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# THE ROLE OF SMADS IN BMP SIGNALING

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## 1. ABSTRACT

Bone morphogenetic proteins, BMPs, are members of the transforming growth factor-β (TGF-β) superfamily, which are implicated in embryogenesis, organogenesis, skeletogenesis, osteogenesis, cellular differentiation and apoptosis by regulating the expression of specific target genes. Recent progresses in studying the BMP signaling reveal that a cytoplasmic protein family, Smad, plays a central role in mediating the biological effects of BMPs. Smad transduces the signal from the cytoplasm to the nucleus where Smad regulates the transcription of the target genes through the direct association with the specific biding elements or with assistance of other transcription factors or co-activators such as p300/CBP. In addition, the signals mediated by Smad are also positively or negatively controlled by cross-talks with other hormone, growth factor or cytokine signalings, thereby modulating the biological actions of BMPs. Moreover, Smad signaling has negative feedback regulations at the cytoplasmic or nuclear level, which are important to restrict or terminate the biological effect of BMPs. Here we provide an overview of recent knowledge about the roles of Smad family in the regulation of BMP signaling.

## 2. ACTIVATION OF SMAD

BMP exerts its diverse biological effects through two types of transmembrane receptors, BMP type I (BMPRI) and type II receptors (BMPRII) (1, 2), both of which posses intrinsic serine/threonine kinase activity (1, 2) (Figure 1). Upon binding to the type II receptors, BMP induces hetero-dimerization between BMP type I and type II receptors, and subsequently BMPRI is phosphorylated by BMPRII, recruits and phosphorylates R-Smad (receptor-regulated Smad), including Smad1, Smad5 and Smad8 (3-5) (Figure 1 and 2). In contrast to BMPs, other members of TGF-β superfamily such as TGF-β or activin activates other R-Smad such as Smad2 and Smad3 (3-5) (Figure 2). Until BMP or TGF-β elicits the receptors, R-Smad remains inactive by forming intra-molecular association between MH1 and MH2 domain, and is anchored to the cell membrane through the binding to SARA (Smad-anchor for receptor activation) (6) (Figure 1). Activated R-Smad forms the heterocomplex with Smad4, which is categorized as Co-Smad (Common-partner Smad) (Figure 2), and then the complex translocates into the nucleus and regulates the transcription of specific target genes (Figure 1). To date, only one Co-Smad has been cloned and characterized in mammals. In *Xenopus*, Smad4-β (also known as Smad10) was identified as the second Co-Smad that lacks the nuclear export signal, resulting in constitutively localizing in the nucleus (7). Both R-Smad and Co-Smad contain two conserved domains named as MH1 (Mad homology 1) and MH2 domains spanned by a linker region (3-5) (Figure 2). MH1 domain serves as DNA binding domain, whereas MH2 domain has a capacity to associate with type I receptor, Co-Smad, transcription factors, p300/CBP, and SARA (Figure 2). Structural study revealed that the sequence motif in L3 loop of R-Smad that associates with L45 loop of type I receptor defines the ligand-specificity (8). R-Smad but not Co-Smad has SSxS motif at the C-terminal region (Figure 2), which is specifically phosphorylated by corresponding type I receptor (Figure 1 and 2). On the other hand, Smad6 and Smad7 have MH2 domain but lack MH1 domain (Figure 2), consequently Smad6 and Samd7 act as inhibitory Smad (I-Smad) for R-

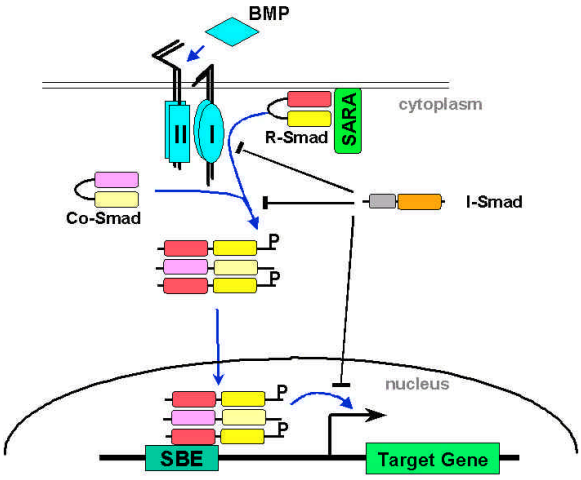


**Figure 1.** Activation and inactivation of Smad signaling.Receptor-regulated Smad (R-Smad), which is recruited to cell membrane by SARA, is ready to respond to ligand stimulation. After activation of type I receptor, R-Smad, is phosphorylated and forms hetero-complex with common Smad (Co-Smad). Subsequently, the hetero-complex translocates into the nucleus and regulates the transcription of target genes. Inhibitory Smad (I-Smad) negatively regulates Smad signaling by blocking the binding of R-Smad to type I receptor, hetero-complex formation between R-Smad and Co-Smad, and the transcriptional regulation by R-Smad in the nucleus.

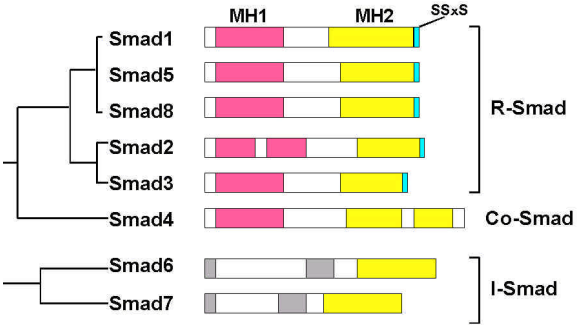


**Figure 2.** Schematic diagram of Smad family. Smad family is classified into receptor-regulated Smad (R-Smad), common-partner Smad (Co-Smad) and Inhibitory Smad (I-Smad). R-Smad contains specific phosphorylation motif, SSxS, at the c-terminal region.

mad and/or Co-Smad by inhibiting the association of R-Smad with type I receptors (9, 10) or its complex formation with Co-Smad (11) (Figure 1 and 2). Interestingly, expression of Smad6 or Smad7 is up-regulated or induced by BMPs or TGF-β (12, 13). Samd7 blocks both BMP and TGF-β signaling, whereas Smad6 seems to be specific for BMP signaling It is also reported that Smad6 function as repressor in the nucleus (14) (Figure 1). Furthermore, I-Smad is involved in degradation of type I receptor through ubiquitin-proteasome system as described below. Thus, I-Smad restricts the magnitude of Smad signaling elicited by BMPs or TGF-β through several different mechanisms (Figure 1). These feedback regulations provide an important fine-tune mechanism for the BMP signaling and control the cellular functions.

## 3. REGULATION OF TRANSCRIPTION BY SMAD

### 3.1. Direct binding of Smad to DNA

Like TGF-β, BMPs mediate the biological effects by stimulating transcriptional actions on the target genes. R-Smad and Co-Smad binds target genes directly through β-hairpin loop present in MH1 domain (15) (Figure 3). Biochemical studies show that CAGAC or AGAC motif is optimal for the binding. Indeed, these Smad binding elements (SBE) exist in the promoter region of plasminogen activator inhibitor-1 (PAI-1), JunB, and IgC α genes that are responded to TGF-β, activin, or BMPs (16-18). However, it is unlikely that this Smad-SBE binding alone is sufficient to define the specificity of Smad signaling based on the following reasons; First, there is no SBE motif in the *goosecoid* gene that is strictly regulated by TGF-β (19). Second, the binding affinity for SBE is not strong enough to achieve the transcriptional regulation of many target genes (15). Third, the sequence of β-hairpin loop is highly conserved in all R-Smad and Co-Smad, and the binding affinities for Smad1, Smad3 and Smad4 to the SBE are similar (15). Fourth, the incidence of SBE sequence in genome is not rare, suggesting that SBE is not able to determine the tissue specific effects of BMPs or TGF-β. Thus, other mechanisms should be required for determining the specificities of Smad and augmenting its binding affinity for the target genes, and the binding to SBE appears to provide the scaffold for Smad.

### 3.2. Association of Smad with tissue-specific transcription molecules

A winged-helix transcription factor family, Fast-1 was identified as a molecule which specifically binds to activin response element (ARE) present in *Xenopus* Mix.2 gene in activin-dependent manner (20) (Figure 3). Since ARE is sufficient to transactivate Mix.2 gene (20), Fast-1 is a key regulator for this transcriptional regulation. Two mammalian homologues of Fast-1, Fast-1 and Fast-2 (19, 21), were also isolated from specifictissues where activin signaling is necessary for embryonic development. The Fast molecules physically associate with α-helix region of MH2 domain of Smad2, and consequently regulate the transcription of the target gene such as Mix.2, *goosecoid* and *Lefty2* genes (19, 21) (Figure 3). Biochemical studies indicate that Smad2 is unable to directly bind these target genes but assists both Smad4 and Fast-2 to bind *goosecoid* gene by forming the complex (19). Collectively, in this paradigm, Smad2/Smad4, which serves as the turning-on/off switch, and Fast family functions determine the specificity of the signals (Figure 3). The transcriptional studies using Smad4 deficient cells support the necessity of complex formation between Smad2 and Smad4 for the transactivation of Mix.2 gene by Fast-1 even though Smad2 does not directly bind to the gene (22). Of interest, Smad3 also physically associates with Fast-2, but inhibits the transcriptional activation of Fast-2 by blocking Smad4 to bind to *goosecoid* gene (19). These observations provide a model that shows the functional

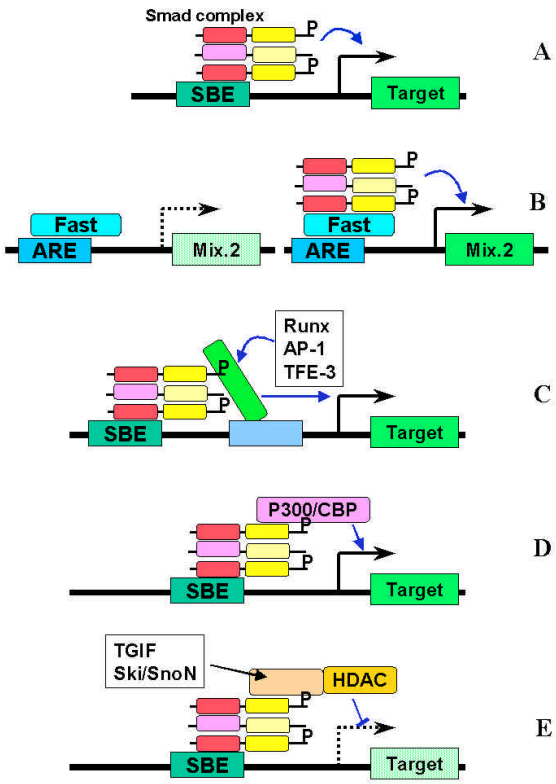


**Figure 3.** Transcriptional regulation by Smad. A: Hetero-complex of R-Smad and Co-Smad binds to Smad binding element (SBE) in the target genes and regulates their transcription. B: Tissue specific transcription factor such as Fast binds to ARE elements in the target genes but Fast itself is not able to transactivate them. When ligand stimulates Smad signal, Smad complex associates with Fast and assists it to exhibit transcription. C: Smad complex bound to SBE interacts with the partner transcription factors including Runx, AP-1 and TFE-3, and synergistically controls the transcription of target genes. D: Smad complex efficiently regulates the transcription of the target genes by association with coactivator P300/CBP. E: When Smad complex associates with repressor, TGIF, Ski or SnoN, the transcription of target genes are shut off.

differences between Smad2 and Smad3 and the bifunctional roles of Fast-2. A zinc-finger protein OAZ is also a bifunctional transcriptional regulator in controlling Xvent-2 gene that is one of the specific target genes for BMPs and controls mesoderm ventralization (23). OAZ is able to associate with activated Smad1, and Smad1/OAZ complex binds to BMP response element (BRE) in Xvent-2 gene and increases Xvent-2 gene promoter activity. In contrast, when a transcription factor Olf-1/EBF exists, OAZ preferentially binds Olf-1/EBF, and transactivates the genes that are involved in development of olfactory epithelium or pre-B lymphocyte through binding to CCGCCC motif, consequently decreasing the transcriptional activity on Xvent-2 gene (23).

### 3.3. Cooperative role of Smad and other transcription factors

Several studies have indicated the functional cooperation between Smad and general transcription factors that enhances their transcriptional activity on the target genes. ATF-2 and c-Jun were shown to be common targets for Smad3 (24-26). Cooperative role of Smad3 and Smad4 with c-Jun/c-Fos complex in the augmentation of AP-1 activity has also been described (26, 27) (Figure 3). In addition, synergistic cooperation of TFE3 and Smad has been found to increase PAI-1 promoter activity in response to TGF-β stimulation (28, 29). Consistent with this notion, the binding region of TFE3 to PAI-1 gene was identified as E-box, which is located adjacent tothe SBE site (28) (Figure 3). Thus, Smad is capable of enhancing the functions of these transcription factors by assembling the formation of this complex.

Smad is also able to associate with the members of a transcriptional family, Runx/Pebp2α/AML/Cbfa (30, 31) (Figure 3). As one of the important aspects in this molecular assembly, Smad3 has been shown to assist Cbfa3 to control the IgA class switching in B lymphocytes by forming the complex (30). Similarly, Smad1 interacts with Runx2/Cbfa1, which is an essential transcriptional factor for bone formation, in a BMP-dependent manner (32, 33), and this complex formation enhances the transcriptional activity of Cbfa1 on osteocalcin promoter (33).

The identification of binding of Smad to SIP1 provides another molecular mechanism by which Smad stimulates the transcription of some genes through releasing the repression. SIP1 works as repressor in basal conditions. When Smad is activated and interacts with SIP1, Smad blocks the SIP1 function and releases SIP1 from DNA, thus transactivating the promoter of specific genes (34, 35).

### 3.4. Recruitment of Co-activator by Smad

To efficiently activate the transcription of the target genes, Smad also recruits the co-activator p300/CBP that has histone acetyltransferase (HAT) activity (36, 37). By this enzymatic activity, the complex enables to loose the nucleosome structure and increases access of basal transcriptional factors including RNA polymerase II (Figure 3). Since p300/CBP is a large molecule and has ability to associate with several transcriptional regulators through different domains, the association of Smad with p300/CBP can link the BMP signaling to other signaling pathways. As an example, Smad1 and Stat3 can assemble the complex through the binding to p300/CBP as discussed below (38) (Figure 3 and 4). In addition, orphan transcriptional activator MSG1, which has a strong transactivating activity but lacks a DNA-binding activity, binds Smad4, which in turn enhances the transcriptional activity of MSG1 (39, 40). These findings suggest that Smad needs the assistance of these co-activators to function optically in the nucleus, although Smad itself apparently has transactivating activity (41, 42).

### 3.5. Suppression of Smad function in the nucleus

To properly mediate the biological effects during cellular differentiation or embryonic development,

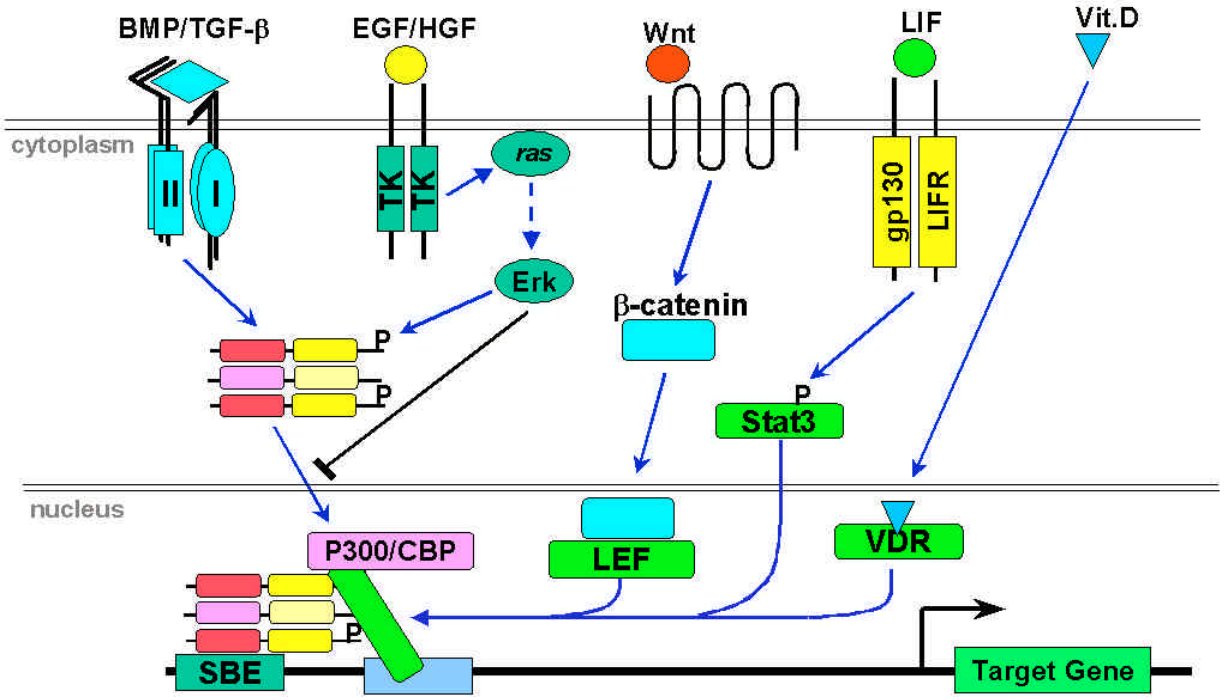


**Figure 4.** Cross-talk between Smad and other signaling. Smad complex directly or indirectly associates with LEF/β-catenin, Stat3 or vitamin D receptor (VDR) which is elicited by Wnt, LIF or vitamin D, respectively. Erk kinase activated by EGF or HGF positively or negatively regulates activation of Smad by phosphorylating c-terminal or linker region, respectively.

BMPs or TGF-β signaling needs to suppress the transcription of some genes or restrict the magnitude of the signals at the transcriptional level. To achieve this, Smad also recruits the co-repressors. A homeo-domain repressor, TGIF, which interacts with histone deacetylases (HDAC), binds to Smad2 or Smad3, and suppresses the transcription regulated by Smad (43-45) (Figure 3). In this case, TGFI seems to block the association of Smad with p300/CBP by interacting the same region. Since the expression of TGIF is induced by ligand stimulation, it is likely that this inhibitory effect by TGIF functions as a negative feedback loop. Similarly, the members of the oncoprotein family, Ski and its related protein SnoN, also interact with Smad2 or Smad3, and negatively regulate the Smad activity by recruiting N-CoR, mSin3A and HDAC to the complex (46-50) (Figure 3). Interestingly, the expression of Ski is down-regulated by degradation when BMP or TGF-β signal is active and necessary in the nucleus (51). However, in some types of cancer cells, Ski is highly expressed and not degraded in response to TGF-β, resulting in shutting down the tumor-suppressive effects of TGF-β (52). This observation suggests the role of oncoprotein Ski and SnoN in oncogenesis.

Similar to Ski and SnoN, a oncoprotein Evi-1 is likely to exhibit the oncogenic activity in hematopoietic cells by suppressing the effects of TGF-β (53). Evi-1, which encodes a zing-finger protein, physically interacts with Smad3 and suppresses its transcriptional activity presumably as a repressor (53). Evi-1 may be involved in the pathogenesis of leukemia, at least in part, through the association with Smad (54).

## 4. NETWORKS WITH OTHER SIGNALING

The biological actions of BMPs or TGF-β are positively or negatively modulated by other hormones, cytokines and growth factors. Vitamin D receptor (VDR) physically interacts with activated Smad2 or Smad3 in the nucleus, and enhances transcriptional activity of Smad2/Smad3 (55) (Figure 4). Although the functional relationship between TGF-β and vitamin D is currently unknown, the observation suggests the functional cooperation between Smad and other nuclear receptors. Wnt/wingless pathway also synergistically communicates with Smad signaling. When Wnt signaling and TGF-β signaling are active, Smad3 associates with β-catenin/LEF-1 complex, and the assembled complex cooperatively transactivates the Xtwn gene promoter (56) (Figure 4). In *Xenopus*, this interaction between Wnt and TGF-β signaling play an important role in formation of Spemann's organization (57, 58).

As described above, Smad1 is able to form complex with Stat3 through binding p300/CBP (Figure 4). Consistent with this, BMP2 and LIF synergistically transactivate the target gene and induce the differentiation of astrocytes in the presence of p300 (38). In contrast, interferon-γ has been shown to induce Smad7 expression through activation of Stat1, thereby inhibiting the action of

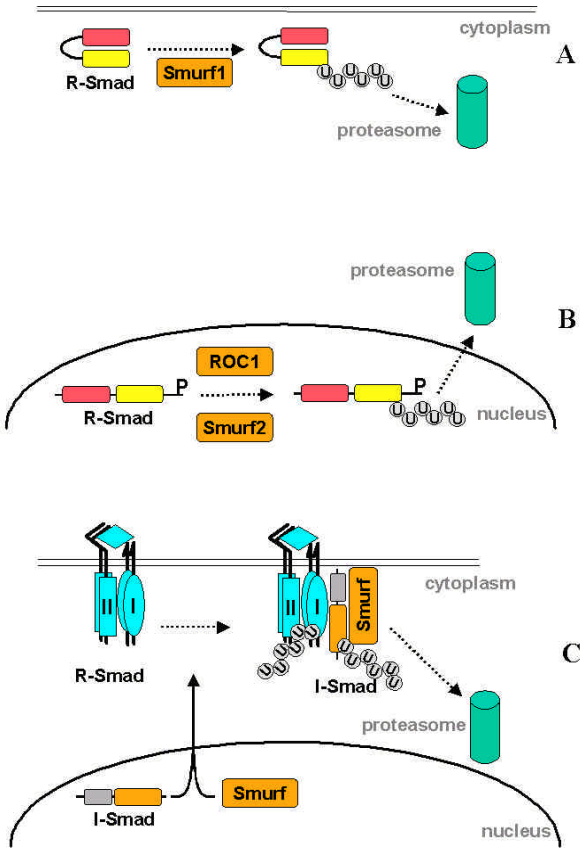


**Figure 5.** Restriction of Smad signaling by ubiquitination-proteasome degradation system. A: Smurf1 interacts with R-Smad and degrades it after ubiquitination. B: In the nucleus, Smurf2 or ROC1 degrades R-Smad after ubiquitination. C: After interaction of Smurf with I-Smad, the complex associates with type I receptor, and Smurf degrades both I-Smad and type I receptor.

TGF-β in the cells (59). These findings indicate that Smad signaling is positively or negatively modulated by the cross-talk with Stat signaling

It is well-known that EGF antagonizes BMP effect. This mechanism has been elucidated, in part, by inactivation of Smad1 (60). A MAP kinase, Erk, which is activated by EGF or HGF stimulation, phosphorylates PxSP motif present in the linker region of Smad1, and this phosphorylated Smad1 is unable to translocate into the nucleus and stimulate the transcription (60) (Figure 4). Consistent with this observation, oncogenic Ras-transformed cells show the resistance to the growth inhibitory effects of TGF-β (61). In contrast, Smad2 has been shown to be phosphorylated at carboxyl-terminal phosphorylation site and activated by HGF stimulation (62) (Figure 4). The reason of this discrepancy between these studies is currently unknown and further investigations are required.

## 5. RESTRICTION OF SMAD SIGNALING BY DEGRADATION

Recently, the evidences that the ubiquitination-proteasome system play roles in regulation of Smad signaling are accumulating. A HECT domain E3 ligase, Smurf1, was initially identified as a molecule that associated with Smad1 (63). Smurf1 has a C2 domain and two WW domains. The WW domain of Smurf1 interacts with PPXY motif in the linker region of Smad1 and Smad5 (63). C2 domain seems to be important for the binding of Smurf1 to the cell membrane (64). After associating with Smurf1, Smad1 and Smad5 are ubiquitinated and degraded in the proteasome (63). It is likely that Smurf1 regulates the magnitude of BMP signaling by controlling the steady-state protein levels of Smad1 and Smad5. Interestingly, since another BMP-regulated Smad, Smad8, lacks PPXY motif, it seems that Smad8 is resistant to Smurf1-dependent degradation (Figure 5). A homologue of Smurf1, Smurf2, plays roles in the degradation of Smad2 in a TGF-β-dependent manner. Unlike Smurf1, Smurf2 interacts only with phosphorylated Smad2 but not unphosphorylated form, and negatively regulates TGF-β signaling by promoting degradation of Smad2 (65) (Figure 5). Smurf2 also degrades SnoN, which is associated with Smad2 (65). Of note, both Smurf1 and Smurf2 also associate with Smad7, which usually exists in the nucleus (66, 67). The complex is exported from nucleus, and recruited to activated TGF-β type I receptor, and Smurfs subsequently degrade TGF-β type I receptor as well as Smad7 (66, 67) (Figure 5). Thus, this paradigm indicates another molecular basis by which I-Smad negatively regulates TGF-β and

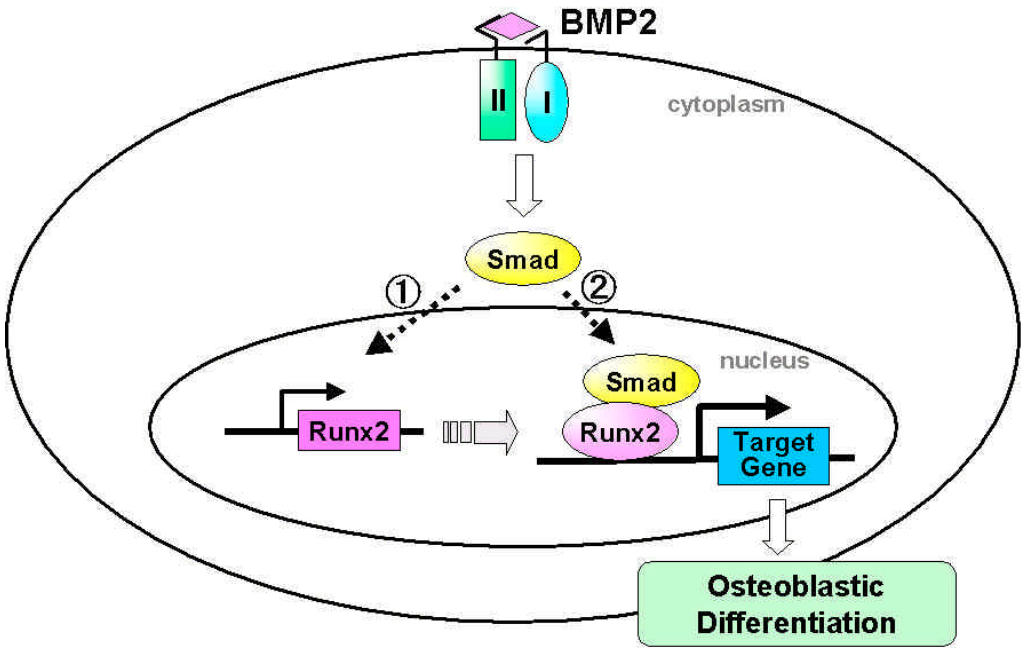


**Figure 6.** Possible molecular mechanisms in induction of osteoblastic differentiation by Smad. After BMP elicits signal, BMP-regulated Smad directly or indirectly up-regulates the expression of Runx2/Cbfa1, and subsequently the Smad interacts with Runx2/Cbfa1 and synergistically controls the transcription of target genes that are necessary for induction of osteoblastic differentiation.

BMP signaling. In addtion to Smurfs, Ring finger protein, ROC1, forms E3 ubiquitin ligase complex with Skp1, Cullin1, and Fbw1a, and degrades Smad2 after ubiquitination (68) (Figure 5). Interestingly, accelerated degradation of mutated Smad2 and Smad4, which are identified in cancer patients, has been reported, and E2 ligase UbcH5 family is likely to be involved in this process (69). This finding suggests the relationship between degradation of Smad and oncogenesis.

## 6. SMAD AND BONE METABOLISMS

BMPs are also known as powerful cytokines that induce bone formation by promoting osteoblast differentiation of mesenchymal stem cells (70, 71). Blockade of Smad signaling by overexpression ofmutant Smad or I-Smad inhibits BMP-induced osteoblast differentiation of undifferentiated mesenchymal cells (72, 73). Consistently, overexpression of Smad1/Smad4 or Smad5/Smad4 is also sufficient to initiate the osteoblast differentiation (74). These observations demonstrate that BMP-regulated Smad, Smad1, Smad5 and probably Smad8 play key roles in osteoblast differentiation of mesenchymal cells. So, the next question would be what is the target of Smad during osteoblastogenesis.

The transcription factor Runx2/Cbfa1/PEBP2αA/Osf2, which belongs to *runt* family, plays important roles in bone formation. Inherited mutations of Cbfa1 cause the cleidocranial dysplasia characterized by severe impairment of osteogenesis in humans (75). Targeted disruption of the Cbfa1 gene resulted in abnormal skeletogenesis with complete lack of osteogenesis in mice (76, 77). In vitro studies have shown that an alternatively-spliced form of Cbfa1 (called Osf2) directly controls the expression of the osteoblast specific genes including osteocalcin, osteopontin and type I collagen during osteoblast differentiation and that overexpression of Cbfa1 promoted osteoblast differentiation in the multipotent mesenchymal C3H10T1/2 cells (78, 79). These findings collectively indicate that the Cbfa1 is an essential transcription factor for osteoblast differentiation of the mesenchymal stem cells and osteogenesis. Importantly, Smad1 and Smad5 physically interact with Runx2 in the nucleus and enhance the osteogenic activity of Runx2 (32, 33). Furthermore, a mutated Cbfa1 found in a patient with the cleidocranial dysplasia is unable to interact with Smad1 (32). These findings suggest the importance of the association between BMP-regulated Smad and Runx2/Cbfa1 in osteoblast differentiation of mesenchymal cells. Expression of Runx2/Cbfa1 during osteoblast differentiation also appears to be controlled by Smad1 and Smad5 (33, 80), although it is not known whether Smad1 and Smad5 directly regulate the transcription of Cbfa1 gene. Thus, Smad1 and Smad5 activated by BMPs control the function and expression of Runx2/Cbfa1, thereby promoting osteoblast differentiation and bone formation (Figure 6). As described above, Smad signaling is negatively controlled at the several levels. Notably, it has been demonstrated that Tob, a member of the emerging family of antiproliferative proteins, is expressed in osteoblasts, and blocks Smad-regulated transcriptional activity through the association with Smad (81). Moreover, orthotopic bone formation in response to BMP-2 is elevated in Tob-deficient mice (81). Collectively, Tob is an inhibitory regulator for BMP-regulated osteogenesis through the interaction with Smad.

Although BMP also promotes and controls the differentiation of chondrocytes, chondrogenesis seems to be independent of Smad signaling because overexpression of Smad6 did not affect the chodrocytic differentiation of ATDC5 cells (73). Since abnormal chondrogenesis has been reported in ATF-2 deficient mice (82), and BMP elicits ATF-2 signaling through p38 kinase, Smad-independent pathway, p38/ATF-2 cascade may be responsible for the regulation of chondrocyte differentiation.

## 7. CONCLUSION

BMPs are a multipotent growth factors and exhibit the tissue- and time-specific effects. As an immediate downstream molecule of BMP signaling, Smad possesses critical functions. First, Smad mediates the signal from corresponding receptor to the nucleus. Second, Smad signaling is tightly and strictly regulated at the several levels. Last, Smad is able to modulate, augment or restrict the signals in the cytoplasm or nucleus through the association with other signaling molecules or transcription factors. Thus, Smad harmonizes the signals by establishing complexand precise regulatory signaling network. Identification of Smad family, the Smad-interacting proteins and regulatory mechanisms have led to a breakthrough in our understanding in the signal transduction and biological functions of BMPs in a variety of tissues and cells.

## 8. ACKNOWLEDGMENTS

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