1 α ,25-dihydroxyvitamin D₃ stimulates human *SOST* gene expression and sclerostin secretion

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Running Title: Vitamin D regulation of *SOST*

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Abstract

Sclerostin, the *SOST* gene product, is a negative regulator of bone formation and a positive regulator of bone resorption. In this study, treatment of human primary osteoblasts, including cells differentiated to an osteocyte-like stage, with $1\alpha,25$ -dihydroxyvitaminD₃ (1,25D) resulted in the dose-dependent increased expression of *SOST* mRNA. A similar effect was observed in human trabecular bone samples cultured *ex vivo*, and in osteocyte-like cultures of differentiated SAOS2 cells. Treatment of SAOS2 cells with 1,25D resulted in the production and secretion of sclerostin protein. *In silico* analysis of the human *SOST* gene revealed a single putative DR3-type vitamin D response element (VDRE) at position -6216 bp upstream of the transcription start site (TSS). This sequence was confirmed to have strong VDRE activity by luciferase reporter assays and electrophoretic mobility shift analysis (EMSA). Sequence substitution in the VDR/RXR half-sites abolished VDRE reporter activity and binding of nuclear proteins. A 6.3 kb fragment of the human proximal *SOST* promoter demonstrated responsiveness to 1,25D. The addition of the evolutionary conserved region 5 (ECR5), a known bone specific enhancer region, ahead of the 6.3 kb fragment increased basal promoter activity but did not increase 1,25D responsiveness. Site-specific mutagenesis abolished the responsiveness of the 6.3kb promoter to 1,25D. We conclude that 1,25D is a direct regulator of human *SOST* gene and sclerostin protein expression, extending the pathways of control of sclerostin expression. At least some of this responsiveness is mediated by the identified classical VDRE however the nature of the transcriptional regulation by 1,25D warrants further investigation.

Keywords: vitamin D; vitamin D response element; *SOST*; sclerostin; osteocyte; gene regulation; promoter

Introduction

Sclerostin (SCL) is the product of the *SOST* gene, mutations in which cause the high bone mass disease in humans, sclerosteosis (Baron et al., 2006). Partial deletion of a distal regulatory region of *SOST* is causative of the high bone mass phenotype seen in Van Buchem's disease (Balemans et al., 2002, Staehling-Hampton et al., 2002, ten Dijke et al., 2008). *SOST*-null mice display a similar high bone mass phenotype (Li et al., 2008). SCL thus appears to be a key regulator of bone mass and is an attractive therapeutic target for treatment of low bone mass diseases, such as osteoporosis (Padhi et al., 2011).

SCL is expressed primarily by osteocytes *in vivo* (Poole et al., 2005, Robling et al., 2006). Stimuli that regulate *SOST* expression exert important influences on bone formation and bone mass. PTH treatment (Bellido et al., 2005, Silvestrini et al., 2007) likely exerts an anabolic effect, at least in part, by suppressing *SOST* expression. Likewise, mechanical loading suppresses *SOST*, consistent with loading-induced increases in bone mass (Robling et al., 2008). In contrast, pro-catabolic stimuli appear to increase SCL expression in bone, for example mechanical unloading (Robling et al., 2006) or exposure of osteoblast-lineage cells to pro-inflammatory cytokines such as TNF α and TWEAK (Findlay and Atkins, Vincent et al., 2009). Another cytokine, oncostatin M, has also been shown to modulate the expression of SCL, depending on whether the exposure is chronic or acute (Brounais et al., 2009, Walker et al., 2010). The hormone 1 α ,25-dihydroxyvitamin D₃ (1,25D) is an important regulator of calcium homeostasis and is thought to act both at the intestine and in the bone itself. Genome-wide analyses indicate that 1,25D, by binding to the vitamin D receptor (VDR), has the potential to regulate the expression of some 3000 genes (Wang et al., 2005). The actions of 1,25D are mediated ultimately by direct effects on individual vitamin D responsive genes. Upon binding of 1,25D with VDR and translocation to the nucleus, the complex forms a heterodimer with the retinoid X receptor (RXR). This induces a VDR conformation that is essential for effective binding to a vitamin D response element (VDRE). This association

serves to recruit nuclear proteins as co-activators or co-repressors, necessary for VDR-mediated transcriptional regulation (Kraichely and MacDonald, 1998). The effects of vitamin D on bone tissue as a whole are not yet fully understood but are likely due to a combination of direct effects *via* VDREs, downstream effects of the induced gene expression and effects at specific stages of bone cell proliferation and differentiation. One direct action that has been clearly demonstrated is the ability of plasma 1,25D to stimulate bone resorption by the activation of osteoclasts (Suda et al., 2003). *In vitro* studies have shown that 1,25D is capable of regulating osteoblast gene transcription, proliferation, differentiation and mineralisation (Atkins et al., 2003, Matsumoto et al., 1991, Rickard et al., 1995). The genes of matrix proteins such as osteopontin and osteocalcin possess VDREs within their promoter regions, suggesting a direct action for 1,25D on their expression. Other important bone matrix associated genes, such as type I collagen and osteonectin, may have non-classical VDREs in their promoters or be indirectly regulated by 1,25D (Chen et al., 1996).

Our previous study (Atkins et al., 2003) showed that in primary human osteoblasts, 1,25D induced the expression of RANKL in phenotypically immature osteoblast precursors, identified by their expression of the marker STRO-1 (Simmons and Torok-Storb, 1991). However, in phenotypically mature osteoblasts, negative for STRO-1 expression, an osteocalcin response predominated (Atkins et al., 2003). This differential response was not related to levels of VDR expression, nor to the overall ability of the cells to respond to 1,25D, evidenced by the expression of other ‘synthetic phase’ 1,25D-responsive genes such as type I collagen and bone sialoprotein-1, which were found to be expressed independently of cell differentiation stage. Similar results were obtained in mineralising cultures of primary mouse osteoblasts, where the 1,25D induction of RANKL expression decreased with increasing maturation of the osteoblast (Thomas et al., 2001).

Osteocytes are the most long-lived and numerous cell type in bone tissue and have emerged over recent years as key controllers of osteoblast behaviour, bone mineralisation and potentially of osteoclast activity (Bonewald, 2007, Bonewald, 2007). While it has not been studied in detail, it is likely that osteocytes are responsible for the majority of osteocalcin synthesis, which as discussed above, is under the control of 1,25D. Other key osteocyte derived proteins appear also to respond to 1,25D, such as the phosphaturic hormone, fibroblast growth factor 23 (FGF23) (Kolek et al., 2005, Tang et al.). Dentin matrix protein 1 (DMP1) is an acidic phosphorylated extracellular matrix protein, and is a member of the small integrin binding N-linked glycoprotein (SIBLING) family (Fisher and Fedarko, 2003). DMP1 is now recognised to be highly expressed in osteocytes and important for the differentiation of these cells (Feng et al., 2006) and is 1,25D responsive (Farrow et al., 2009).

In the current study, we examined the response of human primary osteoblast cultures to 1,25D and found that *SOST* mRNA and SCL protein levels were elevated in treated cultures. *SOST* mRNA was also increased in response to 1,25D in human *ex vivo* bone cultures. We identified and characterised a single putative classical VDRE in the human *SOST* promoter. We conclude that 1,25D represents another example of hormonal regulation of *SOST* expression. Based on our findings it is possible that bone mass may be regulated in part through a 1,25D-SCL pathway.

Materials and Methods

Reagents and plasmids

1,25D was obtained from Wako Pure Chemicals, Japan. DNA sequencing was performed using BigDye[®] Terminator v3.1 cycle sequencing kit (Department of Molecular Pathology, SA Pathology, Adelaide, Australia). Synthetic oligonucleotides were synthesised by Geneworks. (Thebarton, SA, Australia). The pGL2 plasmid vector modified with a minimal thymidine

kinase (TK) promoter cloned ahead of the firefly luciferase gene (pGL2-TK) was obtained from Dr Raman Sharma (Hanson Institute, Adelaide, SA). The pGL3 basic plasmid, pVDR Plasmid containing the sequence for VDR protein, and the pDR3-TK plasmid containing a consensus VDRE sequence was obtained from Dr Prem Dwivedi (Hanson Institute, Adelaide, SA). Dual Luciferase assay kits were purchased from Promega Corp. (Madison, WI). Plasmids were produced in competent JM109 cells (Promega Corp, Madison, WI) and extracted using Qiax II gel extraction kits (Qiagen, Doncaster, Victoria).

Bone tissue, cells and culture media

Human primary osteoblast-like cells, were isolated from femoral trabecular bone and passaged, as described previously (Atkins et al., 2003). The human osteoblast-like osteosarcoma (OS) cell lines MG-63, shown previously to exhibit immature osteoblast characteristics (Stewart et al., 1999), and SAOS2, were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA) and passaged in α MEM containing 10% FCS (α MEM-10). For some experiments, SAOS2 cells were cultured under mineralising conditions for 28d to achieve an osteocyte-like phenotype, as previously described (Prideaux et al., 2014), prior to experimentation. The mouse cell line MC3T3-E1, an immature osteoblast cell model (Quarles et al., 1992), was obtained originally from the American Type Culture Collection (ATCC) and was passaged as for NHBC. Human embryonic kidney cells (HEK-293) cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS in preparation for transfection. The rat osteoblast cell line UMR-106 was obtained from Professor TJ Martin, University of Melbourne (Parkville, VIC, Australia) and passaged in DMEM containing 10% FCS.

Preparation of total RNA and RT-PCR

Total RNA was extracted from human cancellous bone, NHBC and cell lines using Trizol extraction, and cDNA prepared as described previously (Atkins et al., 2011). Gene expression was analysed by real-time RT-PCR, as we have described previously (Atkins et al., 2011). Relative expression between samples was calculated using the comparative cycle threshold method (Δ CT), using GAPDH as the reference gene for comparisons within cell studies, as we have published. Alternatively, 18S rRNA was used as the reference when 18S rRNA expression mirrored total RNA quantities in samples more closely than GAPDH. Oligonucleotide primers were designed in house to flank intron–exon boundaries and were purchased from Geneworks (Thebarton, South Australia, Australia). Real-time oligonucleotide primers for the amplification of genes were as follows: GAPDH (for both mouse and human genes), as described previously (Atkins et al., 2007); 18S (102-bp product), 5'-GGAATTCCCGAGTAAGTGCG-3' (sense), 5'-GCCTCACTAAACCATCCAA-3' (antisense); human SOST (71-bp product), 5'-ACCACCCCTTTGAGACCAAAG-3' (sense), 5'-GGTCACGTAGCGGGTGAAGT-3' (antisense). human BSP-1 (123-bp product), 5' -ATGGCCTGTGCTTTCTCAATG-3' (sense), 5' -AGGATAAAAGTAGGCATGCTTG-3' (antisense); primer sequences for the amplification of human osteocalcin and RANKL were described previously (Atkins et al., 2009).

Oligonucleotides and plasmid constructs

Plasmids containing the minimum predicted VDRE sequence

Putative wild-type and mutant SOST VDRE oligonucleotides were designed to include a ScaI restriction site. This enabled rapid identification of successful insertions into the pGL2-TK parental vector. Oligonucleotides containing the minimum sequence of the predicted VDRE with 3 flanking bases were cloned ahead of the firefly luciferase gene using SacI and NheI restriction sites in the multiple cloning site of the parental vector. The following

oligonucleotide sequences (VDRE sequence is in bold and the mutated bases are underlined) were used to generate double stranded oligonucleotides to be ligated into the parental vector: WT h*SOST* VDRE 5' -AGTACTAAT**GAAACCGGATGACCTTTG**-3' (sense), 5' -CTAGCAA**AGGTCATCCGGTTTC**ATTAGTACTAGCT-3' (antisense). Mutant h*SOST* VDRE 5' -AGTACTAATGAT**TGTCGGAAACA**ATTG-3' (sense), 5' -CTAGCAA**ATTGTTTCCGACATC**ATTAGTACTAGCT-3' (antisense). The plasmid pDR3-TK, containing a DR3 type consensus VDRE sequence AGGTCA, as described previously (Dwivedi et al., 1998), was used as the positive control.

Plasmids containing the native extended SOST promoter

Genomic DNA was extracted using PureLink® DNA extraction kit (Life Technologies), as per manufacturer's instructions. Two different fragments (6333 bp and 6216 bp) of the *SOST* promoter were amplified. The shorter fragment did not include the predicted VDRE sequence which fell between -6231 and -6216 bp from the transcription start site (TSS); this was cloned into the pGL3 parental vector to generate the plasmid pGL3-h*SOST*-6216-WT. Custom designed primers flanked with restriction enzyme recognition sites (underlined) were used to PCR amplify the promoter. The forward primer: 5'-AAAG**CTAGCC**CCTACAAGTGGGAGCAAAATCTG-3' (NheI) was used to generate the 6333 bp product, and the forward primer: 5'-AAAG**CTAGCCTTTG**AGAGTCCTTCCAGATCAAAG-3' (NheI) was used to generate the 6216 bp product. The reverse primer in each instance was 5'-AAA**ACTCGAGT**ACCAGCCAGAGGAGGGC-3' (XhoI). Amplified fragments were ligated into a pCR-XL-TOPO cloning vector from a TOPO® XL PCR Cloning Kit (Life Technologies), as per manufacturer's instructions. pCR-XL-TOPO plasmids containing the *SOST* promoter fragments were transformed into One Shot® TOP10 (Life Technologies)

Chemically Competent *E. coli* competent cells and grown up. Plasmids were extracted using a plasmid MIDI Kit (Qiagen). Cloned *SOST* promoter fragments were excised using NheI and XhoI (NEB) and cloned into the multiple cloning site of a pGL3 parental vector (Promega) digested with the same restriction enzymes to generate pGL3-h*SOST*- 6333-WT (see **Fig. 7A**). The predicted VDRE sequence in pGL3-h*SOST*-6333-WT was mutated using mutagenesis primers:

5'-

CCTCCAGAACCAAGTAGAGAATGATATCGGATAAATTTGAGAGTCCTTCCAGATC

AAA-3' (Forward) and 5'-

TTTGATCTGGAAGGACTCTCAAAATTTATCCGATATCATTCTCTACTTGGTTCTGGA

GG-3' (Reverse), designed using QuickChange primer design software (www.agilent.com/genomics/qcpd), and the Quick change lightning kit (Agilent technologies); the resulting mutant was termed pGL3-h*SOST*-6333-MUT (see **Fig. 7A**). The same primer used in the amplification of genomic DNA (5'-AAAGCTAGCCCTACAAGTGGGAGCAAAATCTG-3') was used to sequence the cloned promoters. Three tandem repeats of the ECR5 sequence (Loots et al., 2005) was cloned ahead of the *SOST* promoter sequence of pGL3-h*SOST*- 6333-WT, pGL3-h*SOST*- 6333-MUT and pGL3-h*SOST*- 6216-WT plasmids to generate pGL3-3xECR5-h*SOST*-6333-WT, pGL3-3xECR5-h*SOST*-6333-MUT and pGL3-3xECR5-h*SOST*-6216-WT (see **Fig. 8A**). The modified vectors were sequenced using the primer 5'-AAAATATTACGCCAGCCCAAGCTA-3'.

Luciferase assays

Transfections were performed in quadruplicate, in 24-well tissue culture plates using FuGENE[®]6 transfection reagent (Roche, Indianapolis, USA), as per manufacturer's recommendations. HEK293 or SAOS2 cells were plated at approximately 60% confluence 24 hours prior to transfection. Four times less pRL-TK (*Renilla* luciferase) plasmid was co-

transfected with each modified pGL2-TK plasmid as an internal control. Cells were transfected for 24 hours and then treated for 24 hours, washed once with PBS and harvested by adding 50 µl of passive lysis buffer for 20 min at 37°C (Promega Corp, Madison, WI). Luciferase activity was determined using a dual luciferase assay system (Promega Corp, Madison, WI) where 10 µl of lysate was assayed using 25 µl of each different luciferase substrate. Luciferase activity was measured using a TD20_20 Luminometer.

Electrophoretic mobility shift assays

MG-63 cells were transfected with pVDR plasmid for 24 hours and treated for 24 hours with 1,25D (10^{-7} M), prior to the extraction of nuclear protein using methods previously described (Lahiri and Ge, 2000). Oligonucleotides containing the wild-type human *SOST* VDRE (5'-GAAACC GGA TGACCT-3'), mutated human *SOST* VDRE (5'-GATGTC GGA AACAAT-3') and wild-type mouse osteopontin VDRE as consensus VDRE (5'-GGTTCA CGA GGTTCA-3') (Noda et al., 1990) were synthesised (Gene Works, Adelaide, Australia). Double-stranded oligonucleotides were labeled by end-filling with α - 32 P-dCTP (Perkin Elmer) using Klenow enzyme (NEB) and purified using ethanol precipitation. Binding reaction for each assay contained 5 µg of nuclear protein, 1µg of poly(dI-dC) to a final volume of 12 µl in modified binding buffer (25 mM Tris-HCL, pH 7.6, 100 mM KCl, 5 mM MgCl₂, 0.5 mM EDTA, and 10% glycerol) and were incubated on ice for 10 min. 200,000 cpm of radiolabeled probe was added to each binding reaction and incubated on ice for another 20 min. Retarded DNA nuclear protein complexes were resolved in 4-12% gradient non-denaturing polyacrylamide gel in a low ionic strength running buffer (0.5 x TBE) at 4 °C. Gels were dried and exposed onto a storage phosphor screen film for 24-48 hours at -70°C.

***Ex vivo* human and mouse bone culture**

Ex vivo cultures of human cancellous bone (proximal femur) were prepared as described previously (Kogawa et al., 2013). Long bones were obtained from wild-type C57BL/6 mice. Long bones were processed into small fragments approximately 2-3 mm³ in PBS. Bone was treated with vehicle (ethanol diluted to the extent present at the highest concentration of 1,25D) or 1,25D in a dose response experiment as indicated, and the bone samples were incubated for 24 hours and 48 hours at 37°C. Samples were then processed for real-time RT-PCR, as described above.

Western blot and ELISA

SAOS2 cells were grown in differentiation media for 28 days to differentiate to an osteocyte like stage (Prideaux et al., 2014) and treated for 24 hours. Supernatants from these experiments were collected for the ELISA assays and cells were lysed using RIPA buffer (Life technologies) with protease inhibitors added (Complete mini; Roche Diagnostics, Mannheim, Germany). Western blotting was conducted using a previously published protocol (Kogawa et al., 2013), loading 40 µg of total protein/track, using mouse anti-human SOST MAb (Clone ID: 220902) (R&D systems) primary antibody at recommended concentrations Mouse monoclonal anti-GAPDH-HRP conjugate antibody (Clone ID:GAPDH-71.1) (Sigma-Aldrich) was used test protein loading. Secreted sclerostin levels were measured by an ELISA, as per the manufacturer's instructions (Biomedica Medizinprodukte GmbH & Co KG).

Statistical Analysis

Student's t-Test was used to analyse the differences in treatments in gene expression experiments and transient transfection assays. One way analysis of the variance (ANOVA) followed by Tukey's post-hoc analysis was used to examine differences in the experiments. A value for $p < 0.05$ was considered to be significant.

Results

The *SOST* gene is responsive to 1,25D treatment

To test the effect of known 1,25D on the transcriptional activity of *SOST* we used a human primary osteoblast model (NHBC), which we have shown are capable of differentiating into mature osteocyte-like cells (Atkins et al., 2011, Atkins et al., 2009, Atkins et al., 2009). Undifferentiated NHBC were treated with 1,25D (10 nM), estradiol (5 nM), and PTH (10 nM). mRNA transcripts of the known vitamin D responsive genes *OCN*, *RANKL* and *BSP1* all increased expression with 1,25D treatment (**Fig. 1A-C**), as did that of *SOST*. Furthermore, the expression of *SOST* decreased with PTH treatment, as expected (Keller and Kneissel, 2005) (**Fig. 1D**). We then performed a dose response for 1,25D in NHBC cultures differentiated to an osteocyte-like stage (28d) and measured *SOST* mRNA levels after a further three days. A clear dose-dependent increase in the expression of *SOST* mRNA was observed, which reached a maximum at 10 nM 1,25D. However, a 1,25D level as low as 1 nM was sufficient to induce a significant increase in the *SOST* mRNA expression (**Fig. 2A**). An initial time course of 1,25D treatment of NHBC cultures differentiated to an osteocyte-like stage showed that *SOST* induction by 1,25D was evident in cultures treated for 48 and 72 hours (**Fig. 2B**). We also examined expression in the human osteosarcoma cell line SAOS2, which we have shown acquires an osteocyte-like phenotype with extended culture under differentiating conditions (Prideaux et al., 2014). The kinetics of *SOST* mRNA induction in this cell line was rapid, with induction of *SOST* mRNA within 3 hours (Fig. 2C). Because of the utility of this cell line to study osteocyte-like behaviour and their abundant expression of *SOST* mRNA (Prideaux et al., 2014), we chose SAOS2 cells as a model to examine sclerostin protein expression in response to 1,25D. Western blot analysis of SAOS2 cells differentiated for 28 d showed robust induction of sclerostin protein after 24 hours of 1,25D treatment (**Fig. 3A**), although we detected an increase as early as 12 hours (data not shown). ELISA analysis of culture supernatants showed

increased secretion of sclerostin protein into the culture media in response to 1,25D treatment after 24 hours (**Fig. 3B**).

To test if the response observed in NHBC, human primary osteocyte-like cells and differentiated SAOS2 cells could be observed at the bone tissue level, we cultured human proximal femur trabecular bone collected at arthroplasty surgery *ex vivo* in the presence of 1,25D. Similar to our findings with the primary bone cell cultures, expression of each of *RANKL*, *OCN* and *SOST* mRNA was increased with 1,25D treatment in human bone samples at the 72 hour time point, in a dose-dependent manner (**Fig. 4A-C**). Likewise, in mouse femoral cortical bone cultured *ex vivo* in the presence of 1,25D, *Sost* mRNA expression also increased at the 72 h time point (**Fig. 4D**). The maximum induction of *SOST* mRNA was approximately 6- and 4-fold at the highest dose of 1,25D (100 nM) in both human and mouse bone.

Identification and characterisation of a putative VDRE

In order to examine whether the responsiveness to 1,25D was due to the presence of a VDRE in the *SOST* promoter, we analysed the 10 kb region of the proximal promoter upstream of the *SOST* TSS, using the web based promoter analysis algorithm, CONSITE (<http://asp.iu.uib.no:8090/cgi-bin/CONSITE/consite>). We identified a strong putative 15 base pair classical DR3-type VDRE, between positions -6231 bp and -6216 bp from the TSS. Our analysis of the *SOST* promoter regions of other species revealed similar predicted VDRE sequences, and the predicted human sequence was shown to have a high degree of conservation within higher mammals, in particular primates (**Fig. 5A**).

To test the ability of the predicted VDRE sequence to respond to 1,25D, we cloned the minimum predicted sequence ahead of a firefly luciferase gene in a modified pGL2 vector containing a constitutively active TK promoter (pGL2-h*SOST*-VDRE-WT). As a positive control, we cloned the minimal mouse *Opn* VDRE, considered to represent the consensus

DR3-type VDRE (Noda et al., 1990), into the same parental vector (pGL2-m*OPN*-VDRE). The empty parental vector (pGL2-TK), pGL2-h*SOST*-VDRE and pGL2-m*OPN*-VDRE plasmids were co-transfected into HEK293 cells together with a plasmid expressing *Renilla* luciferase (pRL-TK). Ratios of firefly to *Renilla* luciferase were used to obtain the relative luciferase activity in transfected and treated cells. 1,25D dose-dependently increased the relative luciferase activity in both pGL2-h*SOST*-VDRE and pGL2-m*OPN*-VDRE but had no effect on the empty parental vector (**Fig. 5B**). The predicted human *SOST* VDRE sequence showed higher luciferase fold-change compared to the consensus VDRE sequence, suggesting that the predicted *SOST* VDRE sequence was the stronger VDRE. The co-transfection of pVDR in HEK293 cells did not influence the fold induction, suggesting that levels of VDR were not limiting in the system (**Fig. 5C**).

To test the ability of the putative VDRE to bind a VDR transcriptional complex, we performed EMSA of MG-63 cells transfected with pVDR and treated for 24 hours with 1,25D. Nuclear proteins from both transfected and non-transfected cells were extracted and used in binding assays with the radiolabeled double-stranded oligonucleotides containing the minimum predicted VDRE sequence. Gel retardation of the radiolabeled DNA demonstrated that the putative sequence was capable of binding protein species from the nuclear extracts (**Fig. 5D**). Nuclear extracts from pVDR transfected cells showed higher intensity of binding to the radiolabeled oligonucleotides, suggesting that VDR protein was interacting with the radiolabeled oligonucleotides. Furthermore, 1,25D treated nuclear extracts interacted at a higher intensity with the oligonucleotides as opposed to extracts from untreated cells. This suggested that, in the presence of 1,25D, increased amounts of ligated VDR/RXR complexes were binding to the putative VDRE sequence.

To test the specificity of the predicted VDRE sequence, we designed mutations at both half sites of the predicted VDRE (as shown in **Fig. 5E**). Sequence analysis using bioinformatics software predicted loss of VDR/RXR binding capability by the mutated sequence. We then

mutated the wild-type VDRE sequence in the pGL2-h*SOST*-VDRE-WT plasmid to generate pGL2-h*SOST*-VDRE-MUT plasmid. pGL2-TK, pGL2-h*SOST*-VDRE-WT, pGL2-h*SOST*-VDRE-MUT and pDR3-TK constructs were transiently co-transfected with pRL-TK into HEK293 cells and treated with either control or 1,25D. Induction of luciferase with 1,25D treatment was observed in pGL2-h*SOST*-VDRE-WT and in pDR3-TK control but not in pGL2-TK or pGL2-h*SOST*-VDRE-MUT (**Fig. 5F**). This suggested that the predicted *SOST* VDRE sequence was critical for the response to 1,25D. The mutation of the predicted VDRE sequence rendered the radiolabeled oligonucleotides incapable of binding to nuclear protein extracts in EMSA experiments. In contrast to the oligonucleotides with the mutant sequence, the wild-type sequence not only bound nuclear proteins, but the level of binding was increased with extracts from 1,25D treated cells (**Fig. 5G**).

The predicted VDRE is critical for the response of the 6.3 kb *SOST* promoter to 1,25D

To test the response of the extended native *SOST* promoter, a 1000 bp region spanning the predicted VDRE from -6333 to -5333 bp from the *SOST* TSS was cloned into a parental pGL2-TK vector ahead of luciferase gene to generate the plasmid pGL2-h*SOST*[-6333;-5333bp] (**Suppl. Fig. S1A**). This construct was co-transfected with pRL-TK into HEK293 cells, which were then treated with 1,25D. pGL2-h*SOST*[-6333;-5333bp] failed to respond to 1,25D treatment except at one dose, whereas the minimum predicted VDRE sequence (**Suppl. Fig. S1B**) showed a prominent dose-dependent response to 1,25D (**Suppl. Fig. S1C**). The existence of other regulatory elements within the extended *SOST* promoter was a possible explanation for this lack of reactivity. To test the native *SOST* sequence including other possible regulatory elements, we cloned the region from -2 to -6333 bp from the *SOST* TSS ahead of a luciferase gene in a pGL3 vector. We mutated each half-site of the predicted VDRE sequence between positions -6231 to -6216, as shown on **Fig. 6A**. Constructs were co-transfected together with pRL-TK into HEK293 cells and treated with 1,25D for 24 hours. Normalised luciferase activity

in the wild-type 6333 bp *SOST* promoter increased with 1,25D treatment. However, in the VDRE mutant we did not observe significant induction of luciferase activity (**Fig. 6B**). Peak induction of the wild-type sequence was approximately 1.3 fold. We hypothesised the *SOST* promoter requires a further enhancer region, such as ECR5, for it to respond to 1,25D at the same levels we observed in our mRNA analysis. Three direct repeats of the ECR5 region (500 bp) were cloned into the pGL3-h*SOST*-6333-WT plasmid directly ahead of the 6333 bp promoter (**Fig. 7A**), as was performed for an earlier study in the context of PTH responsiveness (Loots et al., 2005). In addition, the predicted VDRE sequence was mutated in this construct. As an additional control we generated a plasmid (pGL3-3xECR5-h*SOST*-6216-WT) containing a truncated *SOST* promoter (6216 bp), excluding the predicted VDRE (**Fig. 7A**). Since the ECR5 element has been demonstrated to be important for the expression of *SOST* in bone cells, we transfected the above constructs into UMR-106 cells, and treated with 1,25D prior to measuring luciferase activity. The incorporation of the ECR5 repeats increased basal promoter activity approximately 20-fold (**Suppl. Fig. S2**), confirming the importance of this sequence for the larger *SOST* promoter. Similar to the results we observed with the *SOST* promoter without any additional enhancer regions, we observed a significant increase in luciferase activity in response to 1,25D treatment. While the inclusion of ECR5 repeats did not increase the responsiveness to 1,25D, induction of *SOST* promoter activity was dependent on the predicted VDRE, as no response to 1,25D was evident in either the corresponding VDRE mutant or the truncated 6216 bp *SOST* promoter fragment (**Fig. 7B**).

Discussion

Sclerostin is emerging as a key regulator of bone mass, however what determines the absolute level of sclerostin expression is not entirely clear. Among known regulators, mechanical loading and unloading are important for the regulation of *SOST* (Lin et al., 2009, Spatz et al., 2012) and the hormone PTH is demonstrated to reduce sclerostin expression (Bellido et al.,

2005, Kramer et al., 2010, Leupin et al., 2007). We have previously reported that sclerostin is up-regulated by catabolic stimuli such as TNF and TWEAK (Vincent et al., 2009). Vitamin D plays multiple roles in modulating bone homeostasis and is an important factor affecting key osteocytic genes. In this study we investigated the effect of 1,25D on the regulation of *SOST*.

In a human primary osteocyte model, we observed a clear dose-dependent induction of *SOST* mRNA in response to 1,25D, which was also observed in human and mouse bone cultured *ex vivo*, suggesting a direct effect of 1,25D on the transcription of *SOST/Sost*. The kinetics of the response indicated that *SOST* mRNA expression was regulated by 1,25D similar to that of *RANKL*, a gene for which multiple long range enhancers were demonstrated to be important for the regulatory effect of 1,25D (Kim et al., 2006). *In silico* analysis identified a single predicted DR3 type VDRE sequence -6216 bp from the TSS, which showed high homology to predicted VDRE sequences within the *SOST* proximal promoter regions of higher species, although the position of these within the respective promoters was poorly conserved. This may mean that the conditions under which 1,25D affect *SOST* transcription varies between species. We demonstrated that the predicted minimal human VDRE sequence was capable of responding to 1,25D in a dose-dependent manner in luciferase assays. Furthermore, by mutating the VDRE half sites the response to 1,25D was abolished. The fold-change in activity was almost double that of the response observed in the positive control, validating the predicted VDRE sequence. EMSA experiments confirmed that the putative VDRE sequence was capable of binding nuclear proteins, which was increased in the presence of 1,25D. Mutating the predicted sequence abolished any response to 1,25D in transient luciferase assays as well as binding to nuclear protein extracts in EMSAs.

The extended 6333 bp proximal *SOST* promoter, containing the putative VDRE, also responded to 1,25D in transient luciferase assays and mutating the VDRE sequence within this construct abolished this responsiveness. Cloning a known enhancer element of *SOST*, ECR5 (Loots et al., 2005), ahead of the native 6333 bp promoter increased the basal luciferase

expression by some 20-fold (**Suppl. Fig. S2**) but did not increase the degree of induction by 1,25D. The rat osteosarcoma cell line UMR-106 has been shown to express *Sost* at high levels and has been used previously to characterise the response of *Sost* to PTH (Leupin et al., 2007). In these cells, transient transfection of the human native 6333 bp *SOST* promoter construct together with the ECR5 region showed induction in the presence of 1,25D (**Fig. 7B**). A truncated promoter which excluded the predicted VDRE sequence and the VDRE mutant version of the wild-type promoter did not show any induction in the presence of 1,25D (**Fig. 7B**), consistent with the predicted VDRE contributing to the 1,25D responsiveness of the *SOST* gene.

The lack of an additive effect of ECR5 on the 1,25D response is interesting since this region binds the MEF2 transcription factors that promote *SOST* transcription in osteoblast lineage cells (Loots et al., 2005). The MEF2C/ECR5 interaction is critical for the inhibitory effect of PTH on *SOST* expression (Leupin et al., 2007), and recently has been shown to mediate the effect of histone deacetylase 5 (HDAC5), which interacts directly with the ECR5 and represses MEF2C activity (Johnson, 2015, Wein et al., 2015). However, ECR5 is a distal regulatory sequence residing some 45 kb downstream of the *SOST* coding sequence, whereas the VDRE identified here is 6.2 kb upstream of the TSS. It should be noted that the identification of the VDRE here extends the known upstream promoter of the *SOST* gene beyond the ~2 kb region thus far characterised to contain regulatory elements (Weivoda and Oursler, 2014).

In SAOS2 osteosarcoma cells, which we have shown can be considered to be osteocyte-like when differentiated over a 28d culture period (Prideaux et al., 2014), and whose endogenous *SOST* gene was rapidly responsive to 1,25D and resulted in *de novo* sclerostin production and secretion, the maximum induction observed in luciferase assays, while statistically significant, was only 1.2 fold (**Suppl. Fig. S3A**). However, the measured response of the endogenous *SOST* mRNA from the same cultures showed the response was greater and clearly dose-dependent, with the maximum fold induction observed being 2.2 fold at the highest dose of

1,25D (100 nM) (**Suppl. Fig. S3B**). Furthermore, the 1,25D responsiveness of the endogenous *SOST* promoter in SAOS2 cells was less than we observed for human primary osteoblasts, which sometimes induced up to 15-fold. This implies that in addition to the identified VDRE, additional elements are required for the 1,25D responsiveness, for example non-classical VDREs present within the *SOST/Sost* native promoter. A recent study showed that an increased level of the transcription initiation factor 12 (TAF12), a coactivator of the VDR, was responsible for the 1,25D hypersensitivity of osteoclasts from Paget's disease patients (Teramachi et al., 2013). Other VDR coactivators, such as SRC-1 and TRAM-1, have been demonstrated to be important for the formation of VDR homodimers and subsequent transcription of genes (Takeshita et al., 2000). It remains unknown which VDR and RXR complexes and coactivators are involved in 1,25D mediated transcription of *SOST*. Furthermore, the predicted *SOST* VDRE showed a low degree of conservation between higher mammals and mice, while our experiments indicated that mouse *Sost* is also responsive to 1,25D. Adding to the complexity of this issue, a recent study of the 1,25D-responsive transcriptome in the mouse osteocyte-like cell model IDG-SW3 reported that some 790 responsive genes did not have an identifiable VDRE using a ChIP Seq approach, which included *Sost* (St John et al., 2014): the authors suggested that in many cases, the VDR-mediated regulation of genes may involve a more complex regulatory mechanism (St John et al., 2014). ChIP Seq has also so far failed to identify a VDRE in the human *SOST* locus (Prof. J.W. Pike, *personal communication*). Contrary to our findings, mouse *Sost* mRNA in IDG-SW3 was down-regulated by a 100 nM dose of 1,25D, an effect confirmed by the authors in mouse L5 vertebral bone *in vivo* (St John et al., 2014). Our findings using mouse long bone however, demonstrate that *Sost* mRNA like human *SOST*, is positively regulated by 1,25D. We have also found that *Sost* mRNA is positively regulated by 1,25D in differentiated cultures of mouse neonatal calvarial osteoblasts (data not shown). Despite this intriguing complexity and the possibility that other 1,25D-responsive sequences exist, we have demonstrated in principle

that *SOST* transcription and translation are induced by 1,25D and that the identified VDRE mediates at least some of this activity.

We recently reported two pro-catabolic activities of sclerostin in bone. We demonstrated that sclerostin is capable of regulating *RANKL/Rankl* gene expression in cultured osteocytes and in bone tissue, as well as osteoclastogenesis and osteoclast function *in vitro* (Wijenayaka et al., 2011). We also reported that sclerostin can promote osteocytic osteolysis and regulate the osteocyte microenvironment by inducing the enzyme carbonic anhydrase 2, and potentially cathepsin K and TRAP (Kogawa et al., 2013). Both of these demonstrated mechanisms suggest that elevated levels of SCL can lead to calcium efflux from the bone. Hypervitaminosis D is a condition that leads to excess calcium in the blood; inactivating mutations of the *CYP24A1* gene, encoding the vitamin D-24-hydroxylase that degrades 1,25D, have been demonstrated to cause chronic hypercalcaemia (Colussi et al., 2013, Figueres et al., 2015), however levels of sclerostin have not been reported in this condition to date. A positive correlation between sclerostin expression by osteocytes and serum 1,25D levels was recently reported in a cohort of patients suffering hypercalciuria (Menon et al., 2014). Our data suggest that in such conditions, increased 1,25D levels may increase the level of sclerostin production by osteocytes, which in turn may lead to the release of calcium as suggested by the above catabolic mechanisms. Given the clear importance of sclerostin to bone metabolism, this interesting area of research warrants future investigation.

Acknowledgements

The authors thank Professor J.W. Pike (University of Wisconsin) for helpful advice and discussions regarding the data herein. This work was funded by a Project Grant (ID565372) from the National Health and Medical Research Council of Australia (NHMRC) and the support of the Rebecca Cooper Foundation. ARW was supported by a University of Adelaide International Postgraduate Research Student's Scholarship. GGL was supported by NIH grant

DK075730 and her contribution was performed under the auspices of the U.S. Department of Energy by Lawrence Livermore National Laboratory under Contract DE-AC52-07NA27344.

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Figure list

Figure 1: Regulation of the *SOST* gene in undifferentiated NHBC. Cells were cultured for 3 days alone or in the presence of 1,25D (10 nM), estradiol (E2, 5nM), or PTH (10 nM). Total RNA was prepared and real-time RT-PCR was performed to determine mRNA expression of a range of genes: **A)** *OCN*, **B)** *RANKL*, **C)** *BSPI*, **D)** *SOST*. Data shown are means of triplicate reactions $\pm$ SD normalised to the expression of *GAPDH* mRNA. Statistical difference to the untreated control is indicated by * $p < 0.05$.

Figure 2: 1,25D induces *SOST* expression in human osteocyte-like cells. NHBC differentiated to an osteocyte-like stage as described in Materials and Methods, were **A)** exposed for 3 days to 1,25D at 0.1, 0.3, 1, 3, 10 or 30 nM, or vehicle control. **B)** Osteocyte-like cells were cultured in the presence of 1,25D at 10 nM or vehicle. mRNA was extracted at 24 h, 48 h and 72 h post treatment. **C)** SAOS2 cells cultured under mineralising conditions for 28 days were treated with vehicle control or 1,25D (10 nM) over the time course indicated. Data shown are means of triplicate PCR reactions $\pm$ SD normalised to the expression of a housekeeping gene. Statistical difference to the untreated control is indicated by * $p < 0.05$.

Figure 3: Effects of 1,25D exposure on sclerostin protein expression. **A)** SAOS-2-derived osteocyte-like cells were treated with 1,25D (0.1-100 nM) or recombinant human PTH₁₋₃₄ (50 nM) for 24 hours. Protein lysates were analysed by Western blot analysis and **B)** culture supernatants were analysed by ELISA analysis for sclerostin protein expression. For ELISA, supernatants were diluted 20-fold prior to the assay and values were adjusted accordingly to represent the actual concentration of secreted protein. Data shown are means of quintuplicate biological replicates $\pm$ SEM. Significant difference relative to the untreated control is indicated by * $p < 0.05$.

Figure 4: Effect of 1,25D on gene expression in *ex vivo* cultured bone. Human trabecular bone (**A-C**) and **D**) mouse cortical bone, cultured *ex vivo*, as described in Materials and Methods. Bone samples were cultured in the presence of 1,25D at 0, 1, 10 or 100 nM for 3 days and **A**) *SOST*, **B**) *RANKL* and **C**) *OCN* mRNA expression in human bone and **D**) *Sost* mRNA expression in mouse bone was measured. Data shown are means of triplicate reactions $\pm$ SD normalised to the expression of *GAPDH* mRNA. Statistical difference to the untreated control is indicated by * $p < 0.05$.

Figure 5: Identification and characterisation of a putative *SOST* VDRE. **A**) Predicted DR3 type VDREs (CONSITE algorithm) in the proximal promoters of the *SOST/Sost* genes in human and other species. Conserved bases are indicated by shading and the degree of homology to the single human VDRE is indicated together with the positions of the VDREs in relation to the respective TSS. **B**) Activity of the predicted human *SOST* VDRE. pGL2 expression vector containing the minimum predicted *SOST* VDRE was transfected into HEK293 cells and the cells treated with 1,25D (0-100 nM) for 24 h, and relative dual luciferase levels were measured. A vector containing the mouse osteopontin VDRE was used as a positive control and an empty pGL2-TK was the negative control. **C**) The effect of VDR over-expression on the predicted *SOST* VDRE activity in HEK293 cells transfected as in **B**) with the addition of an empty pBLUESCRIPT vector or the plasmid pVDR. **D**) The minimum predicted *SOST* VDRE binds cell nuclear extract (NE) in EMSA. Binding assays were performed using radiolabeled native predicted *SOST* VDRE with cell lysates from either pVDR or pBLUESCRIPT (negative control) transfected MG-63 cells, which were either untreated or treated with 1,25D (10 nM). **E**) The native predicted *SOST* VDRE minimal sequence and a corresponding *SOST* VDRE sequence where each half-site was disrupted by base substitution (underlined). **F**) Relative luciferase activities of expression vectors containing the human native *SOST* VDRE, mutant *SOST* VDRE and DR3 (positive control). Expression vectors were transfected into HEK293

cells, the cells treated with 1,25D (10 nM) for 24 h, and luciferase activities measured. **G)** EMSA using radiolabeled native predicted *SOST* VDRE or the mutant version with cell lysates from MG-63 cells transfected either $\pm$ pVDR, which were either untreated or treated with 1,25D (10 nM). Data in B), C) and F) represent mean fold-change in firefly luciferase activity $\pm$ SD of quadruplicate transfections. Significant difference relative to the untreated control in each case is indicated by * $p < 0.05$.

Figure 6: The response of the extended human *SOST* promoter to 1,25D in transient expression assays. **A)** The design of pGL3 expression vectors containing either the human native 6333 bp *SOST* promoter (pGL3-h*SOST*-6333-WT) or a mutated sequence (pGL3-h*SOST*-6333-MUT) (mutations underlined). **B)** Relative luciferase activities of expression vectors with the native 6333 bp *SOST* promoter or the VDRE mutant were transiently co-expressed with pRL-TK in HEK293 cells and treated for 24 h with 1,25D (0-100 nM). Data represent mean relative firefly/renilla luciferase activity $\pm$ SEM of six replicate transfections. Significant difference relative to the untreated control in each case is indicated by * $p < 0.05$.

Figure 7: Effect of the ECR5 enhancer on *SOST* promoter activity in response to 1,25D in transient expression assays. **A)** The design of the expression vectors containing the 6216 bp (lacking the predicted *SOST* VDRE; pGL3-3xECR5-h*SOST*-6216-WT) and 6333 bp (pGL3-3xECR5-h*SOST*-6333-WT) fragments (of the native human *SOST* promoter, and the 6333 bp sequence in which both predicted VDRE half-sites were mutated (mutations underlined; pGL3-3xECR5-h*SOST*-6333-MUT). Each promoter fragment was downstream of a triple tandem repeat of the human ECR5 sequence. **B)** Relative luciferase levels of expression vectors transiently co-expressed with pRL-TK in UMR-106 cells and treated for 24 h with 1,25D (1, 3 and 10 nM). Data represent mean relative firefly/renilla luciferase activity $\pm$ SEM of six

replicate transfections. Significant difference relative to the untreated control in each case is indicated by * $p < 0.05$.

Supplementary Figure S1: The response of a 1000 bp region in the *SOST* promoter containing the predicted VDRE site to 1,25D in transient expression assays. **A)** Design of the plasmid pGL2-h*SOST*[-6333;-5333bp] containing a 1000 bp region spanning from -6333 to -5333 bp from the *SOST* TSS. The location of the predicted VDRE site with the cloned fragment is indicated. **B)** Design of the plasmid pGL2-h*SOST*-VDRE-WT containing the minimum predicted VDRE sequence **C)** The plasmids pGL2-h*SOST*[-6333;-5333bp] and pGL2-h*SOST*-WT were transiently co-expressed with pRL-TK in HEK-293 cells and treated for 24 h with 1,25D (0-100 nM). Data represent mean relative firefly/renilla luciferase activity $\pm$ SEM of six replicate transfections. Significant difference relative to the untreated control in each case is indicated by * $p < 0.05$.

Supplementary Figure S2: Enhancing effect of the ECR5 region on the basal activity of the 6333 bp human *SOST* promoter. Expression vectors with the parental vector pGL3, 6333 bp*SOST* promoter and the 6333 bp*SOST* promoter with the 3 tandem repeats of the ECR5 region were transiently co-expressed with pRL-TK in undifferentiated SAOS2 cells. Relative luciferase activity was measured at 48 h post transfection. Fold-changes in basal activity compared to the parental vector is indicated on top of the bars. Data represent mean relative firefly/renilla luciferase activity $\pm$ SEM of six replicate transfections.

Supplementary Figure S3: Comparison of the response of the expression vector pGL3-3xECR5-h*SOST*-6333-WT and the endogenous human *SOST* promoter in undifferentiated SAOS2 cells. pGL3-3xECR5-h*SOST*-6333-WT was transiently co-expressed with pRL-TK in

SAOS2 cells and treated with 1,25D (1,10 and 100 nM), PTH (25, 50 and 100 nM) and a combination of 1,25D (100 nM) and PTH (50 nM) for 24 h. Relative luciferase activity was measured (A). Data represent mean relative firefly/renilla luciferase activity $\pm$ SEM of six replicate transfections. (B) mRNA was extracted from the remaining cell lysate for real-time RT-PCR analysis. Fold induction of endogenous *SOST* mRNA expression normalised to *18S* mRNA levels was measured. Data shown are mean relative expression levels of triplicate PCR reactions $\pm$ SD. Significant difference relative to the untreated control in each case is indicated by * $p < 0.05$.

Figure

Figure 1

Figure 2

Figure 3

Figure 4

A

			5' HS	SP	3' HS		% homology	Position relative to TSS
Human	A	T	G A A A C C	G G A T	G A C C T	T T		-6216
Chimpanzee	A	T	G A A A C C	G G A T	G A C C T	T T	100	-4344
Macaque	A	T	A A A C	A A G A T	G A C C T	T T	80	-7035
Bovine VDRE 1	G	T	A A T C	C A A A	G A C C T	C A	60	-3955
Bovine VDRE 2	C	C	A G C C	T G T T	G A C C T	C T	66	-719
Equine VDRE 1	T	T	A A C C	C T T T	G A A C	C T T	53	-9482
Equine VDRE 2	C	G	T G A A C	T A G A C A	A C C T	T C	60	-5537
Equine VDRE 3	C	A	C A A A A	T T G T T	G A C C	C T C	60	-3133
Canine	G	T	T G A A T	T C C T T	T G T C C	C A T	40	-7035
Mouse	T	G	G G G T C	A A T G G	G A T T C	C C T	26	-712
Opossum	A	G	A G G T C	A T C T A	G C T C	A A A	20	-5157

B

C

D

E

NATIVE hSOST VDRE: 5'-GAAACC GGA TGACCT -3'

MUTANT hSOST VDRE: 5'-GATGTC GGA AACAAT-3'

F

G

Figure 5

A

B

Figure 6

A

B

Figure 7