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Differential Expression of Sclerostin In Adult and Juvenile Mouse Calvaria

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Abstract

Background—An understanding of the molecular mechanisms controlling bone formation is central to skeletal tissue engineering efforts. The observation that immature animals are able to heal calvarial defects while adult animals are not has proven to be a useful tool for examining these mechanisms. Thus, we compared expression of sclerostin, a bone inhibitor, between the calvaria of juvenile and adult mice.

Methods—Parietal bone was harvested from juvenile (6 day old, $n=20$) and adult (60 day old, $n=20$) mice. Sclerostin transcript and protein levels were compared between the parietal bone of juvenile and adult mice using polymerase chain reaction (PCR), Western blotting, and immunohistochemistry (IHC). Finally, osteoblasts from the parietal bone of juvenile and adult mice were harvested and cultured under osteogenic differentiation conditions with and without recombinant sclerostin (200ng/ml). Terminal osteogenic differentiation was assessed at 21 days with Alizarin red staining.

Results—PCR, Western blot analysis, and IHC all confirmed greater expression of sclerostin in the parietal bone of adult mice when compared to that of juvenile mice. Osteoblasts, whether from juvenile or adult parietal bones, demonstrated reduced capacity for osteogenic differentiation when exposed to recombinant sclerostin.

Conclusion—Given sclerostin's role in inhibiting bone formation, our findings suggest that differences in expression levels of sclerostin may play a role in the differential regenerative capacity of calvaria from juvenile and adult animals. These findings suggest it as a potential target to abrogate in future tissue engineering studies.

Keywords

Calvarial Healing; Sclerostin; Parietal Bone

Introduction

Inherent to any biological process are dynamic interactions between molecular agonists and antagonists. The sum of their actions ultimately dictates the biological outcome. The process of bone formation can be viewed through such a lens. The effects of signaling factors such as bone morphogenetic proteins (BMPs), Indian hedgehog (Ihh), and the Wnt family are

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balanced by their respective antagonists. Skeletal tissue engineering seeks to understand and harness these interactions towards bone formation. One such molecule that has recently been identified as an important regulator of bone formation is sclerostin, a glycoprotein encoded by the gene *Sost*. Sclerostin was originally thought to be a BMP antagonist, but recent evidence points to its role as an inhibitor of Wnt signaling via its binding to the Wnt co-receptor, low density lipoprotein receptor-related protein-5 and -6 (LRP5/6).¹⁻³ Of interest, patients with sclerosteosis or Van Buchem disease, both characterized by loss-of-function SOST genotype, display a generalized hyperostosis phenotype.⁴⁻⁶ Conversely, transgenic mice which overexpress sclerostin are characterized by an osteoporotic skeletal system.⁷ Given that juvenile animals and younger children are able to heal calvarial defects while adults are not, this study seeks to investigate whether differences in sclerostin expression underlies this observation.^{8, 9}

Current clinical strategies for addressing critical-sized calvarial defects in patients include the use of autogenous bone grafts, allogeneic bone materials, and prosthetic substitutes, each with their inherent disadvantages, including donor site morbidity, disease transmission, infection, and hardware failure, respectively.¹⁰ Efforts in regenerative medicine are directed at fostering robust bone formation to circumvent these issues. Although the focus of such an approach is often aimed at identifying an osteoprogenitor cell population or an appropriate scaffold, an understanding of the molecular mechanisms which regulate bone formation is without question of equal importance. Finding key, molecular regulators of osteogenesis will facilitate their incorporation in future skeletal regenerative applications as an adjunct to cell-based therapies.

Given the ability of juvenile animals and young humans to heal calvarial defects and the inability of adults to do so, we sought to examine whether differences in sclerostin expression underlie this observation in the mouse model.^{8, 9} We hypothesized that sclerostin expression may be higher in the parietal bone of adult mice when compared to juvenile mice. Gene transcript analysis, Western blotting, and immunohistochemistry were employed to investigate this question. (Figure 1)

Materials and Methods

Animals

All experiments were performed in accordance with Stanford University Animal Care and Use Committee guidelines. Six-day-old (juvenile) and 60-day-old (adult) CD-1 mice were purchased from Charles River Laboratories, Inc. (Wilmington, MA). Animals were housed in a light and temperature controlled environment and given food and water *ad libitum*.

Harvest of Parietal Bone

Juvenile and adult mice (n=20 per age group) were euthanized using hyperbaric carbon dioxide. The calvaria were excised and placed in phosphate buffered saline (PBS) on ice. With the aid of a dissecting microscope (Zeiss; Germany), the parietal bone was sharply excised from the calvaria, with care to remove the surrounding sutures. Under microscopic visualization, the dura mater and periosteal tissue were carefully removed from the parietal bone. Parietal bone from juvenile and adult mice were then pooled into their respective groups and finely minced into small fragments with sterile scissors.

RNA Isolation and Gene Transcript Analysis

For RNA isolation, minced parietal bone was homogenized in Trizol reagent (Invitrogen; Carlsbad, CA) using a mortar and pestle, followed by a repeated aspiration with 20 gauge needle and syringe. Total RNA was extracted using chloroform and 2-propanol.

RNA was reverse transcribed into cDNA using Multiscribe Reverse Transcriptase and T7 oligo dT primers (Applied Biosystems; Foster City, CA). Polymerase chain reaction was then performed to assess differences in expression of sclerostin. *Sost* and *Gapdh* primer sequences for *mus musculus* were obtained from Primer Bank:

Sost (5' → 3'): AGCCTTCAGGAATGATGCCAC

Gapdh (5' → 3'): AGGTCGGTGTGAACGGATTTG

PCR products were separated on a 2% agarose gel and bands visualized with ethidium bromide. Quantitative real-time PCR was then performed to analyze fold differences in sclerostin expression between juvenile and adult calvaria using Sybr Green Master Mix.

Protein Isolation and Western Blot Analysis

Bone lysates were prepared as described by Bellido *et al.*¹¹ In brief, harvested parietal bone were pooled from the respective age groups (n=20 mice/age group). The bones were mechanically homogenized on ice with a mortar and pestle in 2% sodium dodecyl sulfate, 2M urea, 10% glycerol, 10 mM Tris-HCl(pH 6.8), 10 mM dithiothreitol, and 1 mM phenylmethylsulfonylfluoride. This suspension was centrifuged at 15,000 rpm and the supernatant collected. The protein lysate was then quantified using the BCA protein assay (Pierce Biotechnology; Rockford, IL).

Fifty micrograms of protein lysate were electrophoresed on a 12% SDS-polyacrylamide gel (Biorad; Hercules, CA). The separated proteins were transferred to a PVDF membrane which was blocked in 5% non-fat milk for 1 hour. The membrane was incubated overnight with a polyclonal, goat anti-mouse sclerostin antibody at a 1:1000 dilution overnight at 4° C (R&D; Minneapolis, MN). The lower portion of the membrane was separately incubated with a monoclonal beta-actin antibody as a control for even loading. Following the primary antibody incubation, the membrane was incubated with the appropriate horse-peroxidase linked secondary antibody (Jackson Immunoresearch; West Grove, PA) at a 1:10,000 dilution for one hour and chemiluminescence detected using the ECL Plus System (Amersham; Waukesha, WI).

Immunohistochemistry

Parietal bone from juvenile and adult mice (n=3 per age group) were harvested and fixed overnight in 0.4% paraformaldehyde. Decalcification of the parietal bones was accomplished using 19% ethylenediaminetetraacetic acid (EDTA) for approximately two weeks. Once the calvaria were adequately decalcified, the parietal bones were prepared for imbedding in OCT. Five micron-thick cryosections of juvenile and adult mouse parietal bones were obtained. Cryosections were then subjected to boiling in 10 mM sodium citrate buffer (pH 6.0) for antigen retrieval for 10 minutes. Endogenous peroxidase activity was quenched using a 3% hydrogen peroxide solution for 10 minutes. Following this, specimens were blocked in rabbit normal serum and avidin/biotin blocking solution (Vector; Burlingame, CA) for 30 minutes. The sections were then incubated at 4° C overnight with a biotinylated anti-mouse SOST antibody (R&D). Color development was achieved the following day using the Vecatastain Elite® ABC Kit (Vector). The sections were analyzed and photographed with a Zeiss Axioplan 2 microscope equipped with an Axiocam HRC digital camera.

Osteoblast culture and differentiation

Parietal bones, excluding dura mater, periosteum, and suture-associated bone, were harvested from the skulls of juvenile and adult mice (n=20 per group), mechanically minced, and subjected to enzymatic digestion with 0.2% dispase II and 0.1% collagenase A (Roche

Diagnostics; Indianapolis, IN) in serum-free medium. The digestion was repeated 6 times for 10 min each for parietal bones from juvenile mice and 15 min each for parietal bones from adult mice, at 37°C in a waterbath shaker. The first 2 digestions were discarded. The latter 4 digestions were pooled, pelleted and resuspended in alpha-minimal essential medium (alpha-MEM) supplemented with 10% FBS. First-passage osteoblasts were plated at equal density and cultured to subconfluence in growth media (alpha-MEM, 10% FCS, 100 IU/ml penicillin and streptomycin).

First passage osteoblasts from juvenile and adult mice were cultured in osteogenic differentiation media (alpha-MEM media, 10% FBS, 100 µg/ml ascorbic acid, 10 mM β-glycerophosphate, 100 IU/ml penicillin, and 100 IU/ml streptomycin) with or without recombinant sclerostin (200ng/ml) for 21 days.

Mineralization of extracellular matrix (ECM) was assessed using Alizarin red S staining methods. Alizarin red S binds selectively to calcium, staining it red. Cells were washed with PBS twice and fixed with 70% ethanol at 20°C for 1 h followed by several washes with deionized water and then stained with 0.2% Alizarin red S for 20 min at room temperature followed by three washes with PBS. Thereafter, cell culture plates were air-dried and observed by light microscopy using a DMIL (DC 300) inverted microscope (Leica; Solms, Germany), and images were acquired using a ScanJet 5370C scanner and software. Alizarin red S staining was quantified by colorimetric assay. Briefly, Alizarin red S stained cells were incubated with 2 mL solution of 20% methanol 10% acetic acid under gently shaking conditions for 15 min at room temperature. Supernatants were collected and OD was measured at 450 nm. All values were normalized against protein concentration obtained from duplicate wells.

Statistics

Mean and standard deviation were calculated from numerical data, as presented in the text, figures, and figure legends. In graphs, columns represent means, whereas error bars represent 1 SD. Statistical analysis was performed using the Welch's two-tailed t test. A p value less than or equal to 0.05 was considered to be significant.

Results

PCR demonstrates increased sclerostin transcript expression in adult parietal bone

RT-PCR of transcripts isolated from parietal bones revealed a more intense band for sclerostin in the juvenile group when compared to the adult group. Quantitative real time PCR confirmed this difference and revealed sclerostin transcript levels to be nearly 2 fold greater in the adult than juvenile parietal bone when normalized to *Gapdh* expression. (Figure 2)

Western blot analysis confirms sclerostin protein expression in adult parietal bone

Immunoblotting of whole bone lysates obtained from the parietal bone of juvenile and adult mouse skulls again revealed greater expression of sclerostin in adult mice. The band detected in the adult and juvenile lanes were of the same molecular weight as the recombinant sclerostin detected in the control lane. Finally, probing the bony lysates with beta actin confirmed equal loading in all wells. (Figure 3)

Immunohistochemistry localizes expression in parietal bone

Immunohistochemistry (IHC) revealed sclerostin expression to be confined to the lacunae of the parietal bone. (Figure 4) This is consistent with previous reports of sclerostin expression

being specific to mature osteocytes. IHC did not reveal any significant expression of sclerostin in the dura mater or periosteum surrounding the parietal bone.

Notably, IHC revealed sclerostin expression to be pervasive throughout parietal bones from adult mice. In contrast, sclerostin expression was found to be relatively scant in the parietal bone of juvenile mice. This finding was consistent with our hypothesis that sclerostin expression is more highly expressed in adult mice.

Recombinant sclerostin equalizes osteogenic potential of juvenile and adult mouse osteoblast

To further confirm the role of sclerostin in mediating differences in osteogenic potential between juvenile and adult mice, we examined the effect of recombinant sclerostin on osteogenic differentiation between osteoblasts harvested from the parietal bone of juvenile and adult mice. It is known that osteoblasts from juvenile mice possess greater ability for osteogenic differentiation *in vitro*. After culturing juvenile and adult osteoblasts under osteogenic differentiation conditions for 21 days, osteoblasts that were differentiated in the presence of recombinant sclerostin (200ng/ml) demonstrated a reduced capacity for terminal osteogenic differentiation as shown by decreased Alizarin red staining. (Figure 5a) Quantification of Alizarin red staining also demonstrated that the increased capacity of juvenile osteoblasts for osteogenic differentiation compared to adult osteoblasts is negated by recombinant sclerostin. Osteoblasts treated with recombinant sclerostin, regardless of the age of the mice from which they were harvested, demonstrated equivalent and reduced capacity for extracellular mineralization. (Figure 5b)

Discussion

Shedding light on the molecular profile underlying the regenerative response of calvaria from young animals to injury is of great interest to skeletal tissue engineering efforts. In mice, this comparison is based on the observation that juveniles are able to nearly heal defects up to five millimeters in their parietal bone, while the same size defects made in adults resulted in marked absence of healing.⁸ This differential ability in calvarial regeneration has also been observed clinically. Paige and colleagues in a retrospective study of patients undergoing frontoorbital advancement and reconstruction found a statistical correlation between age of surgery and the ability of the patient to heal their calvarial defect.⁹ Calvarial defects resulting from these surgeries in children older than 9–11 months of age were unlikely to spontaneously close. Because of these observations in mice and humans, we utilized the mouse model to investigate whether differences in sclerostin expression between juvenile and adults exists.

Sclerostin, a glycoprotein within the DAN family encoded by the *Sost* gene, was originally thought to be a BMP antagonist because of its inhibition of BMP stimulated bone formation. However, *in vitro* studies have shown that sclerostin binds to BMPs with low affinity and does not bind preferentially to any of the different BMPs.^{7, 12} Data from several groups have pointed towards sclerostin's role as an antagonist of Wnt activity and, hence, indirectly inhibiting BMP action, given that BMPs are downstream of Wnt signaling.^{2–3, 13}

In vitro studies have yielded an unclear picture on the exact role of Wnt signaling on bone biology. Studies that demonstrate Wnt signaling to encourage osteoblast differentiation^{14, 15} are countered by studies that assert Wnt proteins inhibit the ability of mesenchymal stem cells to enter an osteogenic lineage.^{16, 17} Accounting for these different results is made challenging by the variety of experimental conditions and cell lines employed in these *in vitro* studies.

Nevertheless, the role of Wnt signaling on osteogenesis is strongly implicated in several genetic conditions and the development of key transgenic mice. Related to the findings of this paper, patients with sclerosteosis and Van Buchem disease, marked by nonsense mutations and deletions of the SOST gene respectively, are characterized by a generalized increase in bone mass.^{4, 5} In contrast, mice which over-express sclerostin, generalized osteopenia is observed.⁷ Further *in vivo* evidence for the role of Wnt signaling in regulating bone mass can be found in patients with loss-of-function mutations in LRP5, a Wnt co-receptor. This genetic mutation leads to osteoporosis pseudo-glioma syndrome, where patients are afflicted with extremely low bone mass. Conversely, patients with gain of function LRP5 mutations exhibit a high bone mass phenotype.^{18–20}

Ultimately, the goal of bone tissue engineering efforts is to accelerate the healing of critical-sized skeletal defects via the implantation of a competent cell population on a compatible scaffold, with the incorporation of key pro-osteogenic molecules. By probing for expression differences between juvenile and adult animals, which may explain their differences in regenerative capacity, we hope to identify potential molecular targets for such therapies. The identification of sclerostin's increased expression in the calvaria of adult mice, which are unable to spontaneously heal defects, may be instrumental towards that goal. Undoubtedly, future functional studies are required to confirm sclerostin as a worthy molecular target for bone tissue engineering efforts.

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Figure 1. Schematic outlining comparison of sclerostin expression between the parietal bone of juvenile and adult mice using gene transcript analysis, Western blotting, immunohistochemistry.

RT-PCR

Juvenile Adult

Sclerostin

GAPDH
QRT-PCR

Figure 2.

- a) RT-PCR revealed a more intense band for sclerostin transcripts in the adult group compared to the juvenile group.
- b) QRT-PCR revealed sclerostin expression to be 1.8 ± 0.2 (mean $\pm$ standard deviation) fold greater in adult parietal bone than juvenile parietal bone (* $p < 0.05$).

Figure 3.

Western blot analysis of bone lysates revealed a greater sclerostin protein levels from adult parietal bone than juvenile parietal bone. The top row of bands represents recombinant sclerostin (left), sclerostin from juvenile parietal bone lysates (middle), and sclerostin from adult parietal bone lysates (right). Western blot analysis for beta actin in the bottom row demonstrated equal protein loading between the juvenile and adult wells.

Figure 4.

Immunohistochemistry staining for sclerostin in juvenile (left) and adult (right) parietal bone cryosections, at low (10x) and high (63x) magnification. Note sclerostin staining (brown) localized to the lacunae of the parietal bone. IHC demonstrates pervasive staining for sclerostin in the adult parietal bone and scant staining in the juvenile parietal bone.

Figure 5.

a) Alizarin red staining of osteoblasts cultured under osteogenic differentiation conditions in the absence (top panels) and presence (bottom panels) of recombinant sclerostin (200ng/ml) demonstrated sclerostin to suppress extracellular matrix deposition by both juvenile and adult osteoblasts.

b) Quantification of Alizarin red staining confirmed that the presence of sclerostin abrogated terminal osteogenic differentiation of both juvenile and adult osteoblasts. Bars represent mean absorbance and error bars represent standard deviation. (* $p < 0.05$)