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# OSTERIX AND RUNX2 ARE TRANSCRIPTIONAL REGULATORS OF SCLEROSTIN IN HUMAN BONE

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**ABSTRACT**

Sclerostin, encoded by the *SOST* gene, works as an inhibitor of the Wnt pathway and therefore is an important regulator of bone homeostasis. Due to its potent action as an inhibitor of bone formation, blocking sclerostin activity is the purpose of recently developed anti-osteoporotic treatments. Two bone specific transcription factors, RUNX2 and OSX, have been shown to interact and co-ordinately regulate the expression of bone-specific genes. Although it has been recently shown that sclerostin is targeted by OSX in mice, there is currently no information of whether this is also the case in human cells. We have identified SP-protein family and AML1 consensus binding sequences at the human *SOST* promoter and have shown that OSX, together with RUNX2, binds to a specific region close to the transcription start site. Furthermore, we show that OSX and RUNX2 activate *SOST* expression in a co-ordinated manner *in vitro* and that *SOST* expression levels show a significant positive correlation with *OSX/RUNX2* expression levels in human bone. We also confirmed previous results showing an association of several SOST/RUNX2 polymorphisms with bone mineral density.

**KEYWORDS.** OSTERIX, RUNX, Sclerostin, SOST, Human, bone mineral density, polymorphisms.

## INTRODUCTION

Bone tissue homeostasis depends on the balance between bone destruction (resorption) and bone formation, and requires the coordinate activity of three different types of cells: osteoclasts (bone resorbing cells), osteoblasts (bone forming cells) and osteocytes (cells detecting mechanical loads applied to the bone). The alteration of the coordinate activities of these of cells can lead to osteoporosis and other diseases. Osteoporosis is characterized by a low bone mineral density (BMD) and alteration of bone microarchitecture, leading to low bone resistance and therefore to a higher risk of fracture. The treatment of bone fracture due to osteoporosis implies a high cost to public health. Contrary to osteoporosis, two other bone dysplasias, sclerosteosis and Van Buchem's disease, are characterized by an increased bone formation [1, 2]. The studies of these two diseases led to the discovery of sclerostin, a protein encoded by the *SOST* gene. Sclerosteosis is associated to mutations in the *SOST* gene that, either introduce stop codons or affect *SOST* gene splicing [3, 4] leading to reduced sclerostin levels and increased bone formation, whereas Van Buchem´s disease is linked to the deletion of a 52 kb of a noncoding region harbouring the *SOST* ECR5 enhancer[5].

In humans, sclerostin is mainly produced and secreted *in vivo* by osteocytes and other terminally differentiated cells immersed in a mineralized matrix (such as cementocytes or hypertrophic chondrocytes) [6]. Sclerostin binds to the low-density lipoprotein receptor related proteins 4, 5 and 6 (LRP4/5/6), which inhibit the Wnt signalling pathway [7]. Although the role of sclerostin as an antagonist of the Wnt pathway has been thoroughly studied, there is currently scarce information about the molecular mechanisms and transcription factors regulating sclerostin expression in humans. This is largely due to the lack of human osteocytic cell lines and the difficulty to obtain primary osteocytes cultures. However studies in murine models have revealed that two different transcription factors, Runt-related transcription factor 2 (RUNX2) [8] and, more recently, Osterix (OSX) [9], regulate sclerostin expression. OSX, also known as transcription factor SP7 is an osteoblast-specific transcription factor, belonging to the SP/KLF family, known to exert its regulatory function through the binding to guanine-rich sequences at specific target genes. On de other hand, RUNX2 is an essential regulator of the osteoblastic function. This protein binds to specific DNA

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sequences located at the promoter regions of its target genes and control their transcription. Recently, it has also been shown that RUNX2 can interact with OSX, to co-ordinately induce the expression of the *COL1A1* gene encoding the main component of collagen type I [10]. On the other hand, the importance of OSX in bone formation has been highlighted by the fact that *Osx*-null embryos completely lack bone formation [11]. Using a murine model, Yang et *al.* (2010) showed that OSX controls the transcriptional levels of *Sost* through its direct interaction with the *Sost* promoter [9]. It has not been confirmed yet whether this gene-gene interaction is preserved in human cells.

The binding of regulatory proteins to promoter regions can be affected by genetic factors. Single nucleotide polymorphisms (SNPs) can directly alter regulatory sequences in the promoter of a given gene and therefore modify the expression levels of that gene, the folding of the mRNA or the subcellular localization of the protein, which may then influence tissue homeostasis. *RUNX2*, *OSX* and *SOST* encode for proteins with an important role in bone homeostasis. These three genes also share the fact that they harbour, in their DNA sequences, SNPs that have been associated with BMD [12-16]. Indeed, our group, amongst others, has shown an association between some SNPs located at the *SOST* promoter and BMD[16]. On the other hand, a recent meta-analysis confirmed a relationship between SNPs located at the *SOST* and *OSX* promoters and BMD [17]. In this current work, we aimed to confirm whether OSX and RUNX2 bind to the *SOST* promoter and regulate *SOST* expression in humans. Additionally, we explored if there was an interaction between the genetic variants of these genes on BMD.


## MATERIALS AND METHODS

### Plasmids

Human *SOST* promoter reporter constructs were generated by PCR and subcloned into the pGL2 basic vector. Human *RUNX2* expression vector was kindly provided by Dr. Svante Paabo. Human *OSX* expression vector was purchased from Origene (Clone OHu19040).


### Transfection of expression and reporter vectors

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The human HEK-293T cell line was transfected with *SOST* reporter vectors, transcription factor expression vectors (or empty control vectors) and a β-galactosidase control reporter using the Lipofectamine 3000 reagent (Thermofisher Scientific, Waltham, MA, USA). After 48 hours, the cells were harvested and lysed. Luciferase activity was quantified using the Luciferase 1000 assay system 45050 (Promega, Madison, WI, USA). Luciferase values were normalized using β-galactosidase activity measured with Galactosidase Reporter Gene Assay System, t1007, (Thermofisher Scientific).

**Chromatin Immunoprecipitation**

SaOS-2-derived sclerostin superproducer cells were maintained in culture as previously described [18]. Chromatin immunoprecipitation (ChIP) was performed using the Red ChIP Kit (Diagenode, Seraing, Belgium) following the instructions of the manufacturer. Crosslinked cells were sonicated for 15 cycles (30s on/30s off) with the Bioruptor (Diagenode, Seraing, Belgium). The antibodies used included anti-RNA Polymerase (H-224 from Santa Cruz Biotechnology, Dallas, TX, USA), anti-Osx antibody (ab22552 Abcam, Cambridge, UK), anti-Runx2 antibody (d1l7f cell signaling, Danvers, MA, USA) and a control antibody against rabbit IgG (SC-2027 Santa Cruz Biotechnology). Ten million cells were used for each immunoprecipitation. DNA concentration values for each ChIP and input DNA sample were calculated from their respective average cycle threshold values in real-time quantitative PCR (qPCR) analyses. Graphs represent percentage of input values. All reactions were performed in triplicate with samples derived from three experiments.

**Gene expression**

RNA was isolated from trabecular bone samples of the femoral heads of patients undergoing hip replacement surgery (*n*=21) and cDNA synthesis was performed as previously described [19]. *SOST* expression levels were measured by qPCR using the Taqman assay Hs00228830_m1 (Thermofisher Scientific). Analysis of the expression of *OSX* and *RUNX2* was performed using the Exiqon universal probe library and primer designer (Roche). All expression data were

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relative to the housekeeping gene encoding TATA Box Binding Protein (*TBP*) as $2^{-\Delta ct}$. Primer sequences are available upon request.

**Genetic association analysis**

We wanted to analyse if there is an interaction between different polymorphisms in these three genes, *SOST, RUNX2* and *OSX*, that have been previously associated to variations in BMD. In brief, we selected a group of 987 postmenopausal women (either healthy controls or women with primary osteoporosis) and lumbar spine BMD was measured with a Hologic QDR 4500 densitometer. We analysed the following SNPs: *SOST*, rs851056; *RUNX2*, rs2819854 and rs7771980; *OSX*, rs2016266. All have been associated with skeletal traits in previous studies [14, 15, 17, 20]. They were analysed by using predesigned Taqman assays (Thermofisher Scientific), following manufacturer's instructions.

**Statistics**

Quantitative data are expressed as mean±SEM. Statistical comparisons of data sets were performed with the two-tailed Student's t test. The correlations of gene expression levels were assessed using the Pearson´s correlation coefficient after log-transformation of the relative expression values. BMD values were adjusted for age and weight. Differences were considered significant when p values <0.05.

**RESULTS**

**Osterix targets *SOST* gene in humans**

In order to determine if endogenous Osterix binds to the native *SOST* promoter in humans, we performed ChIP experiments. Since sclerostin expression is rarely detected in human osteoblasts, we used an osteoblastic cell line, previously characterized by our group, that produces high levels of sclerostin, as the source of chromatin [18]. *In silico* analysis of the promoter region of *SOST* (-1500/+1) showed four putative SP1 binding sites (5´-(G/T)GGGCGGPuPu(C/T)-3` located at positions -1152, -1145, -317 and -124. Since Osterix, also known as SP7 and a member of the SP-family of proteins,

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has been previously shown to bind to SP1 consensus sequences [10], we designed different oligonucleotide pairs to amplify the regions surrounding these putative SP1 binding sites located at the *SOST* promoter. The region previously identified in mouse as an OSX binding site [10] is only partially conserved in the human sequence, and no binding site for SP1 is predicted in this specific sequence *in silico* at the human promoter. In addition to OSX, RUNX2 is also essential for bone formation. RUNX2 is a sequence-specific transcription factor that recognizes the sequence 5´-PuACCPuCA-3´or its complementary sequence [21, 22]. This sequence is likewise recognized for other members of the Runt-related transcription family proteins, and is generally known as AML1 putative binding site. Besides the four putative SP1 binding sites, the *in silico* analysis also detected three putative AML1 binding sites (-1044, -752, -323), one of them located near the predicted SP1 site at position -317 (Figure 1A, upper panel). We therefore decided to perform an additional ChIP analysis using anti-RUNX2 antibodies, in order to confirm the binding of RUNX2 to this region of the human *SOST* promoter. We detected a significant level of enrichment when the amplification of the fragments pulled down with the RUNX2 and OSX antibodies was performed with oligonucleotide pair number 5. This enrichment was also significant when using oligonucleotide pair 6 for the amplification, but only when the sheared chromatin is pulled down with the anti-RUNX2 antibody (Figure 1A, lower panel). According to our results, the DNA region showing high levels of enrichment for both OSX and RUNX2 in the ChIP contains both a putative SP-family protein binding site (at -317) and a putative RUNX2 binding site in close proximity (at -323), as has been previously shown for the *COL1A1* gene in human cells[10]. No enrichment was detected when the DNA pulled down was amplified with oligonucleotide pairs 3 and 4, suggesting that the putative AML1 binding site located at -752 is not actually functional. A low level of enrichment for both OSX and RUNX2, although not statistically significant, was also detected in the 5´end of the promoter sequence, analysed using oligonucleotide pair 2 (Figure 1A, lower panel). Although this enrichment was not significant and the two target sequences for RUNX2 and OSX are located farther apart from each other than those located in a more proximal position, we cannot completely discard a possible binding of these two proteins to their putative target sites predicted *in silico* and therefore a role of this region in the activation of the

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human *SOST* transcription. However, our results clearly indicate that, similarly to what has been shown in mice, both OSX and RUNX2 bind to the promoter of the human *SOST* gene in a region proximal to the transcription start site (TSS). As an additional control to assess the performance of the procedure, a parallel immunoprecipitation using an antibody against a structural Histone (Histone H3) was used at all points (Figure 1, lower panel).

**RUNX2 and OSX co-operatively regulate the expression of human sclerostin**

Having established that both RUNX2 and OSX bind to the promoter of the human *SOST* gene, our next goal was to study whether these two proteins functionally interact and regulate *SOST* expression in human cells as has been shown for the *COL1A1* gene. To this end, we produced a reporter vector carrying the firefly luciferase gene under the control of the human *SOST* promoter (-1440/+1). This construct was used to transfect the human HEK-293T cell line, with or without *OSX*- and *RUNX2*-expression vectors and the corresponding controls. Our results (Figure 2A) show that both RUNX2 and OSX significantly stimulated the human *SOST* promoter, leading to an increase in the reporter gene expression. Interestingly, there was an additive effect when both transcription factors were transfected, which enhanced luciferase activity 1.5-fold in comparison with the effect of each individual transcription factor (Figure 2A).

**Expression of SOST, OSX and RUNX2 in human bone**

In order to explore whether the results obtained with the reporter vectors reflected the situation *in vivo*, we analysed the expression of *RUNX2*, *OSX* and *SOST* in human bone. Although there was a high variability between samples regarding the expression levels of the three genes, we found a significant positive correlation between the expression levels of *OSX* and *SOST* (r=0.55, $p$=0.01) and also between the expression levels of *RUNX2* and *SOST* (r=0.47, $p$=0.03) (Figure 2B). On the contrary, as expected, there was no correlation between *OSX* and *RUNX2* expression levels (r=0.19, $p$=0.43) (Figure 2B).

**Interaction between *RUNX2*, *OSX* and *SOST* allelic variants and bone mineral density**

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We wanted to analyse if there was an interaction between several polymorphisms of these three genes, *SOST*, *RUNX2* and *OSX*, that have been previously associated to variations in BMD. All genotypes were consistent with Hardy-Weinberg equilibrium. *OSX* alleles were not significantly associated with BMD (Table 1). Both *RUNX2* alleles showed a trend for association with age and weight-adjusted BMD (rs2819854, p=0.04; rs7771980, p=0.09). These loci are located 15.2 kb apart and are in strong linkage disequilibrium. Nevertheless, when alleles at both loci were combined into a single score, the number of risk alleles (the minor alleles) showed a linear trend for association with BMD. Thus, the average lumbar spine BMD values for women with 0,1,2 or ≥3 risk alleles were 0.890±0.008, 0.865±0.007, 0.873±0.009 and 0.822±0.023 g/cm$^2$, respectively (*p*=0.022). The previously reported association of the rs851056 polymorphism of the *SOST* promoter with BMD was confirmed. However, no statistically significant interaction between *SOST* and *RUNX* alleles was found.

## DISCUSSION

In the present work, we aimed to explore the role of OSX in the regulation of human *SOST* expression and its relation with the osteogenic transcription factor RUNX2. Our results show that both OSX and RUNX2 bind to the human *SOST* promoter in a region close to its TSS (Figure 1A). A more distal region also shows a small level of enrichment, suggesting the existence of additional regulatory regions at the human *SOST* promoter that could also be targeted by these two factors. The region that shows a high percentage of enrichment for both RUNX2 and OSX harbours indeed two putative SP1 binding sites, previously shown to be targeted by OSX [10], one of them closely located to a RUNX2 binding site also found in the enriched region (Figure 1A). The close proximity of the RUNX2 and OSX binding sites resembles the disposition of the confirmed OSX and RUNX2 binding sites at the human *COL1A1* promoter [23]. Our results further supports the idea that these two factors could bind to adjacent sites and also physically interact, as previously demonstrated for the COL1A1 [10] and osteocalcin genes [24]. Our results provide evidence that RUNX2 and OSX similarly induce the activity of the human *SOST* promoter. Furthermore, using a luciferase reporter system, we show that binding of both OSX and RUNX2 to the *SOST* promoter, increases luciferase activity, but more

importantly, that the action of these two transcription factors has a cooperative effect on *SOST* transcription, co-ordinately achieving significantly higher *SOST* expression levels that those obtained with the individual expression vectors (Figure 2A). These results indicating a crucial role of RUNX2 and OSX in the activation of the human *SOST* gene are further highlighted by the fact that expression levels of these two factors are positively correlated with *SOST* expression levels in human bone, as we show in Figure 2B. *RUNX2* is already expressed in human mesenchymal stem cells, the precursors of cells in the osteoblastic lineage, whereas *OSX* is only expressed once the cells have matured into pre-osteoblasts. The subsequent differentiation of the pre-osteoblasts into the mature osteoblasts would require the concerted action of both *RUNX2* and *OSX*. Therefore, in view of these results we suggest that both *OSX* expression and *RUNX2* expression, are limiting factors for a correct transcriptional activation of the human *SOST* endogenous gene. We were not able to perform ChIP or reporter vector experiments using human osteocytes. However, the correlation of *SOST* and *OSX/RUNX2* expression in bone fragments (in which osteocytes are the major cellular component) suggests that a similar stimulatory interaction between OSX/RUNX2 and *SOST* indeed takes place in human osteocytes.

There are several forms of *RUNX2* transcripts, which depend of two different promoters (P1 and P2). Both are active in cells of the osteoblastic lineage, but the predominant form may vary with the differentiation stage [13, 25]. In this study we found an association of polymorphisms located in the region of promoter P2 with BMD. This observation is in line with previous reports showing an association of P2 polymorphisms with several skeletal phenotypes [12-14, 26, 27]. However, the underlying mechanisms have not been elucidated. In view of our results, it could be speculated that the polymorphisms regulate the transcriptional activity of *RUNX2* P2, and consequently, *SOST* expression. Further studies are needed to confirm this concept. Although we confirmed the association of *RUNX2* and *SOST* polymorphisms with BMD, we did not find statistical evidence for interaction. The relatively small sample size limits the power of our study. On the other hand, given the exploratory nature of this study, we did not apply multiple test corrections. Therefore, the improved association of RUNX2 SNPs when they are used in combination will need replication in other

cohorts. Of course, RUNX2 modulates not only *SOST* but also other genes related to bone formation [28, 29], and they could be involved in the association of *RUNX2* alleles with BMD. It is also possible that other *RUNX2* polymorphisms in linkage disequilibrium with those studied here are the actual drivers of the association.

Additionally, it is worth noting that the regulation of sclerostin is complex. A number of factors, including OSX and RUNX2, shown in this study, but also vitamin D, TGFβ and BMPs act through the binding to motifs in the 5' region of the [30, 31]. However, elements located distal to the *SOST* gene, such as the ECR5 sequence included in the so-called Van Buchem´s region, seem to mediate the regulatory effects of factors such as parathryroid hormone and MEF2 on sclerostin production [32]

In conclusion, our results confirm that RUNX2 and OSX modulate the transcription of the sclerostin human gene and common *RUNX2* polymorphisms may influence bone mass by still unknown mechanisms.

**FIGURE LEGENDS**

**Figure 1.- RUNX2 and Osterix binding to the human SOST promoter.** (A) Upper panel depicts *SOST* promoter region, Exon 1 and Transcription Start Site (TSS). Oligonucleotide pairs used for the amplification of the different regions are indicated. Solid bars indicate putative biding sites for RUNX2 (black) and OSX (grey). Dashed bar indicates the sequence correspondent to Osx biding site in mouse (not preserved in the human DNA sequence). Position of each of these putative binding sites respect to the TSS is shown.  Left lower Panel shows ChIP analysis of RUNX2, OSX binding and RNA Pol II presence at the *SOST* promoter in human primary osteoblasts. Rabbit IgG was used a negative control. Right lower panel shows the results of a ChIP analysis performed in parallel, using an antibody against histone H3 as a positive control of the procedure. Data was obtained by subtracting IgG control values from the corresponding antibody values. Graphs represent percentage of input DNA. Graph shows average values from three different experiments. Bars represent standard error of the mean values. *P* values are as follows, (***, P≤0.0005; **, P≤0.005; *, P≤0.05). *P* values are always referred to those of the Isotype control (Rabbit IgG antibody).

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**Figure 2.- Association of RUNX2 and OSX with SOST expression.** (A) Induction of SOST transcriptional activity by binding of OSX and RUNX2 to its promoter region. HEK-293T cells were transiently co-transfected with SOST-proximal promoter reporter vectors and mock or OSX construct and/or RUNX2 construct for 48h. Luciferase activity was measured and normalized against β-galactosidase activity. Relative luciferase activities were expressed as mean ± SEM of luciferase activity normalized by β-galactosidase values from four independent experiments. (B) Correlation analysis of mRNA expression of SOST/RUNX2, SOST/OSX and RUNX/OSX mRNAs in human bone tissue. There was a significant positive correlation between SOST gene expression and RUNX2 gene expression. Similarly a positive correlation was found between SOST and OSX expression. No significant correlation was found between OSX and RUNX2. Gene expression values are shown in relative fluorescence units.

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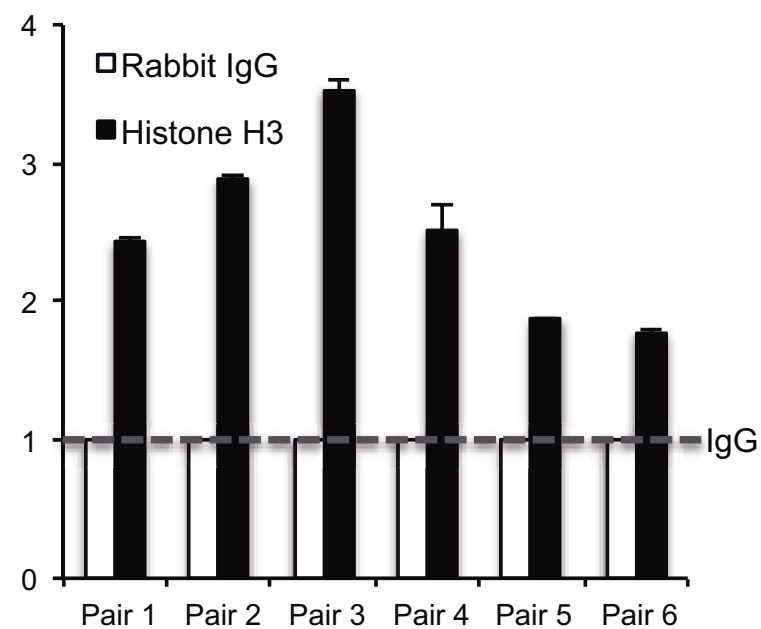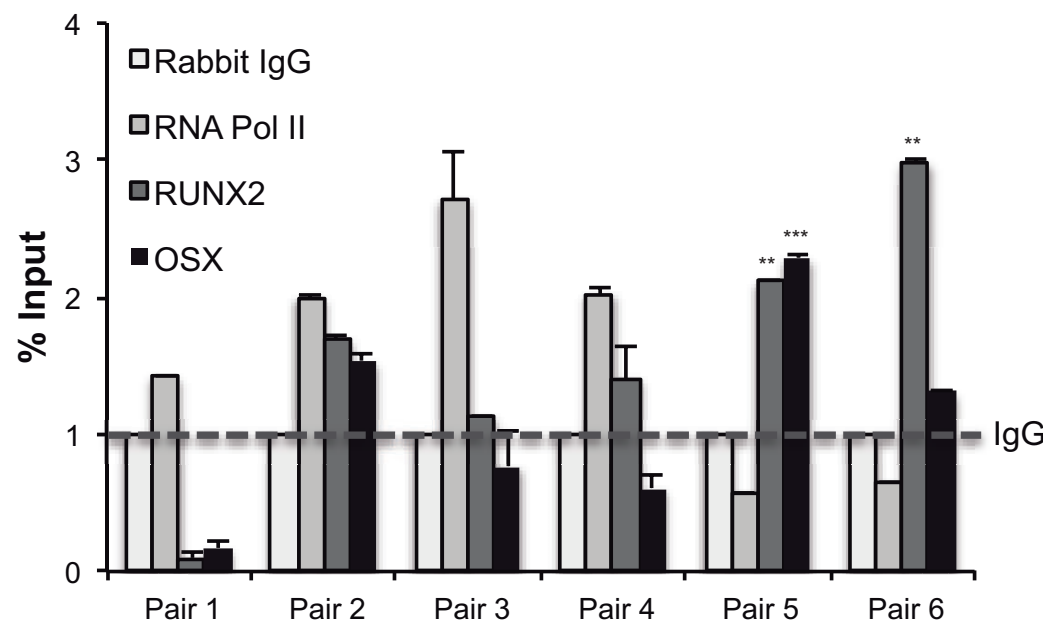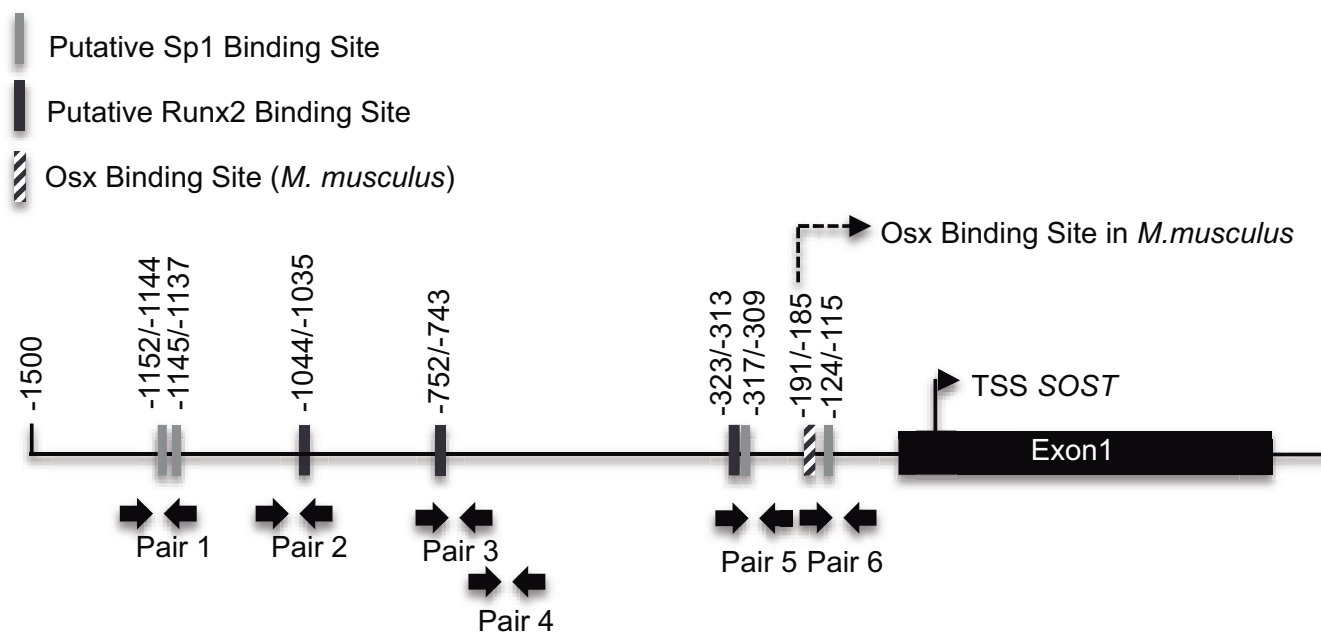[29]    Komori T. Regulation of osteoblast differentiation by Runx2. Adv Exp Med Biol 2010;658: 43-9.

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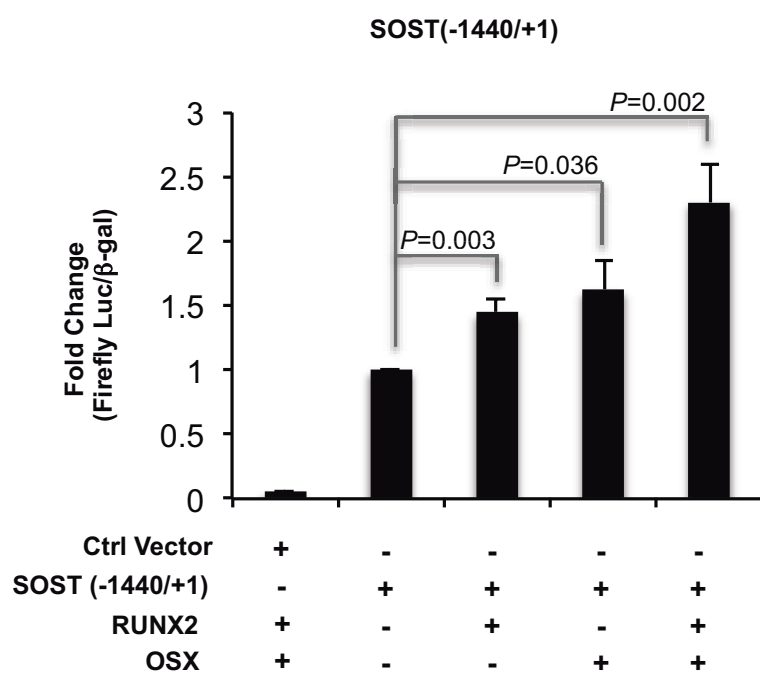
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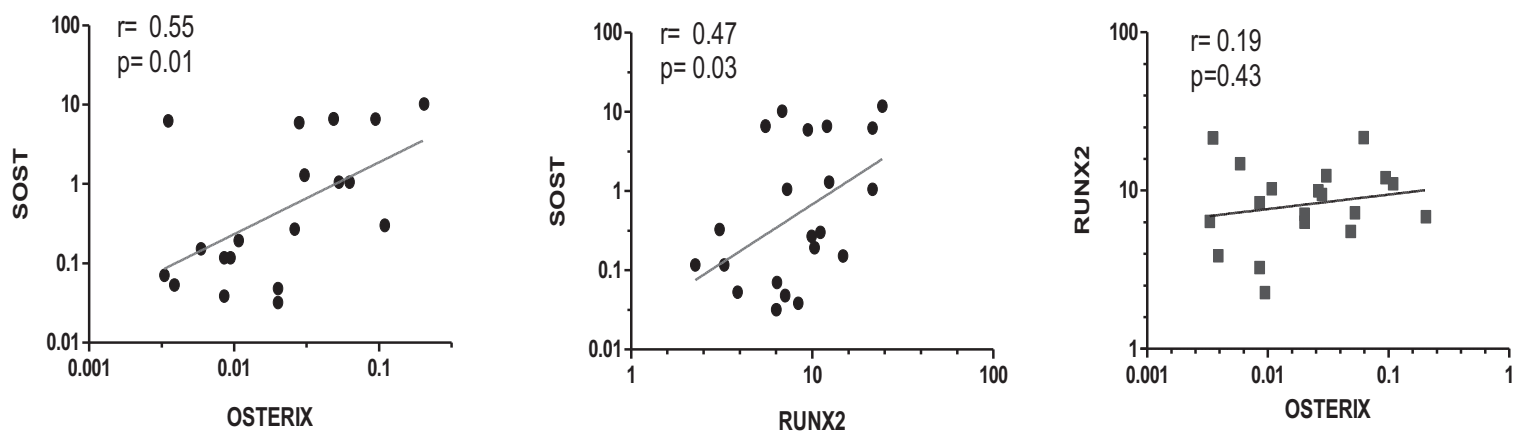
Perez-Campo Figure 2

# A

SOST(-1440/+1)



| | | | | | |
|---|---|---|---|---|---|
| **Ctrl Vector** | + | - | - | - | - |
| **SOST (-1440/+1)** | - | + | + | + | + |
| **RUNX2** | + | - | + | - | + |
| **OSX** | + | - | - | + | + |

# B

**Table 1.-** Genetic Association analysis. Interaction between different polymorphisms at the RUNX2, OSX and SOST genes.

| SNP | GENE | ALLELES | MINOR ALLELE | [a]MAF | [b]MAF-1000G | [c]HWE-p | [d]p | [e]adj-p |
|---|---|---|---|---|---|---|---|---|
| rs2016266 | OSX | A>G | G | 0.340 | 0.344 | 0.72 | 0.259 | 0.143 |
| rs2819854 | RUNX2 | C>T | T | 0.480 | 0.475 | 0.140 | 0.103 | 0.042 |
| rs7771980 | RUNX2 | C>T | C | 0.050 | 0.079 | 0.75 | 0.076 | 0.094 |
| rs851056 | SOST | C>G | G | 0.380 | 0.414 | 0.250 | 0.035 | 0.032 |

[a] **MAF:** minor allele frequency in this series.

[b] **MAF-1000G:** minor allele frequency in the 1000 Genomes project.

[c] **HWE-p:** p-value of the HWE equilibrium test.

[d] **P:** Unadjusted association with BMD.

[e] **Adj-p:** Age- and weight-adjusted association with BMD.

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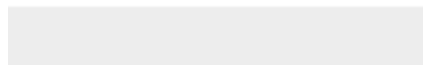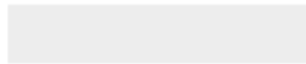**ICMJE Conflict of Interest Form**
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