

JB Review

Bone morphogenetic protein receptors and signal transduction

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Bone morphogenetic proteins (BMPs) exhibit broad spectra of biological activities in various tissues, including bone, cartilage, blood vessels, heart, kidney, neurons, liver and lung. BMPs are members of the transforming growth factor- β (TGF- β) family that bind to type II and type I serine-threonine kinase receptors, and transduce signals through Smad and non-Smad signalling pathways. Recent findings have revealed that BMP signalling is finely tuned by various mechanisms in both positive and negative fashions. Perturbations of BMP signalling pathways are linked to a wide variety of clinical disorders, including vascular diseases, skeletal diseases and cancer. Administration of recombinant BMP ligands and increasing endogenous expression of BMPs provide therapeutic effects on some diseases. The recent development of BMP receptor inhibitors may also prove useful for some clinical diseases induced by hyperactivation of the BMP signalling pathways.

Keywords: BMP/GDF/serine-threonine kinase/Smad/TGF- β

Abbreviations: ActR, activin receptor; ALK, activin receptor-like kinase; bHLH, basic helix-loop-helix; BMP, bone morphogenetic protein; BMPR, BMP receptor; ca, constitutively active; CBP, CREB-binding protein; cGKI, cGMP-dependent kinase I; ChIP-chip, chromatin immunoprecipitation on microarray; co-Smad, common-partner Smad; FOP, fibrodysplasia ossificans progressiva; GDF, growth and differentiation factor; GS domain, glycine and serine-rich domain; GSK3, glycogen synthase kinase 3; HAT, histone acetyl transferase; HHT, hereditary haemorrhagic telangiectasia; HDAC, histone deacetylase; Id, inhibitor of differentiation; I-Smad, inhibitory Smad; MAP, mitogen-activated protein; MH, Mad homology; LIMK1, LIM kinase 1; miRNA, microRNA; MIS, Müllerian inhibiting substance; OP1, osteogenic protein-1; PAH, pulmonary arterial hypertension; RGM, repulsive guidance molecule; R-Smad, receptor-regulated Smad; SSXS motif, Ser-Ser-X-Ser motif; TAK1, TGF- β activated kinase 1; TGF- β , transforming growth factor- β ; T β R, TGF- β receptor; Trb3, Tribbles-like protein 3

Bone morphogenetic proteins (BMPs) were originally identified as factor(s) that induce the formation of bone and cartilage when implanted at ectopic sites in rats. However, BMP-like molecules have been found in vertebrates as well as in invertebrates, and BMPs are now well known to exhibit a wide range of biological effects on various cell types (1). In addition to induction of bone and cartilage tissues, BMPs regulate tooth, kidney, skin, hair, muscle, haematopoietic and neuronal development, and maintain the iron metabolism and vascular homeostasis *in vivo*.

BMPs belong to the transforming growth factor- β (TGF- β) family, which includes 33 members in mammals; these include TGF- β s, activins and inhibins, nodal, myostatin and Müllerian-inhibiting substance (MIS, also known as anti-Müllerian hormone) (2). Among the members of the TGF- β family, more than a dozen molecules have been classified into the BMP subfamily. Although BMPs typically activate BMP type I receptors and Smad1, 5 and 8, they can be further classified into several subgroups, including BMP-2/4 group, BMP-5/6/7/8 group (OP-1 [osteogenic protein-1] group), growth and differentiation factor (GDF)-5/6/7 group and BMP-9/10 group (Fig. 1A) (2, 3). Most of the proteins of the BMP-2/4, OP-1 and BMP-9/10 groups induce formation of bone and cartilage tissues *in vivo*, while GDF-5/6/7 induce cartilage and tendon-like, but not bone-like, tissues. Certain BMPs or GDFs were misnamed and they neither activate BMP type I receptors nor Smad1/5/8. BMP-3 and myostatin (also known as GDF-8) do not activate Smad1/5/8 and fail to induce bone and cartilage tissues *in vivo*. Among 14 different BMPs and GDFs, BMP-2, BMP-6, and BMP-9 were shown to be most potent in the induction of alkaline phosphatase activity and osteocalcin expression in C3H10T1/2 cells, and to induce differentiation of mesenchymal progenitor cells into osteoblasts (4).

Mechanisms for the signal transduction by BMPs have been reviewed in other review articles (3, 5). Signalling mechanisms of serine-threonine kinase receptors and Smads in *Drosophila* and *Caenorhabditis elegans* have also been reviewed by others (6, 7). Activities of BMPs are regulated by various extracellular agonists and antagonists, which have recently been reviewed (8). In this review, we therefore discuss recent advances in the findings on BMP receptors and signalling pathways in mammals.

BMP receptors

Members of the TGF- β family bind to two types of serine-threonine kinase receptors, i.e. type I and type II

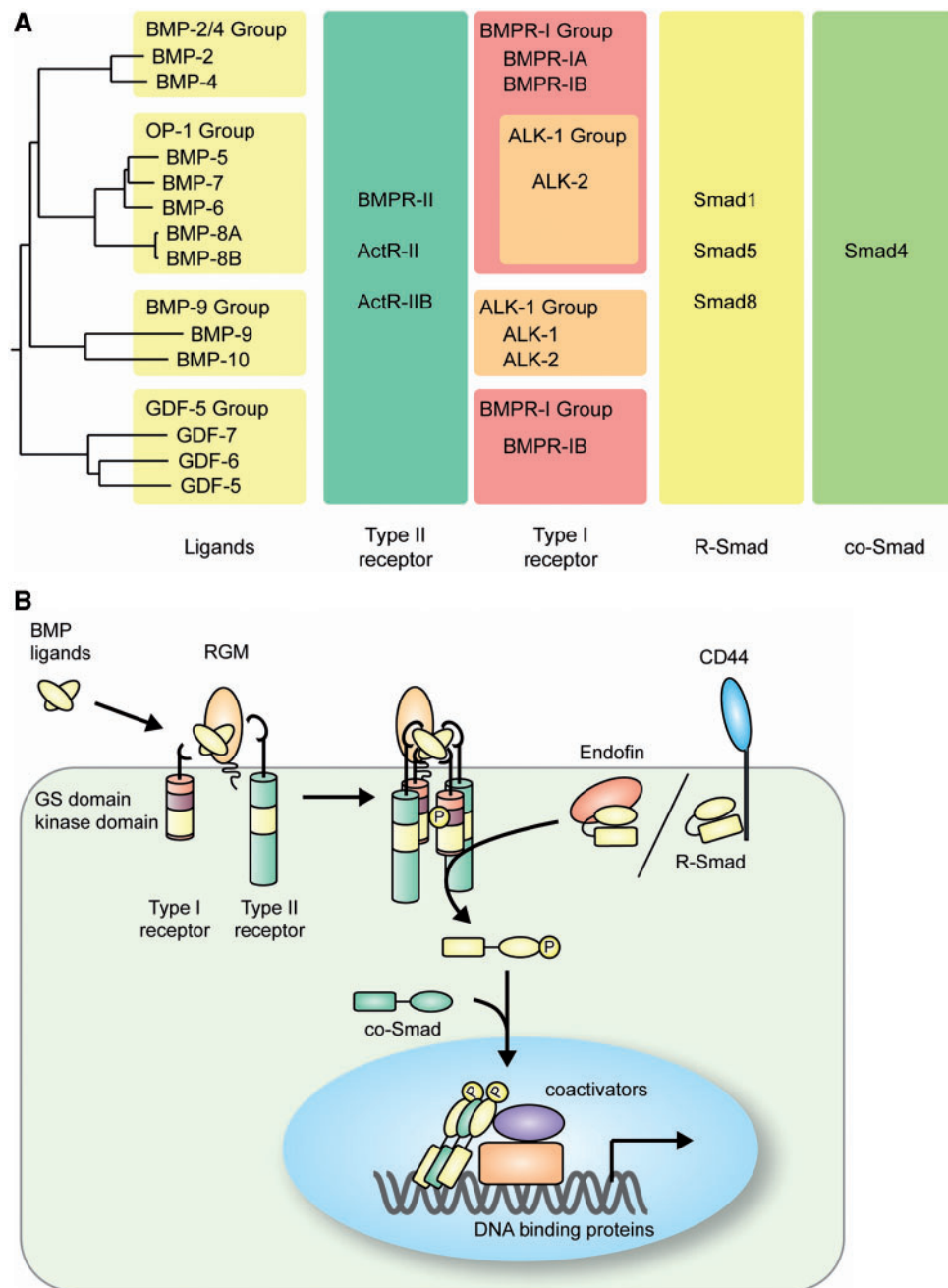


Fig. 1 Signal transduction by BMP receptors and Smads. (A) Relationships between BMP ligands, type II and type I receptors, and Smad proteins in signal transduction. Concerning the binding of BMPs to type I receptors, BMP-2/4 bind to BMPR-IA and BMPR-IB, whereas BMP-6/7 bind strongly to ALK-2, and weakly to BMPR-IB. BMP-9/10 bind to ALK-1 and ALK-2, and GDF-5 preferentially binds to BMPR-IB. Phylogenetic tree of the different BMP subgroups is adapted from Ref. 2. (B) Signalling from BMP receptors at the plasma membrane to the nucleus by Smads. BMP ligands bind to heterotetrameric complex of type II and type I receptors, and RGM proteins serve as coreceptors for BMPs. R-Smads exist in the cytoplasm through interaction with membrane anchoring proteins, e.g. CD44 and endofin. Upon phosphorylation by type I receptors, R-Smads form complexes with co-Smad (Smad4), translocate into the nucleus and regulate transcription of target genes through interaction with transcription factors (DNA-binding proteins) and transcriptional coactivators.

receptors (Fig. 1B) (9, 10). Both type I and type II receptors are required for signal transduction. BMPs bind to type I receptors in the absence of type II receptors. When both types of receptors are present, their binding affinity increases dramatically (11). The type II receptor kinase transphosphorylates the type I receptor, which transmits specific intracellular signals. Type I and type II receptors share similar structural properties, comprised of a relatively short extracellular

domain, a single membrane-spanning domain and an intracellular domain containing a serine-threonine kinase domain.

BMP type I receptors

Seven receptors (activin receptor-like kinases 1 through 7; ALK-1-7), have been identified as type I receptors for the TGF- β family in mammals. ALKs are classified into three groups based on the similarities

in their structures and functions, i.e. the BMPR-I group (BMPR-IA and BMPR-IB; also denoted ALK-3 and ALK-6, respectively), the ALK-1 group (ALK-1 and ALK-2) and the T β R-I group (ALK-4/ActR-IB, ALK-5/T β R-I, and ALK-7) (3). The receptors of the ALK-1 group and those of the BMPR-I group activate Smad1/5/8 and transduce similar intracellular signals, while those of the T β R-I group activate Smad2/3. BMPR-IA and ALK-2 are widely expressed in various types of cells. In contrast, expression of BMPR-IB shows a more restricted expression profile, and that of ALK-1 is limited to endothelial cells and certain other cells.

Specificities of the binding of BMPs to type I receptors are affected by type II receptors (12). Most typically, BMP-2 and BMP-4 bind to BMPR-IA and BMPR-IB (13), whereas BMP-6 and BMP-7 bind strongly to ALK-2 and weakly to BMPR-IB. GDF-5 preferentially binds to BMPR-IB, but not to other type I receptors (14). BMP-9 and BMP-10 bind to ALK-1 and ALK-2 (15–17). BMP type I receptors are shared by certain other members of the TGF- β family. TGF- β binds to T β R-I as well as ALK-1 in endothelial cells (18, 19). MIS binds to ALK-2, BMPR-IA and BMPR-IB in the presence of its specific type II receptor, MISR-II.

BMP type II receptors

Three receptors, BMPR-II, ActR-II and ActR-IIB, serve as type II receptors for BMPs in mammals, and are widely expressed in various tissues. BMPR-II is specific for BMPs, whereas ActR-II and ActR-IIB are shared by activins, myostatin and BMPs. These type II receptors appear to bind most BMP ligands and affect the binding preferences of BMPs to type I receptors (12).

BMPR-II has a unique, long C-terminal tail with 530 amino acids after the kinase domain (11). The long form with the C-terminal tail is predominantly expressed in most types of cells, while the short form lacking the long C-terminal tail may be expressed only in certain types of cells.

Coreceptors for BMPs

Although type II and type I receptors are sufficient for transduction of intracellular signalling by BMPs, binding to receptors and signalling activity of certain ligands is regulated by coreceptors.

Glycosylphosphatidylinositol (GPI)-anchored proteins of the repulsive guidance molecule (RGM) family, including RGMa, b and c, are coreceptors for BMP-2 and BMP-4, and enhance BMP signalling (Fig. 1B) (20–22). RGMb and c are also known as DRAGON and hemojuvelin, respectively. They interact with BMP type I and/or type II receptors, and bind to BMP-2 and BMP-4, but not to BMP-7 or TGF- β 1. In mouse pulmonary artery smooth muscle cells, BMP-2/4 signalling requires BMPR-II, but not ActR-II or ActR-IIB. However, cells transfected with RGMa use both BMPR-II and ActR-II for BMP-2/4 signalling, suggesting that RGMa facilitates the use of ActR-II by BMP-2/4 (23).

BMP-6 plays a key role in iron metabolism in hepatocytes, in which hemojuvelin functions as a signalling component in the BMP signalling pathway. In hepatocytes, BMP binds to hemojuvelin and induces the expression of hepcidin, which in turn decreases iron absorption by the intestine and iron release from macrophages (22). Similar to mutations in the *Hepcidin* gene, those in the human *Hemojuvelin* gene were identified in individuals with juvenile hemochromatosis (type 2A), which is characterized by accumulation of iron in various organs (22).

Endoglin is a transmembrane protein which is expressed in proliferating endothelial cells and other cell types, and binds to various ligands including TGF- β 1/3, activin-A and BMP-2/7 (24). Although its function in TGF- β family signalling has not been fully determined, ectopic expression of endoglin results in inhibition of TGF- β -induced responses, whereas it enhances BMP-7-induced responses (25). Mutations in the human *ENG* gene (encoding endoglin) result in hereditary haemorrhagic telangiectasia (HHT1, also known as Osler–Weber–Rendu disease) similar to those in *ALK1* (which induce HHT2), suggesting that they act in a common signalling pathway (26, 27).

Ligand binding and activation of BMP receptors

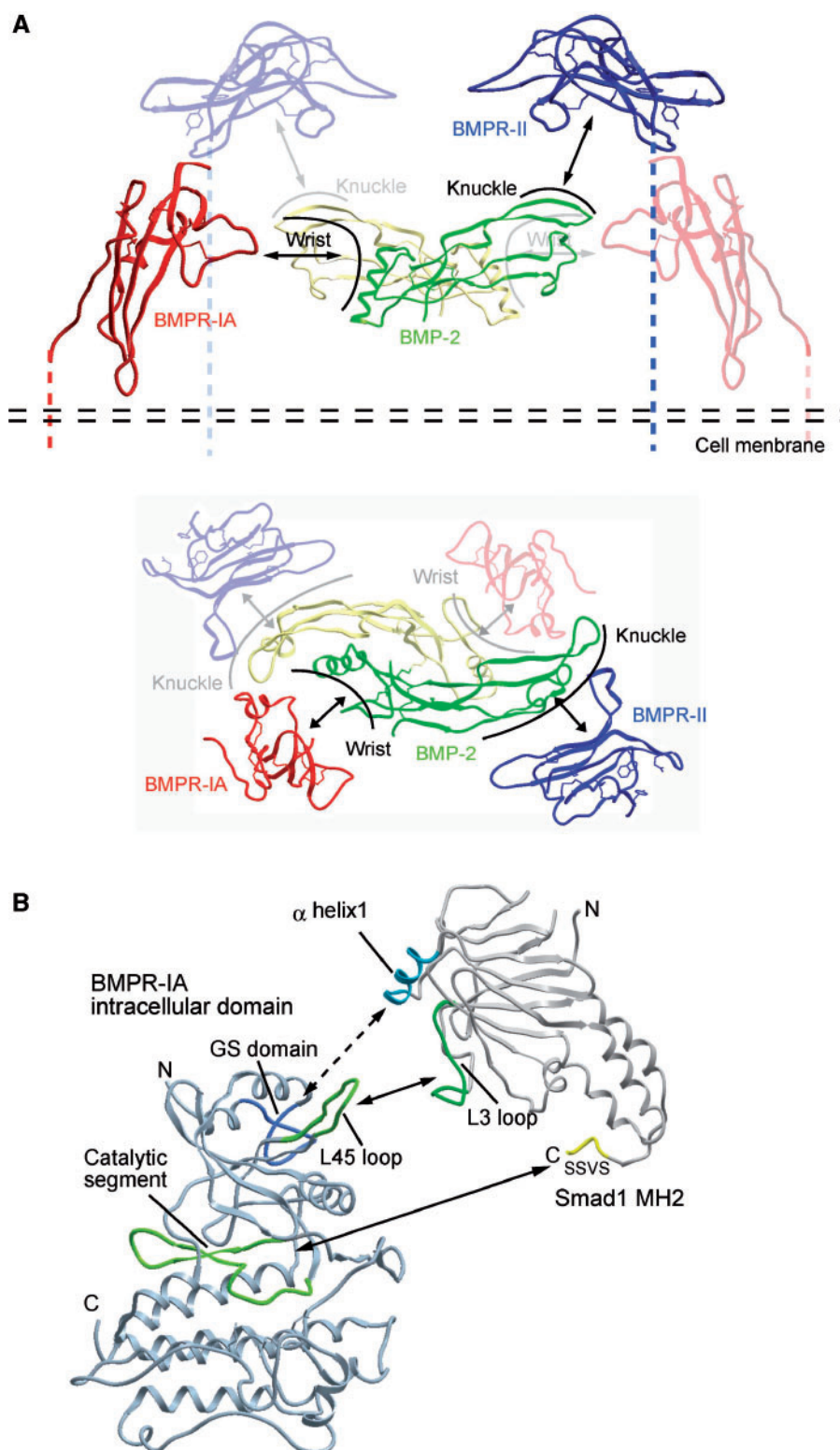
Binding of BMPs to receptor extracellular domains

Several studies showed the structures of binary or ternary complexes containing ligands and receptor extracellular domains (28–30). BMP-2 is a dimeric protein, and two receptor-binding motifs have been identified in the BMP-2 ligand (31). A large epitope 1 (wrist epitope) in BMP-2 is a high-affinity binding site for BMPR-IA, and a smaller epitope 2 (knuckle epitope) is a low-affinity binding site for BMPR-II (Fig. 2A). The wrist epitope, comprising residues from both BMP-2 subunits, contributes to binding to one BMPR-IA molecule, while the knuckle epitope, constituted by residues of one subunit, binds to BMPR-II. The wrist and knuckle epitopes are closely located to each other.

The extracellular domains of type II and type I receptors have several conserved cysteines, which are important for the formation of characteristic three-dimensional structures. The extracellular domain of ActR-II has a structure denoted three-finger toxin fold, composed of three pairs of β -strands projecting from the palm domain with a conserved scaffold of disulfide bridges (32). The extracellular domains of ActR-II and BMPR-IA have similar three-dimensional structures with some differences (28). The BMPR-IA extracellular domain consists of two β -sheets and one α -helix (helix α 1); the latter is missing in ActR-II, and may act as a key element in type I receptors for specific ligand-binding.

Activation of serine-threonine kinases

In the absence of ligand stimulation, small fractions of type II and type I receptors are present as preexisting homodimers and heterodimers on the cell surface. Ligand-binding increases oligomerization of the



receptors, which may induce conformational changes of the receptor molecules.

The intracellular domains of type I receptors have a characteristic GS domain (glycine and serine-rich domain) located N-terminal to the serine-threonine kinase domains. The type II receptor kinase is constitutively active in the absence of ligand. Upon ligand binding, the type II receptor kinase phosphorylates the GS domain of type I receptor (Fig. 1B), which is a critical event in signal transduction by the serine-threonine kinase receptors.

Three-dimensional analysis of the intracellular domain has been reported for T β R-I. The inactive conformation of the T β R-I kinase is maintained by interaction between the GS domain, the N-terminal lobe and the activation loop of the kinase (33). Upon phosphorylation of the GS domain by the type II receptor kinase, the T β R-I kinase is converted to an active conformation. Mutations of Thr-204 in T β R-I and the corresponding Gln in BMP type I receptors to acidic amino acid residues (Asp or Glu) lead to constitutive activation of the type I receptors. The constitutively active type I receptors transduce intracellular signals in the absence of ligands or type II receptors. Type I receptors thus act as a downstream component of type II receptors, and determine the specificity of the intracellular signals.

In the kinase domain, type I receptors have the L45 loop between kinase subdomains IV and V, which protrudes from the kinase domain (33) and specifically interacts with receptor-regulated Smads (R-Smads) (Fig. 2B). Amino acid sequences of the L45 loop are conserved in each type I receptor subgroup, but diverge between different subgroups. The L45 loop of the BMPR-I group is more similar to that of the T β R-I group than that of the ALK-1 group. Interestingly however, receptors of the BMPR-I and ALK-1 groups activate a similar set of Smads, Smad1/5/8, while those of the T β R-I group activate a distinct set of Smads, Smad2/3 (see below).

Regulation of BMP receptor signalling

Functions of the type I and type II receptors of the TGF- β family are regulated by protein modifications, including dephosphorylation, ubiquitination, sumoylation and ectodomain shedding (34). Most of these studies have been done using TGF- β receptors, while not much have been elucidated for modification of the BMP receptor proteins.

BAMBI is a pseudoreceptor for serine-threonine kinase receptors (35). BAMBI is structurally related to type I receptors, but does not have the characteristic intracellular domain. BAMBI stably interacts with various type I and type II receptors and inhibits signalling by these receptors. Expression of BAMBI is induced by BMP and TGF- β (35, 36), and BAMBI is tightly coexpressed with BMP-4 during embryonic development (37).

Some transmembrane tyrosine kinases and cytoplasmic serine-threonine kinases have been reported to interact with BMP receptors and regulate their functions. TrkC, a neuronal tyrosine kinase receptor, directly binds to BMPR-II and interferes with the

interaction with BMPR-I, resulting in the inhibition of downstream signalling (38). A tyrosine kinase receptor Ror2 and BMPR-IB form a heteromeric complex in a ligand independent fashion (39). Ror2 modulates the GDF-5-BMPR-IB signalling pathway by inhibition of Smad1/5 signalling. cGMP-dependent kinase I (cGKI) interacts with and phosphorylates BMPR-II, leading to enhancement of BMP receptor signalling (see below) (40).

Mutations of BMP receptors *in vivo*

Since BMPs, e.g. BMP-2 and BMP-4, play essential roles in mesoderm formation during early embryogenesis, mice lacking *Bmpr1a* or *Bmpr2* gene die by E9.5 due to defects in mesoderm formation during gastrulation (41, 42). Disruption of *Alk2* gene causes embryonic lethality in mouse, although the phenotype of *Alk2*-null embryos is less severe than that of *Bmpr1a*-null embryos (43). Mice lacking *Acvr2* or *Acvr2b* gene (encoding ActR-II or ActR-IIB, respectively) show multiple defects. Some, but not all, *Acvr2*-null mice exhibit hypoplasia of the mandible and other skeletal abnormalities (44). *Acvr2b*-deficient mice exhibit complicated cardiac defects, and abnormalities in lateral asymmetry and vertebral patterning (45).

Since BMPs play important roles in numerous tissues and organs, abnormalities in the BMP receptor genes result in a broad spectrum of phenotypes. Here, we focus on abnormalities in vascular and skeletal tissues, and development of cancer induced by mutations in BMP receptors in mouse and human.

Vascular abnormalities

Primary pulmonary arterial hypertension (PAH or PPH1) is characterized by stenosis of precapillary pulmonary arteries. Proliferation of endothelial cells and smooth muscle cells is observed in the pulmonary arteries of PAH, leading to an increase in pulmonary artery pressure and right ventricular systolic pressure. Familial PAH occurs as an autosomal dominant disorder with reduced penetrance. Mutations in the human *BMPR2* gene are found in ~70% of patients with familial PAH and 10–40% of patients with idiopathic PAH (46, 47). Mutations of the *BMPR2* gene alone are insufficient to initiate the disease process, and additional events are required for the pathogenesis of PAH. Mutations can be observed in various regions of BMPR-II. Mutations of *ALK1* gene have also been reported in some patients with PAH (48).

Conditional heterozygous and homozygous *Bmpr2* deletion in pulmonary endothelium in mice resulted in predisposition to PAH. Wide ranges of right ventricular systolic pressure were observed in *Bmpr2*^{+/-} and *Bmpr2*^{-/-} mice compared with control mice, and a subset of mice with high right ventricular systolic pressure showed right ventricular hypertrophy and an increase in the wall thickness of distal pulmonary arteries (49). Mice expressing a *Bmpr2* mutant lacking C-terminal tail in the pulmonary smooth muscle cells also developed pulmonary vascular lesions relevant to PAH (50). BMP signalling in both endothelial cells and smooth muscle cells may thus play important roles in

the maintenance of vascular integrity in the pulmonary arteries. In another study, Song *et al.* reported that under inflammatory stressed conditions, *Bmpr2*^{+/-} mice showed increase in right ventricular systolic pressure and vascular remodelling. Endothelial injury and enhanced inflammatory response may act in concert with *Bmpr2* heterozygosity to accelerate the development of PAH (51).

Mutations in human *ALK1* gene as well as those in *ENG* gene cause HHT, an autosomal dominant vascular disorder characterized by skin and mucosal telangiectasia, pulmonary, cerebral, and hepatic arteriovenous malformations, and bleeding due to these vascular abnormalities (26, 27). Targeted deletion of *Alk1* or *Eng* gene in mice results in abnormal angiogenesis, showing dilation of major vessels, lack of capillary network and defects in differentiation and recruitment of smooth muscle cells (18, 52–54).

Skeletal abnormalities

Brachydactyly type A2 is an autosomal dominant malformation characterized by shortening and deviation of the index fingers and the first and second toes. Mutations in the GS domain or kinase domain of the *BMPR1B* gene, which act in dominant-negative fashion, are responsible for the malformation (55). Several types of acromesomelic chondrodysplasias are caused by mutations in the *GDF5* gene. Homozygous *BMPR1B* mutation was identified in a patient with a new subtype of acromesomelic chondrodysplasia with genital anomalies (56). *Bmpr1b*-deficient mice are viable, but exhibit short limbs and abnormal digit cartilage, similar to *Gdf5*-null mice (57).

GDF-5 preferentially binds to BMPR-IB (14). A mutation of Arg-438 to Leu in GDF-5 (R438L mutant) is responsible for proximal symphalangism, showing fusion of carpal and tarsal bones and ankylosis of the proximal interphalangeal joints. The R438L mutant of GDF-5 is able to bind to BMPR-IA with a high affinity, indicating that the GDF-5 mutant acquires biological activity similar to BMP-2 (58).

A mutation in *ALK2* (also known as *ACVRI*) causes fibrodysplasia ossificans progressiva (FOP) (59), an autosomal dominant disorder of skeletal malformations and progressive ossification in muscular tissues. A heterozygous mutation in *ALK2* leading to Arg-206 to His is frequently found in individuals with this disorder. Since Arg-206 is located in the GS domain of ALK-2, the mutation results in destabilization of the GS domain and hyperactivation of the ALK-2 kinase. Recently, a Gly-356 to Asp mutation of ALK-2 was identified as a novel mutation in a Japanese FOP patient who had unique clinical features (60). A transgenic mouse model expressing constitutively active (ca) ALK-2 in muscle, which phenocopies key aspects of human FOP, has recently been reported (61). Intramuscular expression of caALK-2 leads to ectopic bone formation, joint fusion and functional impairment, when inflammatory stimuli are induced.

Conditional disruption of *Bmpr1a* gene in osteoblasts during embryonic bone development in mice provided surprising results. In these mice, bone mass was increased with upregulation of canonical Wnt

signalling (62). Osteoclastogenesis was severely reduced at least through activation of the RANK ligand–osteoprotegerin pathway, whereas bone formation was only modestly reduced, resulting in a net increase in bone mass. Expression of sclerostin, an extracellular inhibitor of Wnt signalling, was down-regulated, which may lead to upregulation of Wnt signalling and osteoclastogenesis.

Cancer

Although perturbations of TGF- β signalling are well known to be involved in development of gastrointestinal cancers, BMP signalling also plays a key role in the growth of gastrointestinal epithelium and development of cancers (63). Mutations in human *BMPRIA* as well as those in *SMAD4/MADH4* have been found in some patients with an autosomal dominant syndrome juvenile polyposis (64). Conditional inactivation of the *Bmpr2* gene in the mouse stroma resulted in epithelial hyperplasia in the colon, and they developed hamartomatous polyps in the colorectum and rectal bleeding (65). Knockdown of the expression of *Bmpr2* by RNA interference in mice also resulted in gastrointestinal hyperplasia, as well as vascular dysmorphogenesis and predisposition to angioproliferative diseases (66).

Small-molecule inhibitors for BMP receptors

Small-molecule inhibitors for the receptors of the T β R-I group (ALK-4/5/7) have been developed and some of them are under pre-clinical or clinical studies for treatment of cancer and fibrotic diseases. These ALK-4/5/7 inhibitors do not affect the signalling activity of BMP type I receptors. Yu *et al.* screened for chemical compounds that perturb dorsoventral axis formation in zebrafish, and found that dorsomorphin, originally isolated as a small-molecule inhibitor for AMP-activated protein kinase, selectively inhibits signalling from ALK-2, BMPR-IA and BMPR-IB, and blocks BMP-induced Smad1/5/8 phosphorylation (67). Dorsomorphin also inhibits ALK-1 signalling (68). Through a structure-activity relationship study of dorsomorphin derivatives, an optimized compound (LDN-193189 or DM-3189) with higher activity and specificity on BMP type I receptors has been developed (69).

These small-molecule BMP receptor inhibitors are potentially effective for some clinical disorders. Dorsomorphin inhibited BMP-induced expression of hepcidin *in vitro*, and maintained iron-hepcidin homeostasis *in vivo* (67). Using the mouse model of FOP expressing the caALK-2, LDN-193189 inhibited activation of Smad1/5/8 induced by the caALK-2, leading to a reduction in ectopic ossification and functional impairment in mice (61). Small-molecule BMP receptor inhibitors may also be useful for regenerative medicine. Hao *et al.* (70) reported that dorsomorphin induces myocardial differentiation in mouse embryonic stem cells, when treatment is limited to the initial stages of embryonic stem cell differentiation.

Intracellular signalling from BMP receptors through SMAD signalling pathway

Smads are the major signal transducers for the TGF- β family receptors (9, 10). Type I receptor kinases activated by the type II receptor kinases, phosphorylate R-Smads. R-Smads then form a complex with common-partner Smad (co-Smad) and translocate to the nucleus (Fig. 1B). The oligomeric Smad complexes regulate the transcription of target genes through interaction with various transcription factors and transcriptional coactivators or corepressors. Inhibitory Smads (I-Smads) negatively regulate the action of R-Smads and/or co-Smads.

Eight different Smads have been identified in mammals (Fig. 3A). Smad1, Smad5 and Smad8 are R-Smads in BMP signalling pathways (BMP-specific R-Smads) and Smad2 and Smad3 are those in TGF- β /activin signalling pathways (TGF- β /activin-specific R-Smads). Smad1, Smad5 and Smad8 are structurally

highly similar to each other, and the functional differences between them are largely unknown. Smad4 is the only co-Smad in mammals, shared by both BMP and TGF- β /activin signalling pathways. Smad6 and Smad7 are I-Smads.

Specificity of activation of R-Smads by TGF- β family ligands are not strict as previously thought; Smad1 and Smad5 are activated by TGF- β in endothelial cells and some other cells through activation of ALK-1 and ALK-2 (19, 71). Recent data have revealed that T β R-I can directly induce phosphorylation of Smad1/5 in certain cells (68, 72). However, activation of Smad1/5 by TGF- β through T β R-I or ALK-1/2 occurs only transiently. Similarly, BMP receptors have been shown to phosphorylate Smad2 in certain types of cells (73).

Structures of Smads

Smads have highly conserved N- and C-terminal regions known as Mad homology (MH) 1 and MH2

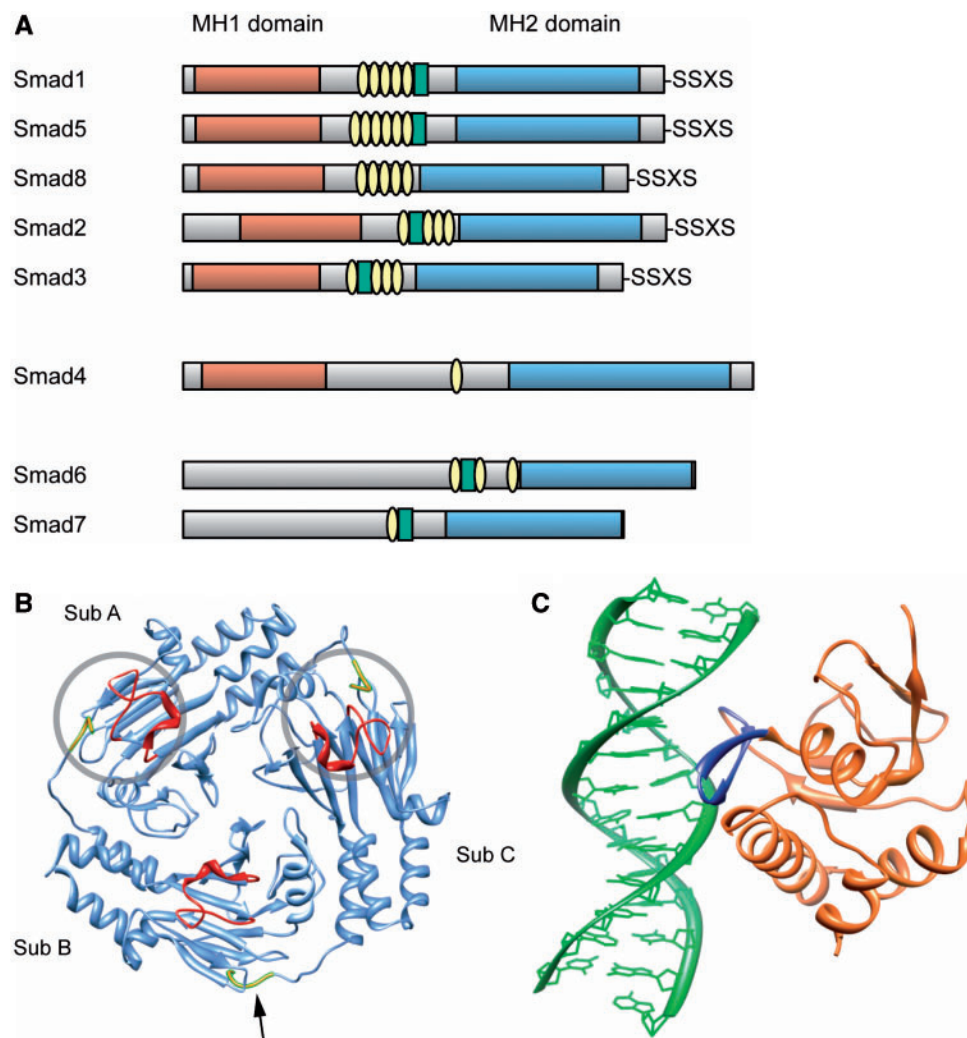


Fig. 3 Structure of Smads. (A) Comparison of the structures of R-Smad, co-Smad and I-Smad. Circles in the linker region indicate the PXS/TP (or S/TP) motif potentially phosphorylated by MAP kinases, and the square indicates the PY motif. (B) Homo-trimer of the MH2 domain of Smad1 (Protein Data Bank entry 1KHU). Each subunit (Sub A–C) represents one MH2 domain. The regions where the C-terminal SXS motifs (yellow) interact with the L3 loop regions of the neighbouring molecules (red) are circled. The C-terminal tail of subunit C (arrow) is not able to interact with L3 loop of subunit B. (C) The MH1 domain of Smad3 and DNA complex (Protein Data Bank entry 1MHD). The MH1 domain is shown by the ribbon representation (orange). Highly conserved 11-residue β -hairpin loop (blue) recognizes major groove of DNA (green) in a sequence-specific manner.

domains, respectively, which are linked by a linker region with a highly variable structure (Fig. 3A). MH2 domains are present in all three types of Smads, while MH1 domains are conserved only in R-Smads and co-Smads. The N-terminal regions of I-Smads are highly divergent from those of the other Smads. R-Smads have a characteristic Ser-Ser-X-Ser sequence (SSXS motif) at the C-terminal ends, which is phosphorylated by the type I receptors.

The MH2 domain is responsible for interaction with receptors, oligomer formation with other Smads, interaction with various DNA-binding proteins, and transcriptional activation. In the absence of receptor activation, the MH2 and MH1 domains physically bind to each other, and suppress the function of each other. Upon phosphorylation of the SSXS motif by type I receptors, this interaction is abolished, and R-Smads become activated, and form an oligomer complex (Fig. 3B) (74). The L3 loop, a region composed of 17 amino acids, protrudes from the surface of the molecule and interacts with the L45 loop of type I receptors (Fig. 2B) (75). The amino acid sequences of the L3 loop are conserved in BMP-specific R-Smads and in TGF- β /activin-specific R-Smads, but diverge between these two groups.

The MH1 domains are responsible for binding to DNA, interaction with certain DNA-binding proteins, nuclear translocation and repression of the function of MH2 domains. Direct-DNA binding of Smads occurs through the β -hairpin loop, a region composed of 11 amino acids, which protrudes from the surface of the molecule (Fig. 3C) (76). The structure of the β -hairpin loop is conserved in R-Smads and co-Smad in mammals, and Smad4 and Smad3 bind to the characteristic Smad-binding elements (SBEs; AGAC or GTCT sequence) through this domain.

The linker regions of BMP-specific R-Smads contain motifs phosphorylated by mitogen-activated protein (MAP) kinases (Fig. 3A) and those by glycogen synthase kinase (GSK) 3, and phosphorylation of these motifs accelerates the degradation of R-Smads through the ubiquitin-proteasome pathway (see below). A PY motif, containing the PPXY sequence, is found in all Smads except Smad4 and Smad8, and responsible for the interaction with proteins containing WW domains, including HECT-type ubiquitin ligases.

Smad signalling from the cytoplasm to the nucleus

R-Smads are anchored at the cell membrane by interacting with various cytoplasmic proteins (Fig. 1B). SARA presents Smad2/3 to type I receptors, and facilitates their activation. SARA does not bind to BMP-specific R-Smads, but endofin interacts with Smad1, and enhances BMP signalling (77). In addition, CD44, a receptor for hyaluronan, was shown to interact with Smad1 in chondrocytes, and present Smad1 to the BMP receptors for activation (78).

Type I receptors phosphorylate R-Smads through physical interaction. The interaction is determined by the L45 loop of type I receptors and L3 loop of the MH2 domains of R-Smads (Fig. 2B) (75). In addition to the L3 loop, the α -helix H1 in the MH2 domain is also required for the interaction of BMP-specific

R-Smads with type I receptors of the ALK-1 group (79). R-Smads then form complexes with co-Smads through their MH2 domains, presumably composed of two molecules of R-Smads and one molecule of co-Smad (Fig. 3B) (80, 81).

Function of Smads in the nucleus

In the nucleus, Smads regulate transcription of target genes through direct binding to DNA, interaction with other DNA-binding proteins and recruitment of transcriptional coactivators and/or corepressors (Fig. 1B). In addition to the characteristic GTCT/AGAC sequence specific for Smad3 and Smad4, BMP-specific R-Smads bind to GC-rich sequences (GCCGnCGC motif) found in the promoter region of Smad6 (82). BMP-specific R-Smads also bind to other GC-rich sequences, e.g. (T)GGCGCC sequence in *Bambi*, *Vent2*, *Smad7* and *hepcidin* (83, 84). In the promoter of *Id1* gene, both SBEs and GC-rich boxes are important for efficient transcriptional activation (85).

Smads interact with various DNA-binding proteins, which is critical for exhibition of specific effects of TGF- β family proteins in different types of cells (86). Many DNA-binding proteins, including Runx, Schnurri, Menin, OAZ, MAN-1, MyoD, Vent2, Hoxc-8 and Msx1, interact with BMP-specific R-Smads (87).

Runx is a family of transcription factors that regulate various biological events, including haematopoiesis and osteogenesis (88). Three mammalian isoforms of Runx, Runx1 through 3, interact with R-Smads. Mice lacking the *Runx2* gene completely lack bone. Haploinsufficiency of the *RUNX2* gene in human is responsible for the development of the autosomal dominant bone disease cleidocranial dysplasia. BMP-specific R-Smads physically interact with Runx2 upon activation by BMP receptors (89), and they cooperatively activate the transcription of target genes, leading to induction of the osteoblast differentiation.

Drosophila Schnurri was originally identified as a zinc finger-type transcription factor that interacts with *Drosophila* R-Smad/co-Smad complex (7). In mammals, three Schnurri homologues, Schnurri-1 to -3, have been identified and are shown to regulate various biological processes, some of which are dependent on Smads. Schnurri-2 translocates into the nucleus upon BMP-2 stimulation and, through interaction with Smad1/4 and C/EBP α , induces the expression of PPAR γ 2, a key transcription factor for adipocyte differentiation (90). In the TGF- β signalling pathway, Schnurri-2 induces nuclear translocation of CLIC4 (chloride intracellular channel 4), leading to protection of phospho-Smad2/3 from dephosphorylation by nuclear phosphatases (91). Schnurri-3 was reported to suppress adult bone mass levels by degradation of Runx2 through E3 ubiquitin ligase WWP1 (92).

p300 and CBP (CREB-binding protein) have histone acetyl transferase (HAT) domains, which upregulate gene transcription by loosening nucleosomal structure and by increasing the accessibility to the general transcription machinery. p300 and CBP interact with various transcription factors; they interact with R-Smads upon ligand stimulation and enhance Smad-dependent

transcription of target genes (93). GCN5 and P/CAF are transcriptional coactivators of the GNAT (GCN5-related *N*-acetyl transferase) superfamily. Similar to p300 and CBP, GCN5 interacts with BMP-specific R-Smads and enhances BMP-induced transcriptional activity.

Transcriptional corepressors, including c-Ski, SnoN and Evi-1, recruit histone deacetylases (HDACs) to Smad complexes (93). They induce nucleosomal condensation and repress the transcription of target genes. c-Ski and SnoN are structurally related to each other and interact with Smads as well as with N-CoR and mSin3A, resulting in recruitment of HDACs to the Smad complexes. c-Ski and SnoN repress BMP signalling through interaction with Smad4 (94, 95). Evi-1 is a sequence-specific zinc-finger transcriptional repressor. Evi-1 binds to Smad2, Smad3 and Smad1, and represses TGF- β as well as BMP signalling (96).

Function of Smads independent of transcriptional control

R-Smads have recently been reported to regulate transcription of certain target genes without forming complex with co-Smad (97). Moreover, R-Smads play a unique role independently of transcription of genes. MicroRNAs (miRNAs) are small non-coding RNAs that regulate synthesis of mRNA and protein, and aberrant miRNA expression leads to progression of some developmental abnormalities and diseases. Davis *et al.* (98) showed that induction of a contractile phenotype in vascular smooth muscle cells by TGF- β and BMPs is mediated by miR-21. miR-21 downregulates PDCD4 (programmed cell death 4), a negative regulator of smooth muscle contractile genes. Both BMP and TGF- β increased the expression of mature miR-21 through a post-transcriptional maturation step by promoting the processing of primary transcripts of miR-21 (pri-miR-21) into precursor miR-21 (pre-miR-21) by the Drosha complex. R-Smads, but not Smad4, were shown to be recruited to pri-miR-21 through the DEAD-box RNA helicase p68, a component of the Drosha complex. Thus, R-Smads are capable of regulating biogenesis of miRNA in transcription-independent fashion.

Target genes for BMPs

By oligonucleotide microarray analyses, numerous BMP target genes have been identified in various cells. During osteoblastic differentiation of early mesenchymal cells, e.g. C2C12 cells, BMP-2 induced various genes, which can be classified into immediate early response genes (2 h after BMP stimulation) and intermediate and late early response genes (6 and 24 h, respectively, after BMP-2 stimulation) (99). Genes involved in signal transduction, including Id1-3 (inhibitor of differentiation or inhibitor of DNA binding 1–3), OASIS, Prx2, TIEG and Snail, have been identified as the immediate early response genes, while those involved in osteoblast differentiation processes, and for transcription factors involved in Notch and Wnt signalling, e.g. Hey1 and Tcf7, respectively, have been identified as the intermediate and late early

response genes. Some negative regulators of BMP signalling, including Smad6 and Smad7, are also induced by BMPs in most cell types.

BMPs induce various genes including Runx2 and Osterix in mesenchymal progenitor cells, which are involved in differentiation into osteoblasts (100). On the other hand, the paired-like homeodomain transcription factor Pitx2 (also termed Rieg), is induced 2 days after BMP stimulation in C2C12 cells, and represses Osterix expression and subsequent osteoblastic differentiation (100).

BMPs stimulate proliferation of certain types of endothelial cells, presumably through expression of vascular endothelial growth factor receptor-2 and Tie2 (101). Oligonucleotide microarray analysis revealed that in addition to Id1, Id2 and Smad6 and Smad7, caALK-1 induced the expression of endoglin, STAT1 and IL1RL1 (interleukin-1 receptor-like 1) in human umbilical vein endothelial cells (102).

Among the various BMP target genes, Id genes are induced by BMP in various types of cells, and are one of the most important targets of BMPs (103). The four isoforms of Id proteins (Id1 through 4) exhibit similar, but not identical biological activities. The ubiquitously expressed basic helix-loop-helix (bHLH) transcription factors, including E2A gene products, associate with tissue-specific bHLH transcription factors, including MyoD and myogenin in muscle, and activate transcription of genes containing E-box sequences in their promoters. Id proteins have a helix-loop-helix dimerization domain, but lack the basic region responsible for DNA binding. By sequestering ubiquitously expressed bHLH transcription factors, they inhibit the transcription induced by bHLH transcription factors. BMPs induce expression of Id proteins, which in turn repress transcription induced by bHLH heterodimers containing MyoD/myogenin, resulting in the inhibition of myogenesis.

Regulation of Smad signalling

Function of I-Smads

I-Smads, i.e. Smad6 and Smad7, function as antagonists of R-Smad/co-Smad signalling (Fig. 4). I-Smads interact with type I receptors activated by type II receptors through their MH2 domains. Unlike R-Smads, however, they are not released from type I receptors, and thus prevent the activation of R-Smads (104). Smad7 inhibits both TGF- β and BMP signalling, whereas Smad6 preferentially represses BMP, but not TGF- β /activin, signalling. Smad7 interacts with type I receptors of the T β R-I, BMPR-I and ALK-1 groups, while Smad6 inhibits signals from the BMPR-I group, but only weakly those from the ALK-1 group (105). Smad6 has also been reported to form a complex with Smad1 and compete with Smad4 for oligomer formation. As a third mechanism, I-Smads interfere with TGF- β family signalling in the nucleus. Smad7 binds to the Smad-responsive DNA element via its MH2 domain, and disrupts the formation of the functional Smad–DNA complex induced by ligand stimulation (106).

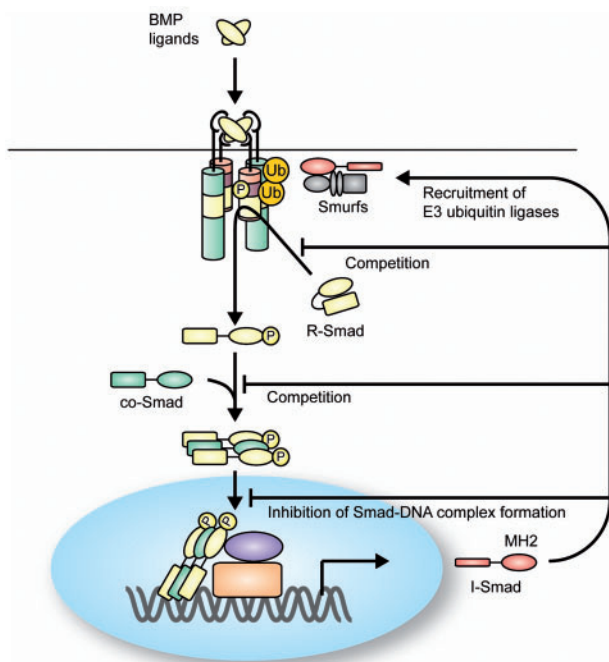


Fig. 4 Modes of action of I-Smads in the membrane, cytoplasm and nucleus. Ligand stimulation activates the Smad signalling pathway and induces expression of I-Smads. I-Smads interact with type I receptors and compete with R-Smads for receptor binding. I-Smads also recruit E3 ubiquitin ligases, e.g. Smurf1 and 2, leading to degradation of receptor proteins by the ubiquitin–proteasome pathway. I-Smads interact with R-Smads and compete with co-Smad for complex formation. I-Smads also act in the nucleus, and inhibit Smad signalling, e.g. by inhibition of the Smad–DNA complex formation.

Expression of I-Smads is strongly induced by TGF- β /activins and BMPs. Transcription of Smad7 and Smad6 is induced by direct effects of TGF- β -specific R-Smads and BMP-specific R-Smads (82). R-Smad/co-Smad and I-Smad thus form a negative feedback loop for regulation of TGF- β family signalling. BMPs induce expression of I-Smads during osteoblastic differentiation in a biphasic fashion. I-Smads are rapidly and transiently induced by BMP within 2 h, followed by sustained induction after 2 days during the osteoblastic maturation phase (107). Endogenous TGF- β signalling is activated during this period, and induces the expression of I-Smads. Osteoblastic differentiation of mesenchymal progenitor cells at the maturation phase can thus be enhanced by a small-molecule T β R-I inhibitor SB431542 (107).

AMSH, originally found as a molecule which associates with the SH3 domain of STAM, interacts with Smad6 upon BMP stimulation, and antagonizes the function of Smad6 by interfering with the association of Smad6 with BMP type I receptors and with Smad1 (108).

Regulation by the ubiquitin–proteasome pathway

R-Smads are degraded by the ubiquitin–proteasome pathway. HECT type E3 ligases, including Smurf1, Smurf2 and NEDD4-2, physically associate with

R-Smads (87). This interaction occurs between the WW motif in Smurfs/NEDD4-2 and the PY motif in the linker region of R-Smads.

Phosphorylation of the Smad1 linker at the MAP kinase sites and the GSK3 sites represses the transcriptional activity of Smad1 by enhancing proteasomal degradation of Smad1 (109, 110). First, BMP signalling induces C-terminal phosphorylation (pSmad1^{Cter}) and nuclear translocation of Smad1. Next, the phosphorylation of Smad1 at the MAP kinase sites (pSmad1^{MAPK}) is induced by Erk, p38 and JNK MAP kinases in the nucleus. Then, GSK3 recognizes the pSmad1^{MAPK} and generates pSmad1^{GSK3}. The triply phosphorylated protein is transported to the centrosome for proteasomal degradation (110). Thus, the Ras-MAP kinase pathway activated by fibroblast growth factors and other growth factors antagonizes BMP action through phosphorylation of the MAP kinase sites, while the canonical Wnt pathway may in part transmit signals through the stabilization of BMP-Smad signals by regulating the phosphorylation of the GSK3 sites.

Smurfs also interact with I-Smads (Fig. 4) (104). Smurfs induce nuclear export of I-Smads, and facilitate the interaction of I-Smads with type I receptors. The enhanced interaction of I-Smads with type I receptors results in suppression of TGF- β family signalling. Moreover, Smurfs induce degradation of the receptors, leading to decrease in the number of cell surface receptors.

Function of Smad4 is controlled by monoubiquitination and deubiquitination. Smad4 is monoubiquitinated at Lys-519 by a RING-type ubiquitin ligase, Ectoderm/TIF1 γ , leading to prevention of its association with R-Smads. FAM/USP9x, a deubiquitinase, reverts this monoubiquitination, and restores the responsiveness of Smad4 to TGF- β family signalling (111).

Phosphatases

Several phosphatases induce dephosphorylation of the SXS motif of R-Smads. PPM1A/PP2Ca, the prototype of PPM (metal ion-dependent protein phosphatase) family phosphatases, interacts with Smad1 and Smad2/3 and dephosphorylates pSmad^{Cter} (87). Other phosphatases, including PDP (pyruvate dehydrogenase phosphatase) and SCPs (small C-terminal domain phosphatases), decrease the level of phospho-Smad1, and suppress BMP signalling. In contrast, PP2A dephosphorylates Smad1, mainly in the linker region, leading to increased nuclear translocation of Smads and amplification of BMP signalling (112).

Other mechanisms

Some other molecules, including Tob, cGKI, SANE and CIZ (Cas-interacting zinc-finger protein), interact with BMP-specific R-Smads and regulate BMP signalling through various mechanisms.

Tob (transducer of ErbB2) is a member of the anti-proliferative gene family, which inhibits growth of some cells. *Tob*-deficient mice show increased bone mass compared to wild-type mouse (113). Tob inhibits

BMP signalling in osteoblasts through interaction with BMP-specific R-Smads.

cGKI plays a unique role in BMP-Smad signalling. In addition to interacting with BMPR-II (see above), cGKI dissociates from BMP receptors upon ligand stimulation, interacts with activated R-Smads and undergoes nuclear translocation. In the nucleus, cGKI binds to Smad1 and the general transcription factor TFII-I at promoters of BMP target genes, and enhances transcriptional activation (40).

In vivo abnormalities associated with Smad genes

In vivo abnormalities induced by mutations of BMP-specific R-Smads, co-Smad and I-Smad are described here. Mutations in mouse Smad genes often show certain interesting phenotypes, whereas diseases related to human Smad gene mutations are uncommon, except for the *SMAD4/DPC4* gene.

Smad5-deficient mice exhibit abnormalities in angiogenesis with dilated vessels and decrease in the number of smooth muscle cells surrounding vessels (114, 115). The phenotype of *Smad5*-null mice is similar to those of the *Alk1*- and *ENG*-null mice. Targeted disruption of the mouse *Smad1* gene demonstrates an essential role for Smad1 in chorioallantoic fusion and primordial germ cell formation (116, 117). Combined loss of Smad1 and Smad5 in cartilage results in severe chondrodysplasia (118). *Smad8*-deficient mice do not exhibit overt abnormalities during embryonic or postnatal development (119, 120). Recently, Huang *et al.* (121) reported that deletion of *Smad8* in mice resulted in changes in distal pulmonary arteries, including medial thickening and smooth muscle hyperplasia, and a subset of *Smad8* mutants had pulmonary adenomas. In agreement with this finding, a nonsense mutation of *SMAD8* has been reported in a patient with idiopathic PAH (122).

Mutations of the human *SMAD4/DPC4* gene are frequently found in pancreatic cancer, metastatic colon cancer and juvenile polyposis (123, 124). *Smad4*-null mice die before E7.5 with gastrulation defects and an abnormal visceral mesoderm, which are similar to the phenotypes of *Bmp4*- and *Bmpr1a*-null mice (125).

Smad6-null mice exhibit cardiac defects with abnormal valve formation and outflow tract septation (126). Some *Smad6*-null mice survive through adulthood, but show aortic ossification and elevated blood pressure.

Most of *Smad7*-null mice are embryonic lethal due to defects in cardiovascular development, including ventricular septal defect, non-compaction and outflow tract malformation (127). Elevated phosphorylation of Smad2/3 is observed in the heart of *Smad7* mutant mice, suggesting that the cardiac defects in the *Smad7*-null mice may be induced by hyperactivation of the TGF- β /activin signalling pathway.

Non-Smad pathways

In addition to the Smad pathways, non-Smad pathways, including p38 and Erk MAP kinase pathways

activated by BMPs, may play important roles in cell proliferation and differentiation. Since non-Smad signalling pathways activated by TGF- β family receptors have been discussed by others (10), we here focus on the signals mediated by TGF- β activated kinase 1 (TAK1), and those transmitted from the molecules associating with the C-terminal tail of BMPR-II.

TAK1, a member of the MAP kinase kinase kinase family, was originally identified as a regulator of MAP kinase activation in TGF- β and BMP signalling pathways (128). TAK1 is activated by BMP-2 and -4, leading to MKK3/6-mediated p38 MAP kinase activation (129). TAK1 is also widely known as a downstream signalling component of interleukin-1, and has critical functions in the regulation of adaptive immunity (130). Targeted disruption of mouse *Tak1* gene causes defects in the intraembryonic vasculature and yolk sac, similar to the phenotypes of *Smad5*-null mice (131), suggesting that TAK1 may be important for Smad activation downstream of BMP stimulation. Conditional targeting of *Tak1* in chondrocytes resulted in chondrodysplasia and joint abnormalities similar to that seen in mice deficient in BMP signalling (132).

Several molecules have been shown to interact with the C-terminal tail of BMPR-II. Nonsense mutations in the C-terminal tail have been found in some patients with familial PAH, indicating that this region plays an important role in signalling activity (46, 47). Mice expressing a *Bmpr2* mutant lacking C-terminal tail in the pulmonary smooth muscle cells also develop PAH-like abnormalities (50). The C-terminal tail of BMPR-II interacts with LIM kinase 1 (LIMK1), Tribbles-like protein 3 (Trb3), Tctex1 and Src. Among them, functions of LIMK1 and Trb3 are described here.

LIMK1 regulates actin dynamics through phosphorylation and inactivation of cofilin. BMPR-II inhibits the ability of LIMK1 to phosphorylate cofilin through interaction with its C-terminal tail (133). LIMK1 has also been reported to be activated by BMPR-II binding and Cdc42, and to modulate BMP-dependent regulation of neurite formation (134).

In addition to regulating the non-Smad pathways, the C-terminal tail may also regulate the Smad signalling pathway. Trb3 is associated with the BMPR-II C-terminal tail, and is released from BMPR-II upon ligand binding. Trb3 then induces degradation of Smurf1, leading to stabilization of BMP-specific R-Smads and facilitation of the Smad signalling pathway (135).

Conclusion and perspectives

Signalling receptors for BMPs were identified by 1995, and Smad signalling pathways, including the three classes of Smads, were discovered by 1997 (9). Since then, numerous studies have unveiled the mechanisms of TGF- β family signalling using various techniques. It should be noted that BMP signalling pathways are conserved in vertebrates and invertebrates, and findings obtained using *Drosophila* and *C. elegans* systems have accelerated the progress in BMP signalling research in mammals (6, 7). Recent genetic analyses

of certain human diseases and analyses of phenotypes of knockout mice have revealed unexpectedly broad spectra of *in vivo* functions of BMPs.

Recent progress in experimental techniques may facilitate further understanding of the signalling mechanisms of BMPs. For example, chromatin immunoprecipitation on microarray (ChIP-chip) analysis is a powerful method for identification of binding sites of transcription factors. The Smad2/3- and Smad4-binding sites involved in TGF- β stimulation were recently examined by ChIP-chip analysis, and the importance of ETS1 and AP-2 at Smad2/3-binding sites and that of AP-1 at Smad4-binding sites have been demonstrated (136, 137). Analyses of Smad1/5-binding sites using ChIP-chip may provide new findings regarding target genes of BMPs and transcription factors that collaborate with BMP-specific R-Smads. Moreover, next-generation high-throughput sequencing systems will enable identification of new BMP target genes as well as miRNAs regulated by BMP signalling in the future.

The recent discovery of BMP receptor inhibitors may be useful for regulation of some human diseases, including FOP and iron-deficiency anaemia. However, since BMPs are pleiotropic factors, more detailed analyses of the *in vivo* functions of BMP inhibitors are required in the future.

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Conflict of interest

Smad6 and uses thereof (United States Patent 6,964,853); Activin receptor-like kinases, ALK-3 and ALK-6, and nucleic acids encoding them (United States Patent 6,207,814); Osteogenesis-promotion enhancer and method of screening the same (United States Patent 7,276,525) awarded to K.M.

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