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THE *DMP1*-*SOST* TRANSGENE INTERACTS WITH AND DOWNREGULATES THE *DMP1*-*CRE* TRANSGENE AND THE *ROSA*^{NOTCH} ALLELE

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Abstract

Activation of Notch1 in osteocytes of *Rosa*^{Notch} mice, where a *loxP*-flanked STOP cassette and the *Nicd* coding sequence were targeted to the reverse orientation splice acceptor (*Rosa*)26 locus, causes osteopetrosis associated with suppressed *Sost* expression and enhanced Wnt signaling. To determine whether *Sost* downregulation mediates the effects of Notch activation in osteocytes, *Rosa*^{Notch} mice were crossed with transgenics expressing Cre recombinase or *SOST* under the control of the dentin matrix protein (*Dmp1*) promoter. *Dmp1*-*SOST* transgenics displayed vertebral osteopenia and a modest femoral cancellous and cortical bone phenotype, whereas hemizygous *Dmp1*-*Cre* transgenics heterozygous for the *Rosa*^{Notch} allele (*Dmp1*-*Cre*;*Rosa*^{Notch}) exhibited osteopetrosis. The phenotype of Notch activation in osteocytes was prevented in *Dmp1*-*Cre*;*Rosa*^{Notch} mice hemizygous for the *Dmp1*-*SOST* transgene. The effect was associated with downregulated Notch signaling and suppressed *Dmp1* and *Rosa26* expression. To test whether *SOST* regulates Notch expression in osteocytes, cortical bone cultures from *Dmp1*-*Cre*;*Rosa*^{Notch} mice or from *Rosa*^{Notch} control littermates were exposed to recombinant human *SOST*. The addition of *SOST* had only modest effects on Notch target gene mRNA levels and suppressed *Dmp1*, but not *Cre* or *Rosa26*, expression. These findings suggest that prevention of the *Dmp1*-*Cre*;*Rosa*^{Notch} skeletal phenotype by *Dmp1*-*SOST* is not secondary to *SOST* expression but to interactions among the *Dmp1*-*SOST* and *Dmp1*-*Cre* transgenes and the *Rosa26* locus. In conclusion, the *Dmp1*-*SOST* transgene suppresses the expression of the *Dmp1*-*Cre* transgene and of *Rosa26*.

Keywords

Sost; Cre recombinase; Rosa26 locus; Dmp1; bone remodeling; osteocytes

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INTRODUCTION

The four Notch receptors and their classical cognate ligands are single-pass transmembrane proteins that mediate signals between adjacent cells and thereby determine cell differentiation and function [Zanotti and Canalis, 2010; Zanotti and Canalis, 2013]. Upon ligand binding, Notch receptors are subjected to sequential proteolytic cleavages that result in the release of the Notch intracellular domain (Nid) in the cytoplasm. Subsequently, the Nid translocates to the nucleus and interacts with the DNA associated protein recombination signal binding protein for immunoglobulin kappa J region. These events result in the displacement of transcriptional repressors by activators of transcription and in the induction of gene expression. Classical target genes of Notch signaling include hairy enhancer of split (*Hes*)1, 5 and 7 and Hes related with YRPW motif (*Hey*)1, 2 and like (*L*) [Kovall, 2008; Nam et al., 2006; Schroeter et al., 1998; Wilson and Kovall, 2006].

Osteocytes are cells of osteoblastic origin that become embedded in the bone matrix as new bone is formed and that communicate with each other and with endosteal lining cells through a canalicular network. Osteocytes translate mechanical forces into intracellular signals that regulate cellular behavior and allow bone to adapt to extracellular stimuli, a process known as mechanotransduction [Kamel et al., 2010; Nakashima et al., 2011; Tatsumi et al., 2007; Turner and Pavalko, 1998; Xiong et al., 2011]. Osteocytes and osteoblasts play different roles in skeletal homeostasis, and activation of regulatory mechanisms shared by osteoblasts and osteocytes may lead to distinct outcomes on bone remodeling. For example, Notch activation in immature and mature osteoblasts causes osteopenia, whereas its preferential activation in osteocytes causes osteopetrosis [Canalis et al., 2013b]. The osteopenia caused by Notch induction in immature and mature osteoblasts is secondary to an inhibitory role of Notch in osteoblast differentiation [Canalis et al., 2013a; Canalis et al., 2013b; Zanotti and Canalis, 2014; Zanotti et al., 2011]. In contrast, the pronounced increase in cancellous bone induced by Notch1 activation in osteocytes is due to a suppression of bone resorption and an increase of cortical bone formation [Canalis et al., 2013a; Canalis et al., 2013b].

Although the mechanisms of Notch1 action in osteoblasts have been subjected to intense study, those determining the effects of Notch activation in osteocytes are less clear. In osteocytes, Notch suppresses *Sost* expression and, as a consequence, it can enhance Wnt/ β -catenin signaling [Canalis, 2013]. In the present study, we sought to determine whether *Sost* downregulation contributes to the skeletal effects of Notch1 activation in osteocytes. To activate Notch1 preferentially in these cells, *Rosa^{Notch}* mice, where a *loxP*-flanked *STOP* cassette was cloned into the reverse orientation splice acceptor (*Rosa*)26 promoter upstream of the coding sequence of the Notch1 Nid, were used [Murtaugh et al., 2003]. These mice were crossed with transgenics expressing Cre recombinase under the control of the 9.6 kilobase (kb) fragment of the dentin matrix protein (*Dmp*)1 promoter (*Dmp1-Cre*) to generate *Dmp1-Cre;Rosa^{Notch}* mice [Lu et al., 2007]. To determine the contribution of *Sost* downregulation to the skeletal effects of Notch activation in osteocytes, *Dmp1-Cre;Rosa^{Notch}* mice were crossed with transgenics expressing *SOST* under the control of the 8 kb fragment of the *Dmp1* promoter to obtain *Dmp1-Cre;Rosa^{Notch};Dmp1-SOST* mice [Tu et al., 2012]. The phenotype of Notch1 activation and SOST overexpression in osteocytes

was determined in male and female mice. These studies were complemented by *in vitro* experiments where osteocyte-enriched cortical bone cultures from *Dmp1-Cre;Rosa^{Notch}* and *Rosa^{Notch}* littermates were exposed to recombinant human SOST.

MATERIALS AND METHODS

Genetically Modified Mice

To express SOST preferentially in osteocytes, *Dmp1-SOST* transgenic mice in a C57BL/6 genetic background were obtained from C. O'Brien and R. Jilka (University of Arkansas for Medical Sciences, Little Rock, AR) [Rhee et al., 2011; Tu et al., 2012]. These mice were generated by oocyte injection of a transgenic DNA construct containing the 8 kb upstream of the putative transcriptional start site, the first exon, the first intron and 17 base pair (bp) of exon 2 of *Dmp1*, followed by the human *SOST* coding sequence [Kalajzic et al., 2004]. Hemizygous *Dmp1-SOST* mice (*Dmp1-SOST*) were crossed with wild type C57BL/6 mice to obtain experimental *Dmp1-SOST* and control wild type littermates.

To induce Notch signaling *in vivo*, *Rosa^{Notch}* mice in a 129SvJ/C57BL/6 genetic background were used (Jackson Laboratory, Bar Harbor, ME) [Murtaugh et al., 2003; Stanger et al., 2005]. In these mice, the *Rosa26* locus was targeted with a DNA construct containing a fragment of the *Rosa26* promoter, a STOP cassette flanked by *loxP* sites and the coding sequence of the Notch1 Nicd. Excision of the STOP cassette by Cre recombination causes expression of *Nicd* from the *Rosa26* locus and subsequent activation of Notch signaling [Buchholz et al., 1996; Sauer and Henderson, 1988]. To activate Notch1 preferentially in osteocytes, homozygous *Rosa^{Notch}* mice were mated with hemizygous *Dmp1-Cre* transgenics (*Dmp1-Cre*) in a C57BL/6 background to create *Dmp1-Cre;Rosa^{Notch}* experimental mice and *Rosa^{Notch}* littermate controls. *Dmp1-Cre* transgenics express Cre under the control of a DNA fragment containing the 9.6 kb upstream of the putative transcriptional start site, the first intron and 17 bp of exon 2 of *Dmp1* (Dr. Feng, Texas A&M Health Science Center, Dallas, TX) [Lu et al., 2007].

To determine the skeletal effects of Notch1 activation in the context of SOST overexpression in osteocytes, *Dmp1-SOST* transgenics were crossed with *Rosa^{Notch}* mice to create *Dmp1-SOST* hemizygotes, homozygous for the *Rosa^{Notch}* allele. The latter were crossed with homozygous *Dmp1-Cre* mice to generate an offspring heterozygous for the *Rosa^{Notch}* allele and hemizygous for the *Dmp1-Cre* transgene, where ~50% of the mice would carry one copy of the *Dmp1-SOST* transgene (*Dmp1-Cre;Rosa^{Notch};Dmp1-SOST*) and 50% would not (*Dmp1-Cre;Rosa^{Notch}*). This mating scheme does not allow for a direct comparison between *Dmp1-Cre;Rosa^{Notch}* and control *Rosa^{Notch}* mice in the same litter. Therefore, data obtained from the *Dmp1-Cre;Rosa^{Notch}* mice generated for this study were pooled with data from studies comparing *Dmp1-Cre;Rosa^{Notch}* and *Rosa^{Notch}* littermate mice, collected over a period of ~4 years. The pooled data from *Dmp1-Cre;Rosa^{Notch}* and *Rosa^{Notch}* mice, and data from the *Dmp1-Cre;Rosa^{Notch};Dmp1-SOST* mice, were subjected to non-parametric statistical testing to determine whether restoration of *SOST* reverses the skeletal phenotype of Notch1 activation in osteocytes. Genotyping of *Dmp1-Cre* and *Dmp1-SOST* transgenes and *Rosa^{Notch}* alleles was carried out by polymerase chain reaction (PCR)

in tail DNA extracts in the presence of specific primers (Table 1, all from Integrated DNA Technologies (IDT), Coralville, IA).

Animal experiments were approved by the Animal Care and Use Committees of Saint Francis Hospital and Medical Center and of UConn Health.

Microcomputed Tomography (μ CT)

Bone microarchitecture of femurs and vertebrae from experimental and control mice was determined using a microcomputed tomography instrument (μ CT 40; Scanco Medical AG, Bassersdorf, Switzerland), which was calibrated weekly using a phantom provided by the manufacturer [Bouxsein et al., 2010; Glatt et al., 2007]. The vertebral body of L3 and femurs were scanned in 70% ethanol at high resolution, energy level of 55 peak kV, intensity of 145 μ A and integration time of 200 ms. Trabecular bone volume fraction and microarchitecture were evaluated starting ~1.0 mm from the cranial side of the vertebral body, or 1.0 mm proximal from the condyles of the distal femur. For L3 vertebrae and femurs, a total of 160 consecutive slices were acquired at an isotropic voxel dimension of 216 μm^3 and a slice thickness of 6 μm and chosen for analysis. Contours were manually drawn every 10 slices a few voxels away from the endocortical boundary to define the region of interest for analysis. The remaining slice contours were iterated automatically. Trabecular regions were assessed for total volume, bone volume, bone volume fraction (bone volume/total volume), trabecular separation, number and thickness, connectivity density, structure model index, and material density using a Gaussian filter ($\sigma = 0.8$) and user-defined thresholds [Bouxsein et al., 2010; Glatt et al., 2007]. For analysis of femoral cortical bone, contours were iterated across 100 slices along the cortical shell of the femoral mid-shaft, excluding the marrow cavity. Analyses of bone volume/total volume, cortical thickness, periosteal perimeter, endosteal perimeter, total cross-sectional and cortical bone area, were performed using a Gaussian filter ($\sigma = 0.8$) and user-defined thresholds. The terminology and units used are in accordance with guidelines published in the Journal of Bone and Mineral Research [Bouxsein et al., 2010].

Osteocyte-enriched Cultures

Cultures of osteocyte-enriched cells were obtained following a modification of a previously described method [Halleux et al., 2012]. One month old *Dmp1-Cre;Rosa^{Notch}* mice and littermate *Rosa^{Notch}* controls were sacrificed by CO₂ inhalation followed by cervical dislocation. Femurs were removed aseptically, the surrounding tissues dissected, the proximal epiphyseal ends excised and the bone marrow removed by centrifugation. The distal epiphysis was excised, and femurs were digested for 20 min at 37°C with bacterial collagenase (Type II collagenase from *Clostridium histolyticum*, Worthington Biochemical Corp., Lakewood, NJ) pretreated with tosyllysine chloromethyl keton hydrochloride (Calbiochem, La Jolla, CA) and subsequently exposed to EDTA 5 mM (Life Technologies, Grand Island, NY) for 20 min at 37°C. The resulting osteocyte-enriched cortical femurs were cultured individually in Dulbecco's modified Eagle's medium (DMEM) supplemented with nonessential amino acids (both from Life Technologies), 100 $\mu\text{g}/\text{ml}$ ascorbic acid (Sigma-Aldrich, St. Louis, MO) and 10% fetal bovine serum (Atlanta Biologicals, Inc., Atlanta, GA) for 3 days at 37°C in a humidified 5% CO₂ incubator. The effects of SOST on

Notch signaling and gene expression in osteocytes were tested with recombinant human SOST (Thermo Scientific, Waltham, MA). SOST was dissolved at a concentration of ~220 μ M in phosphate buffered saline and 0.1% bovine serum albumin (Sigma-Aldrich). Osteocyte-enriched cortical femurs were transferred to culture medium without serum for 24 h and exposed to SOST 40 nM for 6 h or to an equivalent dilution of vehicle solution, as a control.

Quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR)

Total RNA was extracted from calvariae of *Dmp1-SOST* male mice and wild type littermates of the same sex, tibiae of *Dmp1-Cre;Rosa^{Notch}* and *Dmp1-Cre;Rosa^{Notch};Dmp1-SOST* littermates of both sexes following the removal of the epiphyses and bone marrow by centrifugation, and osteocyte-rich preparations. Integrity of total RNA was tested by microfluidic electrophoresis on an Experion automated electrophoresis system (Bio-Rad, Hercules, CA), and RNA exhibiting signs of degradation was excluded from further analysis. To measure mRNA levels, equal amounts of RNA were reverse-transcribed using the iScript RT-PCR kit (Bio-Rad), according to manufacturer's instructions, and amplified in the presence of specific primers (Table 2, all primers from IDT) and iQ SYBR Green Supermix (Bio-Rad) at 60°C for 35 cycles [Nazarenko et al., 2002a; Nazarenko et al., 2002b]. Transcript copy number was estimated by comparison with a serial dilution of cDNA for *Axin2* (GE Healthcare Dharmacon, Lafayette, CO), Cre recombinase (Life Technologies), *Dmp1*, *Notch1* and *Sost* (all from Thermo Scientific), *Hey1*, *Hey2* (both from T. Iso, Los Angeles, CA), *HeyL* (from D. Srivastava, Dallas, TX), tumor necrosis factor receptor superfamily, member 11b (*Tnfrsf11b*, encoding for osteoprotegerin) and Wnt1 inducible signaling pathway protein (*Wisp1*) (both from American Type Tissue Culture Collection (ATCC), Manassas, VA), tumor necrosis factor, member 11 (*Tnfsf11*, encoding for receptor activator of nuclear factor- κ B ligand, Rankl, from Source BioScience, Nottingham, UK), dickkopf-related protein 1 (*Dkk1*, from C. Niehrs, Heidelberg, Germany) and *Rosa26* [Glinka et al., 1998; Iso et al., 2001; Nakagawa et al., 1999]. The latter was generated by cloning a ~200 bp synthetic DNA fragment (from IDT), homologous to expressed sequences from the *Rosa26* locus, into pcDNA3.1(–) (Life Technologies) by isothermal single reaction assembly, using commercially available reagents (New England Biolabs, Ipswich, MA) [Gibson et al., 2009]. Expression of the *Dmp1-SOST* transgene was detected with a forward primer spanning the exon1-exon2 junction of the *Dmp1/SOST* hybrid transcript and a reverse primer homologous to a 5' fragment of the *SOST* coding sequence (Table 2). *Dmp1-SOST* expression was estimated by comparing to a serial dilution of reverse transcribed mRNA from calvaria of a *Dmp1-SOST* transgenic mouse. Reactions were carried out in a CFX96 qRT-PCR detection system (Bio-Rad), and fluorescence was monitored during every PCR cycle at the annealing step. Gene expression was corrected for glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) or for ribosomal protein L38 (*Rpl38*) copy number, estimated by comparison with a serial dilution of cDNA for *Gapdh* (from R. Wu, Ithaca, NY) or *Rpl38* (from ATCC) [Tso et al., 1985]. Presence of a single PCR product was documented by melting curve analysis carried out after the final amplification cycle.

Statistical Analysis

Data for body weight, femoral length and indices of bone microarchitecture are expressed as medians, means, 75th and 25th percentile, and maximum and minimum because of the limited number of observations. Normal distribution for these parameters was not assumed, and statistical differences between groups were determined by Mann-Whitney U-test for pairwise comparisons or by Kruskal-Wallis test for multiple comparisons. Data for mRNA expression are displayed as means $\pm$ SEM, and statistical differences were determined by Mann-Whitney U-test for pairwise comparisons or by ANOVA with Holm-Sidak post-hoc analysis for multiple comparisons.

RESULTS

Femoral Microarchitecture of *Dmp1-SOST* Transgenics

To characterize the impact of the preferential *SOST* overexpression in osteocytes, 1 month old *Dmp1-SOST* hemizygous (*Dmp1-SOST*) mice were compared to wild type littermates. Expression of the *Dmp1-SOST* transgene was confirmed in calvarial bones of *Dmp1-SOST* male mice, and body weight and femoral length were not appreciably different between male and female transgenics and control wild type littermates of the same sex (Figure 1). In accordance with previous observations, *Dmp1-SOST* transgenics displayed modest osteopenia in the femoral cancellous bone compartment, and cortical bone architecture was not different from control littermates [Rhee et al., 2011; Tu et al., 2012]. Confirming a previous report, male and female *Dmp1-SOST* transgenics exhibited a marked decrease of cancellous bone volume in lumbar vertebrae (L3), secondary to reduced trabecular number and thickness (Table 3) [Tu et al., 2012].

Global and Skeletal Phenotype of Preferential Notch1 Activation in Osteocytes and *SOST* Overexpression

Previous work from this laboratory demonstrated that preferential expression of the *Notch1* *Nicd* in osteocytes in *Dmp1-Cre;Rosa^{Notch}* mice causes a marked increase in cancellous bone due to a suppression of bone resorption [Canalis et al., 2013a; Canalis et al., 2013b]. Notch1 activation in osteocytes downregulated *Sost* expression and induced Wnt/ β -catenin signaling, possibly explaining the osteopetrotic phenotype observed [Canalis, 2013; Canalis et al., 2013a]. To establish the contribution of the downregulation of *Sost* to the effects of Notch1 activation in osteocytes, the consequences of Notch1 induction were characterized either in the context (*Dmp1-Cre;Rosa^{Notch};Dmp1-SOST*), or not (*Dmp1-Cre;Rosa^{Notch}*), of *SOST* overexpression.

In agreement with previous findings, *Notch1* *Nicd* induction resulted in a decrease in weight and femoral length in female but not in male mice [Canalis et al., 2013b]. In contrast to the observations in *Dmp1-SOST* mice, *SOST* overexpression in the presence of Notch1 activation resulted in reduced weight and shorter femoral length in male and female mice, so that both parameters were significantly lower in *Dmp1-Cre;Rosa^{Notch};Dmp1-SOST* mice than in *Rosa^{Notch}* controls (Figure 2). These observations suggest that the dual Notch1 activation and *SOST* overexpression in osteocytes causes a reduction in skeletal and body size in male and female mice.

To circumvent the underlying skeletal effects of SOST overexpression in cancellous vertebral bone, which would affect the interpretation of the results, skeletal phenotyping was conducted in femoral cancellous and cortical bone, where the *Dmp1-SOST* transgene caused only a modest phenotype. Microcomputed analysis of the distal femur and of the femoral mid-shaft confirmed former studies reporting the effects of Notch1 activation in osteocytes [Canalis et al., 2013a; Canalis et al., 2013b]. One month old male and female *Dmp1-Cre;Rosa^{Notch}* mice displayed higher trabecular bone volume/tissue volume than littermate *Rosa^{Notch}* controls of the same sex. This effect was secondary to increased trabecular number and was associated with an increase in connectivity density. Although periosteal perimeter, total area and bone area were greater and endocortical perimeter was smaller in the context of Notch1 activation than under control conditions, cortical bone was porous so that cortical bone volume/total volume decreased in *Dmp1-Cre;Rosa^{Notch}* mice of both sexes (Figures 3 and 4). Indices of cancellous femoral bone volume and structure were not different between *Dmp1-Cre;Rosa^{Notch}*; *Dmp1-SOST* male and female mice and *Rosa^{Notch}* controls of the same sex. A modest increase in structural model index (SMI) was noted in the context of SOST overexpression, indicating that trabeculae in *Dmp1-Cre;Rosa^{Notch}*; *Dmp1-SOST* mice were rod-like and more fragile. SOST overexpression partially restored indices of cortical bone microarchitecture and restored bone area in male mice, and precluded the effects of Notch1 on endocortical perimeter in male and female mice (Figures 3 and 4). These findings demonstrate that the *Dmp1-SOST* transgene prevents the cancellous bone osteopetrosis induced by Notch1, and lessens the impact of Notch1 activation in osteocytes on the cortical bone compartment.

Mechanisms of Notch Action in Osteocytes

In previous work, we reported that activation of Notch1 in osteocytes induced *Tnfrsf11b* and suppressed *Sost* and *Dkk1*, which encodes for the Wnt antagonist dickkopf1, possibly explaining the suppression of bone resorption observed in *Dmp1-Cre;Rosa^{Notch}* mice [Canalis et al., 2013a]. To explore the mechanisms whereby SOST overexpression prevents Notch1-induced osteopetrosis, gene expression was analyzed in tibiae from 1 month old *Dmp1-Cre;Rosa^{Notch}* and *Dmp1-Cre;Rosa^{Notch}*; *Dmp1-SOST* littermates. Because the *Dmp1-SOST* transgene prevented the skeletal phenotype of *Dmp1-Cre;Rosa^{Notch}* mice both in male and female mice, these studies were carried out in bones from mice of both sexes, and data from gene expression in male and female mice were pooled.

Expression of the *Dmp1-SOST* transgene was confirmed in *Dmp1-Cre;Rosa^{Notch}*; *Dmp1-SOST* mice and was associated with downregulation of *Tnfrsf11b* and *Tnfrsf11*, and no effects of *Dmp1-SOST* on murine *Sost* and on *Dkk1* expression were noted (Figure 5A–B). The *Dmp1-SOST* transgene downregulated expression of *Axin2* and *Wisp1*, indicating that the transgene suppressed Wnt/β-catenin signaling in the context of Notch induction in osteocytes. To demonstrate whether the *Dmp1-SOST* transgene had an effect on the activation of Notch signaling, transcript levels of *Notch1* *Nicd* and of the Notch target genes *Hey1*, *Hey2* and *HeyL* were measured. *Notch1* *Nicd* and *Hey* transcripts were detected in bone extracts from both experimental cohorts and were suppressed by 60 to 80% in the context of SOST overexpression (Figure 5C).

Levels of *Notch1* *Nicd* transcripts are dictated by the activities of the *Rosa26* locus and of the *Dmp1-Cre* transgene, so that inhibition of Notch signaling by *Dmp1-SOST* could be secondary to either the suppression of *Rosa26* expression or the downregulation of the *Dmp1-Cre* transgene. Analysis of gene expression revealed that *Rosa26*, *Cre* and endogenous *Dmp1* mRNA levels were reduced by ~50 to 90% in *Dmp1-Cre;Rosa^{Notch}* mice compared to *Dmp1-Cre;Rosa^{Notch}* littermates (Figure 5D). Therefore, the reversal of the Notch-induced osteopetrotic phenotype by *Dmp1-SOST* could be due to the inhibition of *Nicd* expression from the *Rosa^{Notch}* allele and not due to the reinstitution of SOST levels. The downregulation of *Notch1* *Nicd* was attributable to the suppression of the *Rosa26* promoter and to a reduction in Cre recombinase.

Effects of Notch1 Activation and of SOST in Osteocyte-enriched Cultures

The inhibition of Notch signaling and downregulation of *Rosa26*, *Dmp1-Cre* and *Dmp1* by *Dmp1-SOST* *in vivo* could be secondary to SOST overexpression or to a genetic interaction between the *Dmp1-SOST* and *Dmp1-Cre* transgenes. To determine whether SOST was responsible for the present observations, osteocyte-enriched cultures from *Dmp1-Cre;Rosa^{Notch}* mice and *Rosa^{Notch}* littermates of both sexes were exposed to recombinant human SOST. *Notch1* *Nicd*, *Hey1*, *Hey2* and *HeyL* mRNA levels were increased in the context of Notch1 induction, confirming activation of Notch signaling in osteocytes. SOST had no effect on *Notch1* *Nicd* and *Hey1* expression either in cultures from *Dmp1-Cre;Rosa^{Notch}* or from *Rosa^{Notch}* mice; and in the context of *Notch1* *Nicd* overexpression, SOST induced *Hey2* mRNA levels and caused a modest suppression of *HeyL* transcripts (Figure 6). These results demonstrate that SOST has modest effects on Notch target gene expression and does not inhibit Notch signaling in osteocytes.

In agreement with the known inhibitory effects of SOST on Wnt/ β -catenin signaling, SOST suppressed the Wnt target genes *Axin2* and *Wisp1* expression in osteocyte-enriched cultures (Figure 6). In contrast to the stimulation of Wnt signaling by Notch1 activation in osteocytes *in vivo* [Canalis et al., 2013a], transcript levels for *Axin2* were suppressed in cultures from *Dmp1-Cre;Rosa^{Notch}* mice and *Wisp1* expression was not affected by Notch1 activation (Figure 6). These observations indicate that suppression of *Sost* and enhancement of Wnt/ β -catenin signaling by Notch1 activation occurs only *in vivo*.

A modest induction of *Rosa26* transcripts was observed in osteocytes from *Rosa^{Notch}* mice treated with SOST, an effect that did not recapitulate the suppression of *Rosa26* expression by *Dmp1-SOST* transgenics *in vivo*. *Dmp1-Cre* transgene expression was documented in cultures from *Dmp1-Cre;Rosa^{Notch}* mice, and the addition of SOST had no effect on *Cre* mRNA levels. However, in accordance with the *in vivo* results, SOST suppressed endogenous *Dmp1* transcript levels (Figure 6). These findings indicate that SOST does not suppress Notch signaling in osteocytes and does not regulate the expression of *Rosa26* or of the *Dmp1-Cre* transgene in these cells, suggesting that reinstitution of SOST levels was not the event that prevented the osteopetrotic phenotype of *Dmp1-Cre;Rosa^{Notch}* mice.

DISCUSSION

Our findings confirm that preferential Notch1 activation in osteocytes causes an osteopetrotic phenotype that can be explained by an induction of osteoprotegerin leading to an inhibition of bone resorption and decreased cancellous bone remodeling [Canalis et al., 2013a; Canalis et al., 2013b]. The effect on osteoprotegerin may be a direct action of Notch or may be secondary to an enhancement of Wnt signaling due to the suppression of *Sost*. Wnt signaling induces osteoblastogenesis and inhibits osteoclastogenesis and bone resorption, and the constitutive activation of β -catenin in the osteoblastic and osteoclastic lineages causes osteopetrosis [Glass et al., 2005; Monroe et al., 2012; Wei et al., 2011]. The inhibitory effect of Wnt signaling on bone resorption has been explained by an increase in osteoprotegerin expression by cells of the osteoblastic lineage and by direct effects of Wnt on osteoclast precursors [Albers et al., 2013; Chen and Long, 2013; Sato et al., 2009]. Although an increase in bone volume is often the result of increased bone formation, it also occurs in conditions where suppressed bone resorption and remodeling are the dominant events, such as the inactivation of the receptor activator of nuclear factor- κ B and the transgenic overexpression of *Tnfrsf11b* [Dougall et al., 1999; Li et al., 2000; Ominsky et al., 2009; Simonet et al., 1997].

We postulated that the downregulation of *Sost* by Notch1 activation in osteocytes could be responsible for the skeletal phenotype of *Dmp1-Cre;Rosa^{Notch}* mice causing an enhancement of Wnt/ β -catenin signaling. An attempt to test this hypothesis was made by crossing *Dmp1-Cre;Rosa^{Notch}* mice with mice harboring conditional alleles for cadherin-associated protein beta (*Ctnnb1*), which encodes for β -catenin, in order to inactivate β -catenin preferentially in osteocytes [Brault et al., 2001]. This approach however, resulted in a pronounced skeletal phenotype and high mouse lethality (Canalis unpublished observations), findings that are in accordance with previously published work documenting a marked osteopenic phenotype and lethality in the context of the conditional *Ctnnb1* inactivation in osteocytes [Kramer et al., 2010]. Therefore, this model could not be used to test the contribution of Wnt/ β -catenin signaling to the phenotype of Notch1 activation in osteocytes.

As an alternative approach to the *Ctnnb1* deletion, we elected to reverse the phenotype of the Notch1 activation by the reinstitution of sclerostin levels using *Dmp1-SOST* transgenics, which exhibit only a modest cancellous and cortical bone phenotype at femoral sites [Rhee et al., 2011; Tu et al., 2012]. Although *Dmp1-Cre;Rosa^{Notch};Dmp1-SOST* mice were smaller and displayed a reduction in femoral length, the *Dmp1-SOST* transgene prevented the osteopetrotic phenotype of *Dmp1-Cre;Rosa^{Notch}* mice. This may suggest that downregulation of *Sost* and upregulation of Wnt signaling was responsible for the skeletal phenotype of Notch1 activation in osteocytes. However, gene expression analysis revealed that the *Dmp1-SOST* transgene suppressed expression of the *Rosa^{Notch}* allele and *Dmp1-Cre* activity, the former possibly secondary to suppression of the *Rosa26* locus. These events were associated with inhibition of *Hey1*, 2 and *L* expression, suggesting that suppression of Notch signaling, and not reinstitution of SOST levels, prevented the Notch1 phenotype.

To determine whether the inhibitory effects of *Dmp1-SOST* on *Dmp1-Cre* and *Rosa26* expression were due to the presence of the *Dmp1-SOST* transgene or to an effect of SOST in

osteocytes, cortical bone cultures from *Dmp1-Cre;Rosa^{Notch}* and *Rosa^{Notch}* mice were exposed to recombinant human SOST. The validity of this system for the study of Notch and SOST signaling in osteocytes was documented by induction of Notch target genes in cultures from *Dmp1-Cre;Rosa^{Notch}* mice, and by the inhibitory effects of SOST on gene markers of Wnt/ β -catenin signaling. It is important to mention that the induction of *Wisp1* in bones from *Dmp1-Cre;Rosa^{Notch}* mice *in vivo* could not be replicated in osteocyte-enriched cultures, indicating that conditions found *in vivo* are required for induction of Wnt signaling by Notch1 activation in osteocytes [Canalis et al., 2013a]. Although these findings indicate that osteocyte-enriched cultures are not suitable to investigate regulation of Wnt/ β -catenin signaling by Notch1, this shortcoming should not alter the interpretation of the direct effects of SOST on Notch signaling and on the activity of the *Dmp1-Cre* transgene and *Rosa26* locus.

Direct addition of SOST to cortical bone cultures did not affect *Notch1*, *Nicd*, *Rosa26* or *Dmp1-Cre* expression, suggesting that the suppression of these transcripts *in vivo* is secondary to genetic interactions among the *Dmp1-SOST* and *Dmp1-Cre* transgenes and the *Rosa^{Notch}* allele. SOST increased *Hey2* expression and decreased *HeyL* transcripts modestly and in the context of Notch1 induction, indicating that suppression of Notch signaling by *Dmp1-SOST* is not the result of a direct effect of SOST in bone cells. These *in vitro* studies would suggest that the reversal of the skeletal phenotype of *Dmp1-Cre;Rosa^{Notch}* mice is an artifact caused by the *Dmp1-SOST* transgene in the context of a *Dmp1-Cre;Rosa^{Notch}* genetic composition. The decrease in body weight and femoral length of *Dmp1-Cre;Rosa^{Notch};Dmp1-SOST* mice is possibly secondary to systemic effects caused by the presence of two *Dmp1* driven transgenes, or to the downregulation of *Dmp1*, since *Dmp1* null mice develop rickets, defective bone mineralization and shortening of long bones [Feng et al., 2006].

Similarly to the effect of the *Dmp1-SOST* transgene, recombinant SOST suppressed *Dmp1* transcripts *in vitro*, but not the *Dmp1-Cre* transgene. This discrepancy points to transcriptional effects of SOST on the *Dmp1* locus, which are mediated by DNA sequences located outside of the 9.6 kb *Dmp1* promoter fragment that governs transcription of Cre from the *Dmp1-Cre* transgene. Post-transcriptional events that increase degradation of the *Dmp1* transcripts when bone cells are exposed to SOST also cannot be excluded. It is not possible to determine whether the suppression of *Dmp1* in tibiae from *Dmp1-Cre;Rosa^{Notch};Dmp1-SOST* mice is due to a direct effect of SOST in osteocytes or to a genetic interaction of the *Dmp1* locus with the *Dmp1-SOST* and *Dmp1-Cre* transgenes. In support of the notion that SOST is indeed responsible for the suppression of *Dmp1* *in vivo*, a recent study demonstrated that administration of a neutralizing SOST antibody to ovariectomized rats increases *Dmp1* mRNA levels in tibiae [Stolina et al., 2014]. *Dmp1* is required for osteocyte maturation, and *Dmp1* null mice exhibit rickets and osteomalacia; consequently, these observations suggest that the detrimental effects of SOST on the skeleton may be mediated in part by a suppression of *Dmp1* and subsequent loss of osteocyte function [Feng et al., 2006].

In conclusion, Notch1 overexpression in osteocytes causes osteopetrosis and suppresses *Sost* expression, and the *Dmp1-Cre* and *Dmp1-SOST* transgenics interact *in vivo* causing artifactual results and phenotypes.

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The abbreviations used are

ATCC	American type culture collection
bp	base pair
BA	bone area
BV/TV	bone volume/total volume
ConnD	connectivity density
Ct.BV/TV	cortical BV/TV
Ctnnb1	cadherin-associated protein beta 1
DMEM	Dulbecco's modified Eagle's medium
Dmp1	dentin matrix protein 1
Dkk1	dickkopf-related protein 1
Gapdh	glyceraldehyde-3-phosphate dehydrogenase
Hes	hairy enhancer of split
Hey	Hes-related with YRPW motif
kb	kilobase
IDT	integrated DNA technologies
μCT	microcomputed tomography
Nicd	Notch intracellular domain
PCR	polymerase chain reaction
P_{endo}	endocortical perimeter
P_{peri}	periosteal perimeter
qRT-PCR	quantitative reverse transcription-PCR
Rankl	receptor activator of nuclear factor kappa B ligand
Rosa26	reverse orientation splice acceptor 26
Rpl38	ribosomal protein L38

SMI	structural model index
TA	total area
Tb.N	trabecular number
Tb.Th	trabecular thickness
Tnfsf11	tumor necrosis factor, member 11
Tnfrsf11b	tumor necrosis factor receptor superfamily 11b
Wisp1	WNT1 inducible signaling pathway protein 1

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Figure 1.

In A, qRT-PCR analysis of *Dmp1-SOST* expression in calvarial total RNA from 1 month old control male mice and *Dmp1-SOST* (SOST) transgenic littermates of the same sex. Data expressed as *Dmp1-SOST* copy number, corrected for *Rpl38* copy number. Values are means $\pm$ SEM; $n = 5$. In B, weight and femoral length of male and female *Dmp1-SOST* transgenics (SOST, dark grey boxes) and littermate controls (white boxes). Horizontal continuous lines represent the median and interrupted lines represent the mean; upper and lower limits of the box represent the 75th and 25th percentile; whiskers (bars) indicate the maximum and minimum values; $n = 4$ to 6.

Figure 2.

Weight and femoral length of male and female 1 month old *Rosa^{Notch}* controls (Control, white boxes), *Dmp1-Cre;Rosa^{Notch}* (Notch, dark grey boxes) and *Dmp1-Cre;Rosa^{Notch};Dmp1-SOST* mice (Notch;SOST, light grey boxes). *Rosa^{Notch}* and *Dmp1-Cre;Rosa^{Notch}* mice were obtained from litters pooled over a period of ~4 years, and the *Dmp1-Cre;Rosa^{Notch}* cohort included mice matched with *Dmp1-Cre;Rosa^{Notch};Dmp1-SOST* littermates (n = 2 to 13). Horizontal continuous lines represent the median and interrupted lines represent the mean; upper and lower limits of the box represent the 75th and 25th percentile; whiskers (bars) indicate the maximum and minimum values. *Significantly different from Control by Kruskal-Wallis test.

Figure 3.

Femoral microarchitecture assessed by μ CT analysis of the distal femoral trabecular compartment (upper panel) and femoral mid-shaft cortical bone (lower panel) of 1 month old control *Rosa^{Notch}* (Control, white boxes), Notch overexpressing *Dmp1-Cre;Rosa^{Notch}* (*Notch*, dark grey boxes), and Notch and SOST overexpressing *Dmp1-Cre;Rosa^{Notch};Dmp1-SOST* (*Notch;SOST*, light grey boxes) male mice. Trabecular μ CT parameters: bone volume/total volume (BV/TV), connectivity density (ConnD), structure model index (SMI), trabecular number (Tb.N) and thickness (Tb.Th). Cortical μ CT parameters: cortical BV/TV (Ct.BV/TV), periosteal perimeter (P.peri) and endocortical perimeter (P.endo), Total (TA) and bone (BA) area. *Rosa^{Notch}* and *Dmp1-Cre;Rosa^{Notch}* mice were obtained from litters pooled over a period of ~4 years, and the *Dmp1-Cre;Rosa^{Notch}* cohort included mice matched with *Dmp1-Cre;Rosa^{Notch};Dmp1-SOST* littermates (n = 4 to 13). Horizontal continuous lines represent the median and interrupted lines represent the mean; upper and lower limits of the box represent the 75th and 25th percentile; whiskers (bars) indicate the maximum and minimum values. *Significantly different from Control; #Significantly different between *Notch;SOST* and *Notch* by Kruskal-Wallis test, $p < 0.05$. Representative μ CT of cancellous and cortical bone from *Dmp1-Cre;Rosa^{Notch}* (*Notch*), *Dmp1-Cre;Rosa^{Notch};Dmp1-SOST* (*Notch;SOST*) and *Rosa^{Notch}* controls are shown on the bottom.

Figure 4.

Femoral microarchitecture assessed by μ CT analysis of the distal femoral trabecular compartment (upper panel) and femoral mid-shaft cortical bone (lower panel) of 1 month old control *Rosa^{Notch}* (Control, white boxes), Notch overexpressing *Dmp1-Cre^{+/-};Rosa^{Notch}* (*Notch*, dark grey boxes), and Notch and SOST overexpressing *Dmp1-Cre;Rosa^{Notch};Dmp1-SOST* (*Notch;SOST*, light grey boxes) female mice. Trabecular μ CT parameters: bone volume/total volume (BV/TV), connectivity density (ConnD), structure model index (SMI), trabecular number (Tb.N) and thickness (Tb.Th). Cortical μ CT parameters: cortical BV/TV (Ct.BV/TV), periosteal perimeter (P_{peri}) and endocortical perimeter (P_{endo}), Total (TA) and bone (BA) area. *Rosa^{Notch}* and *Dmp1-Cre;Rosa^{Notch}* mice were obtained from litters pooled over a period of ~4 years, and the *Dmp1-Cre;Rosa^{Notch}* cohort included mice matched with *Dmp1-Cre;Rosa^{Notch};Dmp1-SOST* littermates (n = 3 to 9). Horizontal continuous lines represent the median and interrupted lines represent the mean; upper and lower limits of the box represent the 75th and 25th percentile; whiskers (bars) indicate the maximum and minimum values. *Significantly different from Control; #Significantly different between *Notch* and *Notch;SOST* by Kruskal-Wallis test, $p < 0.05$. Representative μ CT of cancellous and cortical bone from *Dmp1-Cre;Rosa^{Notch}* (*Notch*), *Dmp1-Cre;Rosa^{Notch};Dmp1-SOST* (*Notch;SOST*) and *Rosa^{Notch}* controls are shown on the bottom.

Figure 5.

qRT-PCR analysis of *Dmp1-SOST* (A), *Tnfrsf11b*, *Tnfsf11*, *Sost*, *Dkk1*, *Axin2* and *Wisp1* (B), *Notch1* *Nicd* (*Nicd*) *Hey1*, *Hey2*, *HeyL* (C) and *Rosa26*, *Cre* and *Dmp1* (D) expression in total RNA from tibiae of 1 month old Notch overexpressing *Dmp1-Cre;Rosa^{Notch}* (*Notch*, white bars), and Notch and SOST overexpressing *Dmp1-Cre;Rosa^{Notch};Dmp1-SOST* (*Notch;SOST*, black bars) littermates. Data for *Dmp1-SOST* expression represent *Dmp1-SOST* copy number corrected for *Gapdh* copy number. Transcript levels for the remaining genes were corrected for *Gapdh* mRNA levels and normalized to expression in *Dmp1-Cre;Rosa^{Notch}* mice; values are means $\pm$ SEM; n = 6 to 7. Data for gene expression was pooled from mice of both sexes. *Significantly different between *Notch* and *Notch;SOST* by Mann-Whitney U-test, $p < 0.05$.

Figure 6.

Osteocyte-enriched femoral diaphyses from 1 month old *Rosa^{Notch}* mice (Control, 2 leftmost bars in each panel) or *Dmp1-Cre;Rosa^{Notch}* littermates (Notch, 2 rightmost bars in each panel) of both sexes exposed to vehicle (white bars) or SOST (black bars). Total RNA was extracted and gene expression quantified by qRT-PCR in the presence of specific primers. Measurements in cultures from mice of both sexes were pooled and data expressed as *Notch1* *Nicd* (*Nicd*), *Hey1*, *Hey2*, *HeyL*, *Axin2*, *Wisp1*, *Rosa26*, *Cre*, *Dmp1* and copy number, corrected for *Rpl38* copy number. Values are means $\pm$ SEM; n = 4 – 5.

*Significantly different between Vehicle and SOST, $p < 0.05$; #Significantly different between Control and Notch, $p < 0.05$; ANOVA with Holm-Sidak post-hoc analysis.

Table 1

Primers used for allele identification by PCR.

Genotyping			
Allele	Strand	Sequence 5'-3'	Amplicon Size (bp)
<i>Dmp1-Cre</i> transgene	Forward Reverse	CCCGCAGAACCTGAAGATG GACCCGGCAAAACAGGTAG	534
<i>Dmp1-SOST</i> transgene	Forward Wild type Reverse <i>Dmp1-Sost</i> Reverse	CAGGTAGAGGAATCGCATTC AAACAACCTTCCTAAACAAGAGGCT CAGGCAGACGAGACACAGG	Wild type <i>Dmp1</i> = 208 <i>Dmp1-SOST</i> = 117
<i>Rosa^{Notch}</i>	Forward Wild type Reverse <i>Rosa^{Notch}</i> Reverse	GGAGCGGGAGAAATGGATATG AAAGTCGCTCTGAGTTGTTATTG GCGAAGAGTTTGTCTCAACC	Wild type = 600 <i>Rosa^{Notch}</i> = 250

Table 2

Primers used for qRT-PCR determinations. GenBank accession number identifies transcript recognized by primer pairs.

Gene	Strand	Sequence 5'-3'	GenBank Accession Number
<i>Axin2</i>	Forward Reverse	CAAGCCCCATAGTGCCCAA CAGACTCCAATGGGTAGCTCT	NM_015732
<i>Cre</i>	Forward Reverse	TGTTAATCCATATTGGCAGAACGA ATCCATCGCTCGACCAGTTTA	NC_005856
<i>Dmp1-SOST</i> transgene	Forward Reverse	AGCCAGAGAGGGTAGAGGAATC TACCAGCAGGCAGACGAGAC	Not applicable
<i>Dkk1</i>	Forward Reverse	CCCTCCCTTGCGCTGAAGATGAGGAGT CGCTTTCGGCAAGCCAGAC	NM_010051
<i>Gapdh</i>	Forward Reverse	CCCCTCTGGAAAGCTGTGGCGT AGCTTCCCGTTCAGCTCTGG	NM_008084 NM_001289726
<i>Hey1</i>	Forward Reverse	ATCTCAACAACACTACGCATCCCAGC GTGTGGGTGATGTCCGAAGG	NM_010423
<i>Hey2</i>	Forward Reverse	AGCGAGAACAAATTACCCTGGGCAC GGTAGTTGTCCGGTGAATTGGACCT	NM_013904
<i>HeyL</i>	Forward Reverse	CAGTAGCCTTTCTGAATTGCGAC AGCTTGGAGGAGCCCTGTTTC	NM_013905
<i>Notch1 Nicd</i>	Forward Reverse	GTGCTCTGATGGACGACAAT GCTCCTCAAACCGGAACCTTC	NM_008714
<i>Rosa26</i>	Forward Reverse	CAGGTCTTCGAGATCAACAT TGAGAACATAAATGGCAACATC	NR_027008 NR_027009 NR_027010
<i>Rpl38</i>	Forward Reverse	AGAACAAGGATAATGTGAAGTTCAAGGTTC CTGCTTCAGCTTCTCTGCCCTTT	NM_001048057 NM_001048058 NM_023372
<i>Sost</i>	Forward Reverse	AGGAATGATGCCACAGAGGTC CTGGTGTCTCAGGAGGAGGCTC	NM_024449
<i>Tnfrsf11b</i>	Forward Reverse	CAGAAAGGAAATGCAACACATGACAAC GCCTCTTCACACAGGGTGACATC	NM_008764
<i>Tnfrsf11</i>	Forward Reverse	TATAGAATCCTGAGACTCCATGAAAAC CCCTGAAAGGCTTGTTTCATCC	NM_011613
<i>Wisp1</i>	Forward Reverse	TCCAGGAGTTAAGTGATTGCTCA CATGTTACATGACACTGGGCTTC	NM_018865

Table 3

Femoral and vertebral microarchitecture assessed by μ CT of 1 month old male and female *Dmp1-SOST* transgenics (*SOST*) and littermate wild-type controls (Control) of the same sex.

	Males		Females	
	Control	<i>SOST</i>	Control	<i>SOST</i>
<i>Distal Femoral Trabecular Bone</i>				
Bone Volume/Tissue Volume (%)	5.9 (5.1–6.8)	4.5 (3.2–5.4)	4.7 (3.6–7.7)	4.3 (2.2–5.0)
Trabecular Separation (μ m)	203 (192–218)	352 (305–372)*	211 (185–258)	365 (320–427)*
Trabecular Number (1/mm)	5.0 (4.6–5.2)	2.9 (2.7–3.4)*	4.8 (4.0–5.5)	2.8 (2.3–3.2)*
Trabecular Thickness (μ m)	22 (21–23)	23 (22–26)	21 (20–23)	25 (24–27)*
Connectivity Density (1/mm ³)	365 (296–494)	306 (126–395)	288 (149–632)	204 (64–278)
Structure Model Index	3.01 (2.88–3.24)	2.90 (2.71–3.01)	3.20 (2.90–3.30)	2.88 (2.84–3.20)*
Density of Material (mg HA/cm ³)	862 (856–879)	860 (854–875)	852 (835–869)	858 (826–870)
<i>Mid-diaphysis Femoral Cortical Bone</i>				
Bone Volume/Tissue Volume (%)	85.4 (82.3–88.0)	84.6 (83.7–86.1)	80.9 (74.9–82.7)	80.6 (74.0–83.3)
Cortical Thickness (μ m)	80.5 (72.0–93.0)	79.5 (71.0–81.0)	70.5 (58.0–79.0)	71.0 (53.0–84.0)
Periosteal Perimeter (mm)	4.2 (3.9–4.3)	4.2 (4.1–4.4)	4.0 (3.6–4.3)	4.1 (3.8–4.3)
Endosteal Perimeter (mm)	3.6 (3.3–3.7)	3.6 (3.4–3.7)	3.4 (3.1–3.6)	3.5 (3.3–3.7)
Total Area (mm ²)	1.4 (1.2–1.5)	1.4 (1.3–1.5)	1.2 (1.0–1.5)	1.3 (1.1–1.5)
Cortical Area (mm ²)	0.39 (0.36–0.39)	0.39 (0.35–0.44)	0.35 (0.29–0.43)	0.37 (0.28–0.42)
<i>Vertebral Trabecular Bone</i>				
Bone Volume/Tissue Volume (%)	6.9 (6.4–7.3)	2.4 (2.0–2.7)*	5.4 (4.2–6.0)	2.2 (1.1–3.4)*
Trabecular Separation (μ m)	177 (167–193)	287 (251–327)*	186 (152–195)	278 (227–339)*
Trabecular Number (1/mm)	5.7 (5.2–6.1)	3.5 (3.1–4.0)*	5.4 (5.1–6.6)	3.6 (2.9–4.5)*
Trabecular Thickness (μ m)	21 (20–23)	24 (21–25)	19 (18–22)	21 (20–24)*
Connectivity Density (1/mm ³)	445 (411–546)	32 (24–67)*	335 (60–401)	29 (10–73)*
Structure Model Index	2.29 (2.25–2.42)	2.99 (2.97–)	2.57 (2.43–3.13)	3.10 (2.88–3.22)*
Density of Material (mg HA/cm ³)	905 (898–920)	886 (865–911)	894 (872–916)	877 (869–888)*

μ CT was performed on femurs and L3 from *Dmp1-SOST* transgenics and control littermates. Values are medians (minimum-maximum); n = 4 to 6.

* Significantly different from controls by Mann-Whitney U-test, $p < 0.05$.