The Wnt Inhibitor Sclerostin Is Up-regulated by Mechanical Unloading in Osteocytes in Vitro*

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Background: Recent studies have suggested osteocytes as key players in mechanosensation and skeletal metabolism.

Results: Simulated microgravity induces an autonomous up-regulation of SOST/sclerostin and RANKL/OPG in a novel osteocytic cell line, Ocy454.

Conclusion: Mechanical loading regulates intrinsic osteocyte responses in concert with hormonal and cytokine inputs.

Significance: Learning how osteocytes sense mechanical loads would enable novel interventions to prevent disuse-induced bone loss.

Although bone responds to its mechanical environment, the cellular and molecular mechanisms underlying the response of the skeleton to mechanical unloading are not completely understood. Osteocytes are the most abundant but least understood bone cell type. Because osteocytes exhibit a dendritic morphology with extensive connectivity throughout the mineralized matrix of bone, they are thought to be responsible for sensing stresses and strains in bone. Sclerostin, a product of the SOST gene, is produced postnataally primarily by osteocytes and is a negative regulator of bone formation. Recent studies show that SOST is mechanosensory at both the mRNA and protein levels. During prolonged bed rest and immobilization, circulating sclerostin increases both in humans and in animal models, and its increase is associated with a decrease in parathyroid hormone levels. To investigate whether SOST/sclerostin up-regulation in mechanical unloading is a cell-autonomous response or a hormonal response to decreased parathyroid hormone levels, we subjected osteocytes to an in vitro unloading environment achieved by the NASA rotating wall vessel system. To perform these studies, we generated a novel osteocytic cell line (Ocy454) that produces high levels of SOST/sclerostin at early time points and in the absence of differentiation factors. Importantly, these osteocytes recapitulated the in vivo response to mechanical unloading with increased expression of SOST (3.4 ± 1.9-fold, p < 0.001), sclerostin (4.7 ± 0.9-fold, p < 0.001), and the receptor activator of nuclear factor κB ligand (RANKL)/osteoprotegerin (OPG) (2.5 ± 0.7-fold, p < 0.001) ratio. These data demonstrate for the first time a cell-autonomous increase in SOST/sclerostin and RANKL/OPG ratio in the setting of unloading. Thus, targeted osteocyte therapies could hold promise as novel osteoporosis and disuse-induced bone loss treatments by directly modulating the mechanosensing cells in bone.

It has been recognized for over a century that mechanical loading is fundamental for the normal development and maintenance of the musculoskeletal system. Reduced loading is prevalent in our aging population, in the setting of spinal cord and other injuries, in prolonged bed rest, as a result of significant weight loss, or as experienced by astronauts during space flight and is invariably associated with bone loss (1–7). Although it has been appreciated for more than a century that bone models itself in response to its mechanical environment (Wolff’s law) (8), the mechanisms underlying this response still need to be fully elucidated. Osteocytes are the most abundant but least understood bone cell type. Because osteocytes exhibit a dendritic morphology with extensive connectivity throughout the mineralized matrix of bone, it is thought that this system forms the bone mechanosensor, acting as the orchestrator of osteoblast and osteoclast activity in response to mechanical stimuli (9–11). Osteocyte ablation results in a resistance to disuse-induced bone loss, highlighting the central role osteocytes play as the mechanosensor of bone (12). Improved understanding of the molecular mechanisms of osteocyte mechanosensation could have significant implications for the treatment of bone disorders including osteoporosis, fracture healing, and disuse-induced bone loss.

The precise mechanisms whereby osteocytes respond to and convert mechanical stimuli to biochemical signals remain elusive because of a lack of appropriate in vitro models. At the molecular level, osteocytes are thought to regulate the response
of bone to mechanical loading by at least two key molecules, sclerostin and receptor activator of nuclear factor κB ligand (RANKL)\(^4\) (9, 13). Mature osteocytes are one of the few cells that postnatally produce sclerostin, which is encoded by the SOST gene. Sclerostin inhibits bone formation both in vitro and in vivo by directly reducing proliferation and differentiation of osteoblasts via the canonical Wnt signaling pathway. Sclerostin is thought to act by binding low density lipoprotein receptors 5 and 6 to inhibit Wnt-β-catenin signaling (14–16). Moreover, sclerostin appears central to the response of bone to mechanical loading. SOST/sclerostin expression increases with mechanical unloading (10, 17) and decreases with loading (10). In addition, SOST knock-out mice are resistant to disuse-induced bone loss (18), and mice treated with sclerostin antibody show an anabolic response in the hind limb unloaded model (19). Furthermore, serum sclerostin is significantly increased during prolonged (90-day) bed rest in healthy volunteers (17), in obese patients undergoing weight loss (20), and acutely in postmenopausal stroke patients (21). In addition to the effects of sclerostin, it was recently shown that soluble RANKL also secreted by osteocytes (9, 13) contributes to the control of bone remodeling. However, RANKL has also been found to be expressed in a variety of other cell types including osteoblasts, bone lining cells, keratinocytes, T and B lymphocytes, mammary epithelial cells, and undefined cell types within the brain (22). Thus, it is currently unknown whether osteocytes can increase RANKL in a cell-autonomous manner, thus potentially serving as an initiator of the cascade of bone resorption seen in mechanical unloading and microgravity.

Regardless of the initiation mechanisms, the hallmark of immobilization and microgravity in humans is an increase in bone resorption (23, 24), resulting in subsequent transient hypercalcemia with persistently increased urinary and fecal calcium loss (23). The endocrine counter-regulatory mechanisms to maintain normal serum calcium are a reduction in serum parathyroid hormone (PTH) and consequently lower 1,25-dihydroxyvitamin D concentrations (23). However, PTH is also a known potent regulator of SOST/sclerostin in osteocytes both in humans and in animal models (25, 26), raising the possibility that the increase in SOST/sclerostin during unloading or bed rest might be a consequence of decreased serum PTH rather than direct mechanical sensing by osteocytes. Indeed, there is an inverse correlation between PTH and sclerostin in male hypoparathyroid subjects (27), and PTH infusion in healthy men induces a decline in circulating sclerostin (28). Both in vivo and in vitro, PTH decreases sclerostin expression via activation of the PTH receptor expressed on osteocytes (29), and mice lacking the PTH receptor specifically in osteocytes have elevated expression of sclerostin (30). Thus, in vivo studies cannot determine whether suppression of PTH or other changes in cytokines, such as prostaglandin E\(_2\) (PGE\(_2\)), are driving the increases in serum sclerostin following unloading. More broadly, there is no evidence to assess whether the increase in SOST/sclerostin is a direct osteocyte response to mechanical unloading as postulated by the mechanostat theory proposed by Harold Frost (31).

Currently available osteocytic cell lines express basally very low levels of SOST/sclerostin and require high cell density with extended time in culture under differentiation conditions to produce detectable SOST/sclerostin (32–34), thus limiting their use for investigating mechanotransduction signaling pathways. To investigate osteocyte responses to unloading, we have isolated and characterized a novel osteocytic cell line (Ocy454), reported herein, that faithfully recapitulates the in vivo response of osteocytes to mechanical stimuli. Ocy454 cells show rapid, high level expression of SOST/sclerostin that is responsive to hormonal (PTH), cytokine (PGE\(_2\)), and mechanical stimuli. Furthermore, \(G\alpha\) knockout in Ocy454 led to significant increases in SOST expression matching known osteocyte in vivo regulation (35), demonstrating the broad utility of this new osteocytic cell line for studying SOST/sclerostin regulation as we have recently reported (36). Ocy454 also showed an enhanced osteocytic phenotype when cultured on a three-dimensional biomaterial by increasing FGF23 expression upon PTH stimulation, highlighting the importance of optimizing in vitro culture conditions for studying certain aspects of osteocyte biology.

The primary hypothesis and objective of this study were to determine whether mechanical unloading is sensed in an osteocyte-endogenous manner and investigate the cellular mechanism(s) osteocytes utilize to regulate SOST/sclerostin. We hypothesized that simulated unloading (microgravity) as achieved in the NASA rotating wall bioreactors would increase SOST/sclerostin in a cell-autonomous fashion and that this increase would be suppressible by negative regulators (PTH and PGE\(_2\)) of SOST/sclerostin. As reported herein, osteocytic cells are indeed capable of responding to reduced mechanical forces with time-dependent increases in SOST/sclerostin expression. In addition, the gene expression profile in simulated microgravity (e.g. SOST, osteocalcin, Phex, and MEPE) is distinct from that seen with mechanical loading as achieved by fluid shear stress. Moreover, the increase in SOST/sclerostin expression is suppressed by PTH and PGE\(_2\), suggesting upstream mechanistic overlap between mechanical sensing and G-protein-coupled receptor signaling and the potential to use targeted therapies in these signaling pathways as treatments for disuse-induced bone loss.

**Experimental Procedures**

**Osteocytic Cell Lines**—Mice expressing the green fluorescent protein (GFP) under the control of dentin matrix protein 1 (8-kb DMP1-GFP) (kindly provided by Dr. Ivo Kalajzic, University of Connecticut Health Center) were mated with mice carrying a ubiquitously expressed SV40 large T antigen (Immortomouse, Charles River), and osteocytes were isolated from the long bones of 4-week-old double transgenic mice. Long bones were cut at the epiphysis; flushed with medium (α-minimum Eagle’s medium) (Gibco) supplemented with 0.1% bovine serum albumin, 25 mM HEPES (pH 7.4) and containing 1 mg/ml collagenase type I/I (ratio, 1:3) (Worthington); and subjected to four sequential collagenase digestions, one EDTA digestion, and a final sixth collagenase digestion, and

\(^4\) The abbreviations used are: RANKL, receptor activator of nuclear factor κB ligand; PTH, parathyroid hormone; OPG, osteoprotegerin; PGE\(_2\), prostaglandin E\(_2\); DMP1, dentin matrix protein 1; hPTH, human PTH.
minced bone fragments were placed in collagen-coated 100-mm tissue discs. Cells were allowed to reach confluence at 33 °C and then grown for an additional 10–12 days at 37 °C prior to FACS for DMP1-GFP expression. Bulk-sorted GFP-positive cells were maintained on collagen-coated flasks grown in α-minimum Eagle’s medium supplemented with 10% FBS (Gibco) and 1% antibiotic-antimycotic (Gibco). Subsequently, two criteria were selected for further identification of a mature osteocyte cell line: sorted GFP-positive were required to 1) have high levels of production of known osteocytic genes (SOST and DMP1) at the early time point of 14 days at the semipermissive temperature in the absence of differentiation and 2) respond to the known effects of PTH stimulation by suppression of SOST and increased expression of RANKL. This method provided a heterogeneous population of DMP1-GFP-positive cells that more faithfully resemble osteocytes in vivo, which are known to be a mixture of cells with various degrees of SOST and DMP1 expression depending on their age/maturity. We performed our experiments in this heterogeneous population. In an effort to establish a more homogeneous osteocytic population, we also performed FACS on Ocy454 to isolate single cell subclones. Ocy454 and several single cell clones (36) have the same osteocyte marker expression and hormonal (PTH, PGE2, and shear stress) response.

For two-dimensional cell culture, cells (Ocy454, IDG-SW3 (33), and primary long bone osteoblasts isolated from 4-week-old SV40 large T antigen mice) were plated at 10⁵ cells/ml and allowed to reach confluence at the permissive temperature (33 °C) for 3 days. Subsequently, cells were either differentiated at the permissive temperature or switched to the semipermissive temperature (37 °C) for the indicated time points. MLO-Y4 cells were plated at 10⁵ cells/ml, and RNA was extracted at 4 days (32). For primary osteocytes, cells were isolated from 4-week-old DMP1-GFP long bones. In brief, long bones were flushed of bone marrow with PBS, subjected to sequential collagenase digestions, and minced, and bone chips were placed in tissue culture plates. Subsequently, at the 2-week time point, cells were subjected to FACS. GFP- and GFP+ populations were directly collected into RNA extraction buffer (Qiagen).

The routine culturing conditions to maintain the Ocy454 osteocytic phenotype were twice weekly subpassages (1:5) for up to 4 months from a frozen stock. For three-dimensional cell culture, 1.6 × 10⁶ Ocy454 cells were plated on 200-μm polystyrene Alvetex (Reinervate) well insert scaffolds. Scaffolds were collagen-coated according to the manufacturer’s protocols for the indicated experiments. All other chemicals were from Sigma-Aldrich or Fisher Scientific.

Quantitative Real Time PCR—Total RNA was isolated (RNAEasy, Qiagen, Valencia, CA) according to the manufacturer’s recommendations, and RNA was quantified (NanoDrop, Thermo Scientific, Rockford, IL). cDNA synthesis was performed (Qiagen or Taraka Clontech) on 0.5–1 μg of total RNA followed by SYBR quantitative PCR (StepOnePlus, Life Technologies). Primer sequences are available upon request. β-Actin (ACTB) or HPRT1 was used for normalization of gene expression. ΔCT was computed within each sample to the housekeeping reference, and ΔΔCT was computed across experimental conditions. Experiments were run in triplicates unless otherwise indicated.

Western Blot—Whole cell lysates (Mammalian Protein Extraction Reagent, Thermo Scientific) from two-dimensional cell culture conditions were prepared according to the manufacturer’s recommendations. Protein concentrations were quantified (Bio-Rad Protein Assay, Bio-Rad), and 10 μg was separated on a 4–20% Tris-glycine denaturing gel (Life Technologies) and transferred to a PVDF membrane using the Trans-Blot Turbo (Bio-Rad) system according to the manufacturer’s recommendations. The membrane was blocked with 3% BSA and 5% nonfat milk in Tris-buffered saline containing 0.05% Tween 20 (TBST) for 1 h and then incubated with goat polyclonal mouse sclerostin antibody (1:200; R&D Systems, Minneapolis, MN) overnight at 4 °C (30). After washing, secondary antibody (1:5000) was incubated for 1 h at room temperature and then developed using enhanced chemiluminescence (Thermo Scientific) (30). For Gα immunoblotting, similar procedures were followed using an anti-Gα antibody (Millipore, catalogue number 06-237).

Sclerostin Immunohistochemistry—Three-dimensional scaffolds were washed once with phosphate-buffered saline (Life Technologies) and frozen embedded (OCT, Tissue Tek), and 10-μm sections were cut onto standard microscope slides. In brief, proteinase K was used for antigen retrieval for 15 min followed by a quench in 3% H₂O₂, methanol for 10 min, washing in H₂O, and rinsing in 1× TBS. Next, biotinylated anti-sclerostin antibody (R&D Systems, BA1580) diluted 1:50 in Tris-NaCl blocking buffer was incubated for 1 h and washed three times with 1× Tris-NaCl-Tween buffer. Streptavidin-HRP diluted 1:100 in Tris-NaCl blocking buffer was then added to slides and incubated for 30 min, washed three times with 1× Tris-NaCl-Tween buffer, and incubated with 3,3’-diaminobenzidine HRP substrate (Vector Labs) for 5 min, and the slide was coverslipped.

Sclerostin ELISA—Four milliliters of cell culture supernatants from slow turning rotating wall bioreactor experiments at the indicated time points were spun at 850 rpm for 4 min, and the volume was reduced to 250 ml with a 10-kDa centrifugal filter unit (Millipore, Billerica, MA) according to the manufacturer’s recommendations. Supernatants were assayed for sclerostin using a commercially available assay (ALPCO, Salem, NH) according to the manufacturer’s recommendations. For additional sclerostin ELISA experiments, an antibody matched pair ELISA was used (34). In brief, for the matched pair sclerostin ELISA, conditioned medium (36–48 h) was harvested from Ocy454 cells as indicated in the figure legends and stored at −80 °C until further use. High binding 96-well plates (Fisher, 21-377-203) were coated with sclerostin antibody VI capture antibody (3 μg/ml) in PBS for 1 h at room temperature. Plates were washed (PBS plus 0.5% Tween 20) and blocked with wash buffer supplemented with 1% BSA and 1% normal goat serum for 1 h at room temperature. Samples (60 μl/well) were then added along with a standard curve of murine recombinant sclerostin (ALPCO), and plates were incubated overnight at 4 °C. Plates were washed three times and incubated with HRP-coupled sclerostin antibody VII detection antibody (0.5 μg/ml) for 1 h at room temperature. After washing, signal detection
was performed using Ultra 3,3',5,5'-tetramethylbenzidine ELISA (Pierce, 34028), stopped by 2 N sulfuric acid, and read at 450 nm. Prior to harvesting supernatant, cell number per well was always determined using the PrestoBlue assay (Life Technologies) according to the manufacturer’s instructions.

For shRNA experiments, shRNA (Broad Institute, Cambridge, MA) lentiviral particles in puromycin-resistant vector technologies) according to the manufacturer’s instructions. For shRNA target sequences, shRNA (Broad Institute, Cambridge, MA) lentiviral particles in puromycin-resistant vector targeted against luciferase (control; shLuciferase) or shGα were used to infect cells plated 1 day prior at 0.5 \times 10^5 cells/ml. Subsequently, infected cells were puromycin-selected (2 µg/ml) at the permissive temperature (33 °C) for 7 days and subsequently allowed to differentiate for 14–16 days at the semipermissive temperature. Table 1 provides the shRNA target sequences.

Simulated Microgravity—Ocy454 cells were plated on threedimensional scaffolds as described above and allowed to grow at the permissive temperature (33 °C) for 3 days. Subsequently, scaffolds were moved to the semipermissive temperature (37 °C) for an additional culturing time before being loaded into the bioreactor. Scaffolds were cut into 3-mm discs using disposable biopsy punches (Integra Millex, Plainsboro, NJ) and placed into non-rotating (static) or rotating (simulated microgravity) 110-mL slow turning lateral vessels (Synthecon, Houston, TX) for 3 days. For the rotating vessels, rotation speed was set to 18.6 rpm for the first 24 h and increased to 20.9 rpm to maintain solid body rotation kinetics throughout the experiment (38).

Two-dimensional Laminar Fluid Shear Stress—Ocy454 cells were plated on glass microscope culture slides (Flexcell International Corp.) at 2 \times 10^5 cells/ml and allowed to grow at the permissive temperature (33 °C) for 3 days. Subsequently, slides were moved to the semipermissive temperature (37 °C) for an additional culturing time (11–14 days). Medium was changed for static slides, or slides were loaded into the laminar fluid flow shear stress device (Flexcell Streamer, Flexcell International Corp.) connected to an electronically controlled peristaltic pump with pulse dampers integrated into the flow circuit to allow for continuous unidirectional shear stress. Cells were exposed to 0.5 or 2 dynes/cm² for 2 h or 3 days (39–41).

Three-dimensional Fluid Shear Stress—Alvetex scaffolds were seeded with 1.6 \times 10^6 cells and allowed to grow at the permissive temperature (33 °C) for 2 days prior to transferring to (37 °C) for differentiation. Cells were differentiated for 14 days prior to transferring to the Reinnervate perfusion plate. The perfusion plates were attached to a Masterflex peristaltic pump (catalog number 7520-57) with a Masterflex standard pump head (catalog number 7014-20) and exposed to either 0.5 or 2 dynes/cm² for either 1 or 3 days.

Statistical Analysis—All values are reported as the mean ± S.D. unless otherwise noted. Group mean differences were evaluated with Student’s t test and considered significant at \( p < 0.05 \).

Results

Osteocyte (Ocy454) Cell Line Basal and Hormonal Characterization—Our method for osteocyte cell line development coupled fluorescent sorting for an osteocytic marker (DMP1) with functional hormonal screening to accurately ensure the cell line possessed the key functional responses of mature osteocytes in vivo. Of several preparations, one population of sorted DMP1-GFP-SV40 large T antigen (Ocy454) cells was selected for further characterization on the basis of its high expression of SOST at early time points at the semipermissive temperature. Ocy454 osteocytic cells displayed a dendritic morphology (Fig. 1A) similar to other osteocytic cell lines (32, 33) and at 2 weeks at the semipermissive temperature (37 °C) expressed the DMP1-GFP transgene (Fig. 1, B and C).

After 2 weeks at 37 °C, Ocy454 cells expressed significantly higher levels of SOST and DMP1 compared with long bone primary osteocytes as well as the only other available osteocytic cell lines, MLO-Y4 and IDG-SW3 (Fig. 2, A and B). Upon further study, we also observed that Ocy454 differentiated upon contact inhibition at the permissive temperature (Figs. 1C and 2B). However, Ocy454 differentiated at a slower pace at the permissive temperature. For example, at the 1-week time point, there were lower levels of SOST at the permissive temperature compared with the semipermissive temperature (Fig. 2B). In addition, Ocy454 expressed levels of SOST that were significantly higher than those expressed by long bone osteoblasts (Fig. 2B) as early as 1 week at 37 °C in the absence of differentiation factors. Sclerostin was detected by ELISA in the cell culture supernatant at day 11, and its concentration continued to increase with time in culture (Fig. 2B). Interestingly, after 2 weeks at the semipermissive temperature (37 °C), Ocy454 cells expressed higher levels of other characteristic osteocytic genes, such as DMP1 (Fig. 2B).

In contrast, these cells had low levels of expression of genes characteristic of immature osteocytes and late osteoblasts, such as keratocan (Fig. 2C, D) that were significantly higher than those expressed by long bone osteoblasts (Fig. 2B) as early as 1 week at 37 °C in the absence of differentiation factors. Sclerostin was detected by ELISA in the cell culture supernatant at day 11, and its concentration continued to increase with time in culture (Fig. 2B). Furthermore, after 2 weeks at the semipermissive temperature (37 °C), Ocy454 cells expressed high levels of other characteristic osteocytic genes, such as DMP1 (Fig. 2B).

We next assessed Ocy454 cell responsiveness to known osteocyte regulators. Short term (4-h) treatment with human (h)PTH(1–34), forskolin, or (16-h) PGE2 induced a statistically significant down-regulation of SOST (Fig. 3A; \( p < 0.001 \) for all) and sclerostin both in whole cell lysate and conditioned medium (Fig. 3, B and C). These results are consistent with the known inhibitory effects of these agents on SOST expression. In contrast, a PGE2 inhibitor, indomethacin, caused an increase in SOST (Fig. 3B), showing that Ocy454 cells have an intact hormonal axis that increases SOST expression.

### Table 1

| shRNA Target Sequences | Target Sequences |
|------------------------|------------------|
| LacZ                   | CCACAGCGACCTATCCCATTA |
| Luciferase             | AGAATCTCCTGATATCAGTGA |
| GNAS E3                | CCCAGCTGAGAAGCCATCA |
| GNAS B2                | GCCCAAGTATCCTTCCGGGAGT |
| GNAS G2                | TCGGAAGTGGTTTTGGGAAAT |
| GNAS G9                | CCTGACATGTTATAGGSSSATA |
| GNAS C2                | CCTGACAGGATCTGGCATTT |

### Osteocyte-autonomous Responses to Mechanical Unloading

Ocy454 cells were plated on three-dimensional scaffolds as described above and allowed to grow at the permissive temperature (33 °C) for 3 days. Subsequently, scaffolds were moved to the semipermissive temperature (37 °C) for an additional culturing time before being loaded into the bioreactor. Scaffolds were cut into 3-mm discs using disposable biopsy punches (Integra Millex, Plainsboro, NJ) and placed into non-rotating (static) or rotating (simulated microgravity) 110-mL slow turning lateral vessels (Synthecon, Houston, TX) for 3 days. For the rotating vessels, rotation speed was set to 18.6 rpm for the first 24 h and increased to 20.9 rpm to maintain solid body rotation kinetics throughout the experiment (38).

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Three-dimensional Fluid Shear Stress—Alvetex scaffolds were seeded with 1.6 \times 10^6 cells and allowed to grow at the permissive temperature (33 °C) for 2 days prior to transferring to (37 °C) for differentiation. Cells were differentiated for 14 days prior to transferring to the Reinnervate perfusion plate. The perfusion plates were attached to a Masterflex peristaltic pump (catalog number 7520-57) with a Masterflex standard pump head (catalog number 7014-20) and exposed to either 0.5 or 2 dynes/cm² for either 1 or 3 days.

Statistical Analysis—All values are reported as the mean ± S.D. unless otherwise noted. Group mean differences were evaluated with Student’s t test and considered significant at \( p < 0.05 \).
In addition, hPTH(1–34) dose- and time-response experiments showed Ocy454 to be sensitive to down-regulation of SOST in as short as 2 h (100 nM; Fig. 3D) and a 50% suppression at doses as low as 0.1 nM hPTH(1–34) (Fig. 3D). Similarly, hPTH(1–34) (4 h), forskolin, and PGE2 caused concurrent increases in RANKL mRNA (Fig. 3E). hPTH(1–34) suppressed Me2C mRNA (Fig. 3F), consistent with previous reports (44, 45), and DMP1 mRNA (Fig. 3G). There was no regulation of FGF23 mRNA by 4-h PTH treatment in Ocy454 at 1 or 2 weeks in two-dimensional non-collagen- and collagen-coated 6-well plate culture conditions (data not shown).

We and others have previously reported that mice lacking (35, 46) Gs/H9251 have increased levels of SOST/sclerostin. To confirm these in vivo results in Ocy454 cells, we used shRNA to knock down Gs/H9251 in Ocy454 as was done previously for HDAC5 (36). The range of sclerostin secretion (normalized to cell number) was determined in each experiment using 10 separate control lentiviruses expressing shRNAs against non-expressed genes (LacZ, luciferase, GFP, and red fluorescent protein). Dotted lines indicate two standard deviations above the mean value of sclerostin secretion (normalized to cell number) in the presence of the control hairpins. As shown in Fig. 4A, two of five hairpins tested to achieve lentivirus-mediated shRNA knock-down of GNAS (but not related heterotrimeric G-proteins GNAQ and GNA11) consistently increased sclerostin secretion (individual hairpins labeled next to corresponding data points). The individual hairpins that reduced GNAS mRNA levels accordingly increased SOST expression (Fig. 4B), thereby confirming the expected knockdown/phenotype relationship for this known SOST regulator. GNAS hairpins “G2” and “G9” both effectively reduced Gα protein levels (Fig. 4C), and hairpin G2 was selected for further study. Sclerostin secretion in control (shLuciferase) and GNAS G2 shRNA-expressing cells was determined over time. As shown in Fig. 4D, GNAS shRNA increases basal SOST expression (after 14 days at 37 °C); furthermore, whereas control cells respond to PTH at this time point with suppression of SOST levels, this is not the case when Gα levels are reduced. Taken together, these data confirm a cell-intrinsic role for Gα in osteocytes and further support the use of Ocy454 cells for studying SOST gene regulation.
Three-dimensional Culture Enhances Osteocytic Phenotype—

To evaluate the effects of a three-dimensional culture environment on the expression of osteocyte-specific genes and to provide a scaffold for cell attachment in the rotating wall bioreactor system used to simulate microgravity, Ocy454 cells were seeded onto scaffolds and cultured for an additional 7–14 days. Consistent with our two-dimensional culture results, we also observed a significant down-regulation of SOST (Fig. 5A), increases in RANKL (Fig. 5B), and decreases in DMP1 (Fig. 5D) in three-dimensional cultures (p < 0.001 for all) upon PTH treatment. Previous reports have demonstrated that TGFβ₁-responsive gene, Serpine1, increased 2.3 ± 0.1-fold (p < 0.007). Interestingly, in contrast to two-dimensional cultures, culture in three dimensions with 4-h PTH treatment resulted in a 5-fold (p < 0.001) increase in FGF23 expression (Fig. 5C). In a direct comparison between three-dimensional and two-dimensional culture at an early time point (3 days at 37 °C), Ocy454 had significantly higher amounts of SOST and RANKL than in the two-dimensional setting. Furthermore, Ocy454 displayed dendritic morphology in three-dimensional culture conditions (Fig. 5E), and we observed decreases in sclerostin protein expression with hPTH(1–34) treatment in three-dimensional culture (Fig. 5F).

**FIGURE 2.** A, Ocy454 at 2 weeks (37 °C) (black bars) express characteristic osteocytic markers versus MLO-Y4 in the absence of differentiation medium. B, Ocy454 (Ocy) at 1 and 2 weeks (wk) (37 °C) (black bars) express characteristic osteocytic markers SOST, sclerostin, DMP1, FGF23, and RANKL and lack keratocan (KERA) expression in the absence of differentiation medium compared with long bone osteoblasts (LB-OBs), IDG-SW3 (2 weeks), long bone (LB) DMP1-GFP+ and long bone DMP1-GFP+ osteocytes. *, p < 0.001 for 1 and 2 weeks at semipermissive growth temperature (37 °C) versus permissive growth temperature (33 °C; 3 days (d)); **, p < 0.001 for 1 and 2 weeks at semipermissive temperature versus permissive growth temperature at the indicated time points; #, p < 0.001 for Ocy454 versus long bone osteoblasts; Ψ, p < 0.001 for Ocy454 versus long bone DMP1-GFP+ osteocytes at the indicated time points. ND, not detected. Error bars represent S.D. of 1.
Fluid Shear Stress Regulation of Ocy454 in Two-dimensional Culture—Ocy454 were then subjected to continuous unidirectional fluid shear stress in two-dimensional culture conditions. Consistent with previous reports using UMR 106.01 osteoblast-like cells (39), short term (2-h) fluid shear stress significantly suppressed \( SOST \) mRNA levels at low and high shear stresses (Fig. 6A). Whereas \( RANKL \) was reduced at low shear stress (0.5–2 dynes/cm\(^2\)) \( RANKL \) and \( DMP1 \) were increased at higher shear stress (8 dynes/cm\(^2\)) as shown in Fig. 6B and C. These results demonstrate that Ocy454 cells are exquisitely responsive to mechanical forces with intact \( SOST \), \( DMP1 \), and \( RANKL \) regulation to overloading stimuli. Our results also suggest differential regulation of \( SOST \) and \( DMP1 \) to fluid shear stress but not to simulated microgravity, whereas the response to hPTH(1–34) is the same.

Simulated Microgravity Increases \( SOST \)/Sclerostin and \( RANKL \)—We then utilized the NASA-developed rotating wall bioreactor system to mimic microgravity to assess whether osteocytes can directly sense mechanical unloading and regulate the expression of sclerostin and \( RANKL \), which are known to be involved in the response of bone to unloading. Indeed, under simulated microgravity conditions (3 days), there was a statistically significant increase of 3.5 ± 1.9-fold (\( p < 0.001 \)) in \( SOST \) expression compared with static controls (Fig. 7A). Secreted sclerostin as assessed by ELISA was also increased by 1.4 ± 0.1 at 1 day, 2.7 ± 0.4 at 2 days, and 4.7 ± 0.1 at 3 days (\( p < 0.001 \) for all) (Fig. 7B). There were no significant changes in other osteoblastic genes (osteocalcin, alkaline phosphatase, and osterix mRNAs) between the loaded and unloaded bioreactors, demonstrating that the increase in...
FIGURE 4. A, Ocy454 cells were infected with control shRNA-expressing lentiviruses (shGFP, shLuciferase, shRed fluorescent protein, and shLacZ) and five separate hairpins targeting the indicated gene. Each data point represents sclerostin/cell number values obtained for an individual hairpin. Dotted lines indicate values two standard deviations above and below those of the controls. For GNAS, individual hairpins are labeled on the data plot. B, Ocy454 cells were infected with shGFP and the indicated GNAS shRNA lentiviruses and then switched to 37 °C. 14 days later, RNA was isolated, and RT-quantitative PCR was performed for /H9252-actin, GNAS, and SOST. C, as in B except lysates were generated for immunoblotting. D, as in B except conditioned medium was collected at the indicated times for sclerostin ELISA. E, as in B expect cells were treated with vehicle or hPTH(1–34) (50 nM) for 4 h followed by semi-quantitative PCR for SOST and β-actin. *, p < 0.01 for hPTH(1–34) versus vehicle (VEH); **, p < 0.001 for shGNAS G2 versus shLuciferase and shGNAS C2; ***, p < 0.001 for shGNAS versus shLacZ for all time points. Error bars represent one S.D.
SOST/sclerostin expression was not a consequence of an altered cell state as we observed in our prolonged two-dimensional fluid shear stress experiments. In an effort to identify upstream regulator of SOST/sclerostin expression, we assessed changes in reported and potential regulators of SOST in the Mef2 pathway (Mef2A–D), PGE2 pathway (mPGES-1, 15-HGD, EP2, and EP4), SIRT1, osterix, PTHrP, PTH receptor, and periostin. We observed no changes in mRNA levels for any of these known regulators of SOST (Table 2) following simulated microgravity.

FIGURE 5. Ocy454 cells were grown on collagen-coated (A–D) three-dimensional scaffold (for hPTH(1–34) experiments). Four-hour hPTH(1–34) (100 nM) and 24-h TGFβ (10 ng/ml) treatment at 12–14 days decreases SOST (A) and increases RANKL (B). Four-hour hPTH(1–34) increases FGF23 (C) and decreases DMP1 (D) expression. E, representative H&E stain of Ocy454 cell within the scaffold. F, 4-h hPTH(1–34) (100 nM) treatment decreases sclerostin expression of Ocy454 cells on the scaffold. G, Ocy454 gene expression for SOST, DMP1, and RANKL on three-dimensional scaffolds versus two-dimensional (2D) culture at semipermissive growth temperature for 3 days (d) (37 °C). *, p < 0.001 for hPTH(1–34) or p < 0.007 for TGFβ, versus vehicle (VEH). Error bars represent S.D. of 1.

FIGURE 6. Short term (2-h) fluid shear stress in two-dimensional culture reduces SOST (A), increases DMP1 at high shear stress (8 dynes/cm²) (B), and reduces RANKL at low shear stress (0.5–2 dynes/cm²) and increases RANKL at high shear stress (8 dyne/cm²) (C). *, p < 0.001; **, p < 0.05 for static versus fluid shear stress. Error bars represent S.D. of 1.
Consistent with previous reports of osteoblasts increasing RANKL expression in simulated microgravity conditions (49), we observed increased RANKL mRNA (Fig. 7C) and a concurrent modest reduction in OPG mRNA (Fig. 7C), resulting in a statistically significant increase in the RANKL/OPG ratio in unloaded versus static conditions (Fig. 7C). We also detected a modest increase on mRNAs encoding DMP1, MEPE, and gp38 with no change in Phex or osteocalcin mRNA under simulated microgravity conditions (Fig. 7C). Thus, we report these regulatory changes to osteocytic genes as a signature of osteocytes exposed to simulated microgravity.

G-protein-coupled Receptor Responsiveness: SOST/Sclerostin in Simulated Microgravity—To determine whether activation of PTH receptors (or other G-protein-coupled receptors) could still suppress SOST/sclerostin in microgravity, we tested the effects of PTH and PGE₂ treatment in simulated microgravity. PTH (Fig. 7A) suppressed SOST and sclerostin levels of expression (Fig. 7B) to the same extent in static and unloaded conditions ($p < 0.001$). Similarly, PGE₂ caused the same magnitude of suppression of SOST expression in both static and simulated microgravity conditions. These results demonstrate that, although the increase in SOST expression is not dependent on reductions in G-protein-coupled receptor expression (PTH receptor and EP2/4) or $G_\alpha$ activity (Table 2), modulating G-protein-coupled receptor signaling can still regulate SOST/sclerostin expression in the setting of microgravity or unloading, such as disuse.

Long Term Fluid Shear Stress Regulation of Ocy454—One limitation of the NASA rotating wall bioreactor system is the possible generation of minimal fluid shear stress demonstrated to be on the order of 0.5–2 dynes/cm² (38, 50). To investigate whether the changes in gene expression observed in the NASA bioreactor were indeed due to simulated microgravity and not minimal shear stress, we subjected Ocy454 cells to long term exposure (1 or 3 days) to low laminar fluid shear stresses (0.5–2 dynes/cm²) in three-dimensional (Alvetex) culture condi-
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TABLE 2
Evaluated regulators of SOST/sclerostin in simulated microgravity

| ECRS enhancers | Msf2A, -C, -D, Mef2B (not expressed) (34, 45, 67) |
|----------------|--------------------------------------------------|
| SOST promoter transcription factors | TGFBI (47, 48), Osterix (59), Runx2 (68), SIRT1 (58), Pax6 (51), Periostin (44), MyoD: not expressed (68), Gα (35, 46) |
| PGE2 pathway | EP2, EP4 (54, 56), Cox-2, mPTGES-1, 15-HGPD |
| Cell membrane receptors | PTHR1, PTHrP: not expressed, P2XR1–7 |

At 2 dynes/cm², we observed a significant reduction in SOST mRNA and no change in DMP1 mRNA at 1 day (Fig. 8). At 0.5 dyne/cm², we observed significant suppression of SOST mRNA; a significant increase in DMP1 mRNA; and decreases in OPG, MEPE, gp38 (1 day), and osteocalcin mRNAs with no effect on RANKL or Phex mRNA (Fig. 8). Similar results for 2 dynes/cm² were observed at 3 days (Fig. 8) with the exception of a lack of regulation of DMP1 mRNA. These data clearly indicated that the up-regulation of SOST/sclerostin present in the NASA rotating wall bioreactor system was indeed due to simulated microgravity and not minimal shear stress.

In addition, as shown in Fig. 9, we subjected Ocy454 to two-dimensional long term low fluid flow. These low flow conditions induced changes in the differentiation state of Ocy454 as illustrated by significantly elevated levels of expression of SOST, DMP1, RANKL, OPG (3 days), Phex, MEPE, and gp38 with a reduction of osteocalcin expression (Fig. 9). Overall, these two-dimensional and three-dimensional long term mechanical overloading results demonstrated that our simulated microgravity experiments reflect a unique osteocyte cellular response to mechanical unloading stimuli.

Discussion

The primary objective of this study was to determine whether increases in SOST/sclerostin and RANKL seen in the context of disuse-induced bone loss are an intrinsic osteocytic response to mechanical unloading. Although it has been established that osteocytes are key players in the response of bone to mechanical stimuli (10, 39, 40, 52, 53), it is still unclear whether their response to unloading is a direct response to reduction in load as theorized by Wolff’s law or a consequence of changes in systemic endocrine or paracrine factors. Furthermore, the biochemical response of the osteocytic network to overloading (10, 39, 40) does not in it of itself provide evidence for a direct response to unloading stimuli. Here we present new data showing that osteocytes elicit an intrinsic response to mechanical loading that is independent of the known external hormonal influence of PTH and other factors.

Prior studies in rodents have reported increases in SOST/sclerostin in bone tissue (10) and in circulating sclerostin (19) during unloading. In addition, increased circulating serum sclerostin levels with a concurrent reduction of PTH levels have been reported in the context of disuse-induced bone loss in rodents (55) and humans (17). However, as PTH is a strong negative regulator of SOST/sclerostin, these in vivo studies cannot address the question of whether osteocytes can directly sense mechanical unloading or respond to hormonal changes.

Importantly, our results suggest that the increase in bone resorption in mechanical unloading and microgravity with associated transient hypercalcemia and reduced parathyroid hormone levels is not the driving force for increases in SOST/sclerostin and RANKL expression. Thus, for the first time, we have observed isolated osteocytes sensing mechanical unloading and responding with increases in SOST/sclerostin and the RANKL/OPG ratio.

The transcriptional regulators of SOST/sclerostin in mechanical unloading are currently unknown. However, Mef2 transcription factors have been shown in several contexts to bind a distal enhancer (ECR5) in the SOST locus, resulting in the increased expression of SOST/sclerostin (34, 45). However, we observed no transcriptional changes in the potential regulators of SOST in the Mef2 pathway (Msf2A, -C, and -D) (Table 2). Furthermore, because PGE2 is a known negative regulator of SOST/sclerostin in a Mef2-independent mechanism (56) and reductions in PGE2 production genes (Cox-2) have been observed in osteoblasts exposed to microgravity (57), we assessed changes in the PGE2 production and degradation pathways (mPTGES-1 and 15-HGPD) and receptor expression (EP2 and EP4) as shown in Table 2. Notably, no changes in mRNA of transcripts responsible for PGE2 production, PGE2 degradation, or PGE2 receptors were observed between static and unloaded cultures, implying that the increases of SOST/sclerostin in mechanical unloading are presumably not arising from changes in the PGE2 pathway. Several transcription factors have also been reported to suppress the SOST promoter (like SIRT1 and osterix) (58, 59) or act at the distal enhancer (ECR5) (like TGFBI) (47). However, in the context of mechanical unloading, we observed no change in SIRT1, osterix, or TGFBI mRNAs (Table 2). It has also been proposed that the peristin matricellular protein suppresses SOST in a Mef2C-dependent mechanism that is regulated by PTH (44, 60). However, in Ocy454, we observed no correlation among sclerostin, PTH, and peristin mRNA or protein expression in two-dimensional cultures or in the context of mechanical unloading (data not shown). Thus, future studies investigating the novel transcriptional or post-transcriptional regulation of SOST/sclerostin the context of mechanical unloading and microgravity are warranted.

G-protein-coupled hormonal (PTH) and cytokine regulators (PGE2) were capable of suppressing the increases of SOST/sclerostin seen in mechanical unloading. Thus, although our results show that osteocytes can directly sense mechanical unloading, they also suggest that the overall level of sclerostin in vivo appears to be an integral response of the osteocyte network to mechanical loading, hormonal, and cytokine cues. Of particular note, we have shown that mice lacking PTH receptor in osteocytes lose bone in the hind limb unloading model, consistent with our in vitro findings that G-protein-coupled receptor
signaling may play a minimal role in disuse-induced bone loss. One study has recently reported that SOST regulation in mechanical unloading in rodents could be site-specific with modest (−1.5%) down-regulation in cancellous metaphyseal and cortical bone, whereas up-regulation was seen in diaphyseal cortical (61) regions. Our results are consistent with these findings as our cell lines were isolated from the diaphysis of long bones. However, as the majority of osteocytes in the load-bearing skeleton are located in the diaphysis of long bones and circulating levels of sclerostin are elevated in the setting of disuse-induced bone loss, the clinical significance of the heterogeneous nature of the osteocytic network remains to be further explored. Furthermore, although the NASA rotating wall bioreactor provides a solid body rotation with a minimal fluid shear stress in the range of 0.5–2 dynes/cm² 38, 50, no currently existing in vitro ground-based model of microgravity can fully eliminate the low level of shear stress inherent in our model.

However, short mechanical loading (10, 62) and fluid shear stress (39) are known to cause decreased, not increased, levels of SOST/sclerostin and RANKL as we have observed (Fig. 7). To further investigate this confounding variable of minimal fluid shear stress in the NASA bioreactor, we subjected Ocy454 cells in two-dimensional and three-dimensional culture conditions to low unidirectional fluid shear stress. Importantly, neither two-dimensional nor three-dimensional fluid shear stress matched the pattern of osteocytic gene expression seen in simulated microgravity. In addition, cells on the surfaces of the scaffolds are likely exposed to shear stresses higher in range than cells within the scaffold. However, the same seeding technique was used in all scaffold experiments so non-uniformity in cell distribution could in and of itself not account for the significant down-regulation of SOST in three-dimensional fluid flow (Fig. 8) compared with the increase in SOST (Fig. 7) we observed in the simulated microgravity experiments. Finally, additional variables, such as nutrient availability, could also be acting as confounding factors to our observed results. However, the simulated microgravity experiments utilized a 110-ml bioreactor. Daily changes of 10% volume of medium were also performed to facilitate elimination of bubbles. Thus, for the cell density and number, these culture conditions for both static and microgravity conditions are nutrient-rich. Our interpretation notwithstanding, we acknowledge that such confounding variables specific to osteocytic cell cultures in simulated microgravity will need to be addressed in future experiments under conditions of true microgravity.

To enable these studies, we generated a novel osteocytic cell line Ocy454 that recapitulates known in vivo osteocytic functions without the requirement for long term high density cultures and in the absence of differentiation medium. These cells were isolated from long bones of double transgenic mice expressing both a GFP under the DMP1 promoter and a temperature-sensitive large T antigen. These cells can be cultured for a long period of time at permissive conditions (33 °C) without losing their phenotypic characteristics and then can rapidly recapitulate a mature osteocytic phenotype after 10–12 days in culture at semipermissive conditions (37 °C). As expected for an osteocyte, these cells express high levels of SOST/sclerostin, DMP1, Phex, and E11, whereas they have undetectable levels of the osteoblastic marker keratocan at all time points. Thus, in contrast to currently available osteocytic cell lines (MLO-Y4 and IDG-SW3 for example), the uniqueness of these cells is their expression of mature osteocytic genes in the absence of differentiation factors at early time points, suggesting that these cells display a mature osteocytic phenotype in a shorter experimental time frame. In addition, Ocy454 responded to short term mechanical overloading, achieved via both traditional two-dimensional laminar shear stress and three-di-
mensional fluid shear stress, by reducing SOST as reported previously (39).

Interestingly, RANKL expression is higher at permissive conditions (more undifferentiated state), and this expression rapidly declines upon differentiation (2–3 days at 37 °C), suggesting that high RANKL-expressing cells might belong to a less mature "osteocytic" phenotype. Similar findings were evident for FGF23 as well. In addition, consistent with prior reports that osteocytes have improved characteristics in three-dimensional culture conditions (63, 64), our osteocytic cell line exhibited increased FGF23 upon PTH treatment in three-dimensional culture conditions but not two-dimensional culture conditions. It is well appreciated that for a wide variety of cell types three-dimensional cell cultures mimic to a greater degree the in vivo conditions by preserving the three-dimensional integrity of individual cells, allowing for cell aggregation and direct signaling and enabling cells to create their own niche microenvironment in conjunction with their extracellular matrix (65). Thus, our studies add to the growing body of evidence for the use of three-dimensional in vitro culture conditions to study certain aspects of osteocyte biology. For the experiments we have conducted, our cell line faithfully reflects key characteristics of bona fide in vivo osteocytes.

In conclusion, isolated osteocytes can directly sense a mechanical unloading stimulus, resulting in the increases in expression of both inhibitors of bone formation (SOST/sclerostin) and stimulators of bone resorption (notably RANKL and the RANKL/OPG ratio). Future therapies, aimed at modulating the gravity-sensing pathways of the osteocyte could lead to improved therapies for a range of bone disorders.
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