

# Bone Morphogenetic Proteins, Their Antagonists, and the Skeleton

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**Skeletal homeostasis is determined by systemic hormones and local factors. Bone morphogenetic proteins (BMP) are unique because they induce the differentiation of mesenchymal cells toward cells of the osteoblastic lineage and also enhance the differentiated function of the osteoblast. However, the activity of BMPs needs to be tempered by intracellular and extracellular antagonists. BMPs bind to specific receptors and signal by phosphorylating the cytoplasmic proteins mothers against decapentaplegic (Smad) 1 and 5, which form heterodimers with Smad 4, and after nuclear translocation regulate transcription. BMP antagonists can be categorized as pseudoreceptors that compete with signaling receptors, in-**

**hibitory Smads that block signaling, intracellular binding proteins that bind Smad 1 and 5, and factors that induce ubiquitination and proteolysis of signaling Smads. In addition, a large number of extracellular proteins that bind BMPs and prevent their binding to signaling receptors have emerged. They are the components of the Spemann organizer, noggin, chordin, and follistatin, members of the Dan/Cerberus family, and twisted gastrulation. The antagonists tend to be specific for BMPs and are regulated by BMPs, indicating the existence and need of local feedback mechanisms to temper BMP cellular activities. (Endocrine Reviews 24: 218–235, 2003)**

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## I. Introduction

**T**HE NUMBER AND the function of cells present in the bone microenvironment determine skeletal homeostasis and are regulated by systemic hormones and local signals. Cells of the osteoblastic lineage secrete various growth factors. Some act primarily as cell mitogens, others act by modifying the function of the differentiated cell (1, 2). Bone morphogenetic proteins (BMPs) have the unique functions of inducing the differentiation of cells of the osteoblastic lineage, therefore increasing the pool of mature cells, and of enhancing the differentiated function of the osteoblast (3–6). A secondary consequence of their effects on cells of the osteoblastic lineage is increased osteoclastogenesis, a process tightly coordinated with osteoblastogenesis (7). Growth factors play an essential role in the maintenance of cellular functions, but there is a need to temper their activities to maintain a coordinated bone remodeling. This can be achieved by local feedback mechanisms, growth factor binding proteins, and intracellular factors. Most of our knowledge of extracellular regulators of growth factors is derived from the effects of IGF binding proteins (IGFBP), which are synthesized by osteoblasts and regulate IGF actions in bone (8). Recently, intracellular and extracellular regulators of BMP action were described and were found to modulate BMP action in a variety of cells. This review will address the target skeletal cells of BMPs, the biological actions of BMPs,

Abbreviations: AML, Acute myeloid leukemia; AMSH, associated molecule with SH3 domain of STAM; BAMBI, BMP and activin membrane-bound inhibitor; BMP, bone morphogenetic protein; Cbfa, core-binding factor; C/EBP, CCAAT-enhancer binding protein; CR, cysteine-rich; CSF, colony-stimulating factor; Dan, differential screening-selected gene aberrative in neuroblastoma; FLRG, follistatin-related gene; GDF, growth differentiation factor; IGFBP, IGF binding protein; Ihh, Indian hedgehog; LEF/TCF, lymphoid enhancer binding factor/T cell specific factor; LRP 5, low-density lipoprotein receptor-related protein 5; MFH-1, mesenchymal forkhead-1; MH, mad homology; M<sub>r</sub>, molecular mass; PEBP, polyoma enhancer binding protein; PRDC, protein related to Dan and Cerberus; RANK-L, receptor activator of nuclear factor-κB ligand; Runx, runt-related transcription factor; Shh, Sonic hedgehog; Smad, mothers against decapentaplegic; Smurf, Smad ubiquitination regulatory factor; Sog, short gastrulation; STAM, signal transducing adaptor molecule; Tob, transducer of Erb B-2; Tsg, twisted gastrulation.

with emphasis on their skeletal effects, and the need to restrict the cellular effects of BMPs by specific antagonists.

## II. Osteoblastic Lineage, Osteoblastic Genes, and Regulation of Osteoblastic Function

To understand the role of BMPs and their antagonists in skeletal homeostasis, it is important to define their target cells and possible target genes. Bone marrow stroma contain pluripotential cells with the potential to differentiate into diverse cells of mesenchymal lineage, including osteoblasts, chondrocytes, myoblasts, and adipocytes (9–12). The ultimate cellular phenotype depends on signals present in the cellular microenvironment, including BMPs and their binding proteins. Transcription nuclear factors also play a central role in determining the fate of undifferentiated cells. For instance, members of the CCAAT-enhancer binding protein (C/EBP) family of transcription factors play an essential role in the differentiation of cells toward an adipocytic pathway, whereas runt-related transcription factor (Runx)-2 or core-binding factor (Cbfa)-1 plays a critical role in the differentiation of cells toward an osteoblastic pathway (13–18).

Three Cbfa or Runt domain genes have been identified: Runx-2/Cbfa-1, also called polyoma enhancer binding protein (PEBP)2 $\alpha$ A/acute myeloid leukemia (AML)3; Cbfa-2/PEBP2 $\alpha$ B/AML1; and Cbfa-3/PEBP2 $\alpha$ C/AML-2. The  $\alpha$ -subunit of these nuclear factors binds to DNA via the Runt domain when paired with the  $\beta$ -subunit. Runx-2/Cbfa-1 binding sites are present in the regulatory sequences of the osteocalcin,  $\alpha_1$  I and  $\alpha_2$  I collagen genes, and Runx-2/Cbfa-1 is a positive transcriptional regulator of these genes, which are expressed by the differentiated osteoblast (19–23). Gene-targeted disruption of Runx-2/Cbfa-1 results in disorganized chondrocyte maturation and a complete lack of bone formation due to an arrest of osteoblast development (21, 24). Runx-2/Cbfa-1 also plays a role in mature osteoblastic function, and transgenic animals overexpressing a dominant negative form of Runx-2/Cbfa-1, under the control of the osteoblastic specific osteocalcin promoter, display decreased bone formation due to impaired osteoblastic function (22). This indicates a dual role of Runx-2/Cbfa-1 in cells of the osteoblastic lineage, regulating osteoblastogenesis as well as the function of mature osteoblasts. The role of Runx-2/Cbfa-1 in later stages of differentiation is less clear, and its overexpression under the control of the type I collagen promoter results in osteopenia because of the lack of terminal maturation of osteoblastic cells (25). This could be due to differences in the promoters used and their response to Runx-2/Cbfa-1 or to autoregulation of Runx-2/Cbfa-1 transcription (26, 27). The function of Runx-2/Cbfa-1 in cartilage tissue is evident by studies on Runx-2/Cbfa-1-null mice displaying impaired chondrogenesis and by studies in transgenic mice overexpressing Runx-2/Cbfa-1 under the control of the cartilage-specific type II collagen promoter. Mice overexpressing Runx-2/Cbfa-1 have enhanced endochondral ossification due to early chondrocyte maturation (28, 29). Consequently, Runx-2/Cbfa-1 plays a role in chondroblast differentiation analogous to that observed in cells of the osteoblastic lineage.

There are two Runx-2/Cbfa-1 isoforms, type I and type II. They are derived from different promoters resulting in two

different transcripts and proteins (13). Type I is derived from a proximal promoter upstream of exon 2, and type II from a distal promoter upstream of exon 1 (13, 30). The two Runx-2/Cbfa-1 isoforms have distinct 5' untranslated regions consisting of 1015 nucleotides for type I and 210 nucleotides for type II, and have some differences in the amino-terminal amino acid sequences of their respective coding regions and identical 3' untranslated regions (30). A third Runx-2/Cbfa-1 isoform has been reported, and it has a different translation start site from the type II isoform. In contrast to the type I and type II isoforms, which induce osteoblastic gene expression, Runx-2/Cbfa-1 type III does not regulate osteoblastic genes (31). Although in transfection experiments the function of the type I and type II isoforms is similar, differential expression of Runx-2/Cbfa-1 isoforms during osteoblastic cell differentiation and BMP-2 dependency of the type II isoform have been reported (13, 31). This would suggest a more specific role for the type II isoform in the function of the differentiated osteoblast, although Runx-2/Cbfa-1 isoform expression has varied with the cell line examined (30, 31). BMP-2 induces Runx-2/Cbfa-1 transcripts in osteoblast and chondrocyte cultures, and this nuclear factor, in association with BMP-specific signaling factors, mediates BMP-2 actions on gene transcription in cells of the osteoblastic lineage (13, 32, 33).

Osterix is a novel zinc finger containing transcription factor expressed by osteoblasts and required for endochondral and intramembranous bone formation. Osterix-null mice have normal cartilage development but fail to develop a mineralized skeleton (34). Osteoblast differentiation is arrested, and histological analysis reveals absence of trabecular bone. Osterix-null mice have reduced or absent expression of a variety of bone matrix proteins, including type I collagen, bone sialoprotein, osteonectin, osteopontin, and osteocalcin, confirming a role in the induction of osteoblast differentiation and function. In contrast to Runx-2/Cbfa-1-null mice that do not form osteoblasts, osterix-null mice form cells of the osteoblastic lineage that express Runx-2/Cbfa-1, but the cells do not mature. This would indicate that osterix has effects on skeletal development that are independent of Runx-2/Cbfa-1 and that osterix acts downstream of Runx-2/Cbfa-1 (34).

Recent studies have demonstrated a role for homeobox genes in osteoblast differentiation and skeletal development. Bapx 1 is a homeobox gene that plays a central role in the axial development of the skeleton. Bapx 1-null mice have defective chondrogenesis and osteogenesis in the axial skeleton causing a shortening of the vertebral column (35). Bapx 1-null mice have decreased Runx-2/Cbfa-1 expression in affected, but not in unaffected bones, although it is not certain whether or not Bapx 1 is a direct regulator of Runx-2/Cbfa-1 gene expression. In addition, Bapx 1 maintains Indian hedgehog (Ihh) expression and regulates the levels and pattern of expression of BMP-4 in the skeleton (19, 35).

The Msh family of homeobox genes includes Msx 1, 2, and 3. Msx 3 is expressed in the central nervous system, whereas Msx 1 and 2 are expressed in skeletal tissue and modulate osteogenesis. Msx 1-null mice display cleft palate and craniofacial and dental developmental abnormalities, and Msx 2-null mice have defects in skull ossification, which are enhanced in double Msx 1/Msx 2 mutants (36, 37). Msx 2-null

mice have defective chondrogenesis and osteogenesis due to a decreased number of osteoprogenitor cells. The skeletal abnormalities are associated with decreased expression of Runx-2/Cbfa-1, indicating that Msx 2 is necessary for osteogenesis and acts upstream of Runx-2/Cbfa-1. Postnatally, the expression of markers of osteoblast differentiation, such as osteocalcin and alkaline phosphatase, are decreased, indicating that Msx 2 plays a role in osteoblastic differentiation *in vivo* (37). This is confirmed in studies in C2C12 undifferentiated myogenic cell lines, which have the potential to differentiate into cells of various lineages, including the osteoblastic lineage. In C2C12 cells, BMP-2 induces osteoblastic differentiation, and Msx 2 mediates this effect. However, in chick preosteoblastic cells and in rat osteoblasts, Msx 2 expression declines as cells differentiate, overexpression of Msx 2 prevents chick osteoblast differentiation, and Msx 2 down-regulates the type I collagen promoter (38, 39).

The mammalian homologs of *Drosophila* distalless (Dlx) 5 and 6 are homeobox genes essential for craniofacial and skeletal development (40). Dlx 5 mRNA is expressed in osteoblasts after differentiation, concomitant with a decline in Msx 2 mRNA and with the appearance of osteocalcin transcripts (39). BMP induces Dlx 5 expression in osteoblasts, and murine osteoblastic MC3T3 cells overexpressing Dlx 5 display increased alkaline phosphatase activity, osteocalcin, and mineralization of the extracellular matrix (41). Dlx 5 is a target gene for BMPs that regulate osteogenesis and dorsoventral patterning, and targeted gene inactivation of Dlx 5 and 6 results in severe skeletal abnormalities leading to perinatal lethality (40).

Mesenchymal forkhead-1 (MFH-1) is a member of the helix/forkhead family of transcription factors, and it is induced by BMP-2 in C2C12 myogenic cells as they differentiate toward the osteoblastic pathway and away from myoblasts (42). Lowering MFH-1 expression with antisense MFH-1 sequence precludes the differentiation of C2C12 cells toward osteoblasts, indicating that in these cells, MFH-1, like Msx 2, plays a role mediating the BMP-2 effects on osteoblastic differentiation (42).

Selected genes can down-regulate osteoblastic differentiation. For example, Id genes are BMP-dependent negative regulators of helix-loop-helix transcription factors and have impact on cell growth and differentiation (43). Down-regulation of Id genes is necessary for terminal differentiation of a variety of cellular processes, including bone morphogenesis. Id genes are negative regulators of differentiation and positive regulators of cell proliferation, and their induction by BMPs may serve as a mechanism to reduce BMP action in cells of the osteoblastic lineage (43, 44). *Hoxa 2* is a different gene that suppresses bone formation and Runx-2/Cbfa-1 expression, but its role regulating BMP actions in bone is not established (45).

An undifferentiated mesenchymal cell can differentiate and form osteoblasts, chondrocytes, myoblasts, or adipocytes. Consequently, a mechanism to regulate the number of cells that eventually mature, or not mature, as osteoblasts is by allowing an early cell to differentiate toward an osteoblast or by diverting its differentiation toward a nonosteoblastic pathway. The C/EBPs are a family of transcription factors that play a role in cell differentiation (15, 46, 47). To date, six

C/EBPs have been characterized:  $\alpha$ ,  $\beta$ ,  $\delta$ ,  $\gamma$ ,  $\epsilon$ , and  $\zeta$ . The C/EBP proteins contain a highly conserved DNA-binding domain and a bZIP dimerization domain, and can form homo- and heterodimers that bind to similar sequence motifs. C/EBPs are expressed in multiple cell types, including osteoblasts and adipocytes, and C/EBP  $\alpha$ ,  $\beta$ , and  $\delta$  are essential for the formation of mature adipocytes, because mice carrying null mutations of C/EBP  $\alpha$ ,  $\beta$ , and  $\delta$  have reduced fat and impaired adipocytic differentiation (16–18, 48, 49). Recently, we confirmed that glucocorticoids induce adipogenesis and enhance the expression of C/EBP  $\beta$  and  $\delta$  in osteoblastic cells, and we demonstrated that these two transcription factors play a role in the down-regulation of IGF-I expression by glucocorticoids (50). Because C/EBP  $\beta$  and  $\delta$  are essential for adipogenesis and cortisol shifts a cell population away from osteoblasts, the findings suggest that C/EBP  $\beta$  and  $\delta$  play a role directing mesenchymal cells away from the osteoblastic pathway and toward an adipocytic pathway. This is reaffirmed by the fact that they mediate the down-regulation of IGF-I, a factor that supports the function of the osteoblast (50). BMP-2 does not up-regulate C/EBP expression in stromal cells, and this would be in line with its effects inducing their differentiation toward the osteoblastic pathway and away from the adipocytic pathway (14). However, under selected culture conditions or in synergy with other factors, BMPs have the potential to induce precursor cells toward the adipocytic pathway (51, 52). Similarly, C/EBPs could play a role in selected aspects of osteoblastic function; a variety of genes, including osteocalcin, have C/EBP consensus sequences; and C/EBP  $\beta$  and  $\delta$  interacting with Runx-2/Cbfa-1 can activate osteocalcin transcription (53).

### III. Bone Morphogenetic Proteins

#### A. BMPs and their expression by the osteoblast

BMPs are members of the TGF- $\beta$  superfamily of polypeptides, which includes TGF- $\beta$ s, activins, and inhibins (54–59). BMPs were originally identified because of their ability to induce endochondral bone formation (56, 59). BMPs account for most of the TGF- $\beta$  superfamily of peptides, and the proteins display extensive conservation among species having seven characteristic cysteine knot domains (56). The conserved cysteine domains participate in the formation of an interchain disulfide bond between two monomers to form a dimeric precursor protein. The precursor dimers are secreted as propeptides, which are activated by proprotein convertases, such as the serine endoprotease furin (56, 60). Mature proteins have a molecular mass ( $M_r$ ) in the 20,000–30,000 range. BMP-1 is unrelated to other BMPs and does not regulate the growth and differentiation of skeletal cells. BMP-1 is a protease that cleaves procollagen fibrils as well as chordin, which is a peptide that binds and antagonizes the actions of BMP-2 and -4 (61).

Although BMPs are synthesized by skeletal cells, their synthesis is not limited to bone because they are expressed by a variety of extraskelatal tissues in which they play a critical role in development and cell function. BMP-1 through -6 are expressed by osteoblastic cell lines, but the degree of

expression depends on the cell line studied (3, 62, 64–66). BMP-2, -4, and -6 are the most readily detectable BMPs in osteoblast cultures; BMP-2 and -4 are 92% identical in their amino acid sequence and consequently have virtually identical activities. Experiments using kinase-deficient truncated BMP receptors have demonstrated that the locally synthesized BMPs play an autocrine role in osteoblastic differentiation and function (65).

The transcriptional and posttranscriptional regulation of BMP expression in chondrogenic and osteoblastic cells has not been established. Autoregulation of BMP expression in osteoblasts is apparent, and BMP-4 mRNA levels are BMP-dependent. BMPs cause an early, short-lived, induction of BMP-4 mRNA in osteoblasts followed by an inhibitory effect, suggesting autocrine regulation (62). The transient increase in BMP-4 expression by BMPs may be required to force cell progression toward a differentiated state, whereas the down-regulation suggests a local control mechanism. The down-regulation is secondary to transcriptional and posttranscriptional mechanisms, but the gene elements responsible for either the increase or decrease of BMP-4 expression have not been established. BMP-2 also can be up- and down-regulated by other BMPs in osteoblasts, and it is of interest that BMP-2 and -4 promoters contain Runx-2/Cbfa-1 binding sequences (67–69). This opens the possibility for a positive feedback loop regulating BMP-2 and -4 expression involving Runx-2/Cbfa-1 because BMPs induce Runx-2/Cbfa-1 expression (13). In long-term cultures of osteoblastic cells, there is an increase in BMP-4 mRNA expression after cell maturation, which may be secondary to a larger pool of cells expressing Runx-2/Cbfa-1 (66). BMP-6 expression in osteoblasts is steroid-dependent, and BMP-6 mRNA levels are enhanced by estrogens in osteoblastic cells (64).

### B. BMPs, osteoblast maturation, and osteoclast formation

A fundamental function of BMPs is to induce the differentiation of mesenchymal cells toward cells of the osteoblastic lineage to promote osteoblastic maturation and function (70, 71). This requires interactions of the BMP signaling molecules against decapentaplegic (Smad) 1/5 and Runx-2/Cbfa-1 (33, 72). As osteoblasts undergo terminal differentiation and the cellular matrix mineralizes, they undergo apoptosis (73). This programmed cellular death is an expected result of cell maturation, and the blocking of BMP actions not only arrests osteoblast differentiation but also prevents apoptosis. In cultures of human osteoblasts, BMP-2 induces apoptosis by protein kinase C-dependent, Smad 1-independent mechanisms (74). The effect of BMPs on apoptosis is not limited to mature osteoblasts, and BMPs also induce apoptosis in developing limbs, which is necessary for normal skeletal and joint development (75, 76). Blocking BMP signaling in the developing limb results in reduction in interdigital apoptosis, and as a consequence, soft tissue syndactyly. BMP-induced apoptosis in embryonic cells is duplicated by the BMP-dependent homeobox gene *Msx 2*, which is expressed at sites of cell replication and programmed cell death (77). Furthermore, the effects of BMPs on osteoblastic differentiation and on apoptosis in embryonic cells is blocked

by *Msx 2* antisense oligonucleotides suggesting that *Msx 2* mediates these BMP effects (77, 78).

The genesis and differentiation of bone-forming osteoblasts and bone-resorbing osteoclasts are coordinated events. Receptor activator of nuclear factor- $\kappa$ B ligand (RANK-L) and colony-stimulating factor (CSF)-1 are osteoblast products and are major determinants of osteoclastogenesis (79). Osteoprotegerin, a secreted receptor of the TNF receptor family, acts as a decoy receptor that binds RANK-L, precluding RANK-L binding to RANK and its effects on osteoclastogenesis and bone resorption. BMPs play a direct and indirect role in osteoclastogenesis. Because RANK-L is an osteoblastic product and BMPs induce osteoblast maturation, it is expected that when osteoblastogenesis is blocked by BMP antagonists, osteoclastogenesis is impaired (7). This indirect effect involves Runx-2/Cbfa-1, and absence of Runx-2/Cbfa-1 also results in impaired osteoblastogenesis and osteoclastogenesis (80, 81). The direct effects of BMPs on bone-resorbing cells involves sensitization of osteoclasts to the effects of RANK-L on cell genesis and survival (80, 82). BMPs also induce osteoprotegerin gene transcription, and this may temper their effects on osteoclastogenesis (83). BMPs stimulate osteoprotegerin transcription through two *Hoxc-8* binding sites. The BMP signaling Smad 1 interacts with *Hoxc-8* and dislodges *Hoxc-8* from its binding element, resulting in induction of gene expression (83). In accordance with the induction of osteoprotegerin, BMPs inhibit collagenase 3 expression in osteoblasts, a matrix metalloprotease that cleaves type I and II collagen fibrils and also is required for normal bone resorption (84, 85).

### C. BMPs, chondrogenesis, and myogenesis

BMPs induce endochondral ossification and chondrogenesis in addition to their effects in the differentiation of mesenchymal cells toward cells of the osteoblastic lineage (72). BMPs stimulate chondrocyte maturation and enhance the function of chondrocytes, increasing the expression of type II and X collagens and the incorporation of sulfate into glycosaminoglycans in growth plate cultures (86, 87). Overexpression of BMP-2 and -4 in developing limbs results in an increase in chondrocyte cell number and in matrix cartilage, which may lead to joint fusions (88). The effect of BMPs in chondrogenesis appears to be mediated by *Sox 9*, a gene central to chondrogenesis and to the expression of type II and X collagens (89). BMP-2 and -4 induce *Sox 9*, and *Sox 9* antisense oligonucleotides blunt the induction of type II and X collagen in mesenchymal cells (90). BMPs play a role in the chondrogenic effects of the vertebrate hedgehog genes, which include *Ihh*, *Sonic hedgehog* (*Shh*), and *Desert hedgehog* (*Dhh*). *Ihh* and *Shh* are highly homologous and enhance chondrogenesis and endochondral ossification (86, 91). The anabolic effects of *Ihh/Shh* and BMP-2 and -4 in metatarsal cultures are analogous, and the effects of *Ihh/Shh* are blocked by BMP antagonists indicating that, in this culture model, local BMPs mediate *Ihh/Shh* actions on endochondral ossification (91). However, other effects of *Ihh/Shh* on cartilage are independent of BMPs. *Ihh* overexpression in cartilage up-regulates PTHrP and delays hypertrophic differentiation independent of BMP activity (92). BMP acts in

conjunction or in sequence with *Ihh/Shh* for effects on chondrocytic cell proliferation and chondrogenesis. *Ihh/Shh* induces *Nkx3.2*, which in turn induces the expression of *Sox 9*, which in the presence of BMP induces chondrogenesis (93, 94). Furthermore, in the presence of BMP, *Sox 9* and *Nkx3.2* induce each other's expression, establishing a positive feedback loop to initiate chondrogenesis (94). Interactions between BMP-dependent signaling *Smads* and *Runx-2/Cbfa-1* are necessary for chondrogenesis and type X collagen transcription (72). Consequently, blocking *Runx-2/Cbfa-1* also precludes the effect of *Ihh/Shh* on endochondral ossification (91). The BMP-related growth differentiation factors (GDFs) also are important in chondrogenesis (95, 96). Mice with GDF-5 mutations display brachypodism and exhibit short limbs and joint fusions (96). In humans, mutations in GDF-5 cause the autosomal recessive chondrodysplasias and display a phenotype similar to that of the brachypodism mutant mice. Whereas BMPs induce osteogenesis and chondrogenesis, BMPs prevent terminal differentiation of myogenic cells, inhibiting the transcription of the muscle-specific nuclear factors *MyoD* and *myogenin* (97, 98).

#### D. BMPs and skeletal development

Members of the TGF- $\beta$  superfamily are important in skeletal development, exemplified by the naturally occurring mutant BMP-5 mouse (the *short ear* mouse), which develops multiple cartilage and skeletal abnormalities affecting the skull and axial skeleton (99, 100). To understand the role of selected BMPs in skeletal and nonskeletal development, gene inactivation by homologous recombination was performed for various BMPs. Mice deficient in BMP-2 are not viable because of defects in the amnion/chorion and in cardiac development, and the BMP-4-null mutation is lethal between 6.5 and 9.5 d gestation because of the lack of mesodermal differentiation and patterning defects (101, 102). Mice with disruption of the BMP-signaling *Smad 5* develop multiple embryonic defects, some reminiscent of those observed with BMP-2-null mice, and the mutation is lethal due to significant defects in angiogenesis and cardiac development (103, 104). The lethality of the various mutations prevented the assessment of the impact of BMP-2 and -4 on skeletal development, although BMP-6-null mice were found to have a delay in ossification of the sternum (105). This limited phenotype may be due to compensation by other BMPs. Gene inactivation of various BMPs often results in significant phenotypic changes outside the skeleton, confirming that they are expressed and are active in extraskeletal tissues. For example, BMP-7 or osteogenin protein 1-null mice display lack of eye and glomerular development, leading to renal failure and neonatal death (106–108). These mice also have modest and discrete areas of skeletal abnormalities, including fused ribs, and vertebral, skull, and hind limb defects, in which polydactylism occurs. Targeted disruptions of BMP-8 or OP-2 result in infertility due to defects in spermatogenesis, because this BMP is expressed in testicular tissue (109).

#### E. BMPs and other growth regulators

Although BMPs are members of the TGF- $\beta$  superfamily of polypeptides, TGF- $\beta$ s and BMPs do not have the same bio-

logical activities in cells of the osteoblastic lineage, and their effects on osteoblastic cell differentiation and maturation differ. Whereas BMP-2 induces the differentiation of stromal cells toward the osteoblastic lineage, this is not the case for TGF- $\beta$ , which opposes the effect of BMPs on osteoblastic cell maturation (110). In some cell cultures, TGF- $\beta$  induces the differentiation of cells toward the chondrocytic lineage, but in some cells, such as C2C12 myogenic cells, TGF- $\beta$  simply arrests cell maturation (97).

BMPs act in conjunction with other growth factors. BMPs induce the differentiation of cells of the osteoblastic lineage, increasing the pool of IGF-I target cells, the mature osteoblast. BMPs increase IGF-I and -II mRNA levels in osteoblast cultures, and IGF-I and -II increase osteoblastic function, resulting in a coordinate increase in osteoblastic differentiation and function (111). BMPs also regulate the levels of IGF-BPs in skeletal cells. Although the changes vary with the cell line studied, they may play a role modulating the anabolic activities of BMPs and IGF-I in bone (112, 113).

Wnts, like BMPs, can induce cell differentiation and act by preventing  $\beta$ -catenin degradation by the ubiquitin-proteasome pathway inhibiting glycogen-synthase kinase-3. This results in  $\beta$ -catenin accumulation, its nuclear translocation and association with members of the lymphoid enhancer binding factor/T cell specific factor (LEF/TCF) transcription factor family, and the targeting of specific genes.  $\beta$ -Catenin and LEF/TCF can form a complex with *Smad 4*, and as such have the potential to regulate BMP and TGF- $\beta$  signaling (114). Low-density lipoprotein receptor-related protein 5 (LRP 5) plays a critical role in bone mass accrual during growth and is a coreceptor for Wnt (115, 116). LRP 5 acts synergistically with Wnt in the activation of the canonical Wnt signaling pathway enhancing LEF/TCF (115). Consequently, LRP 5, like Wnt, has the potential to regulate BMP signaling. LRP 5 is expressed by osteoblasts and stromal cells, and its expression is induced by BMP-2 as stromal cells undergo differentiation (116). LRP 5 is required for optimal Wnt signaling in osteoblasts, and mice with a targeted disruption of the LRP 5 gene develop osteopenia (117). The osteopenia is due to decreased bone formation secondary to a decreased number of osteoblasts, confirming a role of LRP 5 and Wnt in osteoblast maturation. Recent clinical findings have documented further the relationship between LRP 5 and Wnt in skeletal tissue. LRP 5 is a target for the inhibitory effects of *Dickkopf* in Wnt signaling (118, 119). LRP 5 mutations that affect Wnt signaling result in decreased bone mass, whereas mutations that create an LRP 5 resistant to *Dickkopf* inactivation result in sustained Wnt signaling and increased bone mass (116, 120).

#### F. BMP-3, an inhibitor of osteogenesis

BMP-3, or osteogenin, appears to be an exception to the stimulatory role of BMPs in osteoblastic differentiation and function (121). BMP-3 opposes the osteogenic effects of BMP-2 in stromal cell lines, and BMP-3-null mice display an unexpected increase in bone mineral density and in trabecular bone volume (122, 123). The mechanism of this increase does not involve changes in osteoblast or osteoclast number, suggesting changes in osteoblastic activity. BMP-3 seems to

act by an activin-mediated pathway to oppose BMP-2 actions and does not prevent BMP-2 binding to its receptors. Neither BMP-3 nor the closely related GDF-10 seem to play a role in skeletal development because embryos and newborn mice from BMP-3 and GDF-10-null mutations do not display a skeletal phenotype (123, 124).

#### IV. Bone Morphogenetic Protein Receptors and Signaling

##### A. BMP receptors

BMPs initiate signaling from the cell surface by interacting with two distinct serine/threonine kinase receptors that can activate the cytoplasmic proteins Smads (125–128). There are two groups of BMP-2 and -4 receptors, type I and type II (DAF-4), and two subclasses of type I receptors, type IA or activin receptor-like kinase (ALK)-3 and type IB or ALK-6 (126). Upon ligand binding, the type II receptor forms a heterodimer with the type I receptor, and the constitutive kinase of the type II activates the type I receptor and initiates the signal transduction cascade by phosphorylating downstream nuclear factors, which then translocate to the nucleus to activate or inhibit transcription. It is important to note that type I and II BMP receptors are present on the cell surface as homomeric and heteromeric complexes, even before BMP activation (129). It appears that binding of BMP-2 to preformed heteromeric receptor complexes results in activation of the Smad pathway, whereas formation of heteromeric receptor complexes induced by BMP-2 results in activation of the MAPK pathway (Ref. 129; Fig. 1).

BMP-2 and -4 bind to type IA and IB receptors with low affinity, but the binding is enhanced in the presence of the type II receptor, which is specific for BMPs. Some BMPs, such as BMP-7 or OP-1, can interact with activin receptors and display activin-like effects, in addition to interacting with BMP-2 receptors (130). Binding affinity of BMPs for type IA and IB receptors differ, and the level of receptor expression varies in different cells. The type IA and IB BMP receptors have similar structures and 95% homology in the kinase domain; however, their functions are different and vary according to the cell examined (131–133). The type IA receptor is essential for osteoblastic differentiation of C2C12 myogenic cells, but not of preosteoblast 2T3 cells in which the type IB receptor determines osteoblastic differentiation and the type IA receptor determines adipocytic differentiation (9, 134). These findings suggest that in addition to the mode of receptor oligodimerization, activation of type IA and IB receptors can determine the signaling pathway (129). They may also suggest the presence of different coactivators or corepressors in different cells.

##### B. BMP signaling, transduction, and Smads

After receptor activation, BMPs, TGF- $\beta$ , and activin signal via Smads. At least eight Smads have been isolated in mammals and two additional Smads, Smads 9 and 10, in lower species (125, 127, 135, 136). There are three classes of Smads: 1) receptor regulated Smads that can be BMP activated, such as Smad 1, 5, and 8, or TGF- $\beta$  and activin activated, such as

Smad 2 and 3; 2) common TGF- $\beta$  and BMP mediator Smads, Smad 4; and 3) inhibitory Smads, Smad 6 and 7 (125, 127, 135, 137). Smad sequences show two large conserved domains, the mad homology (MH)1 or amino-terminal domain and the MH2 or carboxy-terminal domain. The MH1 domain binds to DNA sequences, and the MH2 domain binds to proteins, creating protein-protein interactions important in gene transactivation. The two domains are separated by a less conserved linker sequence.

In unstimulated cells, Smad 1 and the closely related Smad 5 are found in the cytoplasm and on microtubules. After receptor activation by BMPs, Smad 1/5 are carboxy-terminally phosphorylated at serine residues and translocated to the nucleus after heterodimerization with Smad 4, a common partner with TGF- $\beta$  signaling (137–139). The core binding motif for the Smad 1/4 complex is GCCG or CAGA. The stimulatory effect of TGF- $\beta$  is mediated by similar mechanisms, although TGF- $\beta$  signaling is mediated by Smad 2 or 3. These are activated by phosphorylation after receptor ligand binding, and the activated Smads form a heterodimeric complex with Smad 4, which is translocated to the nucleus (139, 140). In the nucleus, the Smad 1/5-Smad 4 or Smad 2/3-Smad 4 complex interacts with other factors, but little is known about downstream nuclear factors responsible for the transcriptional activation or inhibition of BMP-dependent genes. Although there is a significant amount of information on BMP signaling through Smad 1 and 5, there is less information on the role of Smad 8. BMPs induce the expression and phosphorylation of Smad 8, and Smad 8 can dimerize with Smad 4 (141, 142). In the presence of BMP-2, Smad 1, 5, and 8 potentiate the hypertrophic maturation of chondrocytes, suggesting a role of Smad 8 in chondrocytic differentiation (143). Smads can bind to DNA sequences directly, bind and cooperate with other transcription factors, or bind and displace nuclear factors from their DNA binding sites. For example, the amino-terminal MH1 domain of Smads can bind to DNA and have direct positive or negative regulatory activity; Smads can interact with Runx-2/Cbfa-1 or can displace transcriptional repressors, such as *Hoxc-8* from DNA binding sites (33, 144). Overexpression of Smad 1 and 5 in C2C12 myogenic and in chondrogenic mesenchymal cells results in osteoblastic and chondrogenic differentiation, and the effects are enhanced by cotransfection with Smad 4 (145, 146). The effect on osteoblastic differentiation requires interactions of Smads with Runx-2/Cbfa-1, and interactions of these factors are central for their transactivating activity in mesenchymal cells. Runx-2/Cbfa-1 mutations found in cleidocranial dysplasia result in a truncated Runx-2/Cbfa-1 protein that fails to interact with Smads 1, 2, 3, and 5 (147). The mutated protein is unable to induce osteoblastic differentiation of C2C12 cells in the presence of Smads or BMPs (147). The importance of Runx-2/Cbfa-1-Smad interactions is emphasized further by recent studies demonstrating that the presence of Runx-2/Cbfa-1 is required for the targeting of BMP-2 and TGF- $\beta$ -dependent Smads to subnuclear sites (148). In the absence of Runx-2, Smad 1 and 5 are not translocated to the nucleus after BMP activation. Runx-2 allows for the recruitment of Smads to sites of active transcription, and this effect is coupled with the regulation of gene expression (148).

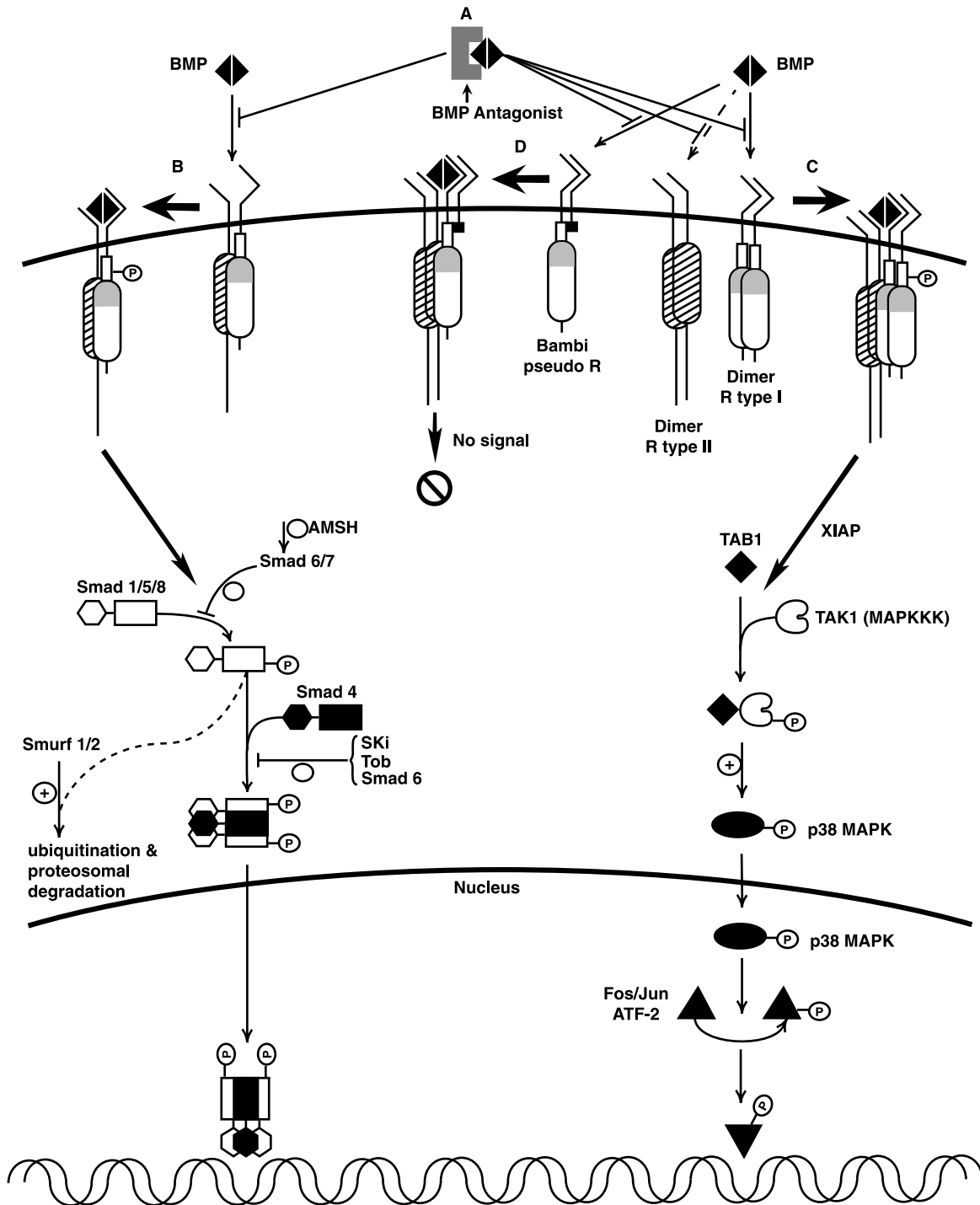


FIG. 1. Mechanisms of BMP signaling revealing multiple levels of regulation, including: 1) secreted extracellular antagonists that bind to their cognate BMPs and prevent receptor binding; 2) signaling from preformed heteromeric complexes of type I and type II BMP receptors in which ligand binding results in activation of the Smad 1/5 pathway, which can be regulated by inhibitory Smads 6 and 7, Smad binding proteins, Ski and Tob, and ubiquitination and degradation by Smurf 1 and 2; 3) signaling from ligand-induced heteromeric complexes of type I and type II BMP receptors, which results in the activation of a p38 MAPK pathway; and 4) nonsignaling BMP pseudoreceptors, BAMBI.

In addition to Smad activation, BMPs and TGF- $\beta$  can activate Smad-independent pathways, such as those dependent on Ras/MAPK signaling (149, 150). In human osteoblasts, activation of Ras/MAPK is responsible for the regulation of specific genes by BMP-2. BMP-2 can stimulate

Ras activity and as a consequence, two MAPKs, ERK and P38 (149, 151). As a result of Ras/MAPK activation, most members of the Fos/Jun family and activating transcription factor-2 (ATF-2) are up-regulated and interact with activating protein-1 (AP-1) sequences in various genes. P38 MAPK

activation is essential in the BMP-2 up-regulation of type I collagen, osteocalcin, and alkaline phosphatase, and P38 MAPK and ERK activation is essential in the up-regulation of fibronectin and osteopontin (150). MAPKs can regulate independent pathways as well as act interdependently with the Smad pathway and phosphorylate Smads (149).

## V. Inhibitors of Bone Morphogenetic Protein Signaling

There is evidence of autoregulation of BMP expression in osteoblasts that can act in a negative feedback loop to decrease cellular exposure to BMPs (62). This protective mechanism does not seem sufficient, and much of the regulation of BMP action occurs by the presence of intracellular and extracellular factors that modulate BMP activity. BMP activity can be suppressed by a variety of mechanisms, including: 1) expression of dominant negative nonsignaling pseudoreceptors; 2) blocking BMP signaling by inhibitory Smads 6/7; 3) blocking BMP signaling by intracellular Smad binding proteins; 4) ubiquitination and degradation of signaling Smads; and 5) blocking BMPs by extracellular antagonist BMP binding proteins (Table 1 and Fig. 1). In addition, intracellular factors may act by down-regulating BMP expression. *Xenopus* brain factor (XBF)-2, a winged helix transcription factor, and its mouse homolog BF-2 repress BMP-4 expression. Furthermore, XBF-2 mRNA levels are increased by BMP-dependent antagonists, indicating a dual role for some BMP antagonists, blocking of BMP activity, and suppression of its expression (152).

### A. Nonsignaling BMP pseudoreceptors

Silencing of TGF- $\beta$  and BMP receptor signaling can occur by the pseudoreceptor BMP and activin membrane-bound inhibitor (BAMBI; Ref. 153). This pseudoreceptor encodes a putative transmembrane protein with an extracellular domain similar to that of type I TGF- $\beta$  and BMP receptors. BAMBI associates with type IA and IB BMP receptors and blunts the effect of the activated receptors without direct interactions with either TGF- $\beta$  or BMP-2. The extracellular domain of BAMBI is necessary to prevent BMP actions because deletions of the domain preclude the inhibitory effects. BAMBI is coexpressed with BMP-4, and BMP-4 is required for its expression, suggesting that this may be a negative feedback loop to temper BMP activity (152).

TABLE 1. Inhibitors of BMP activity

1.	Nonsignaling pseudoreceptors BAMBI
2.	Inhibitory Smads Smad 6 and 7
3.	Smad 1/5 binding proteins Ski and Tob
4.	Degradation of signaling Smads Smurf 1 and 2
5.	Extracellular antagonists of BMPs Noggin, chordin, ventroptin, follistatin, and FLRG Twisted gastrulation Dan/Cerberus family: Dan, Cerberus, PRDC, Caronte, Dante, gremlin/drm, and sclerostin/SOST

### B. Inhibitory Smads

Smad 6 and 7 are inhibitory Smads that interfere with Smad 1/5 phosphorylation and heterodimerization with Smad 4 (125, 137). Smad 6 and 7 have an MH2 domain but not an MH1 domain; therefore, they can bind to proteins but not to DNA. Smad 6 and 7 are phosphorylated after interaction with type I BMP or TGF- $\beta$  receptors, and this interferes with the phosphorylation and activation of signaling Smad 1 and 5 and heterodimerization with Smad 4 and gene regulation (154–156). Smad 6 can inhibit BMP effects by additional mechanisms and modify the interactions of Smad 1/5 with corepressors. For example, a way by which Smad 1 induces transcription is by dislodging transcriptional repressors such as *Hoxc-8*, and Smad 6 can bind to *Hoxc-8* and prevent its dislodging from DNA binding sites so that repression persists (157).

Smad 6 is a more selective inhibitor of BMP action than Smad 7, and Smad 6 mRNA levels are strongly induced by BMP-2 in stromal and myogenic C2C12 cells, whereas BMP-2 induces Smad 7 mRNA levels only in selected cell types (158, 159). The induction of Smad 6 mRNA is not specific to BMPs because TGF- $\beta$  and activin also induce Smad 6 transcripts (159). Because both signaling and non-signaling Smads are BMP-dependent, it is possible that after a short-term exposure of osteoblastic cells to BMPs, there is an increase in the Smad 1/5 complex, whereas after a longer exposure there is accumulation of Smad 6 or 7. The Smad 1/5-4 accumulation would direct an increase in BMP-dependent transcription, whereas the accumulation of Smad 6 or 7 would prevent the transcription of BMP-dependent genes, acting as a local negative feedback mechanism. Smad 9 and 10 have activities opposite to TGF- $\beta$  and BMPs in nonmammalian species, but little is known about their function in mammalian cells (136).

Inhibitory Smads regulate signal-transducing Smads, and their activity also can be modified. Smad 6 is regulated by the associated molecule with SH3 domain of STAM (AMSH) and by histone deacetylases. AMSH is a protein that interacts with the signal transducing adaptor molecule (STAM), and AMSH associates with Smad 6 preventing its inhibitory effects, allowing Smad 1 phosphorylation and downstream gene regulation (160). AMSH does not associate with Smad 7. Histone deacetylases act by binding to the MH2 domain, affecting Smad 6 protein-protein interactions with signaling Smads and allowing the binding of the signaling MH1 domain to DNA and the regulation of transcriptional activity (161).

### C. Intracellular proteins binding signaling Smads

Ski, the transforming protein of the avian homolog of the Sloan-Kettering retrovirus (v Ski), is an oncogene that acts as a Smad-dependent corepressor of BMP, TGF- $\beta$ , and activin signaling (162). The inactivation of TGF- $\beta$  downstream activity by Ski could play a role in oncogenesis by preventing the tumor suppressive actions of TGF- $\beta$  (163). Ski represses BMP signaling and activation of target genes in *Xenopus* and mammalian cells, including stromal and C2C12 myogenic cells, with a consequent failure to express the osteoblastic

phenotype after BMP exposure (164). Ski blocks BMP signaling by associating to the MH2 protein binding domain of Smad 1, 4, and 5. Protein-protein interactions between Ski and Smad 1/5, and 4 are necessary for its inhibitory activity in stromal and myogenic cells, and Ski mutants that fail to bind BMP-signaling Smads fail to inhibit BMP actions (163, 164). The association of Ski with Smad 1 and 5 is dependent on receptor activation and needs to occur before the formation of the Smad 1/5-4 heterodimeric complex, because the formed complex is not displaced by Ski. It is not known whether the association with Smad 4 is receptor activation dependent. The interactions of Ski are not exclusive for BMP-signaling Smads, and Ski also interacts with the TGF- $\beta$  signaling Smad 2 and 3 and represses the transcription of TGF- $\beta$  target genes (163, 165). However, Ski does not interact with the inhibitory Smads 6 and 7. Smad proteins also are affected by Ski-interacting protein (Skip) and Ski-related novel protein (Sno), which regulate the BMP and TGF- $\beta$ -Smad signaling pathway (166, 167). Ski and Sno inactivate, whereas Skip augments TGF- $\beta$  action on target genes.

The ability to antagonize BMP signaling by Ski is critical in embryonic development because Ski-null mutants die at birth (162). Ski plays an important role in the morphogenesis of the central nervous system and craniofacial structures, and Ski-deficient mice display a variety of muscle and skeletal developmental abnormalities. They lack cranial bones and have malformations of basal cranial bones and of the mandible (162). Ski is expressed by skeletal muscle and bone, and transgenic mice overexpressing Ski develop osteopenia (168). This would confirm that the effect of BMPs on osteoblastic cell differentiation plays a fundamental role in the maintenance of bone mass and structure.

The transducer of Erb B-2 (Tob) gene is a member of the PC3/BTG/Tob family of genes, which are involved in cell replication and differentiation (169, 170). The product of the Tob gene is a 345-amino-acid protein that interacts with Erb B-2, a receptor-type protein tyrosine kinase. Tob decreases BMP-2-induced transcriptional activation by binding to MH2, the protein binding domain, of the BMP-dependent Smads 1, 5, and 8 (171). As a result, it modifies their activity and intracellular localization. The Tob gene is expressed by osteoblasts in which its mRNA levels are increased by BMP-2, suggesting a possible negative feedback mechanism to temper BMP action. The expression of Tob in osteoclasts is minimal. Mice with null mutations of the Tob gene have an increased number of osteoblasts and increased bone formation rate, demonstrating that Tob, like Ski, blocks bone formation (171). Furthermore, osteoblast differentiation *in vitro* is accelerated in cultures of immature osteoblastic cells from Tob-null mice, and the cells are more responsive to BMP-2. *In vivo* administration of BMP-2 over the parietal bone of Tob-null mice also results in enhanced bone formation, when compared with wild-type mice. These observations indicate that Tob decreases BMP action *in vitro* and *in vivo*. Although Tob-null mice develop a significant skeletal postnatal phenotype, the mutation is not lethal, and the phenotype appears at 4 months of age. This is possibly due to overlapping functions with Tob 2, a close homolog (172).

#### D. Ubiquitination and degradation of signaling Smads

Smads can be regulated at the level of transcription, activation, binding, and degradation, which is controlled by the ubiquitin-proteasome system. Ubiquitin is a highly conserved protein that covalently binds and recruits target proteins for their degradation by the proteasome (173). Consequently, ubiquitin can regulate the presence of nuclear factors and modify transcription. Smad ubiquitination regulatory factor 1 (Smurf) 1 and Smurf 2 are Smad-specific E3 ubiquitin ligases that selectively interact with BMP receptor-activated Smads. Smurf 1 and 2 trigger their ubiquitination and proteasomal degradation, and therefore their inactivation (174, 175). Smurf 1 interacts with Smad 1 and 5 preferentially, inactivating BMP signaling. Smurf 1 and 2 contain two WW domains that mediate protein-protein interactions and allow binding to the proline-rich PPXY motifs in the linker region of Smad 1 and 5. Smurf 1 and 2 do not bind to Smad 4 because it lacks a PPXY motif, but they bind and induce ubiquitination and degradation of the inhibitory Smad 6 and 7, which have the motif. Although Smurf 1 and 2 are preferential inhibitors of BMP signaling and do not bind directly to the TGF- $\beta$ -signaling Smad 2 or to TGF- $\beta$  receptors, they regulate TGF- $\beta$  signaling by alternate indirect mechanisms. Smurf 1 and 2 can induce the degradation of the TGF- $\beta$  receptor I after the binding to Smad 7. As such, Smad 7 acts as an adapter molecule linking the TGF- $\beta$  receptor I to the ubiquitin-proteasome pathway (176, 177).

## VI. Extracellular Antagonists of Bone Morphogenetic Proteins

In addition to intracellular regulators of BMP signaling, the effects of BMPs can be modulated by a group of secreted polypeptides that limit BMP action and can be induced by BMPs (Table 2). These extracellular BMP antagonists prevent BMP signaling by binding BMPs, therefore precluding their binding to specific cell surface receptors. Extracellular BMP antagonists include noggin, chordin, follistatin and follistatin-related gene (FLRG), ventroptin, twisted gastrulation (Tsg), and the Dan/cerberus family of genes, which is comprised of the head inducer cerberus, the tumor suppressor Dan, gremlin and its rat homolog drm, the protein related to Dan and Cerberus (PRDC), caronte, Dante (Dte) and sclerostin (SOST; Refs. 178–193).

TABLE 2. Expression of extracellular BMP antagonists in osteoblasts

	Expressed	BMP induced
Noggin	+	+
Chordin	+	–
Ventroptin	Not tested	
Follistatin	+	–
FLRG	Not tested	
Tsg	+	–
Dan	+	–
Cerberus	–	–
PRDC	+	+
Dante and Caronte	Not tested	
Gremlin/drm	+	+
Sclerostin/SOST	+	+

### A. *Noggin*, *chordin*, *ventroptin*, *folliculin*, and *FLRG*

Much of our knowledge of BMP antagonists is derived from the developmental effects of the Spemann organizer. The change of an unpatterned cluster of cells to an organized patterned embryo occurs along the anteroposterior and dorsoventral axis. Ventralization and ectoderm development are dependent on BMP-2 and -4 signals, whereas dorsalization and neural formation depend on signals from the Spemann organizer/node, which is composed of *noggin*, *chordin*, and *folliculin* (194, 195). In *Xenopus*, the ventralizing effect of BMP-4 is reproduced by *Smad 1*, indicating that conventional BMP signaling pathways are involved in patterning (196).

The open reading frame of *noggin* resides within a single exon and encodes for a polypeptide with a predicted  $M_r$  of 22 kDa, but it is secreted as a homodimeric glycoprotein with an  $M_r$  of 64 kDa (177). *Noggin* was originally characterized as a component of the Spemann organizer of the amphibian gastrula (178, 179). *Noggin* mimics the actions of the Spemann organizer, and it can induce dorsalization and the formation of neural tissue from ectoderm. The expression and function of *noggin* is widespread and not exclusive to the brain and the Spemann organizer (179). *Noggin* acts by binding BMPs, thus preventing them from binding to their receptors (180). *Noggin* binds with various degrees of affinity BMP-2, -4, -5, -6, and -7, GDF-5, GDF-6, and Vg1, but not other members of the TGF- $\beta$  family of peptides (180, 181). *Noggin* has been used as a tool to block BMP function, because it is a relatively specific inhibitor of BMP activity, and it does not appear to have actions independent of BMP binding.

*Noggin* expression in osteoblasts is limited, but its mRNA and protein levels are induced after exposure of the cells to BMP-2, -4, or -6, suggesting that this may be a protective mechanism to prevent excessive exposure of skeletal cells to BMPs (197). Similar protective mechanisms may exist in chondrocytes in which the expression of *noggin* and *chordin* is up-regulated by *Ihh*, an inducer of endochondral ossification (198). *Noggin* blocks the effect of BMPs in undifferentiated and differentiated cells of the osteoblastic lineage, and the addition of *noggin* to osteoblasts in culture blocks the stimulatory effect of BMPs on collagen, noncollagen protein synthesis, and alkaline phosphatase activity (197). Experiments conducted in stromal cells from transgenic mice overexpressing *noggin* under the control of the osteocalcin promoter and in stromal cells after the addition of *noggin* demonstrate that *noggin* decreases osteoblastogenesis (7, 199). As a consequence, *noggin* prevents osteoclastogenesis, a process dependent on the osteoblastic signals RANK-L and CSF-1. The inhibitory effect of *noggin* on osteoclastogenesis is fundamentally due to a decrease in the number of osteoblasts, and it is reversed by the addition of BMP. However, it is not reversed by the addition of RANK-L and CSF-1, indicating that additional osteoblastic or osteoclastic BMP-dependent signals could be involved. *Noggin* also inhibits membranous ossification and prevents chondrogenesis and limb development (181, 200).

Homozygous null mutations of the *noggin* gene result in serious developmental abnormalities, joint lesions, skeletal abnormalities, and fetal lethality (201). The developmental

abnormalities affect somite and ventral mesoderm development. *Noggin* is essential for the fate of ventral cells in the developing central nervous system and for the survival of neuronal precursors in the neural tube, and the *noggin*-null mutation results in failure of neural tube development and in open neural tubes from the diencephalon to its caudal limit (202). The phenotypic lethality of the *noggin*-null mice has not permitted definition of the function of *noggin* in adult bone. However, mice overexpressing *noggin* under the control of the osteocalcin promoter develop osteopenia and fractures, indicating that *noggin* has detrimental effects in bone integrity either directly or indirectly by binding BMPs (199).

The importance of regulated *noggin* and BMP expression is confirmed by human studies demonstrating that heterozygous mutations of the *noggin* gene result in multiple joint lesions (203). Although heterozygous *noggin*-null mice do not have a distinct phenotype, heterozygous *noggin* missense mutations in humans result in proximal symphalangism and multiple synostosis syndrome. Both syndromes are characterized by joint fusions, and *in vitro* studies have confirmed that the *noggin* gene mutations reported result in decreased *noggin* function (203, 204). The need to temper BMP activity also is confirmed by the demonstration that lymphoblastic cell lines from patients with fibrodysplasia ossificans progressiva, a disease characterized by extraskel-etal ossification, have increased levels of BMP-4 (205). Mutational analysis in these patients failed to demonstrate alterations in the *noggin* gene (206).

*Chordin* is another protein secreted by the Spemann organizer and, like *noggin*, it binds BMPs opposing their activities (182, 183). *Chordin* is the *Xenopus* homolog of short gastrulation (*Sog*) in *Drosophila*. *Chordin* has a predicted  $M_r$  of 105 kDa, although the secreted product has an  $M_r$  of 120 kDa, probably due to posttranslational modifications. *Chordin* has four cysteine-rich (CR) domains of about 70 amino acids each. These domains, particularly CR1 and CR3, determine the function of *chordin* and its ability to bind BMPs (183, 207). *Chordin* binds BMPs specifically, preventing their receptor signaling, and does not bind to other members of the TGF- $\beta$  family of peptides. *Chordin*, like *noggin*, mimics the actions of the Spemann organizer, causing neural induction and mesoderm dorsalization (182). *Chordin* inactivation results in stillborn mice, which have normal early development but show later defects in inner and outer ear development and abnormalities in pharyngeal cardiovascular organization (208). Double mutant *noggin*/*chordin* mice lack extensive areas of the forebrain, eyes, and facial structures, and have disrupted mesoderm development and abnormal left to right patterning (208). This indicates that the anteroposterior, dorsoventral, and left-right patterning are affected and demonstrates that *chordin* and *noggin* are required for the proper specification of the three body axes in the mouse embryo. The neural phenotype of the dual deletion resembles that of *Shh*-null mice, and *noggin*/*chordin*-null mice do not express *Shh*. *Chordin* expression by osteoblasts is limited, and it has not been reported to play a role in osteoblastic function (197). However, *chordin* is expressed by chondrocytes and regulates chondrocytic maturation (209).

*Ventroptin* is a BMP-4 antagonist that is fundamentally expressed in the ventral retina, where it plays a role in retinal

patterning (185). Ventroptin has three CR domains, which are significantly homologous with those of chordin. Ventroptin binds BMP-4 and has BMP neutralizing activity similar to that of chordin and noggin (185). Although ventroptin is also expressed in forebrain, diencephalon, and limb buds, its role in skeletal development and function is unknown.

Follistatin was initially identified as an activin binding protein that precluded activin signaling, but it also can repress BMP-4 expression and signaling by binding BMP-4 (210, 211). Activin plays a role in anteroposterior patterning, and follistatin, like noggin, can induce dorsalization (212). Follistatin-null mice develop a variety of deficiencies in multiple tissues, including the skeleton, resulting in neonatal death (213). Unlike noggin, follistatin expression is down-regulated by BMPs and induced by TGF- $\beta$ , indicating that different signaling pathways can regulate specific BMP antagonists, such as follistatin (214, 215). Activin can induce endochondral bone formation *in vivo*, and the effect is delayed by follistatin (216, 217). Because follistatin is synthesized by proliferating chondrocytes and by osteoblasts, it may act as a local regulator of activin function in skeletal cells. A follistatin-like protein, FLRG, was recently identified and shown to bind activin and BMP-2 (184). FLRG has two instead of three follistatin binding domains present in follistatin, and these domains are growth factor binding motifs. FLRG, like follistatin, binds BMP-2 with lesser affinity than activin, but inhibits BMP-2-induced transcriptional responses (184).

### B. Twisted gastrulation

Tsg is a BMP antagonist that binds chordin/Sog and BMP-4 to form a tertiary complex (218–220). The Tsg-chordin/Sog complex is more efficient than either component in inhibiting BMP signaling. The Tsg gene encodes a secreted protein with an amino-terminal highly conserved CR domain with some analogies with the CR domains of chordin. These domains are likely the sites of primary interaction between BMPs and some of their antagonists, and they appear to be responsible for interactions of Tsg with BMPs. Tsg binds BMPs specifically, and it is unique because it has the potential to have BMP agonistic and antagonistic activity. The BMP agonist effects involve the cleavage of chordin/Sog by tolloid/xolloid, resulting in reactivation of BMP signals, possibly because Tsg competes with the residual anti-BMP activity of chordin fragments (221–223). Tolloid is a metalloprotease with a structure related to BMP-1, a procollagen C-protease (224). The proteolytic activity of tolloid is specific for chordin, and tolloid does not modify the anti-BMP activities of other antagonists, such as noggin and follistatin (221, 222). The relative levels of chordin/Sog, Tsg, and tolloid are likely to dictate whether Tsg acts as a BMP agonist or antagonist. Tsg is expressed by osteoblasts, but it is not regulated by BMPs, and it is not known whether it is also expressed by chondrocytes. Chordin is more prominently expressed by chondrocytes, and tolloid is expressed by osteoblasts and chondrocytes (225).

### C. The Dan family

Differential screening-selected gene aberrative in neuroblastoma (Dan) is a family of secreted, related glycoproteins capable of binding BMPs. At least seven members of the Dan family have been described: Dan, cerberus, PRDC, dante, caronte, gremlin/drm, and sclerostin/SOST (188–193, 226). Protein sequence alignments reveal a region of structural homology among the Dan family of peptides, a carboxy-terminal CR domain, which establishes a functional motif in the tertiary structure of the protein, the cysteine knot (192). This determines the folding of the protein in a structure that exposes specific hydrophobic residues, facilitating the formation of homo- or heterodimers and diverse protein-protein interactions (227). The cysteine knot motif is shared by a number of extracellular signaling molecules, including members of the TGF- $\beta$  superfamily of peptides and their antagonists. Outside the CR domain, members of the Dan family have limited homology. Members of the Dan family bind BMP-2 and -4 with various degrees of affinity and have the capability of inhibiting BMP signaling.

The Dan gene, also called NO3, has tumor-suppressor activity, and it is down-regulated in *v-src* transformed rat fibroblasts (189, 228). The Dan gene encodes a 19-kDa protein, but when secreted it migrates on polyacrylamide gels with a larger  $M_r$  due to homodimerization and glycosylation. Dan is expressed in a variety of adult and embryonic tissues. In *Xenopus* embryos, Dan mRNA injection induces dorsalization and neural formation, events observed with proteins secreted by the Spemann organizer. Surprisingly, mice lacking Dan display only a subtle phenotype (229). These modest phenotypic alterations are different from those observed in the absence of other BMP antagonists, and they argue that not all extracellular BMP antagonists are the same and that each individual factor may have specific and independent functions. The lack of a phenotype may be due to redundancy with other members of the Dan family, but the precise physiological function of Dan in skeletal and extraskeletal tissues is not clear. Furthermore, Dan can bind BMP-2 and -4, but this may or may not have physiological relevance because Dan appears to bind GDF-5 more efficiently, at least in *in vivo* studies. Dan transcripts are expressed by cultured osteoblasts, but they are not induced by BMPs.

Cerberus was identified in the Spemann organizer by differential screening for dorsal specific cDNAs. The cerberus gene encodes a 270-amino-acid polypeptide with a predicted  $M_r$  of 31 kDa (173). Cerberus, like Dan and gremlin, undergoes posttranslational modifications and glycosylation. Cerberus plays a critical role in the formation of neural tissue, and acts as a head organizer, binding BMP-4 and overriding its activity (188, 230, 231). Cerberus binds BMPs selectively and does not bind other members of the TGF- $\beta$  family, but it binds and opposes the activity of Wnt 8 (231). Additional antagonists of Wnt, such as Frzb, are expressed by the Spemann organizer and prevent the binding of Wnt to the signaling frizzled (232, 233).

A murine gene related to cerberus (mCer-1) encodes a 273-amino-acid protein with a predicted  $M_r$  of 31 kDa (234, 235). XCer and the murine homolog are about 30% identical, and both have a carboxy-terminal CR domain. Although the

sequence homology is limited, mCer and XCer share functional activities, and mCer-1 is a potent neuralizing factor that can induce forebrain structures. It is important to note that mCer-1 is expressed exclusively during embryogenesis, and consequently it does not have a function after development (236). Cerberus is not detected in osteoblast cultures. Mice with homozygous deletion of mCer-1 do not have an obvious phenotype, so that its precise function has not been determined.

PRDC was identified by gene trapping in embryonic stem cells. The PRDC gene encodes a protein of 168 amino acids that shares limited homology with Dan and cerberus but shares a high degree of sequence identity with gremlin (191). The precise biological function of PRDC has not been established, but it binds and blocks the activity of BMP-2 and -4 (191). PRDC is expressed by osteoblasts, and PRDC mRNA levels are increased after treatment with BMP-2 (E. Canalis, unpublished observations). Caronte is a cerberus-related gene that encodes a 272-amino-acid protein (226). The chick protein is 30% identical to cerberus, although the identity to other members of the Dan family in the cysteine-rich region is about 60%. The expression of caronte is Shh dependent, and caronte regulates left to right asymmetry in vertebrates during development (226). Although caronte binds BMP-4 and can antagonize BMP activity, its role in postnatal cellular functions is not known. There is no information on whether caronte is expressed by osteoblasts, and a function in bone tissue has not been reported. Dante (Dte) is expressed throughout development in a variety of tissues, including cartilage (192). It is not known whether osteoblasts express Dante, and Dante-null mice do not display a bone or cartilage phenotype, indicating that it is not required for skeletal morphogenesis (A. Economides, unpublished observations).

Gremlin was cloned from a *Xenopus* ovarian library as a gene with axial patterning activities (190). The rodent homolog of gremlin is down-regulated by v-mos (drm). The drm gene encodes a 20.7-kDa protein, but the secreted protein undergoes glycosylation and has an  $M_r$  of 28 kDa (237). Gremlin/drm is highly conserved through evolution, and rat drm and *Xenopus* gremlin share 80% amino acid homology. Gremlin has no sequence similarity to noggin, chordin, or follistatin, and it is not expressed by the Spemann organizer. Gremlin/drm is fundamentally expressed in brain, kidney, and testis with limited expression in mesenchymal tissues. Gremlin and BMP-2 and -4 are coexpressed during development, and gremlin modulates BMP activity in neural crest cells (187). Gremlin also regulates limb bud development and inhibits chondrogenesis, and *in vitro* it inhibits cell replication and induces apoptosis (190, 238).

Although unstimulated osteoblasts express modest levels of gremlin, the transcript and protein are induced after BMP exposure (239). Gremlin antagonizes the BMP-2 effects on osteogenic differentiation in stromal cells and in C2C12 myogenic cells and blocks the effects of BMP-2 on collagen synthesis and alkaline phosphatase activity in cultured osteoblasts (237, 239). These observations indicate that gremlin prevents osteoblastic differentiation and function. It is of interest that gremlin is induced by high glucose levels in mesangial glomerular cells, and it is possible that gremlin

modulates the inhibitory effects of high glucose concentration on osteoblastic differentiation (240).

Sclerostin or SOST is a novel member of the Dan/Cerberus family of BMP antagonists (193). Although the biochemical properties of this protein have not been described, homozygous mutations of this gene in humans result in sclerostosis, an autosomal recessive skeletal dysplasia characterized by excessive bone overgrowth (241, 242). Sclerostin is expressed in cultures of stromal cells and osteoblasts and in bones of adult mice. It is also present in arteries, kidney, liver, duodenum, whisker follicles, and parts of the brain (193). In cultures of cells of the osteoblastic lineage, sclerostin expression is more evident after the cells mature and tend to mineralize, and sclerostin mRNA levels are increased by BMP-4 and -6 (193). Sclerostin blocks BMP effects on osteoblasts, and the progressive increase in bone formation found in patients with sclerostin mutations suggests that this gene is an important new regulator of bone homeostasis (193, 241, 242).

## VII. Conclusions

BMPs play a central role in embryonic development, cell differentiation, and osteoblastic function. As such, they are critical for the maintenance of skeletal integrity. In addition, BMPs play a role in fracture healing (243). However, the activity of BMPs needs to be tempered, and unopposed BMP effects are detrimental. The actions of BMPs are regulated by extracellular and intracellular proteins that bind BMPs or components of the BMP signaling pathways. Often their synthesis is BMP-dependent, pointing to the need of local feedback mechanisms to maintain an ideal balance between BMPs and their antagonists. Future investigations should provide valuable information on the physiological role of BMP antagonists in the skeleton and their role in various skeletal disorders.

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