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Enhanced prostacyclin formation and Wnt signaling in sclerostin deficient osteocytes and bone

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ABSTRACT

We show that prostacyclin production is increased in bone and osteocytes from sclerostin (*Sost*) knockout mice which have greatly increased bone mass. The addition of prostacyclin or a prostacyclin analog to bone forming osteoblasts enhances differentiation and matrix mineralization of osteoblasts. The increase in prostacyclin synthesis is linked to increases in β-catenin concentrations and activity as shown by enhanced binding of lymphoid enhancer factor, Lef1, to promoter elements within the *prostacyclin synthase* promoter. Blockade of Wnt signaling reduces prostacyclin production in osteocytes. Increased prostacyclin production by osteocytes from sclerostin deficient mice could potentially contribute to the increased bone formation seen in this condition.

1. Introduction

Excessive bone loss causes osteoporosis and enhances susceptibility to fractures, leading to significant morbidity, mortality and excess health care costs [1]. Understanding mechanisms by which bone mass can be enhanced or maintained could result in new strategies effective in increasing or preserving skeletal integrity and promoting fracture healing, thus reducing costs and co-morbidities associated with osteoporosis and fractures.

The balance between bone loss and deposition is important for normal bone growth and remodeling, and depends on a complex regulatory interplay among resident bone cells such as osteoclasts, osteoblasts, and osteocytes whose activities are altered by serum concentrations of hormones such as parathyroid hormone and 1α,25-dihydroxyvitamin D, and several regulatory proteins such as bone morphogenetic proteins, receptor activator of nuclear factor κ-B ligand (RANKL), and lipid mediators such as prostaglandins produced by such cells [2–9]. Previous work demonstrated that the prostaglandin PGE_2 can either increase bone formation and osteoblastic activity [10] or increase bone resorption [11] depending on experimental conditions. The prostaglandin, PGI_2 or prostacyclin also alters bone formation and bone resorption depending on the experimental model [12–20]. Deletion of prostacyclin synthase, the enzyme responsible for prostacyclin synthesis, results in a decrease in bone mineral density in young mice and an increase in bone mineral density in older mice [14].

Human patients and mice with inactivating mutations of the sclerostin (*SOST*, *Sost*) gene have significantly increased bone density [7,21,22]. The *Sost* gene product, sclerostin, is a secreted glycoprotein that alters Wnt, bone morphogenetic protein and other signaling pathways [23–27]. We previously examined the mechanisms by which bone mass is increased in a novel sclerostin-deficient mouse model [21] which shows an exceptionally dense skeleton and rapid fracture healing [28]. We now show that the production of prostacyclin (PGI_2), a cyclic prostanoid, is greatly increased in bone and osteocytes of sclerostin-deficient mice. We demonstrate that prostacyclin, and a prostacyclin analog, increase osteoblast differentiation and mineralization. In the absence of sclerostin production, β-catenin activity in osteocytes is increased, and PGI_2 production is influenced by Wnt signaling pathways.

2. Materials and methods

2.1. Animal studies

Animal research was conducted according to National Institutes of Health and the Institute of Laboratory Animal Resources,

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National Research Council guidelines. The Mayo Clinic Institutional Animal Care and Use Committee approved all animal studies.

2.2. Generation of Sost knock-out mice

Mice were generated as described earlier [21].

2.3. Isolation of osteocytes from mouse femurs

Osteocytes were isolated from 8-week-old *Sost* KO and WT mice as described by Stern et al. [29].

2.4. Immortalization of osteocytes

Osteocytes were immortalized using an SV40 T antigen viral construct as described earlier [30]. Clonal populations of osteocytes from *Sost* KO and WT mice were generated via dilution cloning.

2.5. Preparation of decalcified bone for immunohistochemistry

Femurs were isolated and sectioned as described earlier [21,28]. Immunohistochemistry was performed with antibodies to prostaglandin I_2 synthase (Cayman Chemical, 1:50 dilution), or with an IgG isotype control (Vector Laboratories I-1000). Chromogens were developed using a polyvalent mouse and rabbit specific secondary HRP detection kit (Abcam), followed by incubation in 3,3-diaminobenzidine.

2.6. Measurement of prostaglandins

Prostaglandin (6-keto PGF1α, PGE_2, PGE Metabolite, $PGF_{2\alpha}$, PGD_2, and $TBXB_2$) measurements were performed in bone extracts, 24 h urine samples, and cell lysates by enzyme immunoassay (EIA) using kits from Cayman Chemical (Ann Arbor, MI).

2.7. Microscopy (E11/podoplanin, Lef1, β-cat staining)

WT and *Sost* KO osteocytes were grown on 12-well, collagen treated, glass bottom plates (MatTek Corporation). Cells were fixed in 4% PFA, washed in PBS, and blocked in 10% goat serum in PBS. The following primary antibodies were used: podoplanin (8.1.1, Santa Cruz Biotechnology, 1:50 dilution), Lef-1 (N-17, Santa Cruz Biotechnology, 1:50 dilution), β-catenin (Cell Signaling Technologies, 1:1000 dilution). After several PBS washes, secondary antibody was added. For podoplanin: Alexa Fluor 488-labeled goat anti-hamster IgG, 1:200 dilution; for Lef-1: Alexa Fluor 488-labeled donkey anti-goat IgG, 1:200 dilution; for β-catenin: AlexaFluor 594-labeled goat anti-rabbit IgG, 1:200 dilution (all from Life Technologies). Cells were washed with PBS, and counterstained with Vectashield Hard Set Mounting Medium containing 4′,6-diamidino-2-phenylindole (Vector Laboratories). Cells were photographed on an Axio Observer inverted microscope; fluorescence micrographs were taken using a Zeiss Super Resolution Elyra microscope system with Structured Illumination and a Plan-Apochromat 63X/1.4NA oil objective controlled by ZEN 2010 software.

2.8. C59 inhibitor experiments

Serum-free medium (α-MEM, 1% P/S) containing either 100 nM Wnt inhibitor, C59 (2-(4-(2-methylpyridin-4-yl)phenyl)-*N*-(4-(pyridine-3-yl)phenyl) acetamide) or vehicle, was added to cultures of *Sost* knockout osteocytes for 48-h. 6-keto $PGF_{1\alpha}$ was measured in the medium.

2.9. SDS–PAGE and immunoblot analyses

Cell lysates were electrophoresed, transferred to PVDF membranes which were blocked and probed with primary antibodies in 5% BSA, TBST: 1:1000 dilutions of antibodies recognizing non-phosphorylated (active) β-catenin, all β-catenin forms, phosphorylated Ser33/37/Thr41-β-catenin (all from Cell Signaling Technology, Inc.), or 1:1500 dilution prostaglandin I synthase polyclonal antibody made in rabbit (Cayman Chemical). After washing with TBST, 1:2000 dilution horseradish peroxidase labeled goat anti-rabbit secondary antibody in 0.5× Roche block/TBST was applied and blots were visualized using chemiluminescent substrate (Roche). PVDF membranes also probed with a β-actin monoclonal antibody (I2E5) (Cell Signaling Technologies). Densitometric analysis of scanned X-ray films from chemiluminescent detection was performed using ImageJ (NIH).

2.10. RT-PCR analyses of osteocyte markers

Reverse transcription of isolated RNAs was carried out using oligo(dT) primers and SuperScript® III First-Strand Synthesis System for RT-PCR. (Life Technologies). PCR for osteocyte markers was carried out using platinum TAQ polymerase (Life Technologies) and appropriate intron spanning primers designed using the Roche Universal Probe Library Assay Design Center (Roche) (Supplemental Table 1). PCR reactions were electrophoresed on 4% agarose gels that were later stained with ethidium bromide.

2.11. RNA preparation for RNA-seq and QPCR

RNA was prepared using RNA/protein spin columns (Clontech). Lysis solution was added to cells, frozen cell pellets or frozen bone powder. Individual clarified lysates were purified on RNA spin columns. Eluted RNA was characterized by UV absorbance (absorbance 260 nm/280 nm ratio), quantitated and frozen at −80 °C.

2.12. Quantitative PCR

qPCR was carried out using a Roche LightCycler 480 QPCR apparatus using intron-spanning qPCR primer pairs for mouse genes (Roche) (Supplemental Table 2). A SuperscriptIII RT-PCR kit (Life Technologies) was used to generate template DNA from RNA. Reverse transcribed Superscript III product was used to generate PCR products with each primer pair. Product was used to generate standard QPCR curves. QPCR data were quantitated against murine Rpl13a run for each primer pair.

2.13. Chromatin immunoprecipitation (ChIP) assays

ChIP assays were performed as described earlier [31] on osteocytes isolated from WT and *Sost* KO mice. Immunoprecipitations were performed with 2 μg Lef1-specific antibody or an isotype-matched IgG control (Millipore). Purified DNA was added to PCRs containing primers (5′-GCACTGAGACACGGGAAGA-3′ and 5′-GTCTCTGCCTCCCAAGCTC-3′) that flank the putative Lef1 binding site identified in the *Ptgis* promoter (5′-CCTTTGAT-3′, beginning 1860 bp upstream of the translational initiation codon). ChIP DNA was measured by real-time PCR, with threshold values normalized to input DNA and the isotype control immunoprecipitation.

2.14. Mineralization studies

These were performed as described earlier [32]. MC3T3 cells (1×10^4 cells/well) were seeded in 24-well plates. Upon confluence, ascorbic acid (100 μg/mL) was added. After 72 h, 5 mM

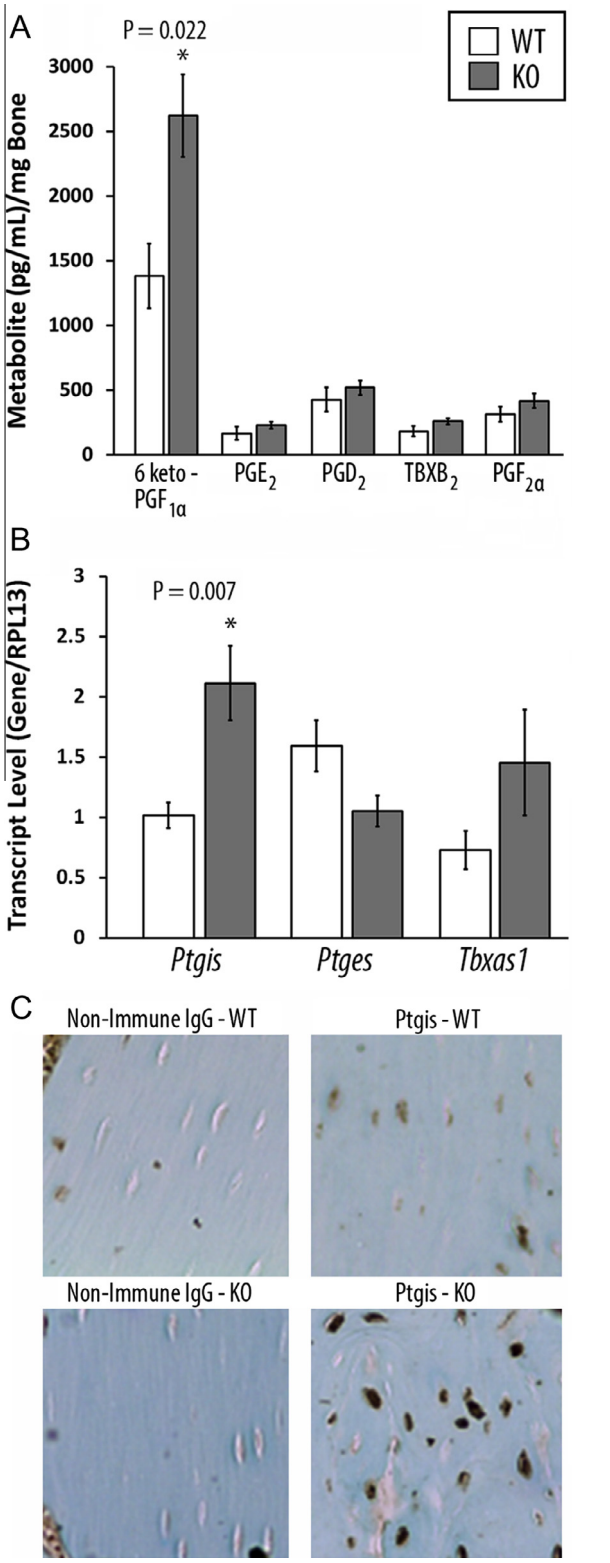

Fig. 1. Prostaglandins, mRNAs for prostaglandin synthases, and prostacyclin synthase in extracts of bones or decalcified bone sections of *Sost* WT or KO mice. (A) Concentrations of prostaglandins in bone. (B) Concentrations of mRNA transcripts for the PG synthases. (C) Ptgis in osteocytes present in decalcified bone sections from *Sost* KO (lower right panel) mice and *Sost* WT mice (upper right panel). * Statistically significant from WT.

β-glycerophosphate, $^{45}CaCl_2$ (18.5 kBq/well), and either vehicle (PBS), prostacyclin (10 μM), or carbaprostacyclin (10 μM) were added. Vehicle, prostacyclin (10 μM) or carbaprostacyclin (10 μM) were added daily, and media were changed every 3.5 days. At days 7 and 14 post-addition of β-glycerophosphate, cells were rinsed with PBS, and frozen at −20 °C. Frozen cells were lysed with 500 μL of 10 mM Tris–HCl (pH 7.4), 10 mM $MgCl_2$, and 0.1% (w/v) Triton X-100, and centrifuged at 14,000×*g* for 5 min. Alkaline phosphatase activity was measured by fluorescence assay (Quanti-Fluo, BioAssay Systems) in the supernatant. Radioactivity (^{45}Ca) was measured with a TopCount NXT (Perkin Elmer) scintillation counter by adding 50 μL of the cell lysate to 150 μL of scintillant (Microscint 40).

2.15. Statistical methods

Statistical differences between samples were analyzed using Student's two-tailed *t* test assuming equal variance. $P < 0.05$ was regarded as statistically significant.

3. Results

Arachidonic acid (AA), the precursor to prostaglandins, is converted via the cyclooxygenase pathway to prostaglandin PGG_2 and subsequently to PGH_2 [33]. The latter is converted to PGI_2, PGD_2, PGE_2, PGF_2 and thromboxane A_2 by specific synthases; PGF_2 is also directly produced from PGE_2 [33]. In femoral bone extracts, higher concentrations of 6-keto $PGF_{1\alpha}$, the stable metabolite of PGI_2, were detected in bone from *Sost* KO mice compared with those measured in WT mice (Fig. 1A). Concentrations of PGE_2, PGD_2, TXB_2 (the stable metabolite of TXA_2) and $PGF_{2\alpha}$, were similar in *Sost* KO and WT mice (Fig. 1A). Messenger RNA levels for the enzyme, *PGI_2 synthase* (*Ptgis*), were increased while mRNAs for *PGE_2 synthase* (*Ptges*) and *thromboxane A synthase 1* (*Tbxas1*) remained unchanged in bone extracts from *Sost* KO and WT mice (Fig. 1B). The increase in prostacyclin and *Ptgis* mRNA was associated with an increase in prostacyclin synthase protein (Ptgis) in osteocytes of bones from *Sost* KO mice relative to osteocytes of bones from WT mice (Fig. 1C). Levels of urinary prostaglandin metabolites were similar in SOST KO and WT mice (ng/mL prostaglandin/mg urinary creatinine; mean ± SEM: KO vs. control: 6-keto $PGF_{1\alpha}$, 27. 74 ± 4.55 vs. 24.15 ± 2.22, $P = 0.241$; PGE, 21.22 ± 5.7 vs. 27.80 ± 4.22, $P = 0.391$; PGD_2, 149.04 ± 11.86 vs. 132.52 ± 35.69, $P = 0.683$; $PGF_{2\alpha}$, 9.04 ± 2.05 vs. 21.47 ± 4.35, $P = 0.061$; and $TBXB_2$ 23.56 ± 3.79 vs. 28.08 ± 3.65, $P = 0.830$).

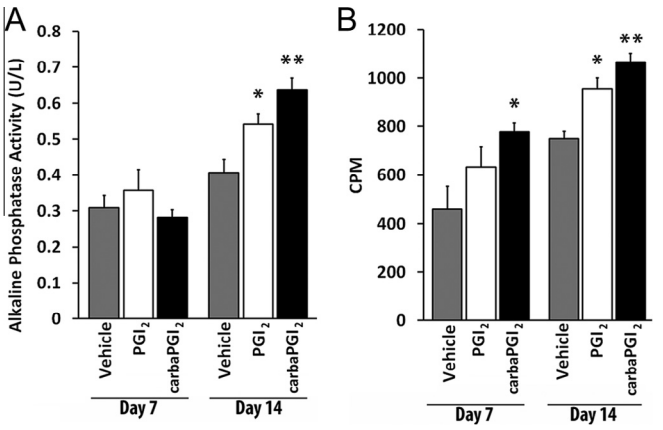

Fig. 2. Effect of prostacyclin and carbaprostacyclin on differentiation and mineralization of MC3T3 osteoblasts. (A) Alkaline phosphatase activity in cells at 7 days and 14 days in the presence of vehicle, PGI_2 or carbaPGI$_2$. (B) ^{45}Ca uptake in cells and matrix at 7 days and 14 days in the presence of vehicle, PGI_2 or carbaPGI$_2$. $^{*}P < 0.05$, $^{**}P < 0.001$.

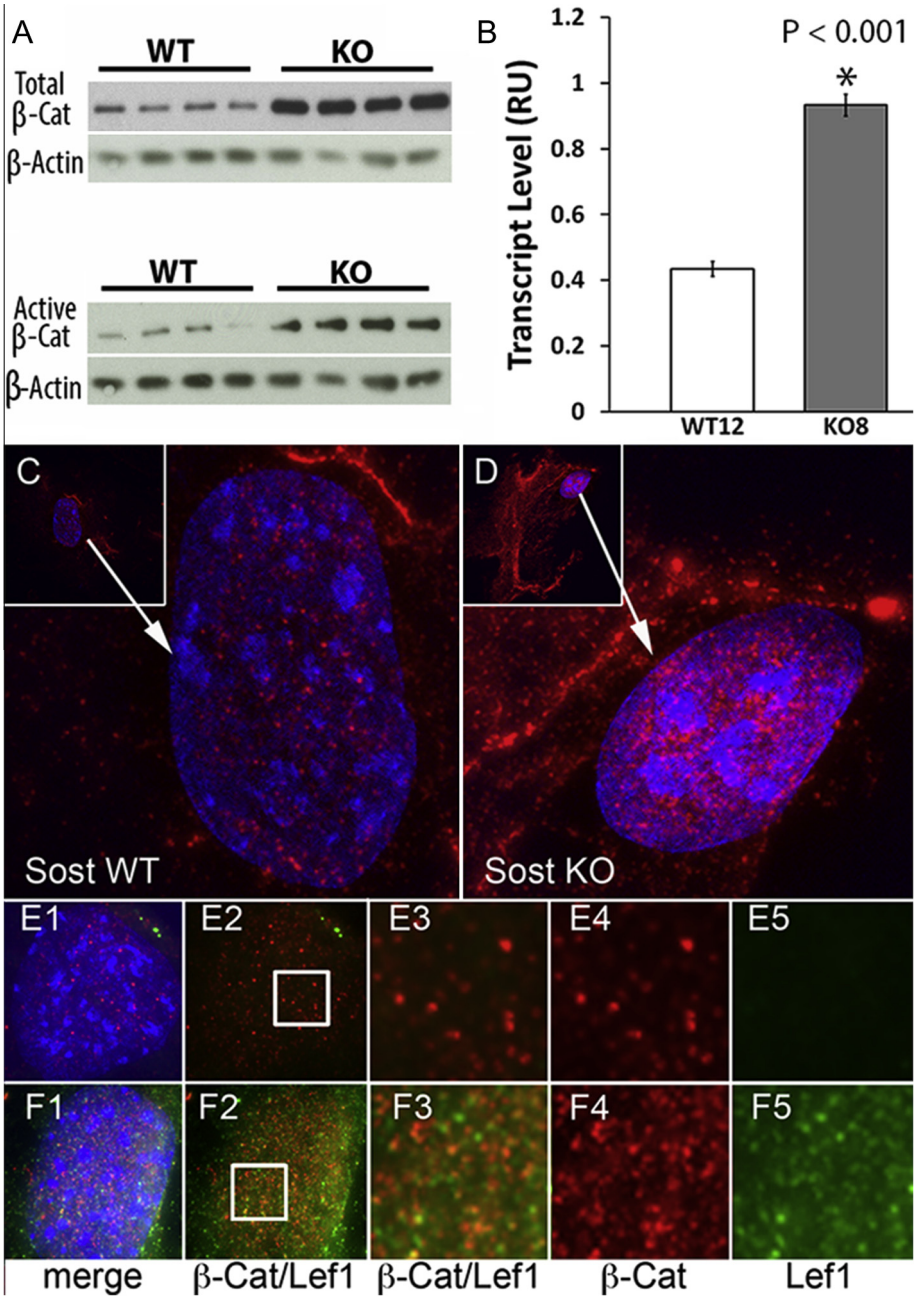

Fig. 3. β-Catenin protein and mRNA and Lef1 protein in clonal osteocytes from *Sost* KO and WT mice. (A) (Upper panel) Immunoblot of cellular protein from WT (left 4 lanes in panel) and KO (right 4 lanes in panel) clonal osteocytes with total β-catenin specific antibody. (Lower panel) Immunoblot of cellular protein from WT (left 4 lanes in panel) and KO (right 4 lanes in panel) clonal osteocytes with non-phosphorylated (active) β-catenin specific antibody. (B) Assessment of β-catenin transcript levels in WT and KO clonal osteocytes. (C) β-Catenin immunofluorescence (IF, red) in WT osteocyte. The nucleus of the cell is stained blue. (D) β-Catenin IF (red) in KO osteocyte. The nucleus of the cell is stained blue. (E). Panels 1–5, localization of β-catenin (red, panel 4) and Lef1 (green, panel 5) in the nucleus of a clonal *Sost* WT osteocyte. In panel 2 and 3, co-localization of β-catenin and Lef1 are shown. (F) Panels 1–5, localization of β-catenin (red, panel 4) and Lef1 (green, panel 5) in the nucleus of a clonal *Sost* KO osteocyte. In panel 2 and 3, co-localization of β-catenin and Lef1 are shown. More intense IF is noted in the clonal KO than in the WT clonal osteocytes. This is especially apparent in panels F3 vs. E3. * Statistically significant from WT.

We established primary and immortalized osteocyte cultures to determine whether the high bone prostacyclin content in *Sost* KO mice was produced in the bone cells that normally express sclerostin. 6-keto $PGF_{1\alpha}$ was increased 3-fold in primary osteocytes isolated from bone of *Sost* KO compared to WT mice (93.68 ± 23.39 vs. 31.24 ± 8.44 pg 6-keto $PGF_{1\alpha}$/mg protein, $P = 0.024$,), whereas PGE_2 concentrations were similar (1.52 ± 0.37 vs. 1.94 ± 0.47 pg PGE_2/mg protein, $P = 0.52$). In mixed populations of immortalized osteocytes, concentrations of 6-keto PGF1α were increased in *Sost* KO compared to WT osteocytes (2,823.51 ± 485.64 pg/mL KO vs. 163.41 ± 10.49 pg/mL WT, $P = 0.005$), whereas PGE_2 concentrations were similar. These changes are mirrored by the increase of mRNA for *Ptgis* by 100-fold, $P < 0.001$, in *Sost* KO osteocytes compared to WT osteocytes. A 1.7-fold increase in *Ptges* mRNA was also observed, $P < 0.001$. Prostaglandin production in clonal populations of *Sost* KO (KO8) and WT (WT12) osteocytes showed a substantial increase in 6-keto $PGF_{1\alpha}$ in *Sost* KO8 compared to WT12 cultures (3,302.41 ± 27.97 pg/mL vs. 178.89 ± 66.49 pg/mL, $P < 0.001$, respectively), whereas PGE_2 concentrations were only slightly but statistically higher in *Sost* KO8 (1,298.9 ± 43.3 pg PGE_2/mL vs.

1,093.2 ± 31.2 pg PGE_2/mL, $P = 0.003$). The mRNA for *Ptgis* increased >400-fold, $P \leqslant 0.001$. A considerably smaller, 13-fold change in *Ptges* mRNA was also observed, $P \leqslant 0.001$. Ptgis protein is increased in *Sost* KO8 osteocytes compared to WT12 osteocytes by western blotting.

Previous experiments have shown that prostacyclin enhances bone mineralization and osteoblast enyzymatic function. To further assess the role of prostacyclin in osteoblast differentiation and mineralization, we treated MC3T3 osteoblasts with prostacyclin or carbaprostacyclin. Alkaline phosphatase activity was enhanced in MC3T3 osteoblast-like cells at 14 days after the exogenous addition of prostacyclin or carbaprostacyclin (Fig. 2A). There was an increase in *Alpl* mRNA in treated cells at day 14 (ratio *Alpl* in PGI_2 or vehicle treated cells = 1.46, $P = 0.037$; carbaPGI_2 or vehicle treated cells = 1.43, $P = 0.006$). Mineralization assessed by the uptake and deposition of ^{45}Ca in matrix was enhanced in cells treated with prostacyclin or carbaprostacyclin when compared to vehicle (Fig. 2B).

To determine whether Wnt signaling was responsible for changes in prostacyclin expression in *Sost* KO osteocytes, we assessed expression and localization of β-catenin in *Sost* KO8 and WT12 osteocyte clones. We found a 2-fold ($P < 0.001$) increase in *β-catenin* mRNA and a corresponding 6-fold ($P < 0.001$) increase in total β-catenin in *Sost* KO8 compared to WT12 (Fig. 3A and B). *Sost* KO8 cells showed a nearly 9-fold ($P = 0.013$) increase in the non-phosphorylated, active form of β-catenin. While both WT12 and KO8 cells showed cytoplasmic and cell membrane associated β-catenin, the increased protein level in *Sost* KO8 cells was accompanied by a substantial increase in nuclear β-catenin (Fig. 3C and D). The nuclear β-catenin showed predominantly euchromatic co-localization with a Wnt effector protein, lymphocyte enhancer protein (Lef1) (Fig. $3F_{1-5}$). WT12 osteocytes showed substantially less nuclear β-catenin and virtually undetectable nuclear Lef1 (Fig. $3E_{1-5}$).

To determine if increased Lef1 localization on chromatin influenced *Ptgis* gene function, we performed chromatin immunoprecipitation experiments using *Sost* KO8 and WT12 cells, and a specific antibody against Lef1, to localize Lef1 binding sites on the *Ptgis* gene. A Lef-binding site was found using *in silico* analysis at −1860 to −1853 bp upstream of the translation initiation site of the *Ptgis* gene promoter. Chromatin immunoprecipitation with a Lef1 antibody, followed by real-time PCR performed with primers flanking this site, showed higher Lef1 occupancy of this site in *Sost* KO8 as compared to WT12 osteocytes (Fig. 4).

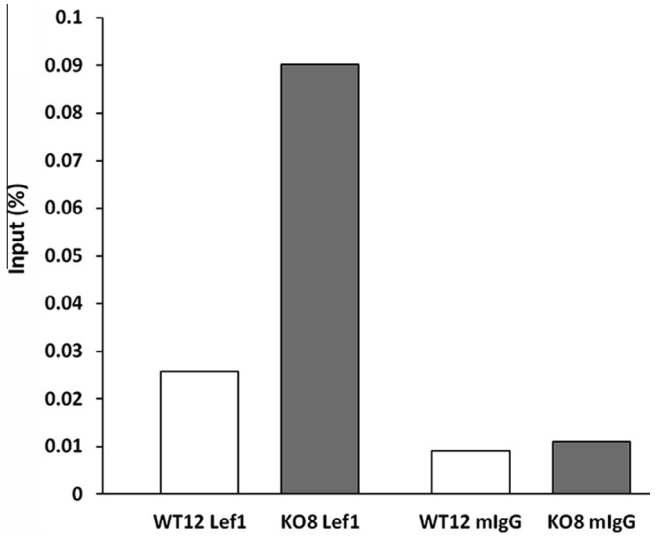

Fig. 4. ChIP analysis of the *Lef1* sites of WT12 and *Sost* KO8 osteocytes. Total percent input of both clones, and associated mouse IgG controls.

The functional importance of β-catenin signaling in sclerostin-mediated increases in PGI_2 synthesis was assessed by treating *Sost* KO8 cells with a Wnt inhibitor, C59 (2-(4-(2-methylpyridin-4-yl)phenyl)-*N*-(4(pyridine-3-yl)phenyl) acetamide). Following treatment of cells with C59 there was a decrease in β-catenin protein concentrations in *Sost* KO8 osteocytes ($P < 0.05$), as well as a concomitant decrease in 6-keto $PGF_{1\alpha}$ (536.41 ± 60.69 pg/mL vehicle vs. 274.96 ± 6.01 pg/mL experimental, $P = 0.013$).

4. Discussion

Relative rates of bone formation and resorption determine the amount of mineralized bone present in the skeleton. These processes are under cellular control and the activities of osteoblasts and osteoclasts that form and resorb bone, respectively, are dependent on physiological needs and mechanical stimulation. Among the physiological regulators are endocrine factors, such as parathyroid hormone and 1α,25-dihydroxyvitamin D and the availability of calcium and phosphorus in the extra-cellular fluid. The activity of several locally produced peptides (e.g. RANKL and the BMPs) and lipid mediators (e.g. PGE_2) whose concentrations and activities are influenced by the aforesaid systemic hormones and factors such as mechanical force and fluid flow are also crucial [2–9,34–38].

With regard to the effects of prostanoids on bone, previous reports demonstrated that PGE_2 is released form bone cells in response to changes in fluid flow [39] and that PGE_2 increases bone mass when administered to rodents [10]. The observation that the production of prostacyclin or PGI_2, a prostaglandin previously only shown to play a role in modulating platelet, vascular and immune function [33,40–43], is greatly increased in bones and osteocytes of sclerostin-deficient mice suggests that this short-lived prostanoid contributes to bone formation in the context of this high bone mass syndrome. The effectiveness of prostacyclin in enhancing osteoblast maturation, as evidenced by increases in alkaline phosphatase expression and mineralization of osteoblast cultures treated with exogenous prostacyclin, indicates that prostacyclin may be effective in increasing bone mass in the normal bone as well.

We demonstrate the expression of prostaglandin synthase, the enzyme responsible for the synthesis of prostacyclin, is regulated by β-catenin and its downstream effector, Lef-1, which binds to the *Ptgis* promoter and increases *Ptgis* mRNA. In *Sost* KO mice, enhanced bone formation has been attributed to increased availability of the Wnt/frizzled pathway co-receptor, LRP 5/6, which facilitates activation of Wnt pathways and β-catenin stabilization when Wnt ligands are present [28]. The work presented here suggests that prostanoid metabolism is regulated by Wnts and β-catenin and may contribute to high bone mass in sclerostin deficient mice.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.04.092.

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