SCLEROSTIN IMMUNOREACTIVITY INCREASES WITHIN THE CORTICAL BONE
OSTEOCYTES IN THE FEMUR OF AGING MICE

By

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Abstract

Sclerostin, a secreted glycoprotein, is known to down-regulate osteocyte differentiation from osteoblasts and acts as a negative modulator of bone formation. It is well established that serum sclerostin levels increase with age but the relationship between changes of cellular expression of sclerostin with age is not well known. Immunohistochemical staining and confocal microscopic analysis of sclerostin immunoreactivity (sclerostin-IR) in the femurs of 4, 9, and 24 month old adult C3H/HeJ male mice was performed. Detectable levels of sclerostin-IR were found in cortical bone osteocytes of the femur in all age groups using an antibody directed against sclerostin. Phalloidin and DAPI were used to mark all osteocytes in cortical bone to detect osteocyte/ sclerostin-IR colocalization. Sclerostin-negative and sclerostin-positive expressing osteocytes were detected in close proximity throughout the cortical bone. Only a subset of osteocytes expressed sclerostin and this ratio of sclerostin positive osteocytes increased with age, from 38.5 ± 1.6% to 43 ± 3.6% to 49 ± 2.3% in young, middle-aged, and old mice, respectively. Understanding the potential mechanisms that drive these age-related changes may influence the therapeutic potential of age-related diseases like osteoporosis.

Introduction

Aging inevitability comes with the heightened likelihood of bone degeneration, as bones become weaker (Zioupos 1998). As a result, age brings the increased risk of bone diseases like osteoporosis, the most common bone disease in aging humans. Osteoporotic patients show symptoms of significantly weakened bone strength, low bone mineral density (BMD), and an increased predisposition to fracture (Recker 2004; Pietschmann 2008). The two current primary
treatment options for osteoporosis are bone anabolics, which stimulate bone formation and anti-resorptive drugs, which inhibit bone resorption (Montagnani 2014). Both approaches have proven to be successful at strengthening bone but with long term use, they have adverse effects. Bone anabolics currently available are limited to PTH, a therapy that has been shown to increase the risk of osteosarcoma in the experimental rat model (Vahle 2002). Clinical trials with the PTH Teriparatide, also yielded adverse effects such as hypercalcemia (Gupta 2012). Anti-resorptives disrupt the bone remodeling balance between osteoblasts and osteoclasts, reducing the turnover rate and decreasing bone formation in patients undergoing treatment (Baron 2012).

Gathering a further understanding of how and why bone quality declines with age requires investigation into properties of the bone that wanes with age. Mice are excellent models for studying human bone degeneration as they have a similar loss in BMD as with aging humans and have conserved disease mechanisms/trends in bone loss suggested by osteoporotic and atherosclerotic mice. Mice have a fast gestation period, making them good for performing studies on a shorter timeline (Jilka 2013).

The loss of BMD among both mice and humans is regulated by the osteocyte in the bone. Osteocytes are further differentiated osteoblasts (bone-building cells), and regulate bone mineralization (Bonewald 2011). The number of osteocytes declines with age, contributing to the loss of BMD with time. Osteocytes are known to secrete a 24kDa glycoprotein called sclerostin, a negative regulator of the Wnt/β-catenin pathway (Brunkow 2001). This pathway is an important regulator of bone formation and resorption, and regulates pluripotent mesenchymal cell differentiation into either osteoblasts or chondrocytes (Li 2005; Chen 2007). The Wnt signal also regulates osteoblast differentiation into an osteocyte. Sclerostin is secreted by the mature osteocyte and acts as a negative regulator to Wnt signaling by inhibiting Wnt interactions with
LRP5/6 receptors early in the pathway, preventing osteoblasts from receiving the signal for differentiation into an osteocyte (Baron 2006).

Inactivation of this pathway can lead to osteopenic diseases and loss of bone integrity (Li 2005). Due to the role of sclerostin in osteocyte differentiation, it is important to consider the potential in targeting the glycoprotein as a means of therapy. Inhibition of sclerostin has shown to increase osteocyte production from osteoblasts, which could counteract many osteopenic diseases many are faced with as they age (Baron 2006). Most osteopenic diseases are some of the most common conditions that come with aging, so it is important to study osteocyte/sclerostin interaction in tandem with age (Pietuchmann 2008).

**Materials and Methods**

*Animals*

Experiments were performed with naïve femurs obtained from young adult (4 month old; n=9), middle-aged adult (9 month old; n=3) and old (24 month old; n=4) adult male C3H/HeJ animals. The mice were housed in accordance with the National Institutes of Health guidelines under specific pathogen-free conditions in autoclaved cages maintained at 22°C with a 12-hour alternating light/dark cycle and access to food and water *ad libitum*. All procedures adhered to the guidelines of the Committee for Research and Ethical Issues of the IASP (Zimmernann, 1983) and were approved by the Institutional Animal Care and Use Committee at the University of Arizona (Tucson, AZ).
**Preparation of tissue for immunohistochemistry and histology**

At 4, 9, and 24 months of age, animals were sacrificed and perfused, as previously described (Jimenez-Andrade et al., 2008). After perfusion, the left hind limbs were removed and post-fixed for 24 hours at 4°C in the same perfusion fixative solution. The femurs were decalcified for approximately two weeks in 10% ethylenediaminetetraacetic acid (EDTA) (PBS, pH 7.4 at 4°C; Sigma Aldrich, St. Louis, MO). Decalcification was monitored radiographically with a Faxitron MX-20 digital cabinet x-ray system (Faxitron/Bioptics, Tucson, AZ). Following thorough decalcification, each femur was cryoprotected in 30% sucrose at 4°C for at least 48 hours to allow the sample to equilibrate and sink to the bottom of the tube before being sectioned.

**Immunohistochemistry and histology**

To characterize the sclerostin-IR in young, middle-aged, and old bone, the femur was processed immunohistochemically and histologically as previously described (Chartier et al., 2014). Immunostaining for sclerostin was performed with antibody directed against Gln24-Tyr211 (1:400 dilution; R&D Systems, Minneapolis, MN). This polyclonal antibody detects mouse SOST/Sclerostin in direct ELISAs and Western blots (Kusu et al., 2003). After overnight primary antibody incubation, preparations were washed 3X10 minutes each in PBS and incubated for three hours at RT (21°C) with secondary antibodies conjugated to fluorescent markers (Cy3; 1:600; Jackson ImmunoResearch, West Grove, PA). After three hour incubation, preparations were washed 3X10 minutes each in PBS and incubated with Alexa Fluor 488-conjugated phalloidin (Life Technologies, Grand Island, NY) for 20 minutes. Bone sections were then washed in PBS 3 X 10 minutes and counterstained with DAPI (1:500; Molecular Probes/Thermo Fisher Scientific) for five minutes. Preparations were washed again in PBS.
3X10 minutes each and coverslipped with VectaShield (Vector Laboratories, Inc. Burlingame, CA). Preparations were allowed to dry at RT for 12 hours before being imaged.

As controls for specific and non-specific sclerostin-IR staining, two methods were used: deletion of the primary antibody (data not shown) and use of a blocking peptide (R&D Systems, Minneapolis, MN). The sclerostin antibody (0.5 µg/ml) was diluted in PBS/TBS and added 5-fold (by weight) to the recombinant mouse sclerostin (‘blocked’). In a separate mixture, the same dilution of antibody with the same volume of saline/PBS without the peptide (‘control’) was made. Both the “blocked” and “control” mixtures were allowed to incubate at RT for one hour. Following the blocking/competition incubation period, the “blocked” and “control” samples were diluted with 1%/0.1% Triton-X 100 blocking buffer and incubated on consecutive bone sections overnight at RT. After this primary incubation, the secondary preparation was carried out as described above.

Confocal images were acquired with an Olympus Fluoview FV1000 (Olympus, Center Valley, PA) system equipped with Multiline Argon (458, 488, 515 nm), Green HeNe (543 nm), Red HeNe (633 nm) lasers and with an Olympus Fluoview FV1200 system (Olympus, Center Valley, PA) equipped with LD (405, 440, 473, 559, 635 nm), Multiline Argon (457, 488, 515 nm), and HeNe(G) (534 nm) lasers. Each Fluoview system was equipped with multiple excitation and emission filters. Selected markers were visualized using excitation beams of 488 and 599 and emissions were detected using BA505-540 and B575-620 emission filters. DAPI was visualized using an excitation beam of 405 nm and emissions were detected using a BA430-470 emission filter. Sequential acquisition mode was used to reduce bleed-through from fluorophores. Images were obtained using Olympus UPlanApo 40x/1.30 and 60x/1.42 (FV1000)
and UPlanFL N 40x/1.30 and PlanApo N 60x/1.42 (FV1200) oil objectives (Olympus, Center Valley, PA).

Histology

Slides of 10 µm bone sections were stained with hematoxylin and eosin for anatomical reference. Histology slides were imaged at 200X using bright-field microscopy on an Olympus BX-51 microscope equipped with an Olympus DP71 digital camera (Olympus, Center Valley, PA). In order to acquire an image of the whole tissue sample (femur), several images were acquired from each region then compiled in Adobe Photoshop. The images were cropped and saved as tiff files.

Quantifications

For all imaging purposes, the images for each cell type were obtained with identical acquisition exposure-time conditions. The percentage of osteocytes with detectable sclerostin-IR in the femoral cortical diaphyseal bone was quantified using immunohistochemical images obtained from young (4 month old), middle-aged (9 month old) and old (24 month old) adult femurs. The left limb from each animal was analyzed at three sites along the diaphysis: proximal diaphysis (4 mm from the top of the femoral head), mid-diaphysis (approximately 8 mm from the top of the femoral head) and distal diaphysis (3 mm from distal end of the femur). The approximate area of bone compartment that was analyzed was 280 µm (length) x 310 µm (width) X 20 µm (depth). For young animals, the sample size was n=3, 9, and 3 for the proximal diaphysis, mid-diaphysis, and distal diaphysis, respectively. For the middle-aged animals, the sample size was n=3 for all three sites. For old adult animals, the same sample size was n=4 for all three sites.
Three slides from each animal were imaged at 20x magnification. Only one of the two sections per slide was analyzed and at least 100 µm between each section was examined to prevent duplicate counting of osteocytes. The total number of osteocytes in the field of view was determined by counting the number of nuclei visible through DAPI staining and through phalloidin staining of the osteocytes. Positive DAPI nuclei forming vascular clusters were not included in the quantifications as they presumably are nuclei from endothelial cells. Then the number of sclerostin-positive osteocytes was determined by counting the number of sclerostin/DAPI/phalloidin overlays visible in each microscopic field. Finally, the total number of sclerostin-expressing cells was divided by the total number of cells to determine the percentage of osteocytes expressing sclerostin. The percent of sclerostin-positive osteocytes were averaged among the three slides examined per animal.

To determine periosteal thickness, under the DAPI channel, three images per middiaphyseal periosteum per age group were taken at 40x magnification. The thickness (length measure) of the total periosteum was determined with Image J software (NIH) and averaged for each animal at each age group. For young, middle-aged, and old adult animals, the sample size was 4, 3 and 4, respectively.

Statistical analysis

All statistical analyses were calculated in SigmaPlot software (San Jose, CA). One way-ANOVA was performed followed by a Tukey’s post-hoc test comparing each group to each other at each age. Significance level was set at p<0.05. In all cases, the investigator responsible for counting phalloidin-IR/DAPI and/or sclerostin-IR-positive osteocytes was blind to the age of each animal.
Results

Sclerostin immunoreactivity (sclerostin-IR) in cortical bone osteocytes in femur

Immunohistochemical staining of naïve mouse femur sections treated with a polyclonal antibody raised against sclerostin revealed sclerostin-IR in the cytoplasm and dendrite-like process of the osteocytes of cortical bone. Sclerostin-IR was also found in the periosteum (Fig. 1B, 2A, B) and chondrocytes (data not shown). In searching through other regions of the bone, sclerostin-IR was not detected at the growth plate or in other cell types, including osteoclast, osteoblasts, macrophage/monocytes, or bone marrow (Table 1).

A primary antibody deletion control (data not shown) and an absorption control (Fig.2C, D) were used to ensure the integrity of the staining of sclerostin IR in young (4 months) and old (24 months) mice.

Sclerostin-negative and sclerostin-positive expressing osteocytes present in close proximity in cortical bone

Previous studies have shown that sclerostin is expressed in mature osteocytes (Bonewald, 2011). To help identify all osteocytes, the marker Phalloidin, which stains for F- actin, was used (Fig. 3). Considering that more than 90% of cells in the cortical bone are osteocytes, DAPI was also used to stain the nuclei (Bonewald, 2011). Thus both phalloidin and DAPI could be used interchangeably to mark osteocytes. When anti-sclerostin was co-localized with Phalloidin and/or DAPI, two subtypes of osteocytes were identified: sclerostin positive and sclerostin negative. In the field of view at the mid-diaphysis (280 µm × 310 µm × 20 µm), out of 176.5 ± 11.5 osteocytes counted, only 68 ± 4.1 were sclerostin-positive (38%). Interestingly, both
subtypes were immediately adjacent to each other, and were found scattered throughout the cortical bone.

*Osteocytes expressing detectable levels of sclerostin-IR increases with age*

It must be noted that with age, the total number of osteocytes declines\(^{18}\) [Frost, 1960]. Three different age groups were used to quantify the total number of osteocytes: At the mid-diaphysis, young animals (4 month old) showed an average of $176.5 \pm 11.5$ osteocytes; middle-aged animals (9 month old) had $138.7 \pm 12.0$ osteocytes, and old animals (24 month old) had $83 \pm 7.5$ osteocytes. The data shows that with age, the number of total osteocytes in the mid-diaphysis declines.

To determine if the subtype of sclerostin-positive osteocytes also changed with age, the same three age groups were examined at the mid-diaphysis. Sclerostin positive osteocytes showed a slight decline with age [68.1 ± 4.1; 60.5 ± 1.7; 41.4 ± 4.0 for young middle-aged and old adults, respectively, per field of view (280 μm × 310 μm × 20 μm)]. Overall, the ratio of sclerostin positive osteocytes to sclerostin negative osteocytes increased with age, from $38.5 \pm 1.6\%$ to $43 \pm 3.6\%$ to $49 \pm 2.3\%$ in young, middle-aged, and old mice, respectively (Fig.4). To determine if this phenomenon was site-specific, quantities were also taken from proximal diaphysis and distal diaphysis of the femur, with a 1.3–1.4-fold change between areas all areas examined.

**Discussion**

The results from the study demonstrated that osteocytes located in the cortical bone of the mouse femur expressed sclerostin-IR in young, middle aged and old male mice, aged 4 months, 9 months, and 24 months old, respectively (Fig.1). The percentage of sclerostin-IR positive
expression was revealed to be higher in osteocytes of older mice than young mice (Table 1; Fig 4E). Finally, sclerostin positive and sclerostin negative expressing osteocytes were located in close proximity to each other in the cortical bone (Fig. 3).

*With age, number of total osteocytes declines, but serum sclerostin levels rise* (Modder 2011)

Previous studies have shown that in both men and women, osteocyte density declines with age (Busse, 2010). In this study, we show that only a subset of those osteocytes express sclerostin and that proportion increases with age. Furthermore, prior studies have also shown that serum sclerostin levels increase with age in humans (Modder 2011). This all suggests that there is a correlative nature between sclerostin and age, thereby an ever-increasing inhibitor of bone formation. While our data is consistent with prior findings, more research on the correlative nature between sclerostin expression in osteocytes, serum, and age is needed to gain further insight into potential applications of sclerostin related therapies.

*Sclerostin inhibition of osteoblast differentiation declines bone integrity*

Sclerostin acts by inhibiting the LRP6/5 receptor, blocking downstream Wnt signaling, preventing the signal that initiates osteoblast to osteocyte differentiation (Van Dinther, 2013). The two key cells in regulating bone mass are osteocytes and osteoblasts and they are in constant communication in order to maintain a healthy skeletal system. Yet with age, there is a lower osteocyte count, which would consequentially result in a decline in bone mineral density (BMD) (Recker, 2001; Pietschmann, 2008; Bonewald, 2011; Holman, 2005). Sclerostin has also been observed to directly contribute to a lower osteoblast count by regulating the apoptosis of bone
cells and acting as an antagonist of the growth factors Wnt and BMP (Winkler, 2003; Galea, 2013).

Furthermore, our results show an increase in sclerostin-IR levels with age (Table 1; Fig. 4E), which likely leads to a decrease in osteocyte differentiation in older individuals (Mullender, 1996). These lowered osteocyte counts consequentially result in a decline in bone mineral density (BMD) making the bone more prone to fracture and osteopenic states (Recker, 2001; Pietschmann, 2008; Bonewald, 2011; Holemen, 2005).

*Sclerostin positive and sclerostin negative osteocytes found in close proximity*

Sclerostin acts as a negative regulator in bone formation by inhibiting osteoblast proliferation and differentiation, so mineralization of osteoblastic cells declines as a consequence creating lower bone mass and bone density (Li, 2009). The mechanism by which sclerostin could be preventing this action is by opposing the effects of growth factors such as BMD and Wnts, or by binding to unknown receptors resulting in inhibition of osteoblast proliferation and differentiation (Fig. 5) (Winkler, 2003; Galea, et al 2013). Sclerostin could act on osteoblast progenitors, preventing the initial matrix formation that spurs osteoblast differentiation, or act on the actual process of osteoblast differentiation itself (Dijke, 2008). Results from the study showed that sclerostin expression among osteocytes was not uniform, suggesting a more singular means of sclerostin regulation in individual osteocytes.

Sporadic distribution of sclerostin positive and sclerostin negative osteocytes distributed throughout the cortical bone suggests a means of regulation of sclerostin expression that differs in each osteocyte (Fig. 3). MiRNAs, non-coding small RNAs, allow for a degree of independent cell-to-cell regulation. Studies have shown miRNAs do have a role in regulating sclerostin...
expression in cells (Chen, 2013; Johnson, 2014). Understanding the extent of the role of miRNA regulation of sclerostin expression in osteocytes can reveal possible mechanisms for the sclerostin positive and negative osteocyte expression, as well as what variables are important in initiating such a response in osteocytes.

Therapeutic implications - how sclerostin could be regulated in osteoporotic patients

In humans, a similar trend to that seen in mice is noted, with a positive increase in sclerostin levels with age. Bone degeneration in mice and humans occurs by a similar mechanism. Like humans, cancellous bone loss begins at early adulthood in mice as indicated by decline in bone mineral density, and progresses with age. A decline in bone wall width, an indication of inadequate osteoblasts, also appears in cancellous bone loss. (Jilka, 2013; Recker, 2004; Almeida, 2007). Because osteoblasts are precursors to osteocytes, which were quantified in each age group, we see the decline in osteocytes with age and is likely related to this initial decline in osteoblasts (Baron, 2006). Sclerostin levels in humans have also been found to increase with age, and seeing as this trend is paralleled in mice, using the mouse model for studying sclerostin effects on osteopenic states is warranted (Modder, 2011).

By regulating the action of sclerostin (i.e. inhibition), the Wnt co-receptors can proceed, and osteoblast to osteocyte differentiation can occur (Baron, 2006; Canalis, 2007). By doing so, a healthy BMD can be restored in osteoporotic bones (Li, 2009). Studies in which sclerostin regulation is controlled using mono-clonal antibodies against the SOST sclerostin encoding gene have shown bone density to increase in several different animal models (Ominsky, 2010; Li 2009; Tian, 2010; Suen, 2015). Anti-sclerostin antibodies have also been studied as potential
therapies for fracture healing and osteoporosis, showing positive results for bone healing in both circumstances (McDonald, 2012; Agholme, 2010; Li, 2009).

These findings have yet to be observed in the scope of age, and while this study has revealed how sclerostin expression increases with age, inhibition of sclerostin in various different age groups could reveal more about potential age-related sclerostin therapies.

Conclusions

The presence of sclerostin-IR expression in young, middle-aged, and old osteocytes located in the cortical bone indicates a degree of conservation among the expression of the glycoprotein with age. The means by which (and why) sclerostin-IR expression increases with age remains unclear. Further investigation into how osteocyte expression of sclerostin is regulated (and how age plays a role in this expression), as well as reasons for why only some osteocytes are sclerostin positive need to be further investigated.

Acknowledgements

Special thanks to Michelle Thompson and everyone at the Mantyh lab.
Figures and Tables

Figure 1 Sclerostin-immunoreactivity (sclerostin-IR) detected in osteocyte cell body and dendrite-like processes located in the cortical bone of the mouse femur. (A) An H & E section of a young (4 month old) male mouse femur at low power magnification. Images 1B and 1C of the mid-diaphyseal region are taken from the boxed section of the femur seen in (A). Image (B) was taken mid-power using confocal microscopy, and shows the expression of sclerostin-IR (red hot) in both the osteocytes and the periosteal region. Image (C) was taken at mid-diaphyseal region with high-powered confocal microscopy, illustrating the sclerostin-IR in the dendrite-like network of the osteocytes.
Figure 2 Images (A) and (B) show sclerostin-IR in mineralized bone of young (4 month old) and old (24 month old) animals, respectively. Images show presence of sclerostin-IR in osteocyte cell body, the dendrite/ canaliculi network, and the periosteum of both young and old animals. Images (C) and (D) are the serial sections of (A) and (B), and were processed in the same manner, except the sclerostin antibody is replaced with the recombinant mouse SOST- therefore acting as the absorption control and showing the specificity of the sclerostin staining.
<table>
<thead>
<tr>
<th>CELL TYPE</th>
<th>SCLEROSTIN IMMUNOREACTIVITY</th>
<th>YOUNG ADULT 4-month old</th>
<th>MIDDLE AGED ADULT 9-month old</th>
<th>OLD ADULT 24-month old</th>
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<td>HYPERTROPHIC CHONDROCYTES</td>
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+= 30%  ++=40%  +++=50%

* = levels of immunoreactivity  - = Not detected
N.A.: Not analyzed due to poor tissue quality of the proximal epiphysis of middle aged adult

Table 1 Sclerostin immunoreactivity levels in mouse osteocytes increased with age, showing 30% young adult, 40% in middle aged adult, and 50% in old adult mice. Sclerostin immunoreactivity was not detected for any age group in the osteoclasts, osteoblasts, bone marrow, or macrophage/monocytes. Detectable levels were seen in across all age groups in the periosteum, and in the young and old models of the hypertrophic chondrocytes.
Figure 3 Phalloidin and DAPI co-stained with sclerostin identify sclerostin-positive and sclerostin-negative osteocytes. Mid-power confocal image of cortical bone show colocalization of phalloidin (green) and sclerostin (red) in a young (4 month old) animal (A). Solid arrowheads point to sclerostin-positive osteocytes, where open arrowheads indicate sclerostin-negative osteocytes. Scale bar (A) 30μm. (B-D) High power confocal image of co-localization of phalloidin (green), DAPI (blue), and sclerostin (red hot), that allows distinction between sclerostin-IR positive and sclerostin-IR negative osteocytes. Note that sclerostin is expressed in the cytoplasm of the osteocyte as well as the dendrite-like processes. Also note the close proximity of sclerostin-positive and sclerostin-negative osteocytes. Scale bar (B-D) 8μm.
Figure 4 Osteocytes expressing sclerostin increase with age. (A) H & E section of a young (4 month old) male mouse femur at low power magnification, with boxed regions to show where osteocyte sclerostin-IR was analyzed. Three sites were used along the diaphysis of the femur- the proximal (4mm from proximal head), mid-diaphysis (8mm from the proximal head), and the distal (3mm from the distal head). Scale (A) 1mm. High- power confocal images were taken of the cortical bone in young (B), middle-aged (C), and old (D) mice, with a sclerostin (red)/ DAPI(blue) overlay. Scale (B-D) at 5 μm. (E) Histogram representing the percentage of osteocytes expressing detectable levels of sclerostin-IR in diaphyseal bone for young, middle-age, and old mice at the proximal, mid-diaphysis, and distal location of the bone. The numbers above the bars indicate how many animals from each age group were analyzed.
All age groups showed significant statistical difference from each other, except middle-aged v. old on the proximal diaphysis (with $p > 0.06$). Error bars represent mean ± SEM; $p < 0.05$ after a one-way ANOVA.
Figure 5 Schematic model illustrating the potential mechanisms of how blocking sclerostin would affect osteoblast proliferation and differentiation. MicroRNAs may also play a significant role in regulating the sclerostin-positive and sclerostin-negative osteocytes found in bone. Figure modified from Dijke et al., 2008.
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