Distinct Modes of Inhibition by Sclerostin on Bone Morphogenetic Protein and Wnt Signaling Pathways*\$  

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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-induced but not direct BMP7-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 BMP7 secretion. Sclerostin interacts with both the BMP7 mature domain and pro-domain, leading to intracellular retention and proteosomal degradation of BMP7. 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. BMP7 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 antagonizing BMP signaling selectively in the osteocytes that synthesize simultaneously both sclerostin and BMP7 proteins.

Sclerosteosis (OMIM accession number 269500) and van Buchem disease (OMIM accession number 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 (1–8). 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 (9–13). Because sclerostin binds, albeit weakly, to mature bone morphogenetic proteins (BMPs),4 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 (9, 14, 15).

Canonical Wnt signaling has been described to play a crucial role in several bone mass disorders. Wnt proteins transduce their signals via seven-transmembrane-spanning receptors of the frizzled family and lipoprotein receptor-related protein-5/6 (LRP5/6), thereby controlling the stability of cytoplasmic β-catenin. In the absence of Wnt ligands, β-catenin forms a complex with APC (adenomatous polyposis coli), axin, GSK3 (glycogen synthase kinase 3), and CK1 (casein kinase I). This complex facilitates phosphorylation and subsequent proteosomal 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 (16). The importance of Wnt signaling in bone formation is illustrated by the low bone mass osteoporosis-pseudoglioma syndrome or high bone mass phenotype caused by missense loss or gain of function mutations in LRP5, respectively (17–20). Sclerostin was found to act as a direct extracellular antagonist of canonical Wnt signaling by binding to LRP5 and LRP6 (21, 22). Several mutants that cause the LRP5 high bone mass trait are actually defective in sclerostin binding, thereby making them resistant to sclerostin-mediated inhibition (22, 23).

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 (24–26). BMPs are expressed in an inactive pro-form, and proteolytic cleavage by furin proteases is required to release the mature BMP proteins (27).

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, Smad1, -5, and -8, at two serine

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4 The abbreviations used are: BMP, bone morphogenetic protein; CM, conditioned medium; luc, luciferase; BRE, BMP-responsive element; BAT, β-catenin-activated transgene.
residues at their C termini. Activated R-Smads can associate with the Co-Smad (common mediator 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 (28, 29).

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 BMP7 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.

**EXPERIMENTAL PROCEDURES**

**Cells**—SAOS-2 human osteosarcoma cells, HEK293 and HEK293T cells, and C2C12 cells stably transfected with the BRE-luciferase (BRE-luc) reporter (30) were cultured in 4.5 g/liter glucose Dulbecco’s modified Eagle’s medium (Invitrogen). Saos-2 cell lines were laterally transduced with shRNA constructs in order to express either a non-targeting control shRNA (shRNA control, CCGGCAAAAGATGAAGACCCACTTCAGAGTTCGGTCTCTTACATCTTGTTGGTTT; Sigma), or shRNA targeting SOST (shRNA 1, CCGGCCCAAGGATGAAGACCCACTTCAGAGTTCGGTCTCTTACATCTTGTTGGTTT; Sigma) or shRNA targeting GAPDH (shRNA 2, CCGGCGTGGAGAAGCCAGACCATCCTGAGATGTAGCTGGTTGTGTGTGTGTTT; Sigma), followed by puromycin selection (5 μg/ml) to obtain SOST and GAPDH knockdown Saos-2 cells. Mouse mesenchymal KS483 cells were cultured in α-minimum essential medium (Invitrogen) (31). All media were supplemented with 10% fetal bovine serum (Integro) 100 IU/ml penicillin and 100 IU/ml streptomycin (Invitrogen).

**Expression Plasmids**—The expression construct pcDNA3-hBMP7 and the pGEM3Zf-mWnt3a expression construct have been described previously (32). C-terminally 3× HA-tagged human BMP7 pro-peptide-expressing construct was generated using a PCR approach. Primers 5’-ATTGGTACCTAGAGCCGGCGCGATGCACG-3’ (5’, containing the ATG codon and KpnI restriction site) and 5’-GCGCGGCTGGAGAACAAGACCATCCTGAGATGTAGCTGGTTGTGTGTGTGTTT (3’, containing the HindIII restriction site) were used to amplify a fragment of BMP7 cDNA that contains the signal peptide and a pro-peptide part. The purified PCR fragment was cloned in frame 5’ of the 3× HA-encoding sequence in 3× HA-pBlueScript vector. The pcDNA3-mLRP5-N-MycΔ expression construct was kindly provided by Xi He (Children’s Hospital, Harvard) (22), and pcDNA3-hLRP5 (WT-LRP5) was from 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 described previously (33, 34). The expression constructs for Smad1 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) (35). 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 described previously (36).

**Reagents and Recombinant Proteins**—Recombinant human BMP7 was kindly provided by K. Sampath (Creative Biomolecules, Inc., Hopkinton, MA). Recombinant mouse Wnt-3a and Dkk-1 were purchased from R&D Systems. Recombinant mouse and human sclerostin were prepared as described previously (37). For BIAcore measurements, rec.BMP7 was obtained from Wyeth, LRP6 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 RevertAid™ H Minus First Strand cDNA synthesis kit (Fermentas) according to the manufacturer’s instructions. Expression of human SOST, BMP7, and GAPDH were analyzed using the following primers: SOST, 5’-GAGACGACCATCAAACTCA-3’ and 5’-CAAGTGACCGAGAAGAAAT-3’; BMP7, 5’-CCACCTGTAATCCCAUGCT-3’ and 5’-GACAAAGACACAGGGTCCA-3’; GAPDH, 5’-ATCACTGCCCACCCAGGAAGAC-3’ and 5’-ATGAGTCCACCCACCTCCTT-3’. All conditions were measured in duplicate, and Taqman PCRs were performed using the StepOne Plus™ real-time PCR system (Applied Biosystems). Gene transcription levels were determined with the comparative ΔΔCt method using GAPDH as a reference, and the non-stimulated condition was set to 1.

**Luciferase Reporter Assays**—Cells were transiently transfected using FuGENE 6 (Roche Applied Science) transfection reagent, with 0.1 μg of firefly luciferase reporter construct, 0.1 μg of β-galactosidase, together with the indicated expression plasmids or empty control plasmid (pcDNA3.1), according to the manufacturer’s instructions. Cells were allowed to recover for 18 h, followed by serum starvation and stimulation with the indicated ligands for 18 h. Afterward, conditioned medium (CM) was collected, and cells were washed with PBS and lysed. C2C12-BRE-luc cells were stimulated with CM for 18 h 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.

**Co-immunoprecipitation, 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 and 24 h. For Western blot, ubiquitination, and co-immunoprecipitation analysis, cells were allowed to grow to 70% confluence, and serum concentration was reduced to 0.5% over-
night. For co-immunoprecipitation, cells were lysed in ice-cold lysis buffer (50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 0.2% Tween 20, and 1 protease inhibitor mixture tablet (Roche Applied Science) per 50 ml). Cell lysates (400 μg of total protein) or CM (50 ml) were incubated with 1 μg/μl anti-FLAG M2 (Sigma) or anti-c-Myc (9E10) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) antibody for 1.5 h at 4 °C. 0.5 μg/μl protein A-Sepharose beads (Amersham Biosciences) was added and incubated for an additional 1.5 h at 4 °C. Beads were extensively washed with ice-cold lysis buffer containing 500 mM NaCl, and eluted protein complexes were subjected to SDS-PAGE and Western blot analysis. Western blot analyses were performed as described previously (38). C-terminal Smad phosphorylation was detected using an antibody specifically recognizing phosphorylated Smad1/5/8 (P-Smad) antibodies obtained from the DeRobertis laboratory (35). BMP7 expression was investigated with a BMP7 antibody kindly provided by Hicham Alaoui (Stryker Biotech, Hopkinton, MA). SOST-N-FLAG expression and LRPS-N-Myc expression were detected using anti-FLAG M2 (Sigma) and anti-c-Myc (9E10) (Santa Cruz Biotechnology, Inc.), respectively. Pro-BMP7-HA and pro-TGF-β-HA were detected via an anti-HA (12CA5) antibody (Roche Applied Science). Ubiquitination assays were performed as described previously (40). The Myc-His-ubiquitin was detected through a control HIS-1 antibody (Sigma). Equal loading was confirmed using an anti-β-actin antibody (Sigma) or anti-Smad1 antibody (Zymed Laboratories Inc.). Secondary horseradish peroxidase-conjugated goat anti-rabbit and sheep anti-mouse IgG antibodies (Amersham Biosciences) were used for detection using ECL (Amersham Biosciences). 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.

Alkaline Phosphatase Activity Assay—KS483 cells were seeded in 96-well plates and grown until confluent. Cells were stimulated with the indicated ligands in 0.2% FBS-containing α-MEM. Alkaline phosphatase 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 h 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. Afterward, 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 h. After washing, labeled secondary antibodies AlexaFluor 488 and goat anti-mouse IgG (Invitrogen) were used for detection. Nuclei were stained using Hoechst 33258 (Invitrogen) according to the manufacturer’s instructions.

Specimens were visualized with an Olympus IX51 microscope at ×100 magnification using the cell² Soft Imaging System (Olympus).

Sclerostin Knock-out Mice—A knock-out 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 of the details regarding the generation of this allele can be found at the Velocigene Web site. SostLacZ/+ mice were generated using clone VG10069A-G10 (supplemental Fig. S1). This allele has been tested, and it phenocopies other published knock-out lines of Sost (supplemental Fig. S2).

Immunohistochemistry, Alcian Blue, and 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% H₂O₂ 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, pH 8.0) for 10 min at 37 °C was followed by three washes with 0.1 M Tris-buffered saline (pH 7.4) containing 0.02% Tween 20 (TNT). Thereafter, slides were incubated with 5% species-specific serum (Dako) in 0.5% Boehringer blocking reagent (Roche Applied Science) 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-BMP7 antibody (1:100) (Stryker), mouse anti-β-catenin antibody (1:100) (BD Transduction Laboratories), rabbit anti-PS1 antibody directed against C-terminal phosphorylated Smad1/5/8 (1:50) (39), and goat anti-sclerostin antibody (1:400) (R&D Systems). 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 biotinylated tyramine. Stainings were carried out using amino-9-ethyl-carbazole (Sigma) and Mayer’s hematoxylin (Merck) according to the manufacturer’s instructions. A water-based mounting solution was applied, and stainings were visualized with an Olympus IX51 microscope using the cell² Soft Imaging 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 manufacturer’s instructions.

To detect apoptosis, a Dead End Colorimetric TUNEL system (Promega) was used according to the manufacturer’s instructions. The 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—LRP6, BMP7, IL-4R, and AbD09095 proteins were biotinylated using NHS–LC-Biotin (Pierce) according to the manufacturer’s recommendation. To minimize 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. Biotinylated proteins were then immobilized to a surface density of 200 (BMP7) to 550 resonance units (LRP6). 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 nonspecific binding of sclerostin (used as analyte) to the biosensor surface. Different running buffer conditions with varying salt concentrations were tested, showing that upon the addition of 300 mM 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 BMP7 and LRP6 was then measured by perfusing the biosensor using six 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 s at a flow rate of 10 μL/min. The dissociation phase was initiated by perfusing only HBS300 buffer and monitored for 200 s. The biosensor surface was regenerated by perfusing a short (60-s) pulse of buffer containing 6 M urea, 1 M sodium chloride, 0.1 M acetic acid. Sensorgrams were evaluated using the software BIACore evaluation version 2.4. Due to the fast association and dissociation kinetics, binding coefficients were deduced from the dose dependence of equilibrium binding.

Statistical Analysis—Values are expressed as mean ± S.D. 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; **, p < 0.01; ***, p < 0.001). Experiments were performed at least three times, and representative results are shown.

RESULTS

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 (32). 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 (9, 15). Some of these experiments were performed not by stimulating cells with exogenous ligands but instead by transfecting expression plasmids or by mRNA injections (32, 41). 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. We stimulated KS483 osteoprogenitor cells with either recombinant BMP7 or recombinant Wnt3a in the presence or absence of recombinant sclerostin. Sclerostin inhibited the BMP7- and Wnt3a-induced activity of the early marker for osteoblast differentiation alkaline phosphatase (ALP) in a dose-dependent manner (Fig. 1A). Sclerostin has been shown to inhibit canonical Wnt signaling by binding to the LRP5 and LRP6 co-receptors (21, 22). To confirm the association of sclerostin with LRP5, we co-transfected SOST and LRP5 in HEK293T cells and performed co-immunoprecipitation experiments. Indeed, sclerostin co-immunoprecipitated with LRP5, and vice versa (Fig. 1B). Surprisingly, binding to the sclerostin receptor LRP6, as measured through surface plasmon resonance (BIACore), revealed a rather low binding affinity with \( K_D^{(eq)} \) values ranging from 2 to 8.6 μM for full-length murine and human sclerostin, respectively (supplemental Fig. S3A and Table 1). Moreover, we investigated by immunofluorescence the effect of sclerostin on Wnt-induced changes in subcellular localization of β-catenin in KS483 cells. Upon stimulation with recombinant Wnt3a, β-catenin translocated to the nucleus, which was completely blocked by Dkk1 and attenuated in ~60% of the cells by sclerostin (Fig. 1C and supplemental Fig. S3B). Co-transfection of a Wnt3a expression plasmid in HEK293T cells resulted in a 3-fold activation of the Wnt reporter construct BAT-luciferase (Fig. 1D). As expected, sclerostin antagonized this Wnt3a-induced reporter activity. Interestingly, co-expression of sclerostin and LRP5 did not reduce but significantly increased Wnt reporter activity. When Wnt3a, LRP3, and SOST were co-expressed, a reduction of the Wnt-induced reporter activity was achieved compared with LRP5/sclerostin co-expression (Fig. 1D). Consistent with previous reports (21, 22, 32), we show that sclerostin inhibits Wnt/LRP5 signaling.

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 BMP7, we performed immunoprecipitation experiments on HEK293T cells co-transfected with BMP7 and SOST expression plasmids. BMP7 and sclerostin were found to interact in CM of these cells (Fig. 2A). Notably, a decrease in BMP7 expression was detectable in the presence of sclerostin compared with when BMP7 was expressed alone. However, when expressed separately and immunoprecipitation was performed on mixed CM, no binding of sclerostin to BMP7 could be detected (data not shown). Binding of sclerostin to BMP7 as investigated through BIACore measurements revealed similar low affinity of sclerostin to BMP7 as to LRP6, with \( K_D^{(eq)} \) values ranging from 1 to 3.8 μM for full-length murine and human sclerostin, respectively (supplemental Fig. S3A and 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 BMP7-induced C-terminal Smad1/5/8 phosphorylation and Smad1/Smad4-driven BRE-luc transcriptional response were not affected by recombinant sclerostin (Fig. 2, B and C, lanes 1–7) or transfected SOST expression plasmid (data not shown) (32). Interestingly, however, we observed...
that when SOST and BMP7 are co-transfected, sclerostin can dose-dependently inhibit BMP7/Smad-induced signaling responses (Fig. 2, \textit{B} and \textit{C}, lanes 8–14).

These results together with the inhibition of BMP7 secretion by sclerostin (Fig. 2B) suggested that sclerostin might retain BMP7 intracellularly when co-expressed. Therefore, we investigated whether the pro-domain of BMP7 plays a role in the interaction with sclerostin. We first noted that the pro-domain of BMP7 (lacking the mature domain) is secreted when expressed alone, whereas co-expression with sclerostin eliminated the observed secretion of the BMP7 pro-domain (Fig. 2D).

When co-expressed, binding of sclerostin to the isolated pro-domain of BMP7 was observed in the cell lysates (Fig. 2, \textit{D} and \textit{E}). To rule out nonspecific binding of sclerostin to BMPs, we simultaneously performed the co-immunoprecipitations with pro-TGF-\(\beta\)/H9252. No sequestration of TGF-\(\beta\)-prodomain (data not shown) or binding with sclerostin was detectable (Fig. 2E).

\textbf{Reduced SOST Expression Leads to Elevated BMP Signaling—}

Because 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 (42).

Upon BMP7 stimulation, SOST mRNA levels in Saos-2 cells expressing a non-targeting shRNA were significantly induced (Fig. 3A, lanes 2 and 3). In cells expressing one of two independent SOST-targeting shRNAs, the SOST mRNA expression remained at basal levels during BMP7 stimulation (Fig. 3A, lanes 4–9), whereas mRNA expression of BMP7 remained unaffected (data not shown). Notably, in the background of
the SOST-targeting shRNAs, BMP7-induced Smad1/5/8 phosphorylation and BRE-luc activity were significantly increased in the case of BMP7 overexpression compared with control shRNAs (Fig. 3, B and C, respectively). The inductive properties of recombinant BMP7 were also marginally elevated upon SOST knockdown (Fig. 3, B and C), which can be due to the self-induction of BMP expression (data not shown). Similar results were obtained using SOST-targeting siRNAs in HEK293T and MG63 cell lines (data not shown). No effects of SOST-targeting shRNAs were detected on TGF-β/H92521-induced CAGA12 luciferase transcriptional reporter activity (supplementary Fig. S4). Altogether, these findings suggest that sclerostin is directly involved in the regulation of autocrine BMP-induced responses.

Sclerostin Sequesters BMP7 and Mediates Proteasomal Degradation of Intracellular BMP—To investigate the underlying mechanism of sclerostin-mediated inhibition of BMP signaling, we co-transfected BMP7 and SOST into Saos-2 cells expressing non-targeting or SOST-targeting shRNA. The harvested cell lysates were subjected to immunoprecipitation with FLAG followed by Western blotting with HA antibody. Expression levels of pro-TGF-β1 and pro-BMP7 were checked by direct Western blotting on cell lysates. β-Actin was used as a loading control. The heterogeneity of sclerostin protein (doublet bands) may be caused by glycosylation. When lysate is loaded, sclerostin usually runs as one band, probably corresponding to non-glycosylated sclerostin, and when conditioned medium is loaded, it runs as a double band, probably corresponding to non-glycosylated and glycosylated sclerostin.
Inhibitory Actions of Sclerostin on BMP and Wnt

Thus far, the inhibitory role of sclerostin on BMP actions has been commonly thought to be indirect through the Wnt signaling pathway (13, 32). Our finding that BMP7 overexpression in Saos-2 cells can induce the Wnt luciferase reporter BAT-luc supports this theory (Fig. 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 potentiation of the Wnt signaling cascade could be shown via inhibition of activation through dominant negative TCF4 (dN-TCF4) (Fig. 5A and supplemental Fig. S5). To demonstrate that our current findings of sclerostin effects on BMP signaling are direct effects and are not the consequence of altered Wnt signaling, we also determined BMP7-induced BRE-luciferase reporter activity in the presence of dominant negative TCF4. BMP7-induced BRE-luciferase reporter activity was significantly elevated in lysates derived from cells expressing SOST-targeting shRNAs but was not affected when dominant negative TCF4 was co-transfected (Fig. 5B). Because it has been shown that Wnt signaling, in particular GSK3β-mediated phosphorylation of the linker region of Smad proteins, leads to their proteasomal degradation (35), we investigated whether sclerostin plays a role in the BMP7-induced linker phosphorylation. KS483 cells overexpressing BMP7 showed markedly increased Smad1/5/8 phosphorylation at the C terminus (P-Smad1 C-term) and at the two Smad1 linker sites that depend on MAPK and GSK3β (Smad1 MAP and Smad1 GSK3, respectively), and phosphorylation at the C terminus and linker sites was reduced in the presence of co-transfected SOST (Fig. 5C). To show that the effects of sclerostin on BMP7 are not mediated through the inhibitory action of sclerostin on the Wnt pathway, we performed a BRE-luciferase assay in the presence of overexpressed Smad1 WT or a Smad1 mutant that is not susceptible to GSK3β phosphorylation (Smad1-GM). Indeed, sclerostin exhibited its BMP7 antagonizing effect in the presence of either the Smad1-WT or the Smad1-GM mutant (Fig. 5D), indicating that the inhibitory action of sclerostin occurs indeed directly at the BMP level.
Elevated Wnt and BMP Signaling in Sclerostin Knock-out Mice—To investigate whether our findings on the inhibitory action of sclerostin on BMP signaling occur 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 (Fig. 6, A–C, and supplemental Fig. 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 osteoblasts and osteocytes of tibia and femur with less expression in the osteocytes of the calcaneus of WT mice. Elevated BMP7 expression was detected in osteocytes of sclerostin KO mice (Fig. 6, A–C, and supplemental Fig. S6). Remarkably, a loss of BMP7 expression was detected in the articular cartilage of the patellar surface of the femur in a sclerostin KO background (supplemental Fig. 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 of phosphorylated Smad1 (P-Smad1) in osteocytes. Although in WT mice, BMP-7 expression was detectable, no active BMP signaling, as investigated with P-Smad-1 expression, was visible in osteocytes of the tibia, femur, and calcaneus (Fig. 6, A–C). In sclerostin KO mice, on the other hand, P-Smad1 expression was detectable and coincided with BMP7 expression in osteocytes in tibia and calcaneus (Fig. 6, A–C) but remained undetectable in the femur (supplemental Fig. S6). Additionally, P-Smad1 expression was detected in articular cartilage at the patellar surface of the femur of WT mice but was not found in sclerostin KO mice, which is in agreement with the absence of BMP7 expression in these cells (supplemental Fig. S6).

To study whether the detected elevated BMP and Wnt signaling in sclerostin KO mice correlated with the proapoptotic effects of sclerostin on bone cells, we performed a TUNEL assay. As described by Lin et al. (43), sclerostin KO mice show significantly fewer apoptotic osteocytes in the cortical bone of the tibia compared with their WT littermates (Fig. 6, A–C) (43).

FIGURE 4. Sclerostin sequesters intracellular BMP7, leading to its proteasomal degradation. A, CM from Saos-2 cells expressing either a non-targeting control shRNA or SOST-targeting shRNA that was transfected with BMP7 (0.3 μg) and/or SOST (0.3–1.2 μg) expression plasmids or an empty control vector were harvested 36 h after transfection. Subsequently, these conditioned media were applied to C2C12-BRE-luc cells, and luciferase activity was determined. B, HEK293 cells were transfected with BMP7 (0.3 μg) and/or SOST (0.3–1.2 μg) 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 (5 μM) or DMSO. Thereafter, the conditioned media and cell lysates were subjected to Western blot analysis and probed with the indicated antibodies. β-Actin was used as a loading control. C, to examine the effect of sclerostin on ubiquitination of pro-BMP7, expression plasmids for HA epitope-tagged pro-BMP7 (3 μg) and/or FLAG-SOST (9 μg) or an empty control vector were transfected with Myc-His-ubiquitin expression plasmid in HEK293 cells. Cells were treated with MG132 (5 μM) for 3 h prior to Western blot analysis. Lysates were immunoprecipitated (IP) with HA, followed by Western blotting with His antibody. Expression level of sclerostin and pro-BMP7 in lysates and CM was examined. β-Actin was used as loading control. Error bars, S.D.; *, p < 0.05; ***, p < 0.001.
The most pronounced differences were detected in the calcaneus (perhaps induced by mechanical stress), where a significant elevation of β-catenin, BMP7 expression, and signaling correlates with a significant decrease of apoptotic osteocytes in the sclerostin KO mice (Fig. 6, A–C). From these data, we conclude that the inhibitory action of sclerostin on Wnt signaling occurs potentially, in addition to 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 BMP7 signaling appear to be predominantly found in osteocytes of the calcaneus and the cortical bone of the tibia.

**DISCUSSION**

Although 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 (44). Their importance in bone homeostasis is underscored by the dramatic loss in bone upon their targeted ablation (45). 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 (46, 47). Sclerostin is expressed in mature osteocytes, and its expression changes upon mechanical strain (7, 14, 15, 48). Genetic and (pre)clinical studies in human, monkey, rat, and mice have demonstrated a key role for sclerostin in controlling bone formation (49–51). In the present study, we uncovered that sclerostin, besides antagonizing Wnt/β-catenin signaling in a paracrine and autocrine manner, also antagonizes BMP/Smad signaling cell autonomously in the osteocytes.

![FIGURE 5. The effect of sclerostin on BMP signaling is independent of Wnt signaling.](image-url)
that osteocytes in bones of adult sclerostin KO mice display increased levels of \(\beta\)-catenin compared with wild type littermates. The apparent affinity of sclerostin for LRP6 as measured by BIAcore is rather low. This may indicate that for efficient binding to LRP6, sclerostin may require additional co-receptors, analogous to the situation observed with DKK proteins, which require binding to Kremen1/2 in addition to LRP5/6 in order to mediate internalization and degradation of DKK-bound LRP5/6 (54). Surprisingly, co-expression of sclerostin and LRP5 in HEK293T cells was found to induce Wnt reporter activity, which implies that, in a certain context, sclerostin may actually exert agonistic effects via LRP5. This is similar to what has been described for Sostdc1, which during Xenopus development can either positively or negatively affect Wnt signaling, and DKK2, which activates Wnt signaling unless Krm2 is co-expressed (11, 55).

Although exogenous sclerostin did not inhibit BMP signaling, when sclerostin and BMP7 are co-expressed, sclerostin was found to bind intracellular BMP7, thereby inhibiting its secretion. In addition to BMP7, also BMP6-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 have to be restricted to BMP7. Actually, a similar mechanism of sequestering BMPs intracellularly has previously been shown to be the cause of the inhibitory action of the antagonist Gremlin on BMP4 secretion (56), suggesting that this might be a shared property among various DAN family members. Consistent with previous results (15), our BIAcore measurements demonstrated that sclerostin only weakly interacts with mature BMP7. Intracellularly the interaction between two BMP7 precursor proteins is promoted by the pro-domain of BMP7.

FIGURE 6. Knock-out of SOST expression leads to elevated BMP and Wnt signaling in osteocytes of 4-month-old female sclerostin \(\sim\) mice compared with sclerostin wild type mice in the tibia and calcaneus. A, representative pictures of the immunohistochemical stainings for the presence of sclerostin, BMP7, \(\beta\)-catenin, and phosphorylated Smad1 (P-Smad1) proteins are shown for the cortical bone of the tibia and the calcaneus. Alcian blue/toluylene red staining is used to distinguish cartilage (blue) from bone (red). TUNEL staining was performed to visualize apoptotic cells. Positive osteocytes are indicated by 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 A and in supplemental Fig. 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. Total positive osteocytes in the indicated areas were counted manually, and the percentage of positive cells was calculated. Error bars, S.D.; *, \(p < 0.05\); **, \(p < 0.01\); ***, \(p < 0.001\).
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 that effectively limits the interaction of sclerostin 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 (32, 57), and β-catenin-TCF/LeF1 can interact with Smad1 to cooperate in transcription of target genes (58). Moreover, Wnt signaling can control the duration of BMP signaling by regulating GSK3β-mediated phosphorylation of Smad1 in its linker region, which results in increased proteasomal degradation of Smad1 (35, 59). Consistent with the fact that only activated Smad1 can be phosphorylated on linker region residues, we observed in cells overexpressing BMP7 both increased C-terminal and linker phosphorylation. Expression of a Smad1 mutant in which the GSK3 phosphorylation site is mutated (Smad1-GM) resulted in an increase of both basal and BMP7-induced BRE-luciferase activity compared with when wild type Smad-1 was expressed. Co-transfection of SOST blocked activation of the BMP reporter both in the cases where wild type Smad1 or the Smad1-GM were co-expressed, indicating that the sclerostin-mediated inhibition of BMP signaling is not determined by Smad1 linker phosphorylation. Notably, KS483 cells express various BMPs (60), among which was BMP7, causing some basal activation of the BMP reporter, which was increased in the case of Smad1-GM expression.

Considering that sclerostin antagonizes BMP signaling by intracellular retention and proteasomal degradation of BMP7, 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, which is mediated through its cell-autonomous inhibitory effect on BMP7/Smad signaling.

The physiological outcomes of inhibition of BMP/Smad signaling by sclerostin were demonstrated by the increased BMP7 signaling in vitro in cell culture in response to SOST knockdown and in vivo in SOST KO compared with 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 toward mechanical stress in different bones. It is known that quadruped and bipedal primates and rodents support most of their weight on their distal bones of the limbs during locomotion (61, 62). Thus far, only Wnt/β-catenin and SOST/sclerostin have been demonstrated to be regulated by mechanical strain in osteocytes (48, 63). 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 that regulate mineral homeostasis through their apoptotic death and ultimate activation of osteoclasts (64–66). It has been shown that sclerostin enhances caspase activity and promotes apoptotic death of osteocytes (43, 67). In support of this notion, we observed that sclerostin KO mice reveal significantly fewer apoptotic osteocytes than their wild type littermates, in particular in areas of increased Wnt/β-catenin and BMP7 signaling of the distal tibia and the calcaneus. Both Wnt/β-catenin and BMP7/Smad signaling can act as prosurvival pathways for osteoblasts and osteocytes (68, 69). Sclerostin, whose expression is regulated by mechanical loading and factors such as prostaglandins, estrogen, and parathyroid hormone (48, 67, 70–74), 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 LRPs5/6 at the cell surface, it can also associate with intracellular BMP7, blocking its secretion in osteocytes. Sclerostin interacts with both the mature domain and pro-domain of BMP7, resulting in proteosomal degradation of BMP7 (schematically represented in Fig. 7). Analysis of sclerostin KO mice confirmed an inhibitory action of sclerostin on BMP7 signaling in osteocytes and suggests that sclerostin not only exerts its potent bone catabolic effects by antagonizing Wnt signaling but also negatively regulates BMP signaling in the osteocytes that express sclerostin.

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