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Genomic deletion of a long-range bone enhancer misregulates *sclerostin* in

Van Buchem disease

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Running title:

Sclerostin regulation in Van Buchem disease

ABSTRACT

Mutations in distant regulatory elements can negatively impact human development and health, yet due to the difficulty of detecting these critical sequences we predominantly focus on coding sequences for diagnostic purposes. We have undertaken a comparative sequence-based approach to characterize a large noncoding region deleted in patients affected by Van Buchem disease (VB), a severe sclerosing bone dysplasia. Using BAC recombination and transgenesis we characterized the expression of human *sclerostin* (*sost*) from normal (hSOST^{wt}) or Van Buchem (hSOST^{vbΔ}) alleles. Only the hSOST^{wt} allele faithfully expressed high levels of human *sost* in the adult bone and impacted bone metabolism, consistent with the model that the VB noncoding deletion removes a *sost*-specific regulatory element. By exploiting cross-species sequence comparisons with *in vitro* and *in vivo* enhancer assays we were able to identify a candidate enhancer element that drives human *sost* expression in osteoblast-like cell lines *in vitro* and in the skeletal anlage of the E14.5 mouse embryo, and discovered a novel function for *sclerostin* during limb development. Our approach represents a framework for characterizing distant regulatory elements associated with abnormal human phenotypes.

Deleterious mutations in distant regulatory elements postulated to dramatically impact human development and health have been minimally explored. This problem is in large part due to the fact that there are no simple ways to discern regulatory elements from nonfunctional sequences or to ascertain whether mutant phenotypes are caused by regulatory mutations. Among Mendelian disorders associated with noncoding mutations only a few cases are described that clearly link alterations in distant *cis*-acting regulatory regions to the cause of the disease (Enattah et al. 2002; Ionasescu et al. 1996; Lettice et al. 2003; Tsui et al. 2003; Wang et al. 2000), and these documented cases predominantly correspond to large chromosomal aberrations (Chuzhanova et al. 2003; Cimbora et al. 2000; Curtin et al. 1985; Curtin and Kan 1988; Kleinjan et al. 2001). Structural variation in the human genome described as large-scale polymorphisms has been recently shown to be more common than previously anticipated (Sebat et al. 2004), therefore the extend to which large noncoding duplications and deletions impact human biology remains a largely unanswered question. In this study, we demonstrate that a very important skeletal dysplasia, Van Buchem (VB) disease, associated with a large noncoding deletion is caused by the removal of a bone-specific distant enhancer element.

Van Buchem disease (MIM 239100) is a homozygous recessive disorder (Balemans et al. 2002; Staehling-Hampton et al. 2002; Van Hul et al. 1998) that maps to chromosome 17p21 and results in progressive increase in bone density (Wergedal et al. 2003). The accumulation of bone mass gives rise to facial distortions, enlargement of the mandible and head, entrapment of the cranial nerves, increase in bone strength, and excessive weight (Balemans et al. 2002; Staehling-Hampton et al. 2002; Van Hul et al. 1998). Sclerosteosis (MIM 269500) is a cranio-tubular hyperosteosis that is

phenotypically indistinguishable from Van Buchem disease (VB) except that it is more

severe and occasionally displays syndactyly of the digits (Balemans et al. 1999; Beighton

et al. 1977; Brunkow et al. 2001; Hamersma et al. 2003; Kusu et al. 2003), a trait absent

in VB patients.

An exciting development has been the recent discovery of a novel protein that

acts as a negative regulator of bone formation, *sclerostin* (*SOST*) (Balemans et al. 2001;

Brunkow et al. 2001), whose expression is affected in both sclerosteosis and Van

Buchem disease. Whereas sclerosteosis patients carry homozygous null *sost* mutations,

VB patients lack any *sost* coding mutations (Staehling-Hampton et al. 2002; Van

Bezooijen et al. 2004; Winkler et al. 2003). They do however, carry a homozygous 52 kb

noncoding deletion (vbΔ) ~35kb downstream of the *sost* transcript and ~10kb upstream

of the downstream gene, *meox1*, on human chromosome 17p21 (Figure 1A) (Balemans et

al. 2002; Staehling-Hampton et al. 2002). The shared clinical similarities between VB

and sclerosteosis along with their strong genetic linkage to the *sost* locus on chromosome

17q12 suggests that they are allelic, and that the deletion in VB patients removes an

enhancer element essential for directing the expression of human *sost* in the adult

skeleton. To gain insight into the mechanism by which this newly discovered gene

impacts bone patterning and remodeling in Van Buchem disease, as well as to

characterize the transcriptional regulation of *sclerostin* we have characterized human

BAC *sost* transgenic mice carrying either a normal (hSOST^{wt}) or an allele with the VB

associated deletion (hSOST^{vbΔ}). Only the hSOST^{wt} allele faithfully expresses human *sost*

in the adult bone and impacts bone metabolism, consistent with the model that the VB

noncoding deletion removes a *sost*-specific regulatory element. By exploiting cross-

species sequence comparisons with *in vitro* and *in vivo* enhancer assays we have identified a potential enhancer element that drives human *sost* expression in the skeletal anlage, and discovered a novel function for *sclerostin* during limb development, demonstrating that this very important skeletal dysplasia, Van Buchem disease, is caused by the removal of bone-specific distant enhancer elements and is allelic to sclerosteosis.

Results

Molecular and Phenotypic Characterization of Van Buchem transgenic mouse models

A ~158 kb human BAC (RP11-209M4) (hSOST^{wt}) encompassing the 3'end of the

DUSP3 gene, *sost, meox1,* and the ~90kb noncoding intergenic interval separating *sost*

from the neighboring gene, *meox1*, was engineered using homologous recombination in

bacteria (Lee et al. 2001) to delete the 52kb region missing in VB patients and to create a

construct that mimics the VB (hSOST^{vbΔ}) allele (Figure 1A). Three independent founder

lines of each transgenic construct were generated using standard transgenic procedures

(Nobrega et al. 2003). Similar to the endogenous mouse *sost* expression, and the reported

human expression (Balemans et al. 2001; Brunkow et al. 2001) all lines of hSOST^{wt}

transgenic animals reliably expressed human *sost* in the mineralized bone of neonatal and

adult mice (skull, rib and femur), while all of the hSOST^{vbΔ} lines had dramatically

reduced levels of human *sost* mRNA expression; as determined by rtPCR and qPCR. All

lines (hSOST^{wt} and hSOST^{vbΔ}) also consistently expressed human *sost* in the adult kidney

and heart. Here, we present data on the top expressing lines from each transgenic

construct, which are referred to in the manuscript as A and B version of each transgene

(Figure 1B). Expression in the lung, brain and spleen varied among lines, while no BAC

transgenic line expressed in the liver contrary to the reported human tissue-specific

expression (Brunkow et al. 2001). These data demonstrate that *in vivo,* the VB allele

confers dramatically reduced *sost* expression in the adult bone and suggests that the vbΔ

contains essential bone-specific enhancer elements.

Sclerostin is an osteocyte-expressed negative regulator of bone formation that is

structurally most closely related to the DAN/Cerberus family of BMP antagonists (Van

Bezooijen et al. 2004; Winkler et al. 2003). Several members of this family including

noggin and *gremlin* are expressed embryonically in the developing limb (Brunet et al.

1998; Khokha et al. 2003), therefore we examined human *sost* expression in the early

mouse embryo. rtPCR analysis of RNA isolated from whole embryos showed high levels

of human *sost* expression in all lines from both hSOST^{wt} and hSOST$^{vb\Delta}$ transgenic

animals (Figure 1C). *Sost* expression precedes endochondral ossification, and was

detected as early as E9.5. Since the VB deletion did not impact human *sost* embryonic

expression, we used E10.5 embryonic RNA to quantify the level of transgene expression

in different hSOST^{wt} and hSOST$^{vb\Delta}$ transgenic founder lines (Figure 1D). Comparable

expression levels were also confirmed in the kidneys of hSOST^{wt} and hSOST$^{vb\Delta}$ animals.

This data strengthens the evidence that the lack of human *sost* bone expression in

hSOST$^{vb\Delta}$ animals is dependent on the 52kb noncoding deletion, rather than reflecting an

artifact due to transgene copy number or site of integration.

The availability of BAC transgenic animals carrying wildtype and VB alleles also

allowed us to address whether the other gene flanking the vbΔ region, the transcription

factor *meox1* is affected by removing the 52kb vbΔ. *Meox1* has been previously shown

to be involved in skeletal myogenesis (Mankoo et al. 2003; Petropoulos et al. 2004),

therefore it has been uncertain whether it also plays a role in the phenotypic outcomes of

Van Buchem Disease, particularly since previous experiments using human patient

samples prevented researchers from directly examining the effect of the noncoding

deletion on gene expression (Staehling-Hampton et al. 2002). We have examined *meox1*

expression in all available transgenic lines from both hSOST^{wt} and hSOST$^{vb\Delta}$ constructs,

and unfortunately were not able to detect any significant human *meox1* expression in

adult tissues (data not shown). Based on these results, we have concluded that the

209M4 BAC does not possess sufficient *meox1*-specific regulatory elements to

recapitulate the wildtype human *meox1* expression in the hSOST^{wt} transgenics, therefore,

our experiments can not evaluate the impact the VB deletion has on *meox1* expression.

Since lack of sclerostin causes increased bone density (Brunkow et al. 2001), we

investigated whether elevated levels of human sclerostin have opposite effects on bone

mass. Consistent phenotypic data has been obtained for both lines A and B of hSOST^{wt}

and hSOST^{vbΔ} transgenic constructs (Figure 2; Supplementary Materials). Animals from

these lines expressed equivalent amounts of embryonic human *sost* (Figure 1D; gray

shaded bars). For the rest of the manuscript we will refer for these mice as hSOST^{wt} and

hSOST^{vbΔ}. hSOST^{wt} transgenics grew to skeletal maturity with normal body size and

weight (Figure 2A) however, the animals displayed decreased bone mineral density

(BMD) in the appendicular and axial skeleton, as evaluated by dual energy X-ray

absorptiometry (DEXA) analysis (Figure 2B). Micro-Computed-Tomography (microCT)

analysis of three-dimensional cancellous bone structures revealed that the mice have

decreased bone volume, trabecular number, thickness and increased trabecular separation

(Figure 2C). In contrast, the bone parameters of hSOST^{vbΔ} transgenics were

indistinguishable from non-transgenic littermate controls. The observed osteopenia was

gene dose dependent. hSOST^{wt} transgenic mice bred to homozygosity revealed a further

dramatic decrease in tibial cancellous bone volume (Figure 3A, C). Histomorphometric

analysis revealed that these animals display further decreased bone formation rates at

skeletal maturity reflected in decreased fluorochrome marker uptake into mineralizing

bone both in cancellous (Figure 3B, D) and cortical bone (tibia: non-tg = 0.319 +/- 0.016

μm/day versus hSOST$^{wt/wt}$ = 0.110 +/- 0.027 μm/day p<.001) in both the appendicular (Figure 3B,C) and the axial skeleton. Either the number of terminally differentiated bone forming cells, the osteocytes, or the number of bone resorbing cells, the osteoclasts, were significantly affected by the transgene expression (data not shown).

In contrast to hSOST^{wt} transgenics, hSOST^{vbΔ} animals did not display an osteopenic bone phenotype in neither the appendicular nor the axial mature skeleton, even in the homozygous configuration (Figure 2B,C; Supplementary Materials). These data demonstrate that modulation of *sost* expression dramatically impacts bone formation in the adult mammalian skeleton. Most importantly, these phenotypic data suggest that overexpressing human *sost* under the control of its own proximal promoter elements in concert with the downstream VB region negatively modulates adult bone mass. In contrast, bone mass is unaffected in transgenic animals that lack the 52kb VB region, in a construct that mimics the allele carried by VB patients, consistent with the model that Van Buchem disease is caused by removing a bone-specific regulatory element.

Interestingly, and consistent with the observed embryonic expression, elevated levels of human *sost* result in abnormal digit development in both hSOST^{wt} and hSOST^{vbΔ} BAC transgenics bred to homozygosity. The fore- and hind-limbs of these animals display a wide range of fused and missing digits as visualized by autoradiography (data not shown) and μCT (Figure 4B). rtPCR data correlates *SOST* expression with the severity of digit abnormalities (data not shown). Mouse whole mount *in situ* hybridization revealed *sost* to be expressed as early as embryonic stage 9.5 (E9.5), predominantly in the mesenchymal tissue of the developing limb bud (Figure 4A). These findings imply that *sost* embryonic expression is controlled by a transcriptional

regulatory element different from the one driving the adult bone expression, consistent

with the observation that both sclerosteosis and VB patients suffer from abnormal bone

mass accumulation while only sclerosteosis patients exhibit syndactyly of the digits

(Staehling-Hampton et al. 2002).

Comparative Sequence Analysis and Enhancer Assays

Given the striking bone phenotypes observed in both VB and sclerosteosis

patients, we next focused on the identification of noncoding sequences required for *sost*

bone-specific expression through a combination of comparative sequence analysis and

transient transfection assays. We aligned a ~140kb human *SOST* region

(http://zpicture.dcode.org/) (Ovcharenko et al. 2004) (RP11-209M4; AQ420215,

AQ420216) to the corresponding mouse sequences from chromosome 11 (Mouse

chr11:101,489,231-101,688,385; Oct.03 Freeze) (Figure 5A). A stringent requirement of

at least 80% identity over a 200 base pair (bp) window ($\geq$80%ID; $\geq$200bp) identified

seven evolutionarily conserved regions (ECR2-8) within the vbΔ genomic interval, which

were prioritized for *in vitro* enhancer analysis. ECR2-8 were tested for their ability to

stimulate a heterologous promoter (SV40) in osteoblastic (UMR-106) and kidney (293)

derived cell lines. One element, ECR5, was able to stimulate transcription in UMR106

cells (Figure 5B), but not in the kidney cell line, suggesting that ECR5 enhancer function

is specific to the osteoblastic lineage. We also tested the transcriptional activity of the

human *sost* proximal promoter region (2kb region upstream of 5'UTR) in the two cell

types and compared it to the SV40 and the osteoblast-specific *osteocalcin* promoter

(*OG2*). The SV40 promoter showed comparable activity in both cell lines and, as

expected, *OG2* was only active in the UMR-106 cells. The human *sost* promoter

stimulated transcription in the osteoblastic cells similarly albeit slightly higher activity

than the *OG2* promoter, while it demonstrated a threefold stronger activity in kidney cells

(Figure 5B). These data suggest that *sost* kidney expression may be due to proximal

promoter sequences, whereas strong expression in osteoblast cells requires the activity of

the ECR5 element. Consistent with the results obtained from transfecting SV40 promoter

constructs, only ECR5 was capable of activating the human *sost* promoter (4X) in

UMR106 cells (Figure 5C), while all other ECR-constructs had background level

expression. Thus, a small sequence element within the vbΔ region (ECR5) was identified

that confers *in vitro* osteoblast-specific enhancer activity onto both the human *sost* and

the SV40 heterologous promoter.

To test ECR5's ability to drive expression in the skeletal structures of the mouse

embryo we expressed a ECR-*hsp68-LacZ* construct in transgenic mice (Figure 5D)

(Nobrega et al. 2003). Transient transgenic animals were created using standard

techniques (Mortlock et al. 2003) and F0 pups were stained for *β-galactosidase*

expression at E14.5 (Nobrega et al. 2003). Transgenic embryos expressed *LacZ* in the

cartilage of the ribs, vertebrae and skull plates (Figure 5D) and the expression was

identical in all positive transgenic embryos obtained from two independent injections

(N=2). In parallel we also injected ECR4- and ECR6-*hsp68-LacZ* constructs and assayed

LacZ expression at E12.5 and E14.5. None of the ECR4 (N=6) and ECR6 (N=2) positive

embryos expressed *LacZ* at these time points. These data highly suggest that the 250

basepair (bp) ECR5 element contained within the 52 kb deleted in Van Buchem patients

functions to drive *sost* bone-specific expression *in vivo*.

Discussion

Sclerosing bone dysplasias are rare genetic disorders in which excessive bone formation occurs due to defects in bone remodeling (Van Hul et al. 2001). Identifying the responsible genes, their regulation and mechanisms of action will provide useful insights into bone physiology and potentially benefit the treatment of these disorders, as well as facilitate the development of therapies for replenishing bone loss in osteoporosis. In this study we have demonstrated that the 52 kb noncoding deletion present in Van Buchem patients removes a distant *sost*-specific regulatory element and therefore Van Buchem disease is hypomorphic to sclerosteosis. Currently, we don't have a clear view of how the lack of sclerostin promotes osteogenesis, therefore elucidating its transcriptional regulation is key to understanding the interconnection between its expression pattern in osteogenic cells and its mode of action either as a BMP-antagonist (Winkler et al. 2003) or WNT-antagonist. The elaborate expression pattern we detect along with the multitude of putative enhancer elements that have the potential to positively or negatively impact *sost* in a spatial and temporal precise manner attest to this molecule's complexity and functional versatility. Consistent with this view, our analysis provides robust *in vivo* evidence for the role of sclerostin during bone formation, modulation of adult bone mass and for a novel function during limb development.

In general, the osteopenic phenotype we observed is consistent with reports describing transgenic mice overexpressing BMP-antagonists from cDNA constructs driven by *osteocalcin* (*OG2*) promoter (Devlin et al. 2003; Winkler et al. 2003). Unlike *OG2*>hSOST transgenic animals (Winkler et al. 2003), we never observed architectural disorganization of the lumbar vertebrae. We believe the osteopenic phenotypic variations

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between the cDNA and BAC *sost* transgenic mice are most likely attributed to the transcriptional control of human *sost* in each transgenic construct. hSOST^{wt} BAC transgenics more faithfully mirror the proper regulatory control exerted on the *sost* gene in the endogenous context of the human genome, while the *OG2*>hSOST transgenic expression is ectopic and highlights the transcriptional specificity of the *osteocalcin* promoter.

Since sclerosteosis is caused by *sost* null mutations (Balemans et al. 2001; Brunkow et al. 2001), our results indicate that VB disease and sclerosteosis are allelic, VB patients are hypomorphic for the *sost* gene and lack *sost* expression in the adult bone. Our data suggests that *sost* embryonic expression is unaltered in VB patients, who also never display syndactyly of the digits, indicating that both reduced and elevated levels of human sclerostin negatively impact limb development and digit formation, a novel function attributed to this molecule.

Our findings provide evidence that noncoding regions in the VB deletion control Sclerostin expression levels and modulate BMD in mice, therefore an important question is whether variation in BMD in the general population could also be directly impacted by sequence variants in key noncoding regions of the VB deletion. A recent new study investigated the association between common polymorphisms in the *sost* gene region with BMD in elderly whites (Uitterlinden et al. 2004). From a set of 8 polymorphisms, one 3-bp deletion (SRP3) from the *sost* promoter region was associated with decreased BMD in women, and a polymorphic variant (SRP9) from the VB deletion region was associated with increased BMD in men. Whereas this SRP9 does not map on any human-mouse conserved region in the VB deletion, an important question for future studies is

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whether this SNP is in linkage disequilibrium with ECR5 or if additional functional SNPs could be identified in this or other *sost*-specific enhancer elements.

The genetic factors that contribute to susceptibility to bone loss are extremely heterogeneous, therefore murine models that affect bone development and growth can provide invaluable insights into the molecular mechanisms of progressive bone loss in humans. Human genetic diseases of the skeleton such as sclerosteosis and Van Buchem disease provide a starting point for understanding the modulation of anabolic bone formation, and ultimately have the potential to identify key molecular components that can be used as new therapeutic agents to treat individuals suffering from bone loss disorders. Our study also provides strong support for the utilization of comparative sequence analysis to dramatically filter through nonfunctional regions in the human genome and enhance the discovery of noncoding disease-causing mutations both in discrete enhancer elements or in large noncoding deletions. This study represents a clear and unambiguous case where altering noncoding genomic content deleteriously impacts gene expression, demonstrating that mutations in distant regulatory elements are able to cause congenital abnormalities analogous to coding mutations.

Methods

Generating Transgenic Mice

FRT-kan-FRT cassette was excised from pICGN21 vector (KpnI; SacI) and inserted into

pUC18 to create pUC18.kan.FRT. Homologous arms were PCR-amplified from 209M4

BAC DNA and cloned into pUC18.kan.FRT vector using EcoRI/SacI sites for the left

arm (VBDelH1: fwd 5'-TTGGTACCGGATTGAAGTGATCCCCAGCTGGA-3'; rvd 5'-

TTGAGCTCCAATCTCCTGACCTTGTGATCCGC-3'), and the SmaI site for the right

arm (VbDelH2: fwd 5'-TTCCCGGGCGCTTGAACCCAGTAGGTGGAGG-3'; rvd 5'-

TTCCCGGGTACCAAGGGATGGACAGAAGACAGGCAG-3') to create the

recombination vector pUC18.kan.FRT.VBDel. 200-300 ng KpnI digested VBDelH1-

FRT-kan-FRT-VbdelH2 fragment was electroporated into EL250-209M4 cells.

Recombinant BACs were identified by PCR and pulse-field gel analysis, were isolated at

a final concentration of 1 ng/ml and microinjected into fertilized FVB mouse eggs using

standard procedures. Transgenic mice were genotyped using PCR analysis of DNA

prepared from tail DNA of founder animals using the following primer pair: 5'-

ATGTCCACCTTGCTGGACTC-3' and 5'-GTCTGTGGGCTGGTTTGCAT-3'.

Transgenic mice were maintained on FVB background.

RT-PCR, Quantitative RT-PCR and in situ hybridization

Total RNA was isolated with Trizol reagent (Invitrogen) and reverse-transcribed into

cDNA (Superscript II, Gibco) using standard methods. cDNA was amplified using GC-

Melt PCR kit (Clontech; 65ºC annealing/3 min extension/35 cycles] using human (fwd 5'-

AGAGCCTGTGCTACTGGAAGGTGG-3', rvd 5'-TAGGCGTTCTCCAGCTCGGCC-

3') and mouse (fwd 5'-GACTGGAGCCTGTGCTACCGA-3', rvd 5'-

CTTGAGCTCCGACTGGTTGTGGAA-3') SOST primer sets. Mouse beta-actin (fwd

5'-CCTCTATGCCAACACAGTGC-3', rvd 5'-CTGGAAGGTGGACAGTGAGG-3')

was used as control [58ºC annealing/30 sec extension/25 cycles].   Quantitative rtPCR

expression analysis was performed using an ABI Prism 7900HT sequence detection

system, TaqMan® Universal PCR Master mix, human 18S rRNA pre-developed

TaqMan® assay reagent for normalization and TaqMan® Assay-on-Demand™ products

for mouse, rat and human SOST all from Applied Biosystems. We considered noon on

the day that we found a vaginal plug to be E0.5. We carried out RNA localization by

whole-mount *in situ* hybridization according to established protocols. RNA antisense

probes were labeled with digoxigenin and were synthesized with T7 RNA polymerase as

previously described.

Dual energy X-ray absoptiometry (DEXA) analysis.

Tibial, femoral and lumbar vertebral bone mineral density (in milligrams per square

centimeter) was measured using a regular Hologic QDR-1000 instrument (Hologic,

Waltham, Mass., USA). A collimator with 0.9-cm-diameter aperture and an ultrahigh

resolution mode (line spacing, 0.0254 cm; resolution, 0.0127 cm) were used. The excised

long bones were placed in 70% alcohol onto a resin platform provided by the company

for soft tissue calibration. Daily scanning of a phantom image controlled the stability of

the measurements. Instrument precision and reproducibility had been previously

evaluated by calculating the coefficient of variation of repeated DEXA and had been

found to be below 2%. Coefficients of variation were 0.5 to 2% for all evaluated

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parameters. A set of 5-month-old male mice was analyzed (non-tg = 13 littermates of all

analyzed lines, hSOST^{wt} = 15 (heterozygous mice from 2 hSOST^{wt} BAC lines, hSOST^{vbΔ}

= 14 off-springs of heterozygous matings from 2 hSOST^{vbΔ} lines).

Micro computed tomography (microCT) analysis.

Cancellous bone structure was evaluated in the proximal tibia metaphysis using a Scanco

vivaCT20 (Scanco Medical AG, Bassersdorf, Switzerland). The nonisometric voxels had

a dimension of 12.5µm x 12.5µm x 12.5µm. From the cross-sectional images the

cancellous bone compartment was delineated from cortical bone by tracing its contour at

every 10th section. In all the other slices boundaries were interpolated based on the

tracing to define the volume of interest. 660 slices covering a total length of 0.8mm

within the area of the secondary spongiosa (1.3mm from the proximal end) were

evaluated. A threshold value of 175 was used for the three dimensional evaluation of

trabecular number, thickness, and separation. Both sets of male 5-month-old mice on

which DEXA and histomorphometric analysis has been performed were analyzed. A

voxel size of 25µm x 25µm x 25µm was chosen for visualization of the digits of the fore-

and hind limbs.

Histomorphphometric analysis.

After dissection, the tibia and lumbar vertebrae were placed for 24h in Karnovsky's fix,

dehydrated in ethanol at 4°C, and embedded in methylmethacrylate. A set of 4- and 8-,

microm-thick nonconsecutive microtome sections were cut in the frontal midbody plane

for evaluation of fluorochrome-label-based dynamic and cellular parameters of bone

turnover. The 4 microm-thick sections were stained with TRAP and Giemsa stain. The

sections were examined using a Leica DM microscope (Leica, Glattbrugg, Switzerland)

fitted with a camera (SONY DXC-950P, Tokyo, Japan) and adapted Quantimet 600

software (Leica, Cambridge, UK). Two sections/animal were sampled for all sets of

parameters. Microscopic images of specimens were evaluated semiautomatically digitally

(X400 magnification). All parameters were measured and calculated according to Paritt

et al. 1987 (J Bone Min Res). Fluorochrome label bone formation dynamics were

evaluated on unstained 8 microm-thick sections. Bone perimeter, single- and double-

labeled bone perimeter, and interlabel width were measured. Mineralized perimeter (%),

mineral apposition rates (micrometers/day) (corrected for section obliquity in the

cancellous bone compartment), and daily bone formation rates (daily bone formation

rate/bone perimeter [micrometer/day]) were calculated. Osteoclast numbers (osteoclast

number/bone perimeter [millimeters-1]) and perimeter values (osteoclast perimeter/bone

perimeter [percent]) were determined on the TRAPstained slides, and osteocyte number

(osteocyte number/bone perimeter [millimeters-1]) on the Giemsa stained slides. All

parameters were evaluated in the spongiosa and at the endocortex. A set of 5-month-old

male mice from one hSOSTw line was analyzed (non-tg = 5, hSOSTwt/+ = 7,

hSOSTwt/wt = 4).

In vitro Enhancer Assays

ECRs were PCR-amplified with 5'NheI-linkers, TOPO-cloned into pCR2.1 vector

(Invitrogen) then shuttled into NheI/XhoI sites of pGL3-promoter (Promega) or

HindIII/PstI of hsp68-LacZ (B. Black). The following primers were used to amplify

human DNA (62ºC annealing/30 sec extension/35 cycles): ECR2 (545 bp) 5'-AGCAACGCAGGGCAGGAGCCAAGA-3', 5'-TAGCTGGCCTCTCCTGGGCGTCTT-3'; ECR3 (410 bp) 5'-GGGGGCTGTATGGAAAGGAGACAT-3', 5'-CTTGAGCAGTAGGGCCAAGCCCT-3'; ECR4 (296 bp) 5'-TGACAAACAGGAAGGTGGCAGGGC-3', 5'-CCCCCAACATTCCTGTCCCCTTG-3'; ECR5 (259 bp) 5'-TCCTTGCCACGGGCCACCAGCTTT-3', 5'-CCCCCTCATGGCTGGTCTCATTTG-3'; ECR6 (666 bp) 5'-CCCTGAGAAACATGCCTCTGTCCC-3', 5'-CTTAGCAATCTGGGTGACCCTGGG-3'; ECR7 (568 bp) 5'-AAACTGCCAAGCCCCAGCTGGCTA-3', 5'-GCCCAGGGCTCAGAAATGTGTGGA-3'; ECR8 (352 bp) 5'-TTCCTACCAAGGTGGCTGCCACC-3', 5'-CCTTCAGAGAAGCAAATGGCTGGGG-3'; -2kb promoter 5'-CAGCAGAAGATGTCACAGCAGG-3', 5'-GAGCTGCATGGTACCAGCCAGA-3'. Human SOST promoter sequence (2kb upstream of 5'UTR) was PCR-amplified with SmaI-linkers and transferred into the SmaI site of pGL3basic (Promega). A luciferase reporter plasmid containing mouse *osteocalcin* (*OG2*) promoter sequence from -1323 to +10 in pGL3basic was kindly obtained from B. Fournier (Novartis Basel, Switzerland). Reporter plasmids containing ECR-4, -5 or -6 upstream of the human *sost* promoter were generated by inserting the ECR elements into the NheI site. Plasmid DNA was isolated using standard endotoxin-free methods (Qiagen). FuGene (Roche) and a CMV-bgal reporter plasmid (Clontech) as internal control were used for transient transfections of rat UMR-106 and human 293 cells. Cells were incubated for 24 hours at 37ºC and luciferase and galactosidase expression were measured using standard assay kits (Promega).

Transient transgenic analysis

500 mg of DNA was linearized with NotI, followed by CsCl gradient purification and 2-5 ng was used for pronuclear injections of FVB embryos. E12.5-E14.5 embryos were dissected in ice-cold PBS, and were fixed in 4% paraformaldehyde at 4ºC for 1-2 hours and stained for *LacZ* as described. Transgenic embryos were detected by PCR from tail DNA [fwd 5'-TTTCCATGTTGCCACTCGC-3', rvd 5'-AACGGCTTGCCGTTCAGCA-3'; 55ºC annealing/30 sec extension/25 cycles].

Acknowledgements

REFERENCES

Balemans, W., M. Ebeling, N. Patel, E. Van Hul, P. Olson, M. Dioszegi, C. Lacza, W.
Wuyts, J. Van Den Ende, P. Willems, A.F. Paes-Alves, S. Hill, M. Bueno, F.J.
Ramos, P. Tacconi, F.G. Dikkers, C. Stratakis, K. Lindpaintner, B. Vickery, D.
Foernzler, and W. Van Hul. 2001. Increased bone density in sclerosteosis is due
to the deficiency of a novel secreted protein (SOST). *Hum Mol Genet* **10:** 537-
543.

Balemans, W., N. Patel, M. Ebeling, E. Van Hul, W. Wuyts, C. Lacza, M. Dioszegi, F.G.
Dikkers, P. Hildering, P.J. Willems, J.B. Verheij, K. Lindpaintner, B. Vickery, D.
Foernzler, and W. Van Hul. 2002. Identification of a 52 kb deletion downstream
of the SOST gene in patients with van Buchem disease. *J Med Genet* **39:** 91-97.

Balemans, W., J. Van Den Ende, A. Freire Paes-Alves, F.G. Dikkers, P.J. Willems, F.
Vanhoenacker, N. de Almeida-Melo, C.F. Alves, C.A. Stratakis, S.C. Hill, and W.
Van Hul. 1999. Localization of the gene for sclerosteosis to the van Buchem
disease-gene region on chromosome 17q12-q21. *Am J Hum Genet* **64:** 1661-1669.

Beighton, P., J. Davidson, L. Durr, and H. Hamersma. 1977. Sclerosteosis - an autosomal
recessive disorder. *Clin Genet* **11:** 1-7.

Brunet, L.J., J.A. McMahon, A.P. McMahon, and R.M. Harland. 1998. Noggin, cartilage
morphogenesis, and joint formation in the mammalian skeleton. *Science* **280:**
1455-1457.

Brunkow, M.E., J.C. Gardner, J. Van Ness, B.W. Paeper, B.R. Kovacevich, S. Proll, J.E.
Skonier, L. Zhao, P.J. Sabo, Y. Fu, R.S. Alisch, L. Gillett, T. Colbert, P. Tacconi,
D. Galas, H. Hamersma, P. Beighton, and J. Mulligan. 2001. Bone dysplasia

sclerosteosis results from loss of the SOST gene product, a novel cystine knot-containing protein. *Am J Hum Genet* **68:** 577-589.

Chuzhanova, N., S.S. Abeysinghe, M. Krawczak, and D.N. Cooper. 2003. Translocation and gross deletion breakpoints in human inherited disease and cancer II: Potential involvement of repetitive sequence elements in secondary structure formation between DNA ends. *Hum Mutat* **22:** 245-251.

Cimbora, D.M., D. Schubeler, A. Reik, J. Hamilton, C. Francastel, E.M. Epner, and M. Groudine. 2000. Long-distance control of origin choice and replication timing in the human beta-globin locus are independent of the locus control region. *Mol Cell Biol* **20:** 5581-5591.

Curtin, P., M. Pirastu, Y.W. Kan, J.A. Gobert-Jones, A.D. Stephens, and H. Lehmann. 1985. A distant gene deletion affects beta-globin gene function in an atypical gamma delta beta-thalassemia. *J Clin Invest* **76:** 1554-1558.

Curtin, P.T. and Y.W. Kan. 1988. The inactive beta globin gene on a gamma delta beta thalassemia chromosome has a normal structure and functions normally in vitro. *Blood* **71:** 766-770.

Devlin, R.D., Z. Du, R.C. Pereira, R.B. Kimble, A.N. Economides, V. Jorgetti, and E. Canalis. 2003. Skeletal overexpression of noggin results in osteopenia and reduced bone formation. *Endocrinology* **144:** 1972-1978.

Enattah, N.S., T. Sahi, E. Savilahti, J.D. Terwilliger, L. Peltonen, and I. Jarvela. 2002. Identification of a variant associated with adult-type hypolactasia. *Nat Genet* **30:** 233-237.

Hamersma, H., J. Gardner, and P. Beighton. 2003. The natural history of sclerosteosis. *Clin Genet* **63:** 192-197.

Ionasescu, V.V., C. Searby, R. Ionasescu, I.M. Neuhaus, and R. Werner. 1996. Mutations of the noncoding region of the connexin32 gene in X-linked dominant Charcot-Marie-Tooth neuropathy. *Neurology* **47:** 541-544.

Khokha, M.K., D. Hsu, L.J. Brunet, M.S. Dionne, and R.M. Harland. 2003. Gremlin is the BMP antagonist required for maintenance of Shh and Fgf signals during limb patterning. *Nat Genet* **34:** 303-307.

Kleinjan, D.A., A. Seawright, A. Schedl, R.A. Quinlan, S. Danes, and V. van Heyningen. 2001. Aniridia-associated translocations, DNase hypersensitivity, sequence comparison and transgenic analysis redefine the functional domain of PAX6. *Hum Mol Genet* **10:** 2049-2059.

Kusu, N., J. Laurikkala, M. Imanishi, H. Usui, M. Konishi, A. Miyake, I. Thesleff, and N. Itoh. 2003. Sclerostin is a novel secreted osteoclast-derived bone morphogenetic protein (BMP) antagonist with unique ligand specificity. *J Biol Chem*.

Lee, E.C., D. Yu, J. Martinez de Velasco, L. Tessarollo, D.A. Swing, D.L. Court, N.A. Jenkins, and N.G. Copeland. 2001. A highly efficient Escherichia coli-based chromosome engineering system adapted for recombinogenic targeting and subcloning of BAC DNA. *Genomics* **73:** 56-65.

Lettice, L.A., S.J. Heaney, L.A. Purdie, L. Li, P. de Beer, B.A. Oostra, D. Goode, G. Elgar, R.E. Hill, and E. de Graaff. 2003. A long-range Shh enhancer regulates expression in the developing limb and fin and is associated with preaxial polydactyly. *Hum Mol Genet* **12:** 1725-1735.

Mankoo, B.S., S. Skuntz, I. Harrigan, E. Grigorieva, A. Candia, C.V. Wright, H. Arnheiter, and V. Pachnis. 2003. The concerted action of Meox homeobox genes is required upstream of genetic pathways essential for the formation, patterning and differentiation of somites. *Development* **130:** 4655-4664.

Mortlock, D.P., C. Guenther, and D.M. Kingsley. 2003. A general approach for identifying distant regulatory elements applied to the Gdf6 gene. *Genome Res* **13:** 2069-2081.

Nobrega, M.A., I. Ovcharenko, V. Afzal, and E.M. Rubin. 2003. Scanning human gene deserts for long-range enhancers. *Science* **302:** 413.

Ovcharenko, I., G.G. Loots, R.C. Hardison, W. Miller, and L. Stubbs. 2004. zPicture: dynamic alignment and visualization tool for analyzing conservation profiles. *Genome Res* **14:** 472-477.

Petropoulos, H., P.J. Gianakopoulos, A.G. Ridgeway, and I.S. Skerjanc. 2004. Disruption of Meox or Gli activity ablates skeletal myogenesis in P19 cells. *J Biol Chem* **279:** 23874-23881.

Sebat, J., B. Lakshmi, J. Troge, J. Alexander, J. Young, P. Lundin, S. Maner, H. Massa, M. Walker, M. Chi, N. Navin, R. Lucito, J. Healy, J. Hicks, K. Ye, A. Reiner, T.C. Gilliam, B. Trask, N. Patterson, A. Zetterberg, and M. Wigler. 2004. Large-scale copy number polymorphism in the human genome. *Science* **305:** 525-528.

Staehling-Hampton, K., S. Proll, B.W. Paeper, L. Zhao, P. Charmley, A. Brown, J.C. Gardner, D. Galas, R.C. Schatzman, P. Beighton, S. Papapoulos, H. Hamersma, and M.E. Brunkow. 2002. A 52-kb deletion in the SOST-MEOX1 intergenic

region on 17q12-q21 is associated with van Buchem disease in the Dutch population. *Am J Med Genet* **110:** 144-152.

Sutherland, M.K., J.C. Geoghegan, C. Yu, E. Turcott, J.E. Skonier, D.G. Winkler, and J.A. Latham. 2004. Sclerostin promotes the apoptosis of human osteoblastic cells: a novel regulation of bone formation. *Bone* **35:** 828-835.

Tsui, F.W., H.W. Tsui, E.Y. Cheng, M. Stone, U. Payne, J.D. Reveille, M.J. Shulman, A.D. Paterson, and R.D. Inman. 2003. Novel genetic markers in the 5'-flanking region of ANKH are associated with ankylosing spondylitis. *Arthritis Rheum* **48:** 791-797.

Uitterlinden, A.G., P.P. Arp, B.W. Paeper, P. Charmley, S. Proll, F. Rivadeneira, Y. Fang, J.B. van Meurs, T.B. Britschgi, J.A. Latham, R.C. Schatzman, H.A. Pols, and M.E. Brunkow. 2004. Polymorphisms in the sclerosteosis/van Buchem disease gene (SOST) region are associated with bone-mineral density in elderly whites. *Am J Hum Genet* **75:** 1032-1045.

Van Bezooijen, R.L., B.A. Roelen, A. Visser, L. Van Der Wee-Pals, E. De Wilt, M. Karperien, H. Hamersma, S.E. Papapoulos, P. Ten Dijke, and C.W. Lowik. 2004. Sclerostin Is an Osteocyte-expressed Negative Regulator of Bone Formation, But Not a Classical BMP Antagonist. *J Exp Med* **199:** 805-814.

Van Hul, W., W. Balemans, E. Van Hul, F.G. Dikkers, H. Obee, R.J. Stokroos, P. Hildering, F. Vanhoenacker, G. Van Camp, and P.J. Willems. 1998. Van Buchem disease (hyperostosis corticalis generalisata) maps to chromosome 17q12-q21. *Am J Hum Genet* **62:** 391-399.

Van Hul, W., F. Vanhoenacker, W. Balemans, K. Janssens, and A.M. De Schepper. 2001. Molecular and radiological diagnosis of sclerosing bone dysplasias. *Eur J Radiol* **40:** 198-207.

Wang, H.L., T. Wu, W.T. Chang, A.H. Li, M.S. Chen, C.Y. Wu, and W. Fang. 2000. Point mutation associated with X-linked dominant Charcot-Marie-Tooth disease impairs the P2 promoter activity of human connexin-32 gene. *Brain Res Mol Brain Res* **78:** 146-153.

Wergedal, J.E., K. Veskovic, M. Hellan, C. Nyght, W. Balemans, C. Libanati, F.M. Vanhoenacker, J. Tan, D.J. Baylink, and W. Van Hul. 2003. Patients with Van Buchem disease, an osteosclerotic genetic disease, have elevated bone formation markers, higher bone density, and greater derived polar moment of inertia than normal. *J Clin Endocrinol Metab* **88:** 5778-5783.

Winkler, D.G., M.K. Sutherland, J.C. Geoghegan, C. Yu, T. Hayes, J.E. Skonier, D. Shpektor, M. Jonas, B.R. Kovacevich, K. Staehling-Hampton, M. Appleby, M.E. Brunkow, and J.A. Latham. 2003. Osteocyte control of bone formation via sclerostin, a novel BMP antagonist. *Embo J* **22:** 6267-6276.

Winkler, D.G., C. Yu, J.C. Geoghegan, E.W. Ojala, J.E. Skonier, D. Shpektor, M.K. Sutherland, and J.A. Latham. 2004. Noggin and sclerostin bone morphogenetic protein antagonists form a mutually inhibitory complex. *J Biol Chem* **279:** 36293-36298.

Figure 1. *Generation and Characterization of Van Buchem transgenic mouse models.*
(A) A 158kb human BAC (hSOST^{wt}) spanning *sost* and *meox1* was engineered using *in vitro* BAC recombination in *E.coli* (Lee et al. 2001) by deleting the 52kb noncoding region missing in VB patients (hSOST^{vbΔ}). Human *sost* expression was analyzed by rtPCR in adult tissues (B) and quantitative rtPCR in E10.5 embryos (C) from two independent lines of each hSOST^{wt} and hSOST^{vbΔ} transgene.

Figure 2. *Sost* transgenic expression negatively impacts bone parameters. (A) Body weight measurements of 5-month-old male mice (non-tg = 13, hSOST^{wt} = 15, hSOST^{vbΔ} = 14; animals were pooled from two lines of hSOST^{wt} and two lines of hSOST^{vbΔ}; Supplementary Materials). (B) Bone mineral density in the tibia, femur and lumbar spine as evaluated by DEXA. (C) Bone volume, trabecular number, thickness and separation as evaluated in the cancellous bone compartment of the proximal tibia metaphysis by μCT. (Mean +/- SEM; * p<.05 versus non-tg).

Figure 3. Human *sost* dose effect on bone metabolism in the proximal tibia metaphysis of 5-month-old male mice (non-tg = 5, hSOST$^{wt/+}$ = 7, hSOST$^{wt/wt}$ = 4). (A) Bone volume and (B) bone formation rates as determined by μCT scans and histomorphometric analysis respectively. (Mean +/- SEM; * p<.05 versus non-tg; x p<.05 versus hSOST$^{wt/+}$). (C) Cancellous bone compartment of non-transgenic and hSOST$^{wt/wt}$ mice. (D) Fluorochrome marker uptake at site of active mineralization of bone matrix laid down by

osteoblasts in wildtype and transgenic mice at the interface between endocortex and cancellous bone.

Figure 4. Embryonic *sost* expression and limb deformity in hSOST^{wt} and hSOST^{vbΔ} transgenic mice. High levels of embryonic *sost* expression was predominantly detected in the developing limb bud of E10.5 defective mice, as visualized by whole mount *in situ* hybridization using a *sost* probe that detects human and mouse transcripts (A). µCT scans of defective hSOST^{wt} and hSOST^{vbΔ} adult limbs overexpressing human *sost* (B).

Figure 5. Enhancer activity of evolutionarily conserved noncoding sequences from the Van Buchem deletion region. (A) Human/Mouse genomic alignment generated using zPicture alignment engine (http://zpicture.dcode.org/). Exons are in blue, untranslated regions in yellow, repetitive elements in green and noncoding sequences in red (intragenic) or pink (intronic). Seven highly conserved elements (≥200 bp; ≥80% ID; ECR2-8) within VBΔ and the promoter region were tested in rat-osteosarcoma (UMR-106) and kidney cells (293) for the ability to enhance luciferase expression from the SV40-promoter (B) or human *sost* promoter (C). ECR5 activates the human *sost* promoter in rat osteosarcoma cells (C), and drives the *hsp68* promoter in the skeleton of E14.5 mouse embryos (D).
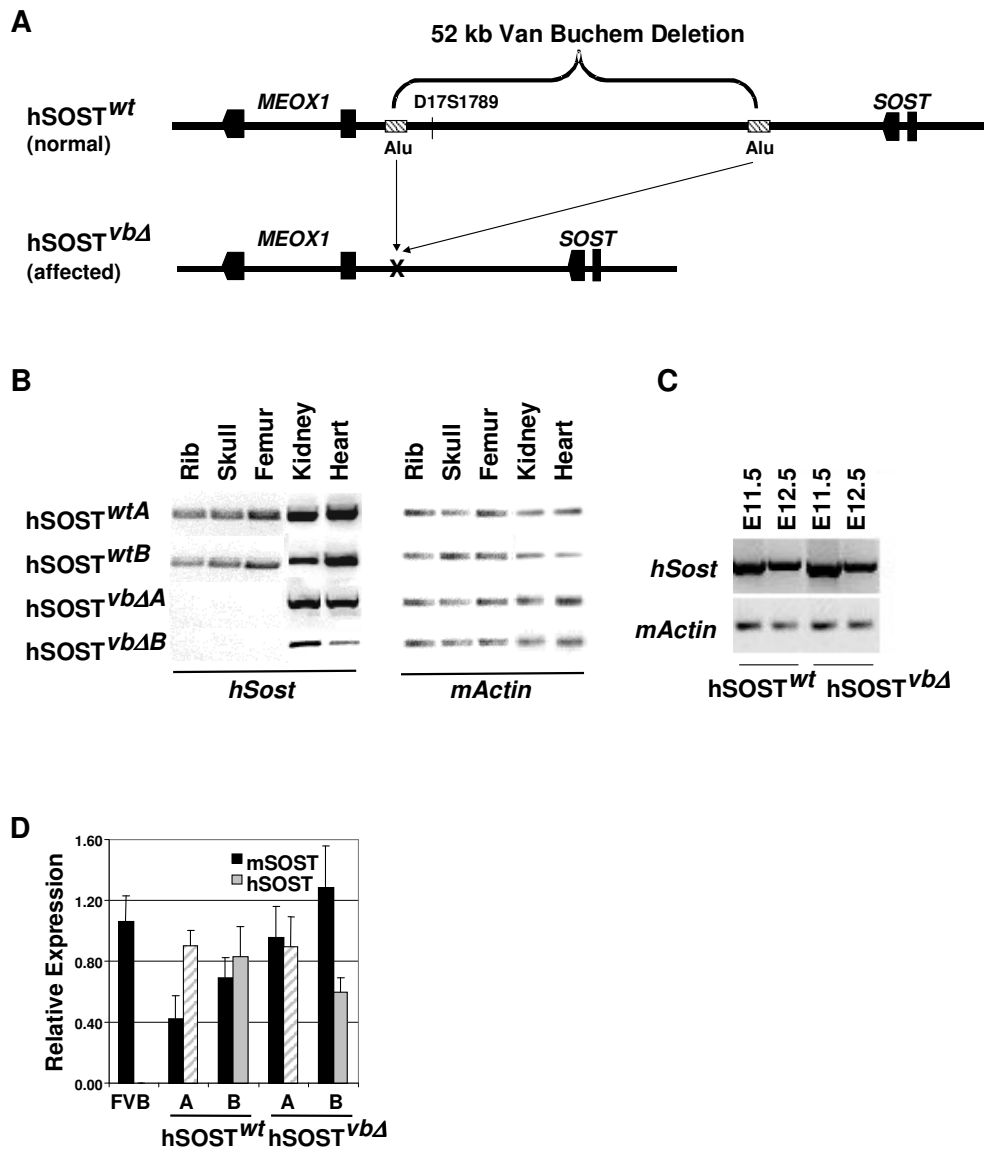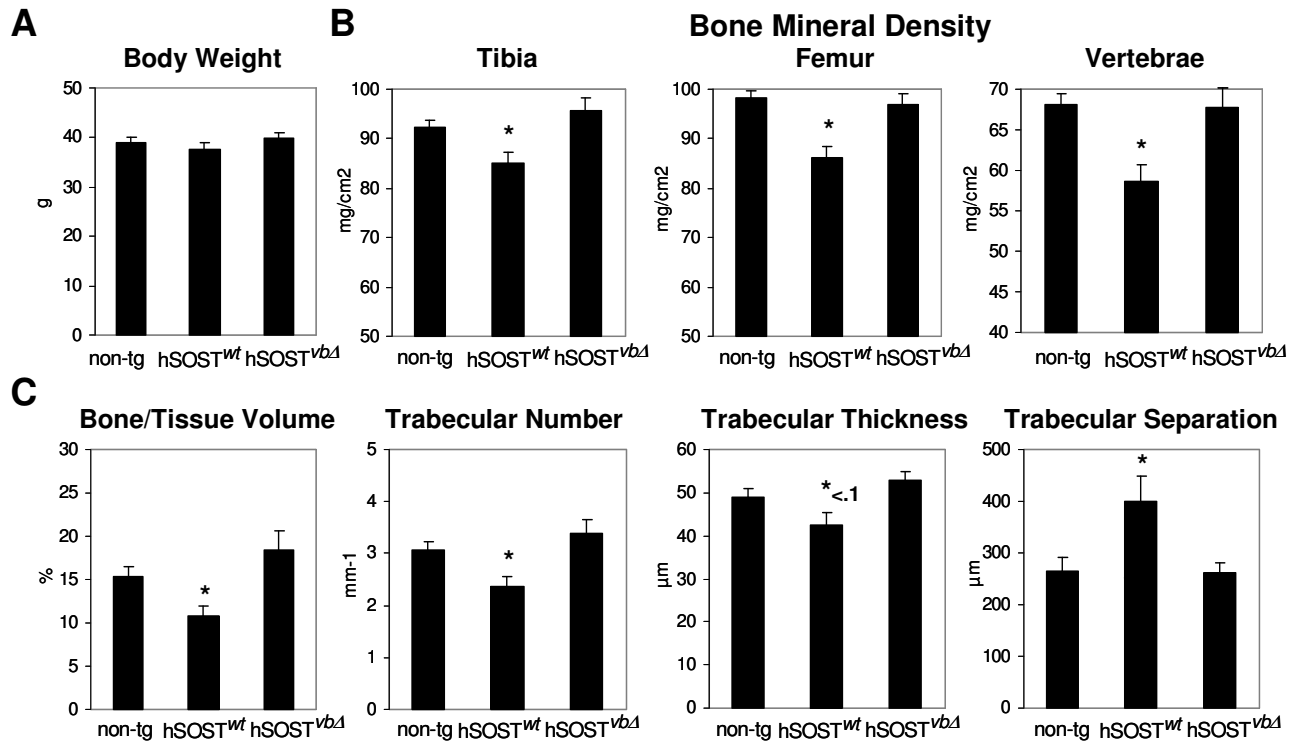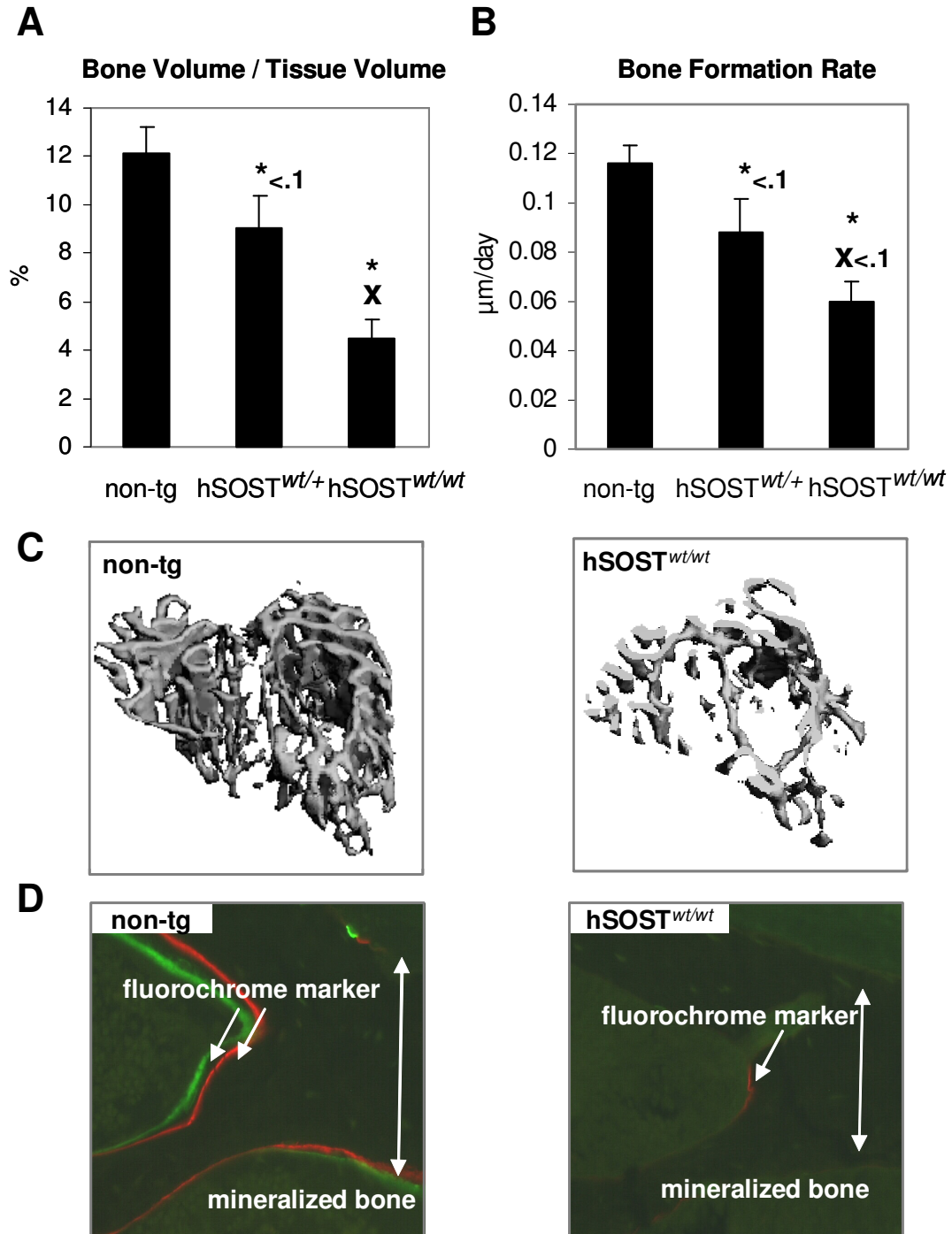
Figure 1

A

B

C

D

Figure 2

Figure 3

A

Bone Volume / Tissue Volume

B

Bone Formation Rate

C

D

Figure 4

Figure 5

A

B

C

D