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Control of the *SOST* Bone Enhancer by PTH Using MEF2 Transcription Factors

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Abstract

Expression of the osteocyte-derived bone formation inhibitor sclerostin in adult bone requires a distant enhancer. We show that MEF2 transcription factors control this enhancer and mediate inhibition of sclerostin expression by PTH.

Introduction—Sclerostin encoded by the *SOST* gene is a key regulator of bone formation. Lack of *SOST* expression is the cause for the progressive bone overgrowth disorders sclerosteosis and Van Buchem disease. We have previously identified a distant enhancer within the 52-kb Van Buchem disease deletion downstream of the *SOST* gene that is essential for its expression in adult bone. Furthermore, we and others have reported that *SOST* expression is suppressed by PTH. The aim of this study was to identify transcription factors involved in *SOST* bone enhancer activity and mediating PTH responsiveness.

Materials and Methods—Regulation of the *SOST* enhancer and promoter was studied by luciferase reporter gene assays. Transcription factor binding sites were mapped by footprint analysis and functional mutation analyses using transient transfections of osteoblast-like UMR-106 cells that exhibit endogenous *SOST* expression. Specific transcription factor binding was predicted by sequence analysis and shown by gel retardation assays and antibody-induced supershifts. Expression of myocyte enhancer factors 2 (MEF2) was detected by in situ hybridization, quantitative RT-PCR (qPCR), and immunohistochemistry. The role of MEF2s in *SOST* expression was assessed by reporter gene assays and siRNA-mediated RNA knockdown.

Results—PTH completely suppressed the transcriptional activity of the *SOST* bone enhancer but did not affect the *SOST* promoter. A MEF2 response element was identified in the bone enhancer. It was essential for transcriptional activation, bound MEF2 transcription factors, and mediated PTH responsiveness. Expression of MEF2s in bone was shown by qPCR, in situ hybridization, and immunohistochemistry. MEF2s and sclerostin co-localized in osteocytes. Enhancer activity was stimulated by MEF2C overexpression and inhibited by co-expression of a dominant negative MEF2C mutant. Finally, siRNA-mediated knockdown of MEF2A, C, and D suppressed endogenous *SOST* expression in UMR-106 osteoblast-like cells.

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Conclusions—These data strongly suggest that *SOST* expression in osteocytes of adult bone and its inhibition by PTH is mediated by MEF2A, C, and D transcription factors controlling the *SOST* bone enhancer. Hence, MEF2s are implicated in the regulation of adult bone mass.

Keywords

myocyte enhancer factors 2 transcription factors; osteocyte; osteoporosis; PTH; *SOST*

INTRODUCTION

In our aging society, osteoporosis is a disease of increasing importance, reaching high prevalence particularly in women but also in men. Osteoporotic bone loss very often progresses undetected until a spontaneous skeletal fracture abruptly reveals weakened bone. Drugs are available to reduce bone loss in osteoporotic patients; however, with the exception of daily subcutaneous PTH peptide injections, there are no therapies available to restore lost bone. Thus, there is an unmet medical need for bone forming therapies capable of rebuilding bone mass.

Recently, molecular and genetic analyses of rare human monogenic sclerosing bone dysplasias have identified novel genes that play pivotal roles in bone formation and hence might provide targets for bone forming osteoporosis therapy.(1) One of these genes is *SOST*, encoding the negative bone formation regulator sclerostin.(2) Loss-of-function mutations in *SOST* cause sclerosteosis (OMIM 269500) and the related Van Buchem disease (OMIM 293100), both of which are autosomal recessive disorders characterized by severe lifelong bone overgrowth of the entire skeleton.(3-6) Heterozygous sclerosteosis gene carriers also have BMDs that are consistently above the mean of age-matched healthy subjects.(7) Bone biopsy and marker analyses have indicated that the increased bone mass and strength in these patients are caused by increased bone formation.(8) In agreement with this, in vitro and in vivo studies have shown that *SOST* is a negative regulator of bone formation. For example, sclerostin was shown to inhibit osteoblast differentiation and mineralization in vitro,(9,10) and mice overexpressing *SOST* exhibited an osteoporotic phenotype.(9,11) Although sclerostin is a distantly related member of the DAN family of secreted bone morphogenetic protein (BMP) antagonists by sequence similarity,(12) it does not seem to inhibit bone formation by directly antagonizing BMP signaling. Rather, accumulating evidence suggests that sclerostin antagonizes Wnt signaling by binding to LRP5 and 6 Wnt co-receptors, which prevents activation by Wnt ligands.(13-15) In agreement with the hypothesis that sclerostin inhibits bone formation by acting as a Wnt signaling antagonist, it was recently shown that human LRP5 gain-of-function mutants, causing high bone mass phenotypes, bind sclerostin less strongly than wildtype LRP5.(16,17) Therefore, inhibition of sclerostin action or expression may provide novel bone-forming osteoporosis therapies.

In adult bone, sclerostin is exclusively expressed in osteocytes, the cells derived from osteoblasts residing within the mineralized bone matrix.(9,10) Mature human osteocytes show strong sclerostin expression, whereas osteocytes freshly embedded in the newly formed bone matrix do not express detectable levels of sclerostin.(18) This has led to the hypothesis that sclerostin may be the long-postulated osteocyte-secreted factor that restricts the bone-forming activity of osteoblasts.(19) Consistent with its restricted expression in mature osteocytes, in vitro osteoblast differentiation systems have shown *SOST* expression only during the terminal mineralization phase.(10,20) Recently, we discovered that rat UMR-106 osteoblast-like cells express high levels of *SOST* comparable to those found in bone.(21) Furthermore, we have shown that *SOST* expression is suppressed by PTH in UMR-106 cells in a manner similar to that observed in vivo in adult bone. Thus, UMR-106 cells seem to be a valid cellular system for studying *SOST* gene regulation. Consistent with this assumption, we recently identified an enhancer element that is required for *SOST* expression in adult bone using UMR-106 cell

enhancer assays.(11) It had been previously postulated that *SOST* expression in adult bone requires an enhancer located downstream of the *SOST* gene, because patients with Van Buchem disease lack *SOST* coding mutations, but instead carry a 52-kb deletion downstream of the *SOST* gene that is devoid of any transcripts.(4,6) Using BAC transgenic mouse models carrying either human wildtype or Van Buchem disease *SOST* alleles, we have shown that this region is indeed required for *SOST* expression in the adult bone.(11) Moreover, by combining cross-species sequence comparisons with in vitro and in vivo enhancer assays, a 255-bp evolutionarily conserved sequence was identified within the Van Buchem deletion that acts as a bone-specific enhancer for *SOST* gene expression.

In this study, we further investigated the structure and function of the *SOST* bone enhancer and determined that its transcriptional activity is PTH-sensitive. Furthermore, we identified a myocyte enhancer factor 2 (MEF2) response element, which is essential and sufficient for enhancer activity and which is suppressed by PTH. MEF2 expression co-localized with *SOST* expression in osteocytes and MEF2A, C, and D expression was shown to be required for endogenous SOST expression in UMR-106 cells. These data suggest that MEF2 transcription factors are essential for the transcriptional activation of the bone formation inhibitor *SOST* using its bone enhancer element, and this activation is controlled by PTH. This study represents the first indication of a role of MEF2 transcription factors in controlling adult bone mass.

MATERIALS AND METHODS

Chemicals

Human PTH(1-34) was obtained from Bachem. An siRNA cocktail targeting rat *SOST* was bought from Qiagen. All other siRNA was designed and synthesized in-house at Novartis. Antibodies against MEF2 (H-300) and p38 were obtained from Santa Cruz Biotechnology and Cell Signaling, respectively. Total rat RNA from tissues other than bone was purchased from Ambion.

Cell culture

Rat UMR-106 osteosarcoma cells were cultured in DMEM:F12 (Gibco) containing 10% FBS (AMIMED) and 1% nonessential amino acids (Gibco) as previously reported.(21) COS-7 were cultured in DMEM, 4.5 g/liter glucose, 10% FCS, and 1% nonessential amino acids. Cells were plated in 48-well tissue culture plates at initial densities of 30,000/well.

RNA preparation and quantitative real-time PCR analysis

Total RNA was isolated using RNeasy (Qiagen) and reverse-transcribed into cDNA using the high-capacity cDNA archive kit (Applied Biosystems) according to the manufacturer's recommendations. Quantitative real-time PCR (qPCR) expression analysis was performed as previously reported using TaqMan probes (Applied Biosystems). Custom TaqMan Gene Expression Assays were ordered for the MEF2 family members. Total RNA from adult female rat femoral cortical bone was isolated as previously reported.(21)

siRNA experiments

Cells were plated in 48-well tissue culture plates at initial densities of 30,000/well. After overnight incubation, cells were transfected with 10 nM siRNA using HiPerfect (Qiagen). Twenty-four hours after siRNA transfection, cells were washed and incubated for a further 24–48 h. Four siRNAs directed against each MEF2A, B, C, and D were synthesized and tested. The two most efficient were selected for further analysis and characterization. Data for one of them is shown in Fig. 6.

Reporter gene assays

All recombinant DNA work was done according to standard procedures. pGL3-based reporter plasmids (Promega) containing ECR5 upstream of the human *SOST* or the SV40 promoter were previously described.(11) For subsequent studies, the constructs were subcloned into the pREP4 vector (Invitrogen). Various deletions and mutations were introduced into the ECR5 enhancer region. Mutations in the MEF2 response element were generated by replacing the consensus CTATAAATAG sequence with GTATACATAG.(22) The human MEF2C expression plasmid was obtained from Origene, and the dominant-negative DN(1–117) hMEF2C was constructed as previously reported.(23) Details of the plasmid constructions are available on request. FuGene (Roche) and a CMV-βgal reporter plasmid (Clontech) as internal controls were used for transient transfections of rat UMR-106 cells and monkey COS-7 cells. Luciferase and galactosidase expression were measured 24 h after transfection using standard assay kits (Promega). In some experiments, stimulation with human PTH(1-34) was performed 8 h after transfection.

Footprint and EMSA analysis

Nuclear extracts from UMR-106 cells were isolated using a Nuclear Extract Kit (Active Motif). Core Footprinting System (Promega) was used to identify transcription factor binding sites, and Gel Shift Assay System (Promega) was used to detect DNA-binding proteins. For antibody-supershift experiments, nuclear extracts and ^{33}P-labeled oligos were incubated with antibodies for an additional 30 min at 4°C. Gels were exposed on a Phosphor Screen (Kodak) and visualized with a Molecular Imager FX (BioRad).

In situ hybridization

In situ hybridization on paraffin mouse embryonic paraffin sections was carried out according to established protocols. Briefly, RNA antisense probes were labeled with digoxigenin (DIG) and were synthesized with T7 RNA polymerase from gel-purified PCR templates amplified from cDNA Image clone 4481227 (Accession no. BC026841) using Roche protocol and MEF2C gene-specific primers (forward: 5′-CACCGGAACGAATTCCACTCC-3′; reverse: 5′-CCGGGTCTGTCCAAACCTCTA-3′; reverse + T7: 5′ TAATACGACTCACTATAGGGCCGGGTCTGTCCAAACCTCTA-3′; T7 sequence is underlined). Signal detection was carried out by horseradish peroxidase (HRP)-conjugated anti-DIG antibody, which reacts with the TSA agent to deposit rhodamine that can be visualized using fluorescent microscopy.

Histology and immunocytochemistry

Primary antibodies used in this study were rabbit anti-MEF2 (Santa Cruz Biotechnology) and goat anti-sclerostin (R&D Systems). UMR-106 cells were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer for 20 min at room temperature, permeabilized in PBS/0.1% TritonX-100 for 5 min, and incubated overnight at 4°C with primary antibodies in 10% normal donkey serum in PBS before incubation with fluorophore-conjugated secondary antibodies (Molecular Probes). Wildtype CD1 mouse calvaria and femora were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer, decalcified in 15% EDTA solution, and frozen in Tissue-Tek OCT compound (Sakura). Cryostat sections obtained with the CryoJane Tape-Transfer system (Instrumedics) were incubated overnight at 4°C with primary antibodies in 10% normal donkey serum in PBS/0.1% TritonX-100 and were processed with fluorophore-conjugated secondary antibodies according to standard procedures. Consecutive sections were stained with 0.6% toluidine blue solution. Images were collected on a Zeiss Axiophot microscope for UMR-106 cells or on a Leica TCS SP2 confocal microscope.

RESULTS

PTH responsiveness and protein-binding sites of the SOST bone enhancer

To determine whether PTH regulates *SOST* expression through the distal bone enhancer element or through the proximal promoter, we tested PTH response of luciferase reporter constructs containing the *SOST* bone enhancer up-stream of the 2-kb human *SOST* proximal promoter or the SV40 heterologous promoter in UMR-106 transient transfections. Consistent with our previous report,(11) the *SOST* bone enhancer stimulated transcription of the *SOST* proximal promoter and the SV40 promoter ~3- and 4-fold, respectively (Fig. 1A). PTH produced only mild inhibitory effects on the *SOST* proximal promoter or the SV40 promoter but completely suppressed the enhancer activity in the context of both the homologous and the heterologous promoter. Thus, the *SOST* bone enhancer activity is negatively regulated by PTH, independently of the endogenous *SOST* promoter.

To identify functional elements within the 255-bp enhancer sequence, we first performed DNase I footprinting experiments to localize transcription factor binding sites (Fig. 1B). Nuclear extracts of UMR-106 cells protected two regions A and B from DNase I digestion in a concentration-dependent manner using either labeled forward (right gel image) or reverse strand (left gel image) enhancer sequences. Region A extended approximately from nucleotides 106–146 and region B from nucleotides 169–192 as indicated by the boxed sequences in Fig. 1C. Two hypersensitive nucleotides (183–184) appeared in footprint B on the reverse strand, indicating a specially exposed DNA topology induced by bound transcription factors rendering these nucleotides more susceptible to DNase I cleavage.

Functional analysis of the SOST bone enhancer

Having identified two putative transcription factor binding sites within the *SOST* bone enhancer, we next performed deletion analyses to determine the functional importance of these sequence elements for transcription activation (Fig. 2). A truncated fragment of the enhancer solely comprising the two footprint regions A and B from base pairs 103–193 (p5-AB) was only slightly less active than the complete enhancer element, whereas the deletion of these regions resulted in a complete loss of enhancer activity (p5-ΔAB). Thus, these two regions correspond to the critical enhancer response elements. To further analyze these regions in greater detail, each individual footprint region was tested for its ability to stimulate transcription. Region A (p5-A) was partially active, as was region B (p5-B), with each retaining ~30–40% activity. Thus, both regions have independent additive enhancer activities. Comparative sequence (human, mouse, and rat orthologous sequences) and transcription factor binding site (TFBS) analysis of the two footprint regions revealed that region A could be composed of two putative TFBS A1 and A2. We constructed deletion mutants pΔA1 and pΔA2 and were able to show that pΔA1 had an increased enhancer activity of ~2-fold above the p5-AB or p5 constructs, whereas the pΔA2 deletion rendered the construct completely inactive. These data support a functional duality of region A and suggest that A1 has negative and A2 has positive transcription regulatory properties. To further substantiate these findings, we tested whether the A2 region alone has higher enhancer activity in the absence of the adjacent A1 repressor element. As expected, the A2 region (p5-A2) was more active and showed almost full enhancer activity. Finally, a construct containing the two enhancer activator regions A2 and B (p5–129/253) was also more active than the intact bone enhancer and showed activity similar to that of the pΔA1 construct. These data showed that the SOST bone enhancer sequence contains one repressor (A1) and two activator elements (A2 and B) that match the regions identified by the footprint analysis.

Because the A2 element alone conferred enhancer activity comparable to the activity of the full-length enhancer sequence, and TFBS analysis predicted a perfect consensus response

element for myocyte enhancer factor-2 (MEF2) transcription factors, we focused our further studies on the A2 element. To determine whether the predicted MEF2 response element is required for enhancer activity, we introduced a two nucleotide mutation known to inactivate the MEF2 binding site into the full-length enhancer sequence (p5-MEF2mut) and in the enhancer element A2 (p5-A2-MEF2mut). Both constructs were transcriptionally completely inactive (Fig. 2). These data suggest an essential role for the MEF2 response element in controlling *SOST* bone enhancer activity.

MEF2 transcription factors bind to the SOST bone enhancer

DNA mobility shift assays were performed to examine transcription factor binding to the A2 enhancer element. A prominent retarded protein/DNA complex was observed using labeled oligonucleotide encoding the A2 sequence and UMR-106 nuclear protein extract (Fig. 3A). This complex was completely prevented by competition with an excess of unlabeled A2 oligonucleotide but was not affected by the addition of oligonucleotide containing a mutated MEF2 binding motif. An excess of unlabeled oligonucleotide containing a MEF2 response element distinct from the one in the *SOST* bone enhancer was also able to prevent complex formation (data not shown). Next, we explored the sequence requirement for the formation of the specific protein/DNA complex by additional competition experiments using oligonucleotides with mutations of four consecutive nucleotides (Fig. 3B). Oligonucleotides with mutations within the putative MEF2 binding site showed no binding activity, whereas those with mutations outside of the MEF2 binding site fully competed, further supporting specific MEF2 binding. To directly address MEF2 binding to the A2 element, we performed antibody-induced supershift experiments (Fig. 3C). Antibodies against MEF2 transcription factors led to further retardation of the protein/DNA complex, whereas antibodies against p38 mitogen-activated protein kinase (MAPK) as a control had no effect on the migration of the complex. Thus, MEF2 transcription factors bind to the predicted MEF2 TFBS in the A2 enhancer element.

Finally, we examined whether the enhancer activity of the A2 activator element containing the MEF2 response element was regulated by PTH in a manner similar to the full-length *SOST* bone enhancer (Fig. 3D). Interestingly, its transcriptional activity was also completely inhibited by PTH(1-34), comparable to the entire enhancer sequence. These data suggest that MEF2s are sensitive to PTH and thus mediate PTH responsiveness of the *SOST* bone enhancer.

MEF2 transcription factors are expressed in bone

In vertebrates, MEF2 transcription factors are encoded by a family of four closely related genes: MEF2A, B, C, and D. Because their expression in adult bone has not yet been described, we first analyzed their expression by qPCR. Cortical bone from adult rat femur and rat UMR-106 osteoblast-like cells showed strong expression of MEF2s comparable to the two known MEF2 target tissues, heart and brain (Fig. 4). Low expression of MEF2s was detected in liver. In femur and UMR-106 cells, MEF2C showed the highest expression level among the four different MEF2 genes, followed by MEF2A and D with about one half as much, whereas MEF2B was only marginally detected. In heart, for comparison, MEF2D expression levels were the strongest, MEF2A and C about one half, and MEF2B was weakly expressed. Furthermore in brain, MEF2C and D were most strongly expressed, whereas MEF2A was about one half as much, and MEF2B was again weakly expressed. In general, MEF2B was the least abundant transcript with similar low expression in all tissues examined. Comparable expression data were obtained using mouse tissues (data not shown). We next performed in situ hybridization on sagittal sections of developing mouse embryos using a MEF2C-specific probe to determine the spatial embryonic expression pattern of this gene (Fig. 5A). At E16.5, MEF2C was expressed in a variety of developing tissues with highest expression detected in the peripheral and central nervous system (dorsal root ganglia and brain), musculature, and future

ossification sites. Developing bone structures such as the jaw, ribs, hip bones, and digits of mouse embryos showed strong MEF2C expression, primarily in proliferating chondrocytes and osteoblasts. Together, our expression data showed that MEF2 genes are expressed in embryonic and adult bones tissues.

Next, we analyzed MEF2 protein expression in UMR-106 osteoblastic cells and in mouse mineralized bone tissue (Fig. 5B). Immunofluorescent co-labeling was used to determine whether osteocytes co-express MEF2 transcription factors and sclerostin. In *SOST* expressing UMR-106 cells, MEF2 proteins were almost exclusively localized to the nucleus, characteristic of transcription factors, whereas sclerostin localized to the Golgi consistent with its described activity as a secreted glycoprotein. In adult and newborn mouse bones, sclerostin was specifically expressed in osteocytes embedded in the bone matrix and in their canalicular protrusions. Osteocytes were abundantly present within the calvaria and the cortical bone compartment of long bones (Fig. 5B and data not shown). Many of the sclerostin-positive osteocytes showed nuclear MEF2 localization, showing that osteocytes co-express MEF2 and *SOST*. As expected, nuclear MEF2 expression was also detected in brain and heart tissues (data not shown). In summary, these data show that MEF2 transcription factors and *SOST* are co-expressed in osteocytes in vivo and in UMR-106 cells.

MEF2s are required for SOST expression

To determine the functional impact of MEF2 transcription factors on *SOST* enhancer activity, we performed co-transfection reporter gene assays in COS-7 cells using a human MEF2C expression vector. As observed in UMR-106 cells, the *SOST* bone enhancer stimulated transcription of the *SOST* proximal promoter ~3-fold (Fig. 6A). Co-transfection of MEF2C expression vector had no effect on the *SOST* proximal promoter activity but strongly increased *SOST* bone enhancer activity by ~300%. Thus, MEF2C stimulated the activity of the *SOST* bone enhancer. To assess the dependence of the *SOST* bone enhancer on MEF2 transcription factors, we analyzed the impact of a dominant-negative MEF2 mutant. A C-terminal truncation of MEF2C containing amino acids 1–117 (dnMEF2C) has previously been shown to act as a dominant–negative MEF2 transcription factor by blocking the binding of endogenous MEF2s to their TFBS. Co-expression of dnMEF2C inhibited *SOST* enhancer activity by 55%, whereas the SV40 basal promoter activity was unaffected (Fig. 6B). A very similar result was obtained when using the SOST promoter instead of the SV40 promoter (data not shown). These data strongly suggest that MEF2 transcription factors control the activity of the *SOST* bone enhancer.

Finally, we determined whether MEF2 transcription factors are required for endogenous *SOST* expression. To this end, we analyzed the impact of individual members of the MEF2 gene family on *SOST* expression in UMR-106 bone cells using RNA interference. UMR-106 cells were transfected with control siRNA or specific siRNA directed against MEF2A, B, C, or D followed by qPCR analysis to determine MEF2A, B, C, and D and *SOST* expression levels. Each siRNA specifically downregulated its own target gene by at least 80% but had no or only minimal effects on the other analyzed genes (data not shown). SiRNA-mediated inhibition of MEF2A, C, and D expression resulted in a strong decrease in *SOST* expression by 65%, 59%, and 84%, respectively, whereas MEF2B inhibition minimally interfered with the endogenous *SOST* expression (Fig. 6C). As none of the MEF2 siRNAs reached the level of *SOST* suppression observed with *SOST* siRNA, we examined synergistic effects of MEF2A, C, and D. Simultaneous knockdown of MEF2A and C, MEF2A and D, or MEF2C and D led to increased inhibition of *SOST* expression reaching levels similar to those observed with *SOST*-specific siRNA. A triple combination of MEF2A, C, and D targeting siRNA resulted in a comparable *SOST* suppression (data not shown). In summary, these data show that MEF2

transcription factors regulate *SOST* bone enhancer activity by binding to the MEF2 response element and that they are required for endogenous *SOST* expression in UMR-106 bone cells.

DISCUSSION

The *SOST* gene is subject to intensive studies in bone research and for therapeutic applications in osteoporosis treatment, because of its key role in controlling bone mass as a negative regulator of bone formation. We and others have reported downregulation of *SOST* expression by PTH and hypothesized that *SOST* suppression may be a critical component of PTH-induced bone formation in vivo.(21,24) Furthermore, we identified a distant enhancer that is required for *SOST* expression in adult bone(11) and thus may confer osteocyte-specific expression and explain the expression restriction of *SOST* to osteocytes in adult bone. Despite its proven role as a key regulator of adult bone mass, the molecular regulatory mechanisms of *SOST* expression are largely unknown. Thus, it is of great interest to elucidate *SOST* gene control mechanisms. Herein, we reveal that the *SOST* bone enhancer mediates PTH responsiveness independently of the proximal promoter. Furthermore, a PTH-inhibitable MEF2 response element was identified as necessary and sufficient for the enhancer activity. This is the first description of a role for MEF2s in mediating *SOST* expression and PTH responsiveness in osteocytes. A schematic model summarizing our findings on *SOST* gene control in adult bone by PTH and MEF2s is presented in Fig. 7.

SOST bone enhancer mediates PTH responsiveness

We showed that PTH fully abolishes *SOST* bone enhancer activity but only marginally influences proximal promoter activity in reporter gene assays, suggesting that PTH control of *SOST* expression in adult bone is principally mediated by the bone enhancer. The bone enhancer was previously identified among several candidate enhancer sequences based on its ability to stimulate reporter gene transcription in *SOST*-expressing UMR-106 cells.(11) Our demonstration that the bone enhancer is PTH-sensitive further strengthens the hypothesis that this *SOST* enhancer is a master regulatory control region that confers both osteocyte-specific expression in adult bone and transcriptional repression mediated by PTH. Full recognition of its role in *SOST* expression control in vivo, however, remains to be confirmed in enhancer-specific knockout mice.

Previously, we have shown that PTH-induced *SOST* downregulation is direct and primarily mediated by the cAMP signaling pathway.(21) Transcriptional regulation by cAMP is principally mediated by protein kinase A–mediated phosphorylation of cAMP response element-binding protein (CREB) transcription factors and related family members, which bind as dimers to a well-defined, conserved cAMP-responsive element (CRE), TGACGTCA.(25) However, sequence analysis did not reveal a CRE within the bone enhancer sequence and, thus, it seems highly unlikely that PTH inhibits *SOST* transcription through the cAMP/PKA/CREB signaling cascade. Other eukaryotic mechanisms of cAMP signaling have recently been discovered involving direct activation of ion channels by cAMP binding and cAMP-regulated guanine nucleotide exchange factors (cAMP-GEFs), which activate the Ras-like small GTP-binding proteins Rap1 and 2.(26,27) Whether these alternative pathways are involved in *SOST* regulation remains to be determined.

Many genes have been reported to be regulated by PTH in bone, most of which are upregulated in response to PTH administration.(28,29) Only few genes are known to be downregulated by PTH, and the molecular mechanisms of their suppression are poorly understood. Besides *SOST*, only the osteoprotegerin (*OPG*) and the PTH receptor 1 (*PTHR1*) genes have been shown to be directly inhibited by PTH.(30) Inhibition of OPG expression involves the cAMP/PKA cascade and seems to be mediated through the proximal 400 bp of the promoter.(30-33) However, CREBs do not seem to be involved in OPG downregulation, because no CREs have

been identified. Suppression of PTHR1 expression by PTH has been shown to involve cAMP but not PKA and to be mediated by a Sp1-like GC-rich sequence element located at the P1 promoter start site.(34,35) This response element is clearly different from a conventional CRE and also does also not resemble any sequence element found within the *SOST* bone enhancer. These data suggest that gene repression by PTH is commonly initiated by cAMP but uses multiple effector mechanisms that are independent of CREBs.

MEF2s control the SOST bone enhancer

The identification of an essential MEF2 response element within the *SOST* bone enhancer comes as a surprise, primarily because MEF2 transcription factors are not obvious mediators of bone-specific transcription, and their function in bone has yet to be fully elucidated.(36) They are best known for their role in muscle cell differentiation(37) and as regulators of neuronal and immune cell differentiation and function.(38) Only recently, MEF2C has been described to play a role in chondrocyte hypertrophy and early bone development.(36) Our data suggest that MEF2A, C, and D are robustly expressed in osteocytes and that they control *SOST* expression in a synergistic manner. These results reveal an unexpected role of MEF2s in osteocytes as direct stimulators of *SOST* transcription. Furthermore, they corroborate the osteocytic nature of UMR-106 cells and further support the validity of these cells as an in vitro model system for the study of osteocyte gene control and PTH responsiveness of the *SOST* gene and potentially other osteocyte-specific genes. The closely related rat osteosarcoma cells Ros17/2.8 also express MEF2s very similarly to the UMR-106 cells but do not express *SOST* (data not shown).(21) Thus, MEF2A, C, and D expression alone is not sufficient for *SOST* expression. Different regulatory mechanisms from those in UMR-106 cells may exist in Ros17/2.8 cells that preclude MEF2s from activating *SOST* expression. Full appreciation of the significance of MEF2s in *SOST* expression in vivo and analysis of the contribution of the individual MEF2 genes A, C, and D will require the generation of targeted osteocyte-specific knock-out mice, because of their widespread expression and lethality, as observed for MEF2A- and C-null mice.(39,40)

The precise mechanism by which PTH suppresses MEF2-stimulated *SOST* bone enhancer activity is currently unclear. A plethora of signaling pathways have been described that control the transcriptional activity of MEF2s involving phosphorylation, sumoylation, and protein–protein interactions of transcriptional regulators.(38) A common theme seems to be calcium signaling. For example, calcium-responsive p38 and extracellular signal-regulated protein kinase (ERK) pathways have been shown to stimulate MEF2 activity by direct phosphorylation of the transactivation domain. There are three conserved phosphorylation sites in MEF2A, C, and D, which are phosphorylated in a cell type– and stimulus-dependent manner.(41) Furthermore, the calcium-activated serine/threonine phosphatase calcineurin (Cn) has been shown to activate MEF2 by directly removing inhibitory phospho-residues or by recruiting dephosphorylated NFAT and subsequently p300 to MEF2 transcription complexes. Finally, calcium/calmodulin-dependent protein kinase (CaMK) signaling is the major stimulator of MEF2 activity in the induction of cardiomyocyte hypertrophy. Interestingly, CaMK's main action is not direct phosphorylation of MEF2, but derepression from class II histone deacetylases (HDACs) through phosphorylation-induced release of HDACs from MEF2s and subsequent nuclear export of HDACs. Any of these signaling pathways are conceivable regulators of MEF2 activity by PTH and PTH may suppress MEF2 activity by interfering with one or more of these proteins. Putative candidates for MEF2 repression in bone could be class II HDACs as observed during muscle cell differentiation, cardiac hypertrophy and early bone development or by the co-repressor cabin as seen in T-cell apoptosis.(36,42-44)

Role of MEF2s in osteocyte-mediated bone mass control

Our results showed a novel role for MEF2s in controlling expression of the bone formation inhibitor *SOST* in osteocytes and thereby the regulation of adult bone mass. MEF2s activate *SOST* expression leading to secretion of sclerostin, which inhibits osteoblast-mediated bone formation, most likely by antagonizing Wnt signaling.(15,45) Although MEF2 activation can affect cell fate in a completely opposite manner depending on the cellular context, distinct MEF2 activation mechanisms may be applied. A similar cell growth repressive effect of MEF2 is known during thymic negative selection of T cells. T-cell receptor–induced calcium/calmodulin/calcineurin signaling removes the repressor cabin1 from MEF2 and instead promotes binding of the transcriptional co-factors p300 and NFAT to induce transcription of nurr77, which triggers T-cell apoptosis.(46) In contrast, MEF2s mediate the activity/calcium-dependent neuronal cell survival during development through p38-induced MEF2 phosphorylation.(47) Thus, although MEF2 activation can differentially affect cell fate depending on the cellular context, distinct MEF2 activation mechanisms may be applied. Whether MEF2 regulation in osteocytes is also mediated by the calcium/calmodulin/calcineurin signaling remains to be elucidated.

Osteocytes are thought to act as sensors and transducers of mechanical loading to cells on the bone surface to affect bone (re)modeling, yet the molecular mechanisms remain poorly understood. A key trigger seems to be strain-induced calcium influx through stretch-sensitive calcium channels. Thus, it is conceivable that calcium/MEF2-regulated *SOST* expression is involved in the control of bone formation by mechanical loading. A major challenge for the elucidation of osteocyte signal transduction pathways is the lack of reliable in vitro cell culture systems that model osteocyte function in vivo. UMR-106 cells seem to offer a promising new alternative because they reproduce *SOST* gene regulation by PTH and express MEF2s as observed in vivo in osteocytes.

Our results suggest that inhibition of MEF2 activity and/or expression in osteocytes leads to decreased *SOST* expression, and thus, constitutes a novel therapeutic venue for stimulating bone formation in patients suffering from osteoporosis and other bone loss disorders. In summary, we provide for the first time evidence that MEF2 transcription factors previously known to act in muscle, neurons and immune cells, are required for osteocyte-specific *SOST* expression and *SOST* downregulation by PTH.

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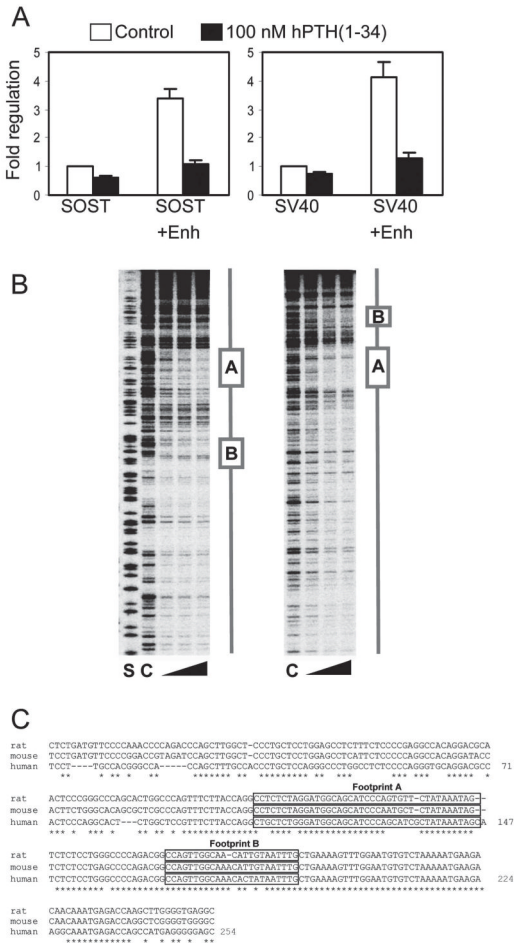

FIG. 1.

PTH responsiveness and transcription factor binding sites in the *SOST* bone enhancer. (A) Analysis of *SOST* promoter and bone enhancer regulation by PTH using reporter gene assays. UMR-106 cells were transfected with reporter plasmids containing 2 kb of the *SOST* proximal promoter (left) or the SV40 promoter (right) with or without upstream *SOST* bone enhancer. Eight hours after transfection, cells were stimulated for 16 h with 100 nM PTH(1-34) (filled bars) or solvent control (open bars). Subsequently, luciferase activity was determined. Shown are means and SE of relative luciferase activity from five independent experiments. (B) Mapping of transcription factor binding sites using DNase I footprint analysis. DNA fragments comprising the human *SOST* bone enhancer were either radiolabeled on the forward (left gel) or reverse strand (right gel) and were incubated with increasing amounts of UMR-106 cell nuclear extract (lanes 3–5). Lane C, control digestion without nuclear extract; lane S, dideoxy cytidine sequencing reaction control lane. (C) Nucleotide sequence of the *SOST* bone enhancer region from mouse, rat, and human. *Sequence identity. Footprint regions A and B are boxed.

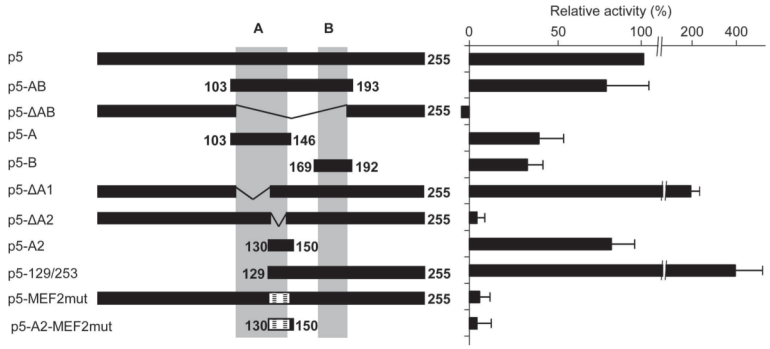

FIG. 2.

Mutation analysis of the *SOST* bone enhancer. Deletion and point mutation constructs as depicted in the figure were tested for transcriptional activation in reporter gene assays using UMR-106 cells. Percent luciferase activity is expressed relative to the level of luciferase activity obtained with the intact *SOST* bone enhancer (p5). Values represent means and SE of five independent transfection experiments. The two protein binding regions A and B identified by footprint analysis are highlighted in gray.

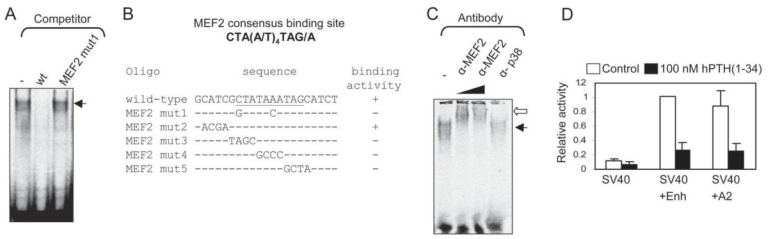

FIG. 3.

The A2 element of the *SOST* bone enhancer binds MEF2 transcription factors. (A) Gel retardation assay using labeled double-stranded wildtype (WT) oligonucleotide comprising the MEF2 binding site and nuclear extract from UMR-106 cells. Competition experiments were performed with 50-fold molar excess of the same unlabeled oligonucleotide (WT) or mutated oligonucleotides as listed in B. The specific protein–DNA complex is indicated by an arrow. (B) Summary of the mutational analysis of the MEF2 binding site using gel retardation competition assays. The MEF2 consensus binding sequence is underlined. (C) Antibody-induced gel retardation supershift assays. Protein–DNA complexes as in A were incubated with two different amounts of a pan-specific MEF2 antibody or a p38 antibody as a control before gel electrophoresis. Protein–DNA complexes are indicated by a solid arrow and the additionally retarded antibody–protein–DNA complexes (supershifts) by an open arrow. (D) Analysis of *SOST* bone enhancer regulation by PTH using reporter gene assay. UMR-106 cells were transfected with reporter plasmids containing the SV40 promoter with or without upstream full-length *SOST* bone enhancer or only the A2 element (MEF2 TFBS). Eight hours after transfection, cells were stimulated for 16 h with 100 nM PTH(1-34) (filled bars) or solvent control (open bars). Subsequently, luciferase activity was determined. Shown are means and SE of relative activity of three independent experiments.

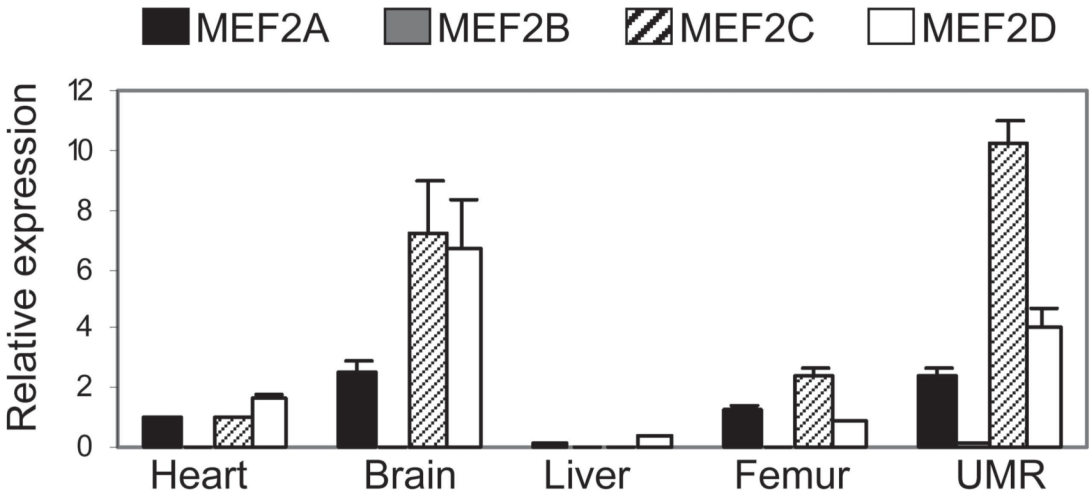

FIG. 4.

MEF2 RNA expression in bone tissues. MEF2A, B, C, and D mRNA expressions were determined by qPCR in adult rat femur and UMR-106 cells using specific TaqMan probes that detect all known isoforms for each *MEF2* gene. For comparison, expression was also measured in heart, brain, and liver. Relative expression levels are shown. Values represent means and SE of three independent experiments.

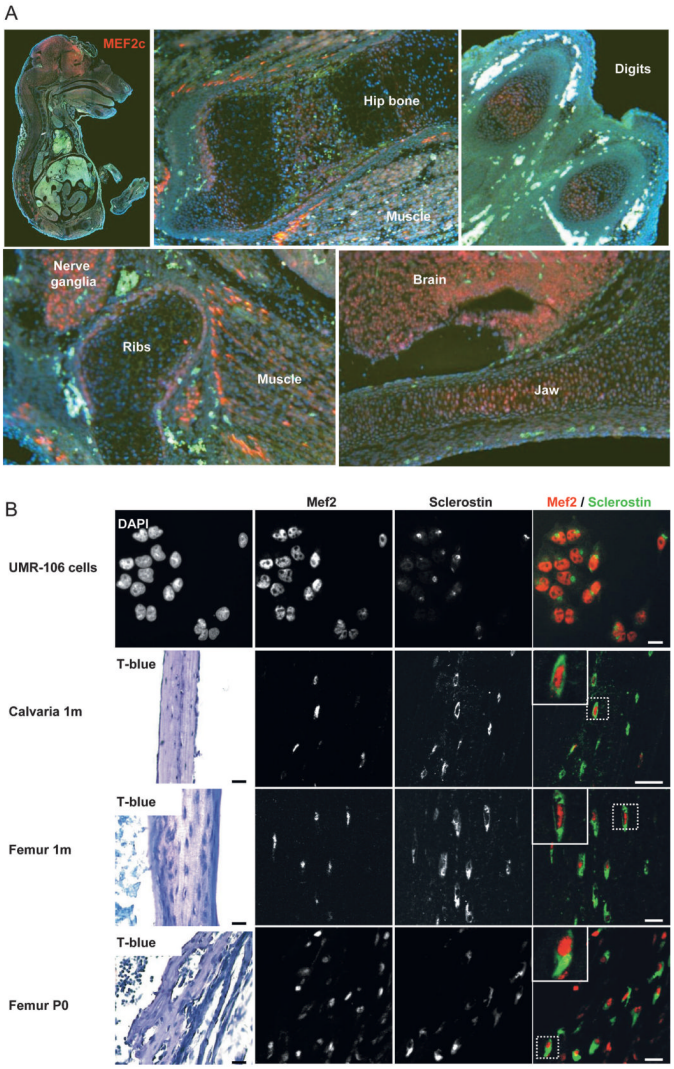

FIG. 5.

Localization of MEF2 RNA and protein in embryonic and adult bone. (A) In situ hybridization of MEF2C in mouse embryo at E16.5. MEF2C expression was detected by in situ hybridization with a fluorescently labeled MEF2C probe (red) and counterstaining with the nuclear stain DAPI. Sagittal sections show expression MEF2C expression in brain, nerve ganglia, and muscle, as well as in ossification sites such as ribs, hip bone, digits, and jaw. (B) Localization of MEF2 transcription factors and sclerostin in UMR-106 cells and osteocytes by immunohistochemistry. MEF2 (red) and sclerostin (green) were detected by double immunofluorescence labeling in permeabilized UMR-106 cells, in calvariae and femurs of 1 m-old (1m) mice, and in femurs of newborn (P0) mice. Insets show higher magnifications of the area indicated by a dotted box. Nuclear DAPI staining of UMR-106 cells and toluidine blue staining (T-blue) of representative bone sections are shown at lower magnification for orientation. Scale bars: 15 μm.

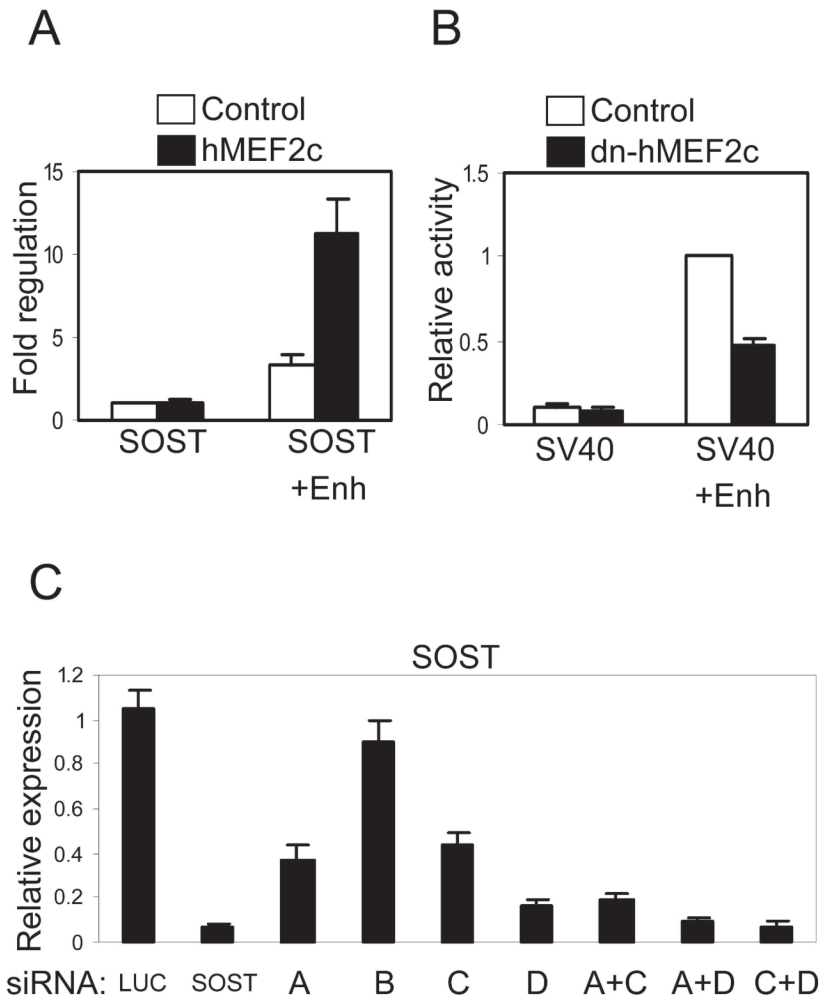

FIG. 6.

MEF2A, C, and D control *SOST* expression. (A) *SOST* bone enhancer activity is stimulated by MEF2. COS-7 cells were co-transfected with a human MEF2C expression plasmid or empty vector and reporter plasmids containing the 2-kb *SOST* promoter with or without upstream *SOST* bone enhancer. Subsequently, luciferase activity was determined. Shown are means and SE of relative luciferase activity from five independent experiments. (B) *SOST* bone enhancer activity is inhibited by a dominant-negative MEF2C mutant. UMR-106 cells were co-transfected with a dominant-negative human MEF2C expression plasmid dn(1–117)-hMEF2C or empty vector and reporter plasmids containing the SV40 promoter with or without the upstream *SOST* bone enhancer. Subsequently, luciferase activity was determined. Shown are means and SE of relative luciferase activity from five independent experiments. (C) Effect of MEF2A, B, C, and D expression inhibition on *SOST* expression. UMR-106 cells were transfected with specific siRNA against luciferase (Luc), *SOST*, MEF2A, B, C, and D, or combinations of MEF2s (A+C, A+D, C+D). Subsequently, *SOST* mRNA expression was determined by qPCR. Relative expression levels and SE are shown from four independent experiments.

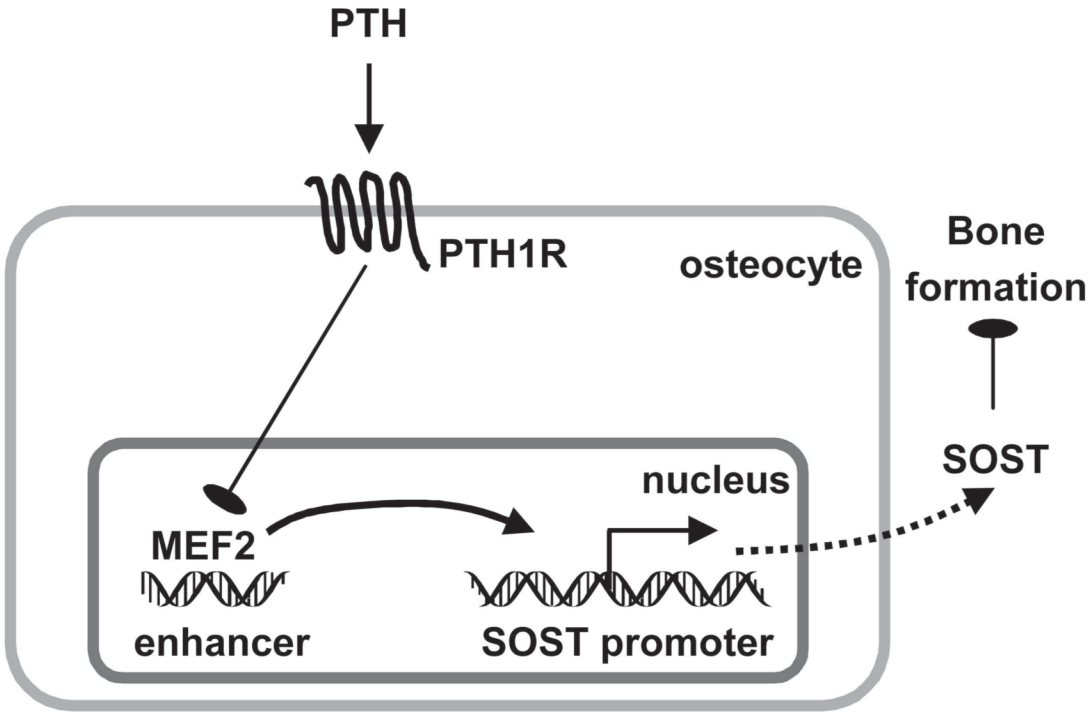

FIG. 7.

Model of *SOST* gene regulation in osteocytes by PTH and MEF2 transcription factors. Expression of the osteocyte-specific bone formation inhibitor sclerostin (SOST) requires activation of the *SOST* bone enhancer by MEF2 transcription factors. Induction of bone formation by PTH-induced downregulation of *SOST* expression is mediated through MEF2 factors.