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Changes In Bone Sclerostin Levels In Mice After Ovariectomy Vary Independently Of Changes In Serum Sclerostin Levels

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Abstract

We examined the effects that ovariectomy had on sclerostin mRNA and protein levels in the bones of 8-week-old mice that were either sham-operated (SHAM) or ovariectomized (OVX) and then sacrificed 3 or 6 wks. later. In this model bone loss occurred between 3 and 5 wks. Post-surgery.

In calvaria OVX significantly decrease sclerostin mRNA levels at 6 wks. post-surgery (by 52%) but had no significant effect at 3 wks. In contrast, sclerostin mRNA levels were significantly lower in OVX femurs at 3 wks. post-surgery (by 53%) but equal to that of SHAM at 6 wks. The effects of OVX on sclerostin were not a global response of osteocytes since they were not mimicked by changes in the mRNA levels for 2 other relatively osteocyte-specific genes: DMP-1 and FGF-23. Sclerostin protein decreased by 83% and 60%, respectively at 3 and 6 wks. post-surgery in calvaria and by 38% in lumbar vertebrae at 6 wks. We also detected decreases in sclerostin by immunohistochemistry in cortical osteocytes of the humerus at 3 wks. post-surgery. However, there were no significant effects of OVX on sclerostin protein in femurs or on serum sclerostin at 3 and 6 wks. post-surgery.

These results demonstrate that OVX has variable effects on sclerostin mRNA and protein in mice, which are dependent on the bones examined and the time after surgery. Given the discrepancy between the effects of OVX on serum sclerostin levels and sclerostin mRNA and protein levels in various bones, these results argue that, at least in mice, serum sclerostin levels may not accurately reflect changes in the local production of sclerostin in bones. Additional studies are needed to evaluate whether this is also the case in humans.

Keywords

Sclerostin; Ovariectomy; Bone; Osteocyte; Serum

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Introduction

Sclerostin, a product of the *SOST* gene (1–3), is a negative regulator of bone formation. It primarily mediates its effects on bone through its ability to inhibit canonical Wnt- β -catenin signaling pathways (4, 5). Wnts are a large family of secreted proteins, which act as paracrine factors that mediate embryonic development as well as cell growth and differentiation (6). To initiate canonical signaling in osteoblast-lineage cells, Wnts bind to a membrane receptor complex containing a frizzled G-protein-coupled receptor and a low-density lipoprotein receptor-related protein (LRP) 5 or 6 (7).

Much has been learned about the role of Wnts in bone biology through studies of individuals who have mutations in critical proteins involved in canonical Wnt signaling. Individuals with autosomal dominant high-bone-mass trait or osteoporosis pseudoglioma syndrome, an autosomal recessive trait with low bone mass, have mutations in *LRP5* (8–10). The most common high bone mass mutation (*LRP5*-G171V) decreases the binding of *LRP5* to sclerostin (11) and another Wnt inhibitory protein, *Dickkopf1* (*DKK1*) (12). Hence, it appears that sclerostin and *DKK1* act as negative regulators by directly interfering with the binding of Wnts to their canonical signaling receptor complex.

Sclerostin-deficient mice have increased bone mass and bone strength (13), whereas overexpression of normal human *SOST* alleles in mice causes osteopenia (14). Similarly, other conditions associated with defective sclerostin production such as sclerosteosis and Van Buchem's disease also present with high bone mass (1, 15). Sclerosteosis is caused by at least 5 different inactivating mutations of the *SOST* gene (1, 2). In contrast, Van Buchem's disease is caused by a deletion of an enhancer element that is normally downstream of the *SOST* gene (15, 16).

The major site of sclerostin production is mature osteocytes in bone (17, 18); although, lesser amounts are found in cementocytes in teeth, mineralized hypertrophic chondrocytes in the growth plate and osteoarthritic cartilage (19, 20). Until recently, sclerostin was thought to act exclusively in a paracrine manner (21). However, the discovery by a number of investigators that sclerostin circulates and its levels in serum are regulated by age (22), gender (22), parathyroid hormone (23–29) and estrogen status (30–33), implies that it may also have endocrine functions. We and others demonstrated that serum sclerostin levels are increased in postmenopausal women compared to premenopausal women, are negatively correlated with serum estrogen status and are decreased by treatment with estrogens (30–33).

In order to better understand the effects that estrogens have on sclerostin production, we examined the effects that ovariectomy (OVX), which markedly reduces estrogen production, had on sclerostin mRNA and protein levels in the calvaria, lumbar vertebrae, humeri and femurs of mice. Changes in sclerostin production were also correlated with the effects that OVX had on bone turnover markers (serum CTX, a measure of bone resorption, and serum osteocalcin, a measure of bone formation) and serum sclerostin levels. Based on the human data, we anticipated that OVX would increase sclerostin production in bone as postmenopausal women have higher serum sclerostin levels than premenopausal women (30). We also anticipated that OVX would increase serum sclerostin levels in mice. However, we found that OVX either had no effect or decreased sclerostin production in bone and this response varied with the time after OVX and the bone that was examined. In addition, the effects of OVX on serum sclerostin levels in mice were minimal.

Materials and Methods

Experimental Animals

Mice in a C57BL/6 background were used for all experiments. Animals were purchased from a commercial vendor (Charles River, Wilmington, MA) and housed in the Center for Comparative Medicine at the University of Connecticut Health Center. The University of Connecticut Health Center's Animal Care Committee approved all animal protocols. At 8 wks. of age, mice were either sham (SHAM) operated or ovariectomized (OVX) and then sacrificed 3 to 7 wks. later. The success of ovariectomy in the mice was confirmed by measurement of at least a 75% reduction in uterine weight in the OVX groups relative to the SHAM groups at the time of sacrifice.

Micro-Computed Tomography

Conebeam X-ray μ CT (μ CT40; Scanco Medical AG, Bassersdorf, Switzerland) was used to quantify the trabecular morphometry within the metaphyses of the distal femur. Serial tomographic images were acquired at 55 kV and 145 μ A, collecting 1000 projections per rotation at 300-ms integration time. 3-dimensional images were reconstructed using standard convolution back-projection algorithms with Shepp and Logan filtering and rendered within a 12.3-mm field of view at a discrete density of 578,704 voxels/mm³ (isometric 12- μ m voxels). Threshold segmentation of bone from marrow and soft tissue was performed in conjunction with a constrained Gaussian filter to reduce noise. Volumetric regions for trabecular analysis were selected within the endosteal borders to include the central 80% of vertebral height and secondary spongiosa of the femur (1 mm from the growth plate and extending 1 mm proximally). Trabecular morphometry was characterized by measuring the bone volume fraction (BV/TV). The measurement, terminology and units that were used for micro-CT analysis of bones were those recommended by Bouxsein et al (34).

Enrichment of Bones for Osteocytes

In some experiments bones were enriched for cortical osteocytes (35) using enzyme digestion to remove their surface cells. In these experiments, one femur and two tibias were cleaned of adherent connective tissue, their ends cut off and their bone marrow flushed by injecting 1ml of MEM-alpha medium without serum through a 25 gauge needle into their shaft. Bones were then incubated with 5 ml of collagenase solution (0.2% collagenase P, Boehringer Mannheim, in 70mM NaCl, 10mM NaHCO₃, 30mM KCl, 3mM K₂PO₄, 1mM CaCl₂, 0.1%BSA, 0.5% glucose and 25mM HEPES) for 3 successive rounds of 20 min, 1 hour and 1 hour, respectively at 37° C and 400 RPM in an incubator shaker. Finally, the remaining bones were washed with sterile PBS and incubated while shaking with 5ml of EDTA solution (5mM EDTA, 0.1% BSA in PBS) for 30 min at 37° C to stop the collagenase digestion. The digested bones, denuded of surface cells, were then extracted for RNA by homogenization in TRI reagent (Molecular Research Center, Inc.) according to the manufacturer's recommendations.

Determination of the number of osteocytes per unit bone area

Femurs from mice that were either sham-operated or ovariectomized at 8 weeks of age and then sacrificed at 7 weeks post surgery. Bones were prepared for histology as previously described (36, 37) and embedded and cut as paraffin sections. One field of either cortical or trabecular bone was selected from a femur of a mouse in either the diaphysis (cortical bone) or the area 2 mm from the growth plate (trabecular bone) and its bone area determined by computer-aided analysis of digitalized images. An operator, who was blinded to the treatment groups, then visually determined the number of osteocytes in this area of bone. The ratio of osteocyte number per μ m² of bone area was then determined for each bone.

Real-Time RT-PCR

Total RNA was extracted from femurs and calvaria using a tissue homogenizer (PowerGen Model 1000, Fisher Scientific) and TRI-reagent. It was then converted to cDNA by reverse transcriptase (High Capacity cDNA Reverse Transcription Kit, Applied Biosystems) using random hexamer. Real-time PCR amplification was performed with multiple samples using gene-specific PCR primers and gene-specific Taqman probes (Applied Biosystems). The PCR mixture (including Taqman primer) was run in an ABI PRISM 7500 Sequence Detection System (Applied Biosystems). Reagents used for the PCR reaction were purchased from Applied Biosystems. Each sample was run in duplicate in a volume of 20 μ l using temperature cycling according to the manufacturer's recommendation. The relative quantification of target gene expression was normalized to the expression of a housekeeping gene, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), for each sample using the $\Delta\Delta$ CT method. Data were expressed as fold change in mRNA level compared to a control group.

Western Blot Assays

Murine calvaria, second to fourth lumbar vertebrae and femurs were dissected free of adhering tissue. For femurs, bone ends were removed, and the marrow cavity was flushed with α -minimum essential medium (without serum) by slowly injecting medium into one end of the bone using a sterile 25-gauge needle. All bones were homogenized in a cocktail of protease inhibitors (RIPA and HALT, Thermo Scientific) using a tissue homogenizer (PowerGen Model 1000, Fisher Scientific). The resultant mixture was spun (12,000 g) for 10 minutes to pellet any bone fragments. Supernatants were collected and assayed for protein (Pierce BCA assay, Thermo Scientific). Equal amounts of protein (20–40 μ g for femurs and lumbar vertebrae and 60–80 μ g for calvaria) were then diluted in sample buffer (62.5 mM Tris-HCl, pH 6.8, 10% (v/v) glycerol, 2% SDS, 0.1% bromophenol blue), and run in 10% SDS-PAGE gels. Samples were transferred onto nitrocellulose membranes by electroblotting (BioRad). Filters were blocked for 2 h in a blocking buffer containing 5% powdered milk and TBS + 0.1% Tween. Filters were then incubated with a primary antibody (polyclonal goat anti-murine sclerostin, R&D Systems, Minneapolis MN or polyclonal rabbit anti-murine β -actin, Cell Signaling Technology, Beverly, MA) at 4°C overnight. After washing, membranes were incubated for 1 h with appropriate secondary antibodies (Rabbit anti-Goat, or Goat-anti-Rabbit, R&D Systems) that were complexed to horseradish peroxidase. Reactive bands were detected by enhanced chemiluminescence using Chemiluminescence LumiGLO (Cell Signaling Technology). Detection was by autoradiography. Quantification was achieved by digitalizing the blots using an imaging station and then measuring band density.

ELISAs

Serum from mice that were without food but with free access to water overnight was obtained for measurement in various ELISAs at the time of sacrifice.

Carboxy-terminal collagen crosslinks (CTX) (RatLaps, ImmunoDiagnostic Systems) and osteocalcin (Biomedical Technologies Inc.) measurements were performed on serum using ELISA kits according to the manufacturer's recommendations.

Measurement of Sclerostin

Sclerostin levels in murine serum were measured in the serum used for the ELISAs as previously published (38, 39). This assay employs a custom-made rat/mouse-specific single-plex LuminexTM antibody-immobilized microbead platform immunoassay (Millipore, Cat#SPR336) according to the manufacturer's protocol. This overnight assay required 25 μ l

sample volume, recombinant mouse sclerostin was used for the generation of a standard curve (standard curve range 20,000 – 4.9 pg/ml). None of the measured values of sclerostin in serum samples was below the limit of detection of the assay (LOD = 5 pg/ml). To confirm the specificity of the assay, we measured sclerostin levels in the serum of sclerostin deficient mice and found them to be undetectable (data not shown).

Preparation of Cryosections

Bone tissues were fixed for 1–2 days in 4% paraformaldehyde/PBS (pH 7.4) at 4°C, decalcified for 3 days, placed in 30% sucrose/PBS overnight, and embedded (Cryomatrix, Thermo Shandon, Pittsburgh). Bones were cryosectioned at 5 µm sections using a CryoJane tape transfer system (Instrumedics, NJ).

Immunostaining

For Immunohistochemical labeling, cryosections were warmed to 65°C for 5 min and then rehydrated through xylene and a series of graded ethanol solutions. Endogenous peroxidase was blocked by incubating sections with 0.3% H₂O₂ in PBS for 15 min. Sections were then washed 3X in PBS. Blocking was with 10% rabbit serum in PBS for 1 h at room temperature. Primary anti-sclerostin antibody (Goat IgG anti-mouse SOST Affinity Purified Ab, R&D Systems Minneapolis, MN, diluted 1:200 with 2% rabbit serum in PBS) was incubated with the sections overnight at 4°C. Negative controls were without primary antibody. Secondary antibody (biotinylated rabbit anti-goat IgG diluted 1:200 with 2% rabbit serum in PBS) was then incubated with the sections for 1 h at room temperature. The slides were developed with VECTASTAIN Elite ABC reagents (Vector Laboratories, Burlingame, CA) according to the manufacturer's recommendations.

Statistical Analysis

Statistical analysis was performed by Student's *t*-test or one way analysis of variance (ANOVA) and the Bonferroni post hoc test when ANOVA demonstrated significant differences. In all experiments only one femur, vertebrae or calvaria per mouse was examined. All experiments were repeated at least twice and representative experiments or pooled data are shown.

Results

We first examined the effects that ovariectomy (OVX) had on bone in this model by measuring femoral trabecular bone mass (BV/TV) using micro-CT (Supplemental Figure 1). Significant decreases relative to sham-operated (SHAM) groups (by approximately 50 %) were detected in OVX mice by 5 wks. post-surgery without significant additional loss through 7 wks. Post-surgery. We have previously studied C56BL/6 mice that were either Sham-operated or ovariectomized at 5–8 weeks of age and demonstrated that at 4 weeks post-surgery there were significant decreases in vertebral (L₁), femoral and tibial bone mineral density as measured by dual energy absorptiometry (DXA) as well as decreases in vertebral (L₁), femoral and tibial trabecular bone area and femoral and tibial cortical thickness and cortical bone area as measured by micro-CT in OVX mice relative to SHAM mice (36, 37). We also found that OVX produced significant decreases in femoral and tibial trabecular number and trabecular thickness (36, 37). We have also previously performed histomorphometry at 4 weeks post-OVX on femurs in this model, confirmed the decrease in trabecular bone volume and demonstrated an increase in osteoclast area in OVX bones relative to SHAM with no significant change in percent osteoblast surface, mineral apposition rate or bone formation rate (36, 37). At 7 weeks post-surgery we found significant decreases in femoral cortical bone area and thickness as well as trabecular number and connectivity in OVX bones relative to SHAM with no significant difference in

trabecular thickness (Table 1). We also measured if OVX altered the number of osteocytes per unit bone area in either the cortical or trabecular bone of mice at 7 weeks post-surgery and found no difference in this parameter relative to results in femurs from SHAM mice (Table 2).

We next examined sclerostin mRNA levels in the calvaria and femurs of female mice that were operated on at 8 wks. of age and then examined 3 and 6 wks. post-surgery (Figure 1). At 3 wks. post-surgery there was a trend for OVX calvaria to have lower (by 33 percent) sclerostin mRNA levels ($p=0.09$) compared to SHAM. In contrast, at 3 wks. post-surgery sclerostin mRNA levels in OVX femurs were 53 percent lower than in SHAM ($p<0.01$).

At 6 week post-surgery this response was reversed. In calvaria OVX was associated with a 52 percent lower sclerostin mRNA levels ($p<0.01$) relative to SHAM while there was no significant difference in sclerostin mRNA levels between OVX and SHAM femurs.

We next examined the effects that OVX had on mRNA levels of two additional genes that also are highly expressed in osteocytes: DMP-1 and FGF-23 (40) (Supplemental Figures 2 and 3). These studies were undertaken to determine the relative specificity of the effects of OVX on sclerostin. We found that, in general, mRNA levels of DMP-1 and FGF-23 in calvaria and femur did not vary between SHAM and OVX bones as much as sclerostin mRNA levels. The only significant effect was an approximately 50 percent decrease in FGF-23 mRNA levels in calvaria of OVX mice relative to SHAM at 6 wks. post-surgery, a time when there was a similar effect of OVX on sclerostin mRNA levels (Supplemental Figure 2 and Figure 1).

To further confirm the effects of OVX on sclerostin mRNA expression in cortical osteocytes, we enriched longbones (femurs and tibia) for cortical osteocytes by flushing away trabecular bone and then performing repeated rounds of collagenase incubation to digest surface cells off the bone (35). Results with digested bones from mice at 3 wks. post-surgery (Supplemental Figure 4) were similar to those from undigested femurs at 3 wks. post-surgery (Figure 1) and demonstrated a 50% decrease in sclerostin mRNA levels in OVX bones relative to SHAM bones.

Immunohistochemical assay for sclerostin protein in humeri at 3 wks. post-surgery demonstrated that sclerostin protein was expressed specifically in cortical osteocytes and was markedly decreased in OVX bones relative to SHAM (Figure 2). Similarly, using western blots, we found that OVX decreased levels of sclerostin protein in calvaria by 83 percent at 3 wks. post-surgery ($p<0.01$) and by 60 percent at 6 wks. post-surgery ($p<0.01$) relative to SHAM (Figures 3). In contrast to the result of the immunohistochemistry studies in humeri (Figure 2) and the western blot assays in calvaria (Figure 3), we found no significant differences in sclerostin protein levels in non-digested femurs between SHAM and OVX at either 3 or 6 wks. post-surgery (Figure 4). We also examined sclerostin protein levels in the L 2-4 vertebrae of mice at 6 wks. post-surgery and found it to be 38 percent lower with OVX (ratio of the density of western blot bands for sclerostin relative to β -actin = 0.69 ± 0.07 for SHAM and 0.43 ± 0.03 for OVX, $p=0.01$).

Overall rates of bone resorption in the mice were measured as levels of serum type 1 collagen carboxy-terminal crosslinks (CTX) (Figure 5). This was 36 percent greater with OVX relative to SHAM at 3 wks. post-surgery ($p<0.01$). However, at 6 wks. post-surgery there was no significant difference in serum CTX between SHAM and OVX mice. Overall rates of bone formation were monitored by measuring serum osteocalcin levels (Figure 5). These were increased in OVX mice by 33 percent over SHAM ($p<0.01$) at 3 wks. post-surgery and by 34 percent over SHAM at 6 wks. post-surgery ($p=0.01$).

We also measured mRNA levels for RANKL and OPG in the calvaria and femurs at 3 and 6 wks. post-surgery (Supplemental Figures 5 and 6). In calvaria there were no differences in RANKL or OPG mRNA levels between SHAM and OVX at 3 wks. However, at 6 wks. there were significant decreases in RANKL (by 50%, $p=0.01$) and OPG (by 64%, $p<0.05$) mRNA levels in OVX calvaria relative to SHAM. In femurs there were no differences in RANKL or OPG mRNA levels between SHAM and OVX bones at either 3 or 6 wks. post-surgery. There was also no significant effect of OVX relative to SHAM on the ratio of RANKL to OPG mRNA levels in calvaria or femur (data not shown).

Finally, we measured the effects of OVX on serum levels of sclerostin (Figure 6). However, unlike humans, where estrogen status significantly influences serum sclerostin levels (30–33), we found no significant effect of OVX on this value in mice; although, there was a trend for OVX to increase serum sclerostin by 7.4 percent ($p=0.066$) relative to SHAM at 3 wks. post-surgery. At 6 wks. post-surgery there was no difference in serum sclerostin levels between OVX and SHAM. Curiously, serum sclerostin levels at 6 wks. post-surgery in SHAM and OVX mice were significantly lower (by 20 percent, $p<0.01$) relative to their respective values at 3 wks. Post-surgery.

Discussion

Sclerostin is a potent negative regulator of bone formation, which mediates its effects by inhibiting canonical Wnt signaling pathways (4, 17). Currently, there are clinical trials underway (41, 42) to determine if inhibiting the actions of sclerostin, using antibodies or other agents, is an effective and safe therapy for diseases like osteoporosis, which are caused by low bone mass. Hence, studies of the mechanisms regulating sclerostin are important to better appreciate how estrogen withdrawal stimulates bone loss and the development of osteoporosis. In addition, they may provide a better understanding of the therapeutic potential of sclerostin inhibition.

In the current study we demonstrated by a variety of methods including RT-PCR, immunohistochemistry, and western blot assay that levels of sclerostin mRNA and protein in different bones of OVX mice were either decreased or the same as levels in SHAM mice. The effects of OVX on sclerostin mRNA and protein were variable and dependent on the time after surgery and the bone that was examined. Effects of OVX were most dramatic in the calvaria where a trend for lower sclerostin mRNA levels relative to SHAM was seen at 3 week post-surgery and values were significantly decreased at 6 wks. post-surgery. In addition, sclerostin protein was significantly lower in OVX calvaria relative to SHAM at both 3 and 6 wks. Post-surgery. We also found significantly lower sclerostin protein in OVX humeri relative to SHAM by immunohistochemistry at 3 wks. post-surgery and in OVX lumbar vertebrae at 6 wks. Post-surgery by western blot assay. However, in the femur we found OVX was associated with lower sclerostin mRNA levels relative to SHAM only at 3 wks. post-surgery. In addition, we found no significant difference in sclerostin protein levels in femurs from OVX mice relative to SHAM at either 3 or 6 wks. post-surgery.

To determine if the effects of OVX on sclerostin were a specific osteocyte response to OVX, we examined mRNA levels in bones of two other relative osteocyte-specific genes, DMP-1 and FGF-23 (40). We found that, in general, OVX had little effect on levels of these mRNAs. There was no difference in DMP-1 mRNA levels in either calvaria or femurs. FGF-23 levels were decreased in OVX calvaria by approximately 50 percent at 6 but not 3 wks. post-surgery. There with no differences in femoral values at either time point. Hence, these data argue that the effects of OVX on sclerostin mRNA and protein levels in bones do not represent a generalized effect of OVX on osteocyte gene expression.

Based on its known actions on bone (18), decreases in sclerostin production would be expected to increase bone formation rates since loss or inhibition of sclerostin is associated with increased bone mass in both animal models and in humans (1, 2, 13, 15). Previous studies from our group (30) and from others (22, 31, 32, 43) have compared the serum sclerostin levels of women pre- and post-menopause and found that postmenopausal women have significantly higher levels. In addition, there is a negative correlation between age and serum sclerostin that occurs in both pre- and postmenopausal women. We and others also found that there is a strong negative correlation between serum sclerostin levels and the amount of circulating estrogens in post-menopausal women (22, 31, 32, 43). In addition, Modder et al demonstrated that treating postmenopausal women for 4 wks. with estrogens decreased serum sclerostin levels (31). These authors also showed that treatment of postmenopausal women with estrogen for 4 months decreased sclerostin levels in bone marrow plasma, which is a better measure of sclerostin levels in the bone microenvironment (32). In summary, the published data in humans examining levels of sclerostin in serum and bone marrow plasma argue that estrogen inhibits sclerostin production and/or enhances its degradation or clearance.

Given these data, we anticipated that in mice, OVX, which acutely decreases serum estrogen levels, would be associated with higher rates of sclerostin production and higher serum levels relative to SHAM mice. Instead, we found that in all bones from OVX mice that were examined, sclerostin mRNA and/or protein levels were either similar to or decreased relative to SHAM. This effect depended on the bone that was examined and the time post-surgery. In addition, we found that in this mouse model there was only a trend for a small (7.4 percent) increase in serum sclerostin relative to SHAM at 3 wks. post-surgery and no differences at 6 wks. post-surgery. These data imply that there are significant differences in the effect of ovariectomy and by inference, estrogen withdrawal, on sclerostin levels in serum and, possibly, also on sclerostin production in bone between humans and murine models. They also demonstrate that changes in serum sclerostin levels do not necessarily reflect similar changes in sclerostin production in osteocytes in bone.

There are a number of potential reasons for these discrepancies. We studied mice that were ovariectomized at 8 wks. of age, when they were still growing and compared them to mice of identical age that were sham-operated. However, it is possible that growth had effects on sclerostin production and/or clearance in the mice, which might not be occurring in postmenopausal females who stopped growing many years prior. There is a positive correlation between serum sclerostin levels and age in humans (22) and all studies to date of the effects of estrogen on serum sclerostin have been performed on postmenopausal women who are at least mid-way in their life span. However, our murine model used relatively young mice. Hence, there may be different effects of ovariectomy on sclerostin in aged mice.

In our current model decreases in trabecular bone mass in OVX mice relative to SHAM were detectable by 5 wks. post-surgery. Bone resorption rates (as measured by serum CTX levels) demonstrated increases relative to SHAM at 3 but not 6 wks. In contrast, bone formation rates (as determined by serum osteocalcin levels) were higher in OVX mice relative to SHAM at both times. These findings suggest that accelerated bone resorption relative to SHAM mice does not persist in this model. Curiously, we did not detect changes in femoral RANKL or OPG mRNA levels in this model at 3 or 6 wks. post-surgery nor did we find significant effects of OVX on the ratio of RANKL to OPG mRNA. Since we did find significant trabecular bone loss in the femurs by 5 wks. (Table 1 and Supplemental Figure 1), these results argue that this bone loss was not mediated by differences in mRNA levels of these cytokines in this bone. Alternatively, it is possible that the trabecular bone loss we observed was mediated predominantly by changes in RANKL and OPG in the bone

marrow and trabecular bone compartment, which we flushed away in our studies and, consequently, did not measure. We did find somewhat similar decreases in both RANKL (by 50%) and OPG mRNA levels (by 64%) in OVX calvaria relative to SHAM. These results imply that although there was a decrease in the mRNA for these cytokines in this bone at 6 wks. post-surgery with OVX, the relative ratios of RANKL to OPG remained constant in SHAM and OVX calvaria. Therefore, it is probable that the effect of these changes on osteoclastogenesis was not major.

Since sclerostin inhibits bone formation, through its effects on canonical Wnt signaling in osteoblast-lineage cells (4, 17), the decreases in sclerostin that we observed in this model with OVX may be involved in the relative increases in bone formation that we measured at 3 and 6 wks. post-surgery. It is known that estrogen withdrawal causes an increase in both bone formation and bone resorption, with the rate of resorption exceeding the rate of formation, leading to net bone loss (44). Therefore, our results argue that the decreases in sclerostin that occurred in the bones of OVX mice relative to SHAM were involved in the relative increases in bone formation that occurred with estrogen withdrawal in this OVX model.

It is unclear yet how the results of the current studies in mice relate to measurements of sclerostin in the serum and bones of humans. The levels of serum sclerostin that we detected in mice were lower than those typically measured in humans and this may reflect differences between mice and humans in the microvasculature of cortical bone or in another factor that influences the access of osteocyte-produced sclerostin to the peripheral circulation. Our findings argue that studies, which examine the effects that estrogen treatment has on sclerostin mRNA and protein levels in the bones of postmenopausal women, need to be performed and correlated with serum sclerostin levels. It is possible that there may be significant differences between the effects that estrogen treatment has on sclerostin mRNA and protein levels in various bone in humans and changes in serum sclerostin.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Authors Roles:

SJ and JK performed the majority of the experiments. MS performed the sclerostin assays. HYW performed the measurements of osteocytes per unit bone area. ET and IK performed the immunohistochemistry for sclerostin on frozen sections. FM, SKL and JL designed the experiments, interpreted the data and wrote the manuscript.

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Figure 1.

Relative sclerostin mRNA levels in calvaria and femurs measured by quantitative real time PCR from SHAM and OVX mice at 3 and 6 weeks post-surgery. Values are mean $\pm$ SEM for 8–14 bones per group. Data are compiled from two to 4 independent experiments.

* Significantly different from SHAM p 0.01

Humerus

Figure 2.

Images of sclerostin protein measured in cortical bone from humeri by immunohistochemistry (positive stain is brown). Sections were processed simultaneously using bones from SHAM or OVX mice. Negative control sections were prepared without the primary antibody.

Calvaria

Figure 3.

Relative sclerostin protein levels in calvaria measured by western blot assay from SHAM and OVX mice at three and six weeks post-surgery. Values are mean $\pm$ SEM for 3–4 bones per group and represent the ratio of the band density for sclerostin relative to β -actin. Images of the western blots are presented above each respective group.

* Significantly different from SHAM p 0.01

Femur

Figure 4. Relative sclerostin protein levels in femurs measured by western blot assay from SHAM and OVX mice at three and six weeks post-surgery. Values are mean $\pm$ SEM for 3 bones per group and represent the ratio of the band density for sclerostin relative to β -actin. Images of the western blots are presented above each respective group.

Figure 5.

Relative serum CTX and osteocalcin levels in SHAM and OVX mice at three and six weeks post-surgery, measured by ELISA. Values are mean $\pm$ SEM for 10 to 19 mice per group. Data were compiled from four independent experiments for the 3-week post-surgery data and 2 independent experiments for the six-week post-surgery data.

* Significantly different from SHAM $p < 0.01$

Figure 6.

Serum sclerostin levels in SHAM and OVX mice at three and six weeks post-surgery. Values are mean $\pm$ SEM for 10 to 19 mice per group. They were compiled from four independent experiments for the three-week post-surgery data and two independent experiments for the six-week post-surgery data.

Significantly different from the respective value at three weeks $p < 0.01$

Table 1

Micro-CT analysis of femoral bone at 7 weeks post-surgery

Groups	Cortical Bone Area mm ²	Cortical Thickness mm	Trabecular Thickness μm	Trabecular Number mm ⁻¹	Connectivity mm ⁻³
SHAM	0.63 ± 0.01	0.174 ± 0.003	45.1 ± 1.5	3.60 ± 0.15	56 ± 8
OVX	0.57 ± 0.01 *	0.158 ± 0.002 *	41.8 ± 1.1	2.94 ± 0.07 *	24 ± 3 *

Values are mean ± SEM for 7 to 10 values per group

* Significantly different from SHAM p< 0.01

Table 2

Analysis of the number of osteocytes in femoral bone at 7 weeks post-surgery

Groups	Number of osteocytes per μm^2 trabecular bone area	Number of osteocytes per μm^2 cortical bone area
SHAM	1.17 ± 0.04	0.91 ± 0.09
OVX	1.09 ± 0.10	0.96 ± 0.05

Values are mean $\pm$ SEM for 7 values per group