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Wnt but Not BMP Signaling Is Involved in the Inhibitory Action of Sclerostin on BMP-Stimulated Bone Formation

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ABSTRACT: Sclerostin is an osteocyte-derived negative regulator of bone formation. It inhibits BMP-stimulated bone formation both in vitro and in vivo but has no direct effect on BMP signaling. Instead, sclerostin inhibits Wnt signaling that is required for BMP-stimulated osteoblastic differentiation.

Introduction: Sclerostin is a member of the Dan family of glycoproteins of which many members have been reported to antagonize BMP activity. Sclerostin has been shown to inhibit BMP-stimulated bone formation, but its mechanism of action seems to be different from classical BMP antagonists. In this study, we investigated the mechanism by which sclerostin inhibits BMP-stimulated bone formation.
Materials and Methods: DNA electroporation of calf muscle of mice using expression plasmids for BMP and sclerostin was used to study the effect of sclerostin on BMP-induced bone formation in vivo. Transcriptional profiling using microarrays of osteoblastic cells treated with BMP in the absence or presence of sclerostin was used to find specific growth factor signaling pathways affected by sclerostin. The affected pathways were further studied using growth factor–specific reporter constructs.
Results: BMP-induced ectopic bone formation in calf muscle of mice was prevented by co-expression of sclerostin in vivo. Transcriptional profiling analysis of osteoblastic cultures indicated that sclerostin specifically affects BMP and Wnt signaling out of many other growth signaling pathways. Sclerostin, however, did not inhibit stimulation of direct BMP target genes. Furthermore, we did not obtain any evidence for sclerostin acting as a direct BMP antagonist using a BMP-specific reporter construct. In contrast, sclerostin shared many characteristics with the Wnt antagonist dickkopf-1 in antagonizing BMP-stimulated bone formation and BMP- and Wnt-induced Wnt reporter construct activation.
Conclusions: Sclerostin inhibits BMP-stimulated bone formation but does not affect BMP signaling. Instead, it antagonizes Wnt signaling in osteoblastic cells. High bone mass in sclerosteosis and van Buchem disease may, therefore, result from increased Wnt signaling.
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Key words: sclerostin, SOST, Wnt, BMP, signal transduction, bone formation, microarray

INTRODUCTION

Sclerosteosis and van Buchem disease are closely related, rare sclerosing disorders characterized by progressive bone thickening caused by increased bone formation.[1–3] Both diseases have been linked to deficiency of the *SOST* gene product sclerostin,[4–7] a negative regulator of bone formation secreted by osteocytes.[8,9]

Sclerostin is a member of the DAN family of glycoproteins that share the capacity to inhibit BMP activity.[3,5,10] Sclerostin directly binds BMPs in vitro, although with low affinity,[9,11] and inhibits their bone-forming capacity in vitro without any apparent preference for specific BMPs.[8,9] The mechanism by which sclerostin antagonizes BMP activity is, however, unclear. Winkler et al.[9] reported that sclerostin attenuated BMP-stimulated Smad phosphorylation in mouse mesenchymal C3H10T1/2 cells, whereas we did not find any antagonistic effect of sclerostin on direct BMP-induced responses like Smad phosphorylation and Smad-driven transcriptional reporter activation in mouse mesenchymal KS483 cells.[8]

We previously suggested two possible mechanisms by which sclerostin may inhibit BMP-stimulated bone formation.[8] Sclerostin may either require a co-factor to prevent binding of BMPs to their receptors or it may antagonize the activity of another signaling pathway that cooperates with BMPs in stimulating bone formation. Recently, sclerostin was found to antagonize canonical Wnt signaling by binding

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to the Wnt co-receptors LRP5 and LRP6.[12,13] This Wnt antagonistic activity of sclerostin may explain the inhibitory effect of sclerostin on BMP-stimulated bone formation, because Wnts are known to cooperate with BMPs in stimulating bone formation.[14–16]

In this study, we used transcriptional profiling to identify possible pathways by which sclerostin may inhibit BMP-stimulated bone formation. Subsequently, the effect of sclerostin on the putative target pathways was studied using reporter assays. DAN family members do not only inhibit the activity of BMPs, but also of some others growth factors, including Wnt, Nodal, and Activin.[17–19] We therefore focused our microarray analysis on the effect of sclerostin on growth factor pathways. Sclerostin was found to specifically affect Wnt signaling out of many growth factors pathways, strongly suggesting that it abrogated BMP-stimulated bone formation by antagonizing Wnt signaling.

MATERIALS AND METHODS

Compounds

Recombinant human BMP4, recombinant mouse Wnt3a (rmWnt3a), recombinant human sclerostin, recombinant mouse noggin, and recombinant human dkk1 were all purchased from R&D Systems Europe (Abingdon, UK). The BMP-responsive luciferase reporter construct BRE-luc and the expression vectors for constitutive active BMP receptors mouse Activin receptor-like kinase 2 (caALK2) and mouse caALK6 were previously described.[20] A modified Wnt-responsive luciferase reporter construct TBE-luc consisting of a minimal Wnt responsive promoter (four repeats of TCF-4 binding element) driving expression of a Gal4VP16 fusion product combined with a responsive UAS-luciferase reporter was kindly provided by C Breukel, Department of Human Genetics, Leiden University Medical Center, Leiden, The Netherlands.[21] Expression constructs for mouse Wnt1, human/mouse hybrid Wnt3, and mouse Wnt3a were generously provided by Dr R Nusse, Stanford University Medical Center, Stanford, CA, USA. The expression construct for human *SOST* was provided by Dr D Winkler, Celltech, Seattle, WA, USA. Human BMP7 expression construct (Clone ID 4183402) was obtained from the I.M.A.G.E. Consortium and subcloned into pcDNA3.1 + using *Kpn*I and *Not*I.

Muscle electroporation

Ten-week-old male Swiss Albino mice received water and food (chow diet) ad libitum. Electroporation and radiography were performed under isoflurane anesthesia (0.8 liters/min, isoflurane; Air Products, Waddinxveen, The Netherlands) using the Vapex3 system (VetTech Solutions). pcDNA3.1 + plasmids encoding for human BMP-7 or human *SOST* dissolved in 30 μl TE buffer and 140 mM NaCl were injected in the calf muscles (25 μg plasmid per leg) followed by eight 10-ms electrical pulses at 200 V/cm with a frequency of 1 Hz. The pulses were generated with a Square Wave Electroporator ECM 830 and administered using Caliper Electrodes (BTX; Harvard Apparatus). pcDNA3.1 + empty plasmid was used as control and to make up the total amount of 50 μg plasmid per leg. To increase transfection efficiency, hind legs of the mice were shaved, and calf muscles were injected with 30 μl of hyaluronidase (in total 13.5 U; Sigma, Zwijndrecht, The Netherlands) 1 h before electroporation as previously reported.[22] Electroporations using a plasmid encoding luciferase driven by the ubiquitous active CAG promoter showed a mean luciferase activity of $1.01 \pm 0.36 \times 10^8$ photons per second (range: 3.55×10^6–4.05×10^8) 4 days after electroporation in vivo using Bioluminescent reporter imaging.[23] After 14 days, X-rays were made to analyze ectopic formation bone in the calf muscles. The amount of ectopic bone formation was determined by quantification of the number of pixels of the intramuscular bone area on the X-rays using Image-Pro Plus version 3 (Media Cybernetics, Silver Spring, MD, USA). Tibia, fibula, and surrounding calf muscle were fixed in 3.7% phosphate-buffered formaldehyde (pH 7.2) for 2 days and decalcified in 12.5% EDTA (pH 7.4) for 3 weeks. Five-micrometer-thick sections were prepared from paraffin-embedded blocks and analyzed for bone formation using Goldner staining. The animal experimental protocol was approved by the Leiden University Committee for Animal Experiments (UDEC).

Cell cultures

Mouse mesenchymal KS483 were cultured in αMEM and mouse mesenchymal C3H10T1/2 cells and rat and human osteosarcoma cells, UMR106 and U2OS, respectively, in DMEM (GIBCO BRL, Breda, The Netherlands), supplemented with penicillin/streptomycin (Invitrogen, Breda, The Netherlands) and 10% FCS (Integro, Zaandam, The Netherlands).

Gene expression microarray analysis

KS483 cells were seeded at a density of 15,000 cells/cm^2, and agents were added when cells reached confluence after 4 days of culture. After a 6-h treatment, total RNA was isolated using Trizol LS Reagent (Life Technologies/GibcoBRL, Breda, The Netherlands) followed by RNeasy isolation kit with on-column DNase digestion (Qiagen, Venlo, The Netherlands) as described by the manufacturers. The isolated RNA samples were sent to ServiceXS BV (Leiden, The Netherlands) where they were processed according to Affymetrix protocols. In brief, RNA concentration was determined by absorbency at 260 nm, and quality and integrity were verified using the RNA 6000 Nano assay on the Agilent 2100 Bioanalyzer (Agilent Technologies, Amstelveen, The Netherlands). Next, 2 μg of high-quality total RNA was used with the Affymetrix Eukaryotic One-Cycle Target Labeling and Control reagents to generate Biotin-labeled antisense cRNA. The quality of the cRNA was checked using the Agilent 2100 bioanalyzer. The labeled cRNA was further used for the hybridization to Affymetrix Mouse Genome 430 2.0 Array Genechips. After an automated process of washing and staining, absolute values of expression were calculated from the scanned array using the Affymetrix GCOS software. Experiments were done in duplicate.

Microarray analyses were made in R (version 2.1.1) using functions from Bioconductor (version 1.6).[24] The scanned intensities (.CEL files) from the hybridizations were preprocessed: Background was corrected with robust multiarray average (RMA), mismatch probes were subtracted according to the MAS5 procedure, and the intensities were normalized by quantiles.[25] The probe intensities were used as separate entities in a linear regression model.[26] Per gene, the $\log_2$ signal intensities were used in the model:

$$\text{Signal} = \beta_1 \text{ Probe} + \beta_2 \text{ BMP} + \beta_3 \text{ sclerostin} + \beta_4 \text{ BMP and sclerostin} + \varepsilon$$

for each probe and the two-level (control and addition of the agent) effects of BMP and sclerostin together with their interaction effect. The ε variables are assumed to be independent and random with mean 0 and variance σ^2.[26] Therefore, for each gene, at least 176 probe signals were measured to estimate the β coefficients. The β_1 coefficients estimate the influence of the individual probes, and the β_2 coefficients correspond to the response when adding BMP. β_3 coefficients reflect the response when adding sclerostin. The β_4 coefficients correspond to the interaction between BMP and sclerostin. For each effect, p values for significance were calculated by multivariate ANOVA. To calculate signal to noise-ratios, the multiple probe signals per gene were summarized into an average value using the MAS5 procedure.

Gene sets were used as defined by the Gene Ontology Consortium[27] and KEGG resource.[28] Using the Bioconductor package moe4302 of May 17, 2005, there are 135 KEGG pathways and 4221 GO terms present on the Affymetrix Mouse Genome 430 2.0 Array Genechip. To determine if members of a given gene set are enriched among the differentially expressed genes ($p < 0.1$ from the multivariate ANOVA), we calculated the hypergeometric probability based on the number of genes in a gene set, the number of differentially expressed genes in the gene set, and the proportion of differentially expressed genes for each effect (i.e., BMP, sclerostin, and the combination BMP and sclerostin). Gene sets with an associated $p < 0.005$ were considered significant. Supplementary datasets and complete lists of GO-term and KEGG pathway analyses are available online at http://www.lumc.nl/1050/research/Signaaltransductie/Signaaltransductie_Journal%20of%20bone%20and%20mineral%20research.htm.

Transfections and reporter assays

KS483, C3H10T1/2, or U2OS cells were seeded at a density of 10,000, 20,000, and 20,000 cells/well in 24-wells plates, respectively, and transiently transfected with either BRE-luc or TBE-luc using Fugene6 transfection reagent according to the manufacture's protocol (Roche, Basel, Switzerland). Wnt1, Wnt3, and Wnt3a expression vectors were co-transfected when needed. To correct for transfection efficiency, *Renilla* luciferase vector was co-transfected (pRL-SV40; Promega, Leiden, The Netherlands). Sixteen hours after transfection, medium was changed for medium containing 0.2% FCS, and cells were treated as described in the Results section. Luciferase assays were performed with the Dual-Luciferase Reporter assay system according to the manufacturer's instructions (Promega). Firefly luciferase activity was corrected for *Renilla* luciferase activity to control for differences in transfection efficiency.

Alkaline phosphatase activity assay

KS483 cells were seeded at a density of 15,000 cells/cm^2 in 96-well plates, and agents were added when cells reached confluence after 3 or 4 days of culture. Alkaline phosphatase (ALP) activity was kinetically measured in the cell layer after another 4 days of culture as previously described.[8]

Statistics

Values are expressed as mean ± SE. Except for the microarray analysis, statistical differences between values were examined by one-way ANOVA followed by Bonferroni multiple comparison test and considered to be significant different at $p < 0.05$. Experiments were performed at least three times.

RESULTS

Sclerostin inhibits BMP-induced bone formation in vivo

To study whether the inhibitory effect of sclerostin on BMP-stimulated bone formation in vitro also occurs in vivo, we studied the effect sclerostin on BMP-induced bone formation in calf muscle of mice using DNA electroporation of equal amounts of expression plasmids for BMP and sclerostin. BMP induced bone formation in six of six calf muscles, as seen on X-rays 14 days after electroporation, with a mean intramuscular bone area of 13,868 ± 2823 pixels (Fig. 1). The bone formed was normal. Sclerostin expression itself did not induce bone formation in any of the muscles nor did we observe any histological changes in the muscle. Coelectroporation of BMP with sclerostin, however, completely abrogated BMP-induced bone formation all six calf muscles. In a second experiment, BMP induced bone formation in four of four calf muscles with a mean intramuscular bone area of 25,648 ± 586 pixels, whereas no bone formation was observed in any of the calf muscles of the control, sclerostin, and BMP + sclerostin–treated groups.

Gene expression microarray analysis

Transcriptional profiling using microarrays was used on confluent mouse osteoblastic KS483 cells treated with BMP and/or sclerostin for 6 h. Affymetrix Mouse Genome 430 2.0 Array Genechips were used, containing >39,000 transcripts and variants including >34,000 well-characterized genes. Of note, these osteoblastic cells produce endogenous BMPs that are biologically active.[29] Initial examination of the gene expression profiles after BMP and/or sclerostin stimulation revealed that BMP and sclerostin affected (threshold of $p = 0.001$) the expression of ~2000 genes and the combination of ~1000 genes (Fig. 2). The majority (±70%) of the affected genes was not affected by the other

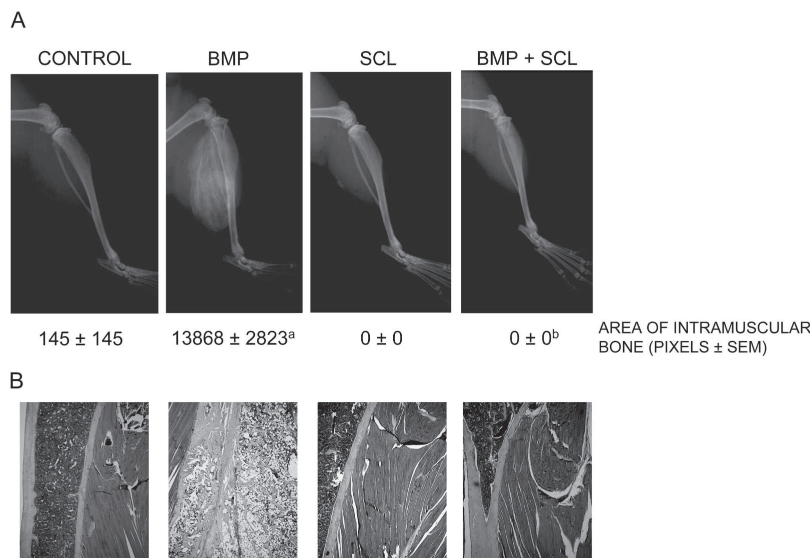

FIG. 1. Sclerostin abrogates BMP-induced bone formation in calf muscles of mice in vivo. Equal amounts of empty, BMP7, and/or sclerostin-containing expression plasmids were transfected into calf muscles of mice using DNA electroporation. BMP7 induced ectopic bone formation in all calf muscles 14 days after electroporation. No bone formation was observed in muscles electroporated with empty, sclerostin, or both BMP7 and sclerostin-expressing plasmids; except for a small piece of bone in one of the muscles in the control group. Shown are (A) representative X-rays and (B) Goldner-stained histological bone sections for each group. Quantification of the area of newly formed bone in the muscle on the X-rays is listed under the representative X-rays per group ($n = 6$ calf muscles per group). Scl, sclerostin. [a]Significant vs. control; [b]significant vs. BMP stimulated.

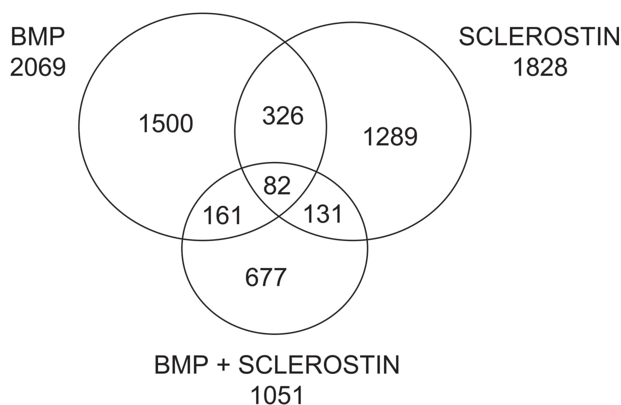

FIG. 2. Venn diagram of number of genes affected by BMP, sclerostin, and/or their combination.

treatments, and only 82 genes were affected by all three treatments, which would not be expected if sclerostin acts as a direct BMP antagonist.

The majority of genes affected by BMP were upregulated (1535 of 2068), whereas sclerostin-affected genes were predominantly downregulated (1492 of 1828). Only 408 genes were affected by both BMP and sclerostin. Of these genes, 65% (266 genes) showed an opposite effect for BMP and sclerostin, as expected when sclerostin acts as a direct BMP antagonist. The remaining 35% of genes (142 genes) were, however, affected by BMP and sclerostin in the same direction. The combination of BMP and sclerostin affected 88% of the genes in a similar way as the cumulative effect of the two compounds separately (1826 of 2069 BMP-affected genes and 1615 of 1828 sclerostin-affected genes), an observation in conflict with sclerostin being a direct BMP antagonist. In total, the combination affected >1000 genes differently from the cumulative effect of BMP and sclerostin separately, including genes that were not affected by either of the compounds separately. Together these findings strongly suggest that sclerostin may have actions distinct from antagonizing BMP signaling.

To identify putative pathways by which sclerostin may inhibit BMP-stimulated bone formation, we performed genome-wide analysis of gene sets defined by the Gene Ontology (GO) Consortium and classified as GO terms.[27] In this analysis, an enrichment of affected genes within a GO term suggests that this GO term is affected by the treatment. The GO database contains 4221 GO terms, but we limited our analysis to the 2335 GO terms containing >10 genes. BMP and sclerostin affected ~400 GO terms (Supplementary Dataset 1). Because we hypothesized that sclerostin exerts its action by antagonizing a specific growth factor signaling pathway, only GO terms that describe such specific pathways were used to identify the signaling pathway(s) affected by sclerostin that may account for its inhibitory effect on BMP-stimulated bone formation. Of the 24 GO terms that describe a specific growth factor pathway, BMP affected the TGF-β/BMP and Notch signaling pathways, two well-known effects of BMP stimulation (Table 1 and Supplementary Dataset 2). Wnt pathway was the only pathway affected by sclerostin. The combination BMP and sclerostin affected almost 300 GO terms differently from the cumulative effect of BMP and sclerostin separately, including BMP signaling.

In a similar approach, we used gene set analysis in which the gene sets consisted of pathways defined by the Kyoto Encyclopedia of Genes and Genomes (KEGG) resource.[28] The KEGG pathway database is a collection of pathway maps of interaction and reaction networks of which 135 were present on the array. BMP and sclerostin affected ~20 pathways (Supplementary Dataset 3). Of the pathways specific for a certain growth factor, BMP affected TGF-β/BMP signaling and sclerostin affected TGF-β/BMP and Wnt signaling (Table 1 and Supplementary Dataset 4). The combination BMP and sclerostin affected 15 pathways differently from the cumulative effect of BMP and sclerostin separately, including Wnt signaling. A restriction of the KEGG database is, however, that it contains only five growth factor specific pathways (i.e., Wnt, Notch, hedgehog, TGF-β/BMP, and insulin). Together, the GO and

TABLE 1. GO TERMS AND KEGG PATHWAYS AFFECTED BY BMP, SCLEROSTIN, AND/OR THEIR COMBINATION

Method	*Treatment*	*Affected pathways*	*Code*
GO	BMP	TGF-β signaling	GO:0007179
		Notch signaling	GO:0007219
			GO:0008013†
	Sclerostin	Wnt signaling	GO:0030111
			GO:0030509†
	BMP + sclerostin	BMP signaling*	GO:0030510
KEGG	BMP	TGF-β signaling	KEGG:4350
	Sclerostin	TGF-β signaling	KEGG:4350
		Wnt signaling	KEGG:4310
	BMP + sclerostin	Wnt signaling*	KEGG:4310

* Different from the cumulative effect of BMP and sclerostin separately.
† Different GO terms representing the same signaling pathway.

TABLE 2. EFFECTS OF BMP, SCLEROSTIN, AND THEIR COMBINATION ON DIRECT BMP TARGET GENES

Direct BMP target genes	*BMP*	*Sclerostin*	*BMP + sclerostin*	*BMP + sclerostin**
CTGF	↑	↓	=	=
Id-1	↑	=	↑	↑
Id-2	↑	=	↑	↑
Id-3	↑	=	↑	↑
Smad-6	↑	=	↑	=
Smad-7	↑	=	↑	=
Dlx-5	↑	=	↑	=
Msx-2	↑	↓	=	=

* Compared with the cumulative effect of BMP and sclerostin separately.

KEGG analyses indicate that sclerostin most likely affects TGF-β/BMP and/or Wnt signaling.

Sclerostin does not antagonize BMP signaling

To address the antagonistic effect of sclerostin on BMP signaling, we analyzed the expression of eight proven direct BMP target genes on the microarrays. As expected, BMP increased their expression levels (Table 2). Sclerostin, however, did not affect their expression; except for CTGF and Msx-2 that were downregulated. The combination of BMP and sclerostin did not affect the stimulated expression of the BMP target genes different from the cumulative effect of BMP and sclerostin alone; only the BMP-stimulated expression of the Id genes was further increased by sclerostin. The latter may indicate that sclerostin removes a suppressor of BMP-stimulated expression of Id genes. Together these findings further support the notion that sclerostin is not a classical BMP antagonist.

These observations are, however, restricted to one cell line, and it may be possible that sclerostin antagonizes BMP signaling in other cells depending on the absence or presence of a co-factor. To look for cell line–specific effects of sclerostin on BMP signaling, we tested three other osteoblastic cell lines including C3H10T1/2 cells, the cell line on which Winkler et al.[9] reported attenuation of BMP-stimulated Smad phosphorylation by sclerostin. However, at a concentration that completely abrogates BMP-stimulated bone formation, sclerostin did not antagonize stimulation of the BMP reporter construct BRE-luc in any of the cell lines, whereas the classical BMP antagonist noggin did (Fig. 3).

Sclerostin may still antagonize BMP-stimulated bone formation by acting as a direct BMP antagonist late during BMP stimulation. To study this, we investigated whether addition of sclerostin 24 h after BMP stimulation inhibited BRE-luc activity, as it inhibited BMP-stimulated ALP activity.[8] Late addition of sclerostin to BMP-stimulated cultures, however, did not inhibit BRE-luc activity measured after 48 and 72 h, in marked contrast to noggin (Figs. 4A and 4B). This showed that sclerostin also did not antagonize direct BMP responses late during BMP stimulation.

To further substantiate this issue, we used an experimental set-up in which the effect of sclerostin on a second pulse of BMP stimulation was studied using BRE-luc as readout. First, we checked whether BRE-luc activity was restored to control level after the first BMP stimulation and removal of BMP stimulus. For this, KS483 cells were treated for 48 h with BMP, which significantly increased BRE-luc activity (Fig. 4C). Removal of BMP stimulus by changing culture medium restored BRE-luc activity to almost control levels after 24 h, and activity was further decreased to control level during the subsequent 24 h. A second BMP stimulation 24 h after medium change induced a renewed activation of BRE-luc. Although the second response was lower, it showed that it was possible to study the effect of sclerostin on a direct BMP response after prior BMP stimulation without the problem of residual BRE-luc activity. The second BMP-stimulated activation of BRE-luc was, however, also not antagonized by sclerostin (Fig. 4D).

Sclerostin antagonizes BMP-stimulated Wnt signaling

The GO and KEGG analyses of our gene expression data suggested that sclerostin may affect Wnt signaling, which is consistent with recent publications showing that sclerostin inhibits canonical Wnt signaling by binding to the LRP5 and LRP6 co-receptors.[12,13] Wnt signaling has been found to be essential for BMP-stimulated ALP activity in several osteoblastic cell lines.[14,15] To study whether Wnts play a role in BMP-stimulated ALP activity in KS483 cells, we tested the effect of the Wnt antagonist Dkk1 and found that it inhibited BMP-stimulated ALP activity similar to sclerostin (Figs. 5A and 5B).[16] In addition, BMP stimulated the Wnt reporter construct TBE-luc (Fig. 5C), and this stimulation was antagonized by both the classical Wnt antagonist Dkk1 and sclerostin (Fig. 5D). Because exogenously added BMPs are still present in the culture system and, therefore, an effect of sclerostin on these BMPs could not be excluded, we studied whether sclerostin also antagonized activation of TBE-luc by constitutive active BMP type I receptors (caALK2 and caALK6) that signal independent of ligand. Similar to the effect on exogenously added BMPs, sclerostin antagonized activation of TBE-luc by both caALK2 and caALK6 (Fig. 5E). Together, these data suggest that sclerostin inhibits late BMP responses like ALP activity by antagonizing Wnt signaling.

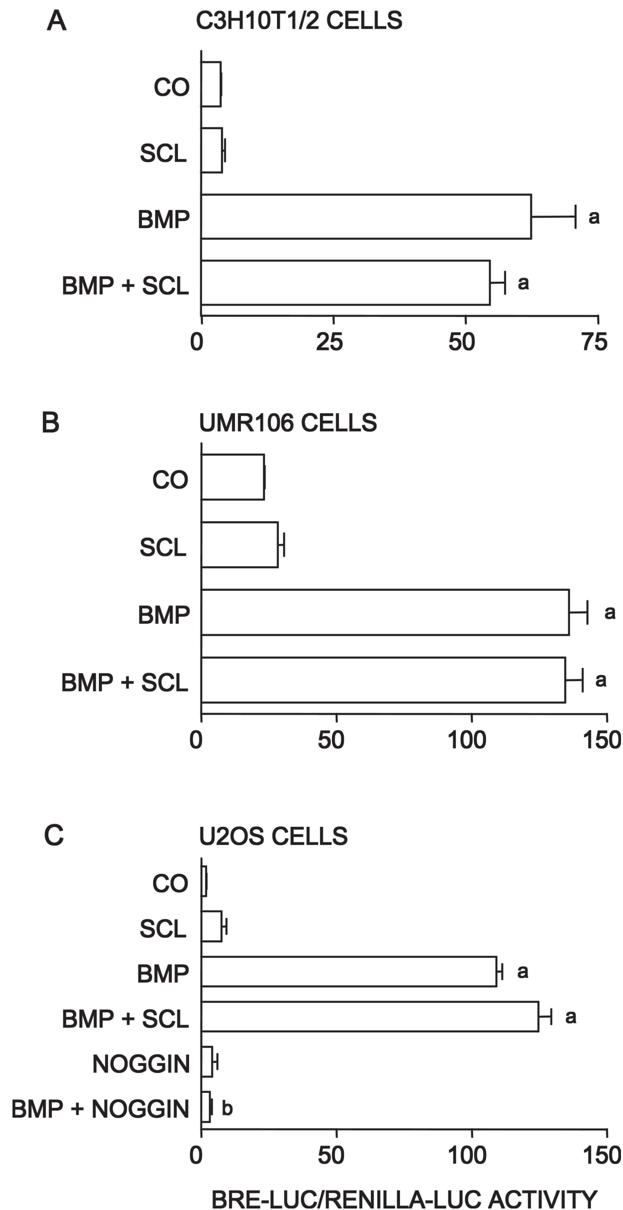

FIG. 3. Sclerostin does not antagonize BMP signaling in several osteoblastic cell lines. (A) BRE-luc transfected C3H10T1/2 cells were stimulated with sclerostin (5 μg/ml), BMP4 (3 ng/ml), or BMP4 + sclerostin for 24 h after which BRE-luc activity was measured. (B) BRE-luc transfected UMR106 cells were treated as described in A, but BMP4 concentration was 100 ng/ml. (C) BRE-luc transfected U2OS cells were treated as described in A, but BMP4 concentration was 10 ng/ml. In addition, cells were stimulated for 24 h with noggin (200 ng/ml) or BMP4 + noggin. In parallel experiments, sclerostin inhibited BMP-stimulated ALP activity in KS483 cells. Scl, sclerostin. [a]Significant vs. control; [b]significant vs. BMP stimulated.

Sclerostin antagonizes Wnt signaling

To address the question of whether sclerostin antagonizes Wnt signaling directly, we studied whether sclerostin antagonized recombinant mouse Wnt3a (rmWnt3a)-induced TBE-luc activity in KS483 cells. Sclerostin, however, did not antagonize rmWnt3a-stimulated activation of this construct, whereas Dkk1 antagonized it dose-dependently (Fig. 6A). Sclerostin, however, may only antagonize direct Wnt responses late during BMP stimulation, for example, after induction of a co-factor. We therefore tested whether sclerostin antagonized rmWnt3a-induced TBE-luc activation after a pretreatment with BMP. First, we checked whether TBE-luc activity induced by BMP stimulation for 48 h was restored to control levels 24 h after removal of the BMP stimulus by medium change, which was the case (Fig. 6B). Of note, TBE-luc activity was also restored to control levels 72 h after BMP stimulation without any medium change (i.e., still in the presence of BMP stimulus). rmWnt3a induced TBE-luc activity to a similar extent in untreated and BMP pretreated cells, independent of a medium change after 48 h of BMP stimulation. This showed that is was possible to study the effect of sclerostin on rmWnt3a-induced TBE-luc activity after 48-h pretreatment with BMP. Sclerostin, however, also did not antagonize rmWnt3a-induced TBE-luc activity after 48-h pretreatment with BMP (Fig. 6C). This was again independent of medium change after 48 h.

It may be, however, that sclerostin does not antagonize Wnt3a, but antagonizes other Wnts. We therefore studied whether sclerostin antagonized TBE-luc activity induced by co-transfection with an expression vector for Wnt1 or Wnt3. In addition, we used an expression vector for Wnt3a as comparison for stimulation with rmWnt3a. Sclerostin completely antagonized Wnt1-induced TBE-luc activity and partly antagonized Wnt3-induced TBE-luc activity (Fig. 7). Remarkably, sclerostin also antagonized TBE-luc activity induced by co-transfection with a Wnt3a expression vector. This suggests that exogenous added rmWnt3a and Wnt3a produced by transfected cells are both biological active, but differently recognized by sclerostin. Dkk1 antagonized TBE-luc activity induced by all three Wnt expression vectors as expected.

DISCUSSION

Sclerostin deficiency that is caused by nonsense mutations in *SOST* or a deletion downstream of the *SOST* gene causes sclerosteosis and van Buchem disease, respectively.[4–7] Absence of this negative regulator of bone formation results in increased bone mass of good quality because of accelerated bone formation, without any significant effects on bone resorption.[3] In patients with sclerosteosis or van Buchem disease, clinical features are all related to the increase in bone formation. This is probably because of the restricted expression of sclerostin; the protein has only been found in osteocytes in the adult.[3,8,9,30] Both diseases are described as autosomal recessive. Measurement of BMD in heterozygotes for the *SOST* mutation among the Afrikaner population in South Africa, however, showed that these persons do have elevated BMD levels without any clinical symptoms.[31] Together these observations suggest that sclerostin may be a good target for the development of therapeutic agents for the treatment of patients with osteoporosis.

The mechanism by which sclerostin inhibits bone forma-

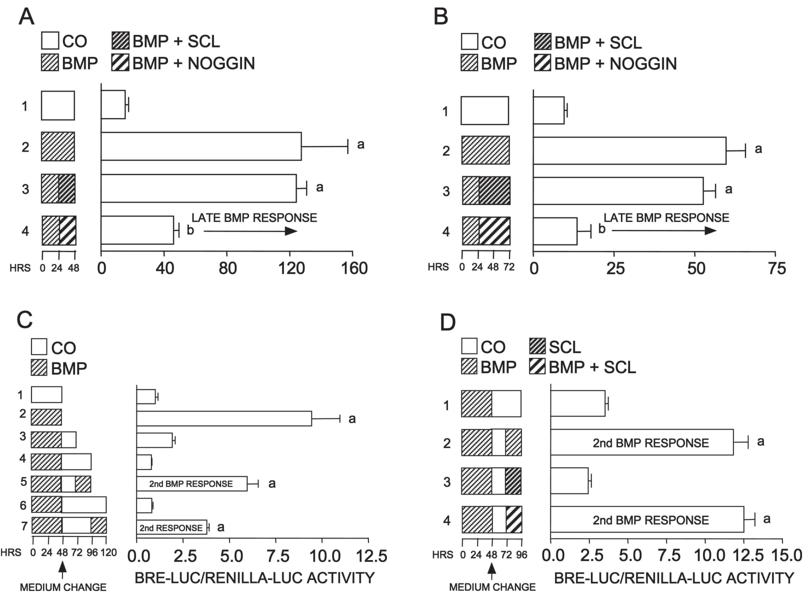

FIG. 4. Sclerostin does not antagonize direct BMP signaling late during BMP stimulation. (A) KS483 cells were stimulated with BMP4 (50 ng/ml) for 48 h and sclerostin (5 μg/ml) or noggin (500 ng/ml) was added during the last 24 h of culture. BRE-luc activity was measured 48 h after BMP4 stimulation. (B) Similar to A, but BMP4 stimulation was 72 h and sclerostin or noggin was added during last 48 h of culture. (C) BRE-luc transfected cells were pretreated with BMP4 (50 ng/ml) for 48 h. Medium was changed to remove BMP stimulus for 24 h before a second BMP4 (50 ng/ml) stimulation for 24 h. BRE-luc activity was measured at 48, 72, 96, and 120 h after first BMP4 stimulation. (D) Cells were pretreated with BMP4 (50 ng/ml) for 48 h. Medium was changed to remove BMP stimulus for 24 h before cells were again stimulated with BMP4 (50 ng/ml), sclerostin (5 μg/ml), or BMP4 + sclerostin for 24 h. BRE-luc activity was measured 96 h after first BMP4 stimulation. In parallel experiments, sclerostin inhibited BMP-stimulated ALP activity in KS483 cells. Scl, sclerostin. [a]Significant vs. control; [b]significant vs. BMP stimulated.

tion is, however, unclear. Originally, sclerostin was suggested to inhibit bone formation by antagonizing BMP activity[5] However, whereas consistent findings have been reported with regard to the inhibitory effect of sclerostin on late BMP responses such as ALP activity, contradictory findings have been published on the direct BMP response of Smad phosphorylation.[8,9]

To identify growth factor specific pathways that may be targeted by sclerostin and account for its inhibitory on BMP-stimulated bone formation, we used genome-wide transcriptional analysis using genes sets consisting of GO terms and KEGG pathways on osteoblastic KS483 cells treated with BMP and/or sclerostin. Sclerostin, either alone or in combination with BMP, influenced many GO terms and KEGG pathways, but of the growth factor–specific pathways, only TGFβ/BMP and Wnt signaling were affected, suggesting that sclerostin may act as a BMP and/or Wnt antagonist. The first option was, however, not supported when analysis was performed at gene level. Thirty-five percent of the genes affected by both BMP and sclerostin were affected in the same direction, and >1000 genes were affected by the combination BMP and sclerostin differently than the cumulative effect of both proteins separately; observations that cannot be explained by sclerostin preventing the binding of BMP to its receptor. Moreover, sclerostin did not counteract upregulation of multiple direct BMP target genes. This lack of direct BMP antagonistic activity was also found on Smad-driven BMP reporter activity in several cell lines (i.e., KS483, C3H10T1/2, UMR106, and U2OS cells), also when the effect of sclerostin on BMP reporter activity was tested late during the BMP stimulation or after a previous BMP stimulation. Our data indicate that sclerostin is not a direct BMP antagonist.

Wnt signaling is required for BMPs to stimulate ALP activity in several osteoblastic cell lines, including KS483 cells.[16,32–34] Inhibition of Wnt signaling by sclerostin may, therefore, account for the inhibitory effect of sclerostin on BMP-stimulated bone formation. Recently, sclerostin was found to antagonize Wnt signaling by binding to the LRP5 and LRP6 co-receptors of canonical Wnt signaling.[12,13] Other DAN family members have also been reported to inhibit Wnt activity.[17–19] Of these members, Coco was shown to antagonize Wnt signaling directly, because it inhibited Wnt8-stimulated reporter construct activity. Sclerostin binds to the first two YWTD-EGF repeats of the Wnt co-receptors LRP5 and LRP6, which is the same region to which Wise binds,[18] but distinct from the third YWTD-EGF repeat, to which Dkk1 binds to exert its antagonistic effect.[35,36] We show here that sclerostin, similar to Dkk1, also antagonized BMP-stimulated Wnt reporter activity. In addition, we observed an antagonistic effect of sclerostin on Wnt reporter construct activation by Wnt1, Wnt3, and Wnt3a expression constructs. Because both sclerostin and Dkk1 inhibit BMP-stimulated ALP activity in our culture system of mouse mesenchymal KS483 cells, these data suggest that sclerostin inhibits BMP-stimulated bone formation by antagonizing Wnt signaling (Fig. 7). Because transcriptional profiling did not reveal any other growth factor pathways affected by sclerostin, it is unlikely that sclerostin abrogates BMP-stimulated bone formation

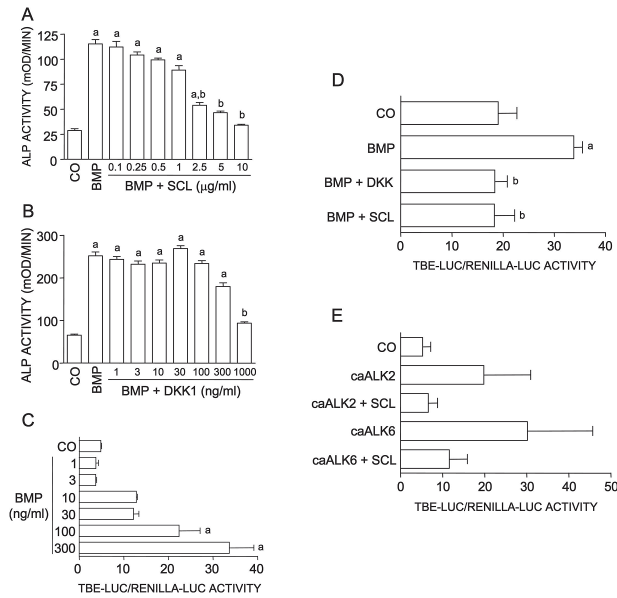

FIG. 5. Sclerostin antagonizes BMP-stimulated Wnt signaling. (A) Confluent KS483 cells were stimulated with BMP4 (50 ng/ml) in the absence or presence of a dose range of sclerostin (0.1–10 μg/ml). (B) Cells were treated as described in A, but in the absence or presence of a dose range of Dkk1 (1–1000 ng/ml). (C) TBE-luc transfected KS483 cells were stimulated with a dose range of BMP4 (1–300 ng/ml) for 24 h. (D) TBE-luc transfected cells were stimulated with BMP4 (50 ng/ml), BMP4 + Dkk1 (1 μg/ml), or BMP4 + sclerostin (5 μg/ml) for 24 h. (E) Cells were co-transfected with TBE-luc and either caALK2 or caALK6 and cultured for 24 h in absence or presence of sclerostin (5 μg/ml). caALK, constitutive active ALK; Scl, sclerostin. [a]Significant vs. control; [b]significant vs. BMP stimulated.

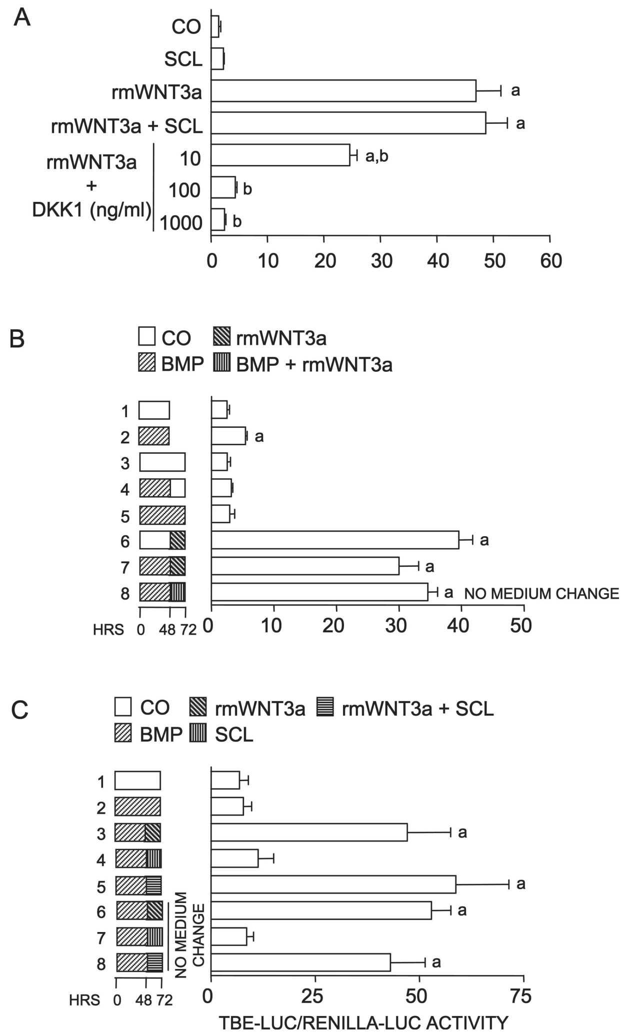

FIG. 6. Sclerostin does not antagonize rmWnt3a signaling. (A) TBE-luc transfected KS483 cells were stimulated with sclerostin (5 μg/ml), rmWnt3a (20 ng/ml), rmWnt3a + sclerostin, or rmWnt3a + Dkk1 (10–1000 ng/ml) for 24 h. (B) Cells were either not stimulated or stimulated with BMP4 (50 ng/ml) for 48 h. TBE-luc activity was measured after 48 h (lanes 1 + 2) or after an additional 24 h in which the cultures were either prolonged (lanes 3 + 5), removed of BMP stimulus by medium change (lane 4) or stimulated with rmWnt3a (20 ng/ml) in the absence (lanes 6 + 7, medium change) or presence of BMP stimulus (lane 8, no medium change). (C) TBE-luc activity was measured 72 h after transfection in nonstimulated (lane 1) or BMP4-stimulated (50 ng/ml) cells (lane 2) or cells stimulated with BMP4 for 48 h followed by a stimulation with rmWnt3a (20 ng/ml; lanes 3 + 6), sclerostin (5 μg/ml; lanes 4 + 7), or rmWnt3a + sclerostin (lanes 5 + 8) with (lanes 3–5) or without (lanes 6–8) medium change. rmWnt3a, recombinant mouse Wnt3a; Scl, sclerostin. [a]Significant vs. control; [b]significant vs. rmWnt3a stimulated.

by antagonizing the activity of growth factors other than Wnts. Of the three Wnts tested in this study and the previous studies by Li et al.[12] and Semenov et al.,[13] only Wnt1 has been reported to be expressed in bone.[14,15] Other Wnts found in bone are Wnt4, Wnt7b, Wnt9a (formerly Wnt14), and Wnt10b, but the exact expression of Wnts in bone and their role in bone metabolism is currently unknown. Wnt10b was found to increase osteoblastogenesis in vitro and trabecular bone mass in vivo.[37] In addition, Wnt10b knockout mice have decreased trabecular bone. Further studies are needed to determine the Wnts antagonized by sclerostin in vivo.

Remarkably, sclerostin did not antagonize activation of the Wnt reporter construct by rmWnt3a, even after BMP pretreatment, whereas Dkk1 did. Similarly, sclerostin has been shown not to antagonize rmWnt3a-stimulated β-catenin stabilization in C3H10T1/2 cells.[38] The difference between Wnt3a produced by transiently transfected cells and rmWnt3a suggests that these molecules are differentially recognized by sclerostin. This may be explained by differences in tertiary structure, glycosylation, membrane-bound presentation, and/or other characteristics of Wnt3a. In addition, it suggests that the mechanism by which sclerostin inhibits canonical Wnt signaling is different from that of Dkk1. Dkk1, in combination with Kremen, antagonizes canonical Wnt signaling by inducing endocytosis and removal of LRP5 and LRP6 from the cell membrane.[39] Sclerostin's mechanism of action may also by different from Wise, because Wise prevented binding of Wnt8 to LRP6,[12] whereas sclerostin binding to LRP5 was not prevented by Wnt3a.[18] Additional studies are required to unravel the exact mechanism by which sclerostin binding to LRP5 and LRP6 inhibits canonical Wnt signaling.

In conclusion, sclerostin inhibits bone formation by a mechanism different from antagonizing direct BMP signal-

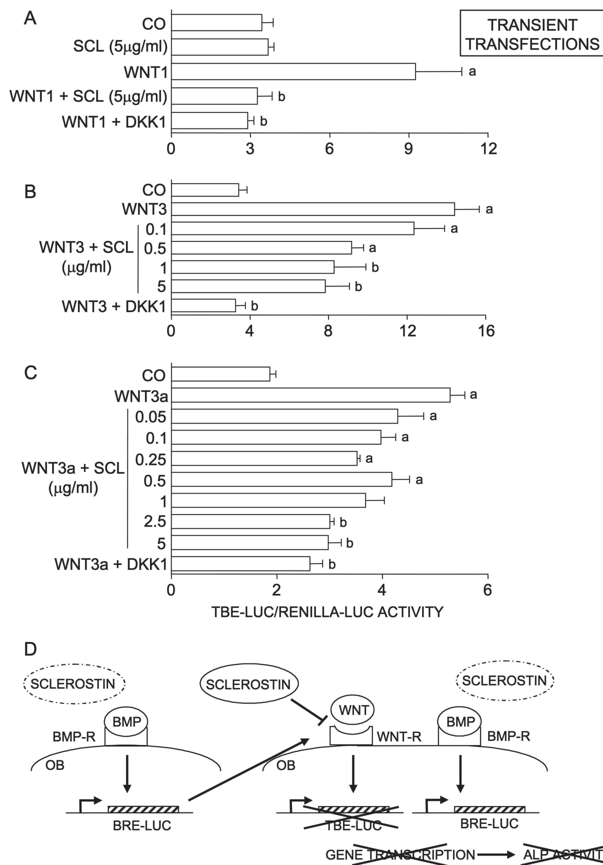

FIG. 7. Sclerostin is a Wnt antagonist. KS483 cells were co-transfected with TBE-luc and expression constructs for (A) Wnt1, (B) Wnt3, or (C) Wnt3a in the absence or presence of sclerostin (0.05–5 μg/ml) or Dkk1 (1 μg/ml). TBE-luc activity was measured 24 h after transfection. (D) Schematic model of sclerostin's mechanism of action. Sclerostin inhibits BMP-stimulated ALP activity by antagonizing BMP-induced Wnt signaling that cooperates with BMP to exert this effect. OB, osteoblast; BMP-R, BMP receptor; BRE-luc, BMP reporter construct; Scl, sclerostin; TBE-luc, Wnt reporter construct; Wnt-R, Wnt receptor. [a]Significant vs. control; [b]significant vs. Wnt stimulated.

ing, which distinguishes sclerostin from classical BMP antagonists such as noggin. Sclerostin antagonized BMP-stimulated Wnt signaling in osteoblastic cells, suggesting that sclerostin inhibits BMP-stimulated bone formation indirectly through antagonizing Wnt signaling. Indeed, sclerostin antagonized direct Wnt1-, Wnt3-, and Wnt3a-stimulated Wnt reporter construct activation. Sclerostin, however, did not antagonize rmWnt3a-stimulated Wnt reporter construct activation, whereas Dkk1 did. This distinguishes sclerostin's mechanism of action from that of the Wnt antagonist Dkk1. Together, these findings indicate that sclerostin, secreted by osteocytes, inhibits BMP-stimulated bone formation by antagonizing Wnt signaling in osteoblasts. The increased bone mass in sclerosteosis and van Buchem disease may, therefore, result from increased Wnt signaling caused by the absence of sclerostin. Another sclerosing disorder, the human high bone mass (HBM) phenotype, is phenotypically very similar to sclerosteosis and van Buchem disease and was found to result from mutations in the *LRP5* gene that make it resistant to Dkk1-mediated inhibition.[36,40,41] This raises the possibility that these skeletal disorders are all caused by increased activity of the same signaling pathway. In HBM, incapability of Dkk1 to inhibit Wnt signaling increases bone formation, whereas in sclerosteosis and van Buchem disease, increased bone formation is caused by enhanced Wnt signaling because of sclerostin deficiency.

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