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ORIGINAL ARTICLE

Sclerostin inhibits osteoblast differentiation without affecting BMP2/SMAD1/5 or Wnt3a/β-catenin signaling but through activation of platelet-derived growth factor receptor signaling *in vitro*

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Sclerostin inhibits bone formation mostly by antagonizing LRP5/6, thus inhibiting Wnt signaling. However, experiments with genetically modified mouse models suggest that a significant part of sclerostin-mediated inhibition of bone formation is due to interactions with other binding partners. The objective of the present work was to identify signaling pathways affected by sclerostin in relation with its inhibitory action on osteogenic differentiation of C3H10T1/2 cells, MC3T3-E1 cells and primary osteoblasts. Sclerostin inhibited BMP2-induced osteoblast differentiation without altering SMAD1/5 phosphorylation and transcriptional activity. Moreover, sclerostin prevented Wnt3a-mediated osteoblastogenesis without affecting LRP5/6 phosphorylation or β-catenin transcriptional activity. In addition, sclerostin inhibited mineralization promoted by GSK3 inhibition, which mimics canonical Wnt signaling without activation of LRP5/6, suggesting that sclerostin can prevent osteoblast differentiation without antagonizing LRP5/6. Finally, we found that sclerostin could activate platelet-derived growth factor receptor (PDGFR) and its downstream signaling pathways PLCγ, PKC, Akt and ERK1/2. PDGFR inhibition could reverse sclerostin-mediated inhibitory activity on BMP2-induced osteoblast differentiation. Therefore, our data suggest that sclerostin can activate PDGFR signaling by itself, and this functional interaction may be involved in the negative effect of sclerostin on osteoblast differentiation.

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Introduction

Characterization of two rare sclerosing bone disorders, sclerosteosis and van Buchem disease, led to the identification of the *SOST* gene as a negative regulator of bone formation.[1–4] The *SOST* gene encodes sclerostin, which is synthetized and secreted postnatally by terminally differentiated osteocytes embedded in the mineralized bone matrix.[5–7] Mice over-expressing the *Sost* gene display an osteoporotic phenotype with reduced osteoblast activity and bone formation but unaffected resorption.[5] Conversely, *Sost* knockout mice exhibit a high bone mass phenotype due to increased bone formation.[8] Because of its restricted expression in the adult skeleton and its inhibitory effect on bone formation, sclerostin has emerged as an attractive therapeutic target to increase bone mass and strength in osteoporotic patients. Consequently, administration of antibodies targeting sclerostin has been shown to augment bone mineral density (BMD) and bone strength in humans, through elevation of bone formation and reduction in bone resorption.[9–12]

During the last decade, a critical effort has been devoted to the elucidation of sclerostin mechanism of action. On the basis of the presence of a cystine-knot motif in its structure, sclerostin has been ranked in the DAN (differential screening selected gene abberative in neuroblastoma) family of secreted glycoproteins that share the ability to antagonize bone morphogenetic protein (BMP) activity.[1,3] As sclerostin inhibits BMP-induced osteoblast differentiation and weakly binds BMP, it has been initially presumed to be a BMP antagonist.[5,13] However, a large body of evidences has since demonstrated that sclerostin antagonizes canonical Wnt signaling by binding low-density lipoprotein receptor-related proteins (LRP) 5 and 6.[14–16] Wnt proteins are secreted glycoproteins that bind to G protein-coupled Frizzled receptors and LRP5/6 co-receptors. This interaction results in the recruitment of Axin at the

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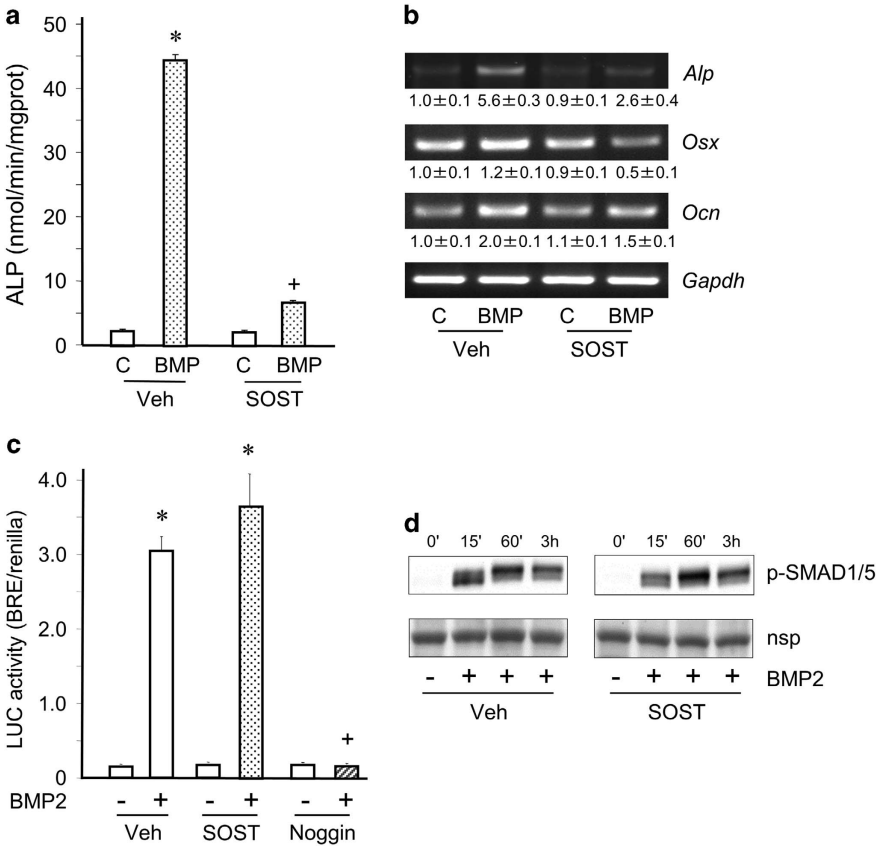

Figure 1 Sclerostin inhibits BMP2-induced osteoblast differentiation without affecting canonical BMP signaling in C3H10T1/2 cells. (**a**) C3H10T1/2 cells in osteogenic medium were pre-incubated with sclerostin (SOST, 5 μg ml^{-1}) or its vehicle (Veh) for 15 min and stimulated with BMP2 (50 ng ml^{-1}) or its vehicle (**c**) for 48 h before measurements of ALP activity. (**b**) Expression of osteoblast marker genes in the same conditions as for (**a**). (**c**) Evaluation of SMAD transcriptional activity in C3H10T1/2 cells pre-incubated with vehicle, 10 μg ml^{-1} sclerostin or 250 ng ml^{-1} noggin and stimulated with 25 ng ml^{-1} BMP2 or its vehicle for 16 h. (**d**) Western blot analyses of SMAD1/5 phosphorylation in C3H10T1/2 cells pre-treated with 10 μg ml^{-1} sclerostin or its vehicle and stimulated with 25 ng ml^{-1} BMP2 or its vehicle for different incubation times (nsp, non-specific protein). *$P<0.01$ for BMP2 versus control; +$P<0.01$ for SOST versus vehicle treatment.

carboxy-terminal domain of LRP5/6 and inhibition of glycogen synthase kinase (GSK) 3β activity toward β-catenin. Unphosphorylated β-catenin is not degraded, accumulates in the cytoplasm, translocates to the nucleus and binds to lymphoid enhancer-binding factor/transcription factor to activate transcription of downstream target genes.[17] The importance of this signaling pathway in regulating bone homeostasis has been pointed out by the identification of gain-of-function mutations of *LRP5* in patients with high bone mass phenotype[18,19] and loss-of-function mutations of *LRP5* in patients with osteoporosis pseudoglioma syndrome.[20]

Interestingly, sclerostin binding to LRP5, and thus inhibition of Wnt signaling, is reduced in *LRP5* gain-of-function mutants,[18,21,22] further indicating that sclerostin inhibits bone formation through LRP5. In fact, it has been recently shown that sclerostin functions only partially through LRP5, as sclerostin inhibition can still increase bone mass accrual in a mouse model of osteoporosis pseudoglioma syndrome (*Lrp5* deficiency).[23,24] In this situation, blocking the LRP6 function with antibodies reverses bone mass gain in the cortical compartment of double *Lrp5*;*Sost* knockout mice to wild-type levels,[24] indicating that sclerostin functions in part by binding to LRP6. However, remaining increased bone mass in the cancellous bone compartment in this experimental mouse model suggests that a significant part of sclerostin-mediated inhibition of bone formation is due to interactions with other binding partners. Sclerostin has been shown to interact with LRP4 *in vivo*.[25,26] This receptor does not seem to be a component of the Wnt signaling system but has been proposed to facilitate sclerostin activity by favoring sclerostin binding to LRP5/6.[25,26] In addition to LRP4/5/6, several other sclerostin-binding partners have been identified on the basis of *in vitro* experiments,[5,27–29] but their respective contribution to sclerostin function remains to be addressed.

The objective of the present work was to investigate signaling pathways affected by sclerostin in relation with its inhibitory action on osteoblast differentiation in different cell culture systems.

Results

Sclerostin inhibits BMP2-induced osteoblast differentiation without affecting SMAD1/5 signaling

First, we analyzed the effects of sclerostin on BMP2-induced osteoblast differentiation of mesenchymal C3H10T1/2 cells. Recombinant sclerostin strongly inhibited BMP2-induced elevations of *alkaline phosphatase* (*Alp*) expression and ALP activity (**Figures 1a and b**). Sclerostin also prevented BMP2-elicited increases in expressions of osteoblast marker genes such as *osterix* and *osteacalcin* (**Figure 1b**). In light

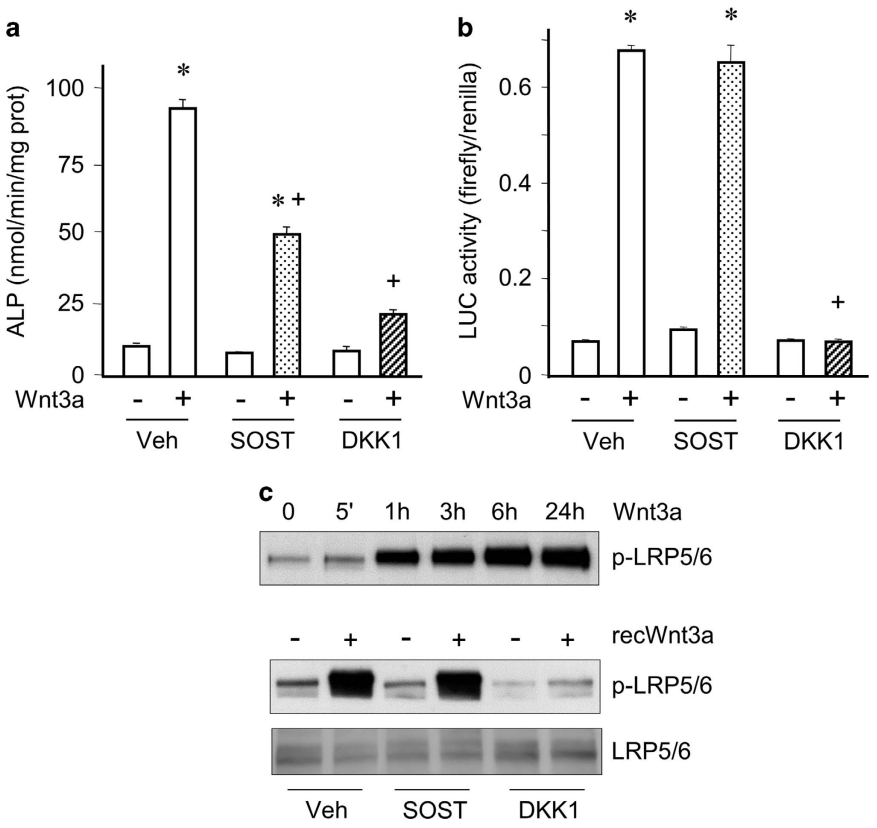

Figure 2 Sclerostin inhibits Wnt3a-induced osteoblast differentiation without affecting canonical Wnt signaling in C3H10T1/2 cells. (**a**) ALP activity of C3H10T1/2 cells pre-incubated with 10 $\mu g\,ml^{-1}$ sclerostin, 1 $\mu g\,ml^{-1}$ dikkopf 1 (DKK1) or vehicle and stimulated with 20% control-conditioned medium or Wnt3a-conditioned medium for 3 days. (**b**) Evaluation of β-catenin transcriptional activity in C3H10T1/2 cells pre-incubated with 10 $\mu g\,ml^{-1}$ sclerostin, 1 $\mu g\,ml^{-1}$ dikkopf 1 or vehicle and stimulated with 20% control-conditioned medium or Wnt3a-conditioned medium for 24 h. (**c**) Western blot analyses of LRP5/6 phosphorylation in C3H10T1/2 cells stimulated with Wnt3a-conditioned medium for different incubation times; and analysis of LRP5/6 phosphorylation in C3H10T1/2 cells pre-incubated with 10 $\mu g\,ml^{-1}$ sclerostin, 1 $\mu g\,ml^{-1}$ dikkopf 1 or vehicle and stimulated with 200 $ng\,ml^{-1}$ recombinant Wnt3a or its vehicle for 3 h. $^*P<0.01$ for Wnt3a versus control; $+P<0.01$ for SOST or DKK1 versus vehicle treatment.

of these results, we next examined whether sclerostin could alter the canonical BMP signaling pathway. Sclerostin did not influence BMP2-induced SMAD transcriptional activity assessed with a BMP-responsive reporter construct, whereas noggin, a natural BMP antagonist, completely blocked it (**Figure 1c**). In addition, sclerostin did not affect BMP2-stimulated phosphorylation of SMAD1/5 (**Figure 1d**). Taken together, those data indicate that sclerostin inhibits BMP-induced osteoblast differentiation without affecting the canonical BMP signaling pathway.

Sclerostin inhibits Wnt3a-induced osteoblast differentiation without affecting canonical Wnt signaling

Next, we evaluated the effects of sclerostin on Wnt3a-induced osteoblast differentiation of C3H10T1/2 cells. Sclerostin significantly reduced the stimulation of ALP activity in response to Wnt3a (**Figure 2a**). Dickkopf 1 (DKK1), a natural antagonistic inhibitor of the WNT signaling pathway, exerted the same but more pronounced effect in this situation (**Figure 2a**). Interestingly, although DKK1 completely blocked Wnt3a-elicited β-catenin transcriptional activity tested with the TOPFlash reporter system, sclerostin did not alter it (**Figure 2b**). Consistent with this result, sclerostin did not affect Wnt3a-stimulated phosphorylation of LRP5/6, whereas DKK1 inhibited this response (**Figure 2c**). Similarly, sclerostin partially inhibited Wnt3a-induced increase in ALP activity without affecting β-catenin transcriptional activity in the pre-osteoblastic MC3T3-E1 cell line (data not shown). Therefore, our data indicate that sclerostin inhibits Wnt3a-induced osteoblast differentiation without affecting the canonical Wnt signaling pathway.

Sclerostin inhibits mineralization induced by GSK3 inhibition

Moreover, we tested the effects of sclerostin on matrix mineralization by C3H10T1/2 cells stimulated by BMP2, Wnt3a or a selective GSK3 inhibitor (SB216763). Those three osteogenic factors significantly increased matrix mineralization (**Figure 3a**). Coherent with its inhibitory effect on osteoblast differentiation of C3H10T1/2 cells, sclerostin inhibited Wnt3a- and BMP2-induced matrix mineralization (**Figure 3a**). More interestingly, sclerostin also dose-dependently inhibited matrix mineralization promoted by the selective GSK3 inhibitor (**Figures 3a and b**). As selective GSK3 inhibitors mimic canonical Wnt signaling without activation of LRP5/6, our findings suggest that sclerostin can prevent osteoblast differentiation without antagonizing LRP5/6, at least in C3H10T1/2 cells.

Sclerostin inhibits osteoblast differentiation through activation of PDGFR signaling

From the latter observation, we hypothesized that sclerostin could function through additional signaling pathways. Therefore, we investigated whether sclerostin could activate

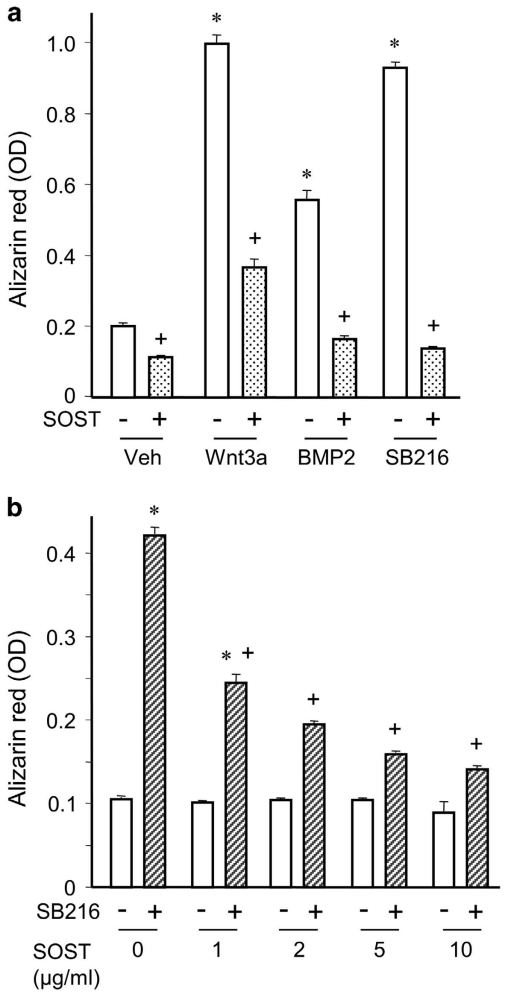

Figure 3 Sclerostin inhibits mineralization induced by GSK3 inhibition in C3H10T1/2 cells. (**a**) Quantification of matrix mineralization evaluated by Alizarin Red-S staining of C3H10T1/2 cells incubated with either vehicle, 20% Wnt3a-conditioned medium, 25 ng ml^{-1} BMP2 or 10 μM SB216763 (a selective GSK3 inhibitor), with or without 10 μg ml^{-1} sclerostin. (**b**) Quantification of matrix mineralization by C3H10T1/2 cells incubated with 10 μM SB216763 or its vehicle, in the presence of various doses of sclerostin. *$P<0.01$ for Wnt3a, BMP2 or SB216763 versus their vehicle; +$P<0.01$ for SOST versus its vehicle.

signaling pathways by itself in C3H10T1/2 and MC3T3-E1 cells. When analyzing phosphorylations of tyrosine residue, we observed that sclerostin was able to induce tyrosine phosphorylation of a protein of approximately 130 kDa in both C3H10T1/2 and MC3T3-E1 cells (**Figure 4**). Reblotting analyses revealed that this protein corresponded to phospholipase Cγ (PLCγ). In addition, sclerostin could activate protein kinase C (PKC), Akt and extracellular signal-regulated kinase 1/2 (ERK1/2; **Figure 4**) in both cell lines. As signaling pathways activated by sclerostin were very similar to those activated by platelet-derived growth factors (PDGFs), we compared signal transductions induced by sclerostin and PDGF in C3H10T1/2 and MC3T3-E1 cells. Sclerostin promoted a rapid and a sustained activation of PDGF receptors (PDGFRs) and its downstream signaling pathways (PLCγ, Akt and ERK1/2), as did PDGF-BB at low concentration (**Figure 5**). PDGFR inhibition with a selective inhibitor significantly reversed the inhibitory effect of sclerostin on BMP2-mediated increase in ALP activity (**Figure 6**), indicating that activation of PDGFR signaling is functionally involved in sclerostin-elicited inhibition of osteoblast differentiation. Finally, sclerostin significantly reduced BMP2-mediated elevation of ALP activity (**Figure 7a**) and could activate PDGFR signaling pathways in primary murine calvarial osteoblasts (**Figure 7b**).

Discussion

Sclerostin inhibits bone formation mostly by antagonizing LRP5/6, thus inhibiting Wnt signaling.[21–24] Sclerostin also binds LRP4, which seems to facilitate sclerostin binding to LRP5/6 in this context.[25,26] However, experiments with genetically modified mouse models suggest that a significant part of sclerostin-mediated inhibition of bone formation is due to interactions with other binding partners.[23,24] Therefore, we aimed to identify signaling pathways affected by sclerostin in relation with its inhibitory action on osteoblast differentiation in different cell culture systems. In the investigation described here, we found that sclerostin itself could activate PDGFR signaling and that this functional interaction inhibited osteoblast differentiation.

First, sclerostin was found to inhibit BMP-induced osteoblast differentiation without affecting SMAD1/5 activation and transcriptional activity, thereby confirming that sclerostin does not function as a classical BMP antagonist.[6] In addition, sclerostin prevented Wnt3a-induced osteoblast differentiation without altering LRP5/6 phosphorylation and β-catenin transcriptional activity. Recent data have highlighted the complexity of Wnt signaling with the discovery of distinct ligand binding domains in LRP5/6 receptors that recognize different classes of Wnt proteins.[30] The Wnt1 class, composed of Wnt 1, 2, 6, 7a, 7b, 9a, 9b and 10b, binds β-propeller 1 of LRP5/6.[30] The Wnt3a class encompasses Wnt3 and 3a and binds β-propeller 3 of LRP5/6.[30] Interestingly, sclerostin has been shown to inhibit the Wnt1 class by binding to the first β-propeller region of LRP5/6 but not the Wnt3a class,[24,30,31] which is coherent with our result. The fact that sclerostin could inhibit BMP2- or Wnt3a-induced osteoblast differentiation without antagonizing their respective canonical signaling suggested that it could function through another signaling pathway. This hypothesis was further strengthened by the observation that sclerostin could prevent osteoblast differentiation induced by GSK3 inhibition (which stimulates canonical Wnt signaling without activating LRP5/6).

Indeed, we demonstrated that sclerostin alone could activate PDGFR and its downstream signaling pathways PLCγ, PKC, Akt and ERK1/2 in two mesenchymal and pre-osteoblastic cell lines and in primary murine osteoblasts. In addition, activation of PDGFR signaling by sclerostin was required for the inhibitory action of sclerostin on osteoblast differentiation. A sclerostin-mediated activation of ERK1/2 in primary human osteoblasts has already been reported,[32] which further supports our findings. Furthermore, we observed that sclerostin could induce a similar pattern of PDGFR signaling pathways in comparison with PDGF-BB. Intriguingly, sclerostin displays a cystine-knot domain similar to that of PDGF-BB, which is essential for its binding to PDGFR.[33,34] However, it is yet to be determined whether sclerostin has the ability to interact with PDGFR. It has been proposed that sclerostin antagonizes canonical Wnt/LRP6 signaling by promoting clathrin-dependent internalization of LRP6.[35] It is likely that sclerostin

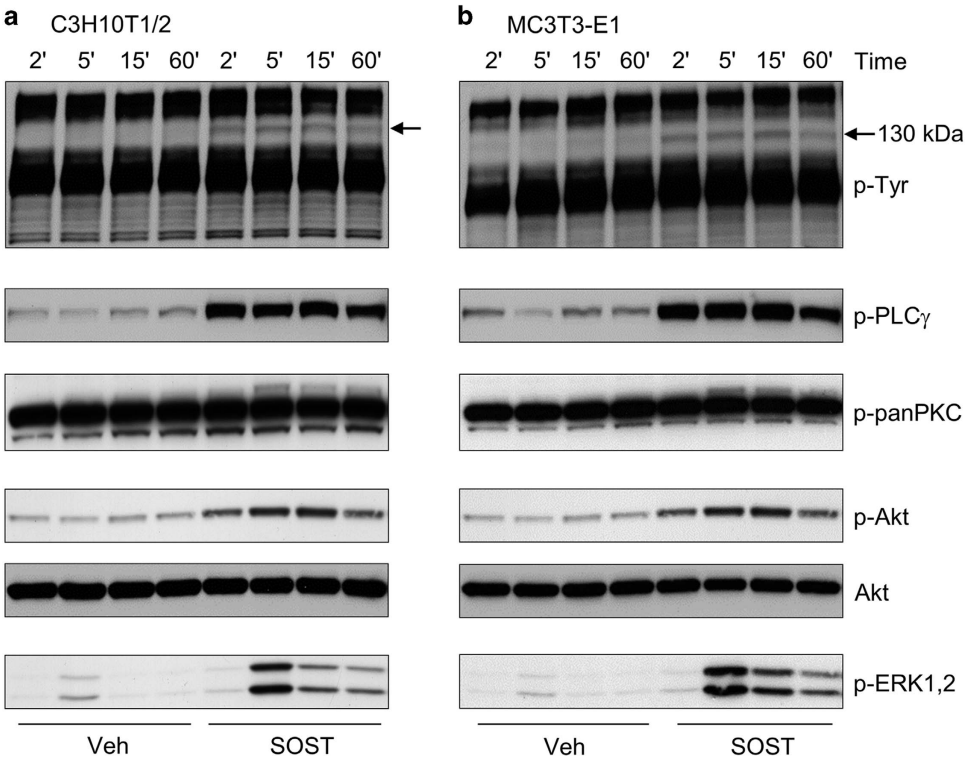

Figure 4 Sclerostin activates PLCγ, PKC, Akt and ERK1/2 signaling in C3H10T1/2 and MC3T3-E1 cells. (**a**) C3H10T1/2 and (**b**) MC3T3-E1 cells were exposed to 10 μg ml^{-1} sclerostin for various incubation times before analysis of signaling proteins by western blotting. The arrows show a protein of approximately 130 kDa, which is phosphorylated on tyrosine residues in response to sclerostin. ERK, extracellular signal-regulated kinase; PKC, protein kinase C; PLCγ, phospholipase Cγ.

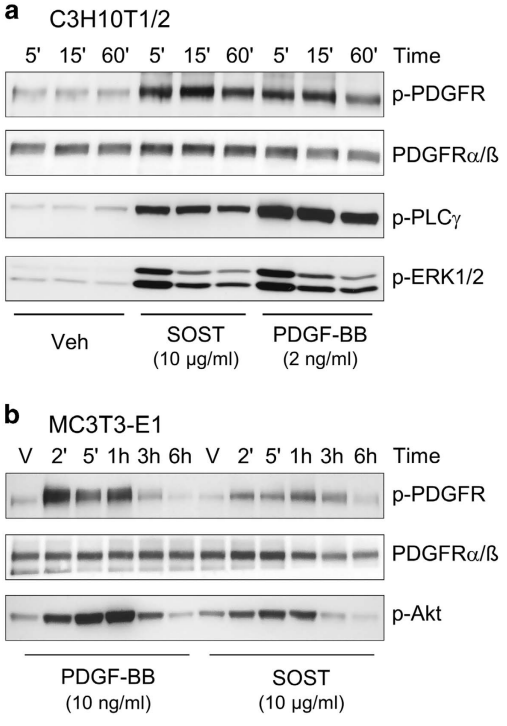

Figure 5 Sclerostin activates PDGFR signaling in C3H10T1/2 and MC3T3-E1 cells. (**a**) C3H10T1/2 and (**b**) MC3T3-E1 cells were treated with vehicle, 10 μg ml^{-1} sclerostin or 2–10 ng ml^{-1} PDGF-BB for various incubation times before analysis of signaling proteins by western blotting. PDGFR, platelet-derived growth factor receptor.

mediates LRP6 endocytosis through binding to a co-receptor, as it has been shown for DKK1. As PDGFR has been shown to be internalized following activation by PDGF and that PDGFR can form complex with LRP6 in other cell type,[36] PDGFR could be the co-receptor involved in sclerostin-elicited endocytosis of LRP6. The fact that sclerostin could activate PDGFR signaling in primary murine osteoblasts suggests that functional interaction between sclerostin and PDGFR may occur *in vivo*.

Treatment with imatinib (an inhibitor of PDGFR, c-kit and c-abl) in patients with chronic myeloid leukemia and treatments with romosozumab or blosozumab (monoclonal anti-sclerostin antibodies) in post-menopausal women display similar and distinct effects on bone metabolism. Both anti-sclerostin antibodies enhanced lumbar spine, total hip and femoral neck BMDs,[11,37] whereas imatinib treatment increased lumbar spine BMD but not that of total hip.[38] The three treatments resulted in similar transitory increases in serum levels of bone formation markers and also promoted reduction in bone resorption markers but with different kinetics; reduction was rapid and sustained with romosozumab or blosozumab treatments, whereas it occurred after long-term therapy with imatinib.[11,37,38] Finally, the three treatments led to slight decreases in serum calcium and elevations in parathyroid hormone concentrations. In contrast, slight reductions in serum phosphate and rises in 1,25-dihydroxyvitamin D3 concentration were only reported in imatinib-treated patients.[11,37,38] Such indirect comparisons must be made with caution because of the differences between the two classes of drugs, the patient populations and data analysis techniques.

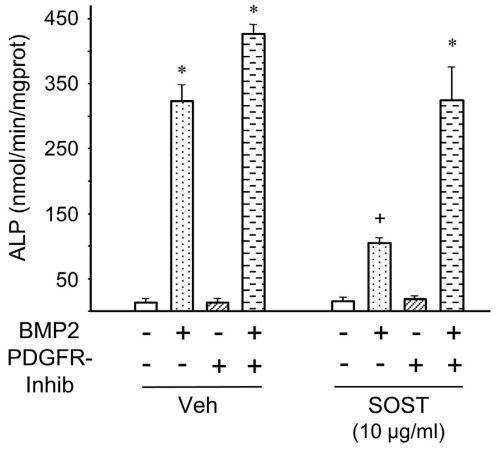

Figure 6 PDGFR inhibition reversed sclerostin-mediated inhibition of osteoblast differentiation. Alkaline phosphatase activity of C3H10T1/2 cells pre-incubated with 1 μM of the selective PDGFR inhibitor III or its vehicle, with sclerostin or its vehicle, and then stimulated with 25 ng ml^{-1} BMP2 or its vehicle for 2 days. *$P<0.01$ for BMP2 versus its vehicle; +$P<0.01$ for SOST versus its vehicle.

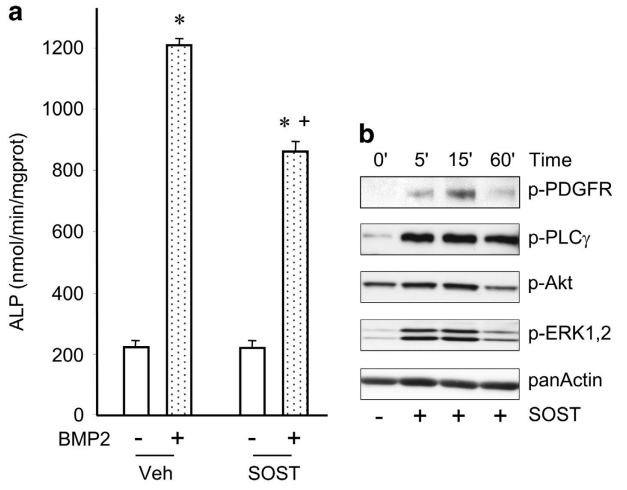

Figure 7 Sclerostin activates PDGFR signaling and inhibits differentiation of primary osteoblasts. (**a**) Primary mouse osteoblasts were pre-treated with 10 μg ml^{-1} sclerostin or its vehicle and stimulated or not with 100 ng ml^{-1} BMP2 for 48 h before evaluation of ALP activity. (**b**) Western blot analyses of PDGFR signaling in primary mouse osteoblasts incubated with 10 μg ml^{-1} sclerostin for different periods. *$P<$ 0.01 for BMP2 versus its vehicle; +$P<0.01$ for SOST versus its vehicle.

Nevertheless, the comparable beneficial effects observed between the two types of treatment on lumbar spine BMD and bone formation, together with our *in vitro* observations, justify to perform a direct comparison between sclerostin and PDGFR inhibitions on bone metabolism in rodent models in future investigations.

In summary, our data demonstrate that sclerostin can activate PDGFR signaling, resulting in the inhibition of osteoblast differentiation *in vitro*. Thus, our results suggest that PDGFR signaling may be an important regulator of bone mass.

Materials and Methods

Reagents and antibodies

Fetal calf serum (FCS), glutamine, antibiotics and trypsin/EDTA were obtained from Gibco (Life Technologies, Basel, Switzerland). Signaling inhibitors were obtained from either Calbiochem (San Diego, CA, USA) or Sigma-Aldrich (Saint Louis, MS, USA). Anti-LRP5/6 antibody was obtained from Biovision (#3801-100, Moutain View, CA, USA). Anti-p-LRP5/6 (#2568), anti-p-ERK1/2 (#9106), anti-p-Tyr (#9106), anti-p-PLCγ (#2821), anti-p-panPKC (#9371), anti-p-Akt (#4060), anti-Akt (#9272) and anti-panActin (#4968) antibodies were obtained from Cell Signaling Technology (Boston, MA, USA). Recombinant human sclerostin, DKK1 and BMP2 were generous gifts from Novartis (Basel, Switzerland), Prostrakan (Romainville, France) and Wyeth Research (Cambridge, MA, USA), respectively. Recombinant mouse Wnt3a was purchased from R&D Systems.

Cell cultures

MC3T3-E1 and C3H10T1/2 cells were purchased from ATCC (Manassas, VA, USA). Primary mouse osteoblasts were isolated from calvaria of 3-day-old C57BL6J mice by sequential digestion with collagenase as previously described.[39] MC3T3-E1 cells, C3H10T1/2 cells and primary osteoblasts were cultured in Minimum Essential Medium Eagle, alpha modifications (α-MEM) containing 10% FCS (vol/vol), 0.5% nonessential amino acids (vol/vol), 100 IU ml^{-1} penicillin and 100 μg ml^{-1} streptomycin, at 37 °C in a humidified atmosphere of 5% CO_2-95% air, and the medium was changed every 2 or 3 days. Confluent L cells and Wnt3a-producing L cells were cultured in Dulbecco's Modified Eagle Medium containing 5% FCS for 12 h for preparation of control and Wnt3a-conditioned media.

Alkaline phosphatase activity

Confluent MC3T3-E1, C3H10T1/2 and primary osteoblast cultures were switched to α-MEM containing 10% FCS, 50 μg ml^{-1} ascorbic acid and 10 mM β-glycerophosphate (osteogenic medium) to induce osteoblast differentiation. The day after, cultures were directly treated with the indicated agents for 2 days. ALP activity was determined as previously described.[40] Essentially, cells were harvested in 0.2% Nonidet P-40 and disrupted by sonication. The homogenate was centrifuged at 1500 *g* for 5 min, and ALP activity was determined in the supernatant by the method of Lowry *et al.*[41]

Mineralization assays

Confluent C3H10T1/2 cells were cultured in osteogenic medium and treated with vehicle, 25 ng ml^{-1} BMP2, 20% (vol/vol) Wnt3a-conditioned medium or 10 μM SB216763, with or without 10 μg ml^{-1} sclerostin for 10 days. Matrix mineralization was analyzed by Alizarin Red-S (AR-S) staining. To quantify mineral-bound AR-S, cell cultures were destained with 10% hexadecylpyridinium chloride, and AR-S concentration was determined by measuring the absorbance at 562 nm.

Cell transfection and transient reporter assays

To analyze β-catenin transcriptional activity, subconfluent C3H10T1/2 cells were exposed to the TCF reporter plasmid (TOPFlash from Upstate Biotechnology, Lake Placid, NY, USA) and the control plasmid pRLTK expressing the Renilla luciferase (Clontech, Takara Bio Europ, France) with 225 μl PolyFect (Qiagen AG, Hombrechtikon, Switzerland) in 2 ml of α-MEM containing 10% FCS for 18 h. Agents were then added for 24 h

before the determination of luciferase activity using the Promega Dual-Luciferase Assay according to the manufacturer's instructions. A similar experimental protocol was used for analyses of SMAD transcriptional activity by using a BRE luciferase reporter plasmid kindly provided by Professor Peter Ten Dijke (Leiden University, Nethertland).

Western blot analyses

Cells treated with indicated agents were rapidly frozen in liquid nitrogen after different incubation times and stored at –80 °C until used for analysis. Cell lysates were prepared by incubating cell cultures in RIPA buffer containing phosphatase and protease inhibitors at 4 °C for 30 min. Lysates were then cleared by centrifugation at 6000 *g* for 30 min. Lysate supernatant was diluted with an equal volume of 2-fold concentrated reducing sample buffer containing 125 mM Tris buffer (pH 6.8), 4% SDS, 20% glycerol, 0.05% bromophenol blue and 200 mM dithiothreitol. The mixture was then heated at + 70 °C for 30 min and subjected to gel electrophoresis on 6% to 15% gels. After SDS–PAGE, proteins were transferred to Immobilon P membranes and immunoblotted with specific antibodies. Detection was performed using peroxidase-coupled secondary antibodies, enhanced chemiluminescence reaction and visualization by autoradiography (Amersham International, Little Chalfont, UK). Re-probed membranes were stripped according to the manufacturer's protocol.

Reverse transcription-PCR

Total RNA was extracted from C3H10T1/2 cell cultures using Tri Reagent (Molecular Research Center, Cincinnati, OH, USA) and purified using an RNeasy Mini Kit (Qiagen AG). Single-stranded cDNA was synthesized from 2 μg of total RNA using a high-capacity cDNA archive kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. Two microliters of cDNA sample was used in a 40-μl-reaction containing a Red Taq PCR reaction buffer (Sigma-Aldrich, Saint Louis, MO, USA), 1.5 mM $MgCl_2$, 0.2 mM dATP, dCTP, dGTP, dTTP, 0.4 μM of gene-specific primers and 1 unit of Red Taq DNA Polymerase (Sigma-Aldrich). Sequences of primers used for reverse transcription-PCR analyses were previously described.[42] PCR products were amplified using a program starting with 5 min of denaturation at 95 °C, followed by 30 cycles with 45 s of denaturation at 95 °C, 45 s of annealing at 58–59 °C and 30–75 s of extension at 72 °C and 5 min of final extension at 72 °C.

Statistical analysis

All experiments were carried out independently at least three times. Each independent functional analyses were performed in triplicates. Results are expressed as mean ± s.e.m. of three independent experiments performed in triplicates. Comparative studies of means were analyzed using one-way ANOVA followed by a *post hoc* test (projected least significant difference Fisher). Differences were considered as significant when $P < 0.05$. No statistical method was used to predetermine sample size.

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgements

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