Distinct modes of inhibition by Sclerostin on bone morphogenetic protein and Wnt signaling pathways


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Chapter 5

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5.1 Abstract

Sclerostin is expressed by osteocytes and has catabolic effects on bone. It has been shown to antagonize bone morphogenetic protein (BMP) and/or Wnt activity, although at present the underlying mechanisms are unclear. Consistent with previous findings, Sclerostin opposed direct Wnt3a but not direct BMP-7 induced responses when both ligand and antagonist were provided exogenously to cells. However, we found that when both proteins are expressed in the same cell, Sclerostin can antagonize BMP signaling directly by inhibiting BMP-7 secretion. Sclerostin interacts with both the BMP-7 mature- and pro-domain, leading to intracellular retention and proteasomal degradation of BMP-7. Analysis of Sclerostin knock-out mice revealed an inhibitory action of Sclerostin on Wnt signaling in both osteoblasts and osteocytes in cortical and cancellous bones. BMP-7 signaling was predominantly inhibited by Sclerostin in osteocytes of the calcaneus and the cortical bone of the tibia. Our results suggest that Sclerostin exerts its potent bone catabolic effects by antagonizing Wnt signaling in a paracrine and autocrine manner, and BMP signaling selectively in the osteocytes that synthesize simultaneously both Sclerostin and BMP-7 proteins.

5.2 Introduction

Sclerosteosis (OMIM: 269500) and van Buchem disease (OMIM: 239100) are closely related, rare high bone mass disorders that have been linked to a deficiency in expression of Sclerostin, encoded by the SOST gene [3, 4, 5, 13, 46, 56, 44, 63]. Sclerostin is an osteocyte derived negative regulator of bone formation belonging to the DAN family of secreted glycoproteins. Members of the DAN family were shown to have the capacity to inhibit BMP- and/or Wnt activity [1, 6, 24, 43, 60]. Since
Sclerostin binds albeit weakly to mature bone morphogenetic proteins (BMPs) it initially was presumed to be a BMP antagonist, however, currently Sclerostin is believed to mediate its inhibitory effect on bone formation by directly blocking the Wnt signaling pathway [1, 61, 70]. Canonical Wnt signaling has been described to play a crucial role in several bone mass disorders. Wnt proteins transduce their signals via 7-transmembrane spanning receptors of the frizzled family and lipoprotein receptor-related protein-5/-6 (LRP-5/-6) thereby controlling the stability of cytoplasmic β-catenin. In the absence of Wnt ligands, β-catenin forms a complex with Adenomatous Polyposis Coli (APC), Axin, Glycogen Synthase Kinase 3 (GSK3) and casein kinase I (CK1). This complex facilitates phosphorylation and subsequent proteasomal degradation of β-catenin. In the presence of Wnt ligands, this complex dissociates and β-catenin accumulates and translocates into the nucleus, where it forms complexes with TCF/LeF1 transcription factors and initiates transcription of target genes [39]. The importance of Wnt signaling in bone formation is illustrated by the low-bone-mass osteoporosis-pseudoglioma syndrome (OPPG) or high-bone-mass (HBM) phenotype caused by missense loss or gain of function mutations in LRP-5, respectively [22, 34, 12, 65]. Sclerostin was found to act as a direct extracellular antagonist of canonical Wnt signaling by binding to LRP-5 and LRP-6 [32, 53]. Several mutants that cause the LRP-5 HBM trait are actually defective in Sclerostin-binding, thereby making them resistant to Sclerostin-mediated inhibition [53, 2]. BMPs were originally identified by their ability to induce bone and cartilage formation. They are required for skeletal development and maintenance of adult bone homeostasis, and play an important role in fracture healing [15, 16, 21]. BMPs are expressed in an inactive pro-form and proteolytic cleavage by furin proteases is required to release the mature BMP proteins [25]. BMPs signal via heteromeric complexes of type-I and type-II serine/threonine receptor kinases. Intracellular signaling is initiated by type I receptor mediated phosphorylation of BMP receptor-regulated Smads, Smad-1, -5 and -8 at two serine residues at their C-termini. Activated R-Smads can associate with the Co-Smad, Smad-4, and translocate into the nucleus. These heteromeric Smad complexes, in cooperation with other transcription factors, co-activators and repressors interact with promoters of target genes and control their transcription [18, 38]. To gain more insight into the molecular mechanisms by which Sclerostin antagonizes bone formation, we investigated the inhibitory effects of Sclerostin on Wnt and BMP signaling. In addition to its negative effect on Wnt/β-catenin signaling, we unexpectedly observed that Sclerostin inhibits bone formation by mitigating the secretion of BMP-7 in osteocytes. Our results reconcile previously published contradictory observations on direct inhibitory effects or lack thereof of Sclerostin on responses elicited by different BMP family members.
5.3 Experimental Procedures

Cells
SAOS-2 human osteosarcoma cells, Hek293 and Hek293T cells and C2C12 cells stably transfected with the BRE-luciferase reporter [75] were cultured in 4.5 g/l Glucose Dulbecco’s modified Eagle’s medium (Gibco). Saos-2 cell lines were lenti-virally transduced in order to express either a non-targeting control shRNA (shRNA control CC GGCAACAAGATGAAGAGCACCAACTCGAGTTGGTGCTTTTCACTTTTGTGGTTTT T; Sigma) or shRNA targeting SOST (shRNA #1 CCGGCCACAACCAGTCGGAGCT AACTCGAGTGAGCTCGACTTGTTGTTTTTG; shRNA #2 CCGGGCTGGA GACCCAAACCATACTCGAGATGGTCTTGTTTCTCTCAGCTTTTTG; Sigma), followed by puromycin selection (5 μg/ml) to obtain SOST-knockdown Saos-2 cells. Mouse mesenchymal KS483 cells were cultured in α-MEM (Gibco) [71]. All media were supplemented with 10% fetal bovine serum (Integro) 100 IU/ml penicillin, and 100 IU/ml streptomycin (Invitrogen).

Expression plasmids
The expression constructs pcDNA3-hBMP-7 as well as the pGEM3zf-mWnt3a expression construct have been previously described [62]. C-terminally 3× HA-tagged human BMP-7 pro-peptide expressing construct was generated using PCR approach. Primers: 5’ATTGGTACCTAGAGCCGGCGCGATGCGACG-3’ (5’, containing ATG codon and KpnI restriction site) and 5’GCCAAGCTTATGCTGCGGAAGTGGACCTC-3’ (3’, containing HindIII restriction site) were used to amplify a fragment of hBMP-7 cDNA that contains the signal peptide and a propeptide part. The purified PCR fragment was cloned in frame 5’ of the 3× HA encoding sequence in 3× HA-pBlueScript vector. The pcDNA3 mLRP-5-N-mycΔ expression construct was kindly provided by Xi He (Children’s Hospital, Harvard, MA) [53] and pcDNA3-hLRP-5 (wtLRP-5) by Wendy Balemans (University of Antwerp, Belgium). The pcDNA3-hSOST as well as the pST-hSOST-N-Flag construct were kindly provided by Brian Kovacevich (Renton, WA). The BMP- and TGF-β-responsive luciferase reporter constructs (BRE-luc and CAGA12-luc, respectively) as well as the Wnt-responsive luciferase reporter construct (BAT-luc) have been previously described [28, 37]. The expression constructs for Smad-1 wild type (wt) as well as the Smad mutant (GM) deficient in phosphorylation of the linker region through GSK3β were kindly provided by Edward De Robertis (Howard Hughes Medical Institute, Los Angeles, CA) [20]. Dominant negative TCF4 (pcDNA-TCF4d/n) and constitutively active β- catenin mutant S33Y were generously provided by Hans Clevers (Hubrecht Laboratory, The Netherlands). Preparation of myc-His-Ubiquitin has been previously described [74].

Reagents and recombinant proteins
Recombinant human BMP-7 was kindly provided by K. Sampath (Creative Biomolecules, Inc., Hoptinton, MA). Recombinant mouse Wnt-3a and Dkk-1 were purchased from
R&D systems. Recombinant mouse and human Sclerostin were prepared as previously described [68]. For Biacore measurements rec.BMP-7 was obtained from Wyeth, LRP-6 was purchased from R&D Systems, the extracellular domain of IL-4R was obtained from Walter Sebald (Biocenter, University Wuerzburg) and the control Fab fragment AbD09095 was obtained from AbD (Morphosys Inc.). MG132 was purchased from Calbiochem.

**RNA isolation and quantitative real-time PCR**

Total RNA was isolated using the RNeasy kit (Macherey-Nagel) according to the manufacturer’s instructions. RT-PCR was performed using the RevertAidTM H Minus First Strand cDNA Synthesis Kit (Fermentas) according to the manufacturer’s instructions. Expression of human SOST, BMP-7 and GAPDH were analyzed using the following primers: SOST, 5'-GAGCAGGCATCACAAAATCA-3' and 5'-CAAGTCCCAGGTGGAAGAAT-3'; BMP-7, 5'-CCACCTGTAATCCCAGCACT-3' and 5'-GACAAAGACCAGAGGTTCCA-3'; GAPDH, 5'-ATCACTGCCACCCAGAGAC-3' and 5'-ATGAGGTCCACCACTTGT-3'. All conditions were measured in duplicate and Taqman PCR reactions were performed using the StepOne PlusTM Real-Time PCR System (Applied Biosystems). Gene transcription levels were determined with the comparative ΔΔCt method using GAPDH as reference and the non-stimulated condition was set to one.

**Luciferase reporter assays**

Cells were transiently transfected using FuGENE 6 (Roche) transfection reagent, with 0.1μg firefly luciferase reporter construct, 0.1μg β- galactosidase, together with the indicated expression plasmids or empty control plasmid (pcDNA3.1), according to manufacturer’s instructions. Cells were allowed to recover for 18 hours, followed by serum-starvation and stimulation with the indicated ligands for 18 hours. Afterwards CM was collected and cells were washed with PBS and lysed. C2C12-BRE-luc cells were stimulated with CM for 18 hours and lysed. Luciferase assays were performed using the Luciferase Reporter assay system (Promega) according to the manufacturer’s instructions using β-galactosidase activity as an internal control. Each transfection was carried out in triplicate and representative experiments are shown.

**CM, ubiquitination and western blot analysis**

Cells were transfected with respective expression constructs or empty control plasmid (pcDNA3.1) using the calcium phosphate precipitation method. The following day, the cells were stimulated with the indicated ligands for 18 hours and lysed. For western blot, ubiquitination and co- immunoprecipitation analysis cells were allowed to grow to 70% confluence and serum concentration was reduced to 0.5% overnight. For co-immunoprecipitation, cells were lysed in ice cold lysis buffer (50mM Tris-HCl (pH 7.5), 100mM NaCl, 0.2% Tween-20, and 1 protease inhibitor cocktail tablet (Roche) per 50 ml). Cell lysates (400μg of total protein) or CM (50ml) were
incubated with 1μg/μl of anti-Flag M2 (Sigma) or anti-c-Myc (9E10) (Santa Cruz Biotechnology) antibody for 1.5 hours at 4°C. 0.5μg/μl of protein A-Sepharose beads (Amersham Biosciences) was added and incubated for additional 1.5 hours at 4°C. Beads were extensively washed with ice cold lysis buffer containing 500mM NaCl and eluted protein complexes were subjected to SDS-PAGE and western blot analysis. Western blot analyses were performed as previously described [51]. C-terminal Smad phosphorylation was detected using an antibody specifically recognizing phosphorylated Smad-1/-5/-8 (P-Smad-1), which has been described previously [42]. The linker phosphorylation of Smads through GSK3β or MAPK signaling was investigated through the use of P-Smad-GSK3 and P-Smad-MAPK antibodies obtained from the DeRobertis laboratory [20]. BMP-7 expression was investigated with a BMP-7 antibody kindly provided by Hicham Alaoui (Stryker Biotech, Hopkinton, MA). SOST-N-Flag expression and LRP-5-N-myc expression were detected using anti-Flag M2 (Sigma) anti-c-Myc (9E10) (Santa Cruz Biotechnology), respectively. pro-BMP-7-HA and pro-TGF-β-HA were detected via an anti-HA (12CA5) antibody (Roche). Ubiquitination assays were performed as previously described [52]. The myc-His-Ubiquitin was detected through a clone HIS-1 antibody (Sigma). Equal loading was confirmed using an anti-β-actin antibody (Sigma) or anti-Smad-1 antibody (Zymed). Secondary horseradish peroxidase-conjugated goat anti-rabbit and sheep anti-mouse IgG antibodies (Amersham Bioscience, Inc.) were used for detection using enhanced chemiluminescence (ECL, Amersham Biosciences, Inc.). Quantitative western Blotting was performed using secondary goat-anti-rabbit IRDye 680 and goat-anti-mouse IRDye 800CW (LI-COR) using the Odyssey Scanner according to the manufacturer's instructions.

**ALP activity assay**

KS483 cells were seeded in 96-well plates and grown until confluent. Cells were stimulated with indicated ligands in 0.2% FBS-containing α-MEM. ALP activity was measured kinetically after another 4 days of culture. Measurements were corrected for protein content using the DC Protein Assay (Bio-Rad).

**Immunofluorescence**

For immunofluorescence staining, KS483 cells were grown on coverslips overnight and stimulated the next day for 3 hours with the indicated ligands. Cells were fixed with 4% paraformaldehyde, washed twice with PBS, quenched with 20 mM NH4Cl and permeabilized with 0.1% Triton X-100. Afterwards cells were incubated in blocking solution (PBS containing 3% bovine serum albumin) for 45 min, followed by incubation with anti-β-catenin antibody (BD Transduction Laboratories), suitably diluted in blocking solution for 1 hour. After washing, labeled secondary antibodies AlexaFluor 488 goat anti-mouse IgG (Invitrogen) were used for detection. Nuclei were stained using Hoechst 33258 (Invitrogen) according to the manufacturer's instructions.
instructions. Specimens were visualized with an Olympus IX51 microscope at 100× magnification using the cellF Soft Imagine System (Olympus).

Sclerostin knock out mice

A knockout allele of Sost was generated via KOMP (line VG10069). The entire protein-coding sequence of Sost was replaced with LacZ and a floxed Neo cassette, in a manner such that the initiating ATG of Sost became the initiating Methionine of LacZ. All the details regarding the generation of this allele can be found at http://www.velocigene.com/komp/detail/10069. Sost-LacZ/+ mice were generated using clone VG10069A-G10 (Supplementary Figure S1). This allele has been tested and it phenocopies other published knockout lines of Sost (Supplementary Figure S2).

Immunohistochemistry, Alcain & TUNEL assay

Dissected bone tissues from 4 month old female Sclerostin KO mice or wild type mice were fixed overnight in 10% paraformaldehyde pH 6.8 at 4°C, decalcified using 10% EDTA pH 7.4, and processed for paraffin embedding and sectioning. Before blocking endogenous peroxidase activity with 40% methanol 1% H_2O_2 in PBS, sections were deparaffinized and rehydrated using Xylene and a descending alcohol series. Antigen retrieval using Proteinase K (2.5 μl in 100 mM Tris pH 9.0 and 50 mM EDTA pH8.0), for 10 min. at 37°C was followed by three washes with 0.1M Tris buffered saline (pH 7.4) containing 0.02% Tween 20 (TNT). Thereafter slides were incubated with 5% species-specific serum (Dako, Denmark) in 0.5% Boehringer Blocking reagent (Boehringer) in TNT for 60 min. at 37°C. Subsequently, the following antibodies diluted in 0.5% Boehringer Milk Powder/TNT were applied overnight at 4°C: rabbit anti-BMP-7 antibody (1:100) (Stryker), mouse anti-β-catenin antibody (1:100) (BD Transduction Laboratories), rabbit anti-PS-1 antibody directed against C-terminal phosphorylated Smad-1/-5/-8 (1:50) [42] and goat anti-Sclerostin antibody (1:400) (R&D). A species-specific biotinylated anti-IgG antibody (1:300 diluted in 0.5% Boehringer Milk Powder/TNT), followed for 45 min. at 37°C. Incubation with streptavidin horseradish peroxidase (1:200 in 0.5% Boehringer Milk Powder/TNT) for 30 min. at 37°C preceded and followed an amplification step using Biotinyl Tyramine. Stainings were carried out using amino-9-ethyl-carbazole (AEC) (Sigma Chemicals, Zwijndrecht, The Netherlands) and Mayers Hematoxyline (Merck, Amsterdam, The Netherlands) according to the manufactures instructions. A water based mounting solution was applied and stainings were visualized with an Olympus IX51 microscope using the cellF Soft Imagine System (Olympus). To discriminate cartilage from bone Alcian Blue staining was performed. Histology sections were incubated in 1% Alcian Blue 8GX (pH 2.5) in 3% acetic acid (Merck) for 15 min. before rinsing the slides with distilled water. Counterstaining with toluylene red (Merck) was performed for 1 min according to the manufactures instructions. To detect apoptosis, a Dead End Colorimetric TUNEL system (Promega) was used according to the manu-
facturer's instructions. The number of total positive osteocytes in the indicated areas were counted manually and the percentage of apoptotic cells was calculated.

In vitro interaction analysis using surface plasmon resonance

LRP-6, BMP-7, IL-4R and AbD09095 proteins were biotinylated using NHS-LC-Biotin (Pierce) according the manufacturer’s recommendation. To minimized inactivation due to multiple biotinylation the molar stoichiometry of protein to NHS-LC- Biotin was kept at 1:1. The surface of a BIAcore CM5 biosensor was first activated via NHS/EDC and streptavidin was subsequently coupled to the activated surface to a final density of 3000 resonance units (RU). Biotinylated proteins were then immobilized to surface density of 200 (BMP-7) to 550 RU (LRP-6). Due to the high positive charge of Sclerostin we used IL-4R, which is highly negatively charged, and the anti-Sclerostin Fab antibody AbD09095 to establish experimental conditions that show no unspecific binding of Sclerostin (used as analyte) to the biosensor surface. Different running buffer with varying salt concentrations were tested showing that upon addition of 300mM NaCl no interaction with the charge-complementary IL-4R is observed and at the same time binding to the anti-Sclerostin antibody AbD09095 is unaltered. Interaction of Sclerostin and variants with BMP-7 and LRP-6 were then measured by perfusing the biosensor using 6 different concentrations for each analyte. All experiments were performed using HBS300 buffer (10 mM HEPES pH 7.5, 300 mM NaCl, 0.005% surfactant P20) as running buffer. The association phase was monitored by injecting the analyte for 300 seconds at a flow rate of $10 \mu l$ per minute. The dissociation phase was initiated by perfusing only HBS300 buffer and monitored for 200 seconds. The biosensor surface was regenerated by perfusing a short (60 seconds) pulse of buffer containing 6 M urea, 1 M sodium chloride, 0.1 M acetic acid. Sensorgrams were evaluated using the software BIAevaluation version 2.4. Due to the fast association and dissociation kinetics binding coefficients were deduced from the dose dependency of equilibrium binding.

Statistical analysis

Values are expressed as mean ± SD. For statistical comparison of two samples, an unpaired two-tailed Student's t-test was used to determine the significance of differences between means. All relevant comparisons were considered to be significantly different ($p < 0.05 = \ast, p < 0.01 = \ast \ast, p < 0.001 = \ast \ast \ast$). Experiments were performed at least three times and representative results are shown.

5.4 Results

5.4.1 Sclerostin inhibits Wnt signaling

Previously we reported that Sclerostin antagonizes BMP-stimulated bone formation, not by direct inhibitory effects on exogenous BMPs, but by targeting the Wnt pathway.
Sclerostin was found to share many characteristics with the Wnt antagonist dickkopf-1 (Dkk1) in antagonizing BMP-stimulated osteoblast differentiation and direct Wnt-induced responses [62]. Results from others had indicated that Sclerostin (and its close homolog Sostdc1) may target BMP signaling directly by sequestering BMPs and thereby preventing receptor binding [1, 70]. Some of these experiments were performed not by stimulating cells with exogenous ligands, but instead by transfecting expression plasmids or by mRNA injections [62, 72]. This made us revisit the interplay between Sclerostin with Wnt and BMP. First we confirmed that Sclerostin has an antagonistic effect on BMP or Wnt-induced osteoblast differentiation.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>$K_p(\text{seq})$ LRP-6</th>
<th>$K_p(\text{seq})$ BMP-7</th>
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<tbody>
<tr>
<td>mSOST FL</td>
<td>2(\mu\text{M})</td>
<td>1(\mu\text{M})</td>
</tr>
<tr>
<td>hSOST FL</td>
<td>8.6(\mu\text{M})</td>
<td>3.8(\mu\text{M})</td>
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Table 1: Binding parameters for Sclerostin binding to BMP-7 and LRP-6. Full-length murine SOST (mSOST FL) and full-length (FL) human SOST (hSOST FL) were tested using plasmon resonance as visualized in supplemental Figure S3A.

We stimulated KS483 osteoprogenitor cells with either recombinant BMP-7 or recombinant Wnt3a in the presence or absence of recombinant Sclerostin. Sclerostin inhibited the BMP-7 and Wnt3a induced activity of the early marker for osteoblast differentiation alkaline phosphatase (ALP) in a dose dependent manner (Figure 1A). Sclerostin has been shown to inhibit canonical Wnt signaling by binding to the LRP-5 and LRP-6 co-receptors [32, 53]. To confirm the association of Sclerostin with LRP-5, we co-transfected SOST and LRP-5 in Hek293T cells and performed co-precipitation experiments. Indeed, Sclerostin co-immunoprecipitated with LRP-5 and vice versa (Figure 1B). Surprisingly, binding to the Sclerostin receptor LRP-6 as measured through surface plasmon resonance (BIAcore) revealed a rather low binding affinity with $K_D(\text{eq})$ values ranging from 2\(\mu\text{M}\) to 8.6\(\mu\text{M}\) for full-length murine and human Sclerostin, respectively (Supplementary Figure S3A and Table 1). Moreover, we investigated by immunofluorescence the effect of Sclerostin on Wnt-induced changes in subcellular localization of $\beta$-catenin in KS483 cells. Upon stimulation with recombinant Wnt3a, $\beta$-catenin translocated to the nucleus, which was completely blocked by Dkk1 and attenuated in approximately 60% of the cells by Sclerostin (Figure 1C, Supplementary Figure S3B). Co-transfection of a Wnt3a expression plasmid in Hek293T cells resulted in a 3-fold activation of the Wnt- reporter construct BAT-luciferase (Figure 1D). As expected, Sclerostin antagonized this Wnt3a- induced reporter activity. Interestingly, co-expression of Sclerostin and LRP-5 did not reduce, but significantly increased Wnt reporter activity. When Wnt3a, LRP-5 and SOST were co-expressed, a reduction of the Wnt-induced reporter activity was achieved compared to LRP-5/Sclerostin co-expression (Figure 1D). Consistent with previous reports [32, 53, 62] we show that Sclerostin inhibits Wnt/LRP-5 signaling.
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Figure 1: Sclerostin inhibits BMP-induced Alkaline Phosphatase (ALP) activity and Wnt/β-catenin signaling pathway. (A) KS483 cells were stimulated with recombinant BMP-7 (2nM) or recombinant Wnt3a (2.5nM) in the absence or presence of recombinant Sclerostin (4−20nM) for 4 days. (B) Co-immunoprecipitation of LRP-5 and Sclerostin. Sclerostin or LRP-5 containing cell lysates of (co)-transfected HEK293T cells were subjected to immunoprecipitation followed by Western Blotting and probed with indicated antibodies (+ = 10μg expression plasmid) (C) Sclerostin and Dkk-1 inhibit Wnt-induced nuclear translocation of β-catenin. KS483 cells were treated for 3 hours with recombinant Wnt3a (2.5nM, 3B) in the presence or absence of recombinant Dkk-1 (5nM, 3D) or recombinant Sclerostin (10nM, 3F). (D) Transfection of SOST expression plasmid inhibits Wnt/LRP-5-induced transcriptional reporter (BAT-luc) activity in Hek293. Cells were (co-)transfected with Wnt3a (0.3μg), LRP-5 (0.3μg) and SOST (0.3μg) expression plasmids (alone or in combinations); empty pcDNA expression plasmid was added to make total amount of DNA in each transfection equal. Luciferase activity was measured after 36 hours after transfection. Normalized reporter activity is shown as the mean ± S.D. of triplicates.

5.4.2 Sclerostin inhibits BMP signaling

Previous studies had shown that Sclerostin could bind BMPs, but that the affinity is rather low. To investigate binding of Sclerostin to BMP-7, we performed immunoprecipitation experiments on Hek293T cells co-transfected with BMP-7 and SOST expression plasmids. BMP-7 and Sclerostin were found to interact in conditioned
media (CM) of these cells (Figure 2A). Notably, a decrease in BMP-7 expression was detectable in the presence of Sclerostin compared to when BMP-7 was expressed alone. However when expressed separately and immunoprecipitation was performed on mixed CM, no binding of Sclerostin to BMP-7 could be detected (data not shown). Binding of Sclerostin to BMP-7 as investigated through BIAcore measurements revealed similar low affinity of Sclerostin to BMP-7 as to LRP-6 with KD(eq) values ranging from 1 μM to 3.8 μM for full-length murine and human Sclerostin, respectively (Supplementary Figure S3A, Table 1). In line with this low affinity interaction and consistent with previous results we showed that Sclerostin does not inhibit direct exogenous BMP-induced Smad-mediated responses; recombinant BMP-7 induced C-terminal Smad-1/-5/-8 phosphorylation and Smad-1/Smad-4 driven BRE-luc transcriptional response, were not affected by recombinant Sclerostin (Figure 2B and 2C; lane 1-7) or transfected SOST expression plasmid (data not shown) [62]. Interestingly, however, we observed that when SOST and BMP-7 are co-transfected, Sclerostin can dose-dependently inhibit BMP-7/Smad induced signaling responses (Figure 2B and 2C; lane 8-14).

These results together with the inhibition of BMP-7 secretion by Sclerostin (Figure 2B) suggested that Sclerostin might retain BMP-7 intracellularly when co-expressed. Therefore we investigated whether the pro-domain of BMP-7 plays a role in the interaction with Sclerostin. We first noted that the pro-domain of BMP-7 (lacking the mature domain) is secreted when expressed alone whereas co-expression with Sclerostin eliminated the observed secretion of the BMP-7 pro-domain (Figure 2D). When co-expressed, binding of Sclerostin to the isolated pro-domain of BMP-7 was observed in the cell lysates (Figure 2D and 2E). To rule out non-specific binding of Sclerostin to BMPs we simultaneously performed the co-immunoprecipitations with pro-TGF-β1. No sequestration of TGF-β1 prodomain (data not shown) or binding with Sclerostin was detectable (Figure 2E).

5.4.3 Reduced SOST expression leads to elevated BMP signaling

Since we found that Sclerostin can inhibit BMP signaling when BMPs and SOST were co-transfected, we wondered whether the investigated effects are truly Sclerostin dependent. We therefore studied the effects of shRNA-mediated SOST knockdown in the human osteogenic sarcoma cell line Saos-2, which displays endogenous SOST expression [54]. Upon BMP-7 stimulation SOST mRNA levels in Saos-2 cells expressing a non-targeting shRNA were significantly induced (Figure 3A; lane 2-3). In cells expressing one of two independent SOST targeting shRNAs, the SOST mRNA expression remained at basal levels during BMP-7 stimulation (Figure 3A; lane 4-9) while mRNA expression of BMP-7 remained unaffected (data not shown). Notably, in the background of the SOST targeting shRNAs, BMP-7 induced Smad-1/-5/-8 phosphorylation and BRE-luc activity were significantly increased in case of BMP-7 overexpression compared to control shRNAs (Figure 3B and 3C, respectively). The inductive properties of recombinant BMP-7 were also marginally elevated upon SOST knockdown (Figure 3B and 3C), which can be due to the self-induction of BMP expression (data
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not shown). Similar results where obtained using SOST targeting siRNAs in Hek293T and MG63 cell lines (data not shown). No effects of SOST-targeting shRNAs were detected on TGF-β1-induced CAGA12 luciferase transcriptional reporter activity (Supplementary Figure S4). Altogether, these findings suggest that Sclerostin is directly involved in the regulation of autocrine BMP-induced responses.

Figure 2: Sclerostin inhibits BMP/Smad signaling and interacts with intracellular BMP. (A) HEK293T cells were (co)-transfected with expression plasmids for BMP-7, SOST alone or together. To visualize interaction between Sclerostin and BMP-7 the CM were immunoprecipitated with Flag antibody followed by Western Blotting with BMP-7 antibody. Secretion of Sclerostin and BMP-7 in CM was analyzed by subjecting it to western blotting with Flag and BMP-7 antibodies, respectively. Sclerostin was provided with N-terminal Flag epitope. β-actin was used as loading control. (+ = 10μg expression plasmid) (B) Left panel: the effect of Sclerostin (4-16 nM) on BMP-7 (2 nM)-induced C-terminal phosphorylation of Smad-1 (P-Smad-1) in Hek293 cells was analyzed by probing cell lysates with P-Smad-1 antibody. Both Sclerostin and BMP-7 were added 36 hours prior to cell lysis. Right panel: the effect of SOST expression plasmid on BMP-7 expression plasmid-induced P-Smad-1 was analyzed by probing cell lysates 36 hours after transfection with P-Smad-1 antibody. BMP-7 (0.3μg), SOST (0.3-1.2μg) and/or empty control vector were used for transfection. β-actin was used as loading control. (C) Left panel: the effect of Sclerostin (4 nM - 16 nM) on BMP-7 (2 nM)-induced was analyzed using BRE-luciferase transcriptional assay in Hek293 cells. BRE-luc activity was measured 18 h after stim-
ululation. Right panel: the effect of SOST expression plasmid on BMP-7 plasmid-induced BRE-luc activity was determined. Cells were transfected with BMP-7 expression plasmid (0.3μg), SOST expression plasmid (0.3-1.2μg) and/or an empty control vector. Normalized reporter activity is shown as the mean ± S.D. of triplicates. (D) HA epitope-tagged BMP-7 pro-domain expression plasmids was transfected alone or together with Flag-SOST expression plasmid, and cell lysates (Lysate) or conditioned media (CM) were subjected to immunoprecipitation with Flag followed by Western Blotting with HA antibody. Expression and secretion levels of pro-BMP-7 and/or Sclerostin were checked by direct Western Blotting on cell lysates or CM. β-actin was used as loading control. (E) HA epitope-tagged BMP-7 pro-domain and TGF-β1 pro-domain expression plasmids were transfected alone or together with Flag-SOST expression plasmid, and cell lysates subjected to immunoprecipitation with Flag followed by Western Blotting with HA antibody. Expression levels of pro-TGF-β1 and pro-BMP-7 were checked by direct Western Blotting on cell lysates. β-actin was used as loading control. The heterogeneity of Sclerostin protein (doublet bands) may be caused by glycosylation. When lysate is loaded Sclerostin usually runs as one band, likely corresponding to non-glycosylated Sclerostin and when conditioned media is loaded, it runs as double band, probably corresponding to non-glycosylated and glycosylated Sclerostin.

5.4.4 Sclerostin sequesters BMP-7 and mediates proteasomal degradation of intracellular BMP-7

To investigate the underlying mechanism of Sclerostin-mediated inhibition of BMP signaling we co-transfected BMP-7 and SOST into Saos-2 cells expressing non-targeting or SOST-targeting shRNA. The harvested CM was applied on C2C12 cells stably transfected with the BRE-luciferase reporter (C2C12-BRE-luc), and luciferase activity was determined after one day of incubation. This revealed that CM derived from BMP-7 transfected cells induced BRE-luciferase activity, whereas BRE-activity was dose-dependently reduced when CM derived from BMP-7 and SOST co-transfected cells was added (Figure 4A). This effect was not observed after incubation with CM of SOST shRNA-expressing cells, suggesting that Sclerostin sequestered intracellular BMP-7. Therefore, we explored whether in Saos-2 cells a relation between BMP-7 protein levels and Sclerostin expression existed. Notably, BMP-7 protein levels were reduced in the cell lysates and CM of cells expressing Sclerostin (Figure 4B). This effect was reversed when the cells were incubated with the proteasomal inhibitor MG132 (Figure 4B). Furthermore, enhanced ubiquitination of pro-BMP-7 was detected in the presence of co-transfected SOST in Hek293 cells (Figure 4C) indicating that Sclerostin mediates proteasomal degradation of intracellular BMP-7. No alteration of ubiquitination could be observed when Sclerostin and pro-TGF-β1 were co-transfected (data not shown). Taken together, we observed that the inhibitory action of Sclerostin on BMP-7 signaling is due to sequestering and subsequent proteasomal degradation of intracellular BMP-7.
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5.4.5 Sclerostin’s effect on BMP signaling is independent of Wnt signaling

Thus far, Sclerostin’s inhibitory role on BMP actions has been commonly consented on being indirect through the Wnt signaling pathway [60, 62]. Our finding that BMP-7 overexpression in Saos-2 cells can induce the Wnt luciferase reporter BAT-luc supports this theory (Figure 5A). Furthermore we show that the Wnt luciferase reporter activity can be further potentiated through the presence of SOST-targeting shRNAs. That these activations were indeed through indirect potentiating of the Wnt signaling cascade could be shown via inhibition of activation through dominant negative

Figure 3: Knockdown of SOST expression leads to elevated BMP signaling in Saos-2 cells. Saos-2 cells expressing either a non-targeting control shRNA or SOST targeting shRNAs were stimulated with recombinant BMP-7 (2 nM) or transfected with BMP-7 expression plasmid (0.3μg) and lysed 18 hours after stimulation or 36 hours after transfection, respectively. Subsequently, SOST mRNA expression levels (A), C-terminal phosphorylation of Smad-1/-5/-8 (P-Smad-1) (B) and BRE-luciferase reporter activity (C) were determined. Normalized BRE-luc reporter activity is shown as the mean ± S.D. of triplicates. Representative experiments of at least three experiments are shown.
TCF4 (dN-TCF4) (Figure 5A and Supplementary Figure S5). To demonstrate that our current findings of Sclerostin on BMP signaling are direct effects, and are not the consequence of altered Wnt signaling, we also determined BMP-7 induced BRE-luciferase reporter activity in the presence of dN-TCF4. BMP-7 induced BRE-luciferase reporter activity was significantly elevated in lysates derived from cells expressing SOST-targeting shRNAs, but was not affected when dN-TCF4 was co-transfected (Figure 5B).

Since it has been shown that Wnt signaling, in particular GSK3β-mediated phosphorylation of the linker region of Smad proteins leads to their proteasomal degradation [20], we investigated whether Sclerostin plays a role in the BMP-7 induced linker phosphorylation. KS483 cells overexpressing BMP-7 showed marked increased Smad-1/-5/-8 phosphorylation at the C-terminus (P-Smad-1C-term) and at the two Smad-1 linker sites that depend on MAP kinase and GSK3β (Smad-1MAP and Smad-1GSK3, respectfully), and phosphorylation at the C-terminus and linker sites was reduced in the presence of co-transfected SOST (Figure 5C). To show that the effects of Sclerostin on BMP-7 are not mediated through the inhibitory action of Sclerostin on the Wnt pathway we performed a BRE-luciferase assay in the presence of overexpressed Smad-1 wildtype (wt) or a Smad-1 mutant that is not susceptible to GSK3β phosphorylation (Smad-1-GM). Indeed, Sclerostin exhibited its BMP-7 antagonizing effect in the presence of either the Smad-1-wt or the Smad-1-GM mutant (Figure 5D), indicating that Sclerostin’s inhibitory action occurs indeed directly at the BMP level.

5.4.6 Elevated Wnt- and BMP-signaling in Sclerostin knock out mice

To investigate whether our findings on Sclerostin’s inhibitory action on BMP signaling occurs in vivo, we examined the hind limbs from 4 month old female wt and Sclerostin knock out (KO) mice, with focus on osteocytes in tibia and femur as well as the calcaneus. Sclerostin expression in wt mice was predominantly detected in osteocytes of all investigated bone types as well as in calcified cartilage of the patellar surface of the femur. As expected, no Sclerostin expression was detected in Sclerostin KO mice (Figure 6A-C, Supplementary Figure S6). Wnt signaling, represented through β-catenin expression was detected in osteoblasts and osteocytes of tibia and femur as well as in the osteocytes of the calcaneus of wt mice. Elevated β-catenin expression was detected in osteocytes of Sclerostin KO mice (Figure 6A-6C, Supplementary Figure S6). Interestingly, the expression of β-catenin was not detected in the cartilage of the patellar surface of the femur in wt mice, while in Sclerostin KO mice several chondrocytes were positive for β-catenin expression (Supplementary Figure S6A). BMP-7 expression was detected mainly in osteoblasts and osteocytes of tibia and femur with less expression in the osteocytes of the calcaneus of wt mice. Elevated BMP-7 expression was detected in osteocytes of Sclerostin KO mice (Figure 6A-6C, Supplementary Figure S6). Remarkably, a loss of BMP-7 expression was detected in the articular cartilage of the patellar surface of the femur in a Sclerostin KO background (Supplementary Figure S6A). Although elevated BMP signaling was detected in osteocytes of Sclerostin KO mice we raised the question whether that coincides with active canonical BMP signaling. Therefore we determined the expression
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Figure 4: Sclerostin sequesters intracellular BMP-7 leading to its proteasomal degradation. (A) CM from Saos-2 cells expressing either a non-targeting control shRNA or SOST targeting shRNA were transfected with BMP-7 (0.3μg) and/or SOST (0.3-1.2μg) expression plasmids or an empty control vector were harvested 36 hours after transfection. Subsequently, these CM were applied to C2C12-BRE-luc cells and luciferase activity was determined. (B) HEK293 cells were transfected with BMP-7 (0.3μg) and/or SOST (0.3-1.2μg) expression plasmids or an empty control vector. Three hours prior to harvesting the conditioned media or cell lysates the cells were treated with the proteasomal inhibitor MG132 (5μM) or DMSO. Thereafter the CM and cell lysates were subjected to western blot analysis and probed with the indicated antibodies. β-actin was used as loading control. (C) To examine the effect of Sclerostin on ubiquitination of pro-BMP-7 expression plasmids for HA-epitope tagged pro-BMP-7 (3μg) and/or Flag-SOST (9μg) or an empty control vector were transfected with myc-His-Ubiquitin expression plasmid in HEK293 cells. Cells were treated with MG132 (5μM) for 3 hours prior western blot analysis. Lysates were immunoprecipitated with HA followed by Western Blotting with His antibody. Expression level of Sclerostin and pro-BMP-7 in lysates and CM were examined. β-actin was used as loading control.

of phosphorylated Smad-1 (P-Smad-1) in osteocytes. The P-Smad-1 stainings did not comply with the BMP-7 expression pattern in wt mice with no P-Smad-1 detectable in osteocytes of the tibia, femur and calcaneus (Figure 6A-6C). In Sclerostin KO mice, P-
Figure 5: Sclerostin’s effect on BMP signaling is independent of Wnt signaling. Saos-2 cells expressing either a non-targeting control shRNA or SOST targeting shRNA were co-transfected with the BAF-luciferase (A) or BRE-luciferase (B) reporters together with BMP-7 (0.3μg) and either dominant negative TCF4 (dN-TCF4) (0.3μg) or an empty control vector. Luciferase activity was determined 36 hours after transfection. Normalized reporter activity is shown as the mean ± S.D. of triplicates. (C) The phosphorylation of Smad-1 at the C-terminus (P-Smad-1C-term.) and the two Smad-1 linker sites that depend on MAPK or GSK3β (P-Smad-1MAPK and P-Smad-1GSK3, respectively) were investigated in KS483 cells transfected with BMP-7 (3μg) and/or SOST (9μg) expression plasmid or an empty control vector. Lysates were prepared 36 hours after transfection and probed with the indicated antibodies. (D) KS483 cells were transfected with BRE-luciferase and BMP-7 (0.3μg) expression plasmids in the absence or presence of SOST (0.9μg) expression plasmid or an empty control vector. BRE-luciferase activity was measured 36 hours after transfection. Cells that were either overexpressing Smad-1 wild type (wt) or the Smad-1 mutant (GM) that cannot be phosphorylated by GSK3β, 36 hours after transfection. Normalized reporter activity is shown as the mean ± S.D. of triplicates.
Smad-1 expression did coincide with elevated BMP-7 expression in osteocytes in tibia and calcaneus (Figure 6A-6C), but remained undetectable in the femur (Supplementary Figure S6). P-Smad-1 expression was detected in articular cartilage at the patellar surface of the femur of wt mice, but was not found in Sclerostin KO mice and thereby coincided with the absence of BMP-7 expression in these cells (Supplementary Figure S6). To study whether the detected elevated BMP and Wnt signaling in Sclerostin KO mice correlated to Sclerostin’s pro-apoptotic effects on bone cells we performed a TUNEL assay. As described by Lin et al, 2009 Sclerostin KO mice show significantly less apoptotic osteocytes in the cortical bone of the tibia compared to their wt littermates (Figure 6A-6C) [33]. The most pronounced differences were detected in the calcaneus (perhaps induced by mechanical stress), where a significant elevation of β-catenin, BMP-7 expression and signaling correlates with a significant decrease of apoptotic osteocytes in the Sclerostin KO mice (Figure 6A-6C). From this data we conclude that Sclerostin’s inhibitory action on Wnt signaling occurs potentially besides on osteoblasts also on osteocytes of the tibia, femur and calcaneus. There is no link to a particular bone type detectable for the enhanced Wnt signaling upon loss of Sclerostin function. However, effects on BMP-7 signaling appear to be predominantly found in osteocytes of the calcaneus and the cortical bone of the tibia.

5.5 Discussion

Whereas over 90% of bone cells in the adult skeleton are osteocytes, in contrast to osteoblasts and osteoclasts that have been studied extensively, these mineralized matrix-embedded terminally differentiated cells remain poorly defined [11]. Their importance in bone homeostasis is underscored by the dramatic loss in bone upon their targeted ablation [59]. Osteocytes have dendrites that form a canalicular network through which they communicate with each other and the bone surface, and coordinate remodeling of the skeleton in response to changes of mechanical loading [9, 10]. Sclerostin is expressed in mature osteocytes and its expression changes upon mechanical strain [44, 61, 70, 49]. Genetic and (pre)clinical studies in human, monkey, rat and mice have demonstrated a key role for Sclerostin in controlling bone formation [31, 41, 66]. In the present study we uncovered that Sclerostin besides antagonizing Wnt/β-catenin signaling in a paracrine and autocrine manner, it also antagonizes BMP/Smad signaling cell autonomously in the osteocytes. The inhibitory effects of Sclerostin on canonical Wnt signaling and its interaction with LRP-5/-6 are consistent with previous reports [32, 53, 62, 33, 17, 26]. Importantly, we show that osteocytes in bones of adult Sclerostin KO mice display increased levels of β-catenin compared to wild type litter mates. The apparent affinity of Sclerostin for LRP-6 as measured by BIAcore is rather low. This may indicate that for efficient binding to LRP-6, Sclerostin may require additional co-receptors, analogous to the situation observed with DKK proteins which require binding to Kremen1/2 in addition to LRP-5/-6 in order to mediate internalization and degradation of DKK-bound LRP-5/-6 [36]. Surprisingly, co-expression of Sclerostin and LRP-5 in HEK293T cells was
Figure 6: Knockout of SOST expression leads to elevated BMP and Wnt signaling in osteocytes of 4 month old female Sclerostin\(^{-/-}\) mice compared to Sclerostin wild type mice in the Tibia and Calcaneus. (A) Representative pictures of the immunohistochemical stainings for the presence of Sclerostin, BMP-7, β-catenin and phosphorylated Smad-1 (P-Smad-1) proteins are shown for the cortical bone of the Tibia and the Calcaneus. Alcian Blue/tolylene red staining is used to distinguish cartilage (blue) from bone (red). TUNEL staining was performed to visualize apoptotic cells. Positive osteocytes are indicated through an asterisk. (B) Schematic representation of the orientation of the investigated mice hind limbs, namely femur, tibia and calcaneus. The areas of interest are boxed and correspond to pictures shown in Figure 6A and Supplementary Figure S6A. The close up indicates the location of bone (B), bone marrow (BM), muscle (M), articular cartilage (black area) and calcified cartilage (striped area). (C) Quantifications of the immunohistochemical staining patterns shown in (A). Numbers of total positive osteocytes in the indicated areas were counted manually and the percentage of positive cells was calculated.
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found to induce Wnt-reporter activity, which implies that in a certain context Sclerostin may actually exert agonistic effects via LRP-5. This is similar to what has been described for Sostdc1, which during Xenopus development can either positively or negatively affect Wnt signaling, and DKK2, that activates Wnt signaling unless Krm2 is co-expressed [24, 35] Whereas exogenous Sclerostin did not inhibit BMP signaling, when Sclerostin and BMP-7 are co-expressed Sclerostin was found to bind intracellular BMP-7, thereby inhibiting its secretion. In addition to BMP-7, also BMP-6-induced BRE-luciferase transcriptional activity was blocked by Sclerostin when both were co-expressed (data not shown), demonstrating that this mode of inhibition does not necessarily has to be restricted to BMP-7. Actually, a similar mechanism of sequestering BMPs intracellularly has previously shown to be the cause of the inhibitory action of the antagonist Gremlin on BMP-4 secretion [57], suggesting that this might be a shared property among various DAN family members. Consistent with previous results [70], our BIACore measurements demonstrated that Sclerostin only weakly interacts with mature BMP-7. Intracellularly the interaction between two BMP-7 precursor proteins is promoted by the pro-domain of BMP-7, which was also found to specifically interact with Sclerostin. It is tempting to speculate that the interaction with the BMP pro-domain is a requirement for efficient Sclerostin binding which effectively limits Sclerostin’s interaction with BMPs to the intracellular compartment, at least under physiological conditions. An intensive cross-talk exists between the Wnt and BMP signaling pathways. BMP-induced osteoblast differentiation is mediated by Wnt signaling [62, 45], and β-catenin TCF/LeF1 can interact with Smad-1 to cooperate in transcription of target genes [23]. Moreover, Wnt signaling can control the duration of BMP signaling by regulating GSK3β-mediated phosphorylation of Smad-1 in its linker region, which results in increased proteasomal degradation of Smad-1 [20, 50]. Consistent with the fact that only activated Smad-1 can be phosphorylated on linker region residues, we observed in cells overexpressing BMP-7 both increased C-terminal as well as linker phosphorylation. Expression of a Smad-1 mutant in which the GSK3 phosphorylation site is mutated (Smad-1-GM) resulted in an increase of both basal and BMP-7-induced BRE-luciferase activity compared to when wild type Smad-1 was expressed. Co-transfection of SOST blocked activation of the BMP reporter both in case wild type Smad-1 or the Smad-1-GM were co-expressed, indicating that the Sclerostin-mediated inhibition on BMP signaling is not determined by Smad-1 linker phosphorylation. Notably, KS483 cells express various BMPs [64], among which BMP-7, causing some basal activation of the BMP reporter, which was increased in case of Smad-1-GM expression.

Considering that Sclerostin antagonizes BMP signaling by intracellular retention and proteasomal degradation of BMP-7, the Sclerostin-neutralizing antibodies used in current clinical studies to increase bone formation will only affect the Sclerostin-mediated inhibition of Wnt signaling. Such extracellular directed targeting strategies will not inhibit the catabolic effect of Sclerostin that is mediated through its cell autonomous inhibitory effect on BMP-7/Smad signaling. The physiological outcomes of inhibition of BMP/Smad signaling by Sclerostin was demonstrated by the increased BMP-7 signaling in vitro in cell culture in response to SOST knock down and in vivo
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Figure 7: Schematic representation of the inhibitory action of Sclerostin on the BMP/Smad and Wnt/β-catenin signaling pathways. Inhibition of BMP-7 signaling by Sclerostin occurs intracellular. Sclerostin binds the pro-domain and mature domain of BMP-7 which leads to the sequestration and intracellular proteasomal degradation of BMP-7. Secreted Sclerostin can inhibit Wnt/β-catenin signaling by binding to the LRP-5/-6 Wnt co-receptor.

In SOST KO compared to wild type mice. Although elevated Wnt/β-catenin signaling was recognized throughout the investigated hind limbs, active BMP signaling was only detected in the lateral malleolus of the cortical bone of the distal tibia and the calcaneus in Sclerostin KO mice. This may be caused by different exposure towards mechanical stress in different bones. It is known that quadru- and bipedal primates and rodents support most of their weight on their distal bones of the limbs during locomotion [8, 47]. Thus far only Wnt/β-catenin and SOST/Sclerostin have been demonstrated to be regulated by mechanical strain in osteocytes [49, 48]. Further investigations are necessary to clarify whether a direct link between BMP signaling and mechanical strain in osteocytes exists. Osteocytes are described as key regulators of bone mass which regulate mineral homeostasis through their apoptotic death and ultimate activation of osteoclasts [14, 19, 29]. It has been shown that Sclerostin enhances caspase activity and promotes apoptotic death of osteocytes [33, 58]. In support of this notion, we observed that Sclerostin KO mice reveal significantly less apoptotic osteocytes than their wild type littermates, in particular in areas of increased Wnt/β-catenin and BMP-7 signaling of the distal tibia and the calcaneus. Both Wnt/β-catenin and BMP-7/Smad signaling can act as pro-survival pathways for osteoblasts and osteocytes [69, 67]. Sclerostin, whose expression is regulated by
mechanical loading, and factors such as prostaglandins, estrogen and parathyroid hormone [49, 58, 7, 27, 40, 55, 73] keeps both pathways in check and plays a key role in maintaining bone homeostasis.

In conclusion, we found that besides the inhibitory effect Sclerostin exerts on the Wnt signaling pathway in osteoblasts and osteocytes by binding LRP-5/-6 at the cell surface, it can also associate with intracellular BMP-7 blocking its secretion in osteocytes. Sclerostin interacts with both the mature domain and pro-domain of BMP-7, resulting in proteasomal degradation of BMP-7 (schematically represented in Figure 7). Analysis of Sclerostin KO mice confirmed an inhibitory action of Sclerostin on BMP-7 signaling in osteocytes and suggest that Sclerostin exerts its potent bone catabolic effects not only by antagonizing Wnt signaling, but also negatively regulates BMP signaling in the osteocytes that express Sclerostin.

5.6 Acknowledgments

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5.7 Supplementary Data

Figure S1: The exon/intron structure of Sost is shown, adapted from Ensembl.org. Boxes indicate the two exons and the coding region is shaded in light gray. The overlap of a CpG island with the beginning of the sequence in exon 2 is indicated. To generate the null allele of Sost (VG10069) the coding sequence was replaced with a LacZ/Neo cassette. The open reading frame of LacZ is shown as a hatched arrow followed by a black arrow indicating a polyadenylation region from SV40. The floxed Neo mini-gene is shown as a dark gray box. Scale bar: 500 bp.
Figure S2: (A-B) BMD of femur and lumbar vertebrae as assessed by dual energy X-ray absorptiometry (DXA) in (A) 4 month old and (B) 6 month old mice showing increased BMD in KO mice. \( n = 6 - 8 \) per group ** \( p < 0.001 \) (C-F) \( \mu \)CT analysis of proximal tibial metaphysis and mid-diaphysis in 2 month old female mice. SOST\(^{-/-}\) mice exhibit higher tibial bone mass compared to SOST wt mice. (C) Representative thickness map of tibial trabecular bone in WT and KO mice. (D) Increased tibial trabecular bone fraction (bone volume/ tissue volume) in ko mice. Trabecular bone volume fraction (BV/TV), trabecular thickness (Tb.Th), and trabecular bone density were 77%, 54%, and 5% \( (p < 0.01 \) each) higher in ko mice compared to wt mice, respectively. (E) Representative transverse cross-section of tibial mid-diaphysis. (F) Increased cortical thickness of tibial mid-diaphysis in KO mice. Cortical bone area, cortical bone thickness, and polar moment of inertia were 58%, 42%, and 97% \( (p<0.01 \) each) higher in ko mice compared to wt mice, respectively. Values are mean ±S.D., \( n = 5 \) per group. ** \( p < 0.01 \) vs WT Our Sost knock out mice phenocopies the Sost knock out generated by the Li et al. [30].
Figure S3: (A) Murine Sclerostin (2 mM) was perfused as analyte over a biosensor surface coated with murine LRP-6 (red sensogram) and mature BMP-7 (blue sensogram). Full-length Sclerostin variant = mSclerostin FL. (B) Quantitation of β-catenin distribution in hundred KS483 cells as seen in Figure 1C.
Figure S4: TGF-β signaling is not affected by knockdown of SOST expression. Saos-2 cells expressing either a non-targeting control shRNA or SOST targeting shRNA were transfected with CAGA_{12}-Luc transcriptional reporter with and without a TGF-β1 expression plasmid (0.3μg). Cells were lysed 36 hours after transfection. Normalized CAGA_{12}-luciferase reporter activity is shown as the mean ±S.D. of triplicates.

Figure S5: Saos-2 cells expressing either a non-targeting control shRNA or one of two distinct SOST targeting shRNAs (shRNA #1 and shRNA #2) were transfected with the BAT-luciferase reporter together with Wnt3a (0.3μg) and/or dominant negative TCF4 (dN-TCF4) (0.3μg), β-catenin S33Y (0.3μg) or an empty control vector. Luciferase activity was determined 36 hours after transfection. Normalized reporter activity is shown as the mean ±S.D. of triplicates.
Figure S6: Knockout of SOST expression does not lead to elevated BMP and Wnt signaling in the femurs of osteocytes of 4 month old female Sclerostin \(^{-/-}\) mice compared to Sclerostin wild type (wt) mice. (A) Representative pictures of the immunohistochemical stainings for the presence of Sclerostin, BMP-7, \(\beta\)-catenin and phosphorylated Smad-1 (P-Smad-1) proteins are shown for the cortical and cancellous bone from the Femur. Alcian Staining is used to distinguish cartilage from bone. TUNEL positive cells are undergoing apoptosis. Positive osteocytes are indicated through an asterisk. n.d. = not detectable (B) Quantifications of the immunohistochemical stainings are stated in percentage.
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