PLoS one

Prostaglandin E$_2$ Signals Through *PTGER2* to Regulate Sclerostin Expression

Damian C. Genetos[1]*, Clare E. Yellowley[1], Gabriela G. Loots[2]

1 Department of Anatomy, Physiology, and Cell Biology, School of Veterinary Medicine, University of California Davis, Davis, California, United States of America, 2 Lawrence Livermore National Laboratory, Biology and Biotechnology Division, Livermore, California, United States of America

Abstract

The Wnt signaling pathway is a robust regulator of skeletal homeostasis. Gain-of-function mutations promote high bone mass, whereas loss of Lrp5 or Lrp6 co-receptors decrease bone mass. Similarly, mutations in antagonists of Wnt signaling influence skeletal integrity, in an inverse relation to Lrp receptor mutations. Loss of the Wnt antagonist Sclerostin (*Sost*) produces the generalized skeletal hyperostotic condition of sclerosteosis, which is characterized by increased bone mass and density due to hyperactive osteoblast function. Here we demonstrate that prostaglandin E$_2$ (PGE$_2$), a paracrine factor with pleiotropic effects on osteoblasts and osteoclasts, decreases Sclerostin expression in osteoblastic UMR106.01 cells. Decreased *Sost* expression correlates with increased expression of Wnt/TCF target genes *Axin2* and *Tcf3*. We also show that the suppressive effect of PGE$_2$ is mediated through a cyclic AMP/PKA pathway. Furthermore, selective agonists for the PGE$_2$ receptor EP2 mimic the effect of PGE$_2$ upon *Sost*, and siRNA reduction in *Ptger2* prevents PGE$_2$-induced *Sost* repression. These results indicate a functional relationship between prostaglandins and the Wnt/β-catenin signaling pathway in bone.

Introduction

There remains considerable effort dedicated toward understanding the signaling pathways that promote skeletal anabolism. Prostaglandins (PG), such as prostaglandin E$_2$ (PGE$_2$), mediate osteoprogenitor proliferation [1,2,3] and differentiation [4,5]. Mechanical loading *in vitro* and *in vivo* induces expression of the enzyme responsible for PG synthesis, COX-2 [6,7,8], whose function is required for load-induced bone formation [9,10]. Similarly, PG administration *in vivo* increases bone mass *via* periosteal and endosteal responses [11]. Further, inhibition of PG synthesis delays fracture healing [12,13] and promotes the formation of non-unions [14,15], whereas localized PGE$_2$ enhances bone healing [16,17,18,19].

Osteoblast differentiation is also regulated by the Wnt signaling pathway [20]. Binding of Wnt ligands to a complex of Frizzled and Lrp5 or Lrp6 co-receptors promotes the stabilization of the transcription factor β-catenin, formation of a complex with TCF/LEF, and induction of Wnt target genes like *Axin2* and *Tcf3* [21,22]. Activating mutations in *Lrp5* cause high bone mass [23,24], whereas *Lrp5* deletion decreases bone mass [25,26] and prevents load-induced bone formation [27]. Control of Wnt signaling involves sequestration of Wnts by soluble decoy receptors like sFRPs [28,29], or Lrp5 antagonists, like Dkk1 and Sclerostin.

Deletion of the gene encoding Sclerostin (*Sost*) causes a rare sclerosing bone dysplasia, sclerosteosis (OMIM ID 269500) in both humans and murine knockout models [30,31,32,33]; a related

disease, van Buchem's disease (OMIM ID 239100), is caused by a distal noncoding deletion that removes regulatory elements required for the transcriptional of the *Sost* gene in adult bone [32]. Both sclerosteosis and van Buchem disease are characterized by general skeletal hyperostosis owing to hyperactive osteoblast activity. In contrast, over-expression of *Sost* causes osteopenia [34,35] and limb deformities [36]. Mechanistically, Sclerostin was initially characterized as a BMP antagonist [34,37,49], but more recent reports recognize it as a potent Wnt antagonist that binds to Lrp5 and Lrp6 [38,39,40,41] to increase the rate of receptor internalization [42]. Keller and Kneissel showed that PTH reduces *Sost* expression *via* PKA [43], as did Bellido *et al.* [44], and we have previously demonstrated that the regulatory element ECR5 contained within the van Buchem deletion region is necessary for bone-specific *Sost* expression in transgenic mice [35], and confers PTH responsiveness, *in vitro* [45]. Recently, we have also shown that a *Sost* null mutation partially rescues the $Lrp6^{+/-}$ skeletal phenotype in $Sost^{-/-};Lrp6^{+/-}$ animals [36].

Whereas both prostaglandins and Wnt signaling have parallel functions during bone anabolism, there is limited evidence for cross-talk between these two signaling pathways in pre-osteoblasts and in transformed cells. In this study, we examined the influence of PGE$_2$ on Sclerostin transcription and Wnt signaling, in osteoblastic cells. We demonstrate that prostaglandin E$_2$, a long-recognized regulator of osteoblast and osteoclast formation activity, decreases *Sost* expression and thereby increases Wnt signaling in osteoblastic cells. We also show that PGE$_2$

transcriptional effect on *Sost* is mediated through the EP2 receptor (*Ptger2*) and cAMP, and involves mitigation of endogenous BMP and Mef2 signaling. These results attribute a novel function of prostaglandins in the regulation of Wnt signaling *via* suppression of the Wnt antagonist Sclerostin.

Results

Prostaglandin E$_2$ decreases Sost transcription

Although both prostaglandins and Wnt signaling have been characterized as robust regulators of skeletal formation and homeostasis [9,24,46,47,48], there is sparse evidence whether there is direct interaction between these pathways. To that end, we first sought whether PGE$_2$ demonstrated an effect upon the transcription of Sclerostin. To test this, UMR106.01 cells were chosen, as they phenotypically resemble mature osteoblasts and express high levels of *Sost* [43,50,51]. UMR106.01 cells were treated with 5 nM–5 μM PGE$_2$ for 3 hours, after which time RNA was collected and analyzed *via* quantitative PCR (qPCR) for *Sost* expression. There was no influence of 5 nM PGE$_2$ on *Sost* expression, while there was steady and progressive decrease in *Sost* levels upon 50 nM–5 μM PGE$_2$ treatment (**Figure 1A**). This inhibitory effect upon Sost was not observed when cells were treated with another osteotropic prostaglandin, PGF$_{2\alpha}$ (5 nM– 5 μM; *data not shown*). The inhibitory effect of PGE$_2$ was rapid, with statistically significant suppression of *Sost* observed after one hour of treatment, and this was maintained throughout 24 hours of culture (**Figure 1B**).

Prostaglandin E$_2$ influences Wnt signaling without affecting Dkk1

Functional decrease in the expression of the Wnt antagonist *Sost* should effectively increase markers of β-catenin/TCF signaling, such as *Axin2* and *Tcf3*. To that end, we observed that PGE$_2$, in the same dosing range that decreased *Sost*, significantly increased *Axin2* and *Tcf3* expression after 24 hour culture (**Figure 2A**), suggesting that PGE$_2$-induced decreases in *Sost* removed a suppressive effect of endogenous Wnt antagonists upon osteoblast function. Dickkopf1 (*Dkk1*) inhibits Wnt signaling in the same manner as does Sclerostin [42]. Whereas 50 nM–5 μM PGE$_2$ dramatically reduced *Sost* transcript and protein (*not shown*) levels, PGE$_2$ had no effect on *Dkk1* transcript (**Figure 2B**) nor its protein (**Figure 2C**) expression, suggesting that *Sost* repression is the primary mechanism of enhanced Wnt signaling in response to PGE$_2$ treatment.

PGE$_2$ decreases Sost through cAMP-dependent mechanisms

UMR 106.01 cells express all four classes of PGE$_2$ receptors (EP1–EP4, encoded by *Ptger1–Ptger4*; **Figure 3A**), which are linked to the synthesis or mobilization of cAMP and Ca$^{2+}_i$. EP2 and EP4 increase cAMP levels, while EP1 increases Ca$^{2+}_i$ through a PLC-dependent mechanism; EP3 increases Ca$^{2+}_i$ and decreases cAMP [52]. To define which receptor(s) are responsible for mediating the suppressive effects of PGE$_2$ upon *Sost*, UMR 106.01 cells were treated with 5 μM PGE$_2$ in the presence of antagonists of protein kinase A (H-89, 2.5 μM) or phospolipase C (U73122, 10 μM) for 3 hours, after which time total RNA was collected and analyzed for *Sost* levels. In the absence of PGE$_2$, inhibition of PLC/IP$_3$/Ca$^{2+}_i$ signaling decreased basal *Sost* levels (**Figure 3B**), suggesting that release of intracellular calcium is important for maintaining *Sost* expression. In the presence of PGE$_2$, the addition of H-89 appeared to attenuate PGE$_2$-induced *Sost* suppression (**Figure 3B**) although this did not reach statistical significance ($p < 0.1$ versus
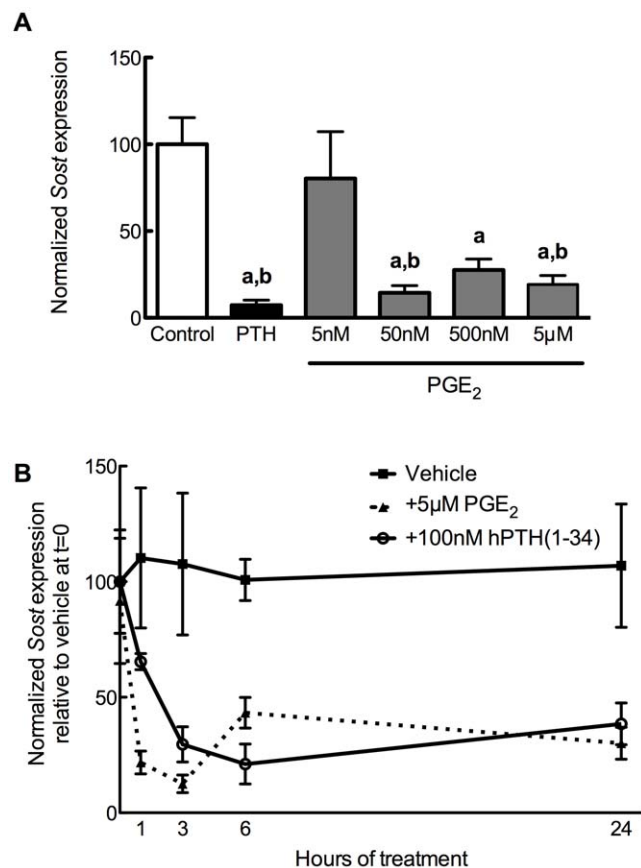

Figure 1. PGE$_2$ decreases *Sost* expression. (A) Human PTH(1–34) (100 nM) or PGE$_2$ (5 nM–5 μM) or vehicle control (0.05% DMSO) was added to UMR 106.01 cells for 3 hours. Total RNA was collected and analyzed for *Sost* and *Rpl32* expression by qPCR. n = 6–10 samples per treatment. **a** indicates $p < 0.05$ *versus* Control; **b** indicates $P < 0.05$ versus 5 nM PGE$_2$. (B) *Sost* mRNA expression was quantified in UMR 106.01 cells after 0, 1, 2, 3, 6, or 24 hours treatment with 5 μM PGE$_2$ or vehicle control. n = 4 samples per treatment. For PGE$_2$, each time point is significantly different ($p < 0.05$) from Control, while for PTH, every time point except 1 hr is significantly different ($p < 0.05$) from Control.
doi:10.1371/journal.pone.0017772.g001

5 μM PGE$_2$ alone). In contrast, the addition of PGE$_2$ to U73122-treated cells demonstrated no change compared to U73122 alone. The role of cAMP and Ca$^{2+}_i$ mobilization in suppressing *Sost* was tested using selective agonists. UMR106.01 cells treated with the cAMP mimetic 8-bromo-cAMP (1 mM) demonstrated similar suppression of *Sost* as 5 μM PGE$_2$-treated cells (**Figure 3C**), whereas 1.3 μM ionomycin treatment significantly increased *Sost* expression. These data indicate that PGE$_2$ receptors linked to increased cAMP—*Ptger2* or *Ptger4*—are involved in the capacity for PGE$_2$ to decrease *Sost*.

Specific agonists for EP2 (butaprost, [53]) or EP4 (CAY10580, [54]) were also tested for their ability to mimic the suppressive effects of PGE$_2$ on *Sost* transcription. Butaprost mimicked the ability of PGE$_2$ to decrease *Sost*, whereas CAY10580 had no effect on *Sost* levels (**Figure 4A**). siRNA directed against *Ptger2* (**Figure 4B**) or *Ptger4* (**Figure 4C**) reproducibly decreased target transcript expression by 60% relative to non-silencing, scrambled siRNAs. Knock-down of *Ptger2*, but not *Ptger4*, significantly impaired the ability of PGE$_2$ to suppress *Sost* expression (**Figure 4D**), indicating the requirement for the *Ptger2* receptor for PGE$_2$-specific activation of Wnt signaling.
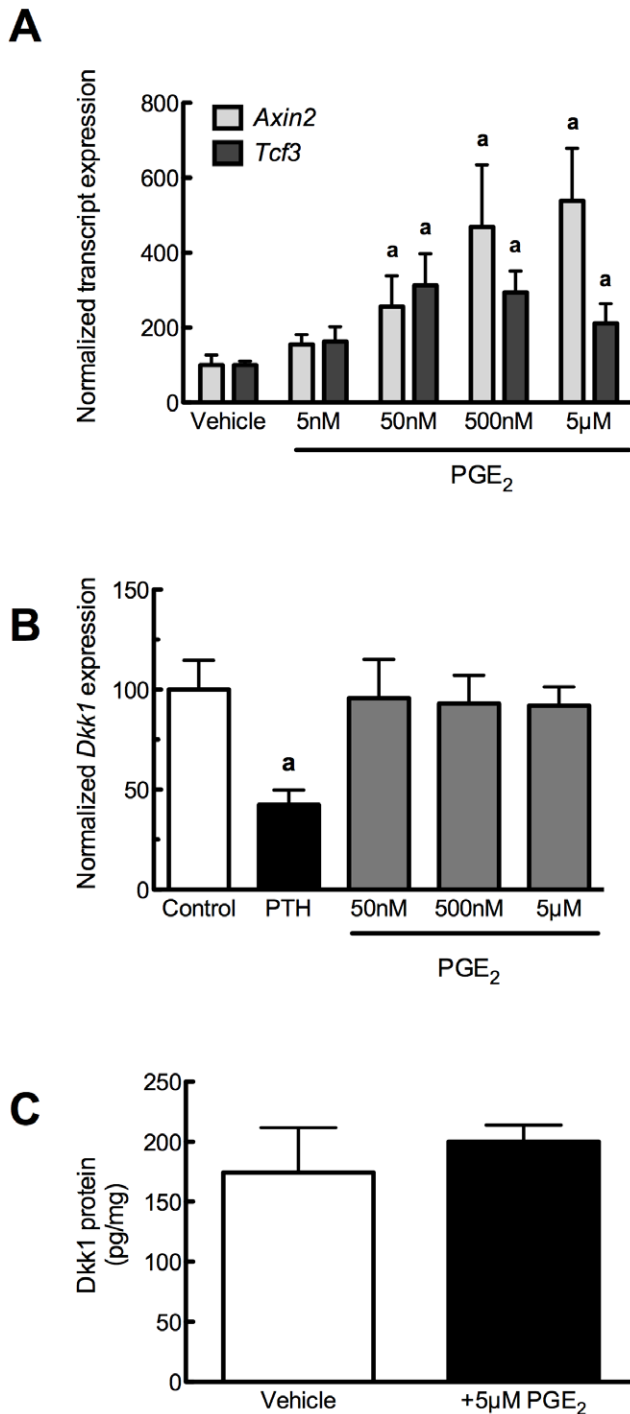
Figure 2. PGE$_2$ increases Wnt signaling without affecting Dkk1.
(**A**) PGE$_2$ (5 nM–5 µM) or vehicle control (0.05% DMSO) was added to UMR 106.01 cells for 24 hours. Total RNA was collected and analyzed for *Axin2*, *Tcf3*, and *Rpl32* expression by qPCR. n = 4 samples. Compared to vehicle control, **a** indicates *p*<0.05. (**B**) Human PTH(1–34) (100 nM) or PGE$_2$ (50 nM–5 µM) or vehicle control (0.05% DMSO) was added to UMR 106.01 cells for 3 hours. Total RNA was collected and analyzed for *Dkk1* and *Rpl32* expression by qPCR. n = 8 samples. Compared to vehicle control, **a** indicates *p*<0.05.
doi:10.1371/journal.pone.0017772.g002

Cycloheximide, but not Actinomycin D, influences PGE$_2$ suppression of Sclerostin

We examined the transcriptional and translational mechanisms whereby PGE$_2$ regulates *Sost* expression. UMR106.01 cells were treated with 5 µM PGE$_2$ for 3 hours in the presence or absence of the RNA polymerase II inhibitor Actinomycin D (2.5 µg/mL), after which time total RNA was collected and analyzed. The suppressive influence of PGE$_2$ on *Sost* transcription was consistent in cells treated with or without actinomycin D (**Figure 5A**), indicating that PGE$_2$ does not promote the degradation of *Sost* transcript.

Next, UMR106.01 cells were treated with or without 5 µM PGE$_2$ for 3 hours in the presence or absence of the protein translation inhibitor cycloheximide (CHX; 10 µg/mL). In the absence of PGE$_2$, CHX increased *Sost* transcript (**Figure 5B**); in cells treated with PGE$_2$ and CHX, there was no suppressive effect of PGE$_2$ upon *Sost* expression, indicating that PGE$_2$ requires *de novo* protein synthesis in order to decrease *Sost*. Similar results were observed after 1 hour of CHX or PGE^2 treatment (*data not shown*).

Transcriptional regulation of Sost by PGE$_2$ does not involve Mef2 or BMPs

We have previously demonstrated that the MEF2 family of transcription factors are responsible for sensitizing the *Sost* distal enhancer ECR5 to PTH [45]. We employed siRNA against *Mef2c* or *Mef2d* in order to determine whether these transcription factors are involved in the capacity for PGE$_2$ to decrease *Sost*. 48 hours after transfection, expression of *Mef2c* (**Figure 6A**) and *Mef2d* (**Figure 6B**) was reduced approximately 70% and 55%, respectively, compared to scrambled siRNA controls. Knockdown of *Mef2c* or *Mef2d* did not alter the ability of 5 µM PGE$_2$ to decrease *Sost* transcript (**Figure 6C**), suggesting that PGE$_2$ does not decrease *Sost* expression by disrupting Mef2 activity.

We, and others, have previously demonstrated the transcriptional influence of bone morphogenetic proteins on *Sost*: exogenous BMPs or constitutively-active BMP receptor 1A increase *Sost* expression [50,51,55], whereas dominant-negative BMP Receptor 1A decreases *Sost* transcription [55]. To examine whether PGE$_2$ signaling disrupted BMP induction of *Sost*, UMR106.01 cells co-cultured with 5 µM PGE$_2$ and BMP-2 (0–500 ng/mL) for 3 hours. BMP-2-treated cells increased *Sost* expression (**Figure 7A**), whereas co-culture with PGE$_2$ prevented *Sost* induction. BMP-2 increased *Id1*, a direct Smad target gene, independent of PGE$_2$ (**Figure 7B**), indicating that BMP signaling was unaffected by PGE$_2$ treatment. These data suggest that PGE$_2$ decreases *Sost* transcription independent of the BMP signaling, and hence the effect of PGE2 upon *Sost* is downstream of BMPs.

PTH does not require prostaglandins to decrease Sost

PTH increases COX-2 expression and subsequent synthesis and release of prostaglandins [56,57]. Because both PTH and PGE$_2$ decrease *Sost* transcription through cAMP/PKA mechanisms, we next examined whether the capacity for PTH to decrease *Sost* required prostaglandins. Cells were treated for 24 hours in reduced serum (2%) conditions in the presence of 0.05% DMSO or 1 µM indomethacin; thereafter, a subset of cells were treated for 24 hours in the presence of 100 nM hPTH(1–34). As shown in **Figure 8**, there was a similar decrease in *Sost* expression in cells treated with PTH with or without indomethacin co-treatment. Thus, PTH does not require prostaglandins to decrease *Sost* transcription, suggesting that PTH and PGE$_2$ function through independent, parallel pathways that converge upstream of Sclerostin's transcriptional regulation.
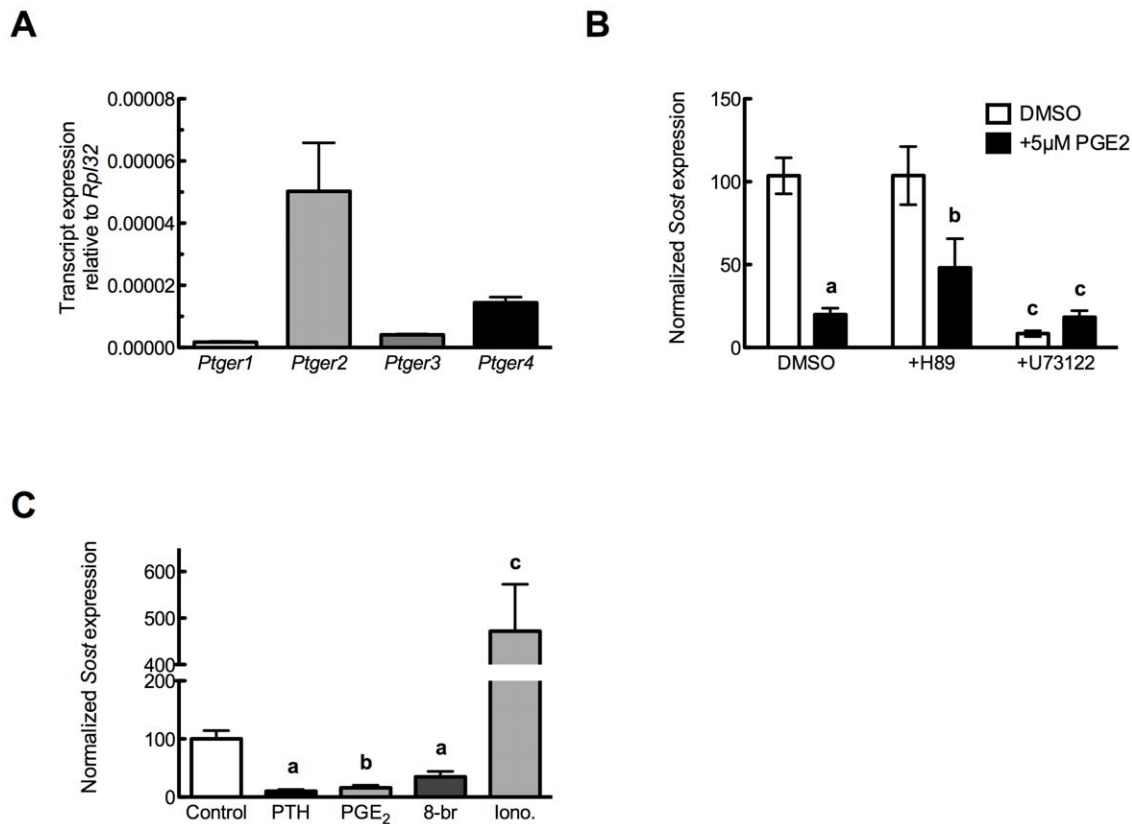
Figure 3. PGE₂ receptor expression and influence of PGE₂ selective agonists upon *Sost* expression. (A) UMR106.01 cells analyzed for *Ptger1*, *Ptger2*, *Ptger3*, and *Ptger4* transcript expression by qPCR. Data are normalized to *Rpl32*. n = 4 samples. (B) UMR106.01 cells were cultured with DMSO as vehicle control, 100 nM hPTH(1–34), 5 μM PGE₂ in the presence and absence of inhibitors of protein kinase A (H-89, 2.5 μM) or phospholipase C (U73122, 10 μM), for 3 hours. Total RNA was analyzed for *Sost* and normalized to *Rpl32*. n = 4–8 samples. Compared to solvent control, **a** indicates $p<.001$ and **b** indicates $p<.05$; **c** indicates $p<.001$. (C) UMR106.01 cells were cultured with DMSO as vehicle control, 100 nM hPTH(1–34), the cell-permeant cyclic AMP analogue 8-br-cAMP (1 mM) or the calcium ionophore ionomycin (1.3 μM) for 3 hours, after which total RNA was collected and analyzed for *Sost* and *Rpl32*. n = 4–8 samples. Versus Control, **a** indicates $p<0.05$, **b** indicates $p<0.01$, and **c** indicates $p<0.001$.
doi:10.1371/journal.pone.0017772.g003

Discussion

Sclerostin is a robust inhibitor of bone formation, such that its absence leads to increased bone formation, and in high amounts it causes bone loss. Thus, regulation of its expression, as well as that of other potent skeletally anabolic or catabolic proteins, is under intensive investigation as a therapy for those afflicted with osteoporosis, or as a means of hastening fracture repair. Indeed, a monoclonal antibody which inhibits Sclerostin function has already been shown to increase bone formation and strength in ovariectomized rats beyond that of non-ovariectomized controls [58] and in aged male rats [59]. Despite its clinical importance in an aging population, the molecular mechanisms controlling Sclerostin expression are only beginning to be unraveled [43,45]. Within this work, we demonstrate that PGE₂, a paracrine signaling agent with diverse effects on skeletal homeostasis, decreases *Sost* transcription through the EP2 receptor subclass (encoded by *Ptger2*). Reductions in *Sost* transcription by PGE₂ was shown to involve cAMP and PKA, *de novo* protein synthesis, and to occur independently of BMP or MEF2 signaling.

PGE₂ decreases Sost expression via PKA and Ptger2

We observed rapid suppression of *Sost* by PGE₂ between 50 nM–5 μM, with a calculated IC_{50} of 41 nM (*data not shown*). Reductions in *Sost* transcript in response to PGE₂ were rapid, occurring within 1 hr of PGE₂ addition, and were sustained, remaining at 30% expression compared to vehicle-treated samples after 24 hours of culture. These results are similar to *in vivo* calvarial and *in vitro* cell culture models treated with PTH [43], as well as murine models of constitutively-active PTHR1 receptor [44,60].

Osteoblastic cells express all *Ptger* receptor genes [61,62], suggesting that PGE₂ can exert biological effects through both cAMP and Ca^{2+}_i signaling pathways. Much of the anabolic effect of PGE₂ is mediated through cAMP *via* EP2 and EP4 [63]. The cAMP analogue 8-bromo-cAMP mimicked the effect of PGE₂ upon *Sost* transcription. Inhibition of PLC/IP₃ with U73122 did not prevent PGE₂ from decreasing *Sost*, indicating that this pathway is not obligate. Interestingly, the calcium ionophore, ionomycin, significantly increased *Sost* transcription nearly 5-fold over vehicle controls after 3 hours of treatment. This would suggest stimulation of MEF2 transcriptional activity in response to increased Ca^{2+}_i, as has been shown in skeletal muscle fibers [64,65]. Keller and Kneissel have shown a modest decrease in *Sost* transcription in response to a similar dose of ionomycin [43], but they measured *Sost* levels after 24 hours of ionomycin treatment (rather than 3 hours, as in the study herein). Whether these contrasting results are due to timing of ionomycin treatment, or are secondary to prolonged cellular stress due to supra-physiologic Ca^{2+}_i [66], remains to be elucidated.
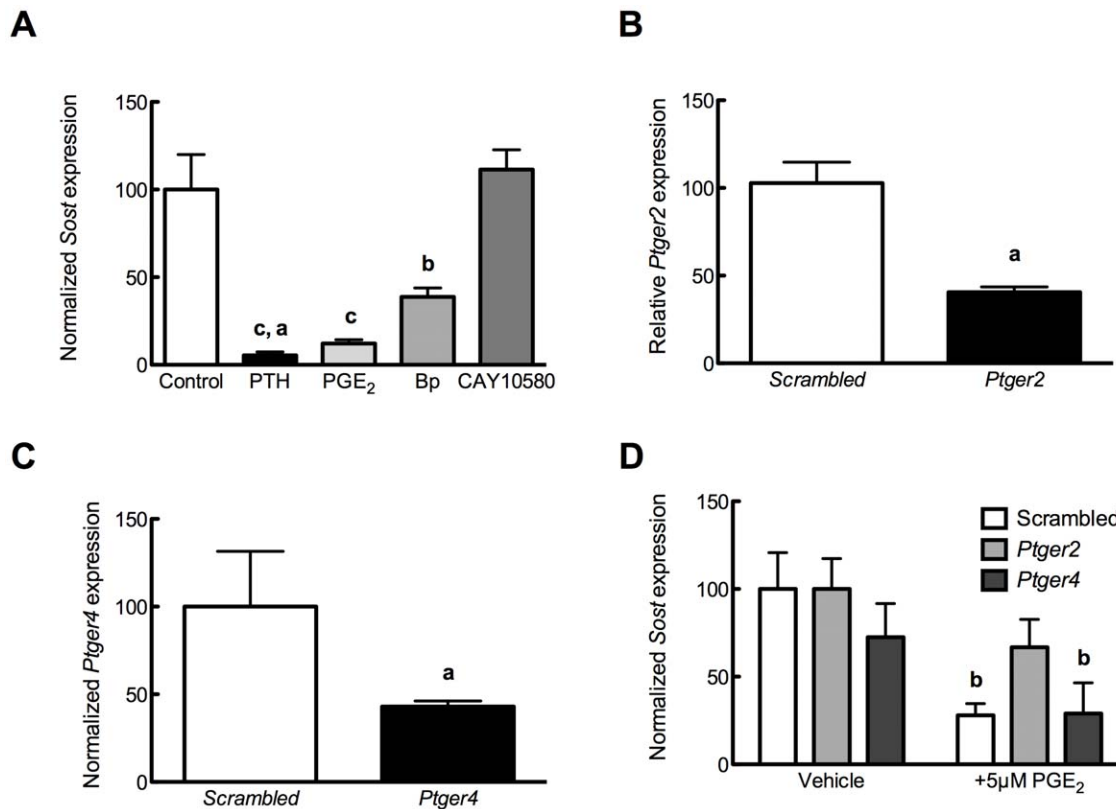
Figure 4. PGE$_2$ signals through *Ptger2* to decrease *Sost* expression. (A) Cells were cultured for 3 hours in the presence of PTH (100 nM), PGE$_2$, EP2 agonist butaprost, or EP4 agonist CAY10580 (each 500 nM). *Sost* expression was analyzed by qPCR and normalized to *Rpl32*. n = 5 samples. Compared to vehicle control, **b** indicates $p < 0.01$ and **c** indicates $p < 0.001$; **a** indicates $p < 0.01$ versus CAY10580. **(B)** UMR106.01 cells were cultured with 50 nM of scrambled or *Ptger2* siRNA for 48 hours, after which *Ptger2* expression was examined by qPCR. n = 4 samples. Compared to vehicle control, **a** indicates $p < 0.05$. **(C)** UMR106.01 cells were cultured with 50 nM of scrambled or *Ptger4* siRNA for 48 hours, after which *Ptger4* expression was examined by qPCR. n = 4 samples. Compared to vehicle control, **a** indicates $p < 0.05$. **(D)** UMR106.01 cells were cultured with 50 nM of scrambled, *Ptger2*, or *Ptger4* siRNA for 48 hours, then with 5 µM PGE$_2$ for 3 hours, after which time total RNA was collected and analyzed for *Sost* and *Rpl32*. n = 5 samples. Compared to vehicle control, **b** indicates $p < 0.01$.
doi:10.1371/journal.pone.0017772.g004

The requirement for cAMP/PKA to decrease *Sost* thus implicated either EP2 or EP4 receptor. Butaprost, a selective agonist for EP2 [67], decreased *Sost* transcription by 70%, whereas CAY10580, an EP4 agonist [54], had no significant effect upon *Sost* levels. The EP2 receptor was further implicated in mediating the suppressive effects of PGE$_2$, as siRNA directed against *Ptger2*, but not scrambled or *Ptger4* siRNA, prevented PGE$_2$-induced decreases in *Sost*. In total, these data indicate that PGE$_2$ signals through EP2 to decrease *Sost* expression.

PGE$_2$ and PTH decrease Sost through parallel pathways

PTH has also been shown to increase COX-2 expression and PG release [56,57]. Because PTH and PGE$_2$ are both capable of mobilizing the same second messengers (cAMP and IP$_3$), and because both PTH and PGE$_2$ decreased *Sost* transcription through similar mechanisms involving cAMP and MEF2, we examined whether PTH required PGE$_2$ (or other PGs) in order to decrease *Sost*. Inhibition of COX-1 and COX-2 function with 1 µM indomethacin increased *Sost* expression (*data not shown*), indicating tonic suppression of *Sost* by endogenously-produced prostaglandins. Cells treated with both PTH and indomethacin continued to demonstrate suppression of *Sost*, indicating that PTH does not require prostaglandins to decrease *Sost* levels in mature osteoblastic cells.

There are several distinctions that must be made regarding *Sost* regulation by PTH and PGE$_2$. Keller and Kneissel [43] demonstrated that PTH rapidly decreases *Sost* expression in UMR106.01 cells through a cyclic AMP-dependent pathway. Leupin *et al.* identified the MEF2 family of transcription factors as a requirement for driving bone-specific *Sost* expression, and as a target of PTH [45]. Within, we demonstrate that PGE$_2$, like PTH, decreases *Sost* in a cyclic AMP-dependent pathway. While PTH decreases *Sost* expression through unknown interactions with MEF2C and MEF2D, we continued to observe *Sost* suppression in cells transfected with *Mef2c* or *Mef2d* siRNA, demonstrating one key difference between PGE$_2$ and PTH. Similarly, inhibition of *de novo* protein synthesis with cycloheximide maintains PTH suppression of *Sost* [43], whereas cycloheximide prevented PGE$_2$ reductions in *Sost*. These data indicate that, while both PGE$_2$ and PTH use cAMP to decrease *Sost*, there is divergence downstream from cAMP/PKA in the signaling pathways utilized by PTH or PGE$_2$ for *Sost* suppression.

Current FDA-approved therapies for combating osteoporosis are limited to drugs, like bisphosphonates, that inhibit bone resorption. Intermittent PTH is the only FDA-approved therapy that promotes bone formation, although treatment is currently limited to 18 months. The sclerosing bone dysplasias sclerosteosis and van Buchem disease are caused by decreased or absent
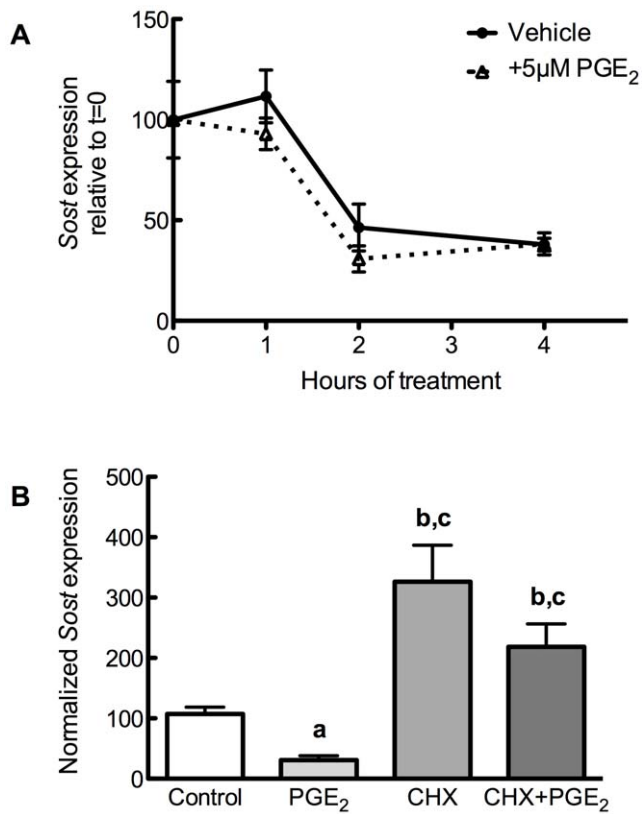
Figure 5. Effects of actinomycin D or cycloheximide upon PGE$_2$ suppression of Sclerostin. (**A**) UMR106.01 cells were serum-starved for 1 hour, treated with 2.5 µg/mL actinomycin D with or without 5 µM PGE$_2$, and collected 3 hours later. cDNA was prepared for qPCR analysis of *Sost* and *Rpl32*. n = 4 samples. (**B**) UMR 106.01 cells were treated with combinations of 10 µg/mL cycloheximide and 5 µM PGE$_2$ for 3 hours. Samples were analyzed by qPCR for *Sost* and *Rpl32*. n = 4 samples. Compared to vehicle control, **a** indicates $p<0.05$ and **b** indicates $p<0.001$; compared to 5 µM PGE$_2$, **c** indicates $p<0.001$.
doi:10.1371/journal.pone.0017772.g005

Sclerostin expression, and thereby implicate Sclerostin as a very potent inhibitor of bone formation. Thus, mechanisms for manipulating *Sost* expression may likely provide a powerful means of increasing bone mass. Within, we have elucidated a novel mechanism of Sclerostin regulation. Continued efforts to modulate its expression and/or activity will likely allow for novel anabolic agents for conditions of bone loss.

Materials and Methods

Cell culture- UMR106.01 cells, which express phenotypic markers of mature osteoblasts [68], were cultured in MEM with Earle's Salts (Invitrogen) supplemented with 10% fetal bovine serum (FBS; Invitrogen) and 1% penicillin and streptomycin (P&S; Invitrogen). Cells were routinely sub-cultured, using 0.05% trypsin/EDTA when 80–90% confluent; for experiments, cells were seeded into 35 mm^2 dishes at 5 k/cm^2, and experiments were performed two days thereafter.

Chemicals and reagents- PGE$_2$ and PGF$_{2\alpha}$ (Cayman Chemical) were dissolved in DMSO as stock concentrations of 10 mM. Human PTH(1–34) (Bachem) was dissolved in HBSS+0.1% BSA and stored at 100 µM aliquots. H-89 or 8-br-cAMP (EMD Biosciences) were dissolved in sterile water; U73122, ionomycin (both EMD Biosciences), butaprost, or CAY10580 (both Cayman Chemical) were dissolved in DMSO.
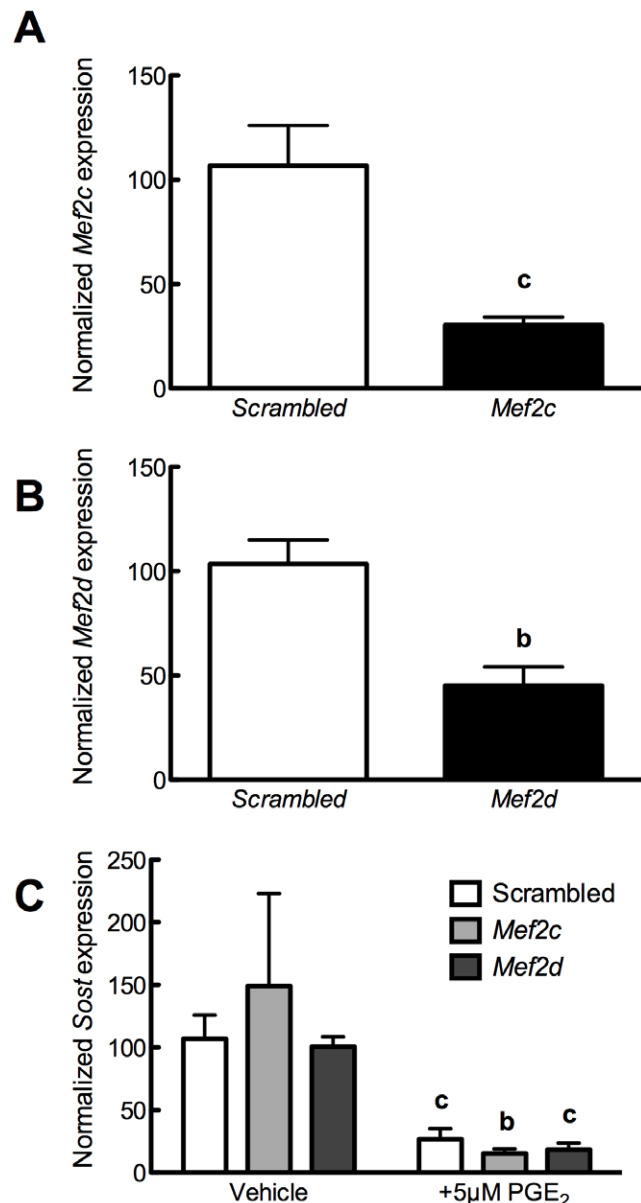

Figure 6. Reductions in MEF2 expression do not impair PGE$_2$ decrease of *Sost*. (**A**) UMR106.01 cells were cultured with 50 nM of scrambled or *Ptger4* siRNA for 48 hours, after which *Mef2c* expression was examined by qPCR. n = 4 samples. Compared to scrambled control, **c** indicates $p<0.001$. (**B**) UMR106.01 cells were cultured with 50 nM of scrambled or *Ptger4* siRNA for 48 hours, after which *Mef2d* expression was examined by qPCR. n = 4 samples. Compared to scrambled control, **c** indicates $p<0.001$. (**C**) UMR106.01 cells were cultured with 50 nM of scrambled, *Mef2c*, or *Mef2d* siRNA for 48 hours, then with 5 µM PGE$_2$ for 3 hours, after which time total RNA was collected and analyzed for *Sost* and *Rpl32*. n = 5 samples. Compared to target siRNA control, **b** indicates $p<0.01$ and **c** indicates $p<0.001$.
doi:10.1371/journal.pone.0017772.g006

Quantitative PCR- At the indicated time, cells were washed with PBS and total RNA was collected using RNeasy Mini kit (Qiagen). Total RNA (200–1000 ng) was reverse-transcribed with QuantiTect Reverse Transcription Kit (Qiagen), which includes a genomic DNA elimination step. qPCR was performed using QuantiFast Probe PCR Kit (Qiagen) on a Mastercycler® realplex2 (Eppendorf). Proprietary primer and TaqMan probe sets were purchased from Applied Biosystems. Amplification conditions
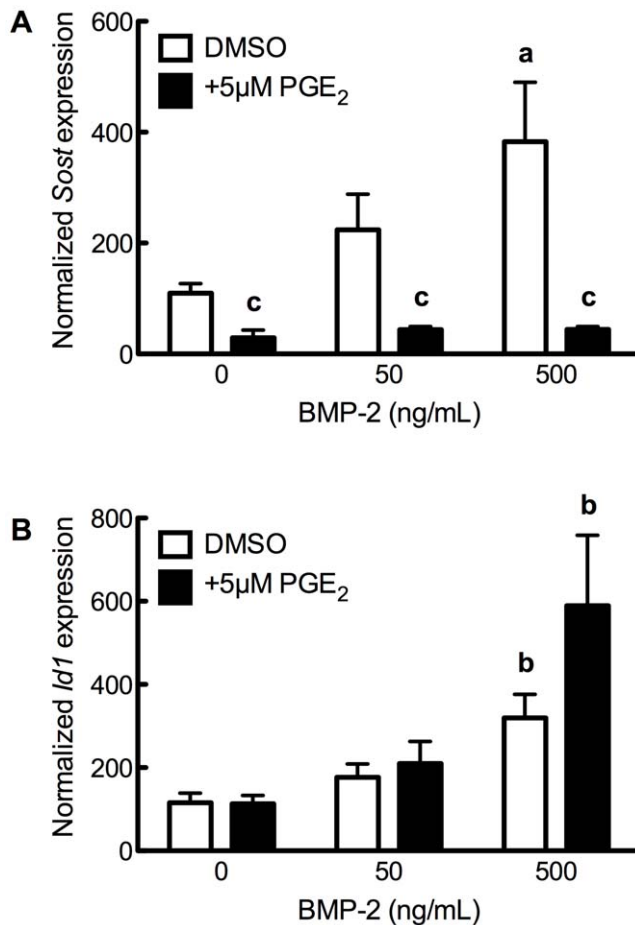
Figure 8. PTH decreases *Sost* expression independent of prostaglandins. Cells were exposed to 1 µM indomethacin for 24 hours, then treated with 100 nM hPTH(1–34) or vehicle control for 24 further hours. *Sost* expression was analyzed by qPCR and normalized to *Rpl32*. Compared to vehicle or indomethacin control, **a** indicates $p<0.05$.
doi:10.1371/journal.pone.0017772.g008

Figure 7. PGE$_2$ decreases *Sost* without affecting BMP signaling. (**A**) Cells were treated with BMP-2 (0–500 ng/mL) in the presence or absence of 5 µM PGE$_2$ for 3 hours, after which (**A**) *Sost* or (**B**) *Id1* expression was monitored. Compared to vehicle control, **a** indicates $p<0.05$ and **b** indicates $p<0.01$; compared to BMP-2 without PGE$_2$; , **c** indicates $p<0.001$.
doi:10.1371/journal.pone.0017772.g007

were 95°C for 3 minutes, followed by 40 cycles at 95°C for 3 seconds and 60°C for 30 seconds. Quantitative PCR results were normalized to loading control (*Rpl32* or *Tbp*) transcript level to yield ΔC_t, then normalized to control conditions to generate $\Delta\Delta C_t$. Relative or fold change in expression was subsequently calculated using the formula $2^{-\Delta Ct}$ or $2^{-\Delta\Delta Ct}$, as described in [69].

Dkk1 ELISA- For measurement of Dkk1 protein production, cells were prepared as described above and cultured for 24 hours in 0.05% DMSO or 5 µM PGE$_2$. Conditioned media and whole cell protein lysates were collected and frozen at −20 C until analysis. Dkk1 protein levels in conditioned media were analyzed using a commercially available ELISA against murine Dkk1 (R & D Systems), and results were normalized to whole cell protein concentration.

siRNA- small, interfering RNA against *Ptger2*, *Ptger4*, *Mef2c*, and *Mef2d* was purchased from Qiagen, as was scrambled, non-silencing control. Cells were seeded at a density of 40,000 cells *per* well in a 24-well plate in media supplemented with 10% FBS and 1% P/S. 30 minutes thereafter, 50 nM scrambled, non-silencing or *Ptger2* was prepared with HiperFect (Qiagen) in 100 µL of serum- and antibiotic-free media. 20 minutes later, siRNA/HiperFect/media was overlayed on top of the cells, which were returned to the incubator. Experiments were performed 48 hours later.

Statistical analysis- Each data set was acquired a minimum of three times, in duplicate. qPCR data were first analyzed relative to the internal control *Rpl32*, then normalized to vehicle control, in order to minimize inter-experimental variation. Results are expressed as mean±standard error of the mean. Data were analyzed by Kruskal–Wallis or ANOVA followed by Dunnet or Tukey post-hoc tests where appropriate. $p<0.05$ was considered statistically significant.

Author Contributions

Conceived and designed the experiments: DCG CEY GGL. Performed the experiments: DCG. Analyzed the data: DCG GGL. Contributed reagents/materials/analysis tools: DCG CEY GGL. Wrote the paper: DCG CEY GGL.

References

1. Feyen JH, Di Bon A, van der Plas A, Lowik CW, Nijweide PJ (1985) Effects of exogenous prostanoids on the proliferation of osteoblast-like cells in vitro. Prostaglandins 30: 827–840.

2. Hakeda Y, Yoshino T, Natakani Y, Kurihara N, Maeda N, et al. (1986) Prostaglandin E2 stimulates DNA synthesis by a cyclic AMP-independent pathway in osteoblastic clone MC3T3-E1 cells. J Cell Physiol 128: 155–161.

3. Minamizaki T, Yoshiko Y, Kozai K, Aubin JE, Maeda N (2009) EP2 and EP4 receptors differentially mediate MAPK pathways underlying anabolic actions of prostaglandin E2 on bone formation in rat calvaria cell cultures. Bone 44: 1177–1185.

4. Hakeda Y, Nakatani Y, Hiramatsu M, Kurihara N, Tsunoi M, et al. (1985) Inductive effects of prostaglandins on alkaline phosphatase in osteoblastic cells, clone MC3T3-E1. J Biochem 97: 97–104.
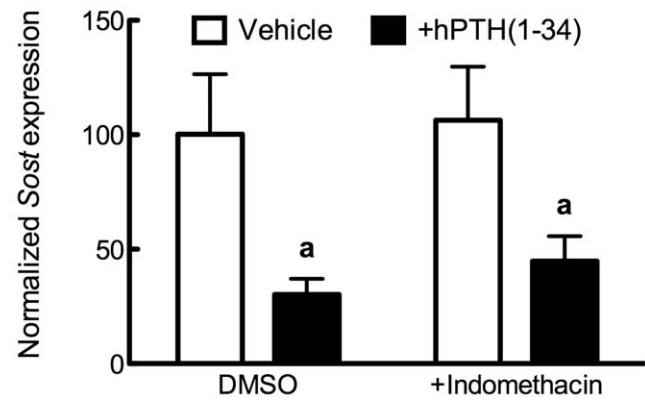
5. Flanagan AM, Chambers TJ (1992) Stimulation of bone nodule formation in vitro by prostaglandins E1 and E2. Endocrinology 130: 443–448.

6. Klein-Nulend J, Burger EH, Semeins CM, Raisz LG, Pilbeam CC (1997) Pulsating fluid flow stimulates prostaglandin release and inducible G/H synthase mRNA expression in primary mouse bone cells. J Bone Miner Res 12: 45–51.

7. Chen NX, Ryder KD, Pavalko FM, Turner CH, Burr DB, et al. (2000) Calcium regulates fluid shear-induced cytoskeletal reorganization and gene expression in osteoblasts. Am J Physiol 278: C989–997.

8. Wadhwa S, Godwin SL, Peterson DR, Epstein MA, Raisz LG, et al. (2002) Fluid flow induction of cyclo-oxygenase 2 gene expression in osteoblasts is dependent on an extracellular signal-regulated kinase signaling pathway. J Bone Miner Res 17: 266–274.

9. Pead MJ, Lanyon LE (1989) Indomethacin modulation of load-related stimulation of new bone formation in vivo. Calcif Tissue Int 45: 34–40.

10. Forwood MR (1996) Inducible cyclo-oxygenase (COX-2) mediates the induction of bone formation by mechanical loading in vivo. J Bone Miner Res 11: 1688–1693.

11. Jee WS, Ma YF (1997) The in vivo anabolic actions of prostaglandins in bone. Bone 21: 297–304.

12. Gerstenfeld LC, Einhorn TA (2004) COX inhibitors and their effects on bone healing. Expert Opin Drug Saf 3: 131–136.

13. Simon AM, Manigrasso MB, O'Connor JP (2002) Cyclo-oxygenase 2 function is essential for bone fracture healing. J Bone Miner Res 17: 963–976.

14. Giannoudis PV, MacDonald DA, Matthews SJ, Smith RM, Furlong AJ, et al. (2000) Nonunion of the femoral diaphysis. The influence of reaming and non-steroidal anti-inflammatory drugs. J Bone Joint Surg Br 82: 655–658.

15. Burd TA, Hughes MS, Anglen JO (2003) Heterotopic ossification prophylaxis with indomethacin increases the risk of long-bone nonunion. J Bone Joint Surg Br 85: 700–705.

16. Li M, Ke HZ, Qi H, Healy DR, Li Y, et al. (2003) A novel, non-prostanoid EP2 receptor-selective prostaglandin E2 agonist stimulates local bone formation and enhances fracture healing. J Bone Miner Res 18: 2033–2042.

17. Paralkar VM, Borovecki F, Ke HZ, Cameron KO, Lefker B, et al. (2003) An EP2 receptor-selective prostaglandin E2 agonist induces bone healing. Proc Natl Acad Sci U S A 100: 6736–6740.

18. Axelrad TW, Kakar S, Einhorn TA (2007) New technologies for the enhancement of skeletal repair. Injury 38 Suppl 1: S49–62.

19. Cameron KO, Lefker BA, Ke HZ, Li M, Zawistoski MP, et al. (2009) Discovery of CP-533536: an EP2 receptor selective prostaglandin E2 (PGE2) agonist that induces local bone formation. Bioorg Med Chem Lett 19: 2075–2078.

20. Bodine PV, Komm BS (2006) Wnt signaling and osteoblastogenesis. Rev Endocr Metab Disord 7: 33–39.

21. Akiyama T (2000) Wnt/beta-catenin signaling. Cytokine Growth Factor Rev 11: 273–282.

22. Nusse R (1999) WNT targets. Repression and activation. Trends Genet 15: 1–3.

23. Gong Y, Slee RB, Fukai N, Rawadi G, Roman-Roman S, et al. (2001) LDL receptor-related protein 5 (LRP5) affects bone accrual and eye development. Cell 107: 513–523.

24. Little RD, Carulli JP, Del Mastro RG, Dupuis J, Osborne M, et al. (2002) A mutation in the LDL receptor-related protein 5 gene results in the autosomal dominant high-bone-mass trait. Am J Hum Genet 70: 11–19.

25. Kato M, Patel MS, Levasseur R, Lobov I, Chang BH, et al. (2002) Cbfa1-independent decrease in osteoblast proliferation, osteopenia, and persistent embryonic eye vascularization in mice deficient in Lrp5, a Wnt coreceptor. J Cell Biol 157: 303–314.

26. Holmen SL, Giambernardi TA, Zylstra CR, Buckner-Berghuis BD, Resau JH, et al. (2004) Decreased BMD and limb deformities in mice carrying mutations in both Lrp5 and Lrp6. J Bone Miner Res 19: 2033–2040.

27. Sawakami K, Robling AG, Ai M, Pitner ND, Liu D, et al. (2006) The Wnt Co-receptor LRP5 is essential for skeletal mechanotransduction but not for the anabolic bone response to parathyroid hormone treatment. J Biol Chem 281: 23698–23711.

28. Bodine PV, Zhao W, Kharode YP, Bex FJ, Lambert AJ, et al. (2004) The Wnt antagonist secreted frizzled-related protein-1 is a negative regulator of trabecular bone formation in adult mice. Mol Endocrinol 18: 1222–1237.

29. Bodine PV, Billiard J, Moran RA, Ponce-de-Leon H, McLarney S, et al. (2005) The Wnt antagonist secreted frizzled-related protein-1 controls osteoblast and osteocyte apoptosis. J Cell Biochem 96: 1212–1230.

30. Balemans W, Van Den Ende J, Freire Paes-Alves A, Dikkers FG, Willems PJ, et al. (1999) Localization of the gene for sclerosteosis to the van Buchem disease-gene region on chromosome 17q12-q21. Am J Hum Genet 64: 1661–1669.

31. Balemans W, Ebeling M, Patel N, Van Hul E, Olson P, et al. (2001) Increased bone density in sclerosteosis is due to the deficiency of a novel secreted protein (SOST). Hum Mol Genet 10: 537–543.

32. Brunkow ME, Gardner JC, Van Ness J, Paeper BW, Kovacevich BR, et al. (2001) Bone dysplasia sclerosteosis results from loss of the SOST gene product, a novel cystine knot-containing protein. Am J Hum Genet 68: 577–589.

33. Li X, Ominsky MS, Niu QT, Sun N, Daugherty B, et al. (2008) Targeted deletion of the sclerostin gene in mice results in increased bone formation and bone strength. J Bone Miner Res 23: 860–869.

34. Winkler DG, Sutherland MK, Geoghegan JC, Yu C, Hayes T, et al. (2003) Osteocyte control of bone formation via sclerostin, a novel BMP antagonist. Embo J 22: 6267–6276.

35. Loots GG, Kneissel M, Keller H, Baptist M, Chang J, et al. (2005) Genomic deletion of a long-range bone enhancer misregulates sclerostin in Van Buchem disease. Genome Res 15: 928–935.

36. Collette NM, Genetos DC, Murugesh D, Harland RM, Loots GG (2010) Genetic evidence that SOST inhibits WNT signaling in the limb. Dev Biol 342: 169–179.

37. Kusu N, Laurikkala J, Imanishi M, Usui H, Konishi M, et al. (2003) Sclerostin is a novel secreted osteoclast-derived bone morphogenetic protein antagonist with unique ligand specificity. J Biol Chem 278: 24113–24117.

38. Li X, Zhang Y, Kang H, Liu W, Liu P, et al. (2005) Sclerostin binds to LRP5/6 and antagonizes canonical Wnt signaling. J Biol Chem 280: 19883–19887.

39. Semenov M, Tamai K, He X (2005) SOST is a ligand for LRP5/LRP6 and a Wnt signaling inhibitor. J Biol Chem 280: 26770–26775.

40. Ellies DL, Viviano B, McCarthy J, Rey JP, Itasaki N, et al. (2006) Bone density ligand, Sclerostin, directly interacts with LRP5 but not LRP5G171V to modulate Wnt activity. J Bone Miner Res 21: 1738–1749.

41. Semenov MV, He X (2006) LRP5 mutations linked to high bone mass diseases cause reduced LRP5 binding and inhibition by SOST. J Biol Chem 281: 38276–38284.

42. Morvan F, Boulukos K, Clement-Lacroix P, Roman Roman S, Suc-Royer I, et al. (2006) Deletion of a single allele of the Dkk1 gene leads to an increase in bone formation and bone mass. J Bone Miner Res 21: 934–945.

43. Keller H, Kneissel M (2005) SOST is a target gene for PTH in bone. Bone 37: 148–158.

44. Bellido T, Ali AA, Gubrij I, Plotkin LI, Fu Q, et al. (2005) Chronic elevation of parathyroid hormone in mice reduces expression of sclerostin by osteocytes: a novel mechanism for hormonal control of osteoblastogenesis. Endocrinology 146: 4577–4583.

45. Leupin O, Kramer I, Collette NM, Loots GG, Natt F, et al. (2007) Control of the SOST bone enhancer by PTH using MEF2 transcription factors. J Bone Miner Res 22: 1957–1967.

46. Chyun YS, Raisz LG (1984) Stimulation of bone formation by prostaglandin E2. Prostaglandins 27: 97–103.

47. Jorgensen HR, Svanholm H, Host A (1988) Bone formation induced in an infant by systemic prostaglandin-E2 administration. Acta Orthop Scand 59: 464–466.

48. Holmen SL, Zylstra CR, Mukherjee A, Sigler RE, Faugere MC, et al. (2005) Essential role of beta-catenin in postnatal bone acquisition. J Biol Chem 280: 21162–21168.

49. Poole KE, van Bezooijen RL, Loveridge N, Hamersma H, Papapoulos SE, et al. (2005) Sclerostin is a delayed secreted product of osteocytes that inhibits bone formation. Faseb J 19: 1842–1844.

50. Papanicolaou SE, Phipps RJ, Fyhrie DP, Genetos DC (2009) Modulation of sclerostin expression by mechanical loading and bone morphogenetic proteins in osteogenic cells. Biorheology 46: 389–399.

51. Genetos DC, Toupadakis CA, Raheja LF, Wong A, Papanicolaou SE, et al. (2010) Hypoxia decreases sclerostin expression and increases Wnt signaling in osteoblasts. J Cell Biochem 110: 457–467.

52. Sugimoto Y, Narumiya S (2007) Prostaglandin E receptors. J Biol Chem 282: 11613–11617.

53. Kiriyama M, Ushikubi F, Kobayashi T, Hirata M, Sugimoto Y, et al. (1997) Ligand binding specificities of the eight types and subtypes of the mouse prostanoid receptors expressed in Chinese hamster ovary cells. Br J Pharmacol 122: 217–224.

54. Billot X, Chateauneuf A, Chauret N, Denis D, Greig G, et al. (2003) Discovery of a potent and selective agonist of the prostaglandin EP4 receptor. Bioorg Med Chem Lett 13: 1129–1132.

55. Kamiya N, Ye L, Kobayashi T, Mochida Y, Yamauchi M, et al. (2008) BMP signaling negatively regulates bone mass through sclerostin by inhibiting the canonical Wnt pathway. Development 135: 3801–3811.

56. Kawaguchi H, Raisz LG, Voznesensky OS, Alander CB, Hakeda Y, et al. (1994) Regulation of the two Prostaglandin G/H Synthases by parathyroid hormone, interleukin-1, cortisol, and prostaglandin E2 in cultured neonatal mouse calvariae. Endocrinology 135: 1157–1164.

57. Maciel FM, Sarrazin P, Morisset S, Lora M, Patry C, et al. (1997) Induction of cyclooxygenase-2 by parathyroid hormone in human osteoblasts in culture. J Rheumatol 24: 2429–2435.

58. Li X, Ominsky MS, Warmington KS, Morony S, Gong J, et al. (2009) Sclerostin antibody treatment increases bone formation, bone mass, and bone strength in a rat model of postmenopausal osteoporosis. J Bone Miner Res 24: 578–588.

59. Li X, Warmington KS, Niu QT, Asuncion FJ, Barrero M, et al. (2010) Inhibition of sclerostin by monoclonal antibody increases bone formation, bone mass and bone strength in aged male rats. J Bone Miner Res.

60. O'Brien CA, Plotkin LI, Galli C, Goellner JJ, Gortazar AR, et al. (2008) Control of bone mass and remodeling by PTH receptor signaling in osteocytes. PLoS ONE 3: e2942.

61. Suda M, Tanaka K, Natsui K, Usui T, Tanaka I, et al. (1996) Prostaglandin E receptor subtypes in mouse osteoblastic cell line. Endocrinology 137: 1698–1705.

62. Lee CM, Genetos DC, You Z, Yellowley CE (2007) Hypoxia regulates PGE(2) release and EP1 receptor expression in osteoblastic cells. J Cell Physiol 212: 182–188.

63. Blackwell KA, Raisz LG, Pilbeam CC (2010) Prostaglandins in bone: bad cop, good cop? Trends Endocrinol Metab 21: 294–301.

64. Wu H, Naya FJ, McKinsey TA, Mercer B, Shelton JM, et al. (2000) MEF2 responds to multiple calcium-regulated signals in the control of skeletal muscle fiber type. EMBO J 19: 1963–1973.

65. Blaeser F, Ho N, Prywes R, Chatila TA (2000) Ca(2+)-dependent gene expression mediated by MEF2 transcription factors. J Biol Chem 275: 197–209.

66. Hamamura K, Liu Y, Yokota H (2008) Microarray analysis of thapsigargin-induced stress to the endoplasmic reticulum of mouse osteoblasts. J Bone Miner Metab 26: 231–240.

67. Gardiner PJ (1986) Characterization of prostanoid relaxant/inhibitory receptors (psi) using a highly selective agonist, TR4979. Br J Pharmacol 87: 45–56.

68. Partridge NC, Alcorn D, Michelangeli VP, Ryan G, Martin TJ (1983) Morphological and biochemical characterization of four clonal osteogenic sarcoma cell lines of rat origin. Cancer Res 43: 4308–4314.

69. Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25: 402–408.