Mechanical Stimulation of Bone in Vivo Reduces Osteocyte Expression of Sost/Sclerostin*

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Sclerostin, the protein product of the Sost gene, is a potent inhibitor of bone formation. Among bone cells, sclerostin is found nearly exclusively in the osteocytes, the cell type that historically has been implicated in sensing and initiating mechanical signaling. The recent discovery of the antagonistic effects of sclerostin on Lrp5 receptor signaling, a crucial mediator of skeletal mechanotransduction, provides a potential mechanism for the osteocytes to control mechanotransduction, by adjusting their sclerostin (Wnt inhibitory) signal output to modulate Wnt signaling in the effector cell population. We investigated the mechanoregulation of Sost and sclerostin under enhanced (unlar loading) and reduced (hindlimb unloading) loading conditions. Sost transcripts and sclerostin protein levels were dramatically reduced by unlar loading. Portions of the unlar cortex receiving a greater strain stimulus were associated with a greater reduction in Sost staining intensity and sclerostin-positive osteocytes (revealed via in situ hybridization and immunohistochemistry, respectively) than were lower strain portions of the tissue. Hindlimb unloading yielded a significant increase in Sost expression in the tibia. Modulation of sclerostin levels appears to be a finely tuned mechanism by which osteocytes coordinate regional and local osteogenesis in response to increased mechanical stimulation, perhaps via releasing the local inhibition of Wnt/Lrp5 signaling.

Low bone mass and poor bone structure are two major risk factors for osteoporotic fracture (1, 2). A simple yet effective means to enhance bone mass and architecture is through mechanical stimulation of the resident bone cell population (3, 4). Mechanical loading (e.g. exercise) improves bone mass and strength by stimulating the addition of new bone onto surfaces experiencing high strains, whereas surfaces that experience small strains largely remain quiescent. This phenomenon occurs both across the skeleton (limb bones adapt to locomotive loading, whereas nonbearing bones (e.g. skull) do not) and within a loaded bone (tension/compression surfaces undergo bone formation, whereas surfaces straddling the neutral bending axis do not). The cellular mechanisms involved in directing new bone formation to the high strain regions of a loaded bone are unclear, but elucidation of these mechanisms would provide an attractive target for pharmaceutical intervention aimed at mimicking the adaptive response to loading (5).

Despite these gaps in our understanding, significant progress has been made in delineating some of the basic mechanisms of mechanotransduction in bone, in large part because of the creation of genetically engineered mice. A key finding in this arena is the requirement for Wnt signaling through Lrp5 (the low density lipoprotein receptor-related protein 5) in mechanically induced bone formation. We reported recently that mice engineered with a loss-of-function mutation in Lrp5 recapitulate the low bone mass phenotype observed in humans with inactivating mutations of LRPS but, perhaps more importantly, were nearly completely unable to respond anabolically to mechanical stimulation (6). These data suggest that Wnt/Lrp5 signaling is an integral part of the mechanotransduction cascade in normal bone tissue (7), and from these observations an obvious question emerges: how does mechanical stimulation enhance Lrp5 signaling? Potential explanations include enhanced agonism of Lrp5 signaling (e.g. enhanced Wnt secretion), reduced antagonism of Lrp5 signaling (e.g. reduced dickkopf homolog 1 (Dkk1) levels, reduced secreted frizzled-related protein (sFrp) levels), or both.

Sclerostin, the protein product of the SOST gene, is an osteocyte-specific cysteine knot-secreted glycoprotein that is a potent inhibitor of bone formation. Mutations in the SOST gene, or in its distant regulatory elements, cause sclerostosis and Van Buchem disease (8–10). Patients with SOST mutations exhibit very high bone density lipoprotein receptor-related protein 5 (Dkk1) levels, reduced secreted frizzled-related protein (sFrp) levels), or both.

Sclerostin, the protein product of the SOST gene, is an osteocyte-specific cysteine knot-secreted glycoprotein that is a potent inhibitor of bone formation. Among bone cells, sclerostin is found nearly exclusively in the osteocytes, the cell type that historically has been implicated in sensing and initiating mechanical signaling. The recent discovery of the antagonistic effects of sclerostin on Lrp5 receptor signaling, a crucial mediator of skeletal mechanotransduction, provides a potential mechanism for the osteocytes to control mechanotransduction, by adjusting their sclerostin (Wnt inhibitory) signal output to modulate Wnt signaling in the effector cell population. We investigated the mechanoregulation of Sost and sclerostin under enhanced (unlar loading) and reduced (hindlimb unloading) loading conditions. Sost transcripts and sclerostin protein levels were dramatically reduced by unlar loading. Portions of the unlar cortex receiving a greater strain stimulus were associated with a greater reduction in Sost staining intensity and sclerostin-positive osteocytes (revealed via in situ hybridization and immunohistochemistry, respectively) than were lower strain portions of the tissue. Hindlimb unloading yielded a significant increase in Sost expression in the tibia. Modulation of sclerostin levels appears to be a finely tuned mechanism by which osteocytes coordinate regional and local osteogenesis in response to increased mechanical stimulation, perhaps via releasing the local inhibition of Wnt/Lrp5 signaling.
recently it has been shown to bind Lrp5/6 with high affinity (13–15). Consequently, sclerostin presents an attractive candidate for regulating mechanically induced signaling through the Lrp5 receptor. Furthermore, sclerostin expression in adult bone is limited to osteocytes (16, 17), which have long been postulated as the “mechanosensor” in bone (18, 19). The osteocyte population density, distribution, and extensive communication networks within bone make these cells ideal mechanosensors in the adaptive process of bone. Despite these attributes, very little data have been generated that implicate the osteocyte network as the primary mechanosensory cell type, to the exclusion of the other cell types (e.g. osteoblasts, bone lining cells). The discovery of a mechanically modulated osteocyte-specific factor, particularly a secreted factor that had the propensity to reach effector cell populations (osteoblasts), with known effects on a crucial mechanotransduction signaling pathway (Wnt/Lrp5), would provide a long sought-after molecular basis for osteocytic reception, initiation, and spatial control of mechanotransduction.

We investigated the regulation of Sost/sclerostin by the mechanical environment using two different rodent models of mechanotransduction: one for enhanced loading (axial ulnar loading) and one for reduced loading (hindlimb unloading). Ulnar loading in rodents induces a consistent pattern of bone deformation (i.e. strain) both along the bone axis and cross-sectionally at the midshaft. We hypothesized that enhanced loading would reduce Sost transcripts and sclerostin protein levels, particularly in the high strain regions of the tissue where enhanced bone formation is maximal. We further hypothesized that hindlimb unloading would increase Sost/sclerostin levels in the unloaded limbs. Our results indicate that sclerostin protein levels were reduced dramatically by ulnar loading. Portions of the ulnar cortex receiving a greater strain stimulus were associated with a greater reduction in the proportion of sclerostin-positive osteocytes. The load-induced reduction in sclerostin levels could be partially explained by a reduction in Sost transcript levels. Conversely, hindlimb unloading, a model of disuse mechanotransduction, yielded an increase in Sost transcript levels in the tibia. Modulation of sclerostin levels appears to be a finely tuned mechanism by which osteocytes coordinate regional and local osteogenesis in response to increased mechanical stimulation, perhaps via releasing the local inhibition of Wnt/Lrp5 signaling.

**EXPERIMENTAL PROCEDURES**

**Animals**—18-week-old C57Bl/6J male mice and 6-month-old virgin female Lewis rats were used for the ulnar loading studies. The unloading studies were performed in 6-week-old male C57Bl/6J mice. The mice were purchased from Jackson Labs, and the rats were purchased from Harlan, Inc. The animals were housed at the Indiana University Animal Care Facility until the proper age for each experiment was reached. Standard rodent chow and water were provided ad libitum. All of the procedures performed in the experiments were in accordance with the Institutional Animal Care and Use Committee guidelines.

**In Vivo Ulnar Loading**—Under isoflurane-induced anesthesia, the right forearms of mice and rats were loaded for 360 cycles/day (2 Hz). The mice used for immunolocalization of sclerostin were loaded for 2 consecutive days and then sacrificed on day 3. The rats and mice used for RNA analysis (in situ hybridization and quantitative PCR) were loaded for a single session (1 day) and sacrificed 24 h later. Loading was conducted on a customized electromagnetic actuator at peak force of 2.7 newtons for mice and 17 newtons for rats, which generates ~2200 μe at the midshaft ulna (Fig. 1A). Strain magnitudes were determined previously using miniature strain gauges bonded to the midshaft ulnar surface (20, 21). The left forearms were not loaded and served as an internal control for loading effects. All of the mice were allowed normal cage activity between loading bouts. Intraperitoneal injections of calcine and alizarin complexone (22 mg/kg body mass; Sigma) were administered to some of the mice 4 and 10 days after the first load day to monitor regional bone formation rates in the ulnae. To collect adequate amounts of ulnar diaphyseal tissue for gene expression analysis, nine adult female virgin Lewis rats were subjected to a single bout of ulnar loading at 17-newton peak force (~2200 μe; 2 Hz; 360 cycles) using a similar device as described for mice. The rat ulnas were harvested 24 h after loading, snap frozen in liquid N₂, and processed for RNA analysis as described below.

**In Vivo Tail Suspension**—To achieve a disuse environment, 6-week-old male mice were outfitted with tail harnesses and suspended from an overhead pulley system in customized cages. The mice can ambulate within the cage using their forelimbs, which remain in contact with the cage floor, but their hindlimbs remain suspended in air and consequently cannot generate ground reaction forces (Fig. 1B). Food and water were provided on the cage floor. The mice were suspended for 72 h, after which they were sacrificed, and their hindlimbs were pro-
Sost/Sclerostin in Bone Mechanotransduction

FIGURE 2. Histological sections from the ulnar midshaft of a mouse that had undergone two brief (1 min long) ulnar loading sessions. The sections in the left panels are stained with hematoxylin and eosin to demonstrate that the osteocytes within the cortex (purple dots) are present and living at the time of sacrifice. The middle panels show sections from the same bones, roughly 30 μm distal to those shown in the left panel, immunolabeled for sclerostin, which can be visualized by the brown staining of the osteocyte cell bodies and cell processes within the calcaneoi. Note the lack of sclerostin staining in all tissues shown (muscle, marrow, vessels, interosseus ligament, and periosteum) except for the osteocytes embedded in the cortex. Loading reduced immunoreactivity in the osteocyte cell bodies and calcaneoi, particularly in the compressive (medial) cortex (shown in the close-up view). To facilitate comparison in sclerostin staining between loaded and nonloaded bones, the right and left ulnae from each animal were embedded side-by-side in the same block, so that identical section thickness and immunolabeling conditions were achieved.

cessed for immunohistochemistry or RNA isolation (see below).

Immunohistochemistry—Minimally dissected mouse ulnae with attached radii were fixed in 4% phosphate-buffered formalin for 48 h at 4°C and then decalcified in a 2:3:1 mixture of 10% EDTA and 4% phosphate-buffered formalin for 10 days. Following decalcification, the right and left ulna/radius from each animal were bundled together with suture so that the right and left ulnae were properly aligned. The ulna bundles were infiltrated and then embedded in paraffin, with six to eight right-left ulna bundles to a block. Sections were taken from the blocks at the ulnar midshaft, 3 mm distal to midshaft, and 3 mm proximal to midshaft, at 8 μm thickness. Tibiae from the tail-suspended and ground control mice were sectioned at the tibiofibular junction. The sections were dried, deparaffinized, reacted for endogenous peroxidase activity, blocked, and incubated in anti-sclerostin primary antibody (R & D Systems). Secondary antibody labeling and detection were accomplished using the Vectastain Elite ABC kit (Vector Labs, Inc.) with diaminobenzidine as the chromogen. The immunolabeled sections were counterstained with methyl green or left without counterstain and then coverslipped.

The immunolabeled sections were photographed using 20× objective and imported into Image-Pro (MediaCybernetics, Inc.) analysis software for quantification. The number of sclerostin positive (Sclr+) osteocytes, defined as those osteocyte cell bodies exhibiting brown staining (Fig. 2), and the number of sclerostin-negative (Sclr−) osteocytes, defined as those osteocyte cell bodies exhibiting (methyl) green staining, were counted on each section. The percentage of sclerostin-positive cells (Sclr+) was calculated as the number of Sclr+ cells divided by the total number of cells (Sclr+ plus Sclr−). The load-induced change in the ulna samples was calculated for each animal as the percentage of Sclr+ cells in the right (loaded) sections minus the percentage of Sclr+ cells in the left (nonloaded control) section. Changes in sclerostin levels along the bone shaft were measured at the three section locations specified above (−3 mm, midshaft, and +3 mm) by quantifying and pooling all of the osteocytes within a section, regardless of their relation to the bending axis. For regional analysis within the ulnar midshaft cortex, each section was divided into three regions, corresponding to high, moderate, and low peak strain values during ulnar loading. To this end, two parallel lines were superimposed on each section, parallel to the neutral bending axis, and the cell counts were tallied separately within each region. The two lines were positioned at roughly ±1200 με, which was located by quantifying the second moment of area and section modulus about the IMIN plane of each section, calculating the peak strain at the outermost fiber using the previously derived relation between strain/force and section modulus (20), and deriving the distance from the neutral axis when strain is equal to 1200 με. Because the ulnar loading model is characterized by bending with an additional axial compression component, the neutral axis is shifted slightly toward the tensile cortex, which has the effect of creating a larger compressive region, characterized by higher mean strains.

Histomorphometry—From the mice that received calcein and alizarin to monitor load induced bone formation, ulnae were cleaned of soft tissue, fixed in 10% neutral buffered formalin for 48 h, dehydrated in graded alcohols, cleared in xylene, and embedded in methyl methacrylate (Aldrich). Using a diamond-embedded wire saw (Histo-saw; Delaware Diamond Knives, Wilmington, DE), transverse thick sections (~70 μm) were removed from the ulnar midshaft. The wafers were ground to a final thickness of ~20 μm and were mounted unstained on standard microscope slides.

The sections were photographed on a Nikon Optiphot fluorescence microscope (Nikon, Inc., Garden City, NY) and imported into Image-Pro (MediaCybernetics, Inc.) analysis software for quantification. The following primary data were collected from the periosteal surface: total perimeter, single label perimeter, double label perimeter measured along the first label, and double label area. From these primary data, we calculated bone formation rates per unit of bone surface (BFR/BS) using standardized protocols (22). To quantify load-induced changes in bone formation rates along the bone length (which is associated with a gradient in peak strains), histomorphometric measurements were collected at three section locations specified above (~3 mm, midshaft, and +3 mm). Three of these locations correspond to low, medium, and high strains during ulnar loading (21). The fluorochrome-labeled midshaft ulnar sections were also subjected to cross-sectional regional analysis (three regions: high, moderate, and low strain) of BFR/BS, as described for the immunolabeled midshaft sections. Load-induced bone formation was calculated for each animal/section/region by subtracting left ulna (nonloaded control) values from right ulna values; this procedure results in a relative (r) bone formation rate (rBFR/BS).

In Situ Hybridization—Sost antisense and sense RNA probes used for hybridization were prepared from an EcoRI- and NotI-linearized T7T3Pac DMP1 subclone and transcribed in vitro in...
the presence of digoxigenin-U-NTP mixture with T3 and T7 polymerase, respectively.

The in situ hybridization was performed using a modification of the procedure described by Wilkinson and Nieto (23). Prior to hybridization, the sections were deparaffinized with xylene and 100% ethanol following rehydration. After treatment with proteinase K solution (protease K, 5 mg/ml in 50 mM Tris, 5 mM EDTA, pH 7.6) for 10 min at 37 °C, the sections were refixed in 4% formaldehyde in phosphate-buffered saline (0.2 M phosphate buffer, 3 mM NaCl), acetylated (100 mM triethanolamine, 0.25% acetic anhydride), and prehybridized in 2× SSC. Hybridization was performed at 55 °C overnight in the hybridization solution (50% formamide, 20 mM Tris-HCl, pH 8.0, 1 mM EDTA, 0.3 M NaCl, 10% dextran sulfate, 1× Denhardt’s solution, 100 μg/ml denatured Salmon Sperm-DNA, 500 μg/ml tRNA) and 1 μg/ml DMP1 UTP-digoxigenin labeled RNA probe. After hybridization, the coverslips were removed in 5× SSC at room temperature, and sections were washed once in 5× SSC and twice in washing solution I (50% formamide, 5× SSC, 1%SDS) at 55 °C for 30 min each. The sections were incubated with RNase (40 mg/ml RNase A1 and 10 units/ml RNase T1) in washing solution II (0.3 M NaCl, 10 mM Tris, 5 mM EDTA, pH 9.0, 150 mM NaCl, 0.1% Tween 20, 2 mM levamisole) and one time at room temperature in TBS-T (100 mM Tris, pH 7.5, 150 mM NaCl, 0.1% Tween 20, 2 mM levamisole) for 1 h, followed by incubation in the washing solution II without RNase for 30 min. Consecutive washes at 55 °C were done twice with washing solution III (50% formamide, 2× SSC) for 30 min each and then three times 5 min each at room temperature in TBS-T (100 mM Tris, pH 7.5, 150 mM NaCl, 0.1% Tween 20, 2 mM levamisole) and one time at room temperature in blocking mix (100 mM maleic acid, 150 mM NaCl, 2 M EDTA, pH 7.6) for 10 min at 37 °C, the sections were refixed overnight at 4 °C. Next day, the sections were washed using TBS-T for 5 min, wash buffer (100 mM maleic acid, 150 mM NaCl, 0.1% Tween 20, 2 mM levamisole) overnight at 37 °C. When detection of hybridization signal was completed, the sections were washed in water three times (5 min/wash), counterstained with eosin for 5 s, and dehydrated in ethanol. Finally, the slides were in xylene and mounted with coverslips using a nonaqueous permount reagent.

The dark purple hybridization signal in the osteocytes was quantitated for intensity using Image J. Average expression was determined at 3 mm proximal to the midshaft, at the midshaft, and at 3 mm distal to the midshaft, by analyzing 50–80 osteocytes/section. In the midshaft sections, Sost staining intensity was quantitated in the three strain regions (high, medium, and low) as described for the immunolabeled sections.

RNA Extraction and Quantitative PCR—Snap frozen rat ulnar diaphyses (central 75% of the bone) and whole mouse tibiae (from tail-suspended mice) were pulverized in liquid N2 using a mortar and pestle. The bone powder was suspended in TRIzol (Invitrogen) and centrifuged for 5 min at 4 °C to pellet the bone grit. From the supernatant, total RNA was isolated and resuspended in H2O according to the manufacturer’s instructions. 1 μg of total RNA from each sample was reverse transcribed using the Superscript II kit with oligo(dT) primers (Invitrogen). From the resulting cDNA, portions of the Sost, Dkk1, sFrp, and β-actin sequences were amplified using Taqman Assay-on-Demand gene expression assay kits (Applied Biosystems, Inc.). Serial dilutions of one of the samples were amplified for each gene to calculate relative expression levels, which were then standardized to β-actin expression to facilitate comparison among samples. The reactions were performed on an ABI Prism 7000 sequence detection system.

Statistical Methods—The data were analyzed using nonparametric statistics to avoid assumptions of normality. Trends across strain magnitudes, both within and along the diaphysis, were analyzed for association using Spearman’s r. Within group (loaded versus nonloaded) comparisons were executed using the Wilcoxon signed rank test. For all tests, the level of significance was set at α = 0.05.

RESULTS

Mechanical Loading Reduces Sclerostin, Particularly in High Strain Regions of the Bone—To investigate the cellular mechanisms involved in load-enhanced Wnt signaling through Lrp5, we probed tissue sections from loaded and control mouse ulnae for sclerostin (Fig. 2). The mice underwent brief ulnar loading sessions for 2 consecutive days and then were sacrificed on day 3. Quantification of the number of Sclr+ osteocytes in the ulnar cortex revealed a dramatic load-induced decrease in the number of Sclr+ osteocyte cell bodies but also in the degree of sclerostin staining within the canicular network (Fig. 2). Sections from the proximal portion of the ulnar diaphysis, which experiences relatively small peak strains during ulnar loading, exhibited a modest (~15%) reduction in Sclr+ cells, whereas sections from the distal diaphysis, which experiences relatively large peak strains during loading, showed a ~60% reduction in Sclr+ cells (Fig. 3B). Sections from the midshaft, which experiences intermediate strains during loading, yielded an intermediate reduction in Sclr+ cells (~30% decrease). The strain dose-responsive reduction in Sclr+ cells along the diaphysis corresponded to the strain dose-responsive increase in bone formation rates after loading (Fig. 3A; p = 0.002). In these experiments, the mice received ulna loading, were given fluorochrome labels 4 and 10 days afterward, and then euthanized on day 17. Quantification of bone formation rates in the loaded and control ulnae revealed small gains in load-induced bone formation proximally, moderate gains at midshaft, and large gains distally. Thus, the diaphyseal locations that underwent large increases in peak strains as a result of mechanical loading were associated with a large reduction in Sclr+ cells and a large increase in bone formation rates.

We further probed the effect of mechanical strain on Sclr expression and bone formation by measuring the load-induced changes in Sclr+ cells and bone formation rates across the mid-
Mechanical Loading Reduces Sost Expression, Particularly in High Strain Regions of the Bone—The observed load-induced reduction in sclerostin protein levels prompted us to explore whether the changes were transcriptionally regulated and whether other Wnt inhibitory transcripts were affected by loading. We measured mRNA levels of Sost, as well as two more Wnt inhibitors, Dkk1 and sFrp1, in loaded and control rat ulnar diaphyses 24 h after loading, using real-time PCR. Mechanical loading reduced Sost mRNA levels by 73% (Fig. 5A). Dkk1 transcripts were also reduced significantly by loading, albeit to a lesser degree than Sost, reaching a 49% decrease induced by loading (Fig. 5B). sFrp1 expression was unchanged by loading (Fig. 5C). We further characterized the load-induced reduction in Sost expression by probing mouse ulnar tissue sections for Sost, using in situ hybridization, 24 h after a loading session. We quantified the level of Sost hybridization signal in 50–80 independent osteocytes at three diaphyseal locations that differed in the peak strain magnitudes endured during loading. As shown in Fig. 3C, loading induced a 24% reduction in sclerostin expression in the proximal location \( (p < 0.001) \) and a 37–51% reduction in the midshaft and distal regions \( (p < 0.001) \). We also analyzed Sost expression/osteocyte in more detail in the midshaft region. There was a greater reduction in Sost expression/osteocyte in the medial regions (high strain) than in the neutral axis (low strain) region or the lateral region (moderate strain) (Fig. 4, right panels). These data suggest that the mechanical loading response of the Sost gene is in part at the transcriptional level and correlates to mechanical strain magnitude. The gene expression pattern of Sost at midshaft is very similar to the sclerostin pattern revealed by immunohistochemistry.

Reduction in Mechanical Loading (Disuse) Has No Effect on Sclerostin Levels—Our observation that enhanced loading results in a loss of sclerostin protein led us to determine whether the opposite is true, i.e. does reduced loading result in increased sclerostin levels? We suspended growing male mice by their tails for 3 or 7 days and then processed their tibiae for immunodetection of Sclr as described for loaded ulnar sections or for RNA extraction and subsequent quantitative PCR for Sost, Dkk1, and sFrp1 transcripts. We were unable to detect a difference histologically between the percentage of Sclr+ osteocytes in the immunolabeled tibial sections from hindlimb suspended mice versus ground control mice at either time point (Fig. 6). We did, however, detect a significant increase in Sost transcript levels at day 3 of tail suspension, which had subsided to a nonsignificant level by day 7 (Fig. 6). Dkk1 transcript levels increased slightly at day 3 of tail suspension, but significance could not be reached \( (p = 0.08) \). sFrp1 transcript levels increased slightly at day 7 of tail suspension, but significance could not be reached \( (p = 0.09) \).

**DISCUSSION**

Our main objective in this study was to determine whether the Wnt inhibitory protein sclerostin was regulated by mechani-

- shaft ulnar cortex. The ulnar axial loading model induces mediolateral bending at midshaft, with a superimposed axial compressive element that results in greater compressive than tensile strains. We took advantage of the unequal distribution of strains by dividing the cortex into a high strain region (compressive, medial portion), a moderate strain region (lateral, tensile portion), and a low strain region (central portion, straddling the neutral bending axis). Quantification of the number of Sclr+ osteocytes in those three cross-sectional regions also revealed a strain dose-responsive reduction \( (p = 0.027) \) in Sclerostin Levels.
ical stimulation. Our results indicate that sclerostin levels are tightly regulated by mechanical strain, both cross-sectionally and longitudinally, in mechanically stimulated long bones. Wnt signaling is a crucial step in the mechanotransduction cascade, as indicated by the observation that the Lrp5-deficient mouse skeleton is unable to respond anabolically to mechanical stimulation (6). Those experiments suggest that in normal bone tissue, the anabolic effects of mechanical stimulation are associated with enhanced Wnt signaling. Our results in the current experiments, implicating sclerostin as a mechanically suppressed signal, in conjunction with its known inhibitory effects on Lrp function and bone formation, provide an attractive regulatory mechanism that permits enhanced Wnt signaling upon mechanical stimulation. Furthermore, the osteocyte-specific expression profile of sclerostin provides a long sought-after molecular mechanism by which osteocytes per se can relay mechanical information biochemically to the effector cell populations (e.g., osteoblasts).

The effects of sclerostin on mechanically induced bone formation are likely to act through the Wnt signaling pathway; direct binding of sclerostin protein to Lrp5 and Lrp6 has been demonstrated repeatedly (13–15, 24). It is possible, however, that the effects of sclerostin are targeted toward inhibition of Bmp signaling. Sclerostin has clear inhibitory
effects on Bmp-induced bone formation and alkaline phosphatase activity, but it remains controversial as to whether those effects are direct (e.g. blockade of Smad phosphorylation) or mediated entirely through the Wnt pathway (25, 26). Furthermore, it is unclear what role Bmps play in mechanically induced bone formation. In vitro, mechanically stimulated osteoblasts up-regulate Bmps 2, 4, 6, 7, and 8b (27, 28), and in vivo load-induced bone formation is enhanced by Bmp-7 (29), but functional studies demonstrating a requirement for Bmp signaling in bone mechanotransduction are lacking. Conversely, both gain-of-function and loss-of-function mutations in Lrp5 have been shown to directly impact mechanotransduction efficiency (6, 30), indicating that the Wnt pathway is a major cascade to be modulated for proper spatial control of load-induced bone formation.

We detected a significant reduction in Sost mRNA levels 24 h after loading, indicating that the observed reduction in sclerostin staining was at least partially fueled by transcriptional regulation. It should be noted, however, that loading also induces the expression of many proteolysis genes (31), so it remains to be determined whether the loss of protein levels resulting from loading also involves enhanced proteosomal degradation. In addition to the down-regulation of Sost, we found that loading also decreased expression of Dkk1, another Wnt signaling inhibitor with direct binding affinity to Lrp5/6. The mechanism of Dkk inhibition of Wnt signaling is different from that for sclerostin (e.g. Kremen binding and endocytosis), but like sclerostin, Dkk1 is a potent inhibitor of bone formation as revealed by the osteopenic Dkk1 overexresser mouse (32) and the high bone mass Dkk1 haploinsufficient mouse (33). Thus, down-regulation of Dkk1 protein after loading could also explain the enhanced Wnt signaling that occurs during mechanotransduction. Relative expression levels of Sost and Dkk1, however, indicate that Sost levels are roughly 10-fold greater than Dkk1. These observations were also borne out at the protein level; we were barely able to detect Dkk1 protein using immunohistochemical techniques in the same sections used for Sost immunolocalization (not shown).

Thus, Sost/sclerostin regulation might represent a much more plentiful, and consequently potent, mechanism for controlling load-induced bone formation. We saw no change in the expression of sFrp1, a Wnt-binding protein that has significant effects on bone accrual (34), indicating that mechanically up-regulated Wnt signaling is enacted via reduced receptor antagonism rather than reduced ligand inhibition.

The immunolabeled sections and the hybridized sections revealed similar strain-driven response in terms of sclerostin and Sost down-regulation, respectively, in the midshaft regional analysis (Fig. 4, center and right columns). However, small discrepancies arose between the results yielded by the RNA and protein analyses along the length of the diaphysis (Fig. 3, B and C). We found slightly but significantly greater Sost reduction at midshaft than at the distal section. The basis for the difference is unclear, but it might be related to the sensitivity of the two different quantification methods employed. The in situ hybridization signal is more amenable to quantification via signal intensity per osteocyte, whereas the immunolabeled sections are more reproducibly quantified using a categorical scoring system of positive or negative
for each cell. This issue might be better addressed in cell culture, once suitable Sost- and sclerostin-expressing cell lines become available.

The Sost expression changes yielded by the ulnar loading studies were complemented by the Sost expression changes yielded by the tail suspension studies, which showed that Sost is up-regulated in a reduced loading environment. These results were recently confirmed by another group that found a similar increase in Sost expression following tail suspension (35). Although we were able to detect a significant increase in Sost transcripts at 3 days of tail suspension, we were unable to detect a difference in the number of sclerostin-positive osteocytes in immunolabeled tissue sections from the same site. Unlike the ulna loading model, which imposes large changes in strain on specific regions of the cortical cross-section, the tail suspension model places the entire tibia in a relatively uniformly reduced loading environment. Thus, there might not be a concentrated region of cells that are affected enough to detect a change at the protein level using immunohistochemistry, as there is in the medial cortex of the loaded ulna. Consequently, the possibility of enhanced tissue levels of sclerostin resulting from disuse is possible but likely would require a detection technique more sensitive than immunohistochemistry.

Tail suspension is a frequently used model for inhibiting bone formation in the rodent caudal skeleton, ultimately producing a low bone mass phenotype. Unloading induces osteocyte and osteoblast apoptosis (36, 37), reduces proliferation (38, 39), and inhibits osteoblast differentiation (40, 41). In light of the observation that at least two of those effects, reduced proliferation and enhanced apoptosis, are induced by sclerostin (42), our data suggest a mechanism for reduced bone formation during weightlessness and/or disuse. Interestingly, Lrp5 HBM G171V transgenic mice do not lose bone in disuse caused by sciatic neurectomy, as was seen in wild-type mice (43), suggesting that disuse-induced bone loss involves modulation of the Wnt signaling pathway.

Modulation of sclerostin, although clearly influenced by mechanical loading, has been reported for other stimuli, including parathyroid hormone and long bone fracture. A single injection of parathyroid hormone results in decreased Sost expression within 2–4 h (44, 45), and continuous infusion of parathyroid hormone results in sustained down-regulation of Sost over several days (44). Healing fracture callus exhibits a time-dependent increase in Sost expression over 28 days, but the same analysis in the fibrous tissue of nonunited fracture sites showed no change in Sost transcript levels (46). Thus, Sost expression has multiple regulatory inputs, of which mechanical load is only one among several.

The effects of sclerostin on bone formation and skeletal phenotype are most readily appreciated in sclerosteosis and van Buchem disease patients, in whom SOST expression is absent or significantly reduced. Sclerosteosis patients exhibit hip and spine bone mineral densities ranging from 8 to 10 standard deviations above age-matched normals, whereas heterozygous carriers exhibit elevated but more normal BMD z-scores, ranging from 0.5 to 5 (11). Although SOST transcripts have been detected in other tissues, including kidney and bone marrow (8), sclerosteosis patients rarely present with clinical symptoms other than those derived from bone overgrowth (47). Those observations suggest that although sclerostin might be found in other postnatal tissues, its role in nonosseous tissues is of minor importance. The predilection for bone overgrowth in the cranium is associated with severe complications, including increased intracranial pressure, and loss of smell, taste, hearing, and vision, arising from cranial nerve foramina stenosis and accompanying nerve impingement (48). The mouse skeleton exhibits nearly 2-fold greater expression of Sost in the skull than in the long bones (femur) (45), which might explain the more severe phenotype in the cranium of sclerosteosis patients. Furthermore, mice harboring loss-of-function mutations in Sost exhibit very high bone mass (49, 50), whereas mice engineered to overexpress Sost are osteopenic (26, 51). Thus, the regulation of Sost and its effects on bone mass are amenable to investigation in mouse models.

It is unclear from the experiments presented here how sclerostin might physically exert its action in mechanotransduction, i.e. whether it acts as an autocrine factor on the osteocytes that released it or whether it makes its way to the osteoblast populations on the bone surfaces as a paracrine factor. Osteoblasts form the effecter cell population that is responsible for bone formation, but we and others (16) have observed that immunolocalization of sclerostin in the osteocyte network is usually confined to the interior, more mature osteocytes, and that a ~10–30-μm band of sclerostin-negative osteocytes is typically found at the bone edges. Sclerostin clearly crosses this border, because one of its main targets, Lrp5, has been most prominently localized to the osteoblasts and bone lining cells on the bone surfaces (52, 53). Thus, it seems likely that sclerostin is normally produced and secreted abundantly by the osteocyte network, keeping local osteoblasts under control from rampant bone formation. We hypothesize that when significant local strains are sensed by this cell population, sclerostin levels are decreased, and the local osteoblast cell populations are released from their suppressed Wnt signaling state, ultimately resulting in new local bone formation in the high strain regions of the bone. Enhanced secretion of Wnt ligand might also comprise a portion of this control mechanism, but that issue has yet to be addressed. Several experiments have demonstrated enhanced Wnt signaling in bone cells as a result of mechanical stimulation. For example, stretched osteoblasts harvested from transgenic mice expressing a T-cell factor/β-catenin transcription reporter (TopGal mice) exhibited activation of canonical Wnt signaling (β-galactosidase expression) after stretching (54). In addition, fluid shear stress stimulates the translocation of β-catenin to the nucleus in MC3T3-E1 cells, UMR cells, and in primary rat calvarial osteoblasts (55). Although they highlight the role of Wnt signaling, those studies do not differentiate between enhanced Wnt ligand secretion and reduced receptor antagonism. Another issue that remains unclear is the identity of the upstream factors that control sclerostin expression and whether sclerostin can be modulated by known classical early mediators of mechanotransduction.
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(e.g., ATP, Ca\(^{2+}\), PGE\(_2\), and NO) or whether sclerostin is controlled by other, novel mechanisms.

In summary, we found that Sost/sclerostin levels were reduced by mechanical stimulation in the rodent ulna, and the reduction was closely related to tissue strain distributions and subsequent bone formation. These data highlight the role of Wnt signaling in mechanically induced bone formation and provide the first evidence for osteocyte-specific control of mechanotransduction, involving a protein with clear effects on osteoblast-mediated bone formation. Furthermore, sclerostin presents an attractive target for modulating bone mass, because we have shown that in specific diaphyseal locations where sclerostin is lost, bone formation occurs. Pharmacologic modulation of sclerostin signaling emerges as an obvious potential therapy for improving bone structure.

REFERENCES

1. Ahlborg, H. G., Johnell, O., Turner, C. H., Ranneyik, G., and Karlsson, M. K. (2003) *N Engl J Med.* 349, 327–334
2. Lane, N. (2006) *Bull NYUL Hosp. Jt. Dis.* 64, 67–71
3. Turner, C. H., and Robling, A. G. (2003) *Exerc. Sport Sci. Rev.* 31, 45–50
4. Turner, C. H., and Robling, A. G. (2005) *J Bone Miner Metab.* 23, (suppl.) 16–22
5. Robling, A. G., Castillo, A. B., and Turner, C. H. (2006)
6. Poole, K. E., van Bezooijen, R. L., Loveridge, N., Hamersma, H., Papapoulos, S. E., ten Dijke, P., and Lowik, C. W. (2007) *J Bone Miner Res.* 22, 19–28
7. Wilkinson, D. G., and Nieto, M. A. (1993) *Methods Enzymol.* 225, 361–373
8. Ellis, D. L., Viviano, B., McCarthy, J., Rey, J. P., Itasaki, N., Saunders, S., and Krumlauf, R. (2006) *J Bone Miner Res.* 21, 1738–1749
9. van Bezooijen, R. L., Svensson, J. P., Eefting, D., Visser, A., van der Horst, G., Karperien, M., Quax, P. H., Vrielig, H., Papapoulos, S. E., ten Dijke, P., and Lowik, C. W. (2007) *J Bone Miner Res.* 22, 19–28
10. Winkler, D. G., Sutherland, M. K., Geoghegan, J. C., Yu, C., Hayes, T., Skonier, J. E., Shpekter, D., Jonas, M., Kovacevic, B. R., Staeling-Hampton, K., Appleby, M., Brunek, M. E., and Latham, J. A. (2003) *EMBO J.* 22, 6267–6276
11. Lau, K. H., Kapur, S., Kesavan, C., and Baylink, D. J. (2006) *J Biol. Chem.* 281, 9576–9588
12. Mitsui, N., Suzuki, N., Maeno, M., Yanagisawa, M., Koyama, Y., Otsuka, K., and Shimizu, N. (2006) *Life Sci.* 78, 2697–2706
13. Cheleine, A. J., Reddi, A. H., and Martin, R. B. (2002) *Bone* 31, 570–574
14. Cullen, D. M., Akhter, M. P., Johnson, M. L., Morgan, S., and Becker, R. R. (2004) *J Bone Miner Res.* 19, (Suppl. 1) 396
15. Xing, W., Baylink, D., Kesavan, C., Hu, Y., Kapoor, S., Chadwick, R. B., and Mohan, S. (2005) *J Cell Biochem.* 96, 1049–1060
16. Li, J., Sarosi, I., Cattley, R. C., Pretorius, J., Asuncion, F., Grisanti, M., Morony, S., Adamu, S., Geng, Z., Qiu, W., Kostenuik, P., Lacey, D. L., Simonet, W. S., Bolon, B., Qian, X., Shalhoub, V., Omins, M. S., Zhu, K., Li, X., and Richards, W. G. (2006) *Bone* 39, 754–766
17. Morvan, F., Bouloukos, K., Clement-Lacroix, P., Roman Roman, S., Sucroyer, I., Vayssiere, B., Ammann, P., Martin, P., Pinho, S., Pognonec, P., Mollat, P., Niehrs, C., Baron, R., and Rawadi, G. (2006) *J Bone Miner Res.* 21, 934–945
18. Bodine, P. V., Zhao, W., Kharode, Y. P., Bex, F. J., Lambert, A. J., Goad, M. B., Gaur, T., Stein, G. S., Lian, J. B., and Komm, B. S. (2004) *Mol Endocrinol.* 18, 1222–1237
19. Tatsumi, S., Ishii, K., Amizuka, N., Li, M., Kobayashi, T., Ito, M., Takeshita, S., and Ikeda, K. (2007) *Cell Metab.* 5, 446–475
20. Aguirre, I. I., Plotkin, L. I., Stewart, S. A., Weinstein, R. S., Parfitt, A. M., Manolagas, S. C., and Bellido, T. (2006) *J Bone Miner Res.* 21, 605–615
21. Dufour, C., Holy, X., and Marie, P. J. (2007) *Exp Cell Res.* 313, 394–403
22. Barou, O., Palle, S., Vico, L., Alexandre, C., and Lafage-Proust, M. H. (1998) *Am J Physiol.* 274, E108–E114
23. Machwate, M., Zerath, E., Holy, X., Hott, M., Modrowski, D., Malouvier, A., and Marie, P. J. (1993) *Am J Physiol.* 264, E790–E799
24. Grano, M., Mori, G., Minielli, V., Barou, O., Colucci, S., Giannelli, G., Alexandre, C., Zallone, A. Z., and Vico, L. (2002) *Calcif Tissue Int.* 70, 176–185
25. Zhang, R., Supowit, S. C., Klein, G. L., Lu, Z., Christensen, D. M., Lozano, R., and Siemens, D. J. (1995) *J Bone Miner Res.* 10, 415–423
26. Sutherland, M. K., Geoghegan, J. C., Yu, C., Turcott, E., Skonier, J. E., Winkler, D. G., and Latham, J. A. (2004) *Bone* 35, 828–835
27. Bex, F. J., Green, P., Marzolf, J., Papapoulos, S. E., and Kharode, Y. (2003) *J Bone Miner Res.* 18, (Suppl. 2) S60
28. Bellido, T., Ali, A. A., Gubri, L., Lu, F., O’Brien, C. A., Manolagas, S. C., and Ilka, R. L. (2005) *Endocrinology* 146, 4577–4583
29. Keller, H., and Knessel, M. (2005) *Bone* 37, 148–158
30. Niikura, T., Hori, M., and Reddi, A. H. (2006) *J Orthop Res.* 24, 1463–1471
31. Hofmeyr, L. M., and Hamersma, H. (2004) *Curr Opin Otolaryngol Head Neck Surg.* 12, 393–397
32. Hamersma, H., Gardner, J., and Beighton, P. (2003) *Clin. Endocrinol.* 69, 192–197
33. Brommage, R., Liu, J., Suwanichkul, A., and Powell, D. R. (2006) *36th International Sun Valley Workshop on Skeletal Tissue Biology, Sun Valley, ID, July 30–August 2, 2006*, pp. 8–9, National Institutes of Health, Bethesda, MD
52. Gong, Y., Slee, R. B., Fukai, N., Rawadi, G., Roman-Roman, S., Reginato, A. M., Wang, H., Cundy, T., Glorieux, F. H., Lev, D., Zacharin, M., Oexle, K., Marcelino, J., Suwaidi, W., Heeger, S., Sabatakos, G., Apte, S., Adkins, W. N., Allgrove, J., Arslan-Kirchner, M., Batch, J. A., Beighton, P., Black, G. C., Boles, R. G., Boon, L. M., Borrone, C., Brunner, H. G., Carle, G. F., Dallapiccola, B., De Paepe, A., Floege, B., Halfhide, M. L., Hall, B., Hennekam, R. C., Hirose, T., Jans, A., Juppner, H., Kim, C. A., Keppler-Noreuil, K., Kohlschuetter, A., LaCombe, D., Lambert, M., Lemyre, E., Letteboer, T., Peltonen, L., Ramesar, R. S., Romanengo, M., Somer, H., Steichen-Gersdorf, E., Steinmann, B., Sullivan, B., Superti-Furga, A., Swoboda, W., van den Boogaard, M. J., Van Hul, W., Vinkkula, M., Votruba, M., Zabel, B., Garcia, T., Baron, R., Olsen, B. R., and Warman, M. L. (2001) *Cell* 107, 513–523
53. Kato, M., Patel, M. S., Levasseur, R., Lobov, I., Chang, B. H., Glass, D. A., 2nd, Hartmann, C., Li, L., Hwang, T. H., Brayton, C. F., Lang, R. A., Karsenty, G., and Chan, L. (2002) *J. Cell Biol.* 157, 303–314
54. Hens, J. R., Wilson, K. M., Dann, P., Chen, X., Horowitz, M. C., and Wysolmerski, J. J. (2005) *J. Bone Miner Res.* 20, 1103–1113
55. Norvell, S. M., Alvarez, M., Bidwell, J. P., and Pavalko, F. M. (2004) *Calci. Tissue Int.* 75, 396–404