effect on RANKL expression, but there was a clear interaction between OSM signaling induction and MMG. When the two were present in combination, osteoprotegerin mRNA levels increased proportionally to the increase seen in RANKL, so that in this case the RANKL:OPG ratio would be preserved. The implication is that the osteoblast recruitment of osteoclasts in free fall may depend on the absence of OSM signaling.

OSM and MMG Have Independent and Opposing Effects on Osteoblast Activity

To determine if the interdependence of osteoblast OSM signaling and MMG extended to their effects on osteoblast maturation and activity, we analyzed samples collected over the course of a week in these conditions by semi-quantitative RT-PCR for several markers of osteoblast differentiation and osteoid production (Fig. 4A). Significant effects were found for collagen α1(I) (Fig. 4B), osteocalcin (Fig. 4C), and sclerostin (Fig. 4D). Independently, OSM and MMG acted on collagen α1(I) and osteocalcin, as would be expected for these components of osteoid and markers of middle and late osteoblast

maturation. Both had mRNA levels increased by OSM and decreased by MMG. Sclerostin was increased by MMG, suggesting an increase in the osteocyte character of these cell cultures and consistent with evidence that free fall inhibits osteoblast differentiation. No significant interaction between these factors was detected by ANOVA, which suggests that spaceflight does not alter the effect of OSM on osteoblast activity.

RCCS and Cytopore Cell Culture Does Not Model Spaceflight's Effect on Osteoid Formation and Mineralization

The effect of OSM on the production of mineralized osteoid was also investigated. The organic dye alizarin red specifically stains mineralization in osteoid (Fig. 6A), allowing visualization of the differences in osteoid production in cell culture. The dye can also be extracted and quantified spectrophotometrically[66]. This aided in the choice of the MC3T3 E1 cell line to study OSM's effect in osteoblasts. The MC3T3 E1 cell line was chosen for these experiments in part because of the clear effect OSM has on culture mineralization, whereas the UMR-106 rat osteosarcoma cell line, for example, shows no effect (Fig. 6b). A different approach to mineral quantification was used for MC3T3 cells cultured on Cytopore microcarriers when the extraction technique proved insufficiently sensitive. Because alizarin red fluoresces when excited by light at 530-560 nm, staining could be visualized with laser confocal microscopy (Fig. 6C). It proved possible to quantify the staining by densitometry, and thus mineralization, on samples of microcarriers (Fig. 6D and F). While reproducible, the results from this technique showed substantially higher mineralization in MMG (Fig. 6F), not at all consistent with the effects of spaceflight[2,3]. During staining, large quantities of what proved to be

mineralizing material were sometimes noted (Fig. 6E). This material—here called extra-vehicular mineralization (EVM) and presumed to be osteoid—was only found in normal gravity control cultures, never in samples from the RCCS. It appears the microcarriers must be stationary for the osteoblasts to produce EVM. In the control gravity samples, the EVM was generally disrupted and lost during staining and washing, so that most of it could not be quantified. When the remnant EVM was quantified, however, it substantially exceeded the mineral found on only the microcarriers from the same samples (Fig. 6F). From these observations, we conclude that the RCCS model of free fall used with Cytopore microcarriers is unable to reproduce the patterns in mineral formation seen in actual spaceflight data.

DISCUSSION

Understanding how spaceflight disrupts the bone remodeling cycle is important for human space exploration and likely to improve health on Earth. The many uncertainties in skeletal physiology are an obstacle to that understanding. Among these uncertainties is the incomplete picture of the action of inflammatory signaling in the regulation of bone remodeling. The results presented here connect the questions of spaceflight's action in bone and the function of the GP130 cytokines. In particular, they support the hypothesis that free fall and ionizing radiation alter the function of OSM signaling in bone. The pattern seen in the disruption of the effects of OSM is consistent with a contribution to the increase in osteolysis and decrease in osteogenesis seen in astronauts.

The most prominent effect of our model of spaceflight on osteoblast OSM signaling was on GP130 signaling itself, particularly the secretion of IL-6. We described here an increase in IL-6 secretion by MMG and the synergistic increase by the combination of MMG and OSM supplementation. The increase in secretion is closely paralleled by an increase in IL-6 mRNA. The degree of increase in IL-6 secretion cannot be explained by a simple combination of the individual effects of MMG and OSM. This is clear evidence that the one effects the other. One possible explanation for this is that MMG interferes with feedback inhibition mechanisms regulating OSM signal transduction or IL-6 secretion. Inhibition of OSM and IL-6 signaling by suppressor of

cytokine signaling (SOCS) protein is an example of an important regulatory pathway that is known to be involved in skeletal health and could be inhibited in spaceflight[67,68]. As IL-6 acts in bone to increase the recruitment of osteoclasts[50,51], this increase in IL-6 secretion can reasonably be expected to contribute to osteolysis in spaceflight. This is particularly significant considered alongside the data collected from astronauts suggesting that GP130 signaling is generally altered in spaceflight and IL-6 levels increased in particular[35–38]. While not dependent on MMG, the observed increase in OSMRβ and IL-6Rα mRNA is consistent with a positive feedback mechanism for OSM and IL-6 signaling that may be important in understanding the general action of these cytokines in bone.

OSM's regulation of osteoblast IL-6 secretion is further complicated in a spaceflight model that includes radiation. Radiation alone or in combination with MMG increased IL-6 secretion, as did OSM treatment. Radiation in combination with OSM, however, diminished the OSM induced secretion by as much as half. Possibly relevant to this effect is a pattern of dose dependence in the response of osteoblasts to ionizing radiation. Exposures at less than 2 Gy, promote osteoblast differentiation and osteoid production[69–71]. Radiation above this level has the opposite effect[43,71–74]. Yumoto et al. have also observed that the combination of irradiation with unloading may also determine the effect of ionizing radiation on osteoblast function[75], as we observed here. Applying these observations to our data, it is reasonable that the combined effect of OSM and radiation differs from their individual effects. It is possible, for example, that the combination of radiation and OSM supplementation at receptor-saturating concentrations, as we used here, crosses a threshold that triggers a protective quiescence in the osteoblast,

similar to the response to high dose radiation described by Kansara et al.[43] It is also noteworthy that factors other than dose affect osteoblast response to radiation. Linear energy transfer (LET) may be an important factor[42,76,77], which should be considered in evaluating our model, which only uses low-LET photons. The radiation from SPE and GCR in spaceflight has a large high-LET component[8]. As we have shown, many factors must be considered in any attempt to understand the osteoblast response to radiation.

In summary, OSM supplementation, modeled free fall, and radiation all independently increase the secretion of IL-6. OSM has the largest and most immediate effect. Free fall and radiation both a have more modest effect on IL-6 secretion that presents more slowly than the effect from high levels of OSM supplementation. The difference in timing may only be apparent, due to the inability of our assays to detect the comparatively smaller early effects, or it may indicate that OSM acts more directly on IL-6 transcription than free fall or radiation. These explanations are not mutually exclusive.

We have also described a dependence on MMG for the action of OSM on osteoblast-mediated osteoclast recruitment. In control gravity conditions, active OSM signaling had no effect on the RANKL:osteoprotegerin ratio. In MMG, OSM signaling increased osteoprotegerin levels. On its own, this is further evidence that free fall alters the action of OSM signaling in the osteoblast, which supports our central hypothesis. It also demonstrates that MMG depends on the presence or absence of other factors for its effects. By implication, the effect of free fall on astronauts may depend on factors that vary between individuals, such as baseline inflammatory cytokine levels. It cannot be determined from these experiments how this feature of OSM signaling in MMG, which would oppose increased osteoclastogenesis, balances with the increase in IL-6 secretion

and its support of osteoclast recruitment. Although these experiments did not support spaceflight acting through OSM signaling to affect osteoblast activity and maturation, it is worth noting that they focused on the already osteoblast-committed MC3T3 cell line. OSM is known to act throughout the osteoblast lineage from uncommitted precursors to osteocytes[59,78,79]. One can imagine that spaceflight would alter the effect of OSM signaling at these other points in the osteoblast lineage. Another possibility is that spaceflight conditions alter the effect of OSM signaling on osteoblast activity, but that these experiments could not detect the effect. Comparatively small effects or post-translational interactions, for example, would not be detected by semi-quantitative RT-PCR. Our results do imply that inflammatory signaling pathways must be considered in understanding the action of spaceflight on osteoclast activity.

The overall picture present here is this: Free fall and radiation act independently of OSM to increase osteoblast-mediated osteoclastogenesis through both the RANKL – osteoprotegerin system and IL-6 production. Meanwhile, free fall inhibits the activity of osteoblasts, leading to an increase in osteolysis and decrease in replacement with new bone. Additionally, free fall synergizes with OSM to increase IL-6 production further without any balancing effect on osteoblast activation, so that the overall effect of OSM on the osteoblast in space may be to promote bone loss. Thus spaceflight may act through OSM signaling to unbalance the bone remodeling cycle. This interpretation of our results is summarized graphically in Figure 7.

It is worthwhile to consider the inability of this system to model changes in osteoid production and mineralization in spaceflight. EVM could not form in MMG, most likely due to the microcarriers' constant motion. The ability of the osteoblasts to move

beyond the microcarrier appears to have been critical for the organization and amount of osteoid they produced. That ability to move and interact in a larger space may also have been critical in successfully modeling the decrease in osteoblast activity seen in spaceflight. Bone is a complex tissue, where many effects depend on the spatial and temporal interaction of many different cells. It is difficult to predict which features of that complexity will be important for a given question, in bone or anywhere in biology. The most valuable contribution of this work may be pointing to GP130 signaling as a feature that must be included in a complete picture of bone in space.

Table 1: PCR Primers and Reaction Conditions

Target	Fwd. Primer Rev. Primer	Prod. Size	Temp. (°C)	Cycles	cDNA dilution
GAPDH	ATCACTGCCACCCAGAAGAC GGTCCTCAGTGTAGCCCAAG	202	57	30	1:10
OSM	AGCAAGCCTCACTTCCTGAG GTGGGCTCAGGTATCTCCAG	200	60	35	1:1
OSMRβ	TAGACTGAACATATCCAACACCA TCCATGGATTGGCTCATCTGGCA	349	60	30	1:1
LIF	CAGACAGACAGGTAGCATAAAG GACACAGAGACAGACAGAGA	487	60	35	1:1
LIFRα	GAAAACTGTAAGGCGCTACA CCAAGTGTTTACATTGGC	483	52	35	1:1
IL-6	CCTCTGGTCTTCTGGAGTACCAT GGCATAACGCACTAGGTTTGCCG	307	55	30	1:10
IL-6Rα	CCAGGTGCCCTGTCAGTATT CCGTGAACTCCTTTGACCAT	317	60	35	1:1
MCSF	CGACTTCCCGTAAAGGCATAAA CAAGGAACACAGCCCAAAGA	530	60	30	1:1
RANKL	GAGAGGTATTCCGATGCTTATG GGTGACCAACATCCTACTTATT	577	60	35	1:1
osteoprotegerin	AGAGTGAGGCAGGCTATT AGTAGTTTCTTCTGGTGCTATG	511	60	35	1:1
RUNX2	CCCTTCCTCTTCCCTTATCTCT GTGCTTCTGCTACCACTCTAAC	509	60	35	1:1
osterix	CTGCTTGAGGAAGAAGCTCACTA GGGGAGCAAAGTCAGATGGG	490	60	35	1:1
collagen I(α1)	AACAAGGTGACAGAGGCATAAA GCTGCGGATGTTCTCAATCT	440	60	30	1:10
osteocalcin	GACCATCTTTCTGCTCACTC TTGCACTTCCTCATCTGAAC	425	60	35	1:1
sclerostin	TTCCACCCAAATGTAAAGCCTGCG ATTTCTGGCCCTTCCACCATCTCT	366	60	35	1:1

Table 2: Summary of Semi-Quantitative RT-PCR

Mean relative expression at 48 hours by densitometry (arbitrary units) where significant by ANOVA. NC indicates no significant change.

OSM	-	+	-	+	-	+	-	+
MMG	-	-	+	+	-	-	+	+
Radiation	-	-	-	-	+	+	+	+
OSM	NC	NC	NC	NC	NC	NC	NC	NC
OSMRβ	0.913	1.338	0.857	1.330	0.942	1.325	0.822	1.350
LIF	NC	NC	NC	NC	NC	NC	NC	NC
LIFRα	NC	NC	NC	NC	NC	NC	NC	NC
IL-6	1.097	13.739	1.834	36.876	1.000	20.271	2.763	88.308
IL-6Rα	1.438	2.038	1.082	2.107	1.424	2.080	1.124	2.235
MCSF	NC	NC	NC	NC	NC	NC	NC	NC
RANKL	0.835	0.980	1.131	1.288	0.937	0.843	1.219	1.291
osteoprotegerin	0.962	1.005	0.760	1.347	0.851	1.110	0.675	1.247
RUNX2	NC	NC	NC	NC	NC	NC	NC	NC
osterix	NC	NC	NC	NC	NC	NC	NC	NC
collagen α1	0.960	1.255	0.603	1.119	1.048	1.360	0.725	1.204
osteocalcin	0.977	1.226	0.602	1.033	0.939	1.192	0.729	1.115
sclerostin	0.832	0.605	1.399	1.209	0.816	0.550	1.151	1.105

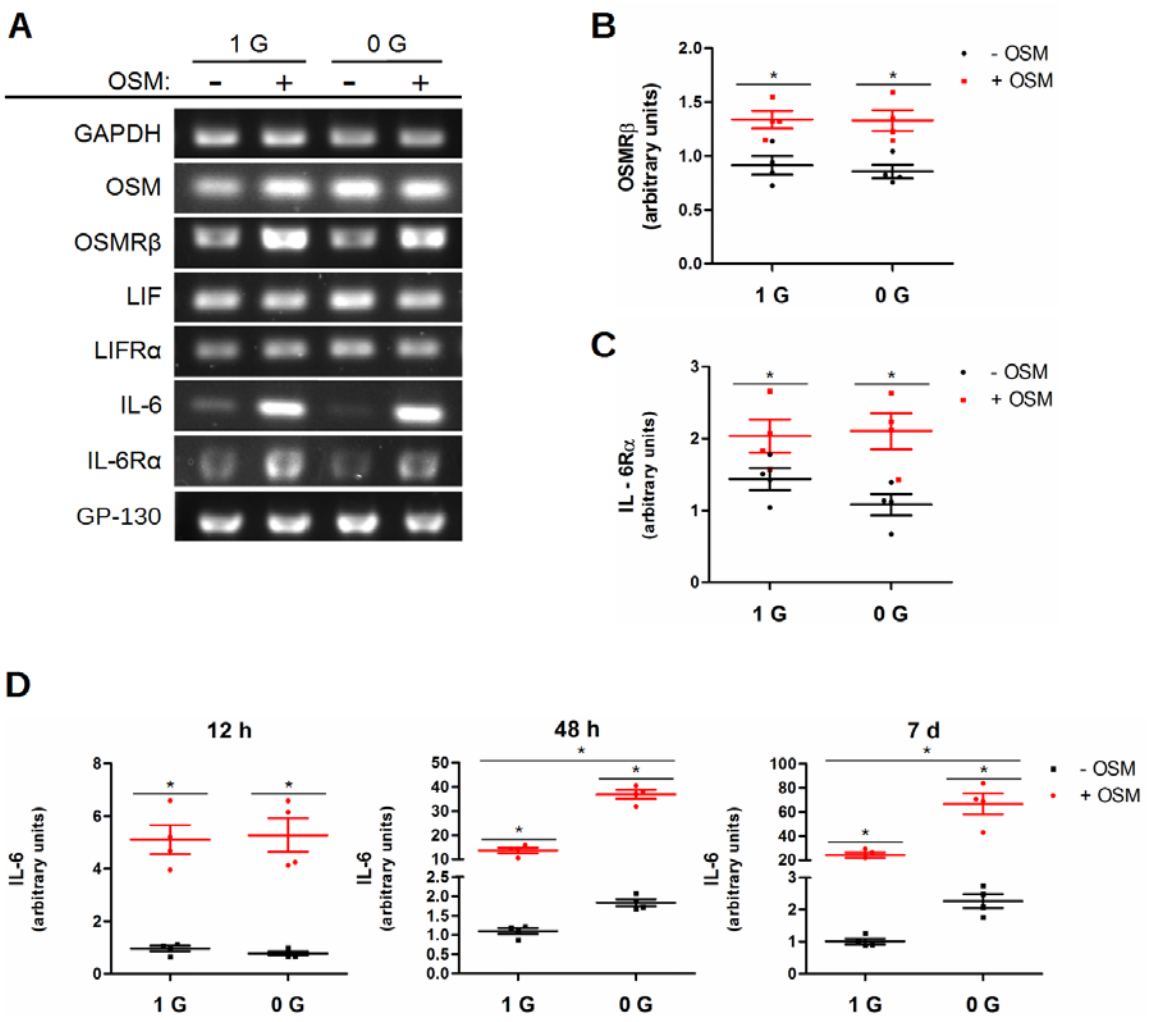


Figure 1. OSM and MMG independently and synergistically induce the transcription of IL-6.

MC3T3 pre-osteoblasts were cultured with OSM supplementation and MMG for 7 days. RNA was collected at 12 h, 48 h, and 7 d. Semi-quantitative RT-PCR was conducted for the cytokines and receptors most closely associated with OSM in osteoblasts, listed in (A) with representative images from 48 h, when effects were seen for the largest number of targets. (B-D) Scatter plots of the densitometry results showing the mean and standard error of the mean (SEM) for the targets with statistically significant regulation by OSM or MMG (arbitrary units). OSM alone induced the (B) OSMRβ and (C) IL-6Rα subunits, shown at 48 h, when the largest effect was seen. (D)

IL-6 was induced independently by both OSM and MMG. Induction by OSM alone progressed from approximately 5-fold at 12 h to 20 fold at 7 d. Induction by MMG was first significant after 48 h and remained stable afterward at approximately 2-fold. The factors interacted significantly to amplify their independent effect, inducing IL-6 transcription approximately 35-fold at 48 h and 70-fold at 7 d.

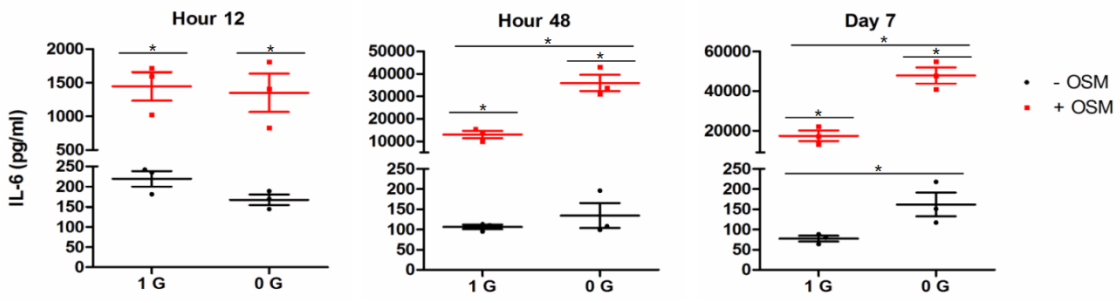
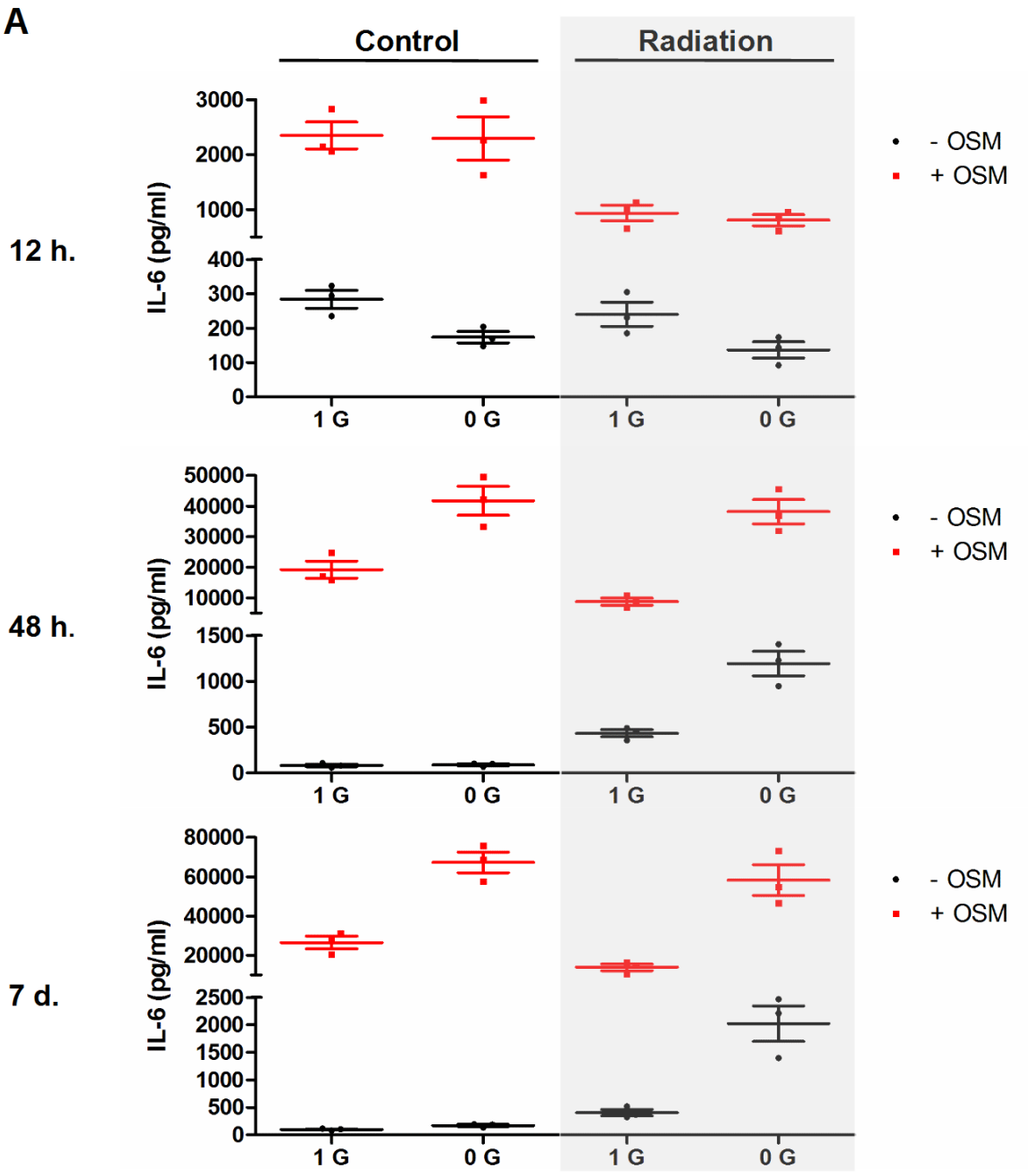


Figure 2. OSM and MMG independently and synergistically induce secretion of IL-6.

MC3T3 pre-osteoblasts were cultured with OSM supplementation and MMG for 7 d. IL-6 secretion was measured in conditioned medium by ELISA; scatter plots show mean and SEM. OSM treatment induces secretion of IL-6 at all time points with a 7-fold induction at 12 h increasing to 200-fold by 7 d. A 2-fold increase in IL-6 secretion by MMG is significant at 7 d, but may be present earlier. The synergistic effect of OSM and

MMG on IL-6 secretion is first significant at 48 h at approximately 350-fold. It increases to approximately 500-fold at day 7.



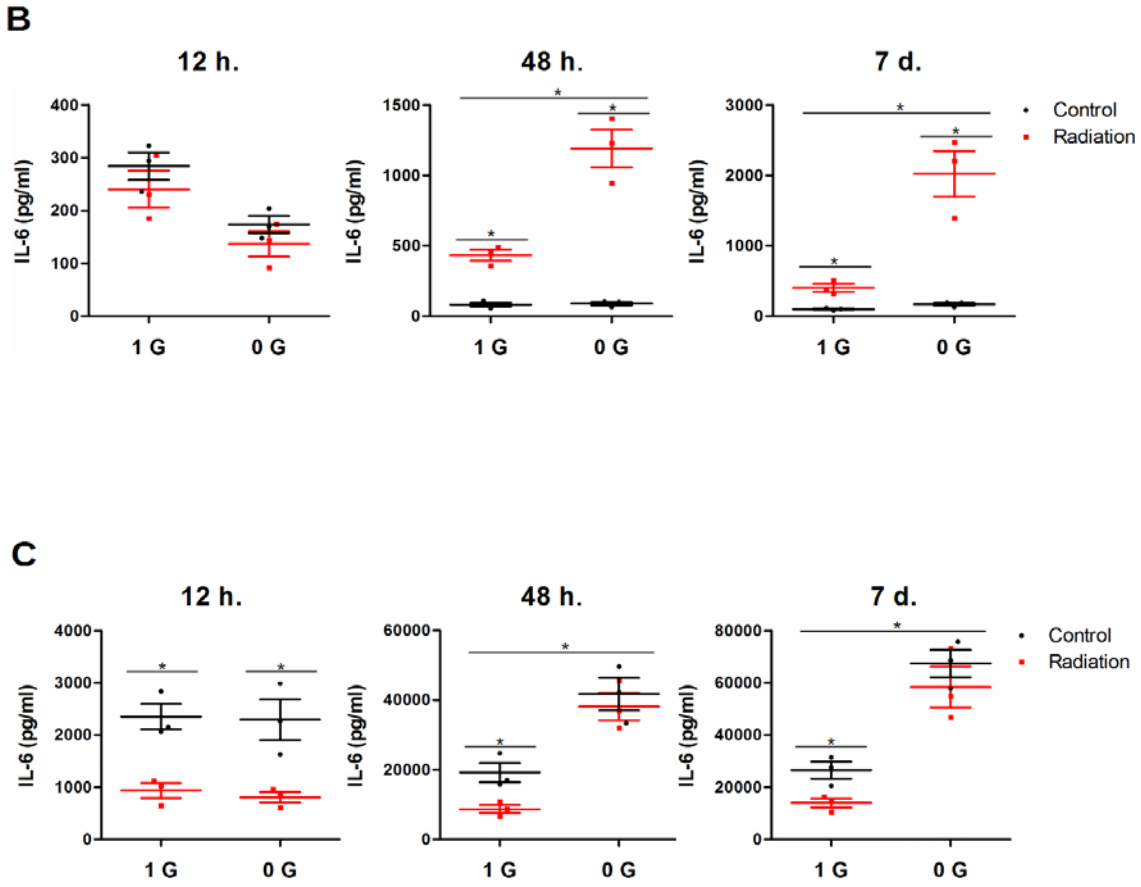


Figure 3. Radiation limits the effect of OSM induction on IL-6 secretion but enhances the effect of MMG.

MC3T3 cells were cultured in a spaceflight model combining culture in the RCCS and radiation representative of a SPE (1 Gy at 364 rad/s from a ^{137}Cs source). Osteoblast IL-6 secretion was measured by ELISA and are shown as scatter plots with mean and SEM. An overview of these data is shown in (A). The effects of OSM and MMG on IL-6 secretion were unaffected by sham irradiation (A, left column). Under all conditions, OSM treatment induced IL-6 secretion by at least 10-fold. Consequently, radiation and MMG effects are broken out into control (B) and OSM treated (C). In the absence of

OSM treatment (B), irradiation alone increased IL-6 secretion relative to sham irradiation by approximately 5-fold from 48 h on. The combination of MMG and radiation treatments (without OSM treatment, B) magnified the induction of IL-6 approximately 10-fold compared to MMG treatment alone. With OSM treatment (C), irradiation without MMG decreased IL-6 secretion at all time points. Irradiation had no effect on the synergistic increase in IL-6 secretion seen with combined OSM induction and MMG. For clarity, statistical results are shown only in (B) and (C).

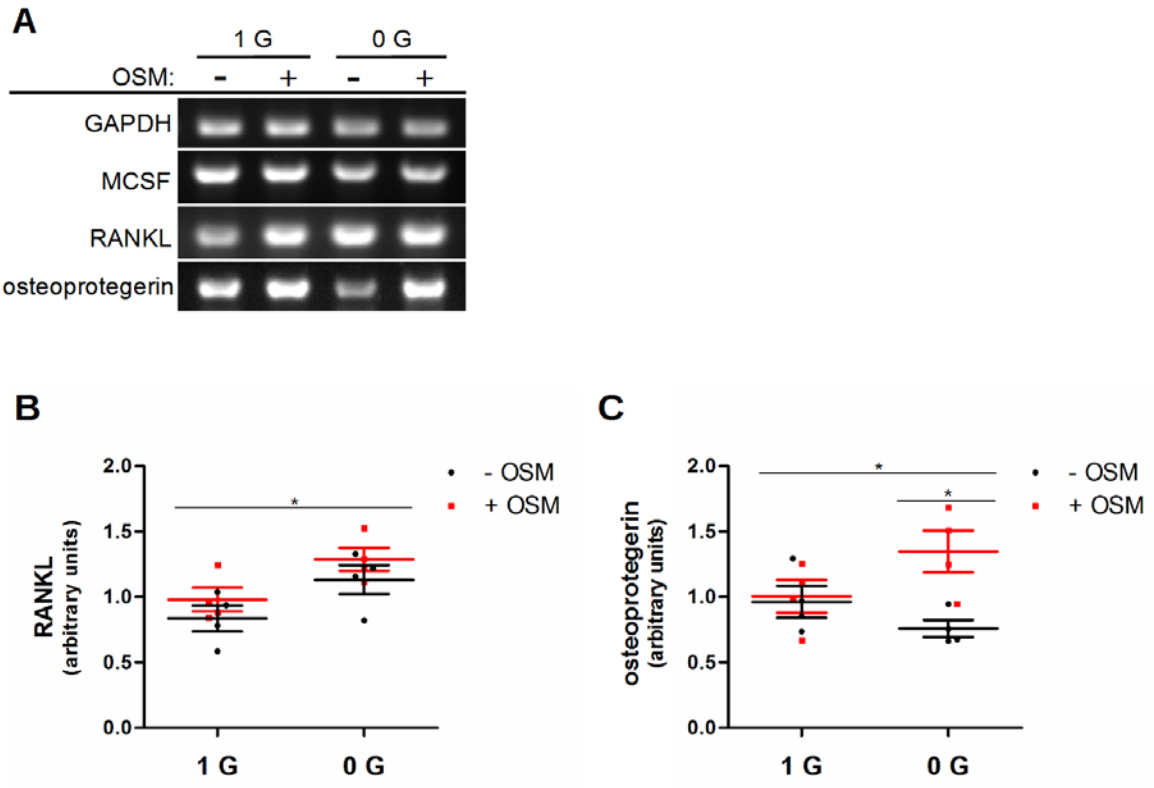


Figure 4. OSM counteracts the effect of MMG on the RANKL: Osteoprotegerin ratio.

Semi-quantitative RT-PCR was used to examine the interaction of OSM and modeled free fall on the osteoblast transcription of MCSF, RANKL, and osteoprotegerin. (A) Representative images from samples collected after 48 h, when the most significant

effects were seen for RANKL and osteoprotegerin. No changes were detected for MCSF or with radiation for any of these targets. Significant effects when quantified by densitometry are shown as scatter plots with mean and SEM for RANKL (B) and osteoprotegerin (C). RANKL transcription increased under MMG (B), without an associated increase in osteoprotegerin (C). The combination of OSM and MMG, however, increased osteoprotegerin proportionally to the increase in RANKL.

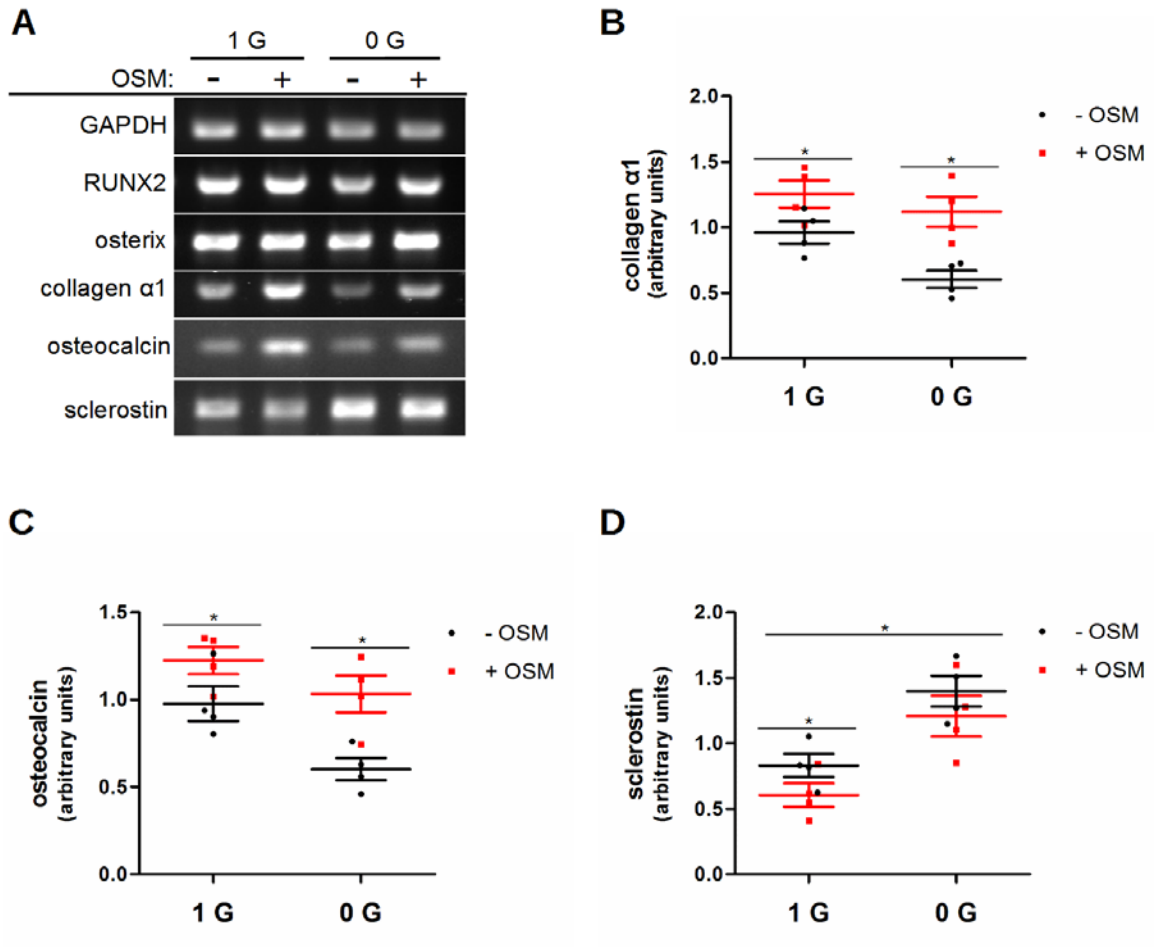


Figure 5. OSM and MMG have independent and opposing effects on osteoblast activity.

Markers of osteoblast maturation and activity were assessed by semi-quantitative RT-PCR for interactions between OSM signaling and MMG. Representative images at 48

h are shown (A), when the largest effects were found. For markers affected by OSM or MMG, the results of densitometry are shown as scatter plots with mean and SEM (B-D). OSM supplementation induced collagen α1 (B) and osteocalcin (C) transcription, but had no significant effect on sclerostin transcription (D). MMG inhibited the transcription of collagen α1 and osteocalcin, while increasing transcription of sclerostin. No significant interaction between the factors was detected by ANOVA.

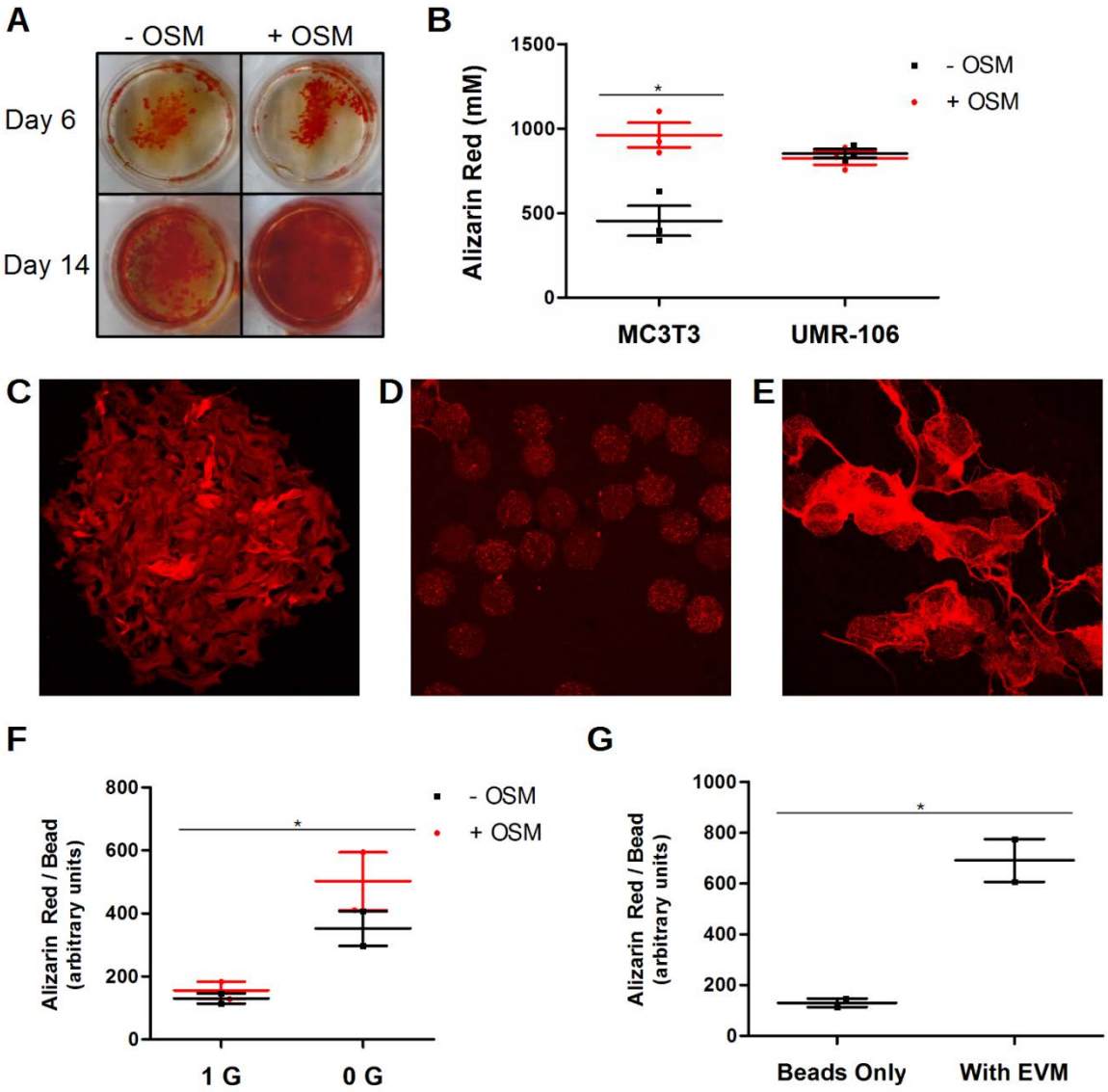


Figure 6. RCCS and Cytopore cell culture does not model spaceflight's effect on osteoid formation and mineralization.

(A) Alizarin red staining of osteoid mineralization after culture of MC3T3 cells for 6 or 14 days in mineralizing culture medium. OSM increases production of mineralizing osteoid. (B) Quantification of the differential effect of OSM on mineralization after 14 d in monolayer culture for the MC3T3 mouse pre-osteoblast and UMR-106 rat osteosarcoma cell lines. (C) Confocal micrograph (400x) of fluorescent alizarin red bound to mineralization in MC3T3 culture on Cytopore microcarriers. (D) Representative confocal micrograph (50x) of MC3T3 cultured 14 d on Cytopore with mineralization stained for quantification by densitometry. (E) Confocal micrograph (50x) of Cytopore MC3T3 culture in control gravity conditions showing extra-vehicular mineralization (EVM). (F) Densitometry showed greater mineralization in MMG cultures. (G) EVM accounts for the majority of mineralization under control gravity conditions.

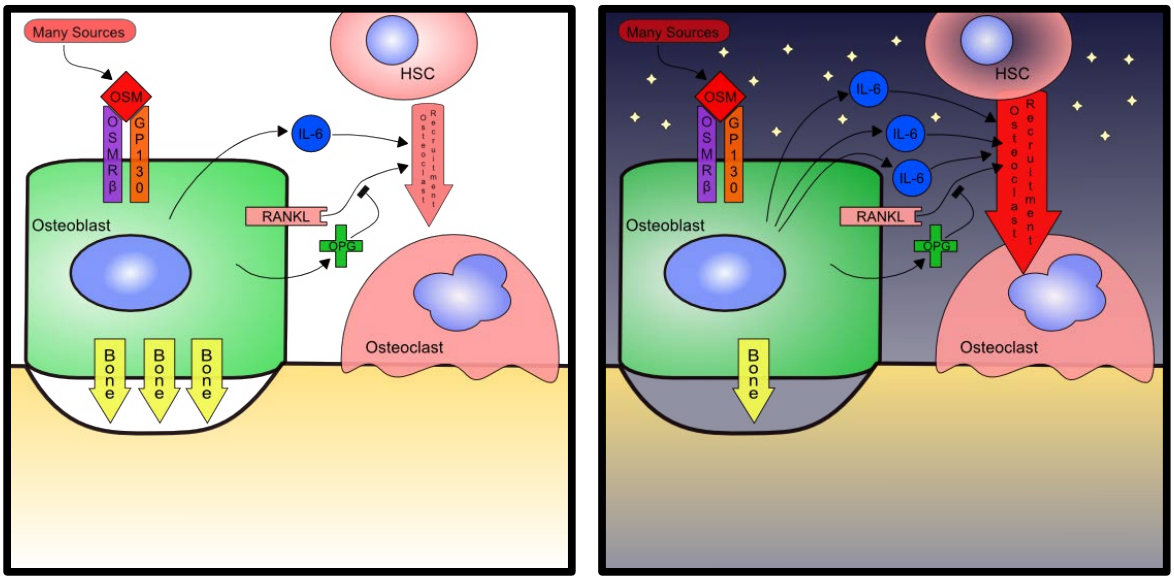


Figure 7. Spaceflight conditions act on bone through OSM signaling.

In normal circumstances (left), OSM signaling has a balanced effect on osteoblast activity and osteoclastogenesis. In spaceflight (right), it is possible that this balance is disrupted. The synergistic increase in IL-6 secretion increases osteoclastogenesis. Meanwhile, there is no matched synergism in the action of OSM on osteogenesis. This leads to an increase in net bone loss.

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