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Bone morphogenetic protein–2 activity is regulated by secreted phosphoprotein–24 kd, an extracellular pseudoreceptor, the gene for which maps to a region of the human genome important for bone quality

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Abstract

The material properties of bone are the sum of the complex and interrelated anabolic and catabolic processes that modulate formation and turnover. The 2q33-37 region of the human genome contains quantitative trait loci important in determining the broadband ultrasound attenuation (an index of trabecular microarchitecture, bone elasticity, and susceptibility to fracture) of the calcaneus, but no genes of significance to bone metabolism have been identified in this domain. *Secreted phosphoprotein–24 kd* (*SPP24* or *SPP2*) is a novel and relatively poorly characterized growth hormone–regulated gene that maps to 2q37. The purpose of this review is to summarize the status of research related to spp24 and how it regulates bone morphogenetic protein (BMP) bioactivity in bone. *SPP24* codes for an extracellular matrix protein that contains a high-affinity BMP-2–binding transforming growth factor–β receptor II homology 1 loop similar to those identified in fetuin and the receptor itself. *SPP24* is transcribed primarily in the liver and bone. High levels of spp24 (a hydroxyapatite-binding protein) are found in bone, and small amounts are found in fetuin-mineral complexes. Full-length secretory spp24 inhibits ectopic bone formation, and overexpression of spp24 reduces murine bone mass and density. Spp24 is extremely labile to proteolysis, a process that regulates its bioactivity in vivo. For example, an 18.5-kd degradation product of spp24, designated spp18.5, is pro-osteogenic. A synthetic cyclized Cys_1-to-Cys_{19} disulfide-bonded peptide (BMP binding peptide) corresponding to the transforming growth factor–β receptor II homology 1 domain of spp24 and spp18.5 binds BMP-2 and increases the rate and magnitude of BMP-2–mediated ectopic bone formation. Thus, the mechanism of action of spp18.5 and spp24 may be to regulate the local bioavailability of BMP cytokines. *SPP24* is regulated by growth hormone and 3 major families of transcription factors (nuclear factor of activated T cells, CCAAT/enhancer-binding protein, Cut/Cux/CCAAT displacement protein) that regulate mesenchymal cell proliferation, embryonic patterning, and terminal differentiation. The gene contains at least 2 single nucleotide polymorphisms. Given its mechanism of action and sequence variability, *SPP24* may be an interesting candidate for future studies of the genetic regulation of bone mass, particularly during periods of BMP-mediated endochondral bone growth, development, and fracture healing.
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1. Introduction

Osteoporosis is the most common metabolic disorder of bone in the developed world [1]. It can be defined as low bone mass and density, poor bone quality with microarchitectural deterioration, and susceptibility to fracture with little or no trauma [1,2]. Although complex interactions between genetic susceptibility, diet, and lifestyle determine the age at onset, severity, and occurrence of osteoporosis, the genetic factors that regulate bone metabolism are important determinants of bone quality, bone quantity, and fracture risk across the life span [3-5].

2. Identifying candidate genes for osteoporosis

The candidate gene approach was initially used to identify specific genes that regulate bone metabolism [4,5]. Regions of the human genome associated with specific calcitropic hormone receptors, cytokines, enzymes, and proteins with

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well-defined roles in bone metabolism were examined for polymorphisms associated with specific skeletal phenotypes, notably extremes in bone mineral density (BMD) [5]. The candidate-gene approach led to the association of more than 10 genes that regulate bone and mineral metabolism (eg, vitamin D, estrogen α- and β- and androgen receptors, growth hormone, insulin-like growth factors I and II, parathyroid hormone and parathyroid hormone receptor 1, and bone morphogenetic protein–2 [BMP-2]) with variations in BMD [1]. However, the results of candidate gene association studies have been contradictory and do not recognize significant associations between specific phenotypes and novel genes, such as *secreted phosphoprotein–24 kd* (*SPP24*), with no previously well-defined functions in regulating bone metabolism [1].

3. Genome screening

More recently, linkage analysis or genome search approaches have been undertaken to identify finite regions of the genome containing markers linked to the transmission of specific bone phenotypes, such as extremes of BMD or osteoporosis [1,4]. Once broad genomic regions linked to the phenotype have been identified, localization vs defined markers can be progressively refined until the candidate genes (quantitative trait loci [QTL]) that determine or regulate the trait or parameter are identified and positional cloning has confirmed the role of the gene [4]. This led to the identification of several regions on human chromosomes, notably 16p, 1p, 3p, 6, 10, 18, 20p, and 22q, linked to low BMD or osteoporosis in multiple studies [1]. A gene of major significance to the regulation of bone formation (BMP2) has been linked to chromosome 20p12, but the association does not hold for all populations [1].

4. Quantitative ultrasound and genes that regulate bone metabolism

Low BMD is only one of the several phenotypes used to screen for genes that regulate bone metabolism and determine bone quantity, quality, and biomechanical strength. Quantitative ultrasound (QUS) measurements of the speed of sound and the broadband ultrasound attenuation (BUA) through trabecular bone provide a rapid, simple estimation of bone quality and quantity that correlates with both BMD and fracture risk [6]. The QUS parameters are of particular interest when identifying skeletal regulatory genes because they are a function of some of the critical material properties of bone, including elasticity, trabecular structure, and density [7]. More specifically, BUA is a function of the structure (pore size, porosity, and permeability), trabecular orientation, mineral content, and density of bone [8-10]. As a result, BUA is strongly correlated with the mechanical properties of bone (ultimate strength, elastic modulus, and energy absorption capacity) at high and low strain rates [11]. Broadband ultrasound attenuation of the calcaneus is a good predictor of BMD at the lumbar spine and femoral neck in middle-aged men and women [12], and low ultrasound values are associated with a high risk of fractures [13-15]. After adjusting for age and body mass index, the heritability of BUA is estimated to be 0.58 (95% confidence interval [CI], 0.41-0.75) vs 0.77 (95% CI, 0.63-0.91) for axial BMD or 0.63 for whole-body BMD (95% CI, 0.26-1.00) [16]. Genetic variations in calcitropic hormones, cytokines, their receptors, and their downstream target proteins, including the vitamin D receptor [17,18], osteocalcin [19], α_2-HS-glycoprotein/fetuin [20], estrogen receptor–α [21], interleukin-6 [22], transforming growth factor (TGF)–β1 [23], methylene tetrahydrofolate reductase [24,25], osteoprotegerin [26], and type I collagen [27], have been correlated with changes in the QUS properties of bone.

5. *SPP24* localizes to a region of the human genome containing QTL for BUA of the calcaneus

As noted above, the genes that have been identified thus far are those for which a clear role in bone metabolism has already been established. Genes with poorly defined functions that contribute to the regulation of bone metabolism may also reside in these areas, but remain unidentified. Recently, a genome screen of a large twin cohort was completed; and the 2q33-37 region of the human genome was found to contain QTL important in determining the BUA of the calcaneus [28]. No gene(s) of interest was identified in this region at the time of the report. However, *SPP24* (alternately known as *secreted phosphoprotein–2* or *SPP2*) maps to chromosome 2q37.1 in the interval 233.64 to 233.67 Mbp of the human genome [29,30]. The object of this review is to summarize recent findings that demonstrate that spp24 is a BMP-2–binding pseudoreceptor that modulates cytokine bioactivity and has significant effects on BMP-2–mediated bone formation in vivo. Therefore, given its gene localization and function, spp24 may be of interest in future studies of the genetic regulation of bone metabolism.

6. Structure: function relationships of spp24

SPP24 is a secreted phosphoprotein that was cloned from chicken [31] and bovine bone and found in the bovine periosteum and liver, but not the heart, lung, kidney, or spleen [32]. Subsequently, it was cloned from the mouse kidney [33] and uterus [34], rat liver (UniProtKB/Swiss-Prot entry Q62740), and human bone (Q13103). To date, 10 *SPP24* genes have been described, all of which are found in vertebrates (protein family PF07448) [30]. The spp24 proteins share 50% to 90% sequence identity and consist of 3 major domains, including (1) a short N-terminal secretory peptide, (2) a cystatin (cysteine protease inhibi-

tor)-like or cathelicidin (neutrophil antimicrobial peptide precursor)-like domain with 2 internal disulfide bonds, and (3) a variable arginine-rich C-terminal region [30,32].

The archetypical spp24 is bovine (b)-spp24, a 203–amino acid residue protein with a calculated mass of 23.1 kd and a theoretical pI of 8.4 (Table 1) (ExPASY ProtParam). A signal peptide (residues 1-23) is cleaved, producing a mature protein of 180 residues (b-spp24, residues 24-203) with a calculated mass of 20.5 kd and a pI before modification of 7.86 (Table 1). By analogy to the cystatins with which it shares sequence homology [32], it is assumed that the 4 absolutely conserved cysteine residues in all spp24 proteins form 2 internal, disulfide-bonded loops [30,32]. In b-spp24, disulfide bonds would link residues 86 and 97 and 110 and 128, respectively (Table 1) [32]. The second internal disulfide-bonded loop of b-spp24 (residues 110-128) is a BMP-2–binding TGF-β receptor II homology 1 (TRH1) domain [35] similar to those described in fetuin and the type II receptor itself [36]. The synthetic peptide corresponding to the TRH1 domain of b-spp24 has been designated *cyclic BMP binding peptide* (*cBBP*), and its function will be described in detail below. The numbering of the residues in Table 1 does not correspond to that in the original description [32] after the recent database correction that extended the N-terminus by 3 amino acid residues (MEK).

Bovine spp24 also contains a serine-rich domain (residues 131-139) that is heavily phosphorylated in the native protein. The phosphoserine domain is postulated to act as a highly charged anionic spacer separating the N-terminal cystatin domain from the arginine-rich C-terminus of the mature protein and modulating its susceptibility to proteolysis or bioactivity [32]. The polyserine domain is interrupted and highly variable in other vertebrates [30], suggesting that a high degree of conservation in this region is not essential for function. The C-terminal domain of b-spp24 (residues 176-197) is rich in arginine, which can form hydrogen bonds with the phosphate groups in apatite [37]. The high arginine content of the C-terminus of spp24 may account for the hydroxyapatite-binding properties of the protein, which is enriched in bone [32,35,38,39].

Initial sequence comparisons suggested that there were comparable levels of identity between bovine spp24 and cystatin (cysteine protease inhibitor) domains 1 and 3 of human kininogen and between spp24 and the neutrophil bactenecin precursor [32]. It was proposed that the N-terminal 107 amino acid residues of mature b-spp24 (residues 24-130; running from the N-terminus of the mature protein to the 11-residue polyserine-rich domain) formed a tertiary structure similar to that of cystatin and that spp24 may function, in part, as an inhibitor of the thiol proteases (eg, cathepsins) involved in bone resorption [32]. However, highly purified recombinant bovine secretory spp24 [40] does not inhibit the enzymatic activities of cathepsins B and K, the 2 major cysteine proteinases in bone [41]. Alternatively, the acidic residues of the cystatin domain of spp24 may form a negatively charged planar *β*-sheet that inhibits basic calcium phosphate precipitation in a manner similar to that observed for the D1 region of the structurally similar cystatin domain of fetuin [42]. This could explain, in part, the observations that overexpression of full-length (fl) b-spp24 inhibits murine bone formation in vivo and that implantation of recombinant His_6-tagged secretory b-spp24 (residues 24-203) dose-dependently inhibits BMP-2–mediated ectopic bone formation [43].

A more recent analysis suggests that the sequences of the spp24s are more similar to those of the cathelicidins than they are to the cystatins (InterPro IRP010892 analysis). Cathelicidins are the precursors of antimicrobial peptides secreted by activated neutrophils [44]. Like the spp24 proteins, cathelicidins consist of a signal sequence, a conserved region of about 100 amino acid residues that contains 4 cysteines involved in 2 disulfide bonds (the cathelin-like domain), and a variable C-terminal domain (IRP001894 annotation). Cleavage of the parental cathelicidin liberates C-terminal peptides with lipopolysaccharide- and phospholipid-binding and antimicrobial properties [44-46]. Cystatin domains are relatively rigid structures in solution, but cathelin domains are rich (>10%) in proline residues and remain highly flexible [47]. Thus, the proline-rich "cystatin-like" domain of spp24 is probably flexible rather than rigid, a property that may facilitate conformational changes upon BMP-2 [35] or apatite [32,35,39] binding or confer lability to proteolysis [32]. Spp24 is exquisitely sensitive to proteolysis [35,38] and is readily cleaved between residues 143 and 144 to produce spp14.5 and residues 157 and 158 to produce spp16 in vitro, liberating the cathelin domain (residues 24-130) from the arginine-rich C-terminus [40]. Both of these labile cleavage sites are N-terminal to the most C-terminal residue (residue 176) identified in the pro-osteogenic 18.5-kd degradation product of spp24 isolated from native bovine bone [35,38]. Thus, a major function of the cathelin domain may be to

Table 1
The amino acid sequence and structural domains of bovine spp24, residues 1 to 203

MEKMAMKMLV$_{10}$IFVLGMNHWT$_{20}$CTG↑*FPVYDYD*$_{30}$*PASLKEALSA*$_{40}$
SVAKVNSQSL$_{50}$*SPYLFRAFRS*$_{60}$*SVKRVNALDE*$_{70}$*DSLTMDLEFR*$_{80}$
IQETTCRRES$_{90}$*EADPATCDFQ*$_{100}$*RGYHVPVAVC*$_{110}$*RSTVRMSAEQ*$_{120}$
VQNVWVRCHW$_{130}$SSSSGSSSSE$_{140}$EMF^FGDILGS$_{150}$
STSRNSY^LLG$_{160}$
LTPDRSRGEP$_{170}$LYEPSREMRR$_{180}$NFPLGNRRYS$_{190}$
NPWPRARVNP$_{200}$GFE$_{203}$

Key features
1. Leader sequence: residues 1-23
2. Disulfide bonds: residues 86-97 and 110-128
3. Serine-rich domain: residues 131-139
4. TRH1 domain ("BBP"): residues 110-128 (underlined)
5. Cystatin (cysteine protease inhibitor)- or cathelicidin-like domain: residues 24-130 (italicized)
6. "^" indicates a labile cleavage site; spp24 is cleaved between residues 143 and 144 to form spp14.5, whereas cleavage between residues 157 and 158 produces spp16.

Residue numbers are displayed as subscripts.

confer a susceptibility to the proteolytic cleavage that has a major effect on the biological activity of spp24 and its derivatives, as outlined below. Thus, for 10 years after it was initially cloned, it was apparent that spp24 was a proteolytically labile apatite-binding extracellular matrix protein found primarily in bone [31,32]. Despite the presence of a cystatin-like domain, it was not a thiol protease inhibitor [41]. Its similarity to cathelicidins suggested that proteolysis could liberate a biologically active fragment [32], but the identity of the fragment and its biological functions were not defined.

7. Spp24 is a precursor of pro-osteogenic proteins

The BMP-binding pseudoreceptor function of spp24 and its derivatives was discovered during the long search for pro-osteogenic proteins in demineralized bone matrix. In 1987, Urist et al [38,48] isolated an 18.5-kd pro-osteogenic protein and a copurifying nonosteogenic 24-kd protein from the noncollagenous proteins in demineralized bone matrix. In 1987, Sen et al [49] also reported isolating a 23-kd osteogenic protein with an N-terminal sequence identical to 12 of the first 14 amino acid residues of secretory spp24, but its similarity to the 18.5-kd protein was not recognized at that time. In addition, b-spp24 was not cloned and sequenced until 1995 [32]. Sequencing errors by Sen's group may account for the 2 residues that are not identical to spp24 [32,49], whereas carbamoylation of the N-terminus of the 18.5-kd protein [40] may account for the inability to sequence it. In 2005, the pro-osteogenic 18.5-kd protein was isolated by differential extraction and precipitation, ceramic hydroxyapatite affinity chromatography, and 2-dimensional sodium dodecyl sulfate–polyacrylamide gel electrophoresis [35]. Matrix-assisted laser desorption/ionization time-of-flight mass spectroscopy and peptide fingerprint analysis of the 18.5-kd protein demonstrated that it was a fragment of spp24 that contained the cystatin domain [35].

Once it was apparent that spp24 was the parental protein from which the 18.5-kd pro-osteogenic protein was derived [35], spp24 was examined to identify features that it shared with related bone proteins, such as fetuin, which might explain its bioactivity. Of particular interest were the cystatin-like domains in fetuin and spp24 because the cystatin domain of bovine fetuin-A contains a disulfide-bonded TRH1 loop (residues 114-132) that mimics the BMPII receptor and specifically binds BMP-2 by surface plasmon resonance (SPR) [36]. Surface plasmon resonance is an optical technique in which the binding of an analyte (eg, fetuin or spp24) to a covalently immobilized ligand (eg, rhBMP-2) on one channel of a glass chip is measured as an electrical signal proportional to the mass of the analyte that binds after subtracting the amount of nonspecific binding observed in a second underivatized channel [50]. The TRH1 domain is a distinctive internal 18– or 19–amino acid residue protein motif with a β-pleated sheet/turn/β-pleated sheet secondary protein structure [36]. The TRH1 domain is cross-linked at its N- and C-termini by a disulfide bond [36]. The TRH1 domain of the type II BMP receptor is important because x-ray crystallography shows that it binds BMP [51]. A manual sequence comparison of fetuin, the BMPII receptor, and spp24 was conducted, leading to the hypothesis that the second disulfide-bonded loop of spp24 (residues 110-128) was a BMP-binding TRH1 domain with a β-pleated sheet/turn/β-pleated sheet secondary protein conformation (Table 2) [35]. Unlike fetuin-A and the receptor, the "turn" domain of the TRH1 domain of spp24 is an α-helix, not a nonspecific structure (Table 2). The cyclized synthetic 19–amino acid residue peptide (designated *cBBP*) corresponding to the TRH1 domain shared by the pro-osteogenic 18.5-kd protein and spp24 specifically binds BMP-2 when assessed by SPR, stimulates the rate and magnitude of BMP-2–mediated ectopic bone formation [35], and significantly increases [^{125}I]-BMP-2 retention in vivo (Hasan Uludag, personal communication). We hypothesize that cBBP regulates BMP-2 bioactivity, in part, by binding to the cytokine and increasing its residence time in mineralized tissues.

We recently confirmed that fl secretory b-spp24 and its truncated derivatives (spp16, spp14.5, and spp18.1) specifically bind BMP-2 by SPR, with K_Ds in the range of 10 nmol/L (data not shown). Although the binding kinetics are similar for each of these molecules, their bioactivity varies depending upon their length. As noted above, overexpression of fl secretory spp24 inhibits bone formation in vivo; and Met-His$_6$–tagged recombinant secretory spp24 (residues 24-203) can completely ablate BMP-2–mediated ectopic bone formation [43], whereas cBBP stimulates it [35] and spp14.5 has no reproducible effect (data not shown). Cyclic BBP is such a potent enhancer of BMP-2 bioactivity that it can reduce the amount of BMP-2 required to achieve spinal fusion by 90% (from 10 to 1 μg) [52]. These observations

Table 2
The structure of the BMP-2–binding TRH1 domains of BBP/spp24, fetuin, and the TGF-β II receptor

Peptide or protein	Residue no.	BMP-binding TRH1 sequence	SSpro secondary structure predicted[a]
BBP (the peptide)	1-19	CRSTVRMSAEQVQNVWVRC	CCCEEEECHHHHEEEEEEC
spp24 (the protein)	110-128	CRSTVRMSAEQVQNVWVRC	CCCEEEECHHHHEEEEEEC
Bovine fetuin-A	114-132	CDIHVL*KQDGQFSVLFTKC	CCEEEEECCCCEEEEECC
Human TGF-β receptor II	84-101	C*VAVWRKNDENIT*LEYVC	CEEEEECCCCCEEEEEEC

The β-pleated sheets of fetuin and the TGF-β receptor II are underlined. H indicates α-helix; E, β-pleated or extended strand; C, the rest.

[a] http://www.igb.uci.edu/tools/scratch.

have led us to hypothesize that, although spp24 and its degradation products are extracellular pseudoreceptors for BMP-2, their biological effects depend, in part, upon the cleavage of the proteolytically labile C-terminus, as previously predicted [32].

8. A role for spp24 in regulating bone metabolism across the life span

Although it is interesting to observe that *SPP24* maps to a region of the human genome associated with QTL linked to BUA in man [28-30], further research is clearly required to clarify the roles, if any, of *SPP24* in determining BUA and the properties (eg, trabecular density and orientation) that contribute to it. The identities of the promoters that regulate *SPP24* gene transcription may provide insight into its fundamental contribution to bone metabolism. *SPP24* is a growth hormone (GH)–regulated gene that was cloned by differential display in normal (*Dw/Dw*) and dwarf (*dw/dw*) chickens and designated *growth hormone–regulated gene–1* [30,31]. *Growth hormone–regulated gene–1* was initially reported to contain a GH response element (GHRE) similar to that found in the serine protease inhibitor 2.1 gene [31]; but more recent analysis refutes this homology, defines a new high-affinity serine protease inhibitor 2.1 GHRE, and places the SPP24 GHRE outside the regions that have been sequenced thus far [30]. The developmental expression of *SPP24* parallels plasma GH levels, and its expression is up-regulated by GH in vivo and during embryonic development [31]. If spp24 functions as a BMP-binding pseudoreceptor as hypothesized, then its up-regulation by growth hormone would have an important impact on BMP gradients and bioavailability during periods of rapid growth and development.

More recently, 3 *cis*-acting promoter domains for nuclear factor of activated T cells (NFAT), CCAAT/enhancer-binding protein (C/EBP), and CCAAT displacement protein (CDP)/Cux/Cut transcription factors were identified in the highly conserved domain 300 base pairs upstream of the human transcription start site for *SPP24* [30]. Demineralized bone powder (which contains BMPs) and BMP-2 induce NFAT gene expression in fibroblasts undergoing chondrogenic differentiation [53], suggesting that NFAT transcription factors are important regulators of endochondral bone formation. The NFAT transcription factors also play a major role in mediating calcium-activated second messenger signaling in osteoblastic cells and coupling osteoblastic and osteoclastic activities [54-56]. The presence of an NFAT promoter upstream of the *SPP24* gene [30] suggests that *SPP24* gene expression is regulated during periods of bone formation [53] and maintenance [54-56]. The C/EBPs or CCAAT enhancer binding proteins are transcription factors that coordinate the molecular genetic actions of cytokines, growth factors, and hormones in bone [57] and are essential downstream mediators of fluid shear stress [58]. The presence of a C/EBP promoter site upstream of the *SPP24* gene is consistent with a role for *SPP24* in the genetic regulation of bone formation, remodeling, and response to mechanical stress. The CDP and *cux* are the human and mouse orthologs of *Drosophila melanogaster* cut protein, a nuclear matrix-associated homeobox transcription factor that regulates limb development [59]. *Cux-1* and *-2* usually act as cell-cycle–dependent transcriptional repressors that modulate the balance between cell proliferation and differentiation by inhibiting the promoter activities of proteins like cyclin-dependent kinase inhibitors p21 and p27 [60]. *SPP24* is expressed at much higher levels in the brain and bone of neonates or weanlings than in adults [30]. The presence of a CDP promoter element upstream of the *SPP24* gene suggests that *SPP24* plays a role in pre/neonatal skeletal development.

In summary, *SPP24* was not previously associated with the genetic regulation of bone mass, although it maps to a region of the human genome associated with QTL linked to BUA [28-30]. Recently published data confirm that spp24 and its derivatives are a new family of extracellular matrix phosphoproteins that contain a BMP-2–binding TRH1 or pseudoreceptor domain capable of modulating the rate and magnitude of bone formation [35,43]. Furthermore, the presence of promoters for GH and 3 differentiation- and development-related transcription factors [30] that regulate mesenchymal cells suggests that *SPP24* may be particularly important in early development and the acquisition of peak bone mass. The significance of the gene to bone metabolism could be obscured in studies in which the parameter of interest, such as BMD, is measured at (post)maturity, when analysis is likely to be confounded by long-term dietary and lifestyle factors. Further studies aimed at elucidating the function of *SPP24*, its degradation products, and the effects, if any, of the single nucleotide polymorphisms observed in the gene [30] may provide valuable insights into how the metabolic processes that determine bone quality and quantity, assessed as BUA, are genetically regulated.

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