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TGF-β regulates sclerostin expression *via* the ECR5 enhancer

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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-βTGF-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.

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 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 occurs within the *SOST* transcript and comprises 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 52 kb non-coding region (also referred to as van Buchem deletion region) that is 35 kb 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),

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.

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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 and Dallas [21], among others). TGF-β_{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 5 k/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 was 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 280 nm. 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 min (5 min for SYBR), followed by 40 cycles of 95 °C for 3 s (10 s for SYBR) and 30 s at 60 °C. qPCR results were calculated relative to internal control (*Rpl32* or *Tbp*; $2^{-\Delta Ct}$) with the exception of Figs. 3A and B, results were further normalized to control, time-matched conditions ($2^{-\Delta\Delta Ct}$) [31].

Table 1
qPCR primers.

Target	Source	Assay no.	Species	Chemistry
Sost	Applied Biosystems	Rn00057791_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

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 6000 cells *per* well in 48-well plates. One hour later, 10 nM siRNA and Fugene 6 (Roche) were diluted into Opti-MEM (Invitrogen) and were gently added to the culture plate. Samples were collected 48 h 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 h with vehicle (HBSS), or SD-208 (60 mg/kg twice daily, one injection every 12 h; Tocris Biosciences) by intraperitoneal delivery (IP). No adverse effects of SD-208 on mouse health were detected during the study. At 24 h 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 2 kb 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 20 k/well into 48-well plates. On the following day, media were removed, replaced with Opti-MEM, and transfected with Fugene6, the reporter of interest (250 ng/well), and pRL-TK (50 ng/well; Promega) as a transfectant control. Twenty-four hours later, media were removed and replaced with TGF-β_1. Samples were analyzed 24 h 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 Dunnett *post-hoc* analysis compared to control (vehicle). $p<0.05$ was considered statistically significant.