

Bone Morphogenetic Proteins

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Bone morphogenetic proteins (BMPs), originally identified as osteoinductive components in extracts derived from bone, are now known to play important roles in a wide array of processes during formation and maintenance of various organs including bone, cartilage, muscle, kidney, and blood vessels. BMPs and the related “growth and differentiation factors” (GDFs) are members of the transforming growth factor β (TGF- β) family, and transduce their signals through type I and type II serine–threonine kinase receptors and their intracellular downstream effectors, including Smad proteins. Furthermore, BMP signals are finely tuned by various agonists and antagonists. Because deregulation of the BMP activity at multiple steps in signal transduction is linked to a wide variety of human diseases, therapeutic use of activators and inhibitors of BMP signaling will provide potential avenues for the treatment of the human disorders that are caused by hypo- and hyperactivation of BMP signals, respectively.

The bone morphogenetic protein (BMP) family of ligands plays important roles in a multitude of processes during embryonic development and adult homeostasis by regulating cellular lineage commitment, morphogenesis, differentiation, proliferation, and apoptosis of various types of cells throughout the body. In this review, we describe biochemical properties and biological activities of BMP family members in development and diseases.

Although BMPs are now known to be multifunctional cytokines identified both in vertebrates and invertebrates, they were first discovered as proteins that induce ectopic bone formation. In 1889, Senn found that aseptic bone cavities can be healed by decalcified bone

(Senn 1889). In 1965, Urist reported that demineralized bone matrix implanted in muscular tissues induces ectopic formation of cartilage and bone tissues with bone marrow (Urist 1965). These findings postulated the presence of bioactive factor(s) in the demineralized bone matrix responsible for inducing bone formation. The factor(s) responsible for ectopic bone formation was named “bone morphogenetic protein,” because this activity was abolished by digestion with trypsin, a typical protease (Urist and Strates 1971). However, the identity of the BMP activity remained elusive until Wang and colleagues reported the isolation of BMP activity from extracts of bovine bone as a single gel band followed by sequencing the pep-

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tides obtained from trypsin digestion of the band (Wang et al. 1988). Subsequently, Wozney and colleagues (1988) cloned cDNAs for human BMP-1 through BMP-4 using the peptide sequence information obtained. Although BMP-1 was found to be a novel metalloproteinase, BMP-2, -3, and -4 were novel members of the transforming growth factor β (TGF- β) family. The corresponding recombinant BMP proteins, including BMP-1, were capable of inducing formation of cartilage or bone in vivo. Subsequently, the coding sequences of additional BMPs were cloned based on amino acid sequence homology (Celeste et al. 1990; Özkaynak et al. 1990; Sampath et al. 1990). Although the bone-inducing activity is unique to BMPs among the TGF- β family members (Sampath and Reddi 1983), it was later shown that BMPs have many other biological activities.

BIOCHEMICAL PROPERTIES OF BMPs AND THEIR INTRACELLULAR SIGNALING

As mentioned above, the name “bone morphogenetic protein” was originally assigned for a unique activity in demineralized bone matrix, which induces heterotopic bone formation in nonskeletal tissues, such as skeletal muscle and subcutaneous tissue (Urist 1965). However, the name “BMP” does not infer the biological activity of all BMP members of the TGF- β family, because they were cloned by homology of DNA or amino acid sequences rather than biological activity. The heterotopic bone-inducing activity in the implantation assay in nonskeletal soft tissues was confirmed for several BMPs and “growth and differentiation factors” (GDFs), but does not apply to TGF- β s, activins, and even several BMPs and GDFs in the TGF- β family, as will be discussed below. The osteogenic and non-osteogenic activities among the TGF- β family members depend on the structures, binding receptors, intracellular signaling molecules, and target genes.

Classification of BMPs

More than a dozen BMPs have been identified in vertebrates, and have highly conserved struc-

tures that are shared by the members of the TGF- β family. Because BMP family members were identified using multiple approaches, some were described with different names such as cartilage-derived morphogenetic proteins (CDMPs), GDFs, osteogenic proteins (OPs), osteogenin, and Vg-related (Vgr), as illustrated in Figure 1. In this article, only the terms “BMP” and “GDF” are used to avoid confusion. Based on structural homology, the BMP family members can be further classified into several subgroups, including the BMP-2/-4 group, BMP-5/-6/-7 (OP-1)/-8 group, BMP-9/-10 group, and BMP-12/-13/-14 (GDF-5/-6/-7) group (Fig. 1). Among BMP family members, only BMP-1 has a metalloproteinase structure and acts as a carboxy-terminal propeptidase for type I collagen (Kessler et al. 1996). BMP family members are found in invertebrates such as deca-entaplegic (Dpp), 60A/ glass bottom boat (Gbb), and Screw in *Drosophila*, and DAF-7 in *Caenorhabditis elegans*. Interestingly, *Drosophila* Dpp and 60A/Gbb, which are structurally similar to BMP-2 and -4 and BMP-6 and -7, respectively, induced ectopic bone formation in rats (Sampath et al. 1993), and human BMP-4 rescued the phenotype resulting from Dpp mutation in *Drosophila* (Padgett et al. 1993), suggesting that the biological activities of BMPs are highly conserved between flies and humans.

Structures and Processing of BMPs

Similar to other members of the TGF- β family, BMP family members are synthesized as inactive large pre-pro-polypeptides that contain signal peptides at their amino termini and mature polypeptides at the carboxyl termini, separated by pro-domains (Xiao et al. 2007). The BMP-4 precursor protein is cleaved by furin, a pro-protein convertase, to liberate the mature BMP polypeptide (Nelsen and Christian 2009). These mature BMP monomers contain seven cysteines, six of which form intramolecular disulfide bonds. The remaining seventh cysteine residue is involved in the dimerization with another BMP monomer through a covalent disulfide bond, resulting in a biologically active dimeric ligand for BMP receptor activation

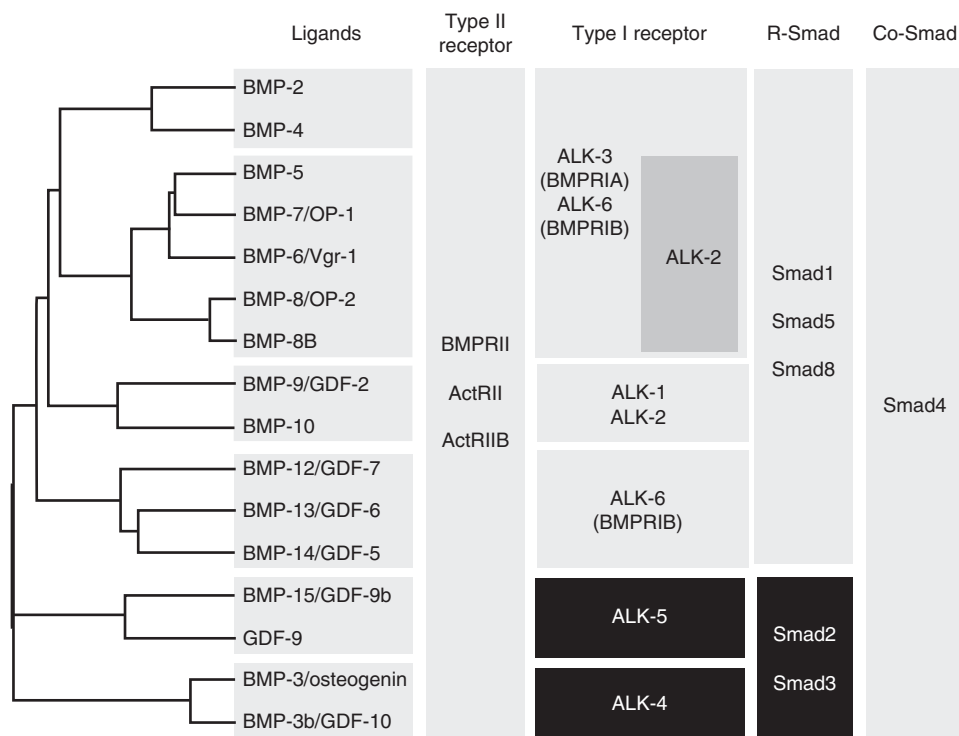


Figure 1. Relationships between bone morphogenetic protein/growth and differentiation factor (BMP/GDF) ligands, type II receptors, type I receptors, and Smad proteins in signal transduction. BMP-1 is a metalloproteinase and is not a member of the transforming growth factor β (TGF- β) family. Structurally related ligands, receptors, and Smad proteins are grouped and shown in the same boxes.

(Bragdon et al. 2011). Although BMP homodimers are produced and examined in vitro and in vivo, some heterodimers show enhanced activities (Israel et al. 1996; Guo and Wu 2012). BMP-2/7 and BMP-4/7 heterodimers have been implicated in mesoderm induction and differentiation of bone marrow cells, respectively (Suzuki et al. 1997; Yuan et al. 2011).

Crystal structures of BMP homodimers have revealed that the core structures of BMP dimers consist of a “cystine-knot” structure, and that the overall structure of BMPs has a “wrist and knuckle” or “two bananas” shape (Griffith et al. 1996; Brown et al. 2005; Schreuder et al. 2005). Studies of the crystal structures of BMPs have also identified possible binding epitopes of BMPs to specific receptors and antagonists (Kirsch et al. 2000; Groppe et al. 2002; Greenwald et al. 2003; Brown et al. 2005; Schreuder et al. 2005).

Receptors of BMPs

BMPs, like other TGF- β family members, elicit their effects through two types of serine—threonine kinase transmembrane receptors, type I and type II receptors. Unlike TGF- β s, BMPs are capable of binding to type I receptors in the absence of type II receptors. However, their binding affinities increase dramatically when both type I and type II receptors are present (Rosenzweig et al. 1995).

There are three type II receptors for BMPs—the BMP type II receptor (BMPRII), the activin type II receptor (ActRII), and activin type IIB receptor (ActRIIB) in mammals. Although BMPRII is specific for BMPs, ActRII and ActRIIB are shared by BMPs, activins, and myostatin. The BMPRII receptor encodes a short form and a long form with a carboxy-terminal tail with 530 amino acids after its kinase domain

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(Rosenzweig et al. 1995). The long form is expressed in most types of cells, whereas the short form is expressed only in certain types of cells. These type II receptors appear to bind most BMP ligands and control the binding preferences of BMPs to type I receptors (Fig. 1). Like other members of TGF- β family, the serine-threonine kinases of type II receptors for BMPs are constitutively active, and phosphorylate the glycine-serine-rich (GS) domain of the type I receptors on ligand binding (Fig. 2).

Among the seven type I receptors (activin receptor-like kinases 1 through 7; ALK-1–7) for TGF- β family proteins, ALK-3 (BMPRIA), ALK-6 (BMPRII), ALK-2, and ALK-1 serve as type I receptors for most of BMPs. ALK-3 and ALK-6 are structurally very similar to each other, and are distantly related to ALK-1 and ALK-2 that are structurally highly similar to each other. ALK-2 and ALK-3 are widely expressed in various types of cells. In contrast, ALK-6 is expressed in a more restricted manner, and ALK-1 expression is limited to endothelial cells

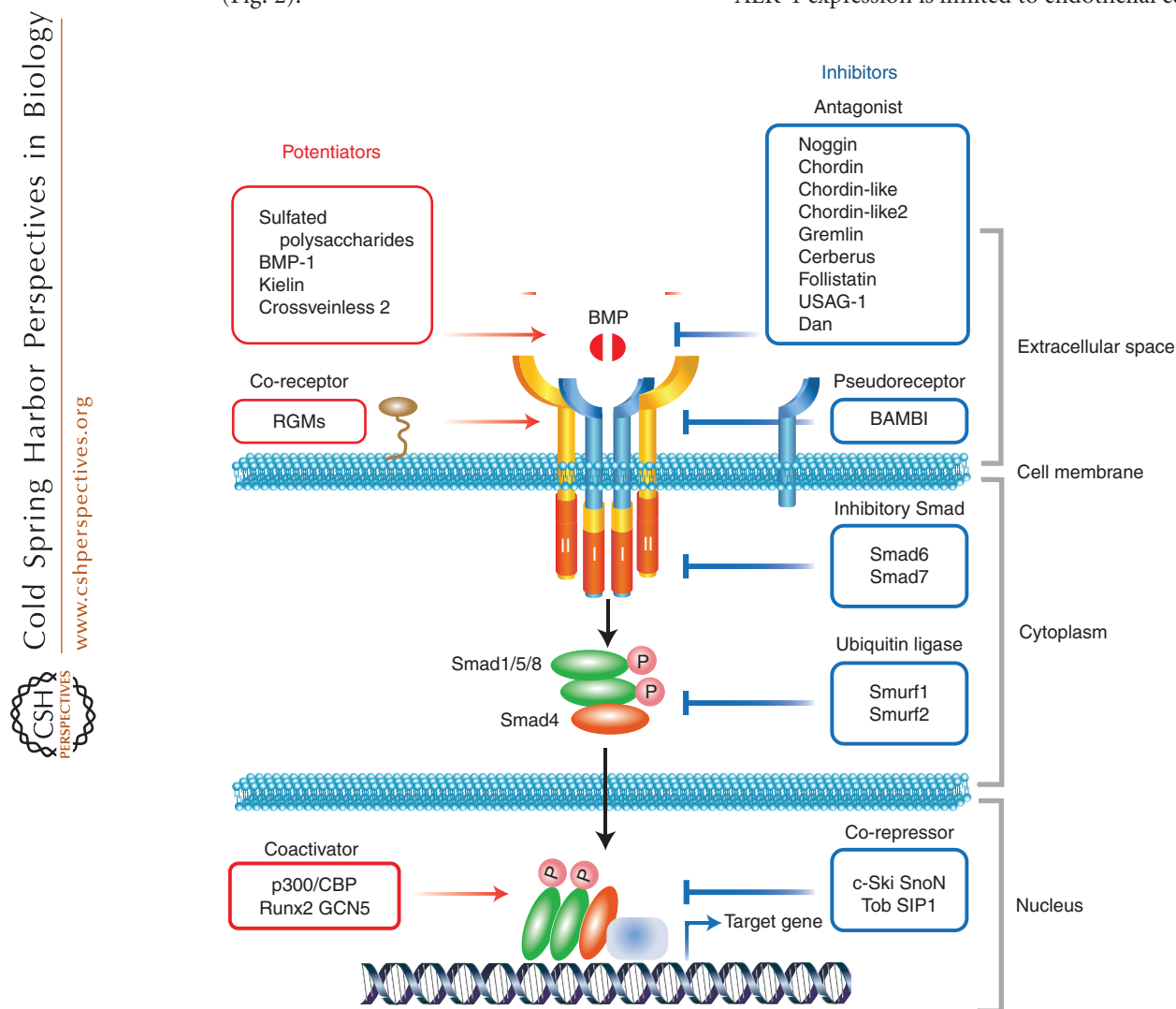


Figure 2. Potentiators and inhibitors of bone morphogenetic protein (BMP) signaling. Potentiators (*left*) and inhibitors (*right*) of BMP signaling are listed. These factors act on signaling extracellularly, at the membrane, in the cytoplasm, or in the nucleus.

and certain other cells. The activated type I receptor kinases then phosphorylate downstream substrates in the cytoplasm, including Smad proteins (Miyazono et al. 2010).

The specificities of the binding of BMPs to type I receptors depend on the identities of the interacting type II receptors and cell types (Fig. 1) (Yu et al. 2005). BMP-2 and BMP-4 bind to ALK-3 and -6 (ten Dijke et al. 1994), whereas BMP-6 and BMP-7 bind weakly to ALK-6 and strongly to ALK-2 (Ebisawa et al. 1999). GDF-5 preferentially binds to ALK-6 (Nishitoh et al. 1996). BMP-9 and BMP-10 bind to ALK-1 and ALK-2 (Brown et al. 2005; David et al. 2007). Some BMP type I receptors are shared by certain other members of the TGF- β family. TGF- β binds to ALK-5 (T β RI) as well as ALK-1 in cultured endothelial cells (Goumans et al. 2003).

In contrast to other BMPs, GDF-9 and BMP-3 have been reported to activate TGF- β and activin type I receptors, that is, ALK-5 and ALK-4, respectively, leading to activation of Smad2 and Smad3 (Fig. 1) (Daluisi et al. 2001; Mazerbourg et al. 2004). Thus, GDF-9 and BMP-3 appear to activate signals similar to TGF- β /activins, but distinct from those induced by other BMPs. BMP-3 has been shown to bind to ActRIIB and suppress BMP-2 and BMP-4 activity (Kokabu et al. 2012).

Intracellular Signaling from Receptors

Eight Smad proteins (Smad1 through Smad8) have been identified in mammals (Smad8 is also known as Smad9). Activated BMP type I receptors phosphorylate receptor-regulated Smads (R-Smads), that is, Smad1, Smad5, and Smad8 (BMP-specific R-Smads) at their carboxy-terminal S-S-X-S motifs. The phosphorylated and activated R-Smad proteins form complexes with Smad4 (common partner Smad: co-Smad), and move into the nucleus. Smad complexes containing two R-Smads and one Smad4 associate with various transcriptional coactivators (p300, CBP, Runx2, and/or GCN5) or co-repressors (c-Ski, SnoN, Tob, or SIP1), and bind to regulatory elements of target genes to regulate their transcription (Fig. 2).

During osteoblast differentiation, BMP-6 induces phosphorylation and nuclear accumulation of Smad1 and Smad5, but not of Smad8 (Ebisawa et al. 1999). Although Smad8 has been shown to be phosphorylated by BMP receptors, and transduce their signals (Kawai et al. 2000), a recent report showed that Smad8 forms complexes with Smad1 and bind to DNA, but suppresses the transcription of a reporter gene of BMP signaling as a dominant negative Smad (Tsukamoto et al. 2014). These findings suggest that Smad8 functions as a transducer and/or a new type of transcriptional regulator of BMP signaling.

BMPs also activate Smad-independent signaling pathways such as mitogen-activated protein kinases (MAPKs), c-Jun amino-terminal kinase (JNK), phosphoinositol-3 kinase (PI3K), Akt, and small GTPases (Derynck and Zhang 2003). These non-Smad pathways cooperate with Smad pathways to regulate various cellular responses.

Target Genes for BMPs

Progress in the genome-wide mapping of binding sites of Smad proteins using chromatin immunoprecipitation (ChIP) approaches, with promoter array analysis (ChIP-chip) and ChIP followed by sequencing (ChIP-seq), has revealed that Smad proteins co-occupy target sites with cell-type-specific master transcription factors (Morikawa et al. 2013). These findings suggest that BMP signals differentially regulate the expression of groups of target genes depending on the cellular contexts to elicit cell-type-specific functions. During osteoblast differentiation of early mesenchymal cells (e.g., C2C12 cells), numerous genes that regulate transcription and signal transduction were identified as immediate early genes (regulated within 2 h after BMP stimulation), including the inhibitor of differentiation or inhibitor of DNA-binding (Id) proteins Id1, Id2, and Id3, Smad6, Smad7, OASIS, Prx2, TIEG, and Snail (de Jong et al. 2004). In contrast, the intermediate (regulated up to 6 h after BMP-2 stimulation) and late (regulated up to 24 h after BMP stimulation) response genes are related to processes of osteoblastic dif-

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ferentiation, including the genes for transcription factors Hey1 and Tcf7, which mediate Notch and Wnt signaling, respectively. During angiogenesis, BMP-4 induces the expression of Id1, which is a common target of BMP signals, and the expression of angiogenesis-related genes including vascular endothelial growth factor receptor 2 (VEGFR2) and Tie2, which are receptors for VEGF and angiopoietins, respectively, and stimulates the proliferation of endothelial cells (Suzuki et al. 2008).

In the promoter region of the *Id1* gene, a GC-rich region located ~1 kb upstream of the transcription start site was identified as the BMP-responsive element, and was found to show 100% identity between human and mouse genes (Katagiri et al. 2002; Korchynskiy and ten Dijke 2002; Lopez-Rovira et al. 2002). Smad1 and Smad4 bind to this element and synergistically activate *Id1* transcription. These results together with the findings that BMPs commonly induce Id1 expression in various types of cells suggest that Id1 is one of the direct targets for BMP signaling via Smad pathways in initiation of downstream events. Id1 through Id4 have similar, although not identical, biological activities. Id proteins interact with the basic helix–loop–helix (bHLH) transcription factors through their HLH domains. Because Id proteins do not bind DNA, they antagonize the transcription induced by bHLH transcription factors. Transcription factors of the bHLH family include MyoD and myogenin, which direct myogenesis, and NeuroD, Mash1, and neurogenin, which regulate neurogenesis. These findings suggest that BMP signals regulate the differentiation of various lineages of cells by inducing the expression of Id proteins. This notion has been confirmed by the finding that BMP induction of *Id1* suppresses differentiation of embryonic stem (ES) cells and sustains their self-renewal (Ying et al. 2003).

Smad6 is another important BMP early response gene (Takase et al. 1998). The proximal BMP-responsive element in the Smad6 promoter, which is important for activation by BMPs, contains a 28-bp GC-rich sequence, including four overlapping copies of the GCCGnCGC-like motif that is recognized by

Smad1 and Smad5 (Ishida et al. 2000). Because Smad6 is an inhibitory Smad (I-Smad), BMP signaling interferes with its own signaling by establishing a negative feedback loop mediated by Smad6, a direct target of BMP signaling, which will be further discussed below. Similar to Smad6, the expression of *Smad8* is increased in response to BMP signaling, but not TGF- β signaling, within 1 h (Tsukamoto et al. 2014).

Regulators of BMP Signaling

BMP signaling is regulated at multiple levels from the extracellular space to the nucleus (Fig. 2). In extracellular compartments, BMP signaling is limited by BMP antagonists, which function through direct binding to BMP, thus preventing their binding to specific receptors (Brazil et al. 2015). Various extracellular BMP antagonists, such as noggin, chordin, chordin-like 1, chordin-like 2, Gremlin, Cerberus, follistatin, ectodin/uterine sensitization-associated gene-1 (USAG-1), and DAN family members, have been identified in various animal species. Expression of some antagonists such as noggin and Gremlin is up-regulated by BMPs, suggesting that the antagonists establish a negative feedback loop (Kameda et al. 1999; Pereira et al. 2000).

BMP signaling is also negatively regulated at the cell membrane by BAMBI (BMP and activin membrane-bound inhibitor), a pseudoreceptor for the TGF- β family. BAMBI lacks the intracellular domain of the serine–threonine kinase receptors, and inhibits ligand-induced signaling by preventing the formation of signaling receptor complexes (Onichtchouk et al. 1999). The expression of BAMBI is induced by BMP and TGF- β , which is another example of negative feedback of TGF- β family signals (Onichtchouk et al. 1999). Intracellularly, BMP signaling is negatively regulated by I-Smads (Smad6 and Smad7), the E3 ubiquitin ligases Smurf1 and Smurf2, and transcriptional co-repressors, such as c-Ski, SnoN, and Tob.

In addition to antagonists, some extracellular potentiators of BMPs have been identified. BMP-1 is a metalloproteinase that cleaves pro-collagens (Kessler et al. 1996). Tolloid and

Xolloid are BMP-1 homologs in *Drosophila* and *Xenopus*, respectively. Because they release active BMPs from inactive BMP–chordin complexes by cleavage of chordin, BMP-1 may also act as an activator of BMPs (Marques et al. 1997; Piccolo et al. 1997). Because mature BMPs are identified as heparin-binding forms, sulfated polysaccharides, such as heparin, heparan sulfate, and dextran sulfate, have been reported to potentiate BMP-2-, BMP-4-, and BMP-7-induced osteoblast differentiation (Ruppert et al. 1996; Irie et al. 2003; Takada et al. 2003). Kielin/chordin-like protein (KCP) was identified from an embryonic kidney cDNA library as a protein, which has 18 cysteine-rich repeats and a von Willebrand factor type D domain (Lin et al. 2005). Although cysteine-rich repeats are frequently found in BMP antagonists, KCP binds BMPs and enhances BMP signaling in a paracrine manner. Crossveinless-2, also known as BMPER, is closely related to chordin and has been shown to function as both a potentiator and antagonist of BMP signals (Moser et al. 2003). When Crossveinless-2/BMPER is depleted in endothelial cells, sprouting phenotypes are diminished with decreased BMP signals (Heinke et al. 2008).

BMP signals are also positively regulated by their coreceptor, glycosylphosphatidylinositol (GPI)-anchored membrane proteins of the repulsive guidance molecule (RGM) family, including RGMa, RGMb (also known as DRAGON), and RGMc (also known as hemojuvelin or HFE2) (Nili et al. 2010). RGMs form complexes with BMP type I receptors, bind selectively to BMP-2 and BMP-4, but not to BMP-7 or TGF- β 1, and enhance BMP signaling (Babitt et al. 2005, 2006; Samad et al. 2005). In the nucleus, transcriptional coactivators, such as p300 and CBP, are required for the transcriptional activity of phosphorylated BMP-specific R-Smads through complex formation. Several transcription factors, including Runx2, interact with Smad1 and 5 and participate in the transcription of some BMP-specific target genes. These potentiators and inhibitors of BMP signals are expressed in a cell-type-specific manner, and play important roles in various biological activities of BMPs.

Expression of BMPs

Although most BMPs are expressed in a variety of tissues during embryogenesis, the expression of some members becomes restricted to specific tissues after birth. For example, BMP-3, -4, -5, and -6 are highly expressed in lung, whereas BMP-7 is abundantly expressed in kidney in adult mice (Özkaynak et al. 1992). Osteoblasts and osteocytes, which are terminally differentiated osteoblasts embedded in secreted bone matrix, are an important source of BMPs in bone matrix, and expression of some BMP mRNAs is induced during bone formation. BMP-3 is abundantly expressed by osteoblasts and osteocytes in mice (Kokabu et al. 2012). BMP-4 expression is transiently induced in callus-forming cells in the early phase of fracture healing (Nakase et al. 1994). The expression of BMP-4 is enhanced by BMP signaling itself and is higher in lymphoblastoid cells established from patients with fibrodysplasia ossificans progressiva (FOP), which is caused by gain-of-function mutation of a BMP receptor, than that in control cells (Shafritz et al. 1996). BMP-6 is highly expressed in hypertrophic chondrocytes, which are cells with cartilage and bone cell characteristics during endochondral ossification (Lyons et al. 1989). BMP-9 is produced by hepatocytes, and circulates in plasma both as an unprocessed inactive form (40%) and as a mature and fully active form (60%) (Bidart et al. 2012). The level of circulating BMP-9 in plasma of healthy adult human is 6.2 ± 0.6 ng/ml with a range variation between 2 and 12, and is enough to induce a constitutive Smad1/5/8 phosphorylation in endothelial cells (David et al. 2008). These results suggest that BMPs are capable of acting not only as local but also as systemic factors.

BIOLOGICAL ACTIVITIES OF BMPs IN SKELETAL TISSUES

BMPs play critical roles in the development and maintenance of various tissues in vertebrates and invertebrates by regulating cell proliferation, differentiation and death. Considering the discovery of BMPs in the context of skeletal

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repair, we first review the major biological activities in skeletal tissues.

Skeletal Development

It is not clear whether all BMPs have bone-inducing activity in vivo. BMP-2, BMP-4, BMP-6, and BMP-7 have been shown to induce formation of bone and cartilage tissues in vivo, whereas GDF-5 induces cartilage and tendon-like tissues in vivo (Fig. 3) (Wozney et al. 1988; Celeste et al. 1990; Sampath et al. 1992; Wolfman et al. 1997). The osteogenic activities of 14 BMPs were evaluated using an adenoviral gene transfer technique in vitro (Cheng et al. 2003). Among them, BMP-2, BMP-6, and BMP-9 were

most potent in inducing alkaline phosphatase activity and osteocalcin expression in murine pluripotent C3H10T1/2 cells, and were found to play important roles in inducing osteoblast differentiation of mesenchymal progenitor cells. In contrast, most BMPs other than BMP-3 and BMP-12 were able to induce alkaline phosphatase activity in human osteosarcoma TE-85 cells, and to induce osteogenesis in mature osteoblasts.

Analyses of mice and humans with skeletal abnormalities have identified loss-of-function or gain-of-function mutations in BMP signaling molecules, confirming that BMPs are important regulators for normal skeletal development. The mutant mouse “*short ear*” has de-

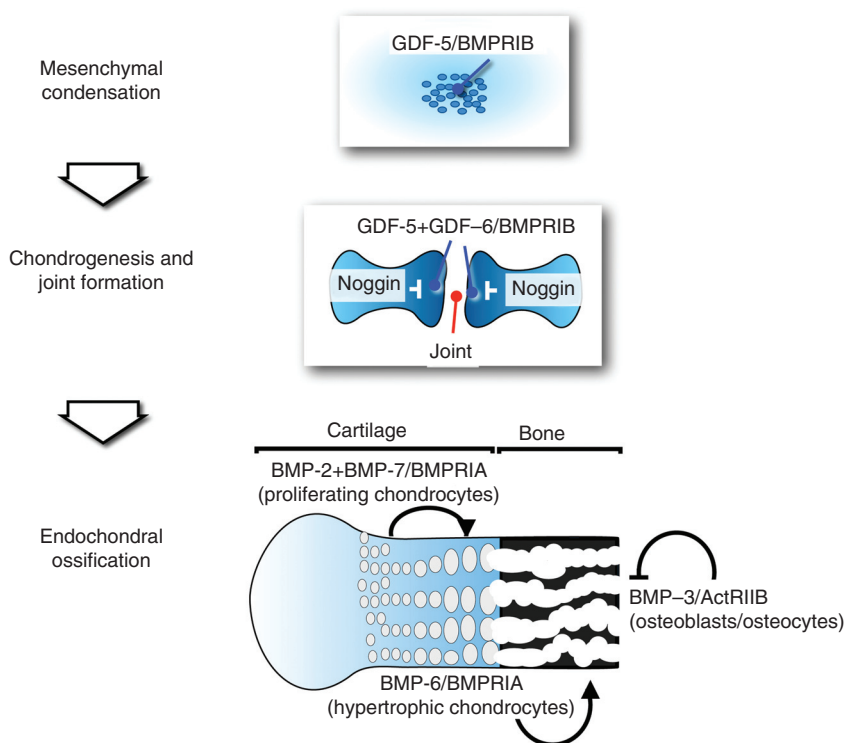


Figure 3. Roles of bone morphogenetic protein (BMP) signaling in skeletal development. Most skeletal elements are formed by endochondral ossification, in which undifferentiated mesenchymal cells condense in response to a GDF-5 signal (*top*). The cells differentiate into chondrocytes and form cartilaginous tissues. GDF-5 and GDF-6 promote cartilage development through BMPRIIB, but noggin suppresses this process as a BMP antagonist to lead a joint formation (*middle*). The proliferating chondrocytes further differentiate into hypertrophic chondrocytes, and then the terminally differentiated chondrocytes are replaced by bone tissue (*bottom*). Chondrocyte differentiation is stimulated by BMP-2, BMP-6, and BMP-7 secreted by the chondrocytes themselves, and bone formation is suppressed by BMP-3 secreted by osteocytes.



fects in growth and patterning of skeletal structures and in repair of bone fractures in adults. In these mice, the *Bmp5* gene is deleted or rearranged in several independent mutations at the short ear locus (Kingsley et al. 1992). *Bmp6*^{-/-} mice are indistinguishable from wild-type mice, but they have mild cartilage phenotypes (Solloway et al. 1998). *Bmp7*^{-/-} mice have skeletal patterning defects restricted to the rib cage, skull, and hindlimbs (Dudley et al. 1995; Luo et al. 1995). Doubly heterozygous *Bmp4*^{+/-}; *Bmp7*^{+/-} mice develop minor defects in the rib cage and the distal parts of limbs (Katagiri et al. 1998). Mutations in the *Gdf5* gene are responsible for skeletal alterations in *brachypodism* (*bp*) mice, which are characterized by skeletal abnormalities restricted to the limbs and limb joints (Storm et al. 1994). Inactivation of the *Gdf6* gene causes defects in joint, ligament, and cartilage formation at sites distinct from those seen in *Gdf5* mutants (Settle et al. 2003). Mice lacking both the *Gdf5* and *Gdf6* genes show additional defects, including severe reduction or loss of some skeletal elements of the limbs, additional fusions between skeletal structures, scoliosis, and alterations of cartilage in the intervertebral joints of the spinal column (Settle et al. 2003). Homozygous *Gdf11* mutant mice show anteriorly directed homeotic transformations throughout the axial skeleton and posterior displacement of the hindlimbs (McPherron et al. 1999). Interestingly, *Bmp3*^{-/-} mice have twice as much trabecular bone after birth as wild-type littermates (Daluisi et al. 2001). BMP-3 suppresses osteoblastic differentiation of bone marrow stromal cells in vitro by binding to ActRIIB without activating BMP intracellular signaling (Kokabu et al. 2012).

A noggin/GDF-5/ALK-6 axis has been identified as a critical signaling pathway for chondrogenesis during limb development (Fig. 3). GDF-5 is abundantly present in mesenchymal condensations before chondrocyte differentiation in embryonic development (Tsumaki et al. 1999). In contrast, noggin, its antagonist, is expressed in joint-forming spaces, which are formed by termination of the chondrogenesis in the cartilage (Brunet et al. 1998). The biological activities of the TGF- β family

members on chondrogenesis have been examined in vitro in high-density micromass cultures of mesenchymal cells prepared from limb bud (Nakayama et al. 2003; Seemann et al. 2005). TGF- β -induced chondrogenesis was inhibited by noggin, suggesting that BMP signaling is involved in this process (Nakayama et al. 2003). Multiple synostoses syndrome, which is characterized by fusion of multiple joints, is caused by an overactivation of BMP/GDF activity as a result of loss-of-function mutations in noggin protein or gain-of-function mutations in GDF-5 (Gong et al. 1999; Dawson et al. 2006). In contrast, a suppression of BMP/GDF activity caused by loss-of-function mutations in GDF-5 or its receptor ALK-6 has been shown to be linked to brachydactyly (Polinkovsky et al. 1997; Thomas et al. 1997; Lehmann et al. 2006).

Osteoblast and Chondrocyte Differentiation

BMPs regulate proliferation and/or differentiation of osteoblasts and chondrocytes, which develop from a common population of undifferentiated mesenchymal stem cells that have pluripotency to differentiate into multiple types of cells such as adipocytes, tenocytes, and myocytes as well. Generally, osteogenic BMPs induce heterotopic bone in soft tissue via endochondral ossification, in which the undifferentiated mesenchymal cells differentiate into chondrocytes secreting cartilage-specific extracellular matrices, such as type II collagen and various proteoglycans, within a week after implantation (Wang et al. 1990). Osteoblasts appear in the perichondrium close to mature hypertrophic chondrocytes in endochondral ossification. In contrast, in intramembranous ossification, the mesenchymal cells directly differentiate into osteoblasts secreting bone-specific extracellular matrices, such as type I collagen, osteopontin, and osteocalcin. Implantation of BMP-2 induces cartilage by day 7 and bone by day 14, with bone formation dependent on the amount of BMP-2 (Wang et al. 1990). Bone formation could be observed at 5 days using higher doses of BMP-2. Although BMP-2 induces differentiation of chondrocytes and osteoblasts

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in vitro, BMP-2 failed to convert the differentiation pathway from chondrocytes to osteoblasts and vice versa (Komaki et al. 1996). The fate of progenitor cells to differentiate into chondrocytes or osteoblasts in response to BMP signaling may be modulated by their microenvironment.

Expression of constitutively active forms of BMP type I receptors, such as BMPRIA, BMPRIIB, ALK-2, and ALK-1, induces osteoblastic differentiation in vitro without adding exogenous ligands (Akiyama et al. 1997; Chen et al. 1998; Fujii et al. 1999; Zhang et al. 2003). FOP is the first identified disorder caused by natural gain-of-function mutations of the BMP receptor ALK-2. FOP is characterized by progressive heterotopic bone formation in soft tissues, such as skeletal muscle, tendon, and ligament, similar to the effects of implantation of BMPs (Katagiri 2010, 2012; Kaplan 2013). Increased expression of the mutant ALK-2 in vitro activates intracellular signaling without adding ligands, and enhances the chondrogenesis in micromass cultures and the osteoblastic differentiation in C2C12 myoblasts (Fukuda et al. 2008, 2009; Shen et al. 2009; Fujimoto et al. 2014, 2015). Twelve types of mutant ALK-2 are found in patients with FOP, and they mildly activate BMP signaling without adding ligands. However, their activities are further enhanced by the presence of type II BMP receptors, such as BMPRII and ActRIIB, but not ActRII (Fujimoto et al. 2015). Activin A induces phosphorylation of Smad1/5 through the mutant ALK-2 responsible for FOP, but not wild-type ALK-2 (Hastell et al. 2015). Moreover, a neutralizing antibody against activin A inhibits heterotopic bone formation in model mice of FOP (Hastell et al. 2015). It is of note that common activating mutations in ALK-2 found in FOP patients are related to the pathogenesis of diffuse intrinsic pontine gliomas (DIPGs), a rare type of glioma that occurs exclusively in children (Pacifi and Shore 2016).

Smad1 and Smad5 are critical effectors of BMP type I receptors, although other signaling pathways induced by BMP receptors, such as MAPK pathways, also affect the BMP-induced osteoblast differentiation. Substitution of two

serine residues at the carboxy-terminal serine–valine–serine (SVS) motif in Smad1 to aspartic acid (DVD) activates the transcriptional activity without the need for phosphorylation by the receptors (Nojima et al. 2010), and expression of such mutant Smad1 induces osteoblast differentiation in C2C12 cells and ventralization in *Xenopus* embryos (Nojima et al. 2010). Similar mutations activate both Smad5 and Smad8, but a Smad8 mutant showed lower activity than the corresponding Smad1 or Smad5 mutants (Tsukamoto et al. 2014). It is still unclear how Smad1 and/or Smad5 induce bone or cartilage formation. The transcription factor Osterix was shown to be expressed in C2C12 cells in response to BMP-2, and is critically required for osteoblast differentiation in vivo, as apparent from the phenotype of *Osterix*^{-/-} mice (Nakashima et al. 2002). Runx2, Dlx-2, Dlx-5, and SOX6 are also involved in the BMP-induced osteoblast or chondrocyte differentiation, suggesting that multiple transcription factors are involved in the Smad1-/5-induced bone and cartilage formation (Ducy et al. 1997; Miyama et al. 1999; Xu et al. 2001; Fernandez-Lloris et al. 2003; Maeda et al. 2004).

BIOLOGICAL ACTIVITIES OF BMPs IN OTHER TISSUES

Skeletal Muscle

Skeletal muscle is one of the target tissues of BMPs, and BMP signaling induces skeletal tissue development in skeletal muscle tissue during embryonic development and in some pathological conditions. Skeletal muscle tissue contains not only multinucleated muscle fibers but also several types of mononuclear cells, including satellite cells, endothelial cells, smooth muscle cells, and mesenchymal interstitial cells, which are potential progenitor cells of chondrocytes and osteoblasts induced by BMPs (Lounev et al. 2009; Medici et al. 2010; Wosczyzna et al. 2012). Satellite cells and myoblasts were believed to be progenitor cells of the chondrocytes and osteoblasts, because osteogenic BMPs induce osteoblastic differentiation in C2C12



myoblasts (Katagiri et al. 1994). However, cell-lineage-tracing experiments in vivo using cell-type-specific fluorescent marker expression showed that MyoD- or Myf5-expressing myogenic cells do not incorporate well into BMP-induced cartilage or bone tissue (Lounev et al. 2009). Heterotopically induced chondrocytes and osteoblasts, but not normal chondrocytes or osteoblasts, are positive for the endothelial marker Tie-2 (Medici et al. 2010). Moreover, BMP treatment induces Tie-2-expressing endothelial cells to differentiate into osteoblasts, chondrocytes, and adipocytes via endothelial-to-mesenchymal transition (EndMT) (Medici et al. 2010). Nonmyogenic interstitial cells in the skeletal muscle, which are positive for Tie-2, platelet-derived growth factor receptor α (PDGFR α) and Sca-1, were identified as progenitors of both chondrocytes and osteoblasts induced by BMP-2 in vivo (Wosczyzna et al. 2012). In these experiments, the CD31- or VE-cadherin-expressing endothelial cells did not differentiate into osteoblasts or chondrocytes in the BMP-induced heterotopic skeletal tissues (Wosczyzna et al. 2012). These findings suggest that several types of progenitor cells could differentiate into osteoblasts and chondrocytes in response to osteogenic BMP signaling in the skeletal muscle.

The skeletal muscle mass is physiologically controlled by the TGF- β family signaling, including osteogenic and nonosteogenic members. Myostatin/GDF-8, a TGF- β family member that is specifically expressed in skeletal muscle, is a negative regulator of skeletal muscle mass, and myostatin-deficient animals, from zebrafish to humans, show marked increase in the skeletal muscle mass rather than fiber numbers. Osteogenic BMP signaling (phosphorylated Smad1/5) was also detected in normal skeletal muscle (Sartori et al. 2013). Surprisingly, adenoviral expression of noggin inhibited the muscle hypertrophy in myostatin-deficient mice, suggesting that BMPs promote increased skeletal muscle mass formation in these mice (Sartori et al. 2013). Moreover, the expression of BMP-14/GDF-5 mRNA was increased in a mouse model of denervation-induced sarcopenia, and overexpression of a constitutively active

BMPRIA-induced muscle hypertrophy in vivo (Sartori et al. 2013; Winbanks et al. 2013).

BMPs are potent inhibitors of myogenesis. Expression of Id1, Id2, and Id3 is induced by the transcriptional complexes consisting of phosphorylated Smad1/5 and Smad4, and they suppress the transcriptional activity of myogenic bHLH factors such as MyoD and Myf5 (Katagiri et al. 1994; Hollnagel et al. 1999; Kowanzetz et al. 2004; Shin et al. 2013). Nuclear localization of Smad4 with E4F1 has been shown to be involved in the expression of Id1 and BMP-induced inhibition of myogenesis (Nojima et al. 2010). MUSA-1, a ubiquitin ligase, has been identified as a target of both BMP and myostatin signaling to control skeletal muscle mass (Sartori et al. 2013). BMP-induced Smad1/5-Smad4 signaling inhibits the expression of MUSA-1 by competing with the formation of Smad2/3-Smad4 complexes induced by myostatin (Sartori et al. 2013). These findings indicate that skeletal muscle mass is regulated in a balance between BMP/GDF and myostatin/activin intracellular signaling through Smad4.

Adipogenesis and Fat Tissues

BMP signaling plays important roles in adipogenesis, not only in maturation of pre-adipocytes, but also in commitment of undifferentiated progenitor cells. BMP-2, BMP-4, and BMP-7 stimulate adipogenesis of C3H10T1/2 cells that show a pluripotency to differentiate into myocytes, adipocytes, chondrocytes, and osteoblast-like cells (Wang et al. 1993; Asahina et al. 1996; Bächner et al. 1998). Similar stimulation of adipogenesis by BMP-2 was observed in a typical pre-adipocyte cell line, 3T3-L1. BMPRIA, but not BMPRII, was shown to promote adipogenesis, suggesting that BMP ligands that bind BMPRIA with high affinity, such as BMP-2 and BMP-4, are involved in adipogenesis (Chen et al. 1998). Gremlin-1, a BMP antagonist, is secreted by pre-adipocytes and prevents maturation into mature adipocytes induced by BMPs (Gustafson et al. 2015).

Although BMPs are important for white adipose tissue formation, BMP signaling also reg-

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ulates brown adipose tissue formation. Both types of adipose tissue are related but have distinct functions, resulting in energy storage and energy expenditure, respectively. Brown adipose tissue develops from Myf5-expressing cells following stimulation with BMP-7 (Tseng et al. 2008). BMP-7 induces commitment of mesenchymal progenitor cells to brown adipocyte lineage cells, and *Bmp7*^{-/-} mice show a reduction of brown fat (Tseng et al. 2008). BMP-7 activates PRDM16, a zinc finger transcription factor that promotes brown adipocyte differentiation, and both the p38 MAPK and Smad1/5 signaling pathways in brown, but not white adipose cells (Tseng et al. 2008). BMP-8b is also expressed in brown adipose tissue and increases thermogenesis not only locally but also systemically through central nerve systems (Whittle et al. 2012). Furthermore, a balance between

BMP and TGF- β signaling regulates brown adipogenesis through a regulation by Bmal1, a circadian clock transcription factor (Nam et al. 2015). These findings indicate that multiple BMP ligands regulate both white and brown adipocyte differentiation in vivo.

Tooth Development

Epithelial and mesenchymal interactions control normal tooth development by regulating the differentiation of enamel-producing ameloblasts and dentin-producing odontoblasts from epithelium and mesenchyme, respectively (Fig. 4). These interactions are controlled by the expression and activities of BMPs, their receptors and antagonists. BMP signaling has been suggested to play an important role in the epithelial-mesenchymal interactions during tooth

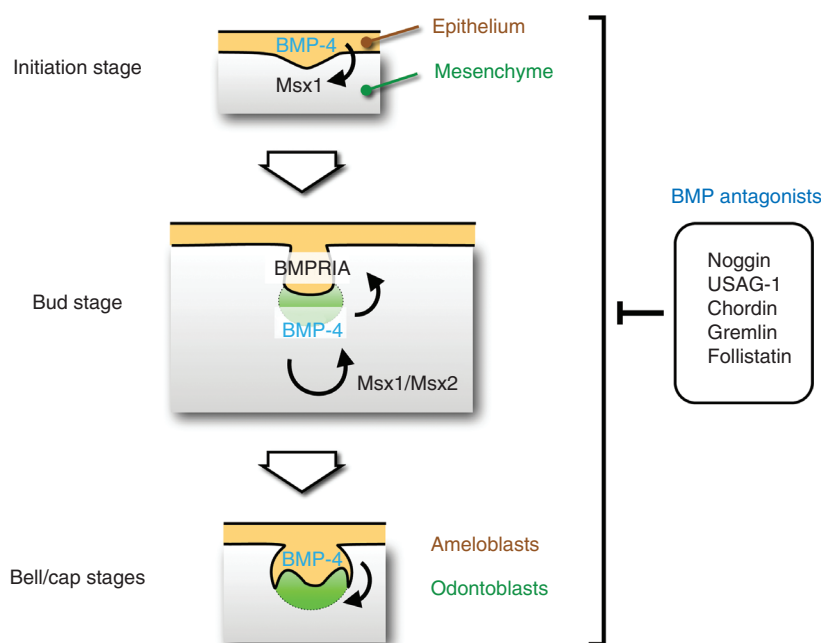


Figure 4. Bone morphogenetic proteins (BMPs) are secreted key regulators of an epithelial–mesenchymal interaction in tooth development. Teeth are formed by an epithelial–mesenchymal interaction during tooth development. At an initiation stage, BMP-4 from epithelial cells initiates tooth development through Msx1, a homeobox-containing transcription factor, in mesenchymal cells (*top*). BMP-2 and BMP-4 from mesenchymal cells act as a positive signal on BMPRIA in initiation of differentiation of epithelial cells at a bud stage (*middle*). At the bell and cap stages, BMP from epithelial cells regulates odontoblast differentiation in mesenchyme (*bottom*). BMP signaling is inhibited by BMP antagonists, including noggin, USAG-1, chordin, Gremlin, and follistatin.

development. BMP-2 and BMP-4 as well as their type I receptor BMPRIA are highly expressed in rat molar development at cap stage (Ikeda et al. 1996). Additionally, beads soaked with BMP-2 or BMP-4 mimicked the interactive signaling between epithelial and mesenchymal cells in tooth development in an ex vivo culture system (Vainio et al. 1993). Depletion of BMPRIA in keratin 5-expressing epithelial cells using a Cre-LoxP system caused enamel defects and induced ectopic cementum-like structures (Yang et al. 2013). The ectopic cement-like phenotype was rescued by conditional depletion of β -catenin in epithelial cells, suggesting that Wnt signaling is involved in the phenotype (Yang et al. 2013). The expression of homeobox-containing transcription factors *Msx1* and *Msx2* was increased by BMP-4 signaling in dental mesenchymal cells (Vainio et al. 1993; Tucker et al. 1998a,b; Zhang et al. 2002). BMP-4 failed to induce its own expression in *Msx1*^{-/-} mice-derived cells and BMP-4 rescued the phenotype of *Msx1*^{-/-} tooth germs, suggesting that BMPs regulate dental epithelial-mesenchymal interactions through the expression of *Msx1* and *Msx2* during early tooth development (Zhang et al. 2002).

BMP antagonists, such as noggin, follistatin, and USAG-1, also regulate tooth development by suppressing BMP signaling. Exogenous noggin in the developing mandible induces ectopic expression of *Barx-1*, a homeobox-containing transcription factor in the incisor mesenchyme, and a change in type of tooth from incisor to molar (Tucker et al. 1998b). Increased expression of noggin in dental epithelium causes a loss of odontogenesis in the epithelium (Wang et al. 2012). In contrast, in *Noggin*^{+/-} mice, a single upper incisor is formed with normal molars and mandibular incisors (Hu et al. 2012). Chordin and Gremlin, which also prevent BMP binding to the receptors, are coexpressed with noggin in the developing lower incisor and molar. Ectopic expression of follistatin in dental epithelium in transgenic mice inhibited ameloblast differentiation in incisors, whereas ameloblasts differentiated ectopically on the lingual surface in *Follistatin*^{-/-} mice (Wang et al. 2004). In cultured tooth explants, follistatin expression

was induced by activin from the surrounding dental follicle (Wang et al. 2004). USAG-1 is abundantly expressed in teeth as a “negative” image of mouse enamel knots (Laurikkala et al. 2003; Kasai et al. 2005). USAG-1-deficient mice showed enlarged enamel knots, markedly altered cusp patterns, extra incisors and molars, and fused molars (Kasai et al. 2005; Yanagita et al. 2006). The supernumerary maxillary incisor formed as a result of the successive development of the rudimentary upper incisor (Murashima-Suginami et al. 2008). Inhibition of BMP signaling rescues supernumerary tooth formation in incisor explants (Murashima-Suginami et al. 2008). These findings indicate that both positive and negative type regulators of BMP signaling control normal tooth development (Fig. 4).

Hair Follicle Development

As described above in the section on tooth development, epithelial-mesenchymal interactions mediated by BMP signals play important roles in the development and regeneration of hair follicles. Hair follicles, which are appendages of skin epithelium, arise from the embryonic ectoderm and undergo cyclic regeneration during postnatal life.

During embryogenesis, skin epithelium arises from the single layer of ectoderm that surrounds the embryo body (Lee and Tumber 2012). Starting around E14.5, the primary hair follicle, which gives rise to guard hair, develops, followed by the formation of secondary hair follicles, which make up the majority of hair follicles. Compartmentalization and differentiation of hair follicles continue up to ~2.5 weeks after birth. During the formation of hair follicles, epidermal keratinocytes are stimulated by instructive signals from the underlying dermal mesenchyme. Such instructive signals for hair follicle morphogenesis include Wnt, Hedgehog, fibroblast growth factor (FGF), and BMPs. During the initiation of hair follicle morphogenesis, suppression of BMP and FGF signaling in combination with activation of Wnt signaling is required for epidermal cells to differentiate to hair follicles. During the maturation of hair follicles,

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BMP signals are necessary for proper hair follicle differentiation. Deletion of *Bmpr1a* results in abnormal hair follicle formation with a lack of lineage-specific differentiation markers (Andl et al. 2004). After birth, the lower portion of the mature hair follicle is regressed by apoptosis, and enters into a quiescent stage, which is regulated by BMP signals. In summary, multiple signals including BMPs are involved in proper follicular development: lineage specification, maturation, and regression. It is of note that the different combinations of the same signaling pathways during specific time windows and in distinct cell populations play important roles in hair follicle morphogenesis.

Around 20 days after birth, hair follicles enter a quiescence stage, but are ready to reactivate on signals from the environment. In adult skin, hair follicles undergo cycles through bouts of active hair growth (anagen), destruction (catagen), and rest (telogen), also known as the “hair cycle.” Hair follicle stem cells reside in the bulge and are the source of all hair follicle lineages (Tumbar et al. 2004). At telogen, on activation signals, some bulge hair follicle stem cells migrate out into hair germ where they lose stem-cell characteristics and become transit-amplifying progenitor cells, followed by proliferation and differentiation, which give rise to differentiated hair follicle lineages and production of hair shaft (anagen). In catagen, cells in the lower hair follicle regress again by apoptosis (Lee and Tumbar 2012). The tightly controlled balance of multiple signaling pathways regulates the hair follicle cycling. Increased evidence suggests that hair follicle development and cycling are regulated by similar mechanisms that involve Wnt, Sonic hedgehog (Shh), and BMP signaling pathways (Lee and Tumbar 2012).

Multiple lines of evidence suggest that BMP signals play important roles in the hair cycle. Postnatal inhibition of BMP signals by ectopic expression of noggin impairs hair follicle formation (Kulessa et al. 2000). During the hair cycle, BMP-4, BMPRIA, and noggin show spatiotemporal changes in their expression patterns, and play important roles in the control of telogen–anagen transition of hair follicles (Botchkarev et al. 2001). During telogen,

BMP-4 is produced by both secondary germ keratinocytes and dermal papilla fibroblasts, and activates intracellular signals via BMPRIA, which is selectively expressed in the secondary germ, leading to prevention of the onset of anagen. Activation of hair growth phase is initiated on up-regulation of noggin in follicular epithelium and mesenchyme. BMP-4 plays inhibitory roles during hair follicle telogen–anagen transition (Botchkarev et al. 2001). Expression of BMP-4 and BMPRIA is down-regulated in the germinative compartment of early anagen hair follicles. Administration of BMP-4 blocks development of anagen in the secondary hair germ of the hair follicles. Of note, the anagen-inducing effect of noggin is partially mediated by activation of Shh signaling, which is essential for hair follicle morphogenesis and initiation of hair cycle. Shh is up-regulated in the hair follicle after noggin treatment, and is down-regulated by BMP-4 (Botchkarev et al. 2001). Furthermore, inhibition of BMP signals promotes Wnt signals in hair follicle stem cells, which induce hair follicle morphogenesis (Jamora et al. 2003). In-depth genomic profiling of hair follicle stem cells and transient-amplifying cells revealed that GATA3, Id1 and Id3, targets of phospho-Smad1/5, play important roles in the specification of hair follicle lineages (Genander et al. 2014). These findings suggest that epithelial and mesenchymal interactions mediated by BMP signals in combination with Wnt and Shh signals play important roles in the regulation of maintenance and lineage specification of hair follicle stem cells during hair cycle.

Iron Homeostasis

Several lines of evidence have suggested that BMP signaling is a regulator in iron homeostasis. Iron is stored mainly in erythrocytes and liver from the circulation. Hepcidin is a critical hormone synthesized in the liver and suppresses the transport of iron from intestinal cells to the circulation. Smad4 was identified as a positive regulator of hepcidin expression in the liver, because the expression of hepcidin mRNA was abrogated in Smad4-deficient hepatocytes (Wang et al. 2005). Juvenile hemochromatosis is



an autosomal recessive disorder characterized by iron overload in various organs and linked to the *HFE2* gene, which encodes HFE2/hemojuvelin/RGMC (Papanikolaou et al. 2004). BMP-2, BMP-4, and BMP-9 increase the expression of hepcidin mRNA in hepatocytes, and BMP-9 shows the most potent stimulation among them (Truksa et al. 2006). Because BMP-9 is highly expressed in liver and is present in circulating plasma (David et al. 2008), BMP-9 has been suggested to act as an autocrine or paracrine regulator of iron homeostasis through HFE2/RGMC/hemojuvelin and Smad4 by regulating hepcidin expression in hepatocytes. The BMP type I receptors, BMPRIA and ALK-2, are expressed in hepatocytes and regulate iron homeostasis. Deletion of the genes encoding either BMPRIA or ALK-2 causes iron overload in mice (Steinbicker et al. 2011). BMPRIA is required for the hepcidin expression induced by interleukin-6 (Mayeur et al. 2014).

Kidney Development

A mammalian kidney contains approximately one million nephrons that consist of glomeruli, proximal tubules, loop of Henle, distal tubules, and collecting ducts. These structures develop through mutual interactions between the ureteric bud and the metanephric mesenchyme that contain nephron progenitors (Dressler 2006). BMP signals have been implicated in many steps of kidney development (Nishinakamura and Sakaguchi 2014). BMP-4 inhibits ureteric bud attraction. During midgestation, BMP-7 plays important roles in the maintenance of the nephron progenitors and, at the same time, sensitizes them to the ureteric bud-derived differentiation signal. Mice deficient for *Bmp7* gene die shortly after birth because of poor kidney development (Dudley et al. 1995; Luo et al. 1995). In *Bmp7*^{-/-} mice, increased apoptosis was observed in the embryonic renal mesenchyme (Luo et al. 1995). BMP-7 is abundantly expressed in the kidney, especially in distal tubule epithelial cells. BMP-7 reversed TGF- β -induced epithelial–mesenchymal transition (EMT) in renal tubular epithelial cells in vitro and in vivo (Zeisberg et al. 2003),

and administration of large doses of BMP-7 reversed renal injury and improved renal function (Vukicevic et al. 1998; Hrusuka et al. 2000; Zeisberg et al. 2003). Furthermore, small peptide agonists of BMP signaling that function through the ALK-3 showed therapeutic benefits in repairing established renal fibrosis (Sugimoto et al. 2012).

Both potentiators and antagonists of BMPs are also expressed in the kidney. Deletion of *Crossveinless-2*, a potentiator of BMP signals, leads to kidney hypoplasia (Ikeya et al. 2010). After birth, when nephron progenitors disappear, Dullard, a phosphatase that inactivates BMP receptors, maintains BMP signals at an appropriate level. Lack of Dullard results in excessive BMP signals, leading to apoptosis of the postnatal nephrons. Mice deficient for KCP, a potentiator of BMP-7, were more susceptible to development of renal interstitial fibrosis and more sensitive to acute tubular injury, suggesting an important role for KCP in preventing renal fibrotic diseases (Lin et al. 2005). Mice lacking USAG-1, a BMP antagonist expressed abundantly in kidney, were resistant to renal injury (Yanagita et al. 2004, 2006). These findings indicate that signals regulated by BMP-7, as well as potentiators and antagonists of BMP signals play important roles in development and pathological conditions in the kidney.

Pluripotent Stem Cells

Pluripotent stem cells possess properties of self-renewal and pluripotency (Hackett and Surani 2014). Mouse ES cells that are derived from inner cell mass of mouse blastocysts, possess “naïve” pluripotency. Proliferation and differentiation of mouse ES cells are regulated by multiple types of signaling cascades, including those mediated by BMPs (Itoh et al. 2014). Pluripotency of mouse ES cells is maintained in serum-containing medium supplemented with leukemia inhibitory factor (LIF). BMP-4 together with LIF is capable of substituting for serum and sustaining self-renewal of naïve mouse ES cells (Ying et al. 2003). Human ES cells are derived from human postimplantation embryos, and show more differentiated charac-

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teristics than naïve mouse ES cells. They are referred to be “primed” for differentiation, and have similar characteristics as mouse epiblast stem cells. Human ES cells and mouse epiblast stem cells are cultured in a LIF-independent condition, and can be maintained in the presence of FGF-2 and activin A, which maintain the primed pluripotent state. Full pluripotency, or a “ground state” of mouse ES cells has been shown to be maintained by a cocktail of two inhibitors of the MAPK kinase 1/2 (MEK1/2) and GSK3 pathways (Ying et al. 2008). However, the BMP–Smad pathway has been shown to be less active in the ground state of mouse ES cells (Boroviak et al. 2014). Furthermore, BMP-4 induces differentiation of primed pluripotent stem cells. These findings suggest that BMP signals play distinct roles in the maintenance and differentiation of pluripotent stem cells.

Mouse ES cell-like cells were generated from mouse embryonic fibroblasts by reprogramming using four transcription factors, Oct4, Sox2, Klf4, and c-Myc (Takahashi and Yamanaka 2006). These pluripotent stem cells are named “induced pluripotent stem (iPS) cells,” share properties with mouse ES cells, such as expression of stem-cell markers, formation of teratomas, capacity to differentiate into all three germ layers, and generation of chimeric mice when iPS cells are injected into blastocytes. This reprogramming method was applied to human somatic cells to generate human iPS cells (Takahashi et al. 2007). In recent years, molecular mechanisms involved in iPS cell reprogramming have been studied, which has led to proposing a model that reprogramming consists of three phases: initiation, maturation, and stabilization (Samavarchi-Tehrani et al. 2010). The first step during the reprogramming is the morphological change from disperse fibroblasts to uniform and tightly packed ES cell-like cells. This morphological process is termed mesenchymal to epithelial transition, which is induced by BMP signals by inducing the expression of miR-205 and the miR-200 family (Samavarchi-Tehrani et al. 2010). BMPs can replace Klf4 in the reprogramming cocktail, allowing mouse embryonic fibroblasts to be reprogrammed using Oct4 alone (Chen et al. 2011). However, repro-

gramming of human somatic cells is inhibited by constitutive activation of BMP signals (Hamasaki et al. 2012). These results also implicate the differential roles of BMP signals during the reprogramming of mouse and human iPS cells.

Formation and Maintenance of Vascular Systems

Tissue fluid homeostasis in vertebrates is maintained by the blood and lymphatic vascular systems. The crucial roles of BMP signals in the formation and maintenance of vascular systems have been identified in human hereditary vascular disorders, including hereditary hemorrhagic telangiectasia (HHT) and pulmonary arterial hypertension (PAH) (Cai et al. 2012). Furthermore, the role of BMP signaling in vascular development has been shown by studies in mouse models using a gene-targeting technique (Goumans and Mummery 2000).

BMPs have been reported to regulate the proliferation and migration of endothelial cells (David et al. 2009). Of the BMPs, BMP-2 and BMP-4 have been reported to regulate the proliferation of endothelial cells both positively (Valdimarsdottir et al. 2002; Suzuki et al. 2008) and negatively (Kiyono and Shibuya 2003). Roles of BMP-9 in the proliferation and migration of endothelial cells are also unclear because, at a high dose, BMP-9 inhibits endothelial cell proliferation (Scharpfenecker et al. 2007; David et al. 2008; Ricard et al. 2012), whereas a low dose of BMP-9 promotes proliferation of various types of endothelial cells in vitro, and angiogenesis in matrigel plug assays and human pancreatic cancer xenografts in vivo (Suzuki et al. 2010). It is likely that BMP-9 has disparate effects on endothelial cells depending on the cellular context and concentration of BMP-9 (Wiley and Jin 2011). BMP-6 and BMP-7 have been implicated in the onset of human diseases, such as cerebral cavernous malformation by inducing EndMT (Maddaluno et al. 2013).

Lymphatic vessels drain interstitial fluid that leaks from blood capillaries and return it to the blood vessels (Karpanen and Alitalo 2008). Dysfunction of the lymphatic system results in lymphedema, which is characterized by

disabling swelling in the affected tissues. Lymphatic vessels also provide a major pathway for tumor metastasis in many types of cancer, and regional lymph node metastasis has been correlated with cancer progression. Inhibition of BMP-9 signals by administration of ALK-1-Fc, a soluble chimeric protein consisting of the extracellular part of ALK-1 fused to a Fc fragment, perturbs the postnatal lymphangiogenesis in the retina, tail, and ear skin, suggesting that BMP-9 signals are involved in lymphangiogenesis (Niessen et al. 2010). Multiple groups reported that BMP-9/ALK-1 signals inhibit the formation of lymphatic vessels in both physiological and pathological conditions by inhibiting proliferation of lymphatic endothelial cells (Levet et al. 2013; Yoshimatsu et al. 2013).

Tumor formation and progression requires newly formed blood vessels that supply cancer cells with oxygen and nutrients. BMP-9/ALK-1 signals have received a lot of attention as an anti-angiogenesis target because BMP-9-induced (tumor) angiogenesis can be pharmacologically inhibited by ALK-1-Fc, which serves as a ligand trap for endogenous BMP-9 and -10 (Cunha et al. 2010). Furthermore, ALK-1-Fc can decrease tumor growth and angiogenesis when combined with VEGFR inhibitor *in vivo*. These results suggest that targeting ALK-1 may be a promising therapeutic strategy for cancer. Roles of BMP signals in tumor microenvironment are further discussed below.

ROLES OF BMPs IN CANCER

Multiple lines of evidence have suggested that BMP signals have divergent roles during the formation and progression of cancer by modulating both cancer cells and tumor microenvironments including tumor vessels (Ehata et al. 2013). It is of note that BMPs function as both suppressors and promoters of cancer in a context-dependent manner (Fig. 5).

Genetic Implication of BMP Signals in Cancer

Germline mutations in genes encoding ALK-3 (*BMPRIA*) and Smad4 (*MADH4*) have been found in subsets of patients with juvenile pol-

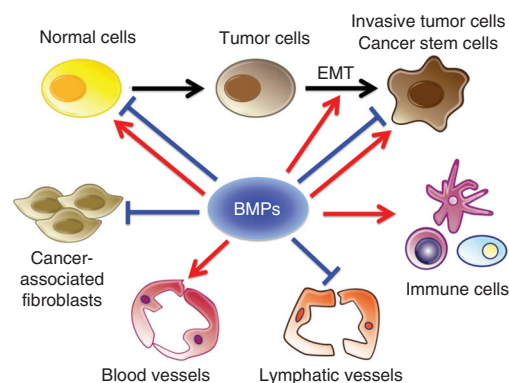


Figure 5. Roles of bone morphogenetic proteins (BMPs) in cancer. Pro- (red) and antitumorigenic (blue) effects of BMPs on various components of cancer microenvironments are shown. BMPs can either promote or suppress the proliferation and progression of cancer cells depending on the cellular contexts. EMT, Epithelial–mesenchymal transition.

yposis syndrome (Howe et al. 2001; Zhou et al. 2001), an autosomal dominant gastrointestinal hamartomatous-polypsis syndrome with risk for development of cancer. Furthermore, mutations in BMP signaling components, such as BMPRII and Smad4, in association with absence of phosphorylation of Smad1/5/8, have been observed in the majority of sporadic colorectal cancers (CRCs) (Kodach et al. 2008; Park et al. 2010). Consistent with these clinical observations, BMPs have been reported to inhibit the *in vitro* or *in vivo* proliferation of CRCs (Hardwick et al. 2004; Lee et al. 2008). In addition to CRC, *in vitro* and *in vivo* proliferation of prostate and diffuse-type gastric carcinoma were inhibited by BMP-7 and BMP-2/-4, respectively, through induction of p21^{CIP1/WAF1}, leading to the hypophosphorylation of the retinoblastoma protein (RB) (Miyazaki et al. 2004; Shirai et al. 2011). These results suggest that BMPs act as tumor suppressors in multiple types of cancer (Ehata et al. 2013), which is also observed with TGF- β .

Tumor Suppression by BMP Signals

Tumor suppressing effects of BMPs are also elicited by their decrease in the size of populations

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of cancer stem cells. Cancer stem (or cancer-initiating) cells have stem-cell-like properties, such as self-renewal and multipotency. Because they show higher tumor-forming abilities and drug resistance than non-cancer stem cells, they have been implicated in growth and recurrence of many types of cancers. Although autocrine TGF- β signals play important roles in maintaining the stem-cell-like properties and tumorigenic activity of glioma-initiating cells (GICs), BMP signals induce the differentiation of these cells (Piccirillo et al. 2006; Lee et al. 2008). BMPs also regulate the differentiation of other types of cancer stem cells, including colorectal and breast cancers (Lombardo et al. 2011; Buijs et al. 2012; Gao et al. 2012; Zhang et al. 2012). Taken together with their inhibitory effects on proliferation of various types of cancer cells, activation of BMP signals is expected to serve as a therapeutic strategy for cancer.

Tumor Promotion by BMP Signals

Although BMPs, similar to TGF- β s, might also act initially as suppressors of many types of cancers, they also behave as tumor promoters during the progression of cancers. Intensity of BMP signaling appears to positively correlate with the degree of cancer malignancy and clinical stage in cancer patients (Helms et al. 2005; Alarmo et al. 2008; Choi et al. 2012; Voorneveld et al. 2013). BMP signals differentially modulate the initiation and progression of CRC depending on the Smad4 and p53 status (Voorneveld et al. 2015). Constitutive activation of Wnt signals plays important roles in the initiation, metastasis and chemosensitivity of CRC. BMP signaling inhibits Wnt signaling in CRC only when p53 and Smad4 are unaffected. In contrast, in Smad4 negative and/or p53 aberrant CRC, activation of Wnt signals (nuclear accumulation of β -catenin) was observed. These results suggest that use of BMPs in CRC therapy should be targeted to individual cancers based on the mutational status of p53 and Smad4. Furthermore, possible involvement of BMP signaling in cancer metastasis has recently been investigated (Alarmo and Kallioniemi 2010). Increasing evidence suggests that BMPs promote the motility

and invasiveness of various types of cancer cells including breast cancer, lung cancer, prostate cancer, colon cancer, and malignant melanoma (Rothhammer et al. 2005; Yang et al. 2005; Bailey et al. 2007; Grijelmo et al. 2007; Katsuno et al. 2008). Of note, BMP-9 induces EMT of hepatocellular carcinoma cells to induce their invasiveness (Li et al. 2013).

Roles of BMP Signals in Tumor Stroma

Tumor stroma plays important roles in cancer progression, and is composed of multiple components including blood vessels, fibroblasts, and inflammatory cells. As discussed, BMP-9/ALK-1 signals promote tumor angiogenesis to alter the tumor microenvironment favorable for cancer growth. Pickup and colleagues investigated the requirement for BMPRII in stromal fibroblasts during the formation and metastasis of mammary carcinoma (Pickup et al. 2015). Genetic ablation of stromal *BMPRII* expression using fibroblast-specific protein 1 (FSP1) promoter-driven Cre resulted in increased tumor metastasis in a transgenic mouse model of mammary carcinoma. Stromal loss of BMPRII results in increased inflammatory cell infiltration via increased secretion of inflammatory cytokines. In human breast cancer patients, the increased expression of chemokines in BMPRII-deleted cancers correlated with poor outcome, suggesting that BMP signals have tumor-suppressive roles in the stroma by regulating inflammation. BMP signals also create “pro-metastatic functions” in bone microenvironments where prostate cancer cells and osteoblasts interact with each other (Nishimori et al. 2012).

BMP Signals as Therapeutic Targets

These findings suggest that BMP signals are promising targets of cancer therapies. It was reported that the resistance of lung squamous cell carcinomas harboring mutations in the epidermal growth factor receptor (*EGFR*) gene to EGFR tyrosine kinase inhibitors (EGFR-TKIs) is partially a result of the activation of BMP signals (Wang et al. 2015). Treatment of these tumor cells with inhibitors specific to BMP re-

ceptors effectively reversed the resistance to EGFR-TKI, suggesting that targeting BMP signals gives clinical benefit for squamous cell carcinomas with EGFR mutations. However, because BMPs have both tumor-promoting and suppressive functions, we need to carefully study the possible outcome when we use BMP ligands or BMP inhibitors, such as noggin and small molecule inhibitors to develop therapeutic strategies for cancers.

CONCLUSIONS AND PERSPECTIVES

BMP was originally identified as an ectopic bone-inducing activity in demineralized bone matrix in 1965. More than 20 BMPs have now been identified in various species, including mammals, *Xenopus*, *Drosophila*, and *C. elegans*. BMP receptors and the three classes of Smad proteins were identified by the late 1990s. Since the discovery of the molecules involved in BMP signaling cascades almost 20 years ago, we still have not fully understood how BMP family signals regulate the formation and maintenance of various organs in vivo. Combination of various ligands, signaling molecules, potentiators, and antagonists makes the BMP signaling cascades complex. BMP ligands have distinct profiles of expression and show distinct biological activities in vivo. Moreover, BMP antagonists and potentiators, which regulate BMP signals, also display distinct spatiotemporal profiles of expression. Thus, the biological activities of BMPs are tightly regulated by complex combination of various factors during various morphogenetic processes. Recent progress in experimental techniques, including ChIP-seq and next-generation high-throughput sequencing systems, may facilitate further understanding of in vivo functions of BMPs.

Studies of the physiological and pathological roles of BMP family members have revealed their clinical potentials not only in bone repair but also in other diseases such as vascular syndromes and cancer. Two BMP products, INFUSE (BMP-2) and OP-1 (BMP-7), have been approved by the U.S. Food and Drug Administration (FDA) for clinical application of fracture healing. However, clinical use of

BMPs has been hampered by several limiting factors such as their short half-lives and the possibility of undesired side effects. To date, usage of recombinant BMP proteins for fracture repair remains a highly expensive procedure with rather limited outcome. On the other hand, small molecule BMP agonists may overcome these limitations. Through a high-throughput screening, a stimulator of BMP signaling has been discovered, while its mode of action has not been elucidated (Balaramnavar et al. 2012). Like BMP agonists, small molecule inhibitors specific for BMP signaling have been developed (Yu et al. 2008a; Hong and Yu 2009; Boergermann et al. 2010; Mohedas et al. 2013; Sanvitale et al. 2013; Tsugawa et al. 2014). These inhibitors appear to be useful for the treatment of various diseases that are caused by activation of BMP signals, such as FOP (Yu et al. 2008b), DIPG (Pacifci and Shore 2016), and metastasis in certain types of cancer.

Thus, therapeutic use of activators and/or inhibitors of BMP signaling will provide potential avenues of opportunity for the treatment of various human diseases that are caused by loss- or gain-of-functions of BMP signals.

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