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Analysis of *SOST* Expression Using Large Minigenes Reveals the MEF2C Binding Site in the Evolutionarily Conserved Region (ECR5) Enhancer Mediates Forskolin, but not 1,25-dihydroxyvitamin D₃ or TGFβ₁ Responsiveness

Hillary C. St John¹, Sydney J. Hansen¹, and J. Wesley Pike^{1,2}

¹Department of Biochemistry, University of Wisconsin-Madison, Madison, WI 53706

Abstract

Transcribed from the *SOST* gene, sclerostin is an osteocyte-derived negative regulator of bone formation that inhibits osteoblastogenesis via antagonism of the Wnt pathway. Sclerostin is a promising therapeutic target for low bone mass diseases and neutralizing antibody therapies that target sclerostin are in development. Diverse stimuli regulate *SOST* including the vitamin D hormone, forskolin (Fsk), bone morphogenic protein 2 (BMP-2), oncostatin M (OSM), dexamethasone (Dex), and transforming growth factor (TGF)β₁. To explore the mechanisms by which these compounds regulate *SOST* expression, we examined their ability to regulate a *SOST* reporter minigene containing the entire *SOST* locus or mutant minigene containing a deletion of the –1 kb to –2 kb promoter proximal region (–1 kb), ECR2, ECR5, or two point mutations in the MEF2 binding site of ECR5 (ECR5/MEF2). Previous reports suggest that both the PTH and TGFβ₁ effects on *SOST* are mediated through ECR5 and that the action of PTH is mediated specifically via the MEF2 binding site at ECR5. Consistent with these reports, the suppressive effects of Fsk were abrogated following both ECR5 deletion and ECR5/MEF2 mutation. In contrast, we found that TGFβ₁ negatively regulated *SOST* and that neither ECR5 nor ECR5/MEF2 was involved. Surprisingly, none of these four deletions/mutations abrogated the suppressive effects of the vitamin D hormone, OSM, Dex, or TGFβ₁, or the positive effects of BMP-2. These data suggest that we need to move beyond ECR5 to understand *SOST* regulation.

Keywords

SOST; MEF2C; ECR5; Osteocyte; BAC

²Please send correspondence to: Professor J. Wesley Pike, Department of Biochemistry, University of Wisconsin-Madison, Hector F. Deluca Laboratories, Room 543D, 433 Babcock Drive, Madison, WI 53706; Phone (608) 262-8229; Fax (608) 263-9609; pike@biochem.wisc.edu.

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Addendum – While this manuscript was in revision, a report by Wijenayaka and colleagues [27] was published that concludes that *Sost* is up-regulated by 1,25(OH)₂D₃.

1. INTRODUCTION

The *SOST* gene product, sclerostin, inhibits osteoblast function and bone formation through antagonizing LRP4/5/6 receptor-mediated Wnt signaling [1–4]. In humans, two very rare diseases are associated with defects in and around the *SOST* gene. Sclerosteosis is the result of mutations within the *SOST* gene [5–7] and van Buchem's disease is caused by a ~53 kb genomic deletion ~35 kb downstream of the *SOST* gene [5, 8, 9]. Consistent with the role of sclerostin as a negative regulator of bone formation, both diseases result in thickened bones throughout the body and symptoms such as facial palsy, hearing loss, and vision difficulties can result from hardening bone damaging cranial nerves [7, 8]. *SOST* genomic deletion mice recapitulate the high bone mass phenotype in humans and overexpression results in osteopenia [10, 11].

Within the van Buchem disease deletion there are several evolutionarily conserved regions (ECRs), of which ECR5 was initially identified as being the most active [12]. A MEF2 binding site within the ECR5 element mediates PTH responsiveness of an ECR5 driven reporter construct and was suggested to be responsible for *SOST* regulation by PTH, although regulation of ECR5 was not linked directly to *SOST* regulation experimentally [13]. Deletion of *Mef2c* in osteocytes results in progressively declining *Sost* expression in cortical bone, suggesting MEF2C is involved in both basal and PTHregulated *Sost* expression [14]. Consistent with the role of ECR5 and its MEF2 component in basal *Sost* expression, mice with deletion of the conserved ECR5 enhancer region have high bone mass due to high rates of bone formation; however, analysis of the effects of this ECR5 genomic deletion on PTH-mediated suppression of *Sost* were not reported [15].

The molecular mechanisms of *SOST/Sost* expression and regulation remain largely unexplored outside of ECR5 and MEF2C. As expected given its osteocyte origins and role in mediating osteoblast function, sclerostin expression is mechanosensitive – decreased following mechanical loading and increased during periods of unloading [16, 17]. Additionally, *SOST* is regulated by cytokines such as transforming growth factor (TGF) β ₁ [18] and oncostatin M (OSM) [19], calcitropic hormones such as the vitamin D hormone (1,25(OH)₂D₃) [20] and PTH [21], bone developmental regulators such as Osterix [22] and bone morphogenic proteins (BMPs) [23], as well as many other natural and synthetic compounds. In this short communication from the proceedings of the 18th Vitamin D Workshop, we employed a bacterial artificial chromosome (BAC) reporter minigene construct to evaluate the contribution of ECR5, the MEF2 site within ECR5, ECR2, and a –1 kb to –2 kb promoter proximal region (–1 kb) to *SOST* regulation by a panel of known *SOST* regulators within the context of the complete *SOST* locus, containing the van Buchem disease deletion and extending from *SOST* to *MEOX1*.

2. MATERIALS AND METHODS

2.1 Reagents

1,25(OH)₂D₃ was obtained from SAFC Global (Madison, WI). Recombinant human BMP-2, CF (355-BM-010/CF), recombinant mouse TGF β ₁ (7666-MB-005), and recombinant mouse OSM (485-MO-025) were purchased from R&D systems (Minneapolis,

MN). Forskolin (Fsk) (F3917) and dexamethasone (Dex) (D4902) were purchased from Sigma (St. Louis, MO). Primers for recombineering were obtained from Integrated DNA Technologies, Inc. (Coralville, IA) and sequences are available upon request.

2.2 BAC Clone Reporter Constructs

A series of large-scale human *SOST* luciferase reporter constructs were established through recombinogenic targeting [24, 25], as described previously [20]. BAC clone RP11-209M4 was purchased through BACPAC Resource Center (Oakland, CA). The targeting construct containing the luciferase reporter was produced by PCR amplification of an IRES-luciferase-TK-neomycin cassette from the pIRESluc plasmid [26]. Galactokinase (galk) positive/negative selection [24] was used to remove an internal NotI site and *MEOX1* (Chr17: 41,717,738–41,738,896) prior to enhancer deletion. The ECR5 deletion removed 252bp of DNA corresponding to chr17: 41,773,921–41,774,172, the ECR2 deletion removed 539bp of DNA corresponding to chr17: 41,784,015–41,784,553, the –1 kb deletion removed 924bp of DNA corresponding to chr17: 41,837,244–41,838,167, and the MEF2C mutation in ECR5 mutated CTATAAATAG to GTATACATAG in ECR5. Primer sequences are available upon request.

2.3 Stable Cell Line Treatments

MC3T3-E1 stable cell lines were established and assayed as described previously [20, 25]. Cells were treated with vehicle, 100nM 1,25(OH)₂D₃, 1μM Fsk, 50μg/mL BMP-2, 25ng/mL OSM, 10nM Dex, or 10ng/mL TGFβ₁.

3. RESULTS

We created a human *SOST*BAC reporter minigene construct containing ~140 kb of the *SOST* locus with *MEOX1* removed as well as mutant BACs in which ECR5, ECR2, or the –1 kb to –2 kb promoter proximal region (–1 kb) had been removed, or a double point mutation in the MEF2 site of ECR5 (ECR5/MEF2). These four constructs were stably incorporated into MC3T3-E1 cells and reporter response was examined following treatment with the vitamin D hormone (1,25(OH)₂D₃), Fsk, BMP-2, OSM, Dex, and TGFβ₁. It has been demonstrated that Fsk and PTH affect *Sost* mRNA levels comparably [21] and as the MC3T3-E1 cell line lacks the PTH receptor, Fsk was used as a substitute PKA activator.

Representative data from one of several stable cell line collections is shown for each BAC construct in Figure 1. Reporter activity of the complete construct was suppressed by 1,25(OH)₂D₃, Fsk, OSM, Dex, and TGFβ₁, while up-regulated by BMP-2. The Dex activity was not statistically significant, although highly reproducible. We demonstrate that ECR5 deletion and the ECR5/MEF2 mutation completely abrogated the inhibitory effects of PTH-signaling (Fsk) on *SOST*. In contrast to a previous report showing that TGFβ₁ up-regulates *Sost* in UMR106.01 cells and activates an ECR5 reporter construct [18], we found that TGFβ₁ down-regulated *SOST* and was not affected by either the ECR5 deletion or ECR5/MEF2 mutation. The ECR2 and –1 kb deletion constructs did not affect response to 1,25(OH)₂D₃, Fsk, OSM, Dex, TGFβ₁, or BMP-2.

4. DISCUSSION

By analyzing BAC clone minigene reporters, which contain the complete *SOST* locus, these studies demonstrate for the first time that dynamic transcriptional regulation of ECR5 is linked directly to *SOST* regulation. Previous studies have examined ECR5 in an isolated or triple repeated (3xECR5) ECR5 reporter construct in which activity and regulation of ECR5 is disconnected to *SOST* [13, 18]. The work presented here confirmed the previously reported model, based upon these isolated ECR5 reporter analyses, that the forskolin responsiveness of *SOST* is mediated through ECR5 and its MEF2 site [13]. The elegant report by Collette and co-workers demonstrates the important link between ECR5 and bone density; however, given the previous work completed on ECR5, evaluation of the PTH effects on *Sost* in the ECR5 KO mouse are noticeably absent [15].

In contrast, our study contradicts another reported regulatory role for ECR5 based upon isolated reporter fragments – regulation of ECR5 by TGF β ₁ [18]. When the *SOST* genomic locus was intact within the BAC context, we found that TGF β ₁ down-regulated *SOST* and that this suppressive effect of TGF β ₁ was not affected by either the ECR5 deletion or ECR5/MEF2 mutation. While these differences may be due to the different species of the host cell line for the isolated or BAC minigene reporter constructs, it more likely reflects the fundamental pitfalls of relying upon isolated reporter constructs that are disconnected from their genomic context.

In addition to analyzing ECR5 and its MEF2C element, we also examined effects of deleting ECR2 and the –1 kb element. Surprisingly, the ECR2 and –1 kb deletion constructs did not affect response to 1,25(OH)₂D₃, Fsk, OSM, Dex, TGF β ₁, or BMP-2. ECR5 has been the focal enhancer of *SOST* regulation studies; however, it is only known to mediate the effects of one of the many regulators of *SOST*. To further our understanding of *SOST* regulation, future studies of *SOST* regulatory enhancers should be performed in context and move beyond ECR5.

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Highlights

- We examined full length and four deletion/mutation *SOST*BAC clone reporters.
- Response to 1,25(OH)₂D₃, OSM, Dex, TGFβ₁, BMP-2, and forskolin was evaluated.
- ECR5 deletion and ECR5/MEF2 mutation eliminated the forskolin response.
- ECR5 deletion and ECR5/MEF2 mutation did not eliminate the TGFβ₁ response.
- 1,25(OH)₂D₃, OSM, Dex, TGFβ₁, and BMP-2 effects were maintained in all reporters.

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