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Methylation of bone SOST impairs SP7, RUNX2, and ER α transactivation in patients with postmenopausal osteoporosis

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Complete List of Authors:	Shan, Yu; Department of Orthopaedics, the Second Affiliated Hospital of Soochow University; the First Peoples' Hospital of Wujiang,, Department of Orthopedics Wang, Liang; Department of Orthopaedics, the Second Affiliated Hospital of Soochow University Li, Guangfei; Department of Orthopaedics, the Second Affiliated Hospital of Soochow University Shen, Guangsi; Department of Orthopaedics, the Second Affiliated Hospital of Soochow University Zhang, Peng; Department of Orthopaedics, the Second Affiliated Hospital of Soochow University Xu, Youjia; Department of Orthopaedics, the Second Affiliated Hospital of Soochow University
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1 **Methylation of bone *SOST* impairs SP7, RUNX2, and ER α transactivation in**
2 **patients with postmenopausal osteoporosis**

3 Yu Shan^{a,b,1}, Liang Wang^{a,1}, Guangfei Li^a, Guangsi Shen^a, Peng Zhang^a, Youjia Xu^{a*}

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5 **Affiliations:**

6 ^aDepartment of Orthopedics, the Second Affiliated Hospital of Soochow University,
7 Suzhou 215004, China

8 ^bDepartment of Orthopedics, the First People's Hospital of Wujiang, Suzhou 215300,
9 China

10 ¹ These authors contributed equally to the manuscript.

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17 *Correspondence to: Youjia Xu

18 Email: xuyoujia@suda.edu.cn

19 Telephone: +86-0512-67783346

20 Fax: +86-0512-67783346

21 **Abstract**

22 Sclerostin (SOST), a glycoprotein predominantly secreted by bone tissue osteocytes, is an
23 important regulator of bone formation, and loss of *SOST* results in Van Buchem disease.

24 DNA methylation regulates *SOST* expression in human osteocytes, although the detailed
25 underlying mechanisms remain unknown. In this study, we compared 12 patients with

26 bone fractures and postmenopausal osteoporosis with eight patients without

27 postmenopausal osteoporosis to understand the mechanisms via which *SOST* methylation

28 affects osteoporosis. Serum and bone *SOST* expression was reduced in patients with

29 osteoporosis. Bisulfite sequencing-polymerase chain reaction (PCR) revealed that the

30 methylation rate was higher in patients with osteoporosis. We identified osterix (SP7),

31 Runt-related transcription factor 2 (RUNX2), and estrogen receptor α (ER α) as candidate

32 transcription factors activating *SOST* expression. Increased *SOST* methylation impaired

33 the transactivation function of SP7, RUNX2, and ER α in MG-63 cells. AzadC treatment

34 and *SOST* overexpression in MG-63 cells altered cell proliferation and apoptosis.

35 Chromatin immunoprecipitation showed that higher methylation was associated with

36 reduced SP7, RUNX2, and ER α binding to the *SOST* promoter in patients with

37 osteoporosis. Our studies provide new insight into the role of *SOST* methylation in

38 osteoporosis.

39 Key words: sclerostin, osteoporosis, DNA methylation, transcription factor.

Introduction

Sclerostin (SOST) is a glycoprotein predominantly secreted by bone tissue osteocytes, and is encoded by *SOST* in humans and other vertebrates (BALEMANS *et al.* 2001; BRUNKOW *et al.* 2001). SOST is an important regulator of bone formation, and loss of *SOST* results in Van Buchem disease (LOOTS *et al.* 2005). Osteoporosis (OP) is a significant aging-associated disease that is characterized by reduced bone mineral density and abnormal bone microarchitecture (CAULEY 2006; ARAKAKI *et al.* 2011). Recently, the disease has also been reported to affect juveniles (ALEJANDRO and CONSTANTINESCU 2017). Studies have revealed an association between OP and altered *SOST* expression. Single nucleotide polymorphisms (SNPs) in the *SOST* promoter correlate significantly with bone mass density (BMD) and OP pathogenesis (REPPE *et al.* 2015; LHANECHÉ *et al.* 2016). Clinically, postmenopausal women tend to be more frequently diagnosed with OP, which is termed postmenopausal osteoporosis (PO). Altered *SOST* expression has also been observed in patients with PO (REPPE *et al.* 2015).

SOST regulates bone formation via the canonical Wnt/ β -catenin signaling pathway (LI *et al.* 2005; TEN DIJKE *et al.* 2008), and its expression has been linked to various transcription factors. Three potential estrogen response elements (EREs) have been identified in the *SOST* promoter region, and estrogen negatively regulates *SOST*

expression through estrogen receptor (ER) β (GALEA *et al.* 2013). Transcription factors osterix (SP7) and Runt-related transcription factor 2 (RUNX2) are both important for osteocyte differentiation and bone formation (SEVETSON *et al.* 2004). In the Saos-2 cell line, SP7 and RUNX2 increase *SOST* expression, and SP7 and RUNX2 binding sites were identified in the *SOST* promoter (REPPE *et al.* 2015). Bone morphogenetic protein 2 (BMP2) regulates *SOST* expression via the BMP signaling pathway, phosphorylating SMAD family members 2 and 3 to increase *SOST* expression. Accordingly, SMAD2/3 binding sites have also been identified in the *SOST* promoter (KAMIYA 2012).

The epigenetic mechanisms of gene expression regulation have been investigated for many years. DNA methylation is a process via which methyl groups are added to CpG sites in the gene promoter region, which usually form clusters called CpG islands (BELL *et al.* 2012). CpG island methylation impairs the binding of transcription factors to promoters, resulting in transcriptional repression (HAN and ZHAO 2009). DNA methylation is essential for normal development and is associated with several key processes, including genomic imprinting, X-chromosome inactivation, repression of repetitive elements, aging, and carcinogenesis (JONES and TAKAI 2001). However, information regarding the effects of DNA methylation during bone remodeling is limited, and the mechanism via which it affects bone differentiation and bone diseases such as OP remains unclear. Delgado-Calle *et al.* reported that alteration in *SOST* methylation regulates the osteoblast-osteocyte transition (DELGADO-CALLE *et al.* 2012). Reppe *et al.* observed that methylation of bone *SOST* correlated strongly with the risk of fracture in

postmenopausal women (REPPE *et al.* 2015). This indicates that *SOST* methylation is related to bone diseases, such as OP. However the detailed mechanism via which DNA methylation regulates *SOST* expression in OP remains unknown.

In this study, we used patients with hip fractures and PO as the experimental group, and patients with hip fractures without PO as the control group to study the mechanisms of *SOST* methylation and its association with OP.

Materials and methods

Primary human samples

Patients with hip fractures were admitted to our clinic. We selected 12 patients with PO as the experimental group and 8 patients without PO as the control group. Patient information is provided in the supplementary tables. Blood and bone tissue samples were collected during Proximal proximal femoral Nail antirotation implantation into the proximal femur. The local ethical review committee and the faculty of the First People's Hospital of Wujiang District approved this study.

SOST enzyme-linked immunosorbent assay (ELISA)

ELISA was performed to quantify the serum SOST level according to the manufacturer's instructions. In brief, microplates precoated with a monoclonal mouse anti-human antibody against SOST were first blocked overnight with 1% bovine serum albumin at 4°C, then inoculated with serially-diluted standards or samples, and finally incubated with a horseradish peroxidase-conjugated polyclonal antibody against SOST. Four washes with Tris-buffered saline containing 0.05% Tween 20 were performed

between each incubation period. The optical density of each well was immediately determined at 450 nm.

Sample processing, mRNA extraction, and quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Total RNA was extracted from bone tissue using TRIzol reagent (Invitrogen) according to the manufacturer's instruction. The RNA (5 µg) was transcribed to cDNA using the first strand cDNA synthesis kit (Takara), and qRT-PCR was performed in an ABI StepOnePlus instrument with the SYBR Green detection system (Invitrogen). PCR involved 40 cycles of 10 s at 95°C and 30 s at 60°C. Experiments were performed in triplicate. All results were normalized to the expression of the housekeeping gene β -actin. The results of qRT-PCR are shown as the relative expression level, calculated using the $2^{-\Delta\Delta CT}$ method (VANGUILDER *et al.* 2008).

Bisulfite sequencing-PCR (BSP) analysis

Genomic DNA was extracted from bone tissue using the genomic DNA isolation kit (Qiagen), and treated with bisulfite conversion reagents of the MethylCode™ bisulfite conversion kit, (Invitrogen, Life Technologies) according to the manufacturer's instructions. Bisulfite-treated DNA was amplified using PCR with BSP-PCR primers designed using the Methyl Primer Express software v1.0 (Table 1). The PCR products were cloned into the pMD-19T vector (Takara) for sequencing with the M13 primer. Ten clones were sequenced for each patient. DNA methylation levels in each sample were

determined using the following formula: (methylated CpG sites/total CpG sites) $\times$ 100.

Luciferase activity assay

A segment of the 2 kb *SOST* promoter was cloned into pGL4.17 (Promega) to generate a *SOST*-luc vector. SP7, RUNX2, and ER α cDNAs were cloned in the pcDNA3.1-myc/His plasmid. The primers used are listed in Supplementary Information. Owing to difficulties in culturing MG-63 cells and low transfection rate, we used easy-to-culture 293T cells with high transfection efficiency for this assay. The reporter and gene expression vectors were co-transfected into 293T cells. After 24 h, luciferase activity was measured using a dual-luciferase reporter assay system (Promega).

***In vitro* methylation of pGL4.17-SOST plasmid reporters**

pGL4.17-SOST plasmids were cytosine-methylated using SssI methylase (New England BioLabs), which methylates all cytosine residues within the double-stranded dinucleotide recognition sequence (59-CG-39), in a buffer containing 10 mM Tris, pH 7.9, 50 mM NaCl, 10 mM MgCl₂, 1 mM dithiothreitol, and 160 mM S-adenosylmethionine at 37°C for 1 h. After methylation, the plasmids were purified by phenol extraction. Successful vector methylation was verified by analyzing the band patterns of the McrBC-digested methylated plasmid DNA after gel electrophoresis according to the manufacturer's instructions (New England BioLabs). Completely methylated plasmids were utilized in transient transfection assays.

Chromatin immunoprecipitation (ChIP)

ChIP was performed using a ChIP assay kit (Millipore) according to the manufacturer's

protocol. Briefly, MG-63 cells were collected after 24 h culture, cross-linked with 2% formaldehyde at 28°C for 30 min, and then treated with a 1/10 volume of 1.25 M glycine to stop the cross-linking reaction, followed by three 10-min washes with phosphate-buffered saline (PBS). Antibodies against SP7, RUNX2, and ER α (Abcam) were used to immunoprecipitate DNA, and purified mouse IgG (Invitrogen) used as the negative control. We performed ChIP PCR using primers flanking the SP7, RUNX2, and ER α binding sites, as well as primers against regions elsewhere in the *SOST* promoter as controls. The primers used for ChIP PCR are listed in Supplementary Table 1. Subsequently, qRT-PCR was performed to determine the binding ability of each transcription factor for the *SOST* promoter.

Proliferation assays

Cell proliferation was analyzed using cell counting kit (CCK)-8 (Dojindo). After 2 days of AzadC treatment, the MG-63 cells were incubated for an additional 24 h. The absorbance was measured at 450 nm using a BioTek microplate reader.

Apoptosis assay

Apoptosis of MG-63 cells was analyzed using the transferase-mediated deoxyuridine triphosphate-biotin nick end labeling (TUNEL) apoptosis detection kit (Beyotime). Cells were collected 2 days after AzadC treatment, washed with PBS, and suspended in 500 μ L binding buffer, followed by staining according to the manufacturer's instructions. The proportion of apoptotic cells was analyzed by counting the TUNEL-positive cells in six randomly selected areas under an optical microscope.

Statistical analysis

Data are presented as mean $\pm$ standard deviation. Statistical differences in serum SOST level, *SOST* methylation, apoptosis, and proliferation were determined using Student's t-test. Statistical differences in bone *SOST* expression, luciferase assays, and ChIP results were determined using one-way analysis of variance, followed by the Student–Newman–Keuls test. P values < 0.05 were considered statistically significant.

Results

Serum and bone SOST levels in patients with and without PO

We used ELISA to measure serum SOST concentrations in 12 patients with PO (experimental group) and eight patients without PO (control group). SOST level was significantly reduced in the serum of patients in the experimental group compared to those in the control group (Fig. 1A). We also investigated SOST mRNA level in bone tissue, and observed lower *SOST* expression in patients of the experimental group than in patients of the control group (Fig. 1B).

DNA methylation of the *SOST* promoter region in patients with PO

We next examined whether SOST expression was associated with changes in DNA methylation in the 12 patients with PO. Two CpG islands were identified in the 2 kb region upstream of the *SOST* transcriptional start site (Fig 2A). CpG island 1 was located from -1560 to -1287, with four identified CpG sites, whereas CpG island 2 was located in from -441 to -181, with 10 identified CpG sites (Fig. 2A). Bone biopsies from 12 patients

with and 8 without PO were analyzed using BSP. CpG island 1 was 77.5% methylated in patients with PO, which was significantly higher than that in patients without PO (control) (62.5%; Fig. 2B). CpG island 2 methylation was also significantly higher in patients with PO (88.2%) than in those without PO (control) (60.1%; Fig. 2C). These results suggest that *SOST* promoter methylation patterns are altered in patients with PO.

We used an online software (<http://jaspar.binf.ku.dk/>) to predict transcription factor binding sites in the two CpG islands. In island 1, SRY-box 9 (SOX9), homeobox protein Hox-C13 (HOXC13), and estrogen response element (ERE) were predicted to be involved in the modulation of *SOST* expression. Sites for ERE, paired box protein Pax-5 (PAX5), NK2 homeobox 2 (NKX2-2), myocyte-specific enhancer factor 2C (MEF2C), RUNX2, E-BOX, CLOCK-related elements, SMAD2, and SP7 were detected in island 2. In CpG island 1, the ER α site was located in CpG4. In CpG island 2, the RUNX2 and SP7 sites were located in CpG7 and CpG10, respectively (Supplementary Fig. 1). As these three transcription factors are related to bone differentiation and remodeling, we next analyzed their effect on *SOST* expression.

Methylation of the *SOST* promoter inhibits transactivation of potential transcription factors

To investigate the potential effects of SP7, RUNX2 and ER α , we co-transfected 293T cells with the methylated *SOST* promoter and each potential transcription factor. SP7, RUNX2, and ER α increased the expression of *SOST*-luc. However, SP7, RUNX2, and

ER α could not activate the expression of methylated *SOST*-luc expression (Fig 3A-C).

These results indicate that methylation of the *SOST* promoter affects its transactivation by potential transcription factors.

Demethylating agent AzadC increases the transactivation of potential transcription factors affecting *SOST* expression

MG-63 is a human osteoblast cell line, which when treated with the demethylating agent AzadC, displays decreased *SOST* methylation and increased *SOST* expression (PAPATHANASIOU *et al.* 2015). We next investigated whether overexpression of SP7, RUNX2, and ER α increased the transcription of demethylated *SOST*. AzadC-treated MG-63 cells displayed significantly increased *SOST* expression, and upon transcription factor overexpression, *SOST* expression was significantly higher than with AzadC treatment alone (Fig. 4A-C). This demonstrates that *SOST* demethylation increases the transactivation of potential regulatory transcription factors.

SP7, RUNX2, and ER α directly bind to the *SOST* promoter in MG-63 cells

To determine whether SP7, RUNX2, and ER α directly bind to the *SOST* promoter, ChIP was performed in MG-63 cells using SP7, RUNX2, and ER α antibodies. Results demonstrate that SP7, RUNX2, and ER α can bind directly to the *SOST* promoter (Fig. 5A-C).

SP7, RUNX2, and ER α display decreased binding to the *SOST* promoter in patients with PO

To determine whether altered methylation levels in patients with PO affect transcription factor binding ability to the *SOST* promoter, ChIP and subsequent qRT-PCR were performed in patients with and without PO. Results show that SP7, RUNX2, and ER α displayed significantly lower binding to the *SOST* promoter in patients with PO than in those unaffected by the disease (control group) (Fig 6A-C). This suggests that methylation of the *SOST* promoter in patients with PO impairs the binding ability of SP7, RUNX2, and ER α .

AzadC-treatment of MG-63 cells and *SOST* overexpression increased apoptosis and decreased proliferation

TUNEL and CCK-8 assays were performed in MG-63 cells after AzadC treatment and *SOST* overexpression. AzadC treatment and *SOST* overexpression significantly increased the number of apoptotic cells compared to untreated cells (Fig 7A-B and Supplementary Figure 2), and cell proliferation was significantly reduced in AzadC-treated and *SOST* overexpressing MG-63 cells (Fig 7C and Supplementary Figure 2). This suggests that altered *SOST* methylation increases apoptosis and inhibits proliferation.

Discussion

High levels of *SOST* promoter methylation decrease *SOST* expression. *SOST*, a potent

249 inhibitor of bone formation (TU *et al.* 2012), is particularly expressed in osteocytes (TEN
250 DIJKE *et al.* 2008). DNA methylation specifically modulates *SOST* expression during
251 osteoblast-osteocyte transition (DELGADO-CALLE *et al.* 2012), and methylation of bone
252 *SOST* correlates strongly with the risk of fracture in postmenopausal women (REPPE *et al.*
253 2015). However, whether *SOST* methylation is altered in OP is not known. We have
254 previously observed that *SOST* methylation is significantly increased in patients with OP.
255 *SOST* is a potent inhibitor of bone formation (BALEMANS *et al.* 2001); however, the
256 relationship between low *SOST* expression and OP pathogenesis requires further
257 investigations.

258 The *SOST* promoter contains two CpG islands with 14 CpG sites, and displayed
259 significantly high methylation levels. Promoter analysis revealed several potential
260 transcriptional factor binding sites in these methylated CpG islands, including those for
261 SOX9, HOXC13, ER α , PAX5, NKX2, MEF2C, RUNX2, E-BOX, SMAD2, and SP7.
262 ER α and RUNX2 have been reported to regulate *SOST* expression (SEVETSON *et al.* 2004;
263 GALEA *et al.* 2013). SMAD2, which acts downstream of BMP signaling, was also
264 identified as a regulator of *SOST* expression (TEN DIJKE *et al.* 2008). However, whether
265 alteration of *SOST* promoter methylation levels is associated with its expression was
266 unknown. To determine the effects of alterations in *SOST* methylation, we selected the
267 bone-related transcription factors SP7, ER α , and RUNX2 for further study.

268 High *SOST* promoter methylation disrupted the transactivation function of SP7, ER α ,
269 and RUNX2. DNA methylation in CpG-rich regions can induce chromatin condensation

and impair transcription factor binding, decreasing transcription. In luciferase assays, SP7, ER α , and RUNX2 activated *SOST*-luc expression. ChIP revealed that SP7, ER α , and RUNX2 directly bind to the *SOST* promoter, with decreased binding ability in patients with OP. SP7 and RUNX2 are bone-specific transcription factors that are required for osteoblast differentiation and bone formation. However, the mechanism via which SP7 and RUNX2 regulate *SOST* expression in these processes was unclear. ER α was first reported as a regulator of *SOST* in 2004 (GALEA *et al.* 2013). Alteration in *SOST* promoter methylation impairs ER α binding and decreases *SOST* expression. In postmenopausal women, estrogen content drops sharply, leading to high risk of postmenopausal OP (CHOU *et al.* 2016; SHANG *et al.* 2016). Whether low estrogen level affects *SOST* expression during osteoporosis remains to be determined.

Reduction in the number of bone cells and bone mass are obvious features of patients with OP (ALEJANDRO and CONSTANTINESCU 2017), and osteoblast differentiation is controlled by changes in the expression of a large number of genes (PITTINGER *et al.* 1999). *SOST* is a marker of osteoblast differentiation into osteocytes (TU *et al.* 2012). In patients with OP, *SOST* secretion is reduced because of increased methylation of the *SOST* promoter, which decreases osteoblast differentiation into osteocytes and results in high bone cell mortality in the body. Therefore, *SOST* plays an important role in osteoblast transformation, and regulation of its activity may offer new possibilities for clinical treatment of orthopedic diseases.

Taken together, our results suggest that low *SOST* expression in patients with OP

was due to high promoter methylation, which hindered the transactivation function of SP7, RUNX2, and ER α . Alteration of *SOST* promoter methylation was also associated with osteoblast proliferation and apoptosis. Our studies provide novel insight into the role of *SOST* methylation in OP.

Acknowledgments

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363 **Figure legends**

364 **Figure 1** Serum and bone *SOST* concentration in patients with osteoporosis. We selected
365 12 patients with PO as the experimental group and eight patients without PO as the
366 control group. (A) ELISA was performed to examine the serum *SOST* concentration. (B)
367 qRT-PCR was performed to investigate *SOST* mRNA expression in bone tissue.

368 **Figure 2** *SOST* promoter methylation is increased in patients with OP. We selected 12
369 patients with PO as the experimental group and eight patients without PO as the control
370 group. BSP was used to investigate *SOST* methylation level. (A) We analyzed two CpG
371 islands located within 2000 bp of the internal *SOST* promoter regions of patients with and
372 without PO. (B) Typical methylation patterns and methylation levels of *SOST* promoter
373 Island 1 in patients with and without PO. (C) Typical methylation patterns and
374 methylation levels of *SOST* promoter Island 2 in patients with and without PO. ● :
375 Methylated; ○: Non-methylated. Statistical significance was determined using Student's
376 t-test.

377 **Figure 3** Transactivation of *SOST* expression by SP7, RUNX2, and ER α . Luciferase
378 assays were performed after co-transfection of (A) SP7, (B) RUNX2, and (C) ER α with
379 unmethylated and methylated *SOST*-luc. Statistical significance was determined using
380 Student's t-test.

381 **Figure 4** AzadC increases *SOST* expression and SP7, RUNX2, and ER α transactivation
382 activity in MG-63 cells. MG-63 cells were transfected with (A) SP7, (B) RUNX2, and

(C) ER α , and treated with the demethylating agent AzadC. SOST expression was assayed using qRT-PCR. Statistical significance was determined using Student's t-test.

Figure 5 SP7, RUNX2, and ER α bind to the *SOST* promoter in MG-63 cells. ChIP was performed to detect the direct binding of (A) SP7, (B) RUNX2, and (C) ER α to the *SOST* promoter. Input: positive control; IgG: negative control (IgG only); negative: primers not flanking the potential binding sites in the *SOST* promoter region.

Figure 6 Reduced binding of SP7, RUNX2, and ER α to the *SOST* promoter in patients with PO. We selected 12 patients with PO as the experimental group and eight patients without PO as a control group, and performed ChIP-qRT-PCR to determine the binding of (A) SP7, (B) RUNX2, and (C) ER α to the *SOST* promoter of patients with and without PO. Colored triangle: relative binding ability of each transcription factor in each individual. Black line: median relative binding of transcription factor in non-osteoporotic and osteoporotic patients.

Figure 7 Effects of AzadC and SOST overexpression on MG-63 cell apoptosis and proliferation. TUNEL and CCK-8 assays were performed to investigate apoptosis and proliferation, respectively. TUNEL-stained images of (A) control cells and (B) AzadC-treated cells are shown. (C) Proliferation of MG-63 cells with and without AzadC treatment. Statistical significance was determined using Student's t-test.

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Figure 1 Serum and bone SOST concentration in patients with osteoporosis. We selected 12 patients with PO as the experimental group and eight patients without PO as the control group. (A) ELISA was performed to examine the serum SOST concentration. (B) qRT-PCR was performed to investigate SOST mRNA expression in bone tissue.

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Figure 2 SOST promoter methylation is increased in patients with OP. We selected 12 patients with PO as the experimental group and eight patients without PO as the control group. BSP was used to investigate SOST methylation level. (A) We analyzed two CpG islands located within 2000 bp of the internal SOST promoter regions of patients with and without PO. (B) Typical methylation patterns and methylation levels of SOST promoter Island 1 in patients with and without PO. (C) Typical methylation patterns and methylation levels of SOST promoter Island 2 in patients with and without PO. ●: Methylated; ○: Non-methylated. Statistical significance was determined using Student's t-test.

Figure 3 Transactivation of SOST expression by SP7, RUNX2, and ER α . Luciferase assays were performed after co-transfection of (A) SP7, (B) RUNX2, and (C) ER α with unmethylated and methylated SOST-luc. Statistical significance was determined using Student's t-test.

Figure 4 AzadC increases SOST expression and SP7, RUNX2, and ER α transactivation activity in MG-63 cells. MG-63 cells were transfected with (A) SP7, (B) RUNX2, and (C) ER α , and treated with the demethylating agent AzadC. SOST expression was assayed using qRT-PCR. Statistical significance was determined using Student's t-test.

Figure 5 SP7, RUNX2, and ER α bind to the SOST promoter in MG-63 cells. ChIP was performed to detect the direct binding of (A) SP7, (B) RUNX2, and (C) ER α to the SOST promoter. Input: positive control; IgG: negative control (IgG only); negative: primers not flanking the potential binding sites in the SOST promoter region.

Figure 6 Reduced binding of SP7, RUNX2, and ER α to the SOST promoter in patients with PO. We selected 12 patients with PO as the experimental group and eight patients without PO as a control group, and performed ChIP-qRT-PCR to determine the binding of (A) SP7, (B) RUNX2, and (C) ER α to the SOST promoter of patients with and without PO. Colored triangle: relative binding ability of each transcription factor in each individual. Black line: median relative binding of transcription factor in non-osteoporotic and osteoporotic patients.

Figure 7 Effects of AzadC and SOST overexpression on MG-63 cell apoptosis and proliferation. TUNEL and CCK-8 assays were performed to investigate apoptosis and proliferation, respectively. TUNEL-stained images of (A) control cells and (B) AzadC-treated cells are shown. (C) Proliferation of MG-63 cells with and without AzadC treatment. Statistical significance was determined using Student's t-test.