

Full-Length Bovine spp24 [spp24 (24-203)] Inhibits BMP-2 Induced Bone Formation

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ABSTRACT: Secreted phosphoprotein 24 kDa (spp24) is a bone matrix protein. It contains a TGF- β receptor II homology 1 (TRH1) domain. A cyclic, synthetic 19 amino acid peptide (bone morphogenetic protein binding peptide or BBP) based on the sequence of the TRH1 domain enhances BMP-2 induced osteogenesis. Many observations suggest that different size forms of this protein have very different effects (inhibiting or enhancing) on BMP-2 induced osteogenesis. Using the stable recombinant Met(His)₆-tagged secretory form of full-length (fl) bovine spp24 [Met(His)₆-spp24 (residues 24–203)] and transgenic (TG) mice expressing fl bovine spp24 (residues 1–203), we have demonstrated that spp24 inhibits BMP-2 induced bone formation. The effects of Met(His)₆-spp24 (24–203) were determined in the ectopic bone-forming bioassay in male mice. Implantation of 5 μ g of BMP-2 stimulated bone formation, assessed densitometrically as bone area and mineral content. When Met(His)₆-spp24 (24–203) was implanted with BMP-2, it elicited a dose-dependent decrease in BMP-2-mediated ectopic bone formation. When added at a 50-fold excess (w/w), Met(His)₆-spp24 (24–203) completely ablated the effects of BMP-2, while addition of a 10-fold excess had no effect. Constitutive expression of fl bovine spp24 (1–203) under the control of the osteocalcin promoter in TG female mice reduced femoral and vertebral bone mineral density at 3 months of age and reduced femoral BMD at 8 months of age, but had no effects in male mice, which can exhibit less osteocalcin-promoter driven gene transcription than females. Histomorphometric analysis demonstrated that bone volume and trabecular thickness were lower in TG female mice at 3 months of age than in sex- and age-matched wild type (WT) controls. Thus, fl spp24 and its secretory isoform (Met(His)₆-spp24 [24–203]), which contain a BMP-binding or TRH1 motif, inhibit ectopic bone formation in male mice and adversely affects BMD and histological parameters related to bone mass and formation in female mice expressing the human transgene. Under these conditions, fl spp24 acts as a BMP antagonist *in vivo*. © 2008 Orthopaedic Research Society. * Published by Wiley Periodicals, Inc. *J Orthop Res* 26:753–758, 2008

Keywords: bone formation; spp24; bone morphogenetic protein

Secreted phosphoprotein-24 (spp24) is a 24 kDa protein of unknown function first cloned from bovine bone matrix.¹ The calculated molecular weight of the full-length unprocessed precursor [bovine spp24 (residues 1–203)] is 23.1 kDa (Swiss-Prot database; AC #Q27967). The first 23 amino acid residues of bovine spp24 (1–203) constitute a signal peptide that is cleaved when the protein is secreted in its mature form [spp24 (24–203)]. The protein contains two internal disulfide bonds linking residues 86 to 97 and 110 to 128, respectively, and contains internal domains with sequence homology to cystatins (cysteine protease inhibitors) and cathelicidins (protease-sensitive precursors of endogenous antibiotic peptides).¹ Recent work demonstrated that an 18.5 kDa protein in the osteogenic fraction that Urist et al.² isolated from demineralized bone matrix (DBM) by hydroxyapatite chromatography appears to be a fragment of spp24.³ Like fetuin, spp24 and its degradation products (e.g., spp18.5)³ may bind members of the TGF- β family of cytokines because they both possess an internal TGF- β receptor II homology-1 (TRH1) domain.^{4,5} The cyclic, synthetic 19 amino acid residue peptide derived from the sequence of the TRH1 domain that is shared by spp24 and spp18.5 binds rhBMP-2

and enhances BMP-2 mediated bone formation.³ This TRH1 domain of spp24 and spp18.5 corresponds to the second internally disulfide-bonded domain of bovine spp24 (residues 110–128).³ To date, the osteogenic properties of the full-length (fl) parental protein (spp24) and its secretory isoform [e.g., bovine spp24 (24–203)] have not been determined. We have generated full-length recombinant bovine spp24 and its stable Met (His)₆-tagged secretory isoform⁵ and report the inhibitory effects of these recombinant spp24 isoforms on BMP-2 stimulated bone formation in two distinct models; in female transgenic mice overexpressing fl spp24 under the control of the osteocalcin promoter and in the ectopic bone-forming bioassay in male mice treated with BMP-2 and bovine Met(His)₆-spp24 (24–203).

MATERIALS AND METHODS

Synthesis of Recombinant Met(His)₆-spp24 (Residues 24–203)

SPP24 specific cDNA was cloned into pET20b (Novagen EMD Biosciences, La Jolla, CA) and expressed in *E. coli* as a stable His₆-tagged secretory protein construct [Met(His)₆-spp24 (24–203)] as described in detail elsewhere.⁵

In Vivo Assay for Bone Formation

The osteogenic activity of combinations of recombinant Met(His)₆-spp24 (24–203) isolated from *E. coli* and recombinant human (rh) BMP-2 was tested using an *in vivo* assay

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approved by the Sepulveda Animal Subjects Committee and the VA Greater Los Angeles Research and Development Committee. Male Swiss-Webster mice aged 8–10 weeks were used (Taconic Farms, Germantown, NY). Lyophilized spp24 was ground to a fine powder and applied to $5 \times 5 \times 13$ mm slices of Helistat collagen sponges (Integra Life Sciences, Plainsboro, NJ) that had been moistened with three small drops of water. The sponges were placed in microfuge tubes and allowed to dry in a tissue culture hood. The sponges containing the spp24 were lyophilized overnight and then loaded into #5 gelatin capsules using a loading funnel (Torpac, Fairfield, NJ). Prior to implantation, the capsules were placed in a sterile rack and the appropriate amount of rhBMP-2 or sterile water was applied to the sponge. The material was allowed to air dry for 30 min prior to sealing and implantation. The capsules were implanted into the quadriceps muscle of the animal subjects as previously described.³ Mice were killed after 28 days and the hindquarters removed for analyses.

Production of Plasmid for Transgenic Animals

The sequence for full length bovine SPP24 (also known as SP2 or secreted phosphoprotein-2) was located in GenBank and used to produce an expression plasmid for the generation of transgenic mice. Bovine liver cDNA was prepared from bovine polyA⁺ RNA using a MasterAmp RT-PCR kit (Epicentre, Madison, WI). The cDNA was amplified using the following primers: [gcgttttctagagccaccatggcgatgaagatgttggtg] and [GCGTTTACCGGTCTCAAAGCCAGGGTTTACTC]. This material was then digested with XbaI-AgeI and cloned into plasmid pcDNA3.1V5HisA (Invitrogen, Carlsbad, CA). The sequence of the 24 kb insert was confirmed using vector primers. The 24 kb insert was amplified using the same primers as shown above and was digested with BglII-BpiI. Plasmid pOC-IGF-I (kindly supplied by the laboratory group of Thomas Clemens, Ph.D.)⁶ was digested with BamHI-EcoRI and then the 4.9 kb EcoRI-BamHI and the 2.0 kb BamHI-BamHI fragments were gel purified. The smaller fragment was dephosphorylated. The following adapter oligos were annealed: [tagcagcatgctgggga] and [aatttccccagcatgct]. Ligation was then performed combining the following components: the 4.9 kb EcoRI-BamHI fragment of pOC-IGF-I; the 2.0 kb dephosphorylated BamHI-BamHI fragment of pOC-IGF-I; the 0.85 kb BglII-BpiI amplification product; and the annealed BpiI-EcoRI compatible adapter oligos. The products were screened for the proper orientation of the 2.0 kb fragment by digestion with EcoRI-XhoI which produced a 780 bp fragment. Sequencing was then conducted to confirm the sequence of the insert as well as the OC (osteocalcin) promoter and rabbit β -globin portions of pOC-IGF-I plasmid. Sequence information was compared to GenBank and an OC promoter- β -globin clone (AB004306) and a human chromosome 1 clone (AL135927, clone RP11-54H19) were used to make corrections to the OC promoter and β -globin portions of the new plasmid, pOC-24kd. This plasmid was digested with SapI-HindIII and the gel purified fragment was sent to the Transgenic Mouse Facility at the University of California, Irvine for the generation of transgenic mice.

Generation and Characterization of Transgenic Mice

Transgenic mice were generated in FVB/N mice at the UCI Transgenic Mouse Facility using their protocols (<http://darwin.bio.uci.edu/~tjf/index.html>). Five founders were obtained. These animals were crossed with wild type FVB/N mice and animals carrying the transgene were identified by

polymerase chain reaction (PCR) of DNA extracted from tissue obtained from the tail. DNA was extracted using a DNeasy kit (Qiagen, Valencia, CA). PCR was conducted using a HotStar DNA polymerase kit (Qiagen). The PCR primers used were identical to those employed by other investigators [M. Zhang, personal communication]: (osteocalcin promoter region) 5'CAAATAGCCCTGGCAGATTC3' and (rabbit β globin region) 5'TGATACAAGGGACATCTTCC3'. The expected band of 230 bp was visualized by agarose gel electrophoresis. The PCR protocol involved 30 cycles of melting (94°C, 0.5 min.), annealing (53°C, 0.5 min.) and extension (72°C, 0.5 min.) and was conducted in a DNA Thermal Cycler (Perkin Elmer, Norwalk, CT). Heterozygous animals of both genders and from all lines were used for the studies described here.

Densitometry

Bone density measurements were performed using a PIXImus2 densitometer designed specifically for use with small animals (GE Lunar Corp., Madison, WI). Bone area and bone mineral content were quantitated in specimens from the hindquarter ectopic bone formation assay by excising the muscle, including the implant site, of the hindquarter and placing them in the specimen tray. A region of interest (ROI) of 51×51 pixels was used to isolate the implant area. Dead 3-month-old transgenic (TG) or wild type (WT) mice were placed in the specimen tray and, after appropriate calibration, duplicate cycles of four scans were obtained. Data from three regions of interest were obtained. "Total body" included all highly dense (osseous) tissues other than the skull and tail which are routinely excluded. "Vertebral" was defined by a rectangle that extended from end plate to end plate for the five center-most thoracolumbar vertebrae and which included the lateral margins of all of the vertebrae involved. "Femoral" was defined as a rectangle 40 pixels in length centered in the mid-shift region of the femur and which included both cortices of the bone.

Histomorphometry

To examine bone turnover in cortical bone, dynamic histomorphometric analyses were performed on the femoral metaphysis of heterozygous TG mice and WT littermates. Bone turnover in trabecular bone was examined by performing histomorphometric analyses on the cancellous bone in an area between the lowest point of the growth plate and 1.5 mm distal to the growth plate. Labeling was accomplished with i.p. injections of 20 mg/kg of tetracycline HCl on days 1 and 2 and on days 9 and 10. Mice were killed on day 12, and one femur from each mouse was excised. The soft tissue surrounding the bone was removed and the specimens were placed in 70% ethanol and stored at 4°C. The femurs were dehydrated in graded ethanol, defatted in xylene, and embedded without demineralization in methyl methacrylate. Five-micrometer-thick coronal sections were cut parallel to the long axis of the femur, using a Leica RM 2155 microtome (Nussloch, Germany) and stained with Goldner's trichrome. Additional 12- μ m-thick unstained sections were prepared for observations under UV light. The following histomorphometric parameters were recorded in these standard sampling sites using the guidelines of the American Society of Bone Mineral Research Histomorphometric Nomenclature Committee: (1) trabecular bone volume (BV/TV, %); (2) mineral apposition rate (MAR, μ m/day); (3) bone formation rate (BFR, %/yr); (4) trabecular thickness (Tb.Th, μ m); (5) trabecular separation (Tb.Sp, μ m); and (6) trabecular number (Tb.N, per mm).⁷

Histomorphometric measurements of bone structure and microarchitectural parameters in cancellous bone were performed using a semiautomatic color image analyzer (ImagePro Plus, Version 4.5.1, Media Cybernetics, Silver Springs, MD) attached to a personal computer.

Serum Osteocalcin and Calcitonin Assays

Serum concentrations of calcitonin and osteocalcin were determined as described previously.⁸ Blood samples were collected by heart puncture between 08:00 h and 12:00 h on the day that the 3-month-old mice were killed. Serum was obtained by centrifuging heparinized, unhemolyzed blood in a microfuge for 10 min at 4°C. The serum was stored at -80°C until the assayed. Serum concentrations were determined using commercial radioimmunoassay (RIA) kits for calcitonin (Incstar, Stillwater, MN) and osteocalcin (Biomedical Technologies, Stoughton, MA). All assays were conducted in triplicate in multiple dilutions of serum and with control serum containing high and low levels of the proteins. Assay results were calculated using AssayZap software (Biosoft, Ferguson, MO).

Numerical Analysis

Means of treatment groups were compared with either Student's *t*-test or one-way ANOVA using InStat software (GraphPad Software, San Diego, CA). All numerical data are presented as mean $\pm$ SEM (standard error of the mean), and (*n*) or sample size.

RESULTS

Preparation of spp24

The preparation of recombinant Met(His)₆-spp24 (residues 24–203) from *E. coli* and the confirmation of the sequence of this protein are described in detail elsewhere.⁵

Effect of spp24 on BMP-2 Induced Ectopic Bone Formation

The effect of Met(His)₆-spp24 (24–203) on BMP-2 induced ectopic bone formation is shown in Table 1. Spp24 alone did not induce bone formation (data not shown). The highest amount of spp24 (2.5 mg per sponge)

completely inhibited BMP-2 induced bone formation. On the other hand, the smallest amount (0.05 mg per sponge) had no significant effect on bone formation. An intermediate dose had an intermediate effect. The relative and absolute amounts of spp24 and rhBMP-2 tested were selected based on the amounts previously shown to demonstrate a stimulatory effect of cBBP (cyclic BMP binding peptide) on rhBMP-2-stimulated ectopic bone formation in the mouse,⁵ where spp24 contains approximately 10-fold more amino acids than BBP.

Effect of SPP24 on Bone Formation in Transgenic Mice

Five independent strains of TG mice were generated in which the entire sequence of SPP24 was expressed under the control of the osteocalcin promoter. Founder animals were crossed to normal animals and heterozygous animals identified by analysis of tail DNA. The bones of heterozygous animals were analyzed at 3 and 8 months of age by densitometry and histomorphometry. The results of the densitometric analysis are shown in Table 2. Female TG mice consistently showed a reduced femoral bone mineral density (BMD) as compared to WT littermates at both 3 and 8 months, whereas male animals did not. Female TG animals also had a reduced vertebral BMD at 3 months but not at 8 months. The vertebral BMD of TG male animals was not different from that of WT littermates.

The microscopic anatomy of femurs from TG mice and WT littermates is shown in Figures 1 and 2. The quantitative histomorphometric analyses are shown in Table 3. Reduced trabecular bone volume accompanied by decreased thickness and increased separation is apparent in the TG mice in comparison to their WT littermates. As in the case with the densitometric measurements, the differences in bone structure were seen primarily in female animals.

Serum concentrations of osteocalcin, an index of bone formation, were lower in TG females than in WT females at 3 months of age (Table 4). Serum concentrations of osteocalcin in male subjects and concentration of calcitonin in all subjects, were not different.

Table 1. Densitometric Analysis of Excised Hindquarter Muscle Samples Containing Implants with Various Amounts of Bovine Met(His)₆-spp24 (24–203) and 5 μ g of rhBMP-2^a

Group	Treatment		Bone Area (cm ²)*	Bone Mineral Content (mg)**
	spp24 (mg)	BMP-2 (μ g)		
B	0.0	5	0.31 $\pm$ 0.065	8.75 $\pm$ 2.29
E	2.5	5	0.0 $\pm$ 0.0 (not detected in any specimen) ^b	0.0 $\pm$ 0.0 (not detected in any specimen) ^b
F	0.5	5	0.12 $\pm$ 0.044	3.5 $\pm$ 1.56
G	0.05	5	0.29 $\pm$ 0.045	8.0 $\pm$ 1.08

^aAll mice were treated for 28 days. Data shown as the mean $\pm$ SEM (*n* = 4). Addition of 2.5 mg spp24 inhibited all BMP-2-induced ectopic bone formation. ^bNo *t*-test can be performed for this data because no bone was detected, and all values for implants in mice treated with 2.5 mg spp24, including means and SEMs, equal zero. **p* values for bone area—B vs. E: N/A, see ^b above; B vs. F: *p* = 0.0518; B vs. G: *p* = 0.8087; E vs. F: N/A, see ^b above; E vs. G: N/A, see ^b above; F vs. G: *p* = 0.0355. ***p* values for bone mineral content—B vs. E: N/A, see ^b above; B vs. F: *p* = 0.1070; B vs. G: *p* = 0.7768; E vs. F: N/A, see ^b above; E vs. G: N/A, see ^b above; F vs. G: *p* = 0.0554.

Table 2. Bone Mineral Density (g/cm²) of Wild Type and Transgenic Mice at 3 and 8 Months of Age^a

Gender/Genotype	3 Months		8 Months	
	Femur BMD (g/cm ²)	Vertebral BMD (g/cm ²)	Femur BMD (g/cm ²)	Vertebral BMD (g/cm ²)
Female/WT	0.0730 ± 0.0022 (5)	0.0729 ± 0.0041 (5)	0.0676 ± 0.0009 (15)	0.0605 ± 0.0016 (17)
Female/TG	0.0654 ± 0.0017 (8)	0.0585 ± 0.0006 (8)	0.0637 ± 0.0015 (7)	0.0591 ± 0.0018 (10)
<i>p</i> Value: WT vs. TG	0.019	0.0010	0.0296	0.5812
Male/WT	0.0676 ± 0.0022 (5)	0.0563 ± 0.0009 (5)	0.0600 ± 0.0018 (12)	0.0520 ± 0.0014 (14)
Male/TG	0.0715 ± 0.0043 (6)	0.0616 ± 0.0037 (6)	0.0609 ± 0.0016 (8)	0.0546 ± 0.0024 (9)
<i>p</i> Value: WT vs. TG	0.4684	0.2356	0.7303	0.3268

BMD, bone mineral density; TG, Transgenic; WT, wild type. ^aMean ± SE (*n*).

DISCUSSION

Deminerized bone matrix (DBM) is a complex mixture of proteins, many of which remain poorly characterized or unidentified. Urist and his collaborators developed a protocol for chemically extracting soluble noncollagenous proteins from defatted DBM, converting collagen to gelatin, and extracting a fraction enriched in BMP activity and noncollagenous proteins ("BMP/NCP") from the insoluble gelatin with 0.5 M CaCl₂ and 6 M urea in the presence of protease inhibitors.² Further purification by detergent extraction, GuHCl precipitation, and hydroxyapatite chromatography led to the resolution of a number of proteins, one of which, an 18.5 kDa protein, exhibited pro-osteogenic activity when implanted with other fractions.² We identified the 18.5 kDa protein as a fragment of spp24 by modern high-resolution 2D SDS-PAGE and matrix-assisted laser desorption ionization/time of flight mass spectroscopy (MALDI/ToF MS).³

The M_rs and tentative identifications of the major proteins that Urist et al.^{2,9} described as co-purifying with the protein which we identified as DBM-derived spp24 include: 34 kDa (osteonectin), 22 kDa (ferritin) to 24 kDa (depending upon reference), 17.5 kDa (histone H2B), 17 kDa (a protein with calmodulin-like activity), and 14 kDa (a protein that renders the BMPs insoluble, possibly matrix Gla protein). We can speculate, but

cannot demonstrate, that the 24 kDa protein that Urist and colleagues observed was fl spp24 and that some of the smaller proteins were spp24 degradation products similar to those identified in *E. coli* (e.g., spp14.5 and spp16).⁵ It should be noted that phosphorylation and glycosylation of eukaryotic proteins in DBM may give rise to spp24 degradation products with higher M_rs than those identified in *E. coli* extracts, where these post-translational processes do not occur. In any case, the 34 kDa, 22 kDa, 17.5 kDa, 17 kDa, and 14 kDa proteins did not form bone alone or in combination, but the semipurified 18.5 kDa (variously reported as 18 kDa to 19 kDa or 18.5 kDa) protein did form bone.^{2,9,10} (Although Urist and his colleagues reported that the 18.5 kDa protein was osteogenic, they also noted that it had lower activity when purified to greater homogeneity.¹⁰ In retrospect, BMP-2 may have been present in the incompletely purified mixture of proteins,^{2,9,10} and it was probably the combination of BMP-2 and spp18.5 that induced osteogenesis.) Furthermore, a combination of the 18.5 kDa protein plus the 14 kDa protein (identified as matrix Gla protein) rendered the 18.5 kDa protein more insoluble and yielded more bone than the 18.5 kDa protein alone, while addition of the 34 kDa protein (identified as osteonectin) or the 34 kDa protein plus the 17.5 kDa protein (identified as histone H2B) to the 18.5 kDa protein "may have" reduced

Table 3. Histomorphometric Analyses of Cancellous Bone in the Femurs of 3-Month-Old Wild Type and fl spp24 Transgenic Mice in Sections Taken between the Lowest Point of the Growth Plate and 1.5 mm Distal to the Growth Plate^a

Parameter	Gender	Wild Type (WT)	Transgenic (TG)	<i>p</i> Value (WT vs. TG)
Bone Volume (%)	Female	15.68 ± 2.19 (5)	7.82 ± 1.18 (7)	0.0066
	Male	10.57 ± 1.92 (5)	10.21 ± 0.90 (7)	0.8552 (NS) ^b
Mineral apposition rate (μm/day)	Female	1.03 ± 0.157 (3)	0.701 ± 0.166 (5)	0.2350 (NS) ^b
	Male	2.14 ± 0.527 (3)	1.77 ± 0.092 (5)	0.3950 (NS) ^b
Bone formation rate (%/year)	Female	37.47 ± 5.72 (3)	32.63 ± 3.12 (5)	0.4430 (NS) ^b
	Male	77.99 ± 19.36 (3)	64.50 ± 3.37 (5)	0.3982 (NS) ^b
Trabecular thickness (μm)	Female	42.37 ± 4.68 (5)	25.15 ± 2.40 (6)	0.0073
	Male	38.01 ± 4.85 (5)	32.97 ± 2.11 (7)	0.3134 (NS) ^b
Trabecular Number (#/mm)	Female	0.36 ± 0.024 (5)	0.34 ± 0.058 (6)	0.7743 (NS) ^b
	Male	0.31 ± 0.089 (5)	0.31 ± 0.029 (7)	0.9999 (NS) ^b
Trabecular separation (μm)	Female	160.01 ± 39.1 (5)	657.61 ± 131.0 (5)	0.0066
	Male	307.0 ± 39.2 (5)	341.6 ± 54.1 (7)	0.6059 (NS) ^b

^aMean ± SEM (*n*). ^bNS, not statistically significantly different.

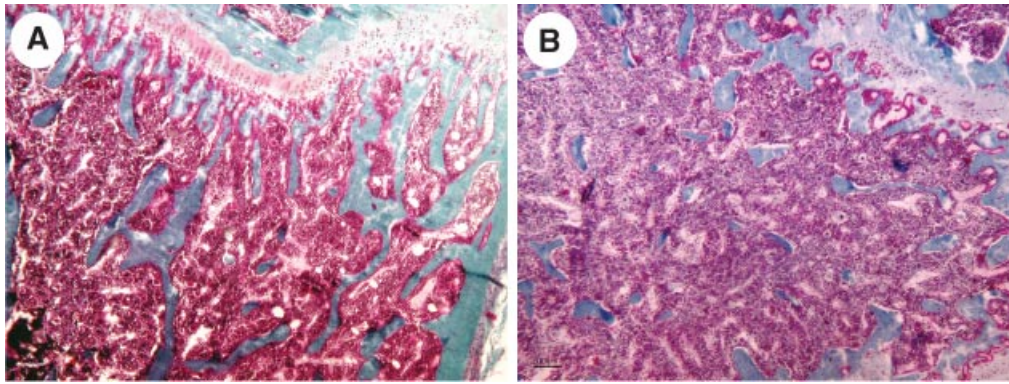


Figure 1. Histological sections of the distal femoral metaphysis from 3-month-old female (A) wild type (WT), and (B) transgenic (TG) mice. When compared to WT mice, the TG animals show reduced femoral cancellous bone volume accompanied by reduced trabecular thickness and increased trabecular separation. Masson-Goldner trichrome stain; 5- μ m-thick undecalcified sections. Bar = 100 μ m.

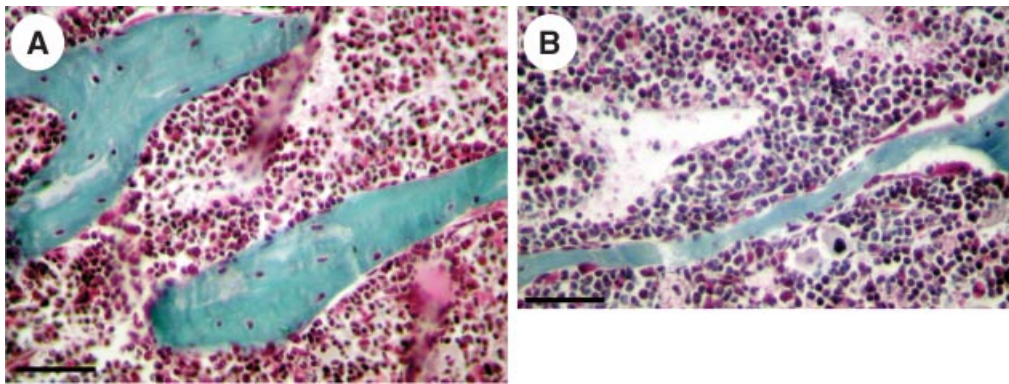


Figure 2. Histological comparison of trabecular thickness in wild type (A) and transgenic (B) female mice. Note that the reduction in cancellous bone volume in female transgenic animals is associated with a significant reduction in individual bone trabecular thickness. Masson-Goldner trichrome stain; 5- μ m-thick undecalcified sections. Bar = 50 μ m.

bone formation when compared to implanting the incompletely purified 18.5 kDa protein.² Finally, a combination of the 22 kDa, 18.5 kDa, and 14 kDa proteins at a ratio of 7:2:1 appeared to be the most osteogenic combination.² The most that can be reliably concluded from the data regarding the interaction of spp24 and BMP-2 (or other BMPs)^{2,9,10} is that the 24 kDa form would be expected to be inhibiting whereas the 18.5 kDa form would be expected to be enhancing with respect to bone formation even though both forms share the TRH1 domain (the sequence of which is the basis of the 19 amino acid synthetic peptide designated BBP that is enhancing).³ The TRH1 domain is critically important because it forms part of the proposed ligand-binding interface for BMPs in type II receptors.¹¹ Sorting these complex relationships out will require a comprehensive and systematic study of the regulated proteolysis and posttranslational modification

of spp24 as well as BMP-2 binding studies and bone formation assays.

In summary, we have demonstrated that fl and secretory spp24 inhibit bone formation in two very different animal models: in TG mice and in ectopic bone-forming assays in the presence of BMP-2. Of interest is the observation that BMD and various histological parameters related to bone formation were reduced in female TG mice expressing fl spp24 under the control of the osteocalcin promoter, but not in male TG mice. Osteocalcin promoter activity can be several-fold higher in females than in males in vivo, while in vitro activity is the same in cells from males and females, suggesting that humoral factors in females upregulate expression at skeletal sites.¹² Different forms of spp24 may perform different physiological functions such as maintaining latency as opposed to providing enhancement of bone formation through a “slow release” mechanism of BMP-2

Table 4. Serum Calcitonin and Osteocalcin in Wild Type and fl spp24 Transgenic Mice^a

Parameter	Gender	Wild Type (WT)	Transgenic (TG)	<i>p</i> -Value (WT <i>vs.</i> TG)
Calcitonin (pg/ml)	Female	183.8 $\pm$ 17.8 (5)	211.8 $\pm$ 25.8 (6)	0.41 (NS) ^b
	Male	209.8 $\pm$ 30.5 (5)	184.7 $\pm$ 21.2 (7)	0.4964 (NS) ^b
Osteocalcin (ng/ml)	Female	36.4 $\pm$ 2.95 (5)	27.9 $\pm$ 1.79 (7)	0.0259
	Male	24.1 $\pm$ 3.65 (4)	25.5 $\pm$ 3.20 (7)	0.7889 (NS) ^b

^aMean $\pm$ SEM (*n*). ^bNS, not statistically significantly different.

or other related proteins. Recent in vitro studies lend credence to this hypothesis.¹³

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