Published in final edited form as:
Bone. 2012 March ; 50(3): 663–669. doi:10.1016/j.bone.2011.11.016.

TGF-β Regulates Sclerostin Expression *via* the ECR5 enhancer

Gabriela G. Loots[1,2,*], **Hansjoerg Keller**[3,*], **Olivier Leupin**[3,*], **Deepa Murugesh**[1], **Nicole M. Collette**[1], and **Damian C. Genetos**[4]

[1]Biology and Biotechnology Division, Lawrence Livermore National Laboratory, Livermore, CA [2]School of Natural Sciences, University of California, Merced, CA, USA [3]Novartis Institute for BioMedical Research, Basel, Switzerland [4]Department of Anatomy, Physiology, and Cell Biology, School of Veterinary Medicine, University of California, Davis, CA, USA

Abstract

Wnt signaling is critical for skeletal development and homeostasis. Sclerostin (*Sost*) has emerged as a potent inhibitor of Wnt signaling and, thereby, bone formation. Thus, strategies to reduce Sclerostin expression may be used to treat osteoporosis or non-union fractures. Transforming growth factor-beta (TGF-β) elicits various effects upon the skeleton both *in vitro* and *in vivo* depending on the duration and timing of administration. *In vitro* and *in vivo* studies demonstrate that TGF-β increases osteoprogenitor differentiation but decreases matrix mineralization of committed osteoblasts. Because Sclerostin decreases matrix mineralization, this study aimed to examine whether TGF-β achieves such inhibitory effects *via* transcriptional modulation of *Sost*. Using the UMR106.01 mature osteoblast cell line, we demonstrated that TGF-β TGF-β_1, -β_2, -β_3 and Activin A increase *Sost* transcript expression. Pharmacologic inhibition of Alk4/5/7 *in vitro* and *in vivo* decreased endogenous *Sost* expression, and siRNA against *Alk4* and *Alk5* demonstrated their requirement for endogenous *Sost* expression. TGF-β_1 targeted the *Sost* bone enhancer ECR5 and did not affect the transcriptional activity of the endogenous *Sost* promoter. These results indicate that TGF-β_1 controls *Sost* transcription in mature osteoblasts, suggesting that Sclerostin may mediate the inhibitory effect of TGF-β upon osteoblast differentiation.

Keywords

Transforming growth factor-beta; Sost; ECR5; Osteoblast; Bone; Wnt

Introduction

Since the discovery in the past decade that the Wnt glycoprotein co-receptor *Lrp5* regulates bone mass [1, 2], tremendous efforts have attempted to elucidate the mechanisms involved. Wnts are ligands for Lrp4, 5, and 6, and a subset of Wnts increase the osteogenic commitment of bone marrow stem cells, enhance matrix formation, and decrease apoptosis of osteoblasts and osteocytes [3, 4]. Regulation of Wnt signaling occurs *via* secreted decoy

Corresponding Author: Damian C. Genetos, Department of Anatomy, Physiology and Cell Biology, School of Veterinary Medicine, 1321 Haring Hall, University of California Davis, Davis CA 95616, Tel: 530-754-0146, Fax: 530-754-0150, dgenetos@ucdavis.edu.
*Equal contribution

receptors (secreted frizzled-related protein [*sFRP*]) or antagonists (Sclerostin [*SOST*], Dickkopf [*DKK*]) that bind to Lrp4 - 6 to prevent Wnt-Lrp interactions, and subsequent signal transduction [5, 6]. As activating mutations in *LRP4-6* promote high bone mass (HBM) phenotypes [1, 2, 7, 8], complementary phenotypes emerge from deletion of Lrp4/5/6 antagonists [9–15]: deletion of *sFRP*s increases trabecular bone [15] and bone mineral density [14], and deletion of *DKK* isoforms or *SOST* increases markers of bone formation and bone mass [9, 10].

The influence of *SOST* on skeletal formation and function is phenotypically observed *via* loss of Sclerostin protein, which is achieved by two distinct genetic mechanisms. One set of mutations occur within the *SOST* transcript and comprise either nonsense mutations in exon 2 or aberrant splice sites resulting in null alleles [10]. These mutations cause sclerosteosis in humans (MIM 269500), which is characterized by generalized cortical hyperostosis accompanied by occasional syndactyly of the digits [10]. A highly similar bone mineral density phenotype is observed in van Buchem disease patients (MIM 239100) who also have severe skeletal hyperplasia, but carry no mutations in the *SOST* gene. Instead, van Buchem results from the deletion of a 52kb non-coding region (also refered to as van Buchem deletion region) that is 35kb downstream of *SOST* [11]; this van Buchem deletion region functions in *cis* to enhance *SOST* transcription in bone. We have previously demonstrated that an evolutionarily-conserved region present within the van Buchem deletion region, termed ECR5, is sufficient to drive reporter assays in bone cells, *in vitro* and *in vivo* [13], and confers responsiveness to parathyroid hormone (PTH) [16].

The TGF-β superfamily is composed of more than 40 structurally and functionally related cytokines that regulate a variety of biological processes including morphogenesis, proliferation, stem cell differentiation, apoptosis, and epithelial-to-mesenchymal transition [17]. The superfamily clusters into the subfamilies TGF-β, bone morphogenetic proteins (BMPs), growth and differentiation factors (GDFs), activins and inhibins, and Mullerian inhibiting factor (MIF) [18]. The TGF-β subfamily contains three distinct proteins—TGF-β_1, -β_2, and -β_3— which exert pleiotropic effects upon cells responsible for maintaining or altering skeletal architecture. Indeed, the TGF-β subfamily demonstrates chemotactic effects on osteoprogenitors during endochondral condensation [19], promotes proliferation and differentiation of early osteoprogenitors, yet it also decreases matrix formation in fully-differentiated osteoblasts (reviewed in Janssens *et al.* [20] and Bonewald *et al.* [21], amongst others). TGF-$\beta_{1–3}$ can interact with osteotropic factors like PTH [22] or prostaglandin E_2 [23] to enhance bone formation. Conversely, factors like BMPs [24–26], PTH [16, 27, 28], and prostaglandin E_2 [29] regulate Wnt signaling *via* manipulation of Wnt or Lrp5/6 antagonist expression. BMP signaling through BMPR1A increases *Sost* expression and decreases Wnt signaling [30], but the influence of other TGF-β superfamily members on Sclerostin expression has not yet been explored. Provided the evidence for a biphasic influence of TGF-β upon osteogenic differentiation, we hypothesized that TGF-β increases *Sost* transcription in mature osteoblasts, and sought the intracellular mechanisms involved.

Materials and methods

Cell culture

UMR106.01 cells were kindly provided by Dr. Alexander Robling (Indiana University School of Medicine). Cells were cultured in MEM with Earle's Salts (Invitrogen), which was supplemented with 10% fetal bovine serum (FBS; Invitrogen) and 1% penicillin-streptomycin (Invitrogen). Cells were maintained in a standard humidified incubator at 37°C/95% air / 5% CO_2, and were routinely sub-cultured with 0.05% trypsin when 75–90% confluent. Unless otherwise indicated, cells were seeded for experiments at 5k/cm^2, and growth factor supplements were added two days later.

Reagents

TGF-β_1, TGF-β_2, TGF- β_3, and Activin A (R&D Systems) and human parathyroid hormone(1–34) (hPTH(1–34); Bachem) were dissolved in 0.1% BSA in PBS and stored at −20°C. Cycloheximide was purchased from Sigma; SIS3 and SB431542 were from Calbiochem. Purified RNA from adult murine calvariae or femora (Zyagen), used to examine *Alk4/5/7* expression were purchased from Zyagen.

Quantitative PCR (qPCR)

At indicated time points, cell culture samples were washed with PBS, collected in RLT buffer with 2-mercaptoethanol (Qiagen), from which RNA was purified using RNeasy Kit (Qiagen). RNA purity was tested by measuring the absorbance at 260 and 280nm. One microgram of RNA was reverse-transcribed with QuantiTect Reverse Transcription kit (Qiagen), which includes genomic DNA elimination. qPCR was performed using primers listed in Table 1 and either QuantiFast Probe PCR Kit (Qiagen) or QuantiFast SYBR Green PCR Kit (Qiagen). Cycling conditions were 95°C for 3 minutes (5 minutes for SYBR), followed by 40 cycles of 95°C for 3 seconds (10 seconds for SYBR) and 30 seconds at 60°C. qPCR results were calculated relative to internal control (*Rpl32* or *Tbp*; $2^{-\Delta Ct}$) with the exception of Figure 3A and 3B, results were further normalized to control, time-matched conditions ($2^{-\Delta\Delta Ct}$) [31].

siRNA transfection

siRNA (Qiagen) were designed against murine *Sost*, *Gapdh*, *Alk4*, *Alk5*, and *Alk7*; specificity was confirmed with BLAST. Cells were seeded at a density of 6,000 cells *per* well in 48-well plates. One hour later, 10nM siRNA and Fugene 6 (Roche) were diluted into Opti-MEM (Invitrogen) and were gently added to the culture plate. Samples were collected 48 hours later and were processed for qPCR analysis of target knock-down.

TGF-βrI kinase inhibitor treatment *in vivo*

Eight-week old male C57BL/6 mice were treated for 24 hours with vehicle (HBSS), or SD-208 (60 mg/kg twice daily, one injection every 12 hours; Tocris Biosciences) by intraperitoneal delivery (IP). No adverse effects of SD-208 on mouse health were detected during the study. At 24 hours after the first injection animals were euthanized humanely. Femoral shafts were scraped of soft tissue and skeletal muscle, flushed with ice-cold HBSS with a 25 gauge needle to remove the bone marrow before placing into RNAlater (Qiagen) and stored at 4°C. For RNA isolation, samples were removed from RNAlater and homogenized in 1 mL Qiazol and purified using Rneasy mini-kit using manufacture's guidelines (Qiagen). Approvals for work conducted on the mice used in this study were granted by Lawrence Livermore National Laboratory Institutional Animal Care and Use Committee, under application no. 168. Animals were treated humanely; housing and experimental procedures followed the guidelines outlined in the National Institute of Health 'Principles of Laboratory Care'.

Reporter gene assays

pGL3-based reporter plasmids (Promega) containing ECR5 upstream of the human SOST or the SV40 promoter were previously described [13, 16]. A putative SMAD site was predicted within a multiple sequence alignment of human and mouse ECR5 sequences using power weight matrices available from TRANSFAC and utilized by MultiTF (http://multitf.dcode.org/). ECR5 was PCR cloned into the EcoR1 site of pGL3-promoter vector, as well as in a pGL3 vector where the SV40 promoter has been replaced by a 2kb fragment of the human sclerostin promoter. Subsequently the SMAD or MEF2 site was deleted using site-directed mutagenesis according to the manufacturer's instructions

(Quickchange Site-directed Mutagenesis kit; Stratagene). UMR106.01 cells were seeded at 20k/well into 48-well plates. On the following day, media was removed, replaced with Opti-MEM, and transfected with Fugene 6, the reporter of interest (250ng/well), and pRL-TK (50ng/well; Promega) as a transfectant control. 24 hour later, media was removed and replaced with TGF-β_1. Samples were analyzed 24 hours later using Dual-Luciferase Reporter System (Promega) and TD-20/20 luminometer (Turner Systems).

Statistical analysis

Each experiment was performed a minimum of 3 times, each time in duplicate or triplicate. Unless otherwise noted, data are presented as mean ± standard error of the mean. Statistical significance was assessed by two-tailed Student's *t* test or ANOVA for non-repeated measurements followed by a Dunnet *post-hoc* analysis compared to control (vehicle). $p < 0.05$ was considered statistically significant.

Results

TGF-β superfamily members modulate *Sost* expression in mature osteoblastic cells

A described mode of modulating Wnt signaling involves altering the expression levels of Lrp5/6 antagonists, as both deletion of *Dkk1* and *Sost* have been shown to promote aggressive bone overgrowth characterized by hyperactive osteoblast activity due to elevated Wnt signaling. We evaluated whether the expression of *Sost* was influenced by members of the TGF-β superfamily. UMR106.01 cells were cultured in the presence of 10ng/mL of TGF-β_1, -β_2, -β_3, Activin A, or Nodal for 6 hours; these growth factors were chosen because they differentially activate signaling through the Alk4, Alk5, or Alk7 TGF-β type I receptors. Alternately, cells were cultured with 100nM hPTH(1–34), which decreases *Sost* expression [27–29]. Each TGF-β isoform increased *Sost* expression nearly 3-fold compared to vehicle control (Figure 1A); Activin A was less effective than TGF-$\beta_{1–3}$, and Nodal did not affect *Sost* expression at the dose examined.

Dose- and time-dependent effects of TGF-β family members upon *Sost* expression

To investigate dose-dependence of *Sost* expression in response to TGF-β_1, -β_2, -β_3, and Activin A, UMR106.01 cells were cultured in the presence of increasing concentrations of growth factor (1–10,000 pg/mL) for 6 hours. TGF-β_1, -β_2, -β_3, effective at *Sost* induction (257 – 301% increase) at 10ng/mL, whereas Activin A was about half as effective (TABLE 2). There were differences in potency amongst the four proteins: TGF-β_1 and -β_3 demonstrated a similar EC_{50} for *Sost* induction (29 and 34pg/mL, respectively), whereas TGF-β_2 (186 pg/mL) and Activin A (1371 pg/mL) were less potent. These data indicate that TGF-β_1, -β_2, -β_3 and Activin A, members of the TGF-β superfamily, are capable of inducing *Sost* expression.

The temporal nature of TGF-β-induced *Sost* expression was examined by treating cells with 10ng/mL of each growth factor, after which RNA was collected 3, 6, or 24h later. TGF-β_1 exerted a rapid increase upon *Sost* expression after 3 hours of culture (Figure 1B); there was a trend ($p<.12$) for decreased *Sost* induction after 6 hours of culture, although expression remained significantly elevated after 6 or 24 hours of culture compared to vehicle control. TGF-β_2 exerted a gradual increase in *Sost* induction over the time course examined (*data not shown*). The effect of TGF-β_3 upon *Sost* expression was transient, as it was significantly elevated after 3 or 6 hours of treatment, but regressed toward baseline after 24 hours of treatment (Figure 1B). The effect of Activin A mimicked that of TGF-β_3 (*data not shown*).

Sost expression is inhibited by antagonists of Alk4/5/7 *in vitro* and *in vivo*

TGF-β and Activin A activate intracellular signaling cascades once bound to type I and type II receptors. TGF-β type I receptors include Alk4, Alk5, and Alk7. The addition of the Alk4/5/7 antagonist SB431542 to UMR 106.01 cells decreased endogenous *Sost* expression (Figure 2A). We next examined whether inhibition of Alk4/5/7 similarly influenced *Sost* expression, *in* vivo. Wild-type mice treated with a related Alk4/5/7 kinase inhibitor, SD-208 (60mg/kg), for 24 hours [32] exhibited a 63% decrease in *Sost* expression compared to PBS-treated controls (Figure 2B).

Alk4 and *Alk5* mediate induction of *Sost*

Expression of the three TGF-β type I receptors (*Alk4*, *Alk5*, and *Alk7*) was confirmed *in vitro* in UMR106.01 osteoblasts (Figure 3A) and *in vivo* from femur and calvariae RNA (Figure 3B). *Alk4* (receptor for Activin A) and *Alk5* (receptor for TGF-β_1, -β_2, -β_3) were consistently expressed at higher levels compared to *Alk7* (receptor for Nodal). siRNA directed against *Alk4*, *Alk5*, or *Alk7* decreased target gene expression by 72–77% relative to non-silencing RNA controls (Figure 3C). siRNA directed against *Alk4* and *Alk5* significantly decreased endogenous *Sost* production by 76 and 81%, respectively, while siRNA directed against *Alk7* only modestly influenced *Sost* levels (Figure 3D). These data indicate that *Sost* expression is in part regulated by *Alk5* and *Alk4* receptors, respectively, whereas in these experiments *Alk7* does not appear to play a significant role in the regulation of *Sost* expression.

TGF-β_1 induction of *Sost* involves Smad3 and is antagonized by PTH

Direct regulation of gene expression by TGF-β involves Smad2 or Smad3 transcription factors. SIS3 is a cell-permeant Smad3 inhibitor that reduces Smad3 phosphorylation and DNA binding, without influencing Smad2 [33]. The addition of 10μM SIS3 to cultures significantly reduced *Sost* expression in both vehicle and 10ng/mL TGF-β_1-treated cells (Figure 4A), indicating that both endogenous and growth factor-induced *Sost* expression is dependent on Smad3 phosphorylation and DNA binding.

We have previously demonstrated that PTH directly inhibits *Sost* expression without the need for *de novo* protein synthesis [28]; thus, we examined the capacity for PTH to interfere with TGF-β_1-induced *Sost* expression. Cells treated with both 100nM hPTH (1–34) and 10ng/mL TGF-β_1 for 6 hours revealed no significant increase in *Sost* expression relative to vehicle controls, suggesting that they regulate *Sost* expression independently (Figure 4B), and blunted each other's effect on *Sost* transcription.

TGF-β_1 targets the ECR5 region of the distal *Sost* enhancer

Regulation of *Sost* by TGF-β_1 could involve activation of the proximal promoter, distal enhancer, or a synergistic effect of both. We examined the responsiveness of Luciferase reporter constructs containing a minimal SV40 promoter, the human *SOST* promoter (h*SOST*), each with or without the ECR5 distal enhancer [13, 16]. Constructs containing either the minimal SV40 promoter or the h*SOST* promoter demonstrated no change in Luciferase expression when treated with 10ng/mL TGF-β_1, whereas constructs containing the ECR5 region increased the Luciferase activity by approximately 3-fold (Figure 5A), independent of promoter choice. There was no additive effect in plasmids containing ECR5 and h*SOST*, indicating that these transcriptional regulatory components do not synergize, and that the TGF-β_1 response is dependent on the distal enhancer, ECR5.

The ECR5 region contains a binding site for the myocyte enhancer family 2 (Mef2) family of transcription factors [34]. This sequence also contains a putative binding site for Smad2/3 [35], transcription factors activated by TGF-β_1. To test the role of the Mef2 and Smad

binding sites in TGF-β-driven Luciferase activity, reporter constructs were generated that contained mutated binding sites for Mef2 or Smads. Deletion of the Smad binding site prevented Luciferase expression in response to TGF-β_1 (Figure 5B); unexpectedly, deletion of the Mef2 binding site also prevented TGF-β_1-driven Luciferase activity.

Discussion

Genetic analyses of the rare sclerosing bone dysplasias sclerosteosis and van Buchem disease that either target the *SOST* transcript, or its distal *cis*-regulatory elements have positioned *SOST* as a robust regulator of skeletal homeostasis. Its role on mediating bone accrual and deposition was further confirmed through the generation of *Sost* [36] or van Buchem region [13] transgenic mice, which are osteopenic compared to wild-type control mice. Sclerostin was initially characterized as a BMP antagonist [36], but was later shown to function through Lrp4-6 [8, 37]. Functionally, a sclerostin-inhibiting antibody enhances bone mineral density and strength [38, 39]. Mechanistically, Sclerostin exerts anti-anabolic effects by decreasing osteoprogenitor proliferation and differentiation, and promoting osteoblast apoptosis [40]. Provided the increasing direct medical costs for osteoporosis-related or other traumatic fractures, it is critical to understand the mechanisms involved in *Sost* expression because of its function *in vivo* as an inhibitor of bone formation.

The TGF-β family represents an important superfamily involved in regulating bone metabolism since a significant number of its members are produced locally within the skeleton [41] where they accumulate within the matrix [42] and are released by osteoclasts during remodeling [43]. Germline deletion of individual TGF-β genes yields multiple, non-overlapping developmental defects [44]. Of the three TGF-β proteins, TGF-β_1 is the isoform most abundantly expressed in bone [45]. It has opposing effects on osteoprogenitors compared to osteoblasts, inducing the migration, proliferation and differentiation of the former, but inhibiting matrix accumulation of the latter [20]. Little is known as to how TGF-β elicits these differential effects, although Alliston *et al.* have demonstrated that TGF-β promotes the degradation of the transcription factor essential for osteoblast differentiation, Runx2 [46]. Later, Balooch *et al.* used a series of transgenic mouse models with alterations in TGF-β signaling to show elevated levels of TGF-β in bone decreased mechanical properties, mineral concentration, and fracture toughness [47]; these trends were reversed in mice expressing a dominant-negative type II TGF-β receptor. Further, Mohammed *et al.* showed that inhibition of Alk4/5/7 with SD-208 *in vivo* had catabolic and anabolic effects on the mouse skeleton [48]. These effects included: increased increased bone mineral density and trabecular bone volume independent of cortical bone changes; increased osteoprogenitor differentiation; and decreased osteoclast number. Functionally, Alk4/5/7 inhibition inceased both bone mechanical and material properties, suggesting that Alk4/5/7 inhibition could be used for conditions of skeletal fragility like osteoporosis. These findings combined with the data presented in this manuscript provide compelling evidence that signaling the the Alk4/5/7 axis positively influences *Sost* expression. We can further speculate that Sclerostin potentially plays an important role in the processes described by Balooch *et al.* and Mohammed *et al.* [48].

TGF-β interacts with a variety of signaling pathways implicated in skeletal homeostasis. For example, PTH increases TGF-β_1 synthesis and secretion and Smad3 phosphorylation to decrease osteoblast apoptosis [22]. TGF-β generally decreases the capacity for BMPs to induce osteogenic differentiation of osteoprogenitors, but de Gorter *et al.* recently demonstrated that this inhibitory effect is very much context dependent with respect to such details as the composition of the culture medium used and the duration of growth factor co-culture [49]. Similarly, TGF-β can regulate expression and activity of Wnt signaling. In mesenchymal stem cells, TGF-β_1 induces proliferation *via* Smad3-dependent β-catenin

nuclear translocation [50] and increases β-catenin expression [51]. Within, we demonstrate that TGF-β isoforms increase *Sost* expression in mature osteoblasts. Provided the inhibitory effect of Sclerostin upon matrix mineralization and differentiation [52], this may provide a mechanism to explain the similarly inhibitory effect of TGF-β_1 upon differentiation of mature osteoblasts, although further studies are required to fully confirm this.

Nonsense mutations in *Sost* cause sclerosteosis [9], and a 52kb deletion 35kb downstream of *Sost* is responsible for van Buchem disease [11]. Using cross-species sequence comparison and enhancer assays, we have previously identified a 255-bp evolutionarily conserved sequence within the van Buchem deletion, termed ECR5, that confers bone-specific expression of *SOST* [13]. Reporter assays containing the ECR5 enhancer, but not the proximal promoter, are responsive to PTH *via* Mef2 transcription factors [16]. Within this work, we demonstrate that, similar to PTH, TGF-β_1 targets the ECR5 enhancer but not the *SOST* promoter (Figure 5). Within the ECR5 enhancer, we identified consensus binding sites for Smad2/3, in addition to the previously identified Mef2 sites; surprisingly, both Smad2/3 and Mef2 sites contribute to TGF-β_1 transcriptional activation of *Sost*. We have not observed any changes in expression of *Mef2c* or *Mef2d* transcription in response to TGF-βs (data not shown), indicating that the regulation is likely post-transcriptional in nature. One candidate mechanism is direct physical interaction between Mef2 and Smad2. Indeed, Quinn *et al*. previously demonstrated direct interaction of Smad2 with Mef2 *in vivo* to enhance Mef2 transcriptional activity [53], as did Ishikawa *et al*. [54]. How TGF-β_1 regulates Mef2 transcriptional activity in osteoblastic cells is currently under investigation.

Similar to TGF-βs, we observed that Activin A, which signals through the Alk4 type I receptor, increases *Sost* expression, although with less potency and efficacy than TGF-β_1,-β_2, -β_3. Despite the decreased potency, siRNA directed against *Alk4* decreased endogenous *Sost* expression to a similar degree as did siRNA against *Alk5*. Similar to TGF-β, the role of Activin A in skeletal homeostasis is conflicting, as various reports demonstrate its capacity to increase or decrease osteoblast- and osteoclastogenesis [55, 56]. Pathologically, Activin A and TGF-β are implicated in the progression of osteolytic metastases. Serum Activin A is elevated in patients with multiple myeloma [57], and a soluble Activin receptor IIA fusion protein decreases bone metastasis and resorption [58]. Interestingly, Dkk1, a Wnt antagonist like Sclerostin, is highly expressed in osteolytic cancers such as multiple myeloma [59]; whether Wnt signaling in osteolytic cancers is dependent upon TGF-β is a possibility worthy of investigation.

In conclusion, we demonstrate that members of the TGF-β superfamily beyond BMPs are also capable of inducing *Sost* expression, as TGF-βs and Activin A demonstrated graded effects on *Sost* transcription. This is not a general effect of the TGF-β superfamily, as Nodal (Alk7 agonist) had no effect on *Sost* expression at the concentration examined. Inhibition of Alk4/5/7 with two different antagonists decreased *Sost* expression *in vitro* and *in vivo*, indicating that use of a clonal mature osteoblastic cell line was not a confounding factor in regulation of *Sost* by TGF-βs. siRNA directed against *Alk4* and *Alk5* decreased *Sost* expression. The specific Smad3 inhitor SIS3 attenuated endogenous and TGF-β-stimulated *Sost* expression, suggesting that traditional TGF-β/Smad, rather than TGF-β/MAPK, signaling is involved in transcription of *Sost*. Luciferase reporter assays indicated that TGF-β_1 targets the ECR5 bone enhancer, and not the proximal promoter, through a mechanism involving both Smad2/3 and Mef2 transcription factors. These results lay the foundation for future work designed to examine the interplay between *Alk4* and *Alk5* function and Wnt signaling *in vivo*, and identification of protein intermediates required for TGF-β—dependent induction of *Sost* transcription.

Highlights

- TGF-β isoforms and Activin A increase *Sost* expression *via* Smad3
- Inhibition of Alk4 and *Alk5* both *in vitro* and *in vivo* reduces *Sost* expression
- TGF-β targets *ECR5* to enhance *Sost*

Abbreviations

TGF-β	Transforming Growth Factor-beta
Lrp5	Low-density lipoprotein-related receptor 5
Wnt	Wingless int
ECR5	Evolutionarily Conserved Region 5
PTH	Parathyroid Hormone

Acknowledgments

This work was supported by Award Number R03AR057547 from the National Institute of Arthritis and Musculoskeletal and Skin Diseases (DCG) and DK075730 from the National Institute of Diabetes and Digestive and Kidney Diseases (DCG, DM, NMC, GGL). This work performed under the auspices of the U. S. Department of Energy by Lawrence Livermore National Laboratory under Contract DE-AC52-07NA27344.

References

1. Gong Y, Slee RB, Fukai N, Rawadi G, Roman-Roman S, Reginato AM, Wang H, Cundy T, Glorieux FH, Lev D, Zacharin M, Oexle K, Marcelino J, Suwairi W, Heeger S, Sabatakos G, Apte S, Adkins WN, Allgrove J, Arslan-Kirchner M, Batch JA, Beighton P, Black GC, Boles RG, Boon LM, Borrone C, Brunner HG, Carle GF, Dallapiccola B, De Paepe A, Floege B, Halfhide ML, Hall B, Hennekam RC, Hirose T, Jans A, Juppner H, Kim CA, Keppler-Noreuil K, Kohlschuetter A, LaCombe D, Lambert M, Lemyre E, Letteboer T, Peltonen L, Ramesar RS, Romanengo M, Somer H, Steichen-Gersdorf E, Steinmann B, Sullivan B, Superti-Furga A, Swoboda W, van den Boogaard MJ, Van Hul W, Vikkula M, Votruba M, Zabel B, Garcia T, Baron R, Olsen BR, Warman ML. LDL receptor-related protein 5 (LRP5) affects bone accrual and eye development. Cell. 2001; l107:513–523. [PubMed: 11719191]
2. Little RD, Carulli JP, Del Mastro RG, Dupuis J, Osborne M, Folz C, Manning SP, Swain PM, Zhao SC, Eustace B, Lappe MM, Spitzer L, Zweier S, Braunschweiger K, Benchekroun Y, Hu X, Adair R, Chee L, FitzGerald MG, Tulig C, Caruso A, Tzellas N, Bawa A, Franklin B, McGuire S, Nogues X, Gong G, Allen KM, Anisowicz A, Morales AJ, Lomedico PT, Recker SM, Van Eerdewegh P, Recker RR, Johnson ML. A mutation in the LDL receptor-related protein-gene results in the autosomal dominant high-bone-mass trait. Am. J. Hum. Genet. 2002; l70:11–19. [PubMed: 11741193]
3. Almeida M, Han L, Bellido T, Manolagas SC, Kousteni S. Wnt proteins prevent apoptosis of both uncommitted osteoblast progenitors and differentiated osteoblasts by beta-catenin- dependent and -independent signaling cascades involving Src/ERK and phosphatidylinositol 3-kinase/AKT. J. Biol. Chem. 2005; l280:41342–41351. [PubMed: 16251184]
4. Bodine PV, Billiard J, Moran RA, Ponce-de-Leon H, McLarney S, Mangine A, Scrimo MJ, Bhat RA, Stauffer B, Green J, Stein GS, Lian JB, Komm BS. The Wnt antagonist secreted frizzled-related protein-1 controls osteoblast and osteocyte apoptosis. J. Cell. Biochem. 2005; l96:1212–1230. [PubMed: 16149051]
5. Williams BO, Insogna KL. Where Wnts went: the exploding field of Lrp5 and Lrp6 signaling in bone. J. Bone Miner. Res. 2009; l24:171–178. [PubMed: 19072724]

6. Choi HY, Dieckmann M, Herz J, Niemeier A. Lrp4, a novel receptor for Dickkopf 1 and sclerostin, is expressed by osteoblasts and regulates bone growth and turnover in vivo. PLoS ONE. 2009; l4:e7930. [PubMed: 19936252]
7. Kato M, Patel MS, Levasseur R, Lobov I, Chang BH, Glass DA 2nd, Hartmann C, Li L, Hwang TH, Brayton CF, Lang RA, Karsenty G, Chan L. Cbfa1-independent decrease in osteoblast proliferation, osteopenia, and persistent embryonic eye vascularization in mice deficient in Lrp5, a Wnt coreceptor. J. Cell Biol. 2002; l157:303–314. [PubMed: 11956231]
8. Leupin O, Piters E, Halleux C, Hu S, Kramer I, Morvan F, Bouwmeester T, Schirle M, Bueno-Lozano M, Fuentes FJ, Itin PH, Boudin E, de Freitas F, Jennes K, Brannetti B, Charara N, Ebersbach H, Geisse S, Lu CX, Bauer A, Van Hul W, Kneissel M. Bone overgrowth-associated mutations in the LRP4 gene impair sclerostin facilitator function. J. Biol. Chem. 2011; l286:19489–19500. [PubMed: 21471202]
9. Balemans W, Ebeling M, Patel N, Van Hul E, Olson P, Dioszegi M, Lacza C, Wuyts W, Van Den Ende J, Willems P, Paes-Alves AF, Hill S, Bueno M, Ramos FJ, Tacconi P, Dikkers FG, Stratakis C, Lindpaintner K, Vickery B, Foernzler D, Van Hul W. Increased bone density in sclerosteosis is due to the deficiency of a novel secreted protein (SOST). Hum. Mol. Genet. 2001; l10:537–543. [PubMed: 11181578]
10. Brunkow ME, Gardner JC, Van Ness J, Paeper BW, Kovacevich BR, Proll S, Skonier JE, Zhao L, Sabo PJ, Fu Y, Alisch RS, Gillett L, Colbert T, Tacconi P, Galas D, Hamersma H, Beighton P, Mulligan J. Bone dysplasia sclerosteosis results from loss of the SOST gene product, a novel cystine knot-containing protein. Am. J. Hum. Genet. 2001; l68:577–589. [PubMed: 11179006]
11. Balemans W, Patel N, Ebeling M, Van Hul E, Wuyts W, Lacza C, Dioszegi M, Dikkers FG, Hildering P, Willems PJ, Verheij JB, Lindpaintner K, Vickery B, Foernzler D, Van Hul W. Identification of a 52 kb deletion downstream of the SOST gene in patients with van Buchem disease. J. Med. Genet. 2002; l39:91–97. [PubMed: 11836356]
12. Kusu N, Laurikkala J, Imanishi M, Usui H, Konishi M, Miyake A, Thesleff I, Itoh N. Sclerostin is a novel secreted osteoclast-derived bone morphogenetic protein antagonist with unique ligand specificity. J. Biol. Chem. 2003; l278:24113–24117. [PubMed: 12702725]
13. Loots GG, Kneissel M, Keller H, Baptist M, Chang J, Collette NM, Ovcharenko D, Plajzer-Frick I, Rubin EM. Genomic deletion of a long-range bone enhancer misregulates sclerostin in Van Buchem disease. Genome Res. 2005; l15:928–935. [PubMed: 15965026]
14. Nakanishi R, Shimizu M, Mori M, Akiyama H, Okudaira S, Otsuki B, Hashimoto M, Higuchi K, Hosokawa M, Tsuboyama T, Nakamura T. Secreted frizzled-related protein 4 is a negative regulator of peak BMD in SAMP6 mice. J. Bone Miner. Res. 2006; l21:1713–1721. [PubMed: 17002585]
15. Bodine PV, Zhao W, Kharode YP, Bex FJ, Lambert AJ, Goad MB, Gaur T, Stein GS, Lian JB, Komm BS. The Wnt antagonist secreted frizzled-related protein-1 is a negative regulator of trabecular bone formation in adult mice. Mol. Endocrinol. 2004; l18:1222–1237. [PubMed: 14976225]
16. Leupin O, Kramer I, Collette NM, Loots GG, Natt F, Kneissel M, Keller H. Control of the SOST bone enhancer by PTH using MEF2 transcription factors. J. Bone Miner. Res. 2007; l22:1957–1967. [PubMed: 17696759]
17. Kanaan RA, Kanaan LA. Transforming growth factor beta1, bone connection. Med Sci Monit. 2006; l12:RA164–RA169. [PubMed: 16865078]
18. Santibanez JF, Quintanilla M, Bernabeu C. TGF-beta/TGF-beta receptor system and its role in physiological and pathological conditions. Clin. Sci. 2011; l121:233–251. [PubMed: 21615335]
19. Origuchi N, Ishidou Y, Nagamine T, Onishi T, Matsunaga S, Yoshida H, Sakou T. The spatial and temporal immunolocalization of TGF-beta 1 and bone morphogenetic protein-2/-4 in phallic bone formation in inbred Sprague Dawley male rats. In Vivo. 1998; l12:473–480. [PubMed: 9827353]
20. Janssens K, ten Dijke P, Janssens S, Van Hul W. Transforming growth factor-beta1 to the bone. Endocr. Rev. 2005; l26:743–774. [PubMed: 15901668]
21. Bonewald LF, Dallas SL. Role of active and latent transforming growth factor beta in bone formation. J. Cell. Biochem. 1994; l55:350–357. [PubMed: 7962167]

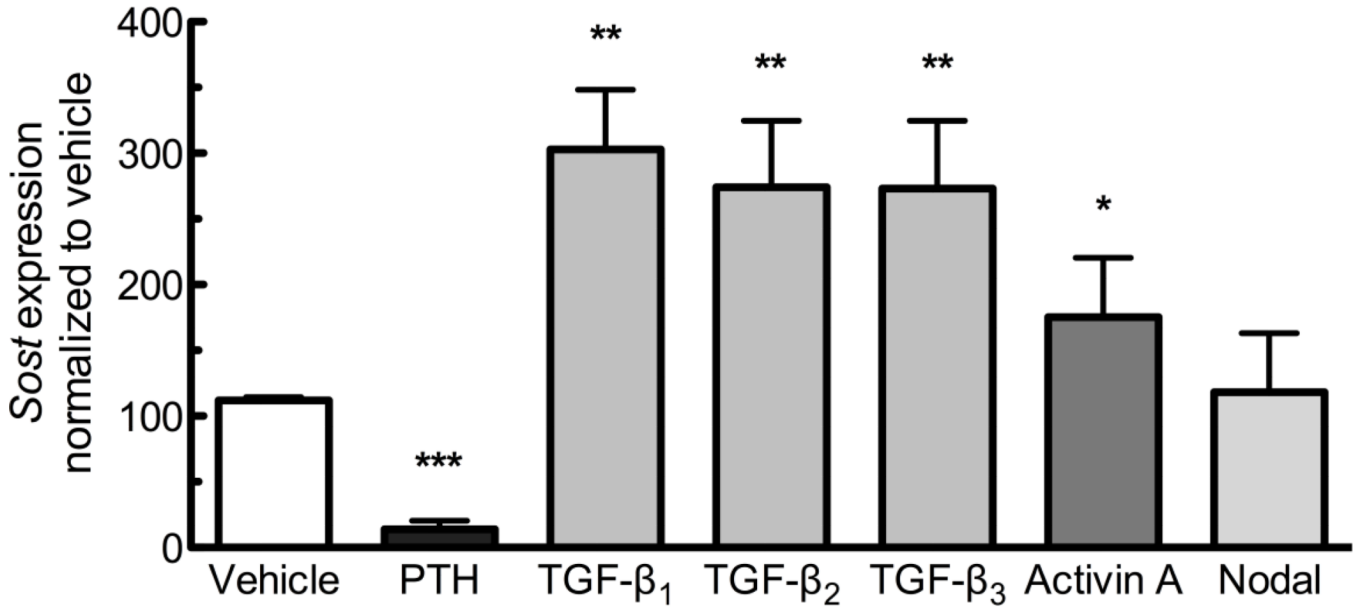
22. Sowa H, Kaji H, Iu MF, Tsukamoto T, Sugimoto T, Chihara K. Parathyroid hormone-Smad3 axis exerts anti-apoptotic action and augments anabolic action of transforming growth factor beta in osteoblasts. J. Biol. Chem. 2003; l278:52240–52252. [PubMed: 14517210]
23. Pilbeam CC, Kawaguchi H, Voznesensky OS, Alander CB, Raisz LG. Regulation of inducible prostaglandin G/H synthase by interleukin-1, transforming growth factors-alpha and - beta, and prostaglandins in bone cells. Adv. Exp. Med. Biol. 1997; l400B:617–623. [PubMed: 9547612]
24. Sutherland MK, Geoghegan JC, Yu C, Winkler DG, Latham JA. Unique regulation of SOST, the sclerosteosis gene, by BMPs and steroid hormones in human osteoblasts. Bone. 2004; l35:448–454. [PubMed: 15268896]
25. Kamiya N, Ye L, Kobayashi T, Mochida Y, Yamauchi M, Kronenberg HM, Feng JQ, Mishina Y. BMP signaling negatively regulates bone mass through sclerostin by inhibiting the canonical Wnt pathway. Development. 2008; l135:3801–3811. [PubMed: 18927151]
26. Papanicolaou SE, Phipps RJ, Fyhrie DP, Genetos DC. Modulation of sclerostin expression by mechanical loading and bone morphogenetic proteins in osteogenic cells. Biorheology. 2009; l46:389–399. [PubMed: 19940355]
27. Kulkarni NH, Halladay DL, Miles RR, Gilbert LM, Frolik CA, Galvin RJ, Martin TJ, Gillespie MT, Onyia JE. Effects of parathyroid hormone on Wnt signaling pathway in bone. J. Cell. Biochem. 2005; l95:1178–1190. [PubMed: 15962290]
28. Keller H, Kneissel M. SOST is a target gene for PTH in bone. Bone. 2005; l37:148–158. [PubMed: 15946907]
29. Genetos DC, Yellowley CE, Loots GG. Prostaglandin E(2) Signals Through PTGER2 to Regulate Sclerostin Expression. PLoS ONE. 2011; l6:e17772. [PubMed: 21436889]
30. Kamiya N, Ye L, Kobayashi T, Lucas DJ, Mochida Y, Yamauchi M, Kronenberg HM, Feng JQ, Mishina Y. Disruption of BMP signaling in osteoblasts through type IA receptor (BMPRIA) increases bone mass. J. Bone Miner. Res. 2008; l23:2007–2017. [PubMed: 18684091]
31. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods. 2001; l25:402–408. [PubMed: 11846609]
32. Yingling JM, Blanchard KL, Sawyer JS. Development of TGF-beta signalling inhibitors for cancer therapy. Nat. Rev. Drug Discov. 2004; l3:1011–1022. [PubMed: 15573100]
33. Jinnin M, Ihn H, Tamaki K. Characterization of SIS3, a novel specific inhibitor of Smad3, and its effect on transforming growth factor-beta1-induced extracellular matrix expression. Mol. Pharmacol. 2006; l69:597–607. [PubMed: 16288083]
34. Fickett JW. Quantitative discrimination of MEF2 sites. Mol. Cell. Biol. 1996; l16:437–441. [PubMed: 8524326]
35. Dennler S, Itoh S, Vivien D, ten Dijke P, Huet S, Gauthier JM. Direct binding of Smad3 and Smad4 to critical TGF beta-inducible elements in the promoter of human plasminogen activator inhibitor-type 1 gene. EMBO J. 1998; l17:3091–3100. [PubMed: 9606191]
36. Winkler DG, Sutherland MK, Geoghegan JC, Yu C, Hayes T, Skonier JE, Shpektor D, Jonas M, Kovacevich BR, Staehling-Hampton K, Appleby M, Brunkow ME, Latham JA. Osteocyte control of bone formation via sclerostin, a novel BMP antagonist. EMBO J. 2003; l22:6267–6276. [PubMed: 14633986]
37. Semenov M, Tamai K, He X. SOST is a ligand for LRP5/LRP6 and a Wnt signaling inhibitor. J. Biol. Chem. 2005; l280:26770–26775. [PubMed: 15908424]
38. Li X, Ominsky MS, Warmington KS, Morony S, Gong J, Cao J, Gao Y, Shalhoub V, Tipton B, Haldankar R, Chen Q, Winters A, Boone T, Geng Z, Niu QT, Ke HZ, Kostenuik PJ, Simonet WS, Lacey DL, Paszty C. Sclerostin antibody treatment increases bone formation, bone mass, and bone strength in a rat model of postmenopausal osteoporosis. J. Bone Miner. Res. 2009; l24:578–588. [PubMed: 19049336]
39. Ominsky MS, Vlasseros F, Jolette J, Smith SY, Stouch B, Doellgast G, Gong J, Gao Y, Cao J, Graham K, Tipton B, Cai J, Deshpande R, Zhou L, Hale MD, Lightwood DJ, Henry AJ, Popplewell AG, Moore AR, Robinson MK, Lacey DL, Simonet WS, Paszty C. Two doses of sclerostin antibody in cynomolgus monkeys increases bone formation, bone mineral density, and bone strength. J. Bone Miner. Res. 2010; l25:948–959. [PubMed: 20200929]

40. van Bezooijen RL, ten Dijke P, Papapoulos SE, Lowik CW. SOST/sclerostin, an osteocyte-derived negative regulator of bone formation. Cytokine Growth Factor Rev. 2005; l16:319–327. [PubMed: 15869900]
41. Robey PG, Young MF, Flanders KC, Roche NS, Kondaiah P, Reddi AH, Termine JD, Sporn MB, Roberts AB. Osteoblasts synthesize and respond to transforming growth factor-type beta (TGF-beta) in vitro. J. Cell Biol. 1987; l105:457–463. [PubMed: 3475276]
42. Seyedin SM, Thompson AY, Bentz H, Rosen DM, McPherson JM, Conti A, Siegel NR, Galluppi GR, Piez KA. Cartilage-inducing factor-A. Apparent identity to transforming growth factor-beta. J. Biol. Chem. 1986; l261:5693–5695. [PubMed: 3754555]
43. Mundy GR, Bonewald LF. Role of TGF beta in bone remodeling. Ann. N. Y. Acad. Sci. 1990; l593:91–97. [PubMed: 2197964]
44. Sanford LP, Ormsby I, Gittenberger-de Groot AC, Sariola H, Friedman R, Boivin GP, Cardell EL, Doetschman T. TGFbeta2 knockout mice have multiple developmental defects that are non-overlapping with other TGFbeta knockout phenotypes. Development. 1997; l124:2659–2670. [PubMed: 9217007]
45. Hering S, Isken E, Knabbe C, Janott J, Jost C, Pommer A, Muhr G, Schatz H, Pfeiffer AF. TGFbeta1 and TGFbeta2 mRNA and protein expression in human bone samples. Exp. Clin. Endocrinol. Diabetes. 2001; l109:217–226. [PubMed: 11453034]
46. Alliston T, Choy L, Ducy P, Karsenty G, Derynck R. TGF-beta-induced repression of CBFA1 by Smad3 decreases cbfa1 and osteocalcin expression and inhibits osteoblast differentiation. EMBO J. 2001; l20:2254–2272. [PubMed: 11331591]
47. Balooch G, Balooch M, Nalla RK, Schilling S, Filvaroff EH, Marshall GW, Marshall SJ, Ritchie RO, Derynck R, Alliston T. TGF-beta regulates the mechanical properties and composition of bone matrix. Proc. Natl. Acad. Sci. U. S. A. 2005; l102:18813–18818. [PubMed: 16354837]
48. Mohammad KS, Chen CG, Balooch G, Stebbins E, McKenna CR, Davis H, Niewolna M, Peng XH, Nguyen DH, Ionova-Martin SS, Bracey JW, Hogue WR, Wong DH, Ritchie RO, Suva LJ, Derynck R, Guise TA, Alliston T. Pharmacologic inhibition of the TGF-beta type I receptor kinase has anabolic and anti-catabolic effects on bone. PLoS ONE. 2009; l4:e5275. [PubMed: 19357790]
49. de Gorter DJ, van Dinther M, Korchynskyi O, ten Dijke P. Biphasic effects of transforming growth factor beta on bone morphogenetic protein-induced osteoblast differentiation. J. Bone Miner. Res. 2011; l26:1178–1187. [PubMed: 21611961]
50. Jian H, Shen X, Liu I, Semenov M, He X, Wang XF. Smad3-dependent nuclear translocation of beta-catenin is required for TGF-beta1-induced proliferation of bone marrow-derived adult human mesenchymal stem cells. Genes Dev. 2006; l20:666–674. [PubMed: 16543220]
51. Maeda S, Hayashi M, Komiya S, Imamura T, Miyazono K. Endogenous TGF-beta signaling suppresses maturation of osteoblastic mesenchymal cells. EMBO J. 2004; l23:552–563. [PubMed: 14749725]
52. Atkins GJ, Rowe PS, Lim HP, Welldon KJ, Ormsby R, Wijenayaka AR, Zelenchuk L, Evdokiou A, Findlay DM. Sclerostin is a locally acting regulator of late-osteoblast/preosteocyte differentiation and regulates mineralization through a MEPE-ASARM-dependent mechanism. J. Bone Miner. Res. 2011; l26:1425–1436. [PubMed: 21312267]
53. Quinn ZA, Yang CC, Wrana JL, McDermott JC. Smad proteins function as comodulators for MEF2 transcriptional regulatory proteins. Nucleic Acids Res. 2001; l29:732–742. [PubMed: 11160896]
54. Ishikawa F, Miyoshi H, Nose K, Shibanuma M. Transcriptional induction of MMP-10 by TGF-beta, mediated by activation of MEF2A and downregulation of class IIa HDACs. Oncogene. 2010; l29:909–919. [PubMed: 19935709]
55. Centrella M, McCarthy TL, Canalis E. Activin-A binding and biochemical effects in osteoblast-enriched cultures from fetal-rat parietal bone. Mol. Cell. Biol. 1991; l11:250–258. [PubMed: 1846021]
56. Kawabata N, Kamiya N, Suzuki N, Matsumoto M, Takagi M. Changes in extracellular activin A:follistatin ratio during differentiation of a mesenchymal progenitor cell line, ROB-C26 into osteoblasts and adipocytes. Life Sci. 2007; l81:8–18. [PubMed: 17512555]

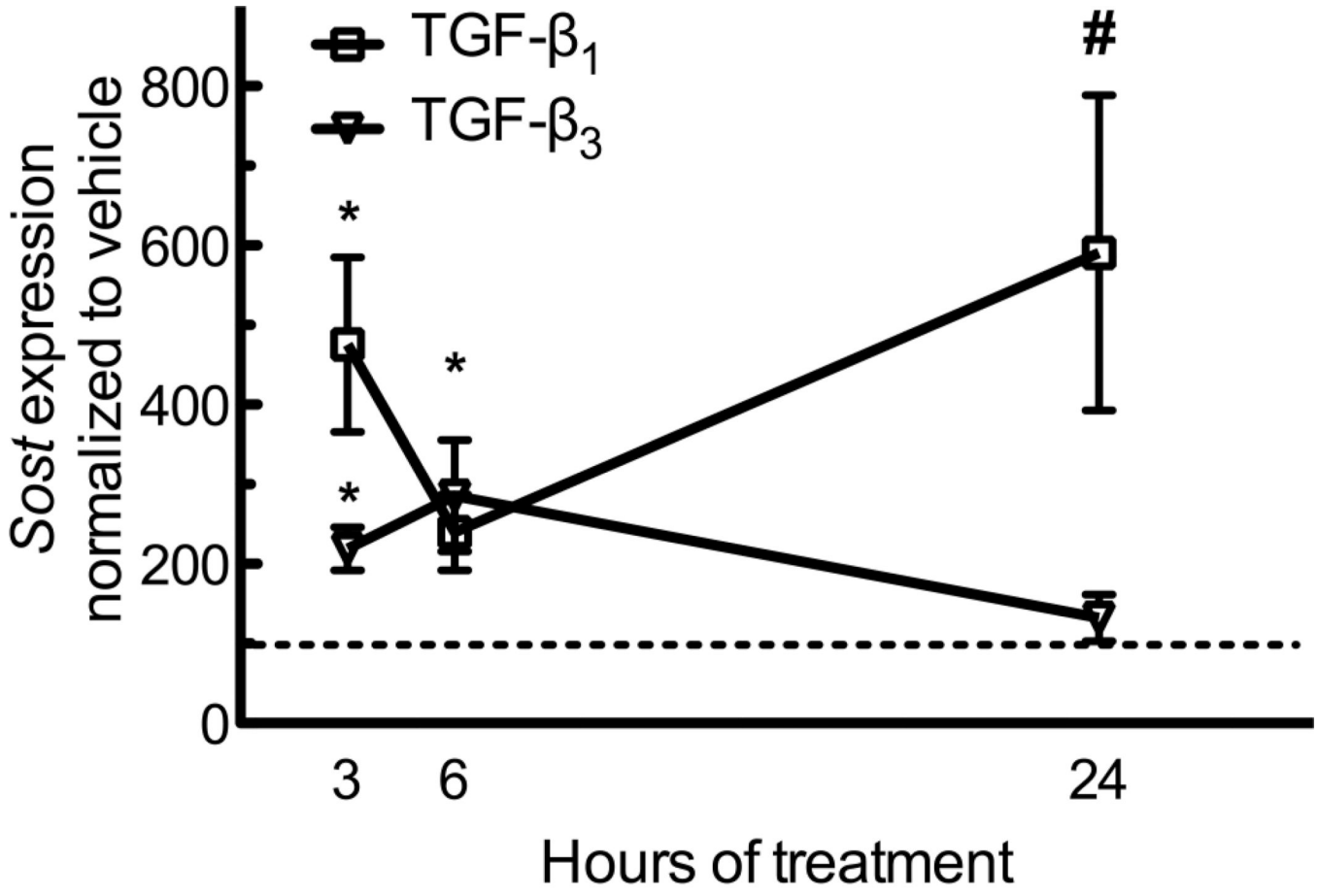
57. Vallet S, Mukherjee S, Vaghela N, Hideshima T, Fulciniti M, Pozzi S, Santo L, Cirstea D, Patel K, Sohani AR, Guimaraes A, Xie W, Chauhan D, Schoonmaker JA, Attar E, Churchill M, Weller E, Munshi N, Seehra JS, Weissleder R, Anderson KC, Scadden DT, Raje N. Activin A promotes multiple myeloma-induced osteolysis and is a promising target for myeloma bone disease. Proc. Natl. Acad. Sci. U. S. A. 2010; l107:5124–5129. [PubMed: 20194748]

58. Chantry AD, Heath D, Mulivor AW, Pearsall S, Baud'huin M, Coulton L, Evans H, Abdul N, Werner ED, Bouxsein ML, Key ML, Seehra J, Arnett TR, Vanderkerken K, Croucher P. Inhibiting activin-A signaling stimulates bone formation and prevents cancer-induced bone destruction in vivo. J. Bone Miner. Res. 2010; l25:2633–2646. [PubMed: 20533325]

59. Hall CL, Daignault SD, Shah RB, Pienta KJ, Keller ET. Dickkopf-1 expression increases early in prostate cancer development and decreases during progression from primary tumor to metastasis. Prostate. 2008; l68:1396–1404. [PubMed: 18561248]



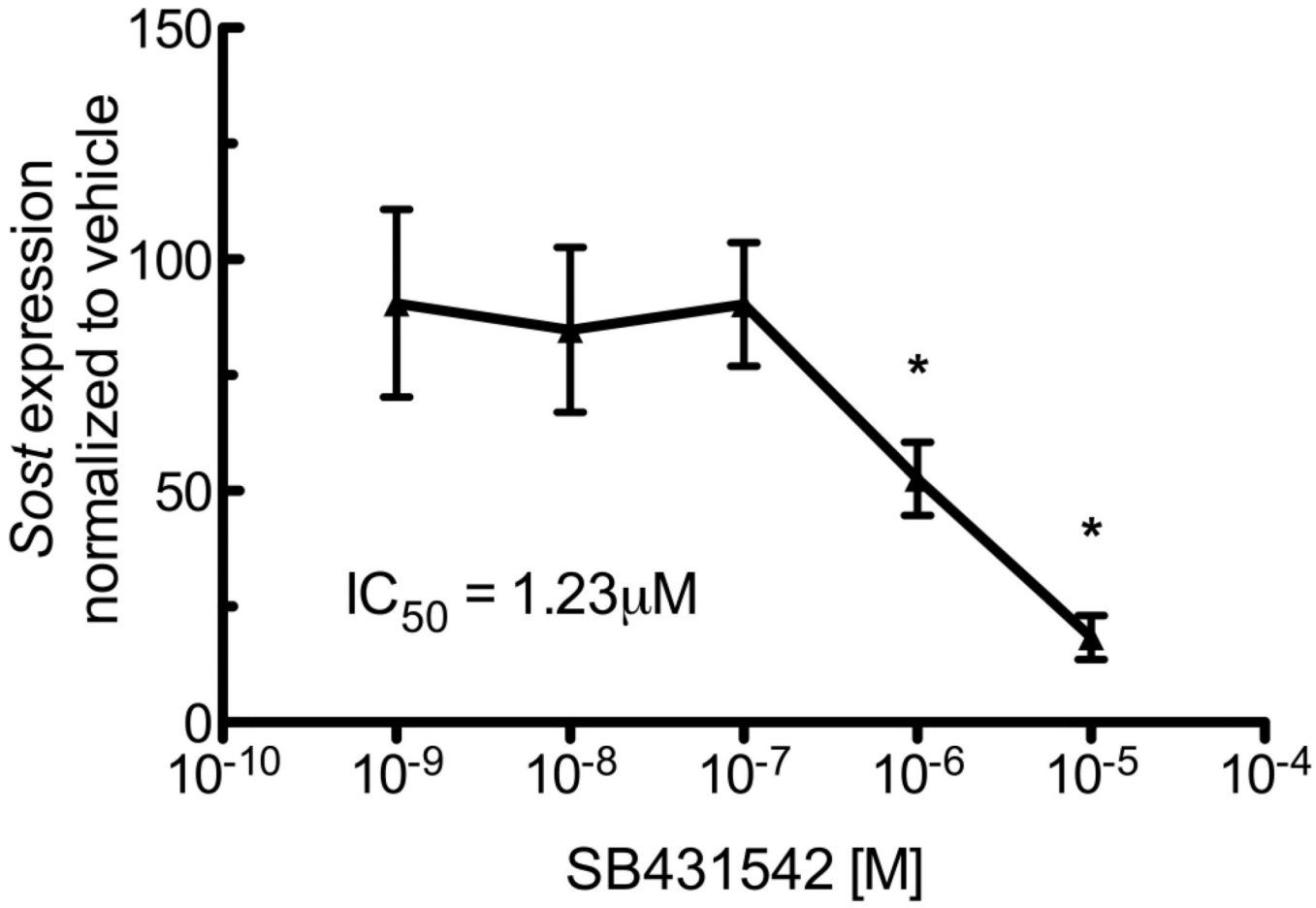
1a



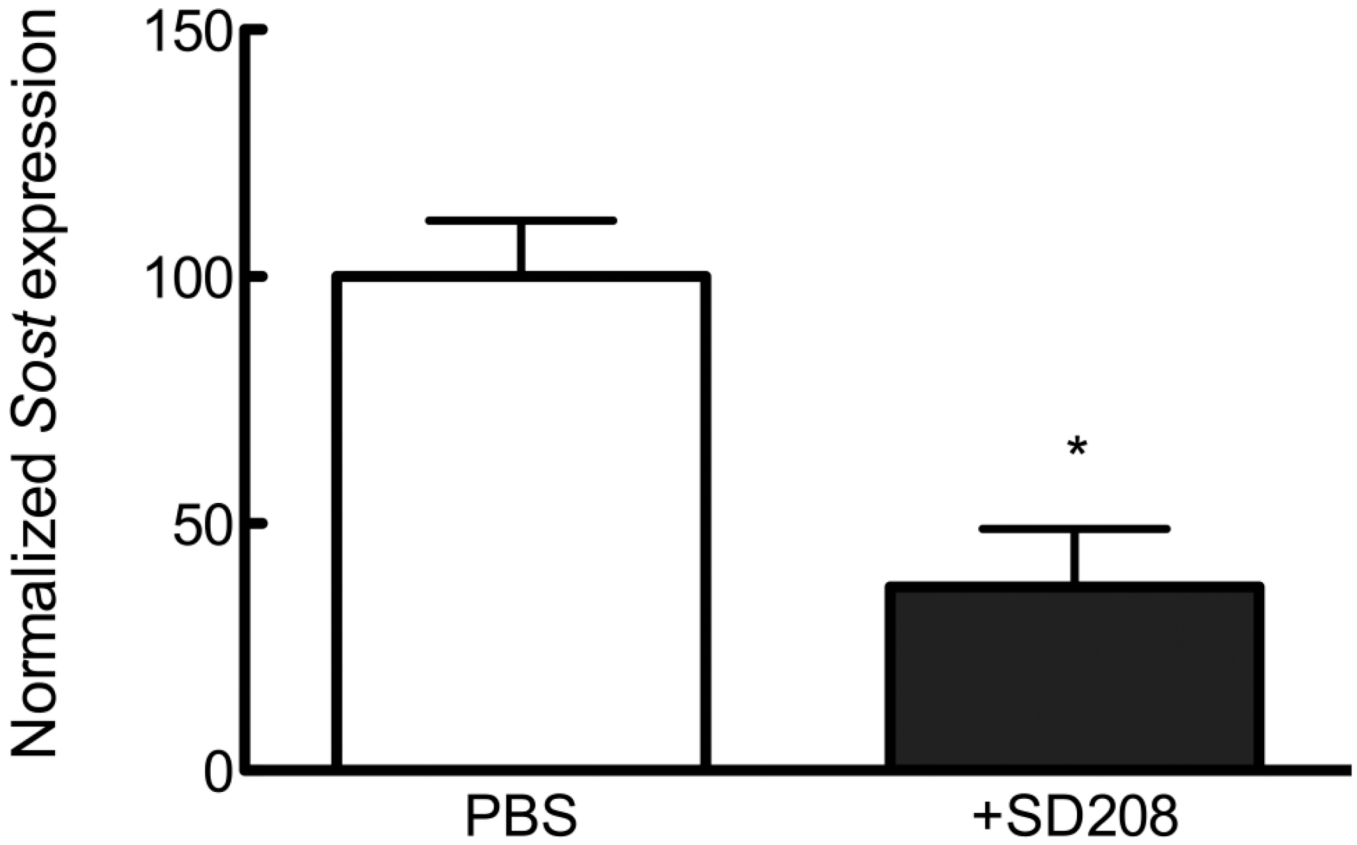
1b

Figure 1. TGF-β superfamily members regulate expression of *Sost*

(A) UMR106.01 mature osteoblasts were cultured for 6 hours with vehicle or 10ng/mL of indicated ligand; 100nM hPTH(1–34) was used as a positive control for *Sost* suppression. RNA was isolated for qPCR analysis of *Sost* expression relative to *Rpl32*. **(B)** UMR 106.01 mature osteoblasts were cultured for 3, 6, or 24h in the presence of 10ng/mL growth factor. For the sake of clarity, only the influence of TGF-β_1 and -β_3 upon *Sost* are shown. Data are mean ± SEM of 5 independent experiments. * indicates $p < 0.05$ compared to Vehicle, ** indicates $p < 0.01$ compared to Vehicle, *** indicates $p < 0.001$ compared to Vehicle, and # indicates $p < 0.05$ compared to TGF-β_3.



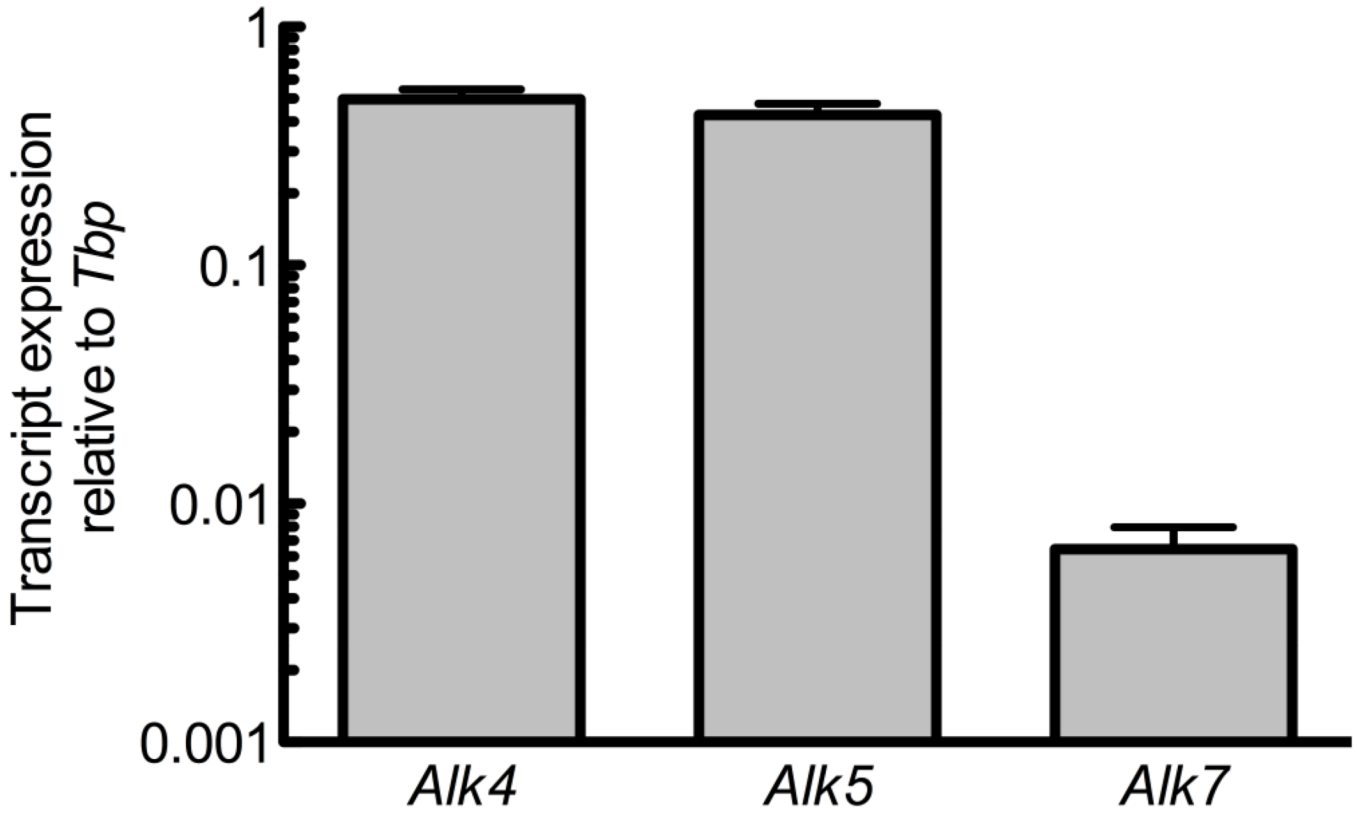
2a



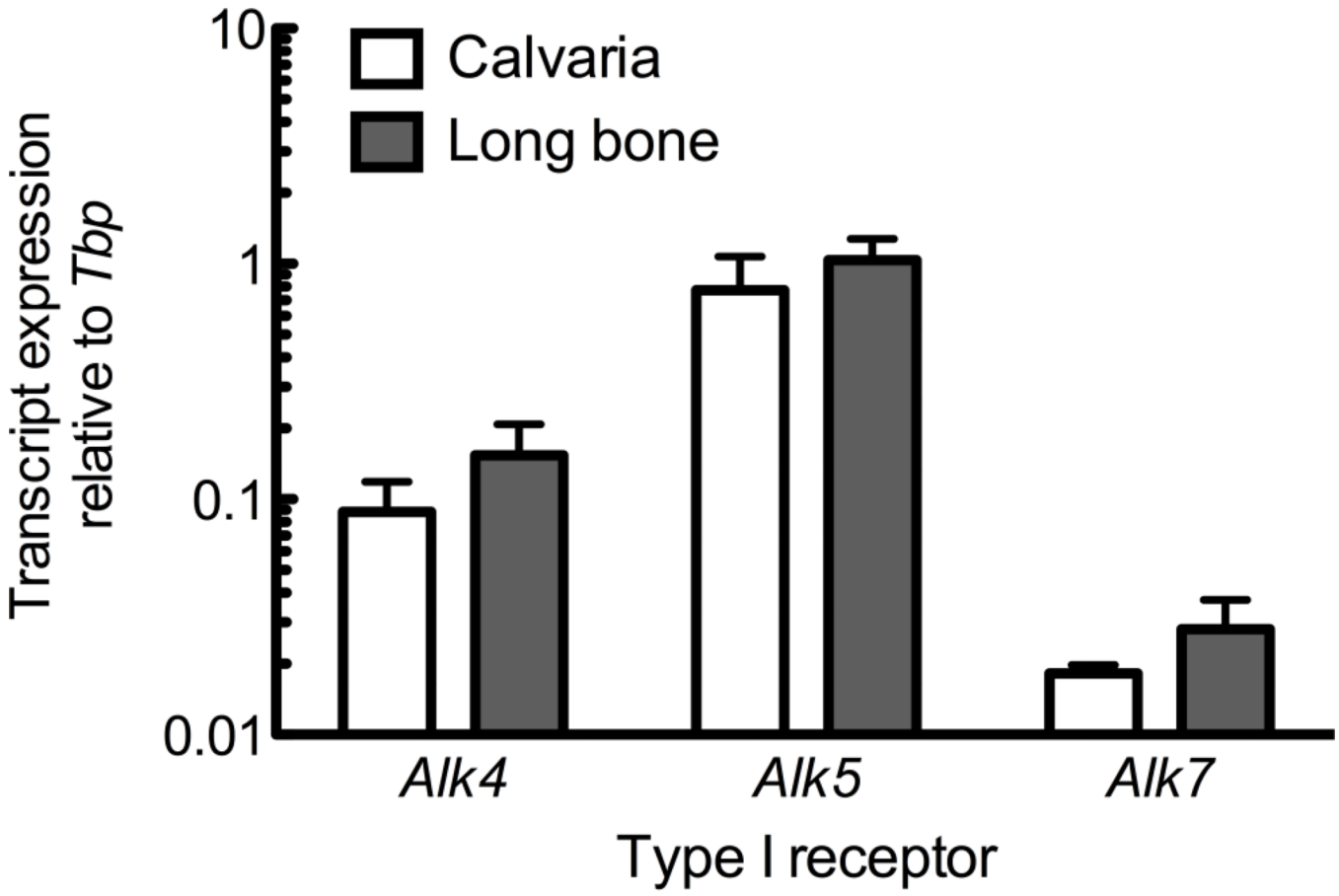
2b

Figure 2. *Sost* expression is inhibited by Alk4/5/7 antagonists

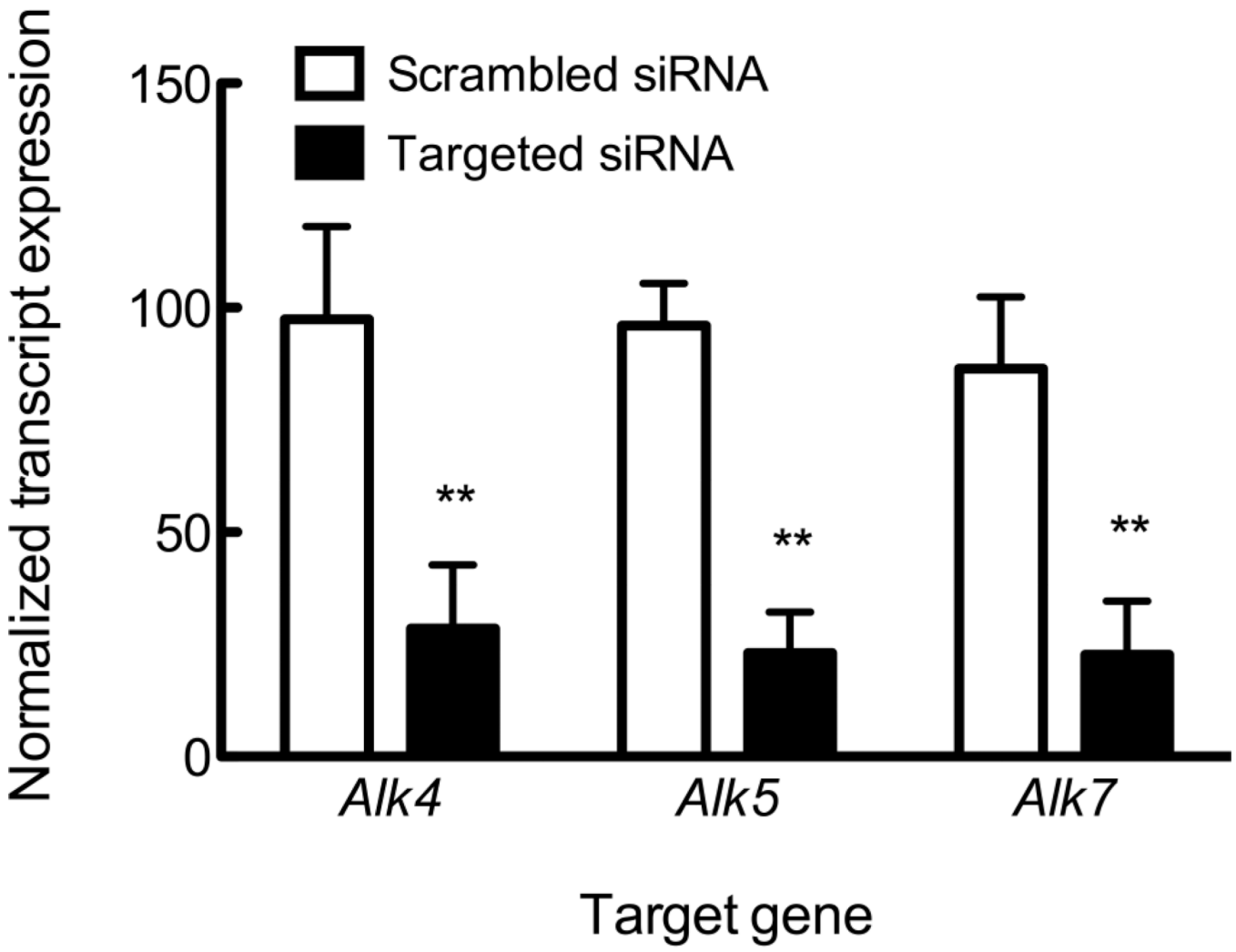
(A) UMR106.01 cells cultured for 6 hours in the presence of the Alk4/5/7 antagonist SB431542. RNA was isolated for qPCR analysis of *Sost* expression relative to *Rpl32*. **(B)** Mice were injected with either PBS or 20mg/kg SD208. 24 hours later, femorae were extracted and processed by qPCR for *Sost* expression relative to *Rpl32*. Data are mean ± SEM of 3 independent experiments. * indicates $p < 0.05$ compared to Vehicle



3a



3b



3c

3D

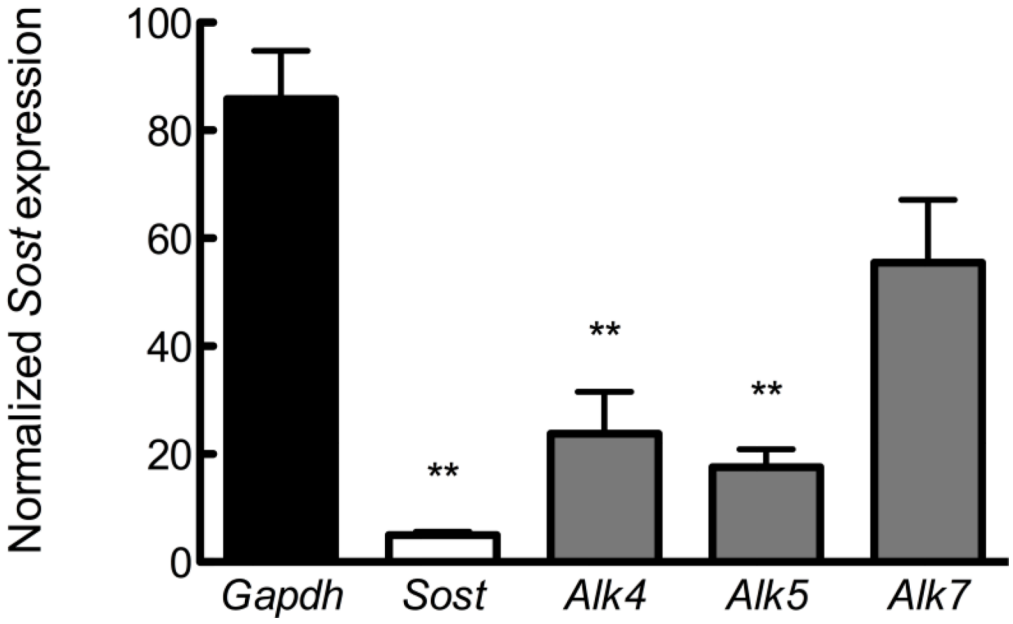
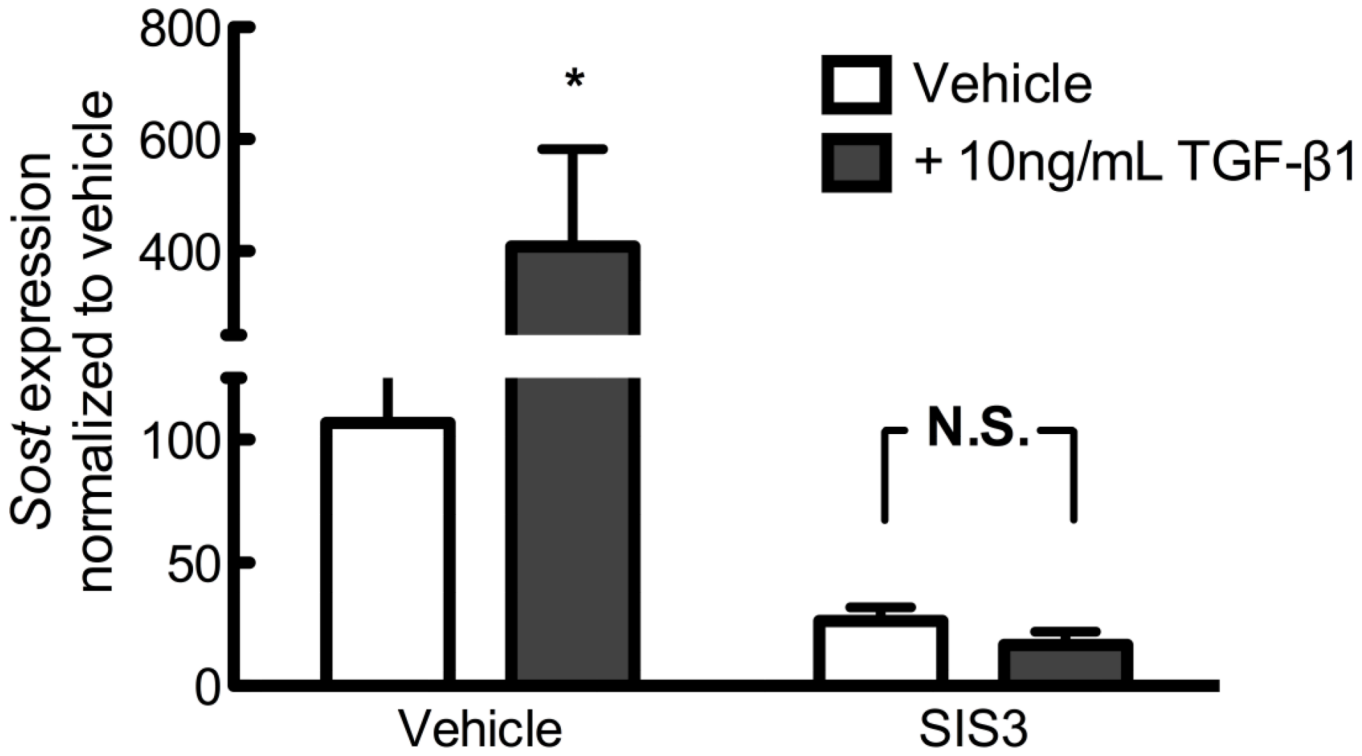
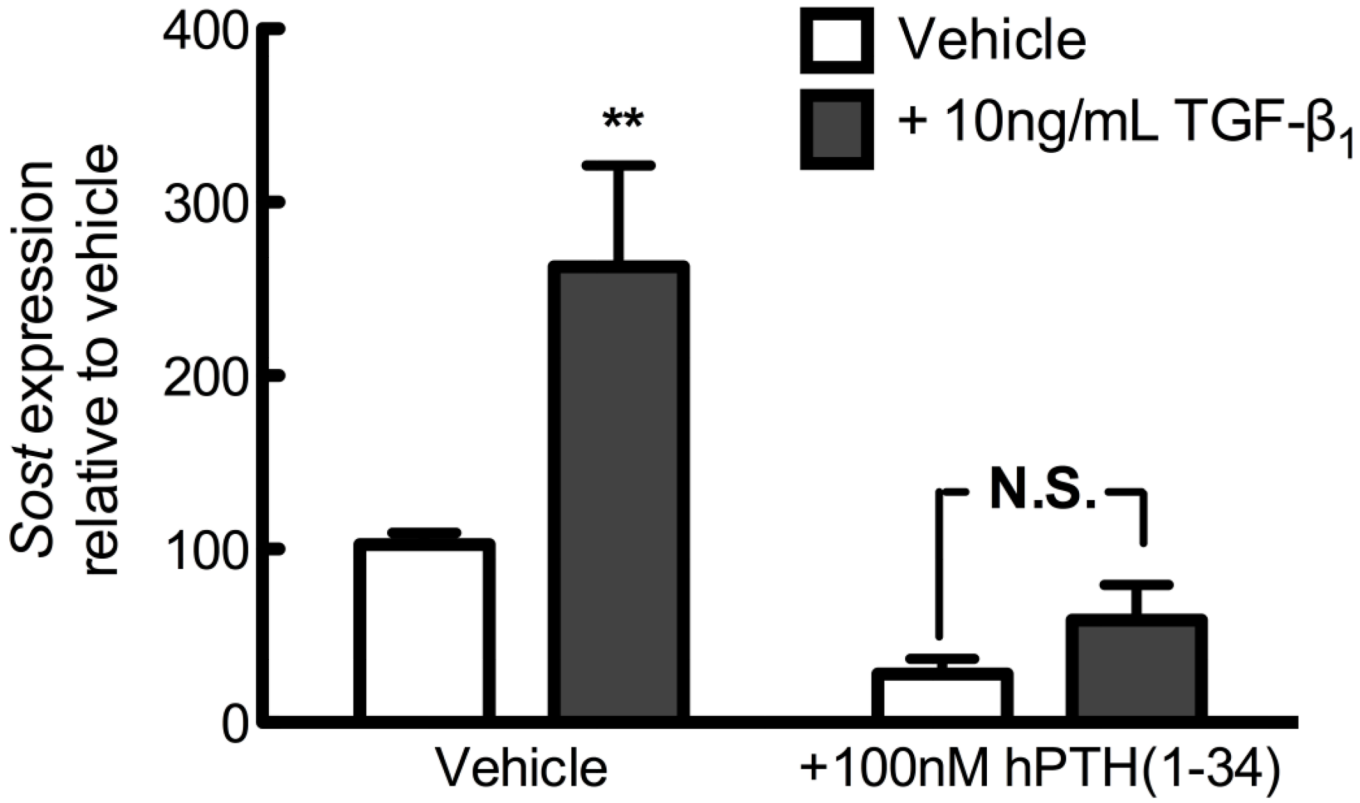


Figure 3. Alk4 and Alk5 regulate *Sost* expression

RNA from UMR106.01 cells **(A)** and **(B)** murine calvaria and long bone was analyzed by qPCR for expression of TGF-β receptors *Alk4*, *Alk5*, and *Alk7* relative to *Tbp*. **(C)** UMR106.01 cells were transfected with custom siRNA against *Alk4*, *Alk5*, or *Alk7*, or scrambled control. 24 hours later, RNA was isolated for qPCR analysis of *Alk4*, *Alk5*, or *Alk7* relative to *Gapdh*. **(D)** RNA from **(C)** was analyzed for *Sost* transcript by qPCR relative to *Gapdh*. * indicates $p < 0.05$ compared to Vehicle, ** indicates $p < 0.01$ compared to Vehicle, *** indicates $p < 0.001$ compared to Vehicle.



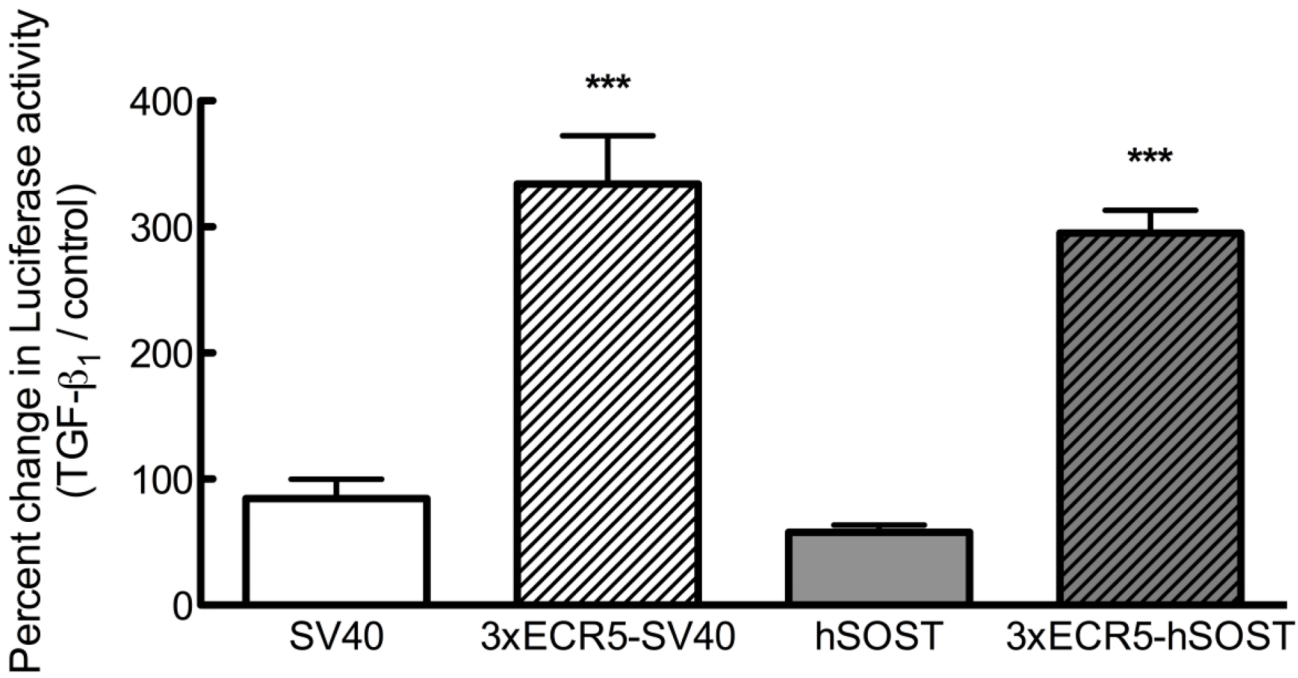
4a



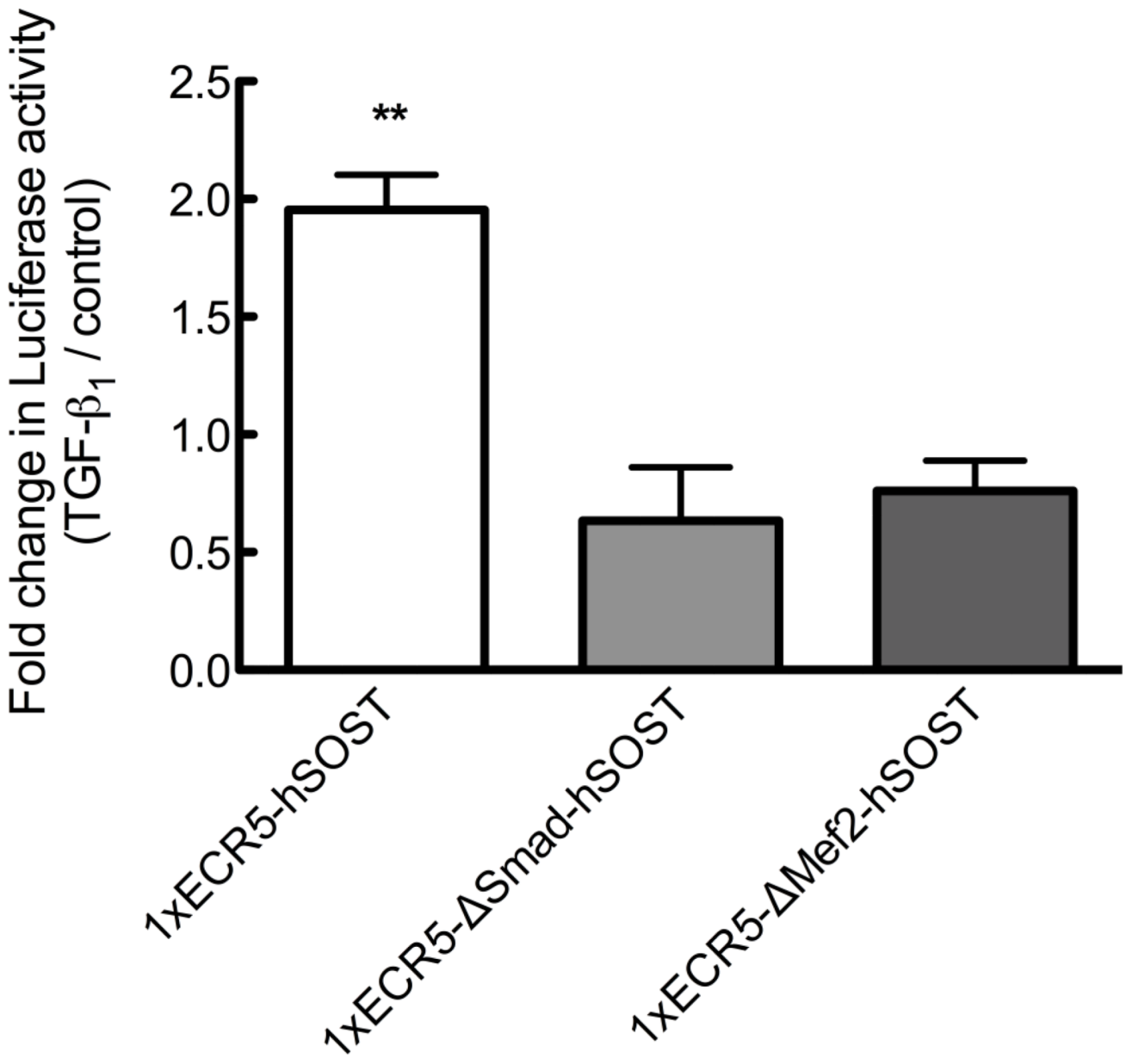
4b

Figure 4. TGF-β induction of *Sost* involves Smad3 and is abrogated by PTH

(A) UMR 106.01 cells were pretreated for 1 hour in the absence or presence of the cell-permeant Smad3 inhibitor, SIS3 (10μM), after which 10ng/mL TGF-β_1 or vehicle control was added for 6 hours. RNA was isolated for qPCR analysis of *Sost* expression relative to *Rpl32*. **(B)** UMR 106.01 cells were cultured for 6 hours in the absence or presence of 100nM hPTH(1–34) with or without 10ng/mL TGF-β_1.



5a



5b

Figure 5. TGF-β_1 induces *Sost via* the ECR5 transcriptional regulatory element through Smad and Mef2

(A) UMR 106.01 cells were transfected with Luciferase constructs containing minimal SV40 promoter, the human *SOST* promoter, each with or without the ECR5 distal enhancer and treated with vehicle or 10ng/mL TGF-β_1 for 24 hours, after which Luciferase activity was measured. **(B)** UMR 106.01 cells were transfected with a wild-type 1xECR5-hSOST Luciferase construct or construct with deletions of the Smad (ΔSmad) or Mef2 (ΔMef2) consensus binding site and treated with vehicle or 10ng/mL TGF-β_1 for 24 hours, after which Luciferase activity was measured. *** indicates < 0.001 compared to plasmids lacking 3xECR5, ** indicates $p < 0.01$ compared to plasmids with deletions.

TABLE 1

qPCR primers

Target	Source	Assay no.	Species	Chemistry
Sost	Applied Biosystems	Rn0057791_m1	Rat	TaqMan
Rpl32	Applied Biosystems	Rn00820748_g1	Rat	TaqMan
Alk4 (Acvr1b)	Qiagen	Mm_Acvr1b_1_SG	Mouse	SYBR Green
Alk5 (Tgfbr1)	Qiagen	Mm_Tgfbr1_1_SG	Mouse	SYBR Green
Alk7 (Acvr1c)	Qiagen	Mm_Acvr1c_2_SG	Mouse	SYBR Green
Tbp	Qiagen	Mm_Tbp_1_SG	Mouse	SYBR Green

TABLE 2

TGF-β isoform effects upon *Sost*

Ligand	*Sost* EC_{50} (ng/mL)	Maximum induction (percent of vehicle)
TGF-β_1	29	257
TGF-β_2	186	301
TGF-β_3	34	297
Activin A	1371	175