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Inhibition of GSK3β Stimulates BMP Signaling and Decreases *SOST* Expression Which Results in Enhanced Osteoblast Differentiation

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ABSTRACT

Both bone morphogenetic protein (BMP) and Wnt signaling have significant roles in osteoblast differentiation and the interaction between BMP and Wnt signaling is well known. Sclerostin is an important inhibitor of bone formation, inhibiting Wnt signaling and downstream effects of BMP such as alkaline phosphatase activity and matrix mineralization in vitro. However, little is known about the effect of BMP and Wnt signaling interaction on the regulation of *SOST*, the gene encoding sclerostin. Possibly, uncoupling of osteoblast differentiation regulators and *SOST* expression could increase osteoblast differentiation. Therefore, we investigated the effect of BMP and Wnt signaling interaction on the expression of *SOST* and the subsequent effect on osteoblast differentiation. Human osteosarcoma cells (SaOS-2) and murine pre-osteoblast cells (KS483) were treated with different concentrations of Wnt3a, a specific GSK3β inhibitor (GIN) and BMP4. Both Wnt3a and GIN increased BMP4-induced BMP signaling and BMP4 increased Wnt3a and GIN-induced Wnt signaling. However, the effect of GIN was much stronger. Quantitative RT-PCR analysis showed that *SOST* expression dose-dependently decreased with increasing Wnt signaling, while BMP4 induced *SOST* expression. GIN significantly decreased the BMP4-induced *SOST* expression. This resulted in an increased osteoblast differentiation as measured by ALP activity in the medium and matrix mineralization. We conclude that GSK3β inhibition by GIN caused an uncoupling of BMP signaling and *SOST* expression, resulting in an increased BMP4-induced osteoblast differentiation. This effect can possibly be used in clinical practice to induce local bone formation, for example, fracture healing or osseointegration of implants. J. Cell. Biochem. 116: 2938–2946, 2015.

KEY WORDS: OSTEOBLAST DIFFERENTIATION; SOST; SCLEROSTIN; BMP; GSK3β INHIBITION

Bone formation is a complex process that involves the differentiation of mesenchymal cells into pre-osteoblasts and osteoblasts that eventually leads to the synthesis and deposition of bone matrix proteins [Bilezikan et al., 2008]. Bone is continuously remodeled by bone-forming osteoblasts and bone-resorbing osteoclasts [Harada and Rodan, 2003; Teitelbaum and Ross, 2003]. An imbalance in the remodeling process can result in bone diseases as sclerosteosis or osteoporosis. Osteoporosis is one of the most prevalent diseases in elderly [Sambrook and Cooper, 2006] and is likely to become more prevalent with the further aging of the population. The expected higher prevalence of osteoporotic fractures and joint replacements due to the increase of the elderly population calls for the identification of regulatory molecules in differentiation of osteoblasts that can potentially serve as targets for treatment of osteoporosis. In addition, these molecules could possibly improve either fracture healing or osseointegration of implants.

Bone morphogenetic proteins (BMPs) and Wnts are well-known regulators of bone formation and have important roles in promoting

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osteoblast differentiation and mineralization. BMPs were identified as the factors responsible for induction of ectopic bone formation [Kingsley, 1994] and the role of BMPs in inducing osteoblast differentiation has been described for several BMPs [Vukicevic and Sampath, 2008; Chen et al., 2012]. BMPs activate the type I and type II BMP receptor complexes, leading to initiation of signaling via phosphorylation of intracellular Smad proteins [Miyazono et al., 2005]. Activated Smads regulate expression of transcriptional factors and transcriptional co-activators important in osteoblast differentiation like Runx2 and Osterix [Chen et al., 2012]. Wnts are a family of secreted proteins that regulate many developmental processes, for example, body axis formation, chondrogenesis, and limb development [Cadigan and Nusse, 1997; Yang, 2003]. Canonical Wnt/β-catenin signaling has been shown to promote osteogenesis by stimulation of Runx2 gene expression [Gaur et al., 2005]. In addition, activation of Wnt/β-catenin signaling promotes osteoblast cell proliferation and mineralization activity, reduces osteoblast apoptosis, and can suppress osteoclast differentiation induced by osteoblasts [Glass et al., 2005]. In the absence of Wnt activation, β-catenin is phosphorylated by glycogen synthase kinase 3β (GSK3β) in a complex with axin and adenomatous polyposis coli (APC), resulting in subsequent degradation. When Wnts bind to the Frizzled receptor and LRP5/6 co-receptor, axin is recruited to the membrane and the destruction complex is disrupted. Consequently, the phosphorylating action of GSK3β is prohibited and β-catenin accumulates in the cytoplasm, translocates into the nucleus, and activates the transcription of Wnt target genes by binding to the TCF/LEF transcription complex [Logan and Nusse, 2004].

Hence, both Wnt and BMP signaling have important roles in promoting osteoblast differentiation and mineralization, and there are many reports showing an interaction between Wnt and BMP signaling [Bain et al., 2003; Mbalaviele et al., 2005; Chen et al., 2007; Fukuda et al., 2010; Miclea et al., 2011]. Wnt signaling has been shown to increase BMP2 and BMP4 expression [Winkler et al., 2005; Zhang et al., 2013] and on the other hand, Wnt1 and Wnt3a expression was increased by BMP2 [Rawadi et al., 2003], suggesting that both BMP and Wnt signaling may synergistically regulate each other. The activity of BMP and Wnt is also controlled by their intrinsic antagonists, which include noggin and sclerostin [Yanagita, 2005; Kamiya et al., 2010]. Apart from natural produced inhibitors, many synthetic inhibitors have been developed to inhibit different aspects of the Wnt signaling pathway. One of these synthetic molecules is XAV939, a tankyrase inhibitor. Tankyrase marks axin for degradation, leading to disruption of the axin/APC/GSK3β complex. Thus, inhibition of tankyrase leads to accumulation of axin, breakdown of β-catenin, and inhibition of the Wnt pathway [Huang et al., 2009]. PNU74654 binds to β-catenin, preventing it from binding to the TCF/LEF transcription complex and subsequently inhibits Wnt signaling [Trosset et al., 2006].

Sclerostin, produced by osteocytes, is an important regulator of bone formation and one of several known Wnt signaling inhibitors. Sclerostin inhibits canonical Wnt signaling in a similar manner as dickkopf-1 (Dkk-1) by binding to the LRP5/6 co-receptor [Semenov et al., 2001; Li et al., 2005; van Bezooijen et al., 2007]. Mutations in the gene encoding sclerostin, *SOST*, or the surrounding regulatory regions lead to sclerostin deficiency and bone overgrowth in sclerosteosis and van Buchem disease, respectively [van Hul et al., 1998; Brunkow et al., 2001; Loots et al., 2005; Moester et al., 2010]. In mice overexpressing *SOST*, there is a significant reduction in osteoblast activity and subsequently bone formation [Winkler et al., 2003; Loots et al., 2005]. In vitro, sclerostin inhibits the differentiation of pre-osteoblast cells [van Bezooijen et al., 2007]. Loss of sclerostin might prolong the bone formation phase of osteoblasts, resulting in the increase of bone mass. Sclerostin physiologically acts as a downstream molecule of BMP signaling to inhibit canonical Wnt signaling and negatively regulates bone mass [Kamiya et al., 2010; Krause et al., 2010].

The fact that sclerostin, a major regulator of bone formation through Wnt and BMP signaling, is limited to skeletal tissue and absence of sclerostin leads to an increase in bone formation, makes it an ideal drug target for bone formation. Recently, it had been shown that treatment with romosozumab, a monoclonal antibody which binds to sclerostin, increases bone formation in patients suffering from osteoporosis [McClung et al., 2014]. BMPs are the most potent inducers of *SOST* expression and strong regulators of osteoblast differentiation [Sutherland et al., 2004]. Uncoupling of osteoblast differentiation regulators and their intrinsic inhibitors could possibly increase or prolong the BMP response, leading to more osteoblast differentiation and subsequent bone formation. Therefore, the goal of this study was to investigate the effect of BMP and Wnt signaling on *SOST* expression and osteoblast differentiation.

MATERIALS AND METHODS

CELLS, MATERIALS, AND REAGENTS

The human osteosarcoma cell line SaOS-2 (ATCC, Manassas, VA) was cultured in DMEM (Gibco, Carlsbad, CA) supplemented with 10% Fetal Calf Serum (FCS; Greiner Bio One, Kremsmünster, Austria), 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco). Murine mesenchymal progenitor cells KS483 were cultured in α-MEM without phenol red (Gibco) supplemented with FCS, penicillin and streptomycin, and glutamax (Gibco).

Recombinant BMP4, Wnt3a, and DKK1 were purchased from R&D Systems (Minneapolis, MN). The specific GSK3β inhibitor 3-imidazo [1,2-a]pyridin-3-yl-4-(1,2,3,4-tetrahydro-[1,4]diazepino-[6,7,1-hi] indol-7-yl) pyrrole-2,5-dione (further referred to as GIN) was kindly provided by Dr. Rawadi (Prostrakan, France) and previously described by Engler et al. [2004] and Miclea et al. [2011]. The Wnt signaling inhibitors XAV939 and PNU74654 were purchased at Sigma (St. Louis, MO). The Wnt-responsive luciferase reporter BAT-luc has been described previously [Maretto et al., 2003; Miclea et al., 2011] as is also the case for the BMP responsive element luciferase reporter BRE-luc [Korchynskyi and ten Dijke, 2002].

LUCIFERASE EXPERIMENTS

SaOS-2 cells were seeded in 96-well plates at a density of 21,000 cells/cm^2 and cultured overnight to 70–80% confluence. The cells were transfected with BAT-luc or BRE-luc reporter construct and a pGL4-CAG renilla luciferase construct using FuGene HD transfection reagent (Promega Fitchburg, WI) according to the manufacturer's instructions. After 24 h of treatment with the

TABLE I. Oligonucleotides Used in RT-PCR

Gene		Forward	Reverse
β2M	Human	5′-TGCTGTCTCCATGTTTGATGTATCT-3′	5′-TCTCTGCTCCCCACCTCTAAGT-3′
	Murine	5′-TGACCGGCTTGTATGCTATC-3′	5′-CAGTGTGAGCCAGGATATAG-3′
SOST	Human	5′-TGCTGGTACACACAGCCTTC-3′	5′-GTCACGTAGCGGGTGAAGTG-3′
	Murine	5′-TCCTCCTGAGAACAACCAGAC-3′	5′-TGTCAGGAAGCGGGTGTAGTG-3′

indicated reagents, luciferase activity was determined using the Dual-Glo Luciferase assay system (Promega, Fitchburg, WI) with a SpectraMax L luminometer (Molecular Devices, Sunnyvale, CA). Relative luminescence was calculated as luciferase/renilla luciferase and expressed as fold change versus control.

DIFFERENTIATION EXPERIMENTS

KS483 cells were seeded at a density of 9,210 cells/cm^2. Every 3 to 4 days, the medium was changed. At confluence (from day 4 of culture onwards), ascorbic acid (50 μg/ml, Merck, Inc., NY) was added to the culture medium. When nodules appeared (from day 11 of culture onwards), β-glycerophosphate (5 mM; Sigma) was added. Every 3 to 4 days, medium samples (25 μl) were analyzed for alkaline phosphatase (ALP) activity by adding 200 μl of 2 mg/ml p-nitrophenylphosphate (Sigma) in 100 mM glycine/ 1 mM $MgCl_2$/ 0.1 mM $ZnCl_2$ buffer (pH 10.5) and reading for 10 min using a VERSAmax Tunable Microplate Reader (Molecular Devices) at 405 nm. ALP activity was determined as the slope of the kinetic measurement (mOD/min). Mineralization of the cultures was quantified using the fluorescent dye Bonetag as described previously [Moester et al., 2014]. Briefly, cells were incubated with 2 nM Bonetag 800 (Perkin Elmer) for 24 h, washed with phosphate-buffered saline (PBS), and fixed with 3.7% buffered formaldehyde. The fixed cells were scanned with the Odyssey Infrared Imaging

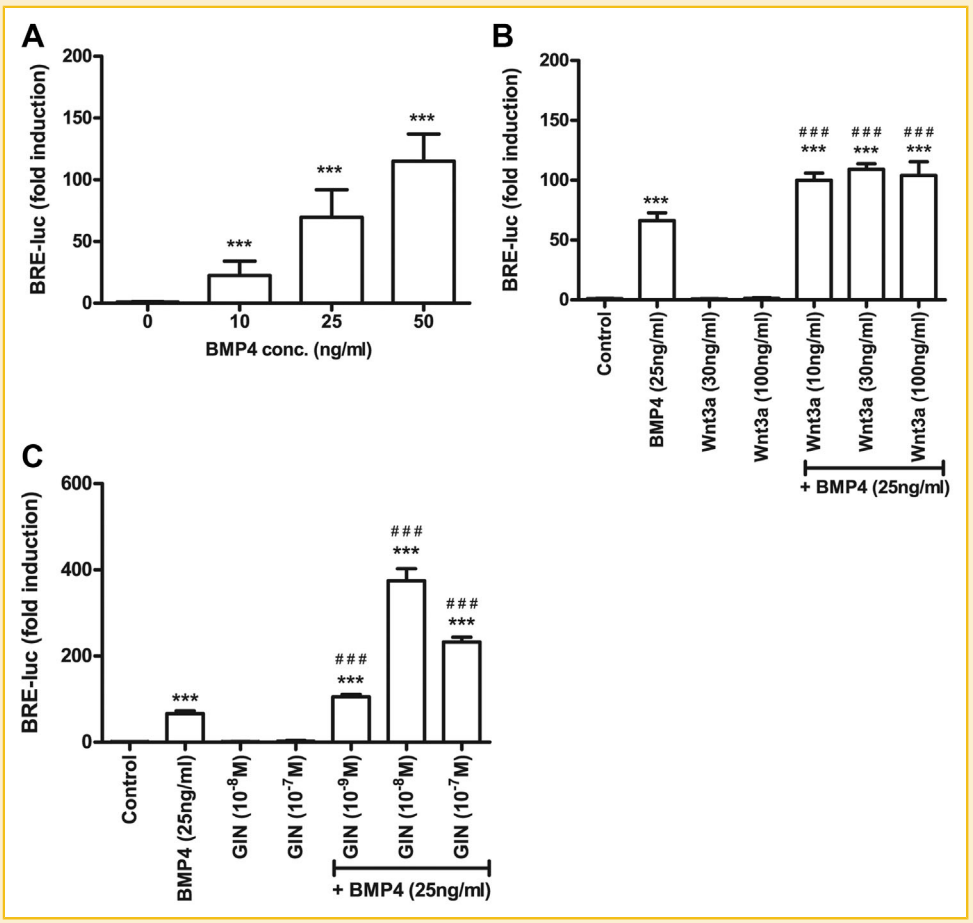

Fig. 1. BMP reporter BRE-luc activity after stimulation with BMP4, Wnt3a, and GIN. SaOS-2 cells were transfected with the BMP reporter construct BRE-luc and were stimulated with the indicated concentrations for 24 h. Luciferase ($n = 6$) was measured, values represent mean ± SD. BRE-luc activity increased dose-dependently with BMP4 (A). Combined Wnt3a and BMP4 increased BRE-luc activation (B). GIN was more potent in increasing BMP4 induced BRE-luc activation (C). $^{***}P < 0.001$ compared to control. $^{###}P < 0.001$ compared to BMP4.

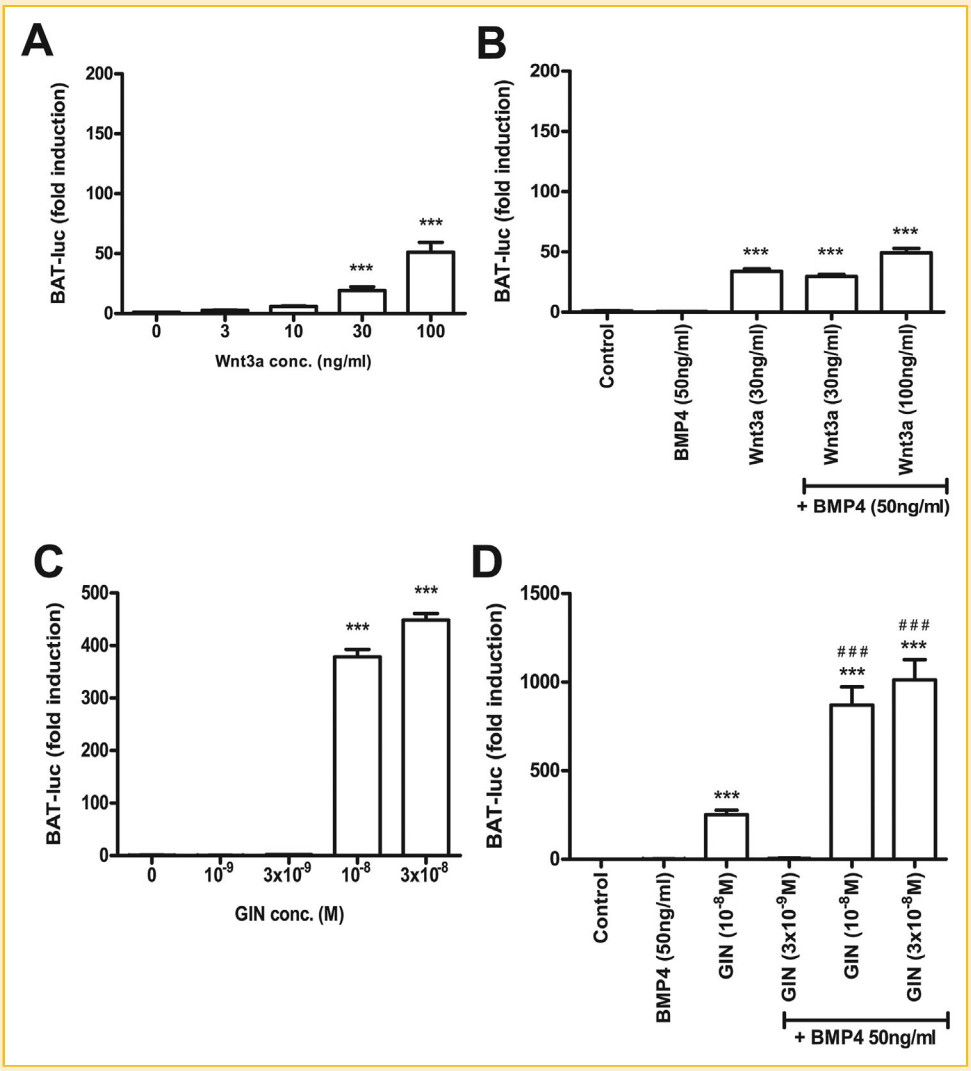

Fig. 2. Wnt reporter BAT-luc activity after stimulation with Wnt3a, GIN, and BMP4. SaOS-2 cells were transfected with the Wnt reporter construct BAT-luc and were stimulated with the indicated concentrations for 24 h. Luciferase ($n = 6$) was measured, values represent mean ± SD. BAT-luc activity increased dose-dependently with Wnt3a (A) and GIN (C). Combined Wnt3a and BMP4 did not increase Wnt3a-induced BAT-luc activity (B), while combined GIN and BMP4 increased BAT-luc activity significantly (D). $^{*}P < 0.05$; $^{***}P < 0.001$ compared to control. $\#\#\#P < 0.001$ compared to GIN 10^{-8} M.

System (Li-COR) at a resolution of 42 μm, medium quality and intensity 5.0–6.5. Integrated intensity (counts/mm^2) of each well was calculated by the Odyssey software.

QUANTITATIVE RT-PCR AND PRIMERS

Total RNA was isolated from SaOS-2 and KS483 cells using TriPure Isolation Reagent (Roche, Penzberg, Germany) 24 h (SaOS-2 cells) or 8–10days (KS483 cells) after treatment with indicated reagents, respectively. cDNA was synthesized using M-MLV reverse transcriptase (Promega) according to the manufacturer's instructions. Quantitative RT-PCR was performed using the Quantitect SYBR-green PCR kit (Qiagen, Venlo, the Netherlands) with an iQ5 PCR cycler (BioRad, Hercules, CA). For used primer sets (Eurogentec, Seraing, Belgium), see Table I. β2-Microglobulin (β2M) was used as an internal control. Measurements were performed in triplicate and analyzed using the ΔΔCt method [Pfaffl, 2001].

STATISTICAL ANALYSIS

Values represent mean ± SD. Differences were tested by one-way analysis of variance (ANOVA) followed by Bonferroni post hoc test using Graphpad Prism 5 software (La Jolla, CA). Results were considered significant at $P < 0.05$.

RESULTS

To address the interaction between Wnt and BMP signaling, different combinations of BMP4, Wnt3a, or the GSK3β inhibitor (GIN) were added to SaOS-2 cells. Unfortunately, we were unable to efficiently transfect KS483 cells; therefore, all transfection experiments were performed in SaOS-2 cells. The effect on BMP signaling was investigated using a BMP-responsive element driving luciferase expression, further referred to as BRE-luc.

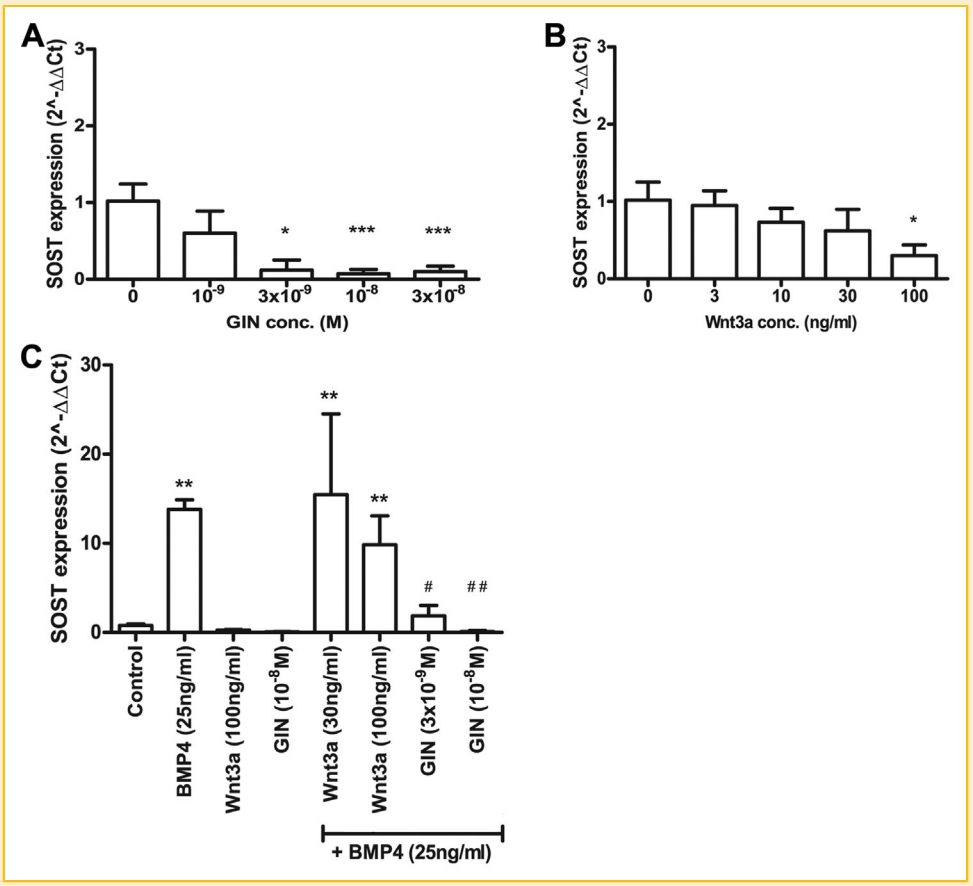

Fig. 3. *SOST* mRNA expression (n = 3) after stimulation with Wnt3a, GIN, and BMP4. Values represent mean ± SD. *SOST* expression decreased dose dependently with GIN (A) and Wnt3a (B). Wnt3a did not decrease BMP4-induced *SOST* expression, while GIN even decreased BMP4-induced *SOST* expression below control levels (C). $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ compared to control. $\#P < 0.05$; $\#\#P < 0.01$ compared to BMP4.

BMP4 alone dose-dependently increased the BRE-luc activity in SaOS-2 cells (Fig. 1A). Similar results on BRE-luc activity were obtained with BMP2 and BMP6 (data not shown). Although Wnt3a alone did not induce BRE-luc activity, it significantly increased BMP4-induced luciferase activity (Fig. 1B). The BMP4-induced luciferase activity increased more than fivefold in combination with 10^{-8} M GIN, even though GIN alone did not have any effect on the reporter (Fig. 1C). Additionally, by performing experiments using BMP4, Wnt3a, or GIN and PNU74654, we found that the increased BRE-luc activity by Wnt3a and GIN was dependent of β-catenin (data not shown).

The influence of the interaction between Wnt and BMP on Wnt signaling was investigated using the Wnt-responsive BAT-luc reporter. We observed a dose-dependent increase of BAT-luc activity after Wnt3a or GIN stimulation (Fig. 2A,C). However, GIN was more potent in inducing BAT-luc activation, stimulating activity more than 400-fold compared to control at 10^{-8} M (Fig. 2C). The GIN-induced BAT-luc activity increased more than fourfold when combined with BMP4, even though BMP4 alone was not able to induce BAT-luc activity (Fig. 2D). The Wnt-induced Bat-luc activity was not significantly increased in combination with BMP4 (Fig. 2B).

To address the interaction between Wnt and BMP signaling on *SOST* expression, we used SaOS-2 cells, since these cells can express constitutively levels of mRNA *SOST*, and therefore, are a good model for studying the effect of GIN and BMP4 on *SOST* expression [Keller and Kneissel, 2005]. GIN dose dependently decreased *SOST* expression levels. Even at a concentration of 3×10^{-9} M GIN, which showed no effect on BAT-luc activity, *SOST* expression was decreased (Fig. 3A). Wnt3a was able to significantly decrease *SOST* expression only at a high concentration (100 ng/ml) (Fig. 3B). Although BMP4 induced *SOST* expression dose dependently (data not shown), a combination of BMP4 and GIN significantly decreased *SOST* expression (Fig. 3C). When Wnt3a was added to BMP4-stimulated cells, only a slight decrease in expression of *SOST* was observed (Fig. 3C). Apart from SOST mRNA expression, we observed a dose-dependent increase of sclerostin protein in the medium of cells treated with BMP4, which could be decreased by the addition of GIN. However, GIN alone could not decrease secretion of sclerostin (data not shown).

Next, we investigated whether the down regulation of *SOST* expression observed after stimulation with GIN was a direct effect of Wnt/β-catenin signaling. To this purpose, three inhibitors of the Wnt

signaling pathway were tested for their ability to counteract the effect of GIN on *SOST* expression. The extracellular inhibitor DKK1 could not inhibit Wnt signaling after stimulation with GIN (Fig. 4A), whereas XAV939 and PNU74654 significantly inhibited the GIN induced BAT-luc reporter activity (Fig. 4A). Both XAV939 as well as PNU74654 alone did not have an effect on BAT-luc reporter activity (data not shown). All three inhibitors were unable to restore *SOST* expression (Fig. 4B).

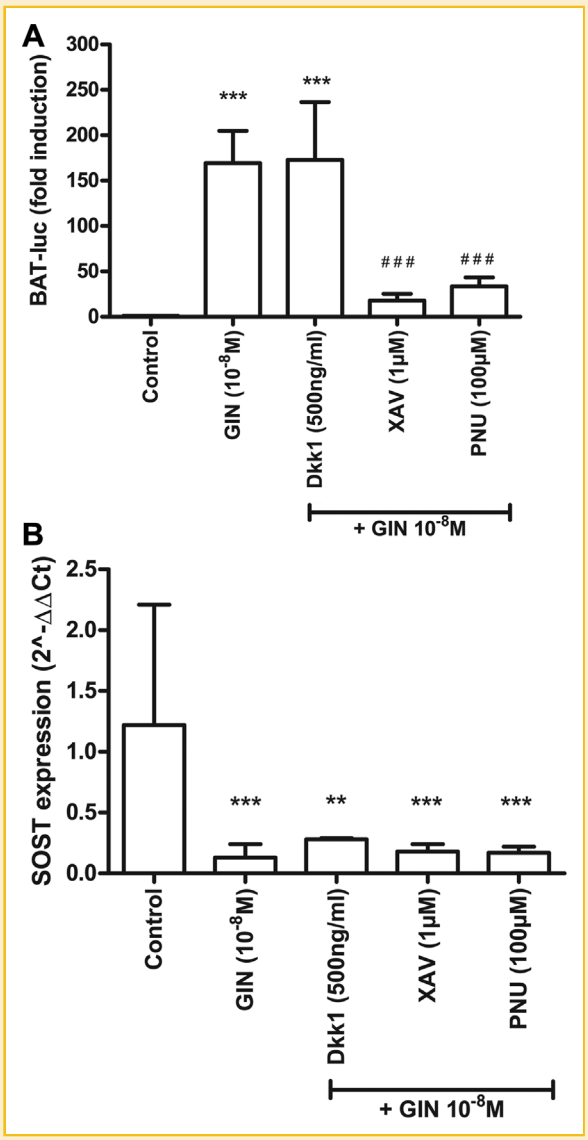

Fig. 4. Wnt reporter BAT-luc activity and *SOST* expression after incubation with GIN and different Wnt signaling inhibitors. A: Wnt reporter BAT-luc activity in SaOS-2 cells after stimulation with GIN ($n = 6$). Values represents mean ± SD. The extracellular inhibitor DKK1 could not inhibit GIN-induced BAT-luc activity. Both XAV939 and PNU74654 significantly inhibited BAT-luc activity. B: RNA from SaOS-2 cells was isolated after 24 h incubation with GIN and other Wnt signaling inhibitors ($n = 3$). Values represent mean ± SD. None of the inhibitors could restore *SOST* expression to control levels. $^{**}P < 0.01$; $^{***}P < 0.001$ compared to control. $###P < 0.001$ compared to GIN.

Finally, we assessed the biological effect of down regulation of *SOST* expression by GIN in KS483 cells. This cell line provides a well-established model for investigating the process of osteoblast differentiation, rather than SaOS-2 cells, which represent human osteogenic osteosarcoma cells with late osteoblast characteristics [van der Horst et al., 2002; Prideaux et al., 2014]. Since *SOST* mRNA expression is restricted to after the onset of mineralization in osteoblastic cultures [van Bezooijen et al., 2004], we investigated the effect of GIN on *SOST* mRNA expression during the first days of mineralization (e.g., after 13 or 14 days of culture). Osteogenic differentiation of the cultures was monitored by measuring alkaline phosphatase activity in the medium and matrix mineralization. As shown in Figure 5A, addition of BMP4 significantly increased ALP activity on days 7, 11, and 14. Addition of GIN even further increased BMP4-increased ALP activity. Treatment of the cells with BMP4 resulted in an increase of mineralization, while addition of GIN increased BMP4-induced mineralization even further (Fig. 5B,D). Consistent with the increase in mineralization by BMP4, *SOST* mRNA expression was also increased by BMP4 (Fig. 5C). GIN alone has a slight but not significant inhibitory effect on both mineralization and *SOST* expression (Fig. 5B,C). However, when GIN was added in combination with BMP4, *SOST* mRNA expression was reduced (Fig. 5C).

DISCUSSION

Both BMP and Wnt signaling have been shown to play important roles in promoting osteoblast differentiation and mineralization [Glass et al., 2005; Chen et al., 2012]. Interaction between both signaling pathways has been found in several studies, suggesting that both BMP and Wnt signaling may synergistically regulate osteoblast differentiation [Bain et al., 2003; Mbalaviele et al., 2005; Chen et al., 2007; Fukuda et al., 2010; Miclea et al., 2011]. Since BMP and Wnt signaling induce their intrinsic antagonists [Kamiya et al., 2010] and *SOST* seems to be involved in both pathways, we investigated the effect of these pathways on *SOST* expression, both separately and in combination.

SaOS-2 cells represent human osteogenic osteosarcoma cells with late osteoblast characteristics, transitioning towards osteocytes [Prideaux et al., 2014]. These cells belong to one of the few cell lines constitutively expressing *SOST*, are easy to transfect, and are therefore, used for studying signaling pathways and the influence on *SOST* expression. However, this cell line is less appropriate to investigate the process of osteoblast differentiation. Therefore, we used KS483 cells, a murine mesenchymal progenitor cell line, which represents a more accurate model to study effects on osteoblast differentiation [van der Horst et al., 2002].

Our results in SaOS-2 cells show that inhibition of GSK3β, either via the Wnt pathway by stimulation with Wnt3a or by direct inhibition using the GSK3β inhibitor GIN, resulted in a decreased expression of the Wnt signaling inhibitor *SOST*. Interestingly, GIN was much more potent in the down regulation of *SOST*. Moreover, BMP4-induced *SOST* expression was decreased by GIN, but not by Wnt3a. We suggest this is the result of the more potent induction of the Wnt pathway by GIN compared to Wnt3a, as was shown by a much higher induction of BAT-Luc by GIN. In addition, when Wnt3a or GIN was

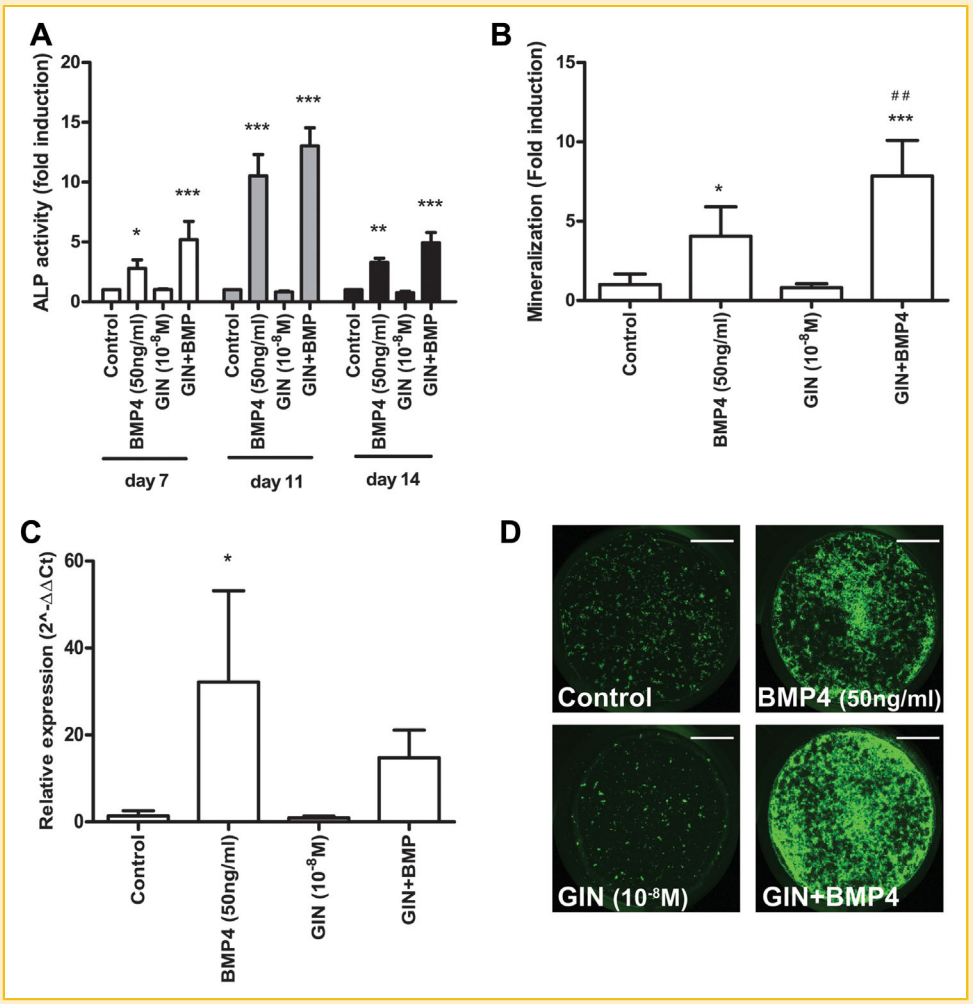

Fig. 5. Osteoblast differentiation of KS483 cells after stimulation with BMP4 and GIN. A: Alkaline phosphatase (ALP) activity in medium. BMP4 increased ALP activity during differentiation, while GIN increased the BMP4 induced ALP activity even further. B, D: Mineralization measured by Bonetag. BMP4 increased mineralization, while GIN slightly inhibited mineralization. A combination of BMP4 and GIN increased the mineralization significantly. C: *SOST* mRNA expression ($n = 4$). BMP4 increased *SOST* expression, while GIN slightly decreased BMP4 induced *SOST* expression. Values represent mean ± SD of four independent experiments, except for (D) which represents pictures from one representative experiment. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ compared to control. $\#\#P < 0.01$ compared to BMP4. Bars represent 5 mm.

combined with BMP4, both Wnt as well as BMP signaling were further increased, suggesting a synergistic mechanism. Again, GIN was much more potent in inducing both pathways in combination with BMP4. In addition, GIN was able to decrease the BMP4-induced sclerostin protein expression in the medium. However, measured protein levels of sclerostin were close to or below the lower detection limit of 1 pg/ml of the assay used and are therefore not shown. The low expression levels of the sclerostin protein seem to be in line with a study of Yu et al. [2011]. They showed that SaOS-cells produce low (non-detectable) levels of sclerostin during the first days of culture. Only after 12 days of culture, detectable levels of sclerostin were found in the medium of the cells.

Because of its clear connection to regulation of bone cells, canonical Wnt signaling seems the most plausible pathway involved in the down regulation of *SOST*. However, *SOST* expression was also decreased at a concentration of GIN where no increase in BAT-luc activity was seen. Moreover, the β-catenin binding inhibitor PNU74654 was not able to restore *SOST* expression after treatment with GIN. This suggests that down regulation of *SOST* by GIN is not a direct effect of the canonical Wnt pathway, but appears to be mediated independent of β-catenin. Although GIN was thoroughly screened for selectivity against a panel of kinases [Engler et al., 2004], further experiments are needed to exclude cross-reactivity or off-target effects of GIN. In addition, we found a connection between GSK3β inhibition and BMP signaling on the down regulation of *SOST*. Therefore, we can rule out the involvement of solely canonical Wnt signaling in the regulation of *SOST*. Previous studies already described a mechanism in which GSK3β phosphorylation primes Smad1 for ubiquitination and degradation. With this mechanism, GSK3β controls the duration of Smad1 activation and therefore BMP signaling [Fuentealba et al., 2007; Eivers et al., 2009]. A similar mechanism may be true for the duration of Smad6 and Smad7 activation, which have been shown to inhibit *SOST* promoter activity [Yu et al., 2011].

The combined effect of GSK3β inhibition and BMP on *SOST* expression observed in SaOS-2 cells was also observed in KS483 cells. The biological effect of downregulation of *SOST* by GIN was measured by alkaline phosphatase (ALP) activity in the medium and mineralization of the matrix in KS483 cells. A combination of GIN and BMP4 increased osteoblast differentiation. Our results are in line with Fukuda et al. [2010] who have shown that BMP4 and canonical Wnt cooperatively induced osteoblast differentiation through a GSK3β-dependent and β-catenin independent mechanism [Fukuda et al., 2010]. Although the decrease in BMP4-induced *SOST* expression by GIN in our experiments was not statistically significant, we hypothesize that the increase in osteoblast differentiation is due to the uncoupling of BMP signaling and *SOST* expression. Our results rely on SOST mRNA levels and these may not be accurately reflecting sclerostin protein production and therefore effects in the cell. In a study by Stolina et al. [2014], rats treated with anti-sclerostin antibody had higher levels of Sost mRNA in osteocytes. The higher levels of Sost mRNA found by Stolina et al. [2014] could be the result of active Sost upregulation in a setting of reduced skeletal strain caused by increased bone mass. However, in our study, we achieved bone formation by reducing SOST mRNA levels (which probably leads to a decrease in sclerostin protein), while sclerostin antibody reduces sclerostin protein levels resulting in bone accrual. Moreover, in a study by Yu et al. [2011], it was shown that SOST mRNA expression was correlated with maturation of osteoblasts in culture. Therefore, regulation of SOST expression seems to be important and associated with transition to an osteocyte-like phenotype. Nevertheless, additional studies are necessary to confirm these results.

In conclusion, this study showed that uncoupling of BMP signaling and *SOST* expression could increase BMP-induced osteoblast differentiation. Furthermore, our results propose the existence of a new regulatory pathway for expression of *SOST*, which is mediated by GSK3β but independent of β-catenin. Further studies are necessary to identify the exact mechanism of regulating sclerostin via GSK3β and the way it interacts with other pathways during bone metabolism.

Inhibition of sclerostin has interesting clinical applications. Recently, a monoclonal antibody inhibiting sclerostin has been shown to enhance bone formation and to prevent implant loosening in preclinical studies [Liu et al., 2012; Suen et al., 2014] and is currently tested in clinical trials phase III (ClinicalTrials.gov number, NCT01631214) [McClung et al., 2014]. Another approach of inhibiting sclerostin and subsequently increasing bone mass would be via GSK3β inhibition as shown in our study. However, we propose that this approach of inhibiting sclerostin would be suitable for local applications only, since a study of Miclea et al. [2011] showed that systemic treatment with GIN induced osteoarthritis-like features in mice. For example, local inhibition of sclerostin via GSK3β could have advantages in fracture healing or could improve osseointegration of implants by local increase of bone growth.

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