BMP Signaling and Skeletogenesis

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ABSTRACT: Bone morphogenetic proteins (BMPs) are members of the transforming growth factor (TGF)-β superfamily of signal molecules that mediate many diverse biological processes ranging from early embryonic tissue patterning to postnatal tissue homeostasis. BMPs trigger cell responses mainly through the canonical signaling pathway where intracellular Smads play central roles in delivering the extracellular signals to the nucleus. While the same Smads are used by BMPs in all types of cells, different transcription factors account in part for the functional diversity of BMPs. These transcription factors are recruited by Smads to regulate the expression of specific subsets of target genes depending on the cell types. Among the transcription factors are Hox proteins. Experimental gain and loss-of-function studies as well as naturally occurring mutations in *Hox* genes demonstrate their central roles in embryonic skeletal patterning. In addition to the interactions with Smads observed for several Hox proteins, there is also evidence that the expression of a number of *Hox* genes is regulated by BMPs. It is suggested that Hox proteins play an important role in the BMP pathway.

KEYWORDS: BMP; Hox; development; transcription factor; skeletogenesis

INTRODUCTION

The term, *bone morphogenetic protein* (BMP), was first introduced to describe the active component(s) in demineralized bone matrix that can induce ectopic bone formation when implanted intramuscularly or subcutaneously into rodents.[1,2] The identity of BMP was not discovered until the late 1980s when three polypeptides with BMP activity were purified—BMP-1, BMP-2A (later known as BMP-2), and BMP-3.[3] With the exception of BMP-1, the sequences of these molecules revealed that they belong to the transforming growth factor β (TGF-β) superfamily of signal molecules.[3] To date, more than 20 BMP members have been characterized. Evidence from extensive studies indicate

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that BMPs are multifunctional growth factors whose activities are essential for embryonic development and tissue homeostasis in adults.[4,5]

BMP molecules are first synthesized as large precursors and then cleaved at a dibasic site so that the C-terminal active domain is released. Seven conserved cysteines within the active domain are involved in intramolecular and intermolecular disulfide bonding. The mature BMP molecules are secreted as either heterodimers or homodimers. Although heterodimers have been generated *in vitro* and, in some cases, had more potent effects than the homodimers, their *in vivo* functions have not been well characterized.[4]

SIGNAL TRANSDUCTION BY BMPS

As members of the TGF-β superfamily, BMPs trigger cellular responses mainly through the Smad pathway,[6] although the signal molecules can also activate the mitogen-activated protein kinase (MAPK) pathway.[7] In the Smad pathway, type II and type I transmembrane serine/threonine receptor kinases and intracellular Smad proteins relay the signal from the cell surface to the nucleus. Three type II receptors have been shown to bind BMP ligands: type II BMP receptor (BMPR-II), and type II and IIB activin receptors (ActR-II and ActR-IIB).[8–11] Three type I receptors for BMPs have also been characterized: type IA and IB BMP receptors (BMPIA or ALK3 and BMPIB or ALK6), and type IA activin receptor (ActRIA or ALK2).[12–14] The expression of both type II and type I receptors is required to achieve high-affinity binding with ligands.[6] Upon ligand binding, the activated type I receptors phosphorylate a subgroup of receptor-regulated Smads (R-Smads, Smad1, 5, and 8) at their C-terminal SSXS motif.[15–17] The phosphorylated R-Smads then disassociate from their respective receptors and form complexes with the common partner Smad, Smad4. The Smad complexes then move into the nucleus where they associate with transcription factors to regulate gene transcription in a cell type-specific manner.[18,19] Smad6 and Smad7 represent another subclass of Smads that negatively regulate cell signaling by blocking phosphorylation of R-Smads and preventing complex formation between Smad1 and Smad4.[20–22]

Several transcription factors have been characterized for BMP signaling, among which are Runx2,[23–25] Menin,[26] OAZ,[27] YY1,[28,29] and Hoxc8.[30–33] All of these factors interact with Smad proteins and mediate BMP responses under different biological situations, thereby accounting for the functional diversity of BMPs.

Runx2, also known as polyomavirus enhancer binding protein 2αA/core binding factor 1 (PEBP2αA/CBFA1), is indispensable for bone formation.[34] The interactions between Runx2 and BMP-specific Smads (Smad1 and 5) suggest their intrinsic cooperation in mediating BMP functions.[23,25] While Runx2 alone does not induce osteoblast differentiation, it synergizes with Smad1 and Smad5 in this biological event.[24,25] Mutant Runx2 with a truncated

transcription activation domain fails to interact with Smad1 and consistently blocks BMP/Smad-induced osteoblast differentiation.[25] Runx2 has also been shown to interact with TGF-β-specific Smads[23,25] and mimics the common effects of TGF-β1 and BMP-2 to block myogenic differentiation.[24] However, it only synergizes with BMP-2, but not TGF-β1, to induce osteoblast differentiation,[24] suggesting that BMP-specific Smads are required for Runx2 activities.[24] In addition to Runx2, menin, the product of the multiple endocrine neoplasia type 1 (MEN1) gene, is required for BMP-induced osteoblast differentiation.[26] Menin interacts with both Runx2 and Smad1/5 in multipotential mesenchymal cells. When menin is knocked down, the cells fail to differentiate into osteoblast lineage. Conversely, menin interacts with TGF-β specific Smad3 in well-differentiated osteoblasts and inhibits BMP-induced late-stage differentiation.[26] Therefore, the function of menin in BMP signaling is stage-dependent.

Nuclear factor Yin Yang 1 (YY1) interacts with Smad1/4 and, depending on the cell types and target genes can act as both a repressor and an activator in the BMP-signaling pathway.[28,29] It inhibits Smad binding to its consensus DNA target *in vitro* and represses certain BMP target gene transcription. Furthermore, YY1 inhibits BMP-induced cell differentiation but has no effect on BMP-mediated growth inhibition.[28] In a different situation, YY1, in response to BMP, serves as an activator to drive the transcription of a key cardiac regulator (*Nkx2.5*) during mouse embryonic development. YY1 and Smad1/4 form a complex and bind to adjacent promotor elements. Disruption of the YY1/Smad1/4 complex eliminates transactivation by BMP.[29] Again, YY1 seems not to be specific for BMP since it also interacts with Smad2 and Smad3, although the biological consequences are different.[28]

Compared with the factors discussed above, Olf-1/EBF-associated zinc finger protein (OAZ) specifically interacts with activated Smad1, but not activated Smad2.[27] Although OAZ lacks transactivation activity alone, its interaction with Smad1/4 is required for BMP-induced homeobox gene *Xvent-2* transcription, an event that is essential for mesoderm ventralization and neural inhibition. The *Xvent-2* gene promotor element contains separate binding sites for OAZ and Smads. The presence of both OAZ and Smad complexes on the element is necessary and sufficient for *Xvent-2* gene transactivation. OAZ does not activate another BMP target gene *Tlx-2* suggesting that it helps Smad proteins distinguish a specific subset of target genes from others.[27]

Hoxc8, a member of the Hox family, has been demonstrated to act as a repressor in the BMP pathway. It binds to the consensus site of the osteopontin (OPN) gene and silences its transcription. The Smad1/4 complex, in response to BMP, interacts with its DNA-binding domain (known as homeodomain) and dislodges it from the OPN promotor element, thereby initiating gene transcription and inducing osteoblast differentiation.[30,31] Importantly, transgenic mice that overexpress the interaction domain of Smad1 with Hoxc8 in bone tissues have enhanced bone density.[33] In contrast, Smad6 Complexed with Hoxc8 on

the promotor element, serves as a transcriptional co-repressor.[32] Similar interactions have also been observed for Hoxa9.[32,35] Recently, Hoxa13 and Hoxd13 have been shown to antagonize Smad-induced transactivation by interacting with both Smad1/5 and Smad2.[36] It seems that Smad/Hox interaction is universal for most Hox proteins. However, due to the molecular diversity of Hox proteins, other interactions are likely to exist. For example, some Hox proteins interact with BMP-specific Smads or TGF-β-specific Smads, or both.

BIOLOGICAL FUNCTIONS OF BMPS

BMPs as Ventralizing Factors during Early Development

As the fertilized egg divides, three germ layers (endoderm, ectoderm, and mesoderm) are established during early development that will ultimately give rise to all types of somatic cells. The ectoderm will become neural tissues and epidermis. The mesoderm will develop into blood, mesenchyme, muscle, and notochord. The endoderm will form the respiratory and digestive tracts.[37,38] Dorsal–ventral patterning of the mesoderm and ectoderm during blastula and early gastrula stages, guided by inductive tissue interactions, is the crucial step for cell-fate determination. Accumulating data indicate that these tissue–tissue interactions are largely mediated by BMP-related factors and their antagonists. Results from *Xenopus laevis* studies will be reviewed according to BMP functions during early development. Tissue patterning of other vertebrates such as zebrafish and mice share similar mechanisms, albeit with certain variations.

Before the blastula stage, the animal and vegetal regions of the embryo are occupied by ectoderm and endoderm, respectively. During blastula and gastrula stages, mesoderm is formed and patterned in the marginal zone between ectoderm and endoderm. Mesoderm patterning is guided by ventralizing and dorsalizing signals. Ventralizing signals induce ventral mesoderm (blood, mesothelium, and mesenchyme), and dorsalizing signals induce dorsal mesoderm (notochord, muscle, and kidney). BMP-4 has been shown to be the most potent ventralizing factor. *Bmp-4* mRNA is present on the ventral side of the developing embryo.[39,40] Overexpression of *Bmp-4* by mRNA injection induces ventral mesoderm structures and marker expression.[39–43] Its effects are strong enough to override dorsalizing factors such as activin and lithium.[39,41,42,44] Conversely, disrupting BMP signaling either by a dominant-negative BMP receptor[40,45–47] or by antisense *Bmp-4* RNA[44] results in dorsalized mesoderm. In addition to BMP-4, BMP-2[48] and BMP-7[49] have also been shown to have ventralizing effects. Interestingly, BMP-3 has dorsalizing activities.[50,51] BMP-3 antagonizes BMP signaling and partially blocks the ventralizing effects of BMP-4, most likely by competing for the receptors since it fails to inhibit the activities of constitutively activated BMP type I receptor (CA-ALK3).[51] The roles of BMPs in ventral mesoderm formation are also strongly supported by

studies on the Spemann organizer (the dorsal mesoderm), which secretes BMP antagonists to counteract BMP function and induce dorsal mesoderm. The Spemann organizer was first characterized by Spemann and Mangold in 1924, who successfully demonstrated that the small dorsal lip of the embryo induces dorsal mesoderm as well as dorsal ectoderm formation when grafted to the ventral side.[52] Seventy years later it was found that the dorsalizing activities of the Spemann organizer are mediated by several secreted signals—noggin, chordin, and follistatin.[53–57] Soon thereafter, noggin[58] and chordin[59] were demonstrated to bind to extracellular BMPs with high affinity, blocking signal transduction by preventing ligand–receptor interactions. Noggin and chordin specifically bind to BMP molecules, since they bind to neither TGF-β1 nor activin.[58,59] Follistatin, on the other hand, primarily serves as an activin antagonist.[57] However, it also binds to BMPs and has similar inhibitory effects.[57,60] Therefore, it is believed that BMPs ventralize mesoderm and their antagonists released from the Spemman organizer dorsalize mesoderm. The balance between these two sets of signals in specific mesoderm regions determines their terminal fates.

During relatively later gastrulation stages, BMPs and their antagonists mediate ectoderm patterning in a manner similar to that of mesoderm patterning. At these stages, the ectodermal cells differentiate into either epidermis (ventral fate) or neural tissue (dorsal fate). BMP ligands serve as epidermal inducers and neural inhibitors.[40,49,56,61–64] In contrast, noggin, chordin, and follistatin favor neural fate.[56,57,59,64,65] Moreover, another family of BMP antagonists (DAN family), including Gremlin, Cerberus, and the tumor suppressor, DAN, has been demonstrated to have similar neural-inducing effects.[66,67] Overall, BMPs and their antagonists serve as the central control that guides dorsal–ventral patterning of mesoderm and ectoderm during early development.

BMPs and Skeletogenesis

As their name indicates, BMP molecules are capable of inducing ectopic cartilage and bone formation, a process that mimics embryonic endochondral bone formation.[68,69] Extensive studies demonstrate that BMPs are important factors regulating chondrogenesis and skeletogenesis during normal embryonic development. Individual BMPs exhibit distinct expression patterns in skeletal elements. *Bmp-2* is expressed in areas surrounding the initial cartilage condensations,[70,71] while *Bmp-4* is expressed in perichondrium.[71] *Bmp-2* is also expressed in periosteal and osteogenic zones.[70] *Bmp-5* is expressed in initial cartilage condensations as well as in perichondrium and periosteum at later stages of bone development.[72] *Bmp-6* is expressed in hypertrophic chondrocytes.[70] High levels of *Bmp-7* mRNA have been observed in the perichondrium, but its expression is absent in the zones of joint formation.[73] In contrast to *Bmp-7*, *Gdf-5* transcripts were detected in regions where joints will form.[74] Distinct expression patterns of individual BMPs indicate that different

BMP molecules mediate specific events during skeletogenesis. For example, BMP-2 recruits mesenchymal cells surrounding the initial cartilage condensations into chondrogenic fate, while BMP-4 recruits perichondrial cells.[71] BMP-6 may be essential for terminal chondrocyte differentiation.[70] BMP-7 is a potential inhibitory factor for joint formation[73] while GDF-5 is required for joint formation.[74]

BMPs are potent inducers of cartilage and bone formation *in vitro*. BMP-2 induces cartilage nodule formation in chick limb bud mesenchyme cultures.[75] BMP-6 accelerates hypertrophic chondrocyte differentiation and mineral accretion.[76] In multipotential mesenchymal cells (MMCs) isolated from human bone marrow, BMP-2 and BMP-9 promote chondrogenic differentiation, possibly through activation of *Sox-9*, a chondrogenic-related transcription factor. BMP-9 appears to have stronger effects than BMP-2.[77] BMP-2 and BMP-7 have been shown to induce both chondrocyte and osteoblast differentiation in C3H10T1/2 cells.[78,79] Although the mechanisms are not fully understood, it has been proposed that BMPs can induce both undifferentiated stem cells and more differentiated multipotent cells along chondrogenic or osteogenic pathways.[80–82]

Genetic studies have provided direct evidence of the specific functions of individual BMPs in cartilage and bone formation. First, strong evidence comes from the naturally occurring mutations of *Bmp-5* and *Gdf-5* genes.[72,83,84] Mutations of *Bmp-5* are the direct cause of skeletal defects observed in *short ear* mice, including a reduction in size of the external ear, malformations of the sternum, rib cage and the sixth cervical vertebra, and defective repair of bone fractures.[72,83] Mutations of *Gdf-5* are responsible for the defects in limb skeletons observed in *brachypodism* mice.[84] Experimental mutation studies provide further evidence. *Bmp-7*-deficient mice exhibit skeletal alterations in the rib cage, hind limbs, and skull.[85] *Bmp-6* mutant mice have delayed sternum ossification.[86] The cumulative data indicate that individual BMPs mediate particular stages during skeletogenesis of specific bone elements. BMP-3 represents an exception. Consistent with its opposite roles to BMP-4 during mesoderm patterning,[50,51] BMP-3 inhibits BMP-2-induced osteogenic differentiation.[50] *Bmp-3* knockout mice exhibit increased bone density.[50] Again, the data indicate that BMP-3 antagonizes BMP signaling *in vivo*. BMP-2 and BMP-4 are the most well-characterized ligands within the family. Homozygous *Bmp-2* mutant embryos die between embryonic day 7.5 (E7.5) and 10.5 (E10.5) with cardiac defects.[87] *Bmp-4* null mice die between E6.5 and E9.5 with mesoderm formation defects.[88]

BMPs and Apoptosis

During embryonic development, BMPs mediate programmed cell death, or apoptosis, the process that removes unnecessary tissues, thereby ensuring proper morphogenesis. BMP-4 was first demonstrated to induce apoptosis in

rhombomeric neural crest cells.[89] Other evidence for BMP-mediated apoptosis mainly comes from studies on limb bud mesenchymal cell death, a notable feature of limb development. In chick limb bud, apoptosis occurs in the anterior necrotic zone (ANZ), posterior necrotic zone (PNZ), and interdigital necrotic zone (INZ) to ensure proper limb structure. *Bmp-4* is expressed in these regions prior to the onset of apoptosis. Its expression is maintained during the course of apoptosis. *Bmp-2* has a similar but weaker expression pattern.[90] Overexpression of dominant-negative BMP type I receptors[90,91] suppresses apoptosis in chick limb bud, leading to the webbing phenotype. Conversely, overexpression of constitutively active BMP type I receptor promotes apoptosis.[92] When BMP-2- or BMP-7-soaked beads were implanted into chick limb buds, they induced apoptosis in the undifferentiated mesenchymal cells.[73] In transgenic mice overexpressing the BMP antagonist, noggin, the interdigital tissue had incomplete regression.[93] BMP-2 and BMP-4 also induce apoptosis in mesenchymal cells isolated from the interdigital regions of chick limb buds. This effect was not observed for either TGF-β1 or activin.[90] Similar results from separate studies support the notion that the apoptotic effect is specific for BMP molecules.[94]

Several lines of evidence indicate that the homeobox-containing gene *Msx2* acts downstream of BMPs to mediate apoptosis. Overexpression of dominant-negative BMPR-IB leads to downregulation of *Msx2* and suppression of apoptosis in the chick embryos.[91] High level of *Msx2* expression, either by stable transfection or induction by BMP-4, results in increased apoptosis in P19 cells. Furthermore, BMP-4 cannot increase apoptosis in cells with forced expression of *Msx2*.[95] On the other hand, *Msx2* was found to be not essential for BMP-mediated apoptosis in mouse limb.[93] It is possible that different mechanisms exist for different species, or that *Msx2* is only one of the factors responsible for the apoptotic effects of BMPs.[93] Dickkopf-1 (Dkk-1), a potent inhibitor of the Wnt/β-catenin pathway, has also been shown to be involved in BMP-mediated apoptosis.[96] It appears that BMP-mediated Dkk-1 activation is mainly via the MAPK pathway.[96]

HOX PROTEINS AND SKELETOGENESIS

Hox proteins are a family of transcription factors that control anterior–posterior body axis patterning during embryonic development.[97] The fact that Hoxc8,[30–32] Hoxa9,[35] and Hoxa13/d13[36] interact with Smads suggests their potential roles as BMP/TGF-β downstream transcription factors. The *Hox* gene family members share well-conserved homeobox sequences that encode the homeodomains and have a characteristic organization along the genome. In mice and humans, 39 *Hox* genes that are arranged into four genomic clusters (*Hoxa-d*) have been identified. According to the sequence similarities and positions along the clusters, the 39 genes are divided into 13 paralogs.[98] During

development, the expression of *Hox* genes follows the rule of temporal and spatial collinearity. The more 3′ *Hox* genes are expressed earlier in anterior regions and the more 5′ *Hox* genes are expressed later in posterior regions. Consistently, unique subsets of *Hox* genes specify the identities of individual regions where they are expressed.[99,100]

The crucial roles of *Hox* genes in embryonic pattern formation have been substantiated by extensive genetic studies. Mutations of *Hox* genes usually result in multiple skeletal defects. *Hoxa2* mutant mice exhibit homeotic transformation of the cranial skeleton. As a result, a set of the first branchial arch derivatives, including malleus and incus, are duplicated at the expense of the second branchial arch elements such as stapes and the lesser horns of hyoid bone.[101,102] *Hoxa2* was further demonstrated to restrict chondrogenic centers as well as inhibit dermal bone formation, likely via excluding *Sox9* expression and downregulating *Cbfa1* expression, thereby providing mechanisms for *Hoxa2*-mediated second branchial arch patterning.[103] In *Hoxc8* null mice, transformations occur in vertebrae (1st lumbar vertebra to 14th thoracic vertebra) and ribs. Additional sternebra is formed. Some ribs attach to the sternum abnormally.[104] Transgenic mice overexpressing *Hoxc8* under its own promotor do not exhibit homeotic transformation, suggesting that the temporal stages of *Hoxc8* expression are critical for its function.[105] Instead of patterning defects, transgenic mice show immature chondrocyte differentiation, with accumulation of proliferating chondrocytes and reduced hypertrophic cartilage. *Hoxd4* expression results in a phenotype similar to that produced by *Hoxc8* expression, indicating comparable functions in chondrocyte differentiation.[105] In contrast to the anteriorized transformations observed in *Hoxa2* and *Hoxc8* mutant mice, *Hoxa11* mutant mice develop a posteriorized phenotype.[106] The 13th thoracic vertebra is transformed into the 1st lumbar vertebra. The possible underlying mechanism is that disruption of the *Hoxa11* gene may change the expression of other *Hox* genes in the regions where *Hoxa11* is normally expressed, thereby changing the Hox code in those regions.[106] In addition to the axial skeletal malformations, limb skeletal defects have also been observed in *Hoxa11* mutant mice.[106]

While most of the evidence for *Hox* gene functions during development comes from experiments with targeted gene disruption, several naturally occurring *Hox* gene mutations have also been identified with distinct developmental defects in mice and humans. The phenotype of Hypodactyly (*Hd*) mice is caused by a 50 base-pair deletion in the first exon of the *Hoxa13* gene; therefore, no functional Hoxa13 protein is made from the mutated allele. As a result, heterozygous *Hd* mice have a shortened digit I in all limbs and homozygous *Hd* mice have more severe defects with only one digit in each limb.[107] The molecular basis of human hand-foot-genital (HFG) syndrome has been found to be a nonsense mutation in the *HOXA13* gene, leading to the production of truncated proteins which, in all likelihood, cannot serve as functional transcription factors. The defects include autopod and urinary tract malformations.[108] Mutation

of *HOXD13* is involved in synpolydactyly (SPD) which is characterized by an increase in the number of digits and digit fusions.[109,110] The mutant HOXD13 proteins contain duplicated polyalanines upstream of the homeodomain.

Several studies reveal an intrinsic linkage between Hox factors and BMP signaling. Hox factors participate in BMP functions by two means. First, Hoxc8[30–33] and Hox13 proteins[36] interact with Smads and serve as BMP/TGF-β downstream transcription factors. Second, *Hox* and *Bmp* gene expression is mutually regulated by each other. BMP-2 has been shown to induce ectopic expression of *Hoxd11* and *Hoxd13* in anterior limb mesenchyme.[111] BMP-2 also induces ectopic *Hoxa13* expression in the anterior region of limb muscle masses, while the BMP antagonists, noggin/chordin, downregulate its expression.[112] There is also evidence that BMP signals regulate the initial expression of a number of *Hox* genes during early *Xenopus* development.[113] On the other hand, Hoxa13 and Hoxd13 can activate *Bmp-4* promotor activity.[114] The cumulative results indicate that Hox proteins and BMP signals cooperate with each other to fulfill their biological missions.

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