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Review

TGF-β and BMP Signaling in Osteoblast Differentiation and Bone Formation

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Abstract

Transforming growth factor-beta (TGF-β)/bone morphogenic protein (BMP) signaling is involved in a vast majority of cellular processes and is fundamentally important throughout life. TGF-β/BMPs have widely recognized roles in bone formation during mammalian development and exhibit versatile regulatory functions in the body. Signaling transduction by TGF-β/BMPs is specifically through both canonical Smad-dependent pathways (TGF-β/BMP ligands, receptors and Smads) and non-canonical Smad-independent signaling pathway (e.g. p38 mitogen-activated protein kinase pathway, MAPK). Following TGF-β/BMP induction, both the Smad and p38 MAPK pathways converge at the Runx2 gene to control mesenchymal precursor cell differentiation. The coordinated activity of Runx2 and TGF-β/BMP-activated Smads is critical for formation of the skeleton. Recent advances in molecular and genetic studies using gene targeting in mice enable a better understanding of TGF-β/BMP signaling in bone and in the signaling networks underlying osteoblast differentiation and bone formation. This review summarizes the recent advances in our understanding of TGF-β/BMP signaling in bone from studies of genetic mouse models and human diseases caused by the disruption of TGF-β/BMP signaling. This review also highlights the different modes of cross-talk between TGF-β/BMP signaling and the signaling pathways of MAPK, Wnt, Hedgehog, Notch, and FGF in osteoblast differentiation and bone formation.

Key words: Osteoblasts, Bone, TGF signaling, BMP signaling, Smad, Runx2

INTRODUCTION

Bone is formed through two distinct phases: endochondral ossification, in which a cartilage model is replaced by bone, and intramembranous ossification, in which bones are shaped directly from condensations of mesenchymal cells without a cartilage intermediate (1). Bone is continuously remodeled throughout life and an imbalance in this process can result in bone disease. The integrity and function of bone are maintained by an exquisite balance between osteoblasts and osteoclasts, the two major bone cells involved in the bone remodeling process. Osteoblasts are responsible for bone formation, while osteoclasts are the bone-resorbing cells (1-2).

The transforming growth factor-beta (TGF-β) superfamily is comprised of over forty members, such as TGF-βs, Nodal, Activin, and bone morphogenetic proteins (BMPs) (3). TGF-β signaling first transmits signals across the plasma membrane through the

formation of heteromeric complexes of specific type I and type II serine/threonine kinase receptors. The type I receptor is phosphorylated following the activation of specific type II receptor (4). Activated type I receptors initiate intracellular signaling through phosphorylation of specific Smad proteins, R-Smads. Activated R-Smads form a complex with co-Smad and Smad4 and then translocate into the nucleus to direct transcriptional response (5) (Figure 1, 2).

TGF-β/BMPs have widely recognized roles in bone formation during mammalian development and exhibit versatile functions in the body (6-7). For instance, a BMP morphogen gradient is established in a multicellular embryo, and BMP drives the differentiation of ectodermal cells and mediates dorsal patterning to establish dorsal-ventral axis (8). Disruptions of TGF-β/BMP signaling have been implicated in multiple bone diseases including tumor metastasis, brachydactyly type A2, and osteoarthritis (9-11). In this review, we will summarize novel discoveries from recent decades regarding the regulations of TGF-β/BMP signaling in bone as well as the different modes of cross-talk between TGF-β/BMP signaling and the signaling pathways of MAPK, Wnt, Hedgehog, Notch, and FGF. These new insights into TGF-β/BMP signaling on bone will open new prospects for generating novel therapies against clinical disorders.

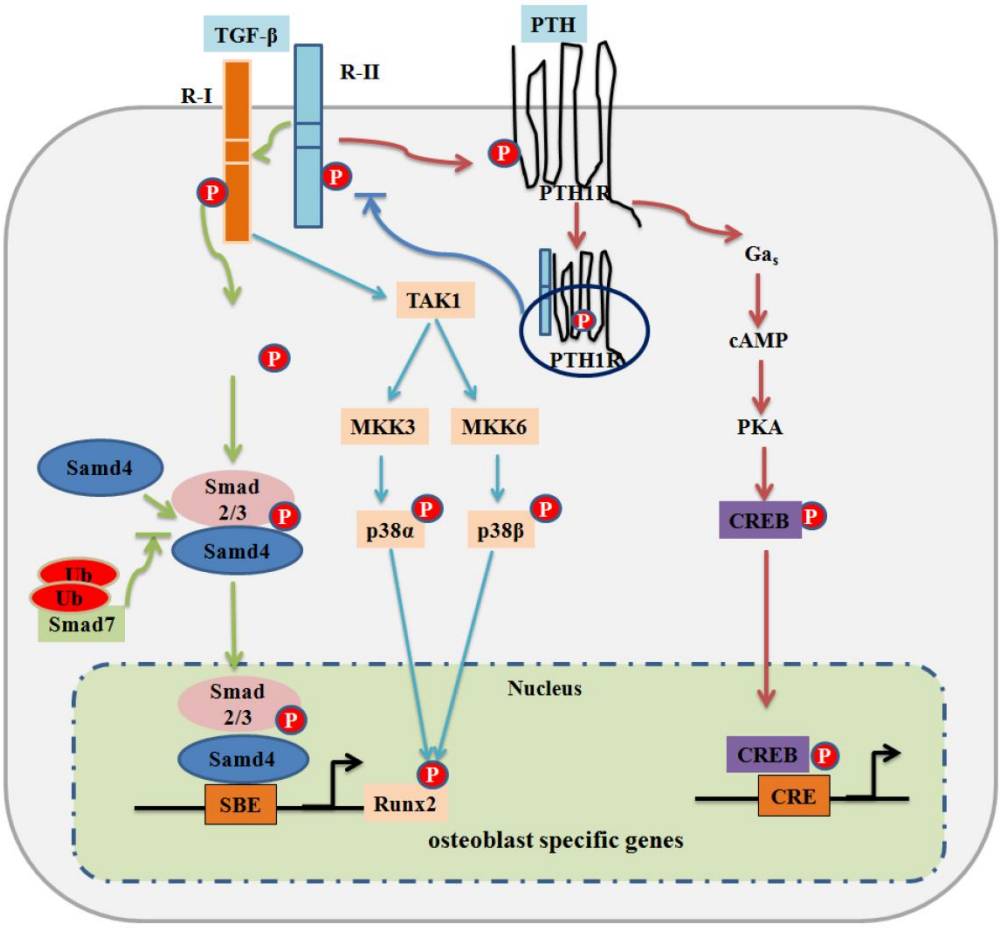

Figure 1. TGF-β signaling and negative regulation in bone formation. Canonical Smad-dependent TGF-β signaling first binds to receptor type II (R-II) and receptor type I (R-I), and then signaling transduces to their Smads. Activated Smads form a complex with Smad4 and then translocate into the nucleus where they interact with other transcription factors to trigger target gene expression. Smad7 disrupts the activated Smad2/3 to form a complex with Smad4. The non-Smad-dependent TAK1 signaling pathway also regulates bone formation. PTH binding activates PTH1R to stimulate several downstream effectors. PTH binding also drives internalization of PTH1R-TGFβRII complex, which attenuates both TGF-β and PTH signaling on bone development. Transcriptional factor cAMP response element binding protein (CREB) mediates PTH signaling in osteoblasts. P: phosphorylation; Ub: ubiquitination.

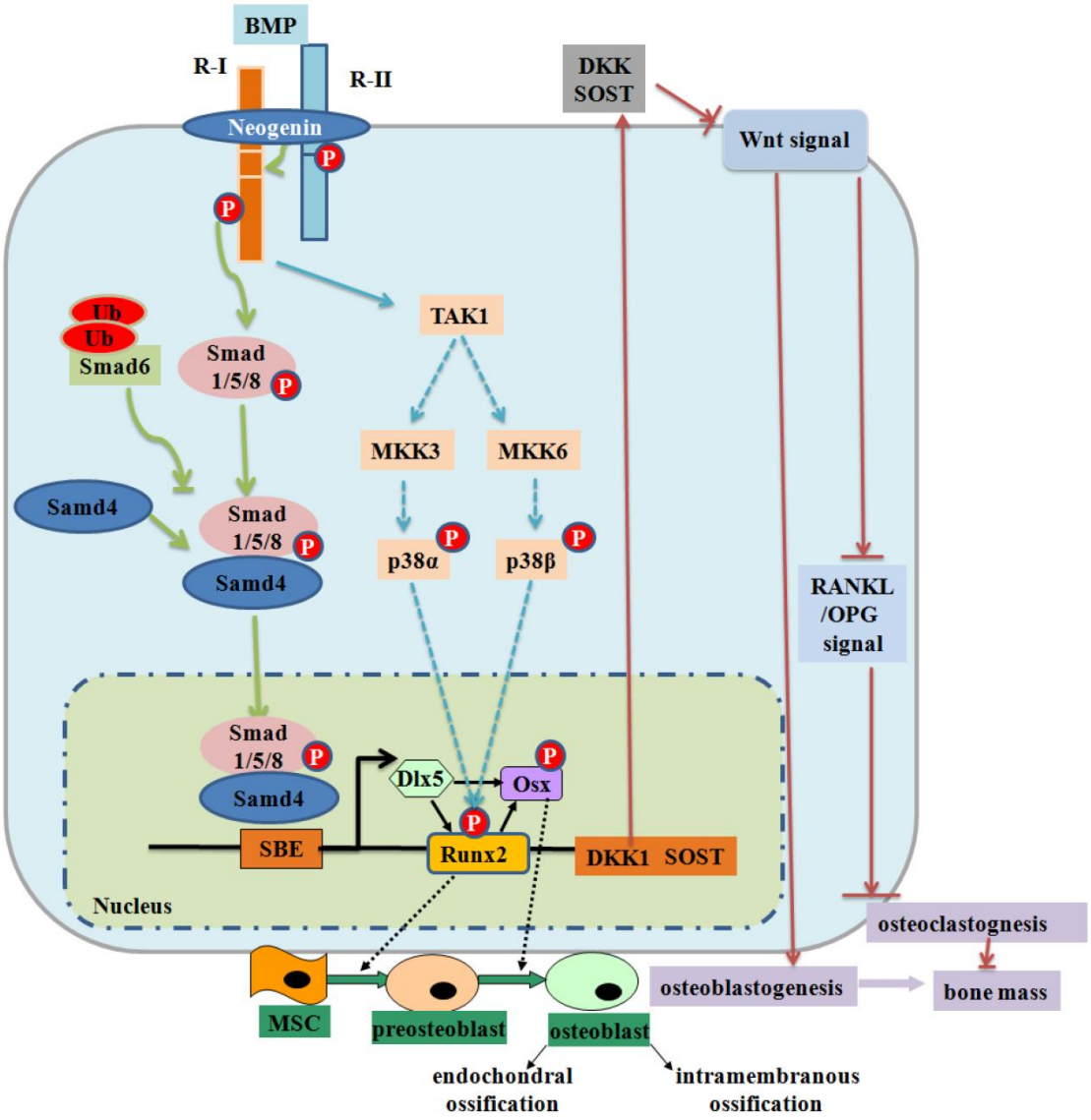

Figure 2. BMP signaling and negative regulation in bone formation. Smad-dependent-BMP signaling binds to receptor type II (R-II) and receptor type I (R-I) and then the signaling transduces to their Smads. Activated Smads form a complex with Smad4 and then translocate into the nucleus where they interact with other transcription factors to trigger target gene expression. Neogenin regulates BMP receptor association and Smad1/5/8 signaling. Activated Smads regulate expression of transcriptional factors and transcriptional coactivators important in osteoblasts (Dlx5, Runx2 and Osx). Smad6 binds type I BMP receptor and prevents Smad1/5/8 to be activated. Non-Smad-dependent TAK1 signaling pathway also regulates bone formation. The interplay between BMPs and Wnt signaling affects bone formation (99). BMPRIA signaling upregulates Sost expression primarily through Smad-dependent signaling, while it upregulates DKK1 through Smad-dependent and non-Smad-dependent signaling. Both Sost and DKK1 inhibit canonical Wnt signaling, leading to a decrease in bone mass. P: phosphorylation; Ub: ubiquitination.

TGF-β SIGNALING IN OSTEOBLAST DIFFERENTIATION AND BONE FORMATION

Autocrine and paracrine stimulation by TGF-β is important in the maintenance and expansion of the mesenchymal stem/progenitor cells, the progenitors of osteoblasts (12). Bone and cartilage contain large amounts of TGF-β and target cells for TGF-β activity. At earlier developmental stages, osteoblast-enriched populations from fetal bone are more sensitive to the mitogenic effect of TGF-β than similar populations from newborns (13). Furthermore, TGF-β signaling also promotes osteoprogenitor proliferation, early differentiation, and commitment to the osteoblastic lineage through the selective MAPKs and Smad2/3

pathways, and the cooperation between TGF-β and PTH, Wnt, BMP, as well as FGF signaling.

Canonical TGF-β signaling functions in bone (TGF-βs, receptors, Smads)

TGF-β isoforms and their receptors, type I receptor (TGFβRI or ALK5) and type II receptor (TGFβRII or Tgfbr2), play important roles in endochondral and intramembranous ossification (Table 1). TGF-β1 deficient mice display reduced bone growth and mineralization (14). TGF-β2 and TGF-β3 double knockout mice display a lack of distal parts of the rib (15). Mice carrying tissue-specific removal of TGFβRI using Dermo1-Cre have short and wide long bones, reduced bone collars, and reduced trabecular bone (16). Tgfbr2 exhibits functions in the maintenance of boundaries in the sclerotome and developing axial skeleton (17). Conditional transgenic mice with a dominant negative form of Tgfbr2 (dnTgfbr2) developed hypoplastic cartilage (18). Deletion of Tgfbr2 via Col2a1-Cre in mice causes multiple defects in the base of the skull and in the vertebrae (19). Removal of Tgfbr2 driven by Prx-Cre results in defects in the long bones, joints (20), and skull vault (21), indicating that Tgfbr2 has a critical role in both intramembranous bone formation and endochondral bone formation. Further evidence from Wnt1-Cre mediated tissue specific disruption, shows that the loss of Tgfbr2 signaling leads to defects in cranial neural crest cells (CNCC)-derived osteoprogenitor cells during intramembranous bone formation (22) (Table 1).

Table 1. Conditional knockout models on TGF-β/BMP signaling on bone formation

Gene	Cre	Defects	Refer
Tgfbr2	*Col2a1-Cre*	obvious defects in long bone formation	(18)
	Wnt1-Cre	osteogenic cell proliferation and differentiation	(22)
	Prx1-Cre	short limbs and fusion of the joints in the phalanges	(20)
	Wnt1-Cre	severe defects in mandibular development	(170)
	Col2a-Cre	defects in the base of the skull and in the vertebrae	(19)
ALK5	*Dermo1-Cre*	the short bones and ectopic cartilaginous protrusions	(16)
TAK1	*Prx1-Cre*	novel embryonic developmental cartilage defects	(171)
	Osx-cre	clavicular hypoplasia and delayed fontanelle fusion	(82)
Smad7	*Prx1-Cre*	poor cartilage formation	(172)
BMP-7	*Prx1-Cre*	normal postnatal limb growth and maintenance of bone mass	(49)
Bmp2/Bmp4	*Col2a1-Cre*	a severe chondrodysplasia phenotype	(53)
Bmp2	*Prx1-Cre*	spontaneous fractures	(52)
Bmp2/Bmp4	*Prx1-Cre*	a severe impairment of osteogenesis	(50)
Bmp4	*Prx1-Cre*	limb skeletogenesis occurs normally in the absence of BMP-4	(51)
BMPR-II	*Prx1-Cre*	normal skeleton	(60)
BMPR-IA	*Col1a1-Cre*	increased bone volume	(61)
	Col1-CreERTM	increased bone mass	(99)
	Prx1-Cre	shortened limbs and almost complete agenesis of the autopod	(62)
Smad1	*Col1a1-Cre*	osteopenic phenotype	(68)
	Col2a1-Cre	calvarial bone development delay	(68)
Smad1/5	*Col2a1-Cre*	chondrodysplasia	(69)
Smad4	*TTR-Cre*	died at E7.5-E9.5 without head-fold and anterior embryonic structures	(72)
	Mu-Cre	misalignment of the cardiac outflow tract	(74)
	OC-Cre	lower bone mineral density, decreased bone volume, decreased bone formation rate	(26)
	Col2a1-Cre	dwarfism	(41)

R-Smads that respond to TGF-β receptors are Smad2 and 3. Small C-terminal domain phosphatases (SCP) were reported to be good regulators of Smad2/3 activation. SCP1/2 knockdown inhibits TGF-β transcriptional responses by dephosphorylating Smad2/3 at the linker (inhibitory) but not the C-terminal (activating) site (23). Activation of Smad7 is another way to control the function of R-Smads, which targets the TGF-β receptor for degradation (24). Smad7 mutants that have impaired the ability for the recruitment of Smurf2 to the receptors are compromised in their inhibitory activity (25). Smad7 also competes with Smad2/3 to form complexes with Smad4, a common mediator for TGF-β signaling, which plays an essential role in coupling bone formation and bone resorption and maintaining normal postnatal bone homeostasis (26).

Recently, Smad2/3 were reported to directly associate with the TRAF6-TAB1-TAK1 molecular complex (27). Once TGF-β signaling is blocked, the TRAF6-TAB1-TAK1 molecular complex is not observed, indicating that TGF-β1 is indispensable in RANKL-induced osteoclastogenesis (28). TGF-β1 promotes matrix production and osteoblast differentiation while it reduces the ability of osteoblasts to secrete osteoclast differentiation factor RANKL, thereby TGF-β1 indirectly limits further osteoclast formation and may affect bone mass.

Non-canonical TGF-β signaling functions in bone

The non-Smad-dependent signaling pathway also contributes greatly to osteoblast differentiation and bone formation. The study of tamoxifen-inducible Cre-ER-mediated ALK5-deficient primary calvarial cell culture revealed that TGF-β signaling promotes osteoprogenitor proliferation, early differentiation, and commitment to the osteoblastic lineage through the selective MAPKs and Smad2/3 pathways (16). TGF-β activation kinase1 (TAK1) and TAK1 binding protein 1 (TAB1) play a pivotal role as upstream signal transducers by activating the MKK3-p38 MAPK signaling cascade that leads to the induction of type I collagen expression by TGF-β1. TAK1 has a novel function in the regulation of the steady-state protein levels of MKK3 and p38 MAPK (29) (Figure 1). Recent results demonstrate that following TGF-β induction, both the Smad and p38 MAPK pathways converge at the Runx2 gene to control mesenchymal precursor cell differentiation (30). In addition, ERK and p38 also differentially mediate TGF-β and BMP-2 function in osteoblasts (31). TGF-β2-induced activation of ERK-MAPK is an important signaling component that stimulates cell proliferation to enrich osteoprogenitor cells, thereby promoting their differentiation into osteoblasts to achieve a rapid calvarial bone expansion (32).

Interplay between TGF-β signaling and PTH, Wnt, FGF, as well as BMP signaling in bone

TGF-β and PTH signaling in bone

Mice with Tgfbr2 deleted in osteoblasts have increased bone mass due to the hyperactivition of PTH type I receptor (PTH1R) (33-34). Disruption of PTH signaling by injection of PTH (7-34) or ablation of PTH1R rescues the bone phenotype of these mutant mice (33). Molecular study shows that Tgfbr2 directly phosphorylates the PTH1R cytoplasmic domain. PTH couples the processes of bone resorption and formation by enforcing simultaneous internalization of Tgfbr2 and PTH1R (34) (Figure 1), which attenuates both TGF and PTH signaling *in vivo*. It is recognized that transcriptional factor cAMP response element binding protein (CREB) mediates PTH signaling in osteoblasts, and PTH-CREB signaling pathway acts as an effective activator of BMP-2 expression (35).

TGF-β and FGF signaling in bone

TGF-βs and FGF-2, -4, and -6 have been proven to be inducers of osteoblast proliferation (a higher extent for TGF-β and FGF-2) and inhibitors of alkaline phosphatase (ALP) activity and osteoblast mineralization (36), indicating potential application for *in vitro* bone growth induction in bone tissue engineering. In addition, FGF acts downstream of TGF-β signaling in regulating CNC cell proliferation and exogenous FGF-2 rescues the cell proliferation defect in the frontal primordium of Tgfbr2 mutants, demonstrating the biological significance of the TGF-β-mediated FGF signaling cascade in regulating frontal bone development (37). Reports also show that FGF/FGFR3 signals mediate some of the effects of TGF-β on embryonic bone formation (38) (Table 3).

TGF and Wnt signaling in osteoblast

TGF-β cooperates with Wnt signaling, and promotes osteoblast differentiation of human mesenchymal stem cells (hMSCs). Knockdown of β-catenin with siRNA stimulated ALP activity and antagonized the inhibitory effects of TGF-β1 on bone sialoprotein expression. TGF-β1 activates β-catenin signaling via ALK5, Smad3, PKA, and PI3K pathways, and modulates osteoblastogenesis via ALK5, PKA, and JNK pathways in hMSCs (39). Recent studies in differentiating osteoblasts indicate that Wnt pathway induction stabilizes β-catenin and increases TCF/LEF-dependent gene expression in parallel with β-catenin-independent complex formation between

TCF-4 and Runx2. Activation of either Runx2 or TCF-4 coenhances TCF and Runx2 activity and increases TGFβRI expression. Overall, Wnt pathway induction has complex stimulatory and inhibitory effects on TGF-β activity (40) (Table 3).

TGF-β and Ihh signaling in bone

Expression of Indian hedgehog/parathyroid hormone-related protein (Ihh/PTHrP) signaling in the growth plate is decreased in Smad4 mutant mice. The cultured mutant metatarsal bones failed to respond to TGF-β1. These findings indicate that Smad4-mediated TGF-β signals are required for maintaining the normal organization of chondrocytes in the growth plate (41), which is important for normal endochondral ossification.

Interplay between TGF-β and BMP signaling in osteoblast and bone

TGF-β1 strongly enhances ectopic bone formation induced by BMP-2, with the resulting bone volume being five-fold greater than that induced by BMP-2 alone (42). Evidence shows that increased BMPR-IB by TGF-β1, FGF-2, and PDGF-AB significantly enhances BMP-2-induced osteogenic functions *in vitro* (43). Results demonstrate that BMPRII and ActRII are the functional type II TGF-β receptors in BMP-9-induced osteogenic differentiation of C3H10T1/2 cells (44), indicating a strong connection between TGF-β1 and BMP signaling in osteoblast differentiation (Table 3).

BMP SIGNALING IN OSTEOBLAST DIFFERENTIATION AND BONE FORMATION

BMPs have widely recognized roles in bone formation during mammalian development and exhibit versatile functions in the body, indicating potential for therapeutic use. Studies have elicited BMPs in mice to promote the rehabilitation of critical-size bone defects, which renders them useful in the field of tissue engineering and regeneration. Recent discoveries will shed light on better understanding of intrinsic BMP signaling and the crosstalk with Wnt, Notch, FGF and Hh signaling on osteoblast differentiation and bone formation.

Canonical BMP signaling in bone (BMPs, receptors, Smads)

BMP-2, 4, 5, 6, and 7 all have strong osteogenic capacity. Addition of BMP-2 vastly increases osteocalcin (45) and a short-term expression of the BMP-2 is necessary and sufficient to irreversibly induce bone formation (46). BMP-7 induced the expression of osteoblastic differentiation markers such as ALP activity and accelerated calcium mineralization (47-48). *In vivo* genetic studies using a Prx1-cre model demonstrated that the absence of locally produced BMP-7 has no effect on postnatal limb growth and maintenance of bone mass, indicating other BMPs present in adult bone are sufficient to compensate for the absence of BMP-7 (49). Loss of both BMP-2 and BMP-4 resulted in severe impairment of osteogenesis (50). However, limb skeletogenesis occurred normally despite the absence of BMP-4, suggesting that BMP-4 is not required for bone formation and function in the limb (51). Mice lacking the ability to produce BMP-2 in their limb bones have spontaneous fractures that do not resolve with time, other osteogenic stimuli cannot compensate for the absence of BMP-2 (52). BMP-2, not BMP-4, plays a crucial role for chondrocyte proliferation and maturation during endochondral bone development (53) (Table 1).

Trabecular bone volume of BMP-3-deficient mice is two-fold greater than that of wild-type mice (54). BMP-3 limits skeletal progenitor cell differentiation to mature osteoblasts, herein regulating adult bone mass (55). Overexpression of BMP-3 in chick wing bud reduces BMP signaling results in expanded skeletal elements (56). BMP-3 transgenic mice are subject to spontaneous rib fractures and have altered signaling through activin receptor type IIB (ActRIIB) in chondrocytes and the periosteum (57).

BMP receptors, such as BMPR-II, differentially modulate the responsiveness of target genes to BMP-2 (58). BMPR-II and ActR-IIB are able to compensate each other functionally in mediating BMP-2 signaling and BMP-2-induced osteoblast differentiation in 2T3 cells (59). Normal skeletons develop in mice with BMPR-II deletion via Prx1-Cre, indicating that BMPR-II is not required for limb development or that the loss of BMPR-II is compensated by BMP utilization of other type II BMP receptors (60). Conversely, mice with deletion of BMPR-IA through Col1-Cre have increased bone volume (61), shortened limbs, almost complete agenesis of the autopod (62), small body size, irregular calcification and low bone mass (63) (Table 1). Decreased osteoclastogenesis through the RANKL-OPG pathway was reported to contribute to the increased bone mass in trabecular bone (64).

Neogenin, a transmembranous protein, was reported to regulate BMP receptor association with lipid raft, where BMP induces canonical Smad1/5/8 phosphorylation (65-66). Overexpressing BMP signaling through ALK2 will lead to ectopic phosphorylation of Smad1/5/8 (67). Osteoblast-specific Smad1 conditional knockout mice developed an osteopenic phenotype and partial inhibition of BMP signaling

(68). Combined loss of Smads 1/5/8 results in severe chondrodysplasia (69) (Table 1). If Smad1 is modified, it can modulate BMP-mediated osteogenesis (70) and the intensity of BMP signals can be determined by BMP receptors via Smad1 C-terminal phosphorylation (71). These finding demonstrated that Smad1/5/8 are intracellular signaling proteins that transduce signals elicited by members of BMP signaling in osteoblasts.

Smad4 is the only common Smad for both TGF-β and BMP signaling. Targeted disruption of Smad4 in mice results in numerous developmental defects and cancer formation in various tissues. Smad4-deficient mice died at E7.5-E9.5 without head-fold and anterior embryonic structures, and with impaired responsiveness to TGF-β-induced gene expression (72). Smad4-mediated TGF-β signaling is also reported to be important in the inhibition of oncogenesis (73). Myocardial deletion of Smad4 in mice causes misalignment of the cardiac outflow tract (74), highlighting essential roles of this co-Smad in mediating signaling of the TGF-β superfamily. Conditional deletion of Smad4 in osteoblast leads to lower bone mineral density, decreased bone volume, decreased bone formation rate, and a reduced number of osteoblasts (26). Control of Smad4 is a good way to regulate bone formation. FAM and Ectodermin/Tif1gamma (Ecto) were reported to respectively regulate the de-ubiquitination and ubiquitination of Smad4 (75-76). On the other hand, Smad6 competes with R-Smad and forms a non-functional complex with Smad4, which will inhibit BMP signaling in bone formation. Smad6 is involved in a negative feedback loop regulating BMP signaling (77) and is required to limit BMP signaling during endochondral bone formation (78). Smad6/Smurf1 double transgenic mice show severely delayed endochondral ossification compared to the Smad6 knockout (79). Smurf1, with its WW domain, specifically binds to the PY motif of Smad6 and transports Smad6 into the cytoplasm. When Smad6 is in its rest state, it is mainly localized in the nucleus (80).

Non-canonical BMP signaling function in bone

The importance of canonical BMP signaling is well established, but the necessity for non-canonical (Smad-independent) signaling during these processes is also important. TAK1 is a factor that is involved in the fine-tuning of BMP effects during osteogenic development (81). Osteoblast-specific deletion of Tak1 resulted in clavicular hypoplasia and delayed fontanelle fusion, a phenotype similar to the cleidocranial dysplasia observed in human haploinsufficient for Runx2 (82) (Table 1). Mechanistic analysis revealed that the TAK1-MKK3/6-p38 MAPK axis phosphorylated Runx2, promoting its association with the coactivator CREB-binding protein (CBP), which is required to regulate osteoblast genetic programs (82). Surprisingly, deletion of TAK1 seems to affect not only activation of the p38 MAPK signaling cascade, but also activation of the BMP-responsive Smad1/5/8 (83). Smad and MAPK pathways act synergistically in the BMP pathway controlling limb development (84). The coordinated activity of Runx2 and BMP/TGF-β-activated Smads is critical for the formation of the skeleton. TGF-β/BMP2 signaling, MAPK dependent phosphorylation, and Runx2 subnuclear targeting converge to induce the osteogenic phenotype (85).

Crosstalk between BMP and Notch, Hh, FGF, as well as Wnt signaling in osteoblasts and bone

Runx2, Dlx5, and Osx in osteoblasts and bone

Runx2 is a master transcription factor, and both endochondral and intramembranous bone formation are totally absent in Runx2-null mice (86). Autocrine BMP production is necessary for the Runx2 transcription factor to be active. Additionally, BMPs and Runx2 cooperatively interact to stimulate osteoblast gene expression (87), and direct evidence shows that Runx2 is essential for the execution and completion of BMP2 signaling for osteoblast differentiation (88). BMP-activated Smads interact with Runx2 to induce osteoblast-specific gene expression in C2C12 cells (86). Smad1 interacts with Runx2 on the promoter of target genes and controls osteoblast gene expression and differentiation (89). Mutations in Runx2 may lead to an autosomal-dominant human bone disease called cleidocranial dysplasia, which is related to impaired Smad signaling and the target the of Runx2 activity during bone formation (90).

However, BMP-Smads can also function independently of Runx2. Osterix (Osx), which is indispensable for preosteoblasts to differentiate into mature osteoblasts and form bone (91), was previously reported to be a Runx2-targeted gene. Osx induction by BMP-2 is completely abrogated by the antisense blocking of Dlx5, indicating BMP-2-induced Osx expression is mainly mediated by Dlx5, but not by Runx2, suggesting that Dlx5 may directly interact with Osx (92). On the other hand, Dlx5 can drive Runx2 expression and osteogenic differentiation in developing cranial suture mesenchyme (93), indicating that Dlx5 can work as an upstream gene of Runx2. Recently, Akt, a member of the serine/threonine-specific protein kinase, was found to

phosphorylate Osx and Dlx5 (94). Akt activation increases protein stability, osteogenic activity, and transcriptional activity of Osx and Dlx5 (95), revealing that Osx regulates bone formation through different signaling pathways, including a novel mechanism involving Akt during osteoblast differentiation.

BMP and Wnt signaling in bone

Wnt3A and BMP-9 enhanced each other's ability to induce ALP in MSCs (96). *In vitro* deletion of the β-catenin gene inhibits osteoblast proliferation, alters osteoblast differentiation, and reduces the responsiveness of osteoblasts to BMP-2 treatment (97). Interactions between β-catenin and Runx2 play an important role in BMP-9-induced osteogenic differentiation of MSCs (96). On the other hand, BMPR1A specifically deleted in osteoblasts leads to a significant increase in bone mass, which is partially due to hyperactivated Wnt signaling (98) and increased expression of sclerostin (Wnt receptor antagonist) and Dkk1 activity. It has been shown that both sclerostin and DKK1 act physiologically as downstream molecules of BMP signaling to inhibit canonical Wnt signaling and therefore negatively regulate bone mass (99) (Figure 2). Loss-of-function of either DKK1 or SOST, which are downstream targets of BMPs, causes a high bone mass phenotype in humans and mice, suggesting an important role of DKK1 and SOST for bone mass regulation (100) (Table 3).

BMP and FGF signaling in bone

FGF signaling was reported to interplay with BMP signaling on bone formation. FGF-2 and FGF-9 increased expression of other osteogenic factors BMP-2 and TGF-β1, and endogenous FGF/FGFR signaling is a positive upstream regulator of the BMP-2 gene in calvarial osteoblasts (101). FGF-2 and BMP-2 have a synergistic effect on fracture healing: FGF-2 has a critical function at early stage while BMP-2 promotes mineralization at later stage (102). FGF-2 null mice have impaired nuclear accumulation of Runx2 and hindered BMP-2 induced bone formation and ALP activity (103). Runx2 is an important mediator of the expression of BMP-2 in response to FGF stimulation in cranial bone development (104). FGF and BMP synergy on osteogenesis may be modulated through Rnnx2 activation (Table 3).

BMP and Notch signaling in osteoblasts

Similar synergy is found in Notch and BMP crosstalk: activating Notch signaling enhanced BMP-induced ALP activity and formation of calcified nodules *in vitro* (105). Notch inhibition results in decreased ALP activity and decreased promoter activity of BMP target genes (106). Notch signal transduction pathway genes, Lfng, Hey1, and Hes1, are differentially regulated by BMP-2 and TGF-β. These genes might function as the focal point for interaction of Smad and Notch signaling during osteoblast differentiation (107). Functional Notch signaling is required for BMP4-induced inhibition of myogenic differentiation (108), which may be beneficial to osteoblast differentiation.

BMP, Hh, and FGF signaling in osteoblasts and bone

Sonic hedgehog (Shh) is involved in osteoblast differentiation by cooperating with BMP-2 (109). Shh stimulates BMP-2 promoter activity and osteoblast differentiation. The effects of Shh are mediated by Gli2, a powerful activator of BMP-2 gene expression, which is required in turn for normal osteoblast differentiation (110). In the developing axial skeleton, sequential Shh and BMP signals are required for specification of a chondrogenic fate in presomitic tissue (111). On the other hand, BMP activity negatively regulates Shh transcription and a BMP-Shh negative-feedback loop serves to confine Shh expression during limb development (112) (Table 3).

Ihh is found to be required for BMP-induced osteogenesis of a limb-bud cell line in culture. Ihh signaling is directly required for the osteoblast lineage in developing long bones. Ihh functions in conjunction with other factors such as BMPs to induce osteoblast differentiation. *In vivo,* Ihh acts on potential progenitor cells to promote osteoblast differentiation and prevent chondrocyte differentiation (113). Ihh and BMP synergistically induce ALP activity. Evidence indicates a stimulatory role for osteoblast-expressed Ihh in bone formation in a positive feedback loop (114). Conditional knockout Smad1/5 mice developed chondrodysplasia and exhibited abnormal growth at the end of bones. The molecular mechanism underlying the defects in conditional knockout Smad1/5 mice appears to be an imbalance in the cross-talk between the BMP, FGF, and Ihh/PTHrP pathways (69), indicating that BMP, FGF, and Ihh/PTHrP is important for normal long bone development (Table 3).

NEGATIVE REGULATION OF TGF-β/BMP SIGNALING IN OSTEOBLAST DIFFERENTIATION AND BONE FORMATION

TGF-β and BMP signaling is under elaborate regulation to maintain correct patterning and morphogenesis of various tissues and organs. TGF/BMP signaling in bone is negatively regulated through a number of mechanisms, including extramembranous

regulation (ligand antagonist), intracellular regulation (Smurf and inhibitory Smads), and transcriptional regulation (transcriptional repressors and epigenetic control).

Ligand antagonists

Antagonists targeting TGF-βs, such as Chordin (115) and Follistatin (116), and BMPs, such as Noggin (117), have been reported. Noggin, a BMP antagonist, negatively regulates BMP activities during vertebrate dorsal-ventral patterning, skeletogenesis, and joint formation (117). Noggin binds the domain that is required for BMP-7 to interact with BMP type I and type II receptors (118). Transgenic mice with overexpressed Noggin in the bone microenvironment are subject to osteopenia and fractures and exhbiti decreased trabecular bone volume and impaired osteoblastic function (119-120). Noggin suppression may be a novel strategy for the treatment of osteolytic bone metastases (121).

Ubiquitin ligase mediated degradation

Smurf1 and Smurf2 are E3 ubiquitin ligases known to suppress TGF-β signaling through degradation of Smads and receptors for TGF-β and BMPs (122). Smurf1 interacts with BMP-activated Smad1 and 5 and to mediate degradation of these Smad proteins as well as Runx2 (123). Smurf1-deficient mice exhibit an age-dependent increase of bone mass (124). Smurf2 has been reported to form a complex with Smad2 and then target SnoN for degradation (125). Smurf2-transgenic mice exhibited decreased articular cartilage area and subchondral sclerosis (126). In addition, some proteins (e.g. CHIP, carboxyl terminus of Hsc70-interacting protein) inhibit the signaling activities of Smad1/5 by recruiting Smad1/5 from the functional R-/Co-Smad complex and further promoting the ubiquitination and degradation of Smad1/5 in a chaperone-independent manner (127). Other proteins, such as the serine/threonine kinase Fused (Fu), can function in concert with the E3 ligase Smurf to regulate ubiquitination and proteolysis of the BMP receptor (128).

Transcriptional repressors

In the nucleus, transcriptional repressing factors Ski and SnoN play a negative role together with I-Smads (129) to disrupt the formation of the TGF-β-induced functional Smad-DNA complex and thereby inhibit target gene expression (130). Since the Ski-binding surface on Smad4 significantly overlaps with the surface required for binding R-Smad phosphorylated tails, binding to Ski or SnoN interferes with the interaction between Smad4 and phosphorylated R-Smads and disrupts the active heteromeric Smad4/R-Smad complex (131). Ski and SnoN also prevent Smads from binding to the transcriptional coactivator p300/CBP (131) which is important in skeletogenesis.

Epigenetic regulation

Epigenetics is an essential mechanism to control gene expression and fundamental cellular processes. DNA methylation is important in the regulation of ALPL expression through the osteoblast-osteocyte transition (132). Smad2-mediated transcription requires the histone acetyltransferase p300, which is required for the ability of Smad2 to mediate activin and TGF-β signaling (133). Mutation of lysine at Lys-19, Lys-20, and Lys-39 abolished Smad2 acetylation *in vivo* and prevented nuclear accumulation of Smad2 and subsequent TGF-β and activin responses (134). Smad7 interacts with specific histone deacetylases (HDACs), which are able to deacetylate Smad7 (135). This is significant since acetylation of Smad7 protects it against ubiquitination and degradation mediated by the ubiquitin ligase Smurf1 (136).

Runx2 is a global regulator of osteogenesis and is crucial for regulating the expression of bone-specific genes. Runx2 is controlled by a dynamic equilibrium of acetylation, deacetylation, and ubiquitination. BMP-2 signaling stimulates p300-mediated Runx2 acetylation, which increases transactivation activity and inhibits Smurf1-mediated degradation of Runx2. HDAC4 and HDAC5 deacetylate Runx2 and lead to a Smurf-mediated degradation (137). BMP-induced non-Smad ERK signaling pathway cooperatively regulates osteoblast differentiation, in part, through increasing the stability and transcriptional activity of Runx2 or increasing Runx2 acetylation by p300 (138). On the other hand, studies suggest that NFATc1 acts as a transcriptional co-repressor of osteocalcin promoter possibly in an HDAC-dependent manner (139). These findings demonstrate the importance of epigenetic regulation on bone formation and its important medical implications since TGF-β/BMPs and Runx2 are of great interest with regard to the development of therapeutic agents against bone diseases.

TGF-β/BMP SIGNALING IN DISEASES AND CLINICAL APPLICATIONS

TGF-β/BMP signaling in human diseases

Basic research has contributed greatly to our understanding of human skeletal diseases caused by mutations related to TGF-β/BMP signaling (Table 2). Fibrodysplasia ossificans progressiva (FOP) is a rare disabling disease caused by mutations in ALK2 (140)

and characterized by heterotopic ossification (141). Mutations in BMPR1B were recently demonstrated in two affected families with brachydactyly type A2 (BDA2) (142). BDA2 is characterized by hypoplasia/aplasia of the second middle phalanx of the index finger and sometimes the little finger. BDA2 was first described by Mohr and Wriedt in a large Danish/Norwegian kindred and GDF5 (growth and differentiation factor 5) was identified as a novel BDA2 causing gene (142-143). Furthermore, two mutations (N445K, T) of GDF5 in patients developed synostosis syndrome (144). An additional functional mechanism for the pathogenesis of BDA2 is duplication of a regulatory element that affects the expression of BMP2 in the developing limb (145). Mutations in BMP antagonist NOGGIN cause brachydactyly type B (BDB), which is characterized by terminal deficiency of fingers and toes (146). Mutations of Sost (antagonist of BMPs) are associated with sclerosteosis which is a disorder featuring increased bone density (147). Mutations of the TGF-β1 gene on chromosome 19q13.1-q13.3 was reported to be the cause for camurati-engelmann disease (CED) characterized by bone pain and osteosclerosis affecting the diaphysis of long bones (148). Recently, a new TGF-β1 mutation (E169K) in exon 2 was identified in a Chinese family (149) which developed CED (Table 2).

Except for the skeletal disorder, disruptions of TGF-β/BMP signaling also cause other human diseases. BMP-15 defects are involved in the pathogenesis of hypergonadotropic ovarian failure in humans, which leads to female infertility (150). Mutation of BMPRII is linked to the development of primary pulmonary hypertension (PPH) and features the widespread occlusion of small pulmonary arteries, which leads to sustained elevation of pulmonary arterial pressure (151) (Table 2). Germline mutations of the gene encoding BMPR-IA results in juvenile polyposis, an autosomal dominant gastrointestinal hamartomatous polyposis syndrome in which patients are at risk for developing gastrointestinal cancers (152). Moreover, BMPs have been implicated in periodontal disease (153), osteoarthritis (154), and the tumor metastasis. Indeed, BMP-2 may facilitate bone metastasis in gastric cancer (155). These findings indicate the importance of TGF-β/BMPs signaling in bone development and homeostasis.

It is hoped that basic research on disease and its underlying mechanism may open new avenues for the generation of antagonists, small inhibitory molecules, or novel delivery systems that target bone diseases. A small molecule inhibitor of BMP type I receptor activity has been demonstrated to be useful in treating FOP and heterotopic ossification syndromes (156). TGF-β type I receptor kinase inhibitor downregulates rheumatoid synoviocytes and prevents the arthritis (157). TGF-β1–induced migration of bone mesenchymal stem cells couples bone resorption with formation, so modulation of TGF-β1 activity could be an effective treatment for bone remodeling diseases (158). The delivery of TGF-β3 with an injectable calcium-phosphate matrix at the supraspinatus tendon footprint has promise to improve healing after soft tissue repair (159).

Table 2. TGF-β/BMP mutations involved in human diseases

Gene	Disease	Defects	Refer
ALK2	fibrodysplasia ossification progressive (FOP)	ectopic bone formation	(173) (140)
NOGGIN(NOG)	Brachydactyly type B (BDB)	terminal deficiency of fingers and toes	(146)
SOST	sclerosteosis	increased bone density	(147)
BMP15	hypergonadotropic ovarian failure	a common cause of female infertility	(150)
BMPR-II	primary pulmonary hypertension (PPH)	obstruction of pre-capillary pulmonary arteries	(151)
GDF5	brachydactyly type A2 (BDA2)	hypoplasia/aplasia of the second middle phalanx of the index finger and sometimes the little finger.	(142)
BMPR1B			(142-143)
conserved regulatory element downstream of BMP2			(145)
BMPRIA	juvenile polyposis	early developing gastrointestinal cancers	(152)
TGF-β 1	camurati-engelmann disease (CED)	characterized by bone pain and osteosclerosis affecting the diaphysis of long bones	(148-149)

Table 3. Crosstalk between TGF-β/BMP signaling and other signaling molecules in osteoblast and bone

Gene	Crosstalk signaling	Results	Refer
Tgfbr2↓	PTH type I receptor↑	increased bone mass	(33)
PTH-CREB→	BMP-2 expression	osteoblastogenesis	(35)
FGF2↑	Tgfbr2 mutant→normal	regulates frontal bone	(37)
FGF-- FGFR3	TGF-beta	mediates embryonic bone formation	(37)
TGF-β 1→	beta-catenin↑	osteoblastogenesis↑	(39)
Wnt and TGF-β	TCF,Runx2↑ TβRI ↑	Runx2↑ osteoblast maturation↑	(40)
Smad4 mutant	Ihh/PTHrP↓	×→TGF-β 1 response	(41)
TGF-β1→	↑BMP-2	→ ectopic bone formation	(42)
Wnt3A→	↑ BMP-9	↑ ALP	(96)
β-catenin ↓	↓responsiveness to BMP-2	alters osteoblast differentiation	(97)
β-catenin and Runx2→	↑ BMP-9-induced	osteogenic differentiation	(96)
BMPR1A↓	↑ Wnt (SOST, Dkk1)	bone mass↑	(99)
FGF-2,-9 →FGF/FgfR→	↑ BMP-2 and TGFbeta-1	↑osteogenic expression	(101)
Notch	↑BMP-induced ALP	→Smad and Notch	(105)
Shh (Gli2)→	↑ BMP-2 promoter activity	normal osteoblast differentiation	(110)
Ihh→	↑BMP-induced osteogenesis	bone formation	(113)
Ihh and BMP→	↑ALP, Ihh expression	long bone development	(114)

Note: ↓decrease; ↑increase; → stimulate; × block

TGF-β /BMP signaling in clinical applications

Currently, two BMP products have been approved by the Food and Drug Administration (FDA) for clinical applications to treat fractures of long bones and improve intervertebral disk regeneration through a purified collagen matrix respectively infused with BMP-2 (Medtronic) or OP-1 BMP-7 (Stryker Biotech) and implanted at the site of the fracture. BMP treatment for acute open tibial fractures may be more favorable economically (160). Combination of BMP-7 with a type-one collagen carrier has been the subject of increasing interest. BMP-7 in combination with osteosynthesis revision and bone grafting, or with bone grafting alone, shows that there is no perioperative or postoperative complications in patients (161). The application of BMP-7 in a total of 19 joint fusions (ankle, subtalar, talonavicular, pubic and sacroiliac) resulted in healing rates of 90% and satisfactory subjective functional outcome in 70% of cases (162).

With the use of BMPs increasingly accepted in spinal fusion surgeries, other therapeutic approaches targeting BMP signaling are emerging beyond applications to skeletal disorders. BMP-7 is also regarded as a strong candidate for the clinical treatment of chronic kidney disease (CKD) (163). Administration of BMP-7 prevents the development of adynamic bone disease in a preclinical model of chronic kidney failure (164). TGF-β/BMPs have also been used as the prognostic biomarkers. For instance, GDF-15 has emerged as a prognostic biomarker in acute coronary syndrome trial populations (165) and in cardiac and vascular dysfunction and disease (166). GDF-11 may be a novel diagnostic and prognostic biomarker in patients with colorectal cancer (167). Additionally, GDF-5 has emerged as a therapeutic target for rheumatic diseases (168). Recent applications graft BMP peptides corresponding to residues 73-92, 89-117, and 68-87 of BMP-2, BMP-7, and BMP-9 as adhesion peptides (GRGDSPC) onto polyethylene terephthatalate (PET) surfaces to enhance osteogenic differentiation and mineralization of pre-osteoblastic cells (169). These engineered biomaterials for enhanced bone regneration are in the initial trial stage of development.

SUMMARY AND PERSPECTIVE

Our understanding of bone is rapidly advancing with the use of new technology, such as conditional knockout mice, high-throughput screening, and the discovery of newly recognized cross-talk between TGF-β/BMP signaling and many other major signaling pathways such as MAPK, Wnt, Hedgehog, Notch, and FGF. TGF-β/BMP signaling plays critical regulatory functions in osteoblast differentiation and bone formation. The signaling relays in each stage (ligands, receptors, Smads) are responsible for the final target gene expression. Perturbations of TGF-β/BMP sig-

naling result in various clinical disorders including cancers, bone diseases, and vascular diseases. Thus, there is great potential for the clinical applications of TGF-β/BMP molecules for the treatment of bone-related diseases, such as FOP, chronic kidney disease, brachydactyly type A2, and osteoporosis. More importantly, with the aging population expected to double over the next decade, the number of people suffering from osteoporosis is likely to increase dramatically and so is the cost of Medicare. The current cost of medical care associated with osteoporosis (especially hip fractures) has been estimated at more than $17 billion a year. As such, there is increased pressure to elucidate the pathophysiology of bone diseases and the molecular mechanisms of skeletal remodeling in health and disease.

So far, BMP-2-and BMP-7-containing osteogenic implants have been used in over one million patients worldwide for the treatment of long bone nonunions, spinal fusions, and acute fractures. Apart from their recognized role in bone regeneration, BMPs have been used systemically to improve skeletal volume, kidney regeneration, glucose, and iron metabolism. There are still much to discover, including small molecule inhibitors with special target sites and better effectiveness, as well as better delivery systems and reliable TGF-β/BMPs retention at target sites *in vivo*.

ABBREVIATIONS

PTH: parathyroid hormone; ActR-II: Activin type II receptor; ActR-IIB: Activin type II B receptor; TβR-II, Tgfbr2: TGF-β type II receptor; BMPR-II: BMP type II receptor; AMHR-II: AMH type II receptor; ALK: Activin receptor-like kinase; BMPR-IA: Type I receptor of ALK-3; BMPR-IB: Type I receptor of ALK-6; TAK1: TGF-β activation kinase1; TAB1: TAK1 binding protein 1.

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Conflict of Interests

The authors have declared that no conflict of interest exists.

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