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Sost downregulation and local Wnt signaling are required for the osteogenic response to mechanical loading

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ABSTRACT

Sclerostin, the Wnt signaling antagonist encoded by the *Sost* gene, is secreted by osteocytes and inhibits bone formation by osteoblasts. Mechanical stimulation reduces sclerostin expression, suggesting that osteocytes might coordinate the osteogenic response to mechanical force by locally unleashing Wnt signaling. To investigate whether sclerostin downregulation is a pre-requisite for load-induced bone formation, we conducted experiments in transgenic mice (TG) engineered to maintain high levels of *SOST* expression during mechanical loading. This was accomplished by introducing a human *SOST* transgene driven by the 8 kb fragment of the DMP1 promoter that also provided osteocyte specificity of the transgene. Right ulnae were subjected to in vivo cyclic axial loading at equivalent strains for 1 min/day at 2 Hz; left ulnae served as internal controls. Endogenous murine *Sost* mRNA expression measured 24 h after 1 loading bout was decreased by about 50% in TG and wild type (WT) littermates. In contrast, human *SOST*, only expressed in TG mice, remained high after loading. Mice were loaded on 3 consecutive days and bone formation was quantified 16 days after initiation of loading. Periosteal bone formation in control ulnae was similar in WT and TG mice. Loading induced the expected strain-dependent increase in bone formation in WT mice, resulting from increases in both mineralizing surface (MS/BS) and mineral apposition rate (MAR). In contrast, load-induced bone formation was reduced by 70–85% in TG mice, due to lower MS/BS and complete inhibition of MAR. Moreover, Wnt target gene expression induced by loading in WT mice was absent in TG mice. Thus, downregulation of *Sost*/sclerostin in osteocytes is an obligatory step in the mechanotransduction cascade that activates Wnt signaling and directs osteogenesis to where bone is structurally needed.

Introduction

The skeleton adapts to meet mechanical needs by changing its mass, shape, and microarchitecture [1–3]. Osteocytes (former osteoblasts buried in the bone matrix) are proposed to act as mechanosensors [4]. Whereas bone-forming osteoblasts and bone-resorbing osteoclasts are present on bone surfaces for relatively short periods of time and in low numbers, osteocytes are by far the most abundant resident cells and are present throughout the entire bone tissue. Osteocytes are also the core of a functional syncytium that extends from the mineralized bone matrix to the bone surface and the bone marrow, reaching the blood vessels. Their abundance and strategic location make osteocytes the most suitable candidates for detecting variations in the level of strain and for distributing signals leading to adaptive responses [5].

Regulation of the expression of sclerostin, a glycoprotein encoded by the *Sost* gene, has emerged as a compelling mechanism by which osteocytes control the activity of bone remodeling cells [6]. This protein is secreted by osteocytes and acts in a paracrine (and potentially autocrine) fashion to inhibit bone formation by antagonizing the pro-differentiating and survival actions of Wnts in osteoblasts. Genetic and pharmacologic evidence supports this mechanism. Loss of *SOST* expression in humans causes the high bone mass disorders Van Buchem's disease [7] and sclerosteosis [8]. Mice with targeted deletion of the *Sost*

Abbreviations: DMP1, dentin matrix protein 1; PTHrP, PTH related protein; BMP, bone morphogenetic protein; Cx43, connexin 43; WT, wild type; TG, transgenic mice; ChoB, ribosomal protein S2; LRP, low density lipoprotein receptor-related protein; Tph1, Tryptophan hydroxylase 1

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gene also display progressive high bone mass and increased bone strength [9,10]; whereas, conversely, transgenic mice overexpressing *SOST* exhibit low bone mass [11–13]. Pharmacologic inhibition of sclerostin with neutralizing antibodies leads to marked anabolic effects in several preclinical osteopenic animal models and has been met with promising results in clinical settings [6,14]. Sclerostin is also regulated by hormonal stimuli that affect the skeleton. In particular, elevation of parathyroid hormone (PTH), either in an intermittent or a continuous mode, downregulates sclerostin expression in osteocytes in mice and decreases the circulating levels of the protein in humans [15–19].

Mechanical forces are essential for the development, growth, and maintenance of the skeleton. Skeletal sites subjected to high mechanical strains exhibit high bone formation, whereas unloaded bones display reduced bone formation. These adaptive responses of the skeleton are thought to be mediated by osteocytes and to result from regulation of the Wnt signaling pathway [4]. Using the murine ulnar loading model [20], we have demonstrated that cortical bone areas exposed to high mechanical strain exhibit a reduction in sclerostin-positive osteocytes that is associated with higher bone formation on adjacent periosteal surfaces [21]. This evidence suggested that osteocytes coordinate the osteogenic response to mechanical force by downregulating sclerostin, thereby locally unleashing Wnt signaling. We now show that mice overexpressing a human *SOST* transgene in osteocytes, which is not downregulated by loading, failed to exhibit activation of the Wnt pathway and the anabolic response to mechanical stimulation. Thus, *Sost* downregulation is an obligatory step for mechanotransduction.

Materials and methods

DMP1-SOST transgenic mice

DMP1-*SOST* transgenic mice were generated by inserting the human *SOST* cDNA (I.M.A.G.E. clone ID: 40009482, American Tissue Culture Collection, Manassas, VA) downstream from a DNA fragment containing 8 kb of the 5′-flanking region, the first exon, the first intron, and 17 bp of exon 2 of the murine dentin matrix protein 1 (DMP1) gene [22], and upstream from a 140 bp fragment containing the rabbit beta-globin polyadenylation sequence, as previously described [13]. Hemizygous DMP1-*SOST* mice or wild type littermates were used in the experiments. Mice were fed a regular diet (Harlan/Teklad #7001, Indianapolis, IN) and water ad libitum and maintained on a 12-h light/dark cycle. Protocols were approved by the Institutional Animal Care and Use Committee of Indiana University School of Medicine.

Bone mineral density (BMD) measurement and micro-computed tomography (Micro-CT) analysis

4, 8, and 16 week-old mice were anesthetized via inhalation of 2.5% isoflurane (Abbott Laboratories, Abbott Park, IL) mixed with O_2 (1.5 l/min) and BMD of the total body, excluding the head and the tail, lumbar spine (L1-6), and femur was measured by dual energy X-ray absorptiometry (DXA) using a PIXImus II densitometer (G.E. Medical Systems, Madison, WI), as previously described [23]. For micro-CT analysis, bones from 6, 10, and 16 week-old mice were dissected, cleaned off soft tissue, fixed in 10% buffered formalin, and stored in 70% ethanol until scanned at 6 micron resolution (Skyscan 1172, SkyScan, Kontich, Belgium). Bone length was measured using a digital sliding caliper after removing the soft tissue.

Ulna strain measurements

Strain levels at the midshaft ulna were measured in a cohort of 6 female mice at 16 weeks of age to derive the relation between applied force and mechanical strain for both genotypes, as previously published [24]. Briefly, the right forearm was dissected to expose the lateral surface of the ulnar diaphysis, and a miniature (EA-015DJ-120; Vishay, Inc.) single element strain gauge was bonded to the midshaft. The forearm was then placed in a computer-controlled electromagnetic mechanical actuator (Bose, Eden Prairie, MN), and exposed to cyclic axial compression using a 2-Hz haversine waveform. The peak force was progressively increased with each cycle, and ranged from 1.2 to 2.4 N. During loading, conditioned voltage output from the strain gauge and output from the load cell were recorded and processed as previously described [20]. Voltage output was converted to strain using previously described calibration procedures. From these data the force:strain relation was derived for both genotypes individually, and low, medium, and high loads for in vivo mechanical loading were calculated so that 2460, 2850, and 3240 με were generated, respectively, in both genotypes.

In vivo ulnar loading

To examine the effect of loading on bone formation, mice were loaded 1 min per day during 3 consecutive days and bones were collected after 16 days of initiation of loading to perform dynamic histomorphometry. Previous extensive evidence had shown that this loading regimen and timing of measurement is the optimal to detect the increased bone formation in areas of cortical bone exposed to high mechanical strain [20,24]. 6 to 8 female mice per group at 16 weeks of age were loaded on the right forearm for 1 min at 120 cycles/day (2 Hz) for 3 consecutive days at low, medium and high magnitude of strain, and sacrificed 16 days after initiation of loading [24]. Mice were injected with calcein (10 mg/kg) and alizarin red (15 mg/kg) (Sigma Chemical Co, St. Louis, MO), 10 and 3 days before sacrifice, respectively, as previously described [23]. The left ulnae served as non-loaded internal controls.

To examine the effect of loading on sclerostin and Wnt target gene expression, bones were collected after a single loading bout for mRNA expression or after 2 loading bouts for protein expression. This approach assures that data represent early changes in gene expression induced by loading rather than changes due to alterations in bone cell populations. We have used these time points for sample collection in our earlier publication demonstrating changes in Sost and sclerostin expression [21]. For mRNA analysis by quantitative PCR, ulnae were loaded once at high strain magnitude and mice were sacrificed 24 h later. Bones were snap-frozen in liquid nitrogen and stored at −80 °C until RNA isolation. For protein analyses by Western blotting and immunohistochemistry, mice were loaded for 2 consecutive days at high strain magnitude and sacrificed 24 h after the second loading bout. Ulnae were dissected and cleaned off soft tissue. Bones were snap-frozen in liquid nitrogen and stored at −80 °C until used for preparation of protein lysates, or fixed in 10% buffered formalin and stored in 70% ethanol at 4 °C until processed for immunohistochemistry.

Dynamic bone histomorphometry

Dynamic bone histomorphometric analysis was done as previously described [13]. Briefly, thick (100 μm) cross-sections at the mid-diaphysis of ulnae embedded in methyl methacrylate were prepared using a diamond embedded wire saw (Histosaw, Delaware Diamond Knives, Wilmington, DE) and ground to a final thickness of around 40 μm. Total, single, and double labeled perimeter, and inter-label width were measured on periosteal surfaces using a semiautomatic analysis system (Bioquant OSTEO 7.20.10, Bioquant Image Analysis Co., Nashville, TN) attached to a microscope equipped with an ultraviolet light source (Nikon Optiphot 2 microscope, Melville, NY). The terminology and units used are those recommended by the Histomorphometry Nomenclature Committee of the American Society for Bone and Mineral Research [25].

Quantitative PCR

Total RNA was extracted from ulnar mid-diaphysis (approximately 1/3 of the bone) using Ultraspec reagent (Biotecx Laboratories,