Regulation of TGF- β Family Signaling by Inhibitory Smads

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Inhibitory Smads (I-Smads) have conserved carboxy-terminal MH2 domains but highly divergent amino-terminal regions when compared with receptor-regulated Smads (R-Smads) and common-partner Smads (co-Smads). Smad6 preferentially inhibits Smad signaling initiated by the bone morphogenetic protein (BMP) type I receptors ALK-3 and ALK-6, whereas Smad7 inhibits both transforming growth factor β (TGF- β)- and BMP-induced Smad signaling. I-Smads also regulate some non-Smad signaling pathways. Here, we discuss the vertebrate I-Smads, their roles as inhibitors of Smad activation and regulators of receptor stability, as scaffolds for non-Smad signaling, and their possible roles in the nucleus. We also discuss the posttranslational modification of I-Smads, including phosphorylation, ubiquitylation, acetylation, and methylation.

Ligands of the transforming growth factor β (TGF- β) family play crucial roles in embryonic development and adult tissue homeostasis. The family includes TGF- β s, activins, bone morphogenetic proteins (BMPs), growth and differentiation factors (GDFs), and Müllerian-inhibiting substance (MIS). These ligands are both structurally related to each other and share the basic machinery for signal transduction. TGF- β family ligands trigger signaling through hetero-oligomerization of two types of transmembrane receptors with intrinsic serine-threonine kinase activities: the type I and type II receptors (Shi and Massagué 2003). Five type II receptors and seven type I receptors, also called activin receptor-like kinase (ALK) 1–7,

have been identified in mammalian cells. In the ligand–receptor complex, the constitutively active type II receptors phosphorylate and activate the type I receptors. The type I receptors then phosphorylate a subgroup of Smad proteins, the receptor-regulated Smads (R-Smads). The R-Smads comprise Smad2 and -3 for TGF- β and activin signaling, and Smad1, -5, and -8 for BMP signaling. Phosphorylated R-Smads form a heterotrimeric complex with a distinct common-partner Smad (co-Smad), Smad4. The complexes then translocate to the nucleus, where they activate or repress gene expression in association with other transcription factors and transcriptional coactivators or corepressors (the Smad signaling pathway). Alternatively, the ac-

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tivated receptors can transmit signals independent of Smad proteins (non-Smad signaling pathways) (Zhang 2009).

TGF- β family signaling is regulated through multiple mechanisms and its amplitude is finely tuned by a variety of positive and negative regulators (Miyazono 2000). Although negative signal regulators are found in other signaling pathways, the TGF- β family signaling systems may be unique, as some negative regulators are structurally related to the components of the signaling pathway. In addition, TGF- β family signaling induces the expression of many of these negative regulators in different types of cells, and these regulators, in turn, repress signaling through negative feedback loops. Lefty 1 and lefty 2 contain cystine-knot motifs and are structurally similar to the TGF- β family ligands, but do not form disulfide-linked dimers (Meno et al. 1999; Thisse and Thisse 1999). Lefty 1 and lefty 2 bind to activin receptors and compete with activins for receptor binding. Inhibins are dimeric proteins composed of an α - and β -chain, and antagonize the effects of activins composed of β -chain dimers (Vale et al. 1988). BAMBI (BMP and activin membrane-bound inhibitor) is a transmembrane protein with extracellular and transmembrane domains structurally similar to those type I receptors, but lacks an intracellular kinase domain (Onichtchouk et al. 1999). BAMBI interacts with type I receptors but is unable to transduce intracellular signals.

Inhibitory Smads (I-Smads) are members of the Smad family with conserved carboxy-terminal MH2 domains, which inhibit intracellular signaling through interactions with activated type I receptors and R-Smads. Smad6 preferentially inhibits Smad signaling by the BMP type I receptors ALK-3 and ALK-6 (Goto et al. 2007), whereas Smad7 inhibits both TGF- β - and BMP-induced Smad signaling (Hanyu et al. 2001). I-Smads also regulate certain non-Smad signaling pathways. Here, we focus on the mechanisms of action of I-Smads in TGF- β family signaling pathways in vertebrates and their relation to certain clinical diseases. We also discuss the functions of I-Smads that are independent of TGF- β family signaling.

STRUCTURES OF I-SMADS

Among the eight different Smad proteins in vertebrates, Smad6 and Smad7 are I-Smads (Hayashi et al. 1997; Imamura et al. 1997; Nakao et al. 1997; Hata et al. 1998; Souchelnytskyi et al. 1998). In *Drosophila*, daughters against DPP (Dad) acts as an I-Smad (Tsuneizumi et al. 1997; Inoue et al. 1998). In *Caenorhabditis elegans*, the TAG68 protein is structurally related to I-Smads, but has not yet been shown to function as an I-Smad (Padgett and Patterson 2006). The carboxy-terminal MH2 domains are conserved between I-Smads and other Smads, but the amino-terminal regions (N domains) of I-Smads diverge from the MH1 domains and linker regions of R-Smads and common-partner Smads (co-Smads) (Fig. 1). I-Smads inhibit TGF- β family signaling through multiple mechanisms, among which interactions with activated type I receptors and activated R-Smads are crucial for the inhibition of Smad-mediated signaling. The MH2 domains are required for interactions with activated type I receptors and R-Smads. R-Smads have an Ser-Ser-X-Ser (SSXS) motif at their carboxyl terminus, which is phosphorylated by type I receptors, whereas I-Smads and the co-Smad lack such a motif and are not phosphorylated by type I receptors.

Only a low level (36.7%) of amino acid sequence identity exists between the N domains of mouse Smad6 and Smad7. In addition, the N domains of Smad6 and Smad7 are not highly conserved between mammals and *Xenopus* (51.3% amino acid sequence identity in Smad6 and 67.4% in Smad7) (Nakayama et al. 1998a,b). A truncated form of Smad6 lacking 235 amino acid residues of the amino terminus is expressed in human endothelial cells (Topper et al. 1997). In this truncated protein (termed Smad6s), the long amino-terminal sequence of Smad6 is replaced with a unique 12 amino acid sequence followed by the carboxy-terminal half of wild-type Smad6. On injection of Smad6s RNA into *Xenopus* embryos, Smad6s antagonizes BMP signaling similarly to full-length Smad6 (Krishnan et al. 2001). Smad6s is expressed in the human coronary artery (Krishnan et al. 2001), with up-regulated expression in athero-

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Figure 1. Structures of Smad proteins. Schematic representation of the structures of Smad6 and Smad7 (I-Smads), Smad3 (R-Smad), and Smad4 (co-Smad). The Leu-rich motif (LRM) is partially conserved in the Smad6 N domain, but its ability to recruit UbcH7 has yet to be determined. The Ser-Ser-X-Ser (SSXS) motif is only present in R-Smads. I-Smads, Inhibitory Smads; R-Smad, receptor-regulated Smad; co-Smad, common-partner Smad; PY, Pro-Tyr; PLDLS, Pro-Leu-Asp-Leu-Ser; Me, methylation site; Ac/Ub, acetylation and ubiquitylation site.

sclerotic vessels (Sandusky et al. 2002; Berg et al. 2005). Similar to most R-Smads, I-Smads have a PY (Pro-Tyr) motif in their middle regions, which is required for recognition by the WW (Trp-Trp) domains of Smurf (Smad ubiquitin regulatory factor) E3 ubiquitin ligases. I-Smads are observed predominantly in the nucleus of most cells, but are also found outside the nucleus in association with plasma membranes. The N domains appear to be a determinant of their subcellular localization (Itoh et al. 1998; Hanyu et al. 2001). TGF- β and BMP-7 induce the nuclear export of Smad7 and Smad6, respectively (Itoh et al. 1998, 2001). In addition, Smurf proteins induce the translocation of I-Smads from the nucleus to the cytoplasm in a nuclear export receptor chromosomal region maintenance 1 (CRM1)-dependent manner (Tajima et al. 2003). Smad6, but not Smad7, has a PLDLS (Pro-Leu-Asp-Leu-Ser) motif,

which recruits the corepressor carboxy-terminal binding protein (CtBP) to Smad6 (Lin et al. 2003). The N domain of Smad7 also has a Leu-rich motif (LRM), which is required for recruitment of the E2 ubiquitin-conjugating enzyme UbcH7 (Ogunjimi et al. 2005). Although the LRM is partially conserved in Smad6, it is unknown whether the LRM of Smad6 interacts with UbcH7.

MECHANISMS UNDERLYING THE FUNCTIONS OF I-SMADS

I-Smads inhibit TGF- β family signaling through various mechanisms (Fig. 2), including interfering with the interactions between R-Smads and type I receptors, down-regulation of cell surface type I receptors in cooperation with other regulators, prevention of complex formation by R-Smads and co-Smads, and tran-

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Figure 2. Regulation of transforming growth factor β (TGF- β) family signaling through I-Smad association with activated type I receptors. (A) Inhibitory Smads (I-Smads) inhibit TGF- β family signaling via interactions with type I receptors and compete with receptor-regulated Smads (R-Smads) for receptor activation. The N domain of Smad7 associates with the MH2 domain and facilitates the interaction with type I receptors. (B) Smad ubiquitin regulatory factors (Smurfs) and other E3 ubiquitin ligases induce the degradation of receptors through interactions with I-Smads. UbcH7 is recruited to the Smad7–Smurf2 complex. Salt-inducible kinase (SIK) cooperates with the complex, whereas ubiquitin-specific peptidase 15 (USP15) counteracts the Smad7–Smurf2 complex. (C) The growth arrest and DNA damage protein 34 (GADD34)–PP1c complex induces dephosphorylation of activated type I receptors through interactions with I-Smads. (D) Toll-interacting protein (Tollip) interacts with Smad7 and ubiquitinated type I receptor to facilitate the endosomal localization of receptors, possibly leading to their lysosomal degradation.

scriptional regulation in the nucleus. I-Smads also regulate non-Smad signaling pathways induced by TGF- β family proteins and control other signaling pathways and transcription factors that do not directly mediate TGF- β family signaling.

Inhibition of TGF- β Family Signaling through Direct Interaction with Type I Receptors

The MH2 domains of I-Smads associate directly with activated type I receptors and thus compete with R-Smads for activation by the receptors (Fig. 2A). The L3 loop in the MH2

domain of R-Smads plays an essential role in determining their specificity for binding type I receptors. Although the L3 loops in I-Smads do not provide binding specificity, they are indispensable for the association of I-Smads with type I receptors and cannot be replaced by the L3 loop of Smad4 (Kamiya et al. 2010). Smad7 uses two distinct protein surfaces in the MH2 domain, both of which include the L3 loop, for its interaction with type I receptors. One surface is the basic groove that includes the L3 loop and α -helix 1, which is also important in R-Smads for their binding specificity with type I receptors (Mochizuki et al. 2004). The other surface

is a three-finger-like structure consisting of residues 331–361, residues 379–387, and the L3 loop (Kamiya et al. 2010). Smad7 can use both surfaces in its interaction with the ALK-2, -3, and -4 receptors, but only the basic groove is used in the interaction between Smad7 and the TGF- β type I receptor (T β RI, also known as ALK-5). In contrast, Smad6 exclusively uses the basic groove in its interaction with the BMP type I receptor ALK-3.

Although the MH2 domains of I-Smads are required for interactions with type I receptors, the Smad7 MH2 domain is not sufficient for maximum inhibition of TGF- β signaling, and the N domain of Smad7 plays an important role in efficiently repressing TGF- β signaling. A chimeric protein composed of the N domain of Smad7 and the MH2 domain of Smad6 is as potent as wild-type Smad7 in inhibiting TGF- β signaling (Hanyu et al. 2001). The R-Smad MH1 domain may physically interact with the MH2 domain, interfering with the R-Smad association with type I receptors and complex formation with Smad4 (Hata et al. 1997). In contrast, the N domain of Smad7 physically associates with the MH2 domain and facilitates interactions between the MH2 domain and type I receptors (Hanyu et al. 2001; Nakayama et al. 2001).

Inhibition of TGF- β Family Signaling through Effector Recruitment to Type I Receptors

In addition to the direct inhibition of type I receptor kinase activity, I-Smads inhibit signaling in cooperation with other proteins by affecting the fate of type I receptors after activation.

BAMBI is a homolog of the type I receptors and functions as a general negative regulator of TGF- β family signaling by interfering with the formation of a functional complex by type I and type II receptors (Onichtchouk et al. 1999; Yan et al. 2009). In addition, through the formation of a ternary complex with Smad7 and the T β RI receptor, BAMBI suppresses the association of R-Smads with the receptors, blocking their phosphorylation. The inhibitory effect of Smad7 on TGF- β signaling is partially attenuated on silencing BAMBI expression and, con-

versely, that of BAMBI is partially attenuated on silencing Smad7 (Yan et al. 2009).

The HECT (homologous to the E6-accessory protein) type E3 ligases Smurf1 and Smurf2 physically interact with I-Smads and enhance their inhibition of TGF- β family signaling. Smurf1 and Smurf2 were originally identified as molecules that associate with and degrade R-Smads (Zhu et al. 1999; Lin et al. 2000; Zhang et al. 2001). The WW domains of Smurfs are involved in their interaction with the PY motifs of Smads (Zhu et al. 1999; Zhang et al. 2001). In addition to targeting R-Smads for degradation, leading to inhibition of TGF- β family signaling, Smurfs associate with Smad6 and Smad7 and assist in their association with type I receptors, interfering with the interactions between R-Smads and receptors (Fig. 2A) (Kavsak et al. 2000; Ebisawa et al. 2001; Murakami et al. 2003). Smurf1 has functional nuclear export signals in the HECT domain and facilitates the export of I-Smads from the nucleus to the cytoplasm in a CRM1-dependent fashion (Tajima et al. 2003). Moreover, Smurf1 has a C2 domain in its amino-terminal region that targets the Smurf1–Smad7 complex to the plasma membrane after nuclear export and enhances the interaction of Smad7 with the activated T β RI receptor (Suzuki et al. 2002). Such cooperative action of Smad6 and Smurf1 has been shown in vivo using transgenic mice expressing Smad6 and Smurf1 (Horiki et al. 2004). Although transgenic mice with increased expression of Smurf1 in chondrocytes do not show significant abnormalities, double-transgenic mice overexpressing Smad6 and Smurf1 in chondrocytes show a greater delay in endochondral ossification than Smad6 transgenic mice, suggesting that Smurf1 enhances the effects of Smad6 in vivo (Horiki et al. 2004).

In addition to promoting R-Smad degradation, Smurfs induce the ubiquitylation and proteasomal degradation of type I receptors and down-regulate the number of receptors at the cell surface, resulting in the suppression of TGF- β family signaling (Fig. 2B). Ubiquitylation is induced by an enzyme cascade, including activating (E1), conjugating (E2), and ligating (E3) enzymes. Smad7 facilitates the interaction be-

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tween Smurf2 and the E2 enzyme UbcH7 (Ogunjimi et al. 2005). The N domain of Smad7 interacts with the HECT domain of Smurf2, as well as UbcH7 via its LRM sequence (see Figs. 1 and 2B). Thus, Smad7 regulates the function of Smurf2 by recruiting UbcH7 to the HECT domain and facilitates the degradation of type I receptors.

Salt-inducible kinase (SIK) is a serine-threonine kinase of the AMP-activated protein kinase family. SIK interacts and cooperates with the Smad7–Smurf2 complex, facilitating the down-regulation of activated T β RI receptor (Kowanetz et al. 2008; Lönn et al. 2012). The protein kinase activity of SIK enhances the down-regulation of T β RI by Smurf2, but the substrates involved in this pathway are unknown. SIK expression is induced by TGF- β and BMP signaling; thus, SIK functions as a negative feedback regulator.

In contrast, ubiquitin-specific peptidase 15 (USP15) is a deubiquitylating enzyme that counteracts the degradation of type I receptors by the complex of Smad7 and Smurf2 (Eichhorn et al. 2012). USP15 is recruited to the T β RI receptor by interacting with the Smad7–Smurf2 complex, then deubiquitylates and stabilizes T β RI. The *USP15* gene is amplified in glioblastoma, breast carcinoma, and ovarian carcinoma, and potentiates their malignant phenotypes by enhancing TGF- β signaling.

Some other HECT type E3 ligases, such as WWP1/Tiul1 and NEDD4-2, also inhibit TGF- β and BMP signaling by promoting the degradation of type I receptors (Komuro et al. 2004; Seo et al. 2004; Kuratomi et al. 2005). AIP4/Itch is another HECT type E3 ligase that interacts with Smad7. Interestingly, AIP4/Itch inhibits TGF- β signaling by enhancing the interaction of Smad7 with the T β RI receptor, but it does not induce the degradation of T β RI (Lallemand et al. 2005). However, AIP4/Itch has been reported to promote the ubiquitylation of Smad2 and enhance TGF- β -induced transcription (Bai et al. 2004), suggesting that AIP4/Itch may modulate TGF- β signaling in a context-dependent fashion.

Smad7 has also been shown to interact with IGADD34 (growth arrest and DNA dam-

age protein 34), a regulatory subunit of the protein phosphatase 1 (PP1) holoenzyme that recruits the catalytic subunit of PP1 (PP1c) (Fig. 2C) (Shi et al. 2004). Smad7 facilitates dephosphorylation of the activated T β RI receptor through recruitment of the GADD34–PP1c complex. The expression of both GADD34 and Smad7 is induced by UV light irradiation, leading to TGF- β resistance in epithelial cells exposed to UV light. In endothelial cells, TGF- β activates both the T β RI/ALK-5 receptor and the ALK-1 receptor, a type I receptor that is activated by either TGF- β or BMP-9/10 and phosphorylates Smad1/5/8 (Goumans et al. 2003). By activating ALK-1, TGF- β induces the expression of Smad7 and PP1 α , a mammalian isoform of PP1c, in endothelial cells. Smad7 then recruits PP1 α to ALK-1 and attenuates the ALK-1-induced activation of Smad1 and 5 (Valdimarsdottir et al. 2006).

Toll-interacting protein (Tollip) is an adaptor protein that consists of a Tom1 binding domain (TBD), a C2 domain, and a coupling of ubiquitin to endoplasmic reticulum degradation (CUE) domain. Tollip interacts with the MH2 domain of Smad7 through its C2 domain. In response to TGF- β , and with the aid of Smad7, Tollip associates with the ubiquitylated T β RI receptor through its TBD and CUE domains, facilitating endosomal localization of T β RI (Fig. 2D). Tollip may promote the degradation of T β RI without affecting Smurf-mediated degradation (Zhu et al. 2012). Thus, Smad7 interacts with activated type I receptors and represses TGF- β family signaling through competition with R-Smads for receptor interaction, promoting proteasomal degradation of receptors by Smurfs, dephosphorylation of activated receptors by PP1, and facilitated endosomal localization by Tollip (Fig. 2).

Interference in R-Smad Complex Formation with Co-Smad

Smad6 interacts with activated Smad1 and inhibits BMP signaling by interfering with the formation of a complex between Smad1 and Smad4 (Hata et al. 1998). In addition, the association of Smurf1 with I-Smads can result in an

indirect association of Smurf1 with Smad1/5, leading to Smad1/5 ubiquitylation and degradation (Murakami et al. 2003). BMP stimulates the interaction of I-Smads with Smad1 and -5, which is further enhanced by Smurf1 (Hata et al. 1998; Murakami et al. 2003). Similarly, Smad7 can inhibit TGF- β signaling by targeting Smad2/3. Smad7 forms a heteromeric complex with activated Smad2/3 and interferes with Smad2/3–Smad4 complex formation. It also recruits the HECT-type E3 ligase NEDD4-2 to the Smad2/3–Smad7 heteromeric complex and facilitates the ubiquitylation and degradation of phosphorylated Smad2/3 (Yan et al. 2016). Consistently, a Smad7 mutant that fails to interact with T β RI still inhibits signaling induced by TGF- β (Kamiya et al. 2010).

Direct Transcriptional Regulation of TGF- β Family Signaling by I-Smads in the Nucleus

I-Smads are predominantly located in the nucleus in most cell types and can act as transcriptional regulators in the nucleus. Smad7 has been reported to interfere with the formation of functional Smad–DNA complex, with Smad7 interacting with the Smad-binding DNA element through its MH2 domain (Zhang et al. 2007). Smad7 fused to the DNA-binding domain of GAL4 represses Gal4 luciferase reporter genes (Pulaski et al. 2001; Yan et al. 2014), and this activity is enhanced in cooperation with YY1 and histone deacetylase 1 (HDAC-1) (Yan et al. 2014), suggesting that Smad7 acts as a transcriptional corepressor.

Smad6 interacts with the transcriptional corepressor CtBP through its PLDLS motif in the linker region (Fig. 1) (Lin et al. 2003). Because the PLDLS motif is not conserved in Smad7, only Smad6 can recruit CtBP. Smad6 possibly associates with the *Id1* promoter DNA through interactions with Smad1 and represses BMP-induced transcription of *Id1* in the nucleus. CtBP represses transcription in HDAC-dependent and -independent fashions depending on the promoter context. Whether the transcriptional repression of *Id1* by Smad6 is dependent on HDACs is unknown.

Smad6 also interacts with the homeobox transcription factors Hoxc-8 and -9 on BMP stimulation and inhibits the transcription of *osteopontin* (Bai et al. 2000). Smad6 interacts with some HDACs, including HDAC-1 and -3, through its MH2 domain. In addition, Smad6 has been reported to bind to DNA through its N domain and recruit HDACs to DNA (Bai and Cao 2002). The HDAC inhibitor trichostatin A abolishes the repressive effect of Smad6 on BMP signaling. Furthermore, Smad6 inhibits the interaction of Smad1 with Hoxc-8 and suppresses transcription by Smad1.

Roles of I-Smads in Non-Smad Signaling Pathways

In addition to the Smad pathway, TGF- β can transmit signals through non-Smad pathways, many of which still remain to be elucidated in detail. Among these, the TRAF6 (tumor necrosis factor [TNF] receptor-associated factor 6) adaptor protein leads to activation of p38 mitogen activated kinase (MAPK) and c-Jun amino-terminal kinase (JNK) signaling (Sorrentino et al. 2008; Yamashita et al. 2008). Smad6 and Smad7 have been shown to play distinct roles in their activation.

In response to TGF- β , TRAF6 associates with the heterotetrameric TGF- β receptor complex through its TRAF homology domain. TRAF6 then induces K63-linked polyubiquitylation of itself, as well as TAK1 (TGF- β activated kinase 1), which is recruited to the T β RI receptor by Smad7. TAK1 is a MAPKKK activated in response to TGF- β , interleukin-1, and several other inducers (Yamaguchi et al. 1995; Nino-miya-Tsuji et al. 1999; Shim et al. 2005). K63-linked polyubiquitylation activates TAK1, which triggers the activation of p38 MAPK or JNK by MAP kinase kinase 3 (MKK3) or MKK6, inducing apoptosis (Edlund et al. 2003) or actin reorganization through activation of small GTPases Cdc42 and RhoA (Edlund et al. 2004). Smad7 is required for the TRAF6 pathway, as its scaffold function allows assembly of TAK1, MKK3, and p38 MAPK, and facilitates the activation of p38 MAPK (Edlund et al. 2003; Jung et al. 2013).

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In contrast, Smad6 negatively regulates the TRAF6 pathway (Jung et al. 2013). TGF- β induces the expression of Smad6 through Smad-mediated transcriptional activation (Afrakhte et al. 1998). Smad6 then associates with TRAF6 and the TNF- α -induced protein A20 (Krikos et al. 1992) via distinct regions of the protein. A20 inhibits several ubiquitin ligases, including TRAF6, and functions as a negative regulator of the NF- κ B pathway. Smad6 suppresses the activation of p38 MAPK and JNK by facilitating the inhibition of TRAF6 by A20 (Jung et al. 2013).

Thus, Smad7 effectively enhances the TGF- β -induced noncanonical TRAF6-p38-JNK pathway, acting as a scaffold to facilitate TAK1-mediated activation of downstream kinases and also inhibiting the induction of Smad6 expression through suppression of the Smad pathway. These findings explain previous reports that increased Smad7 expression leads to the apoptosis of some epithelial cell lines (Landström et al. 2000; Lallemand et al. 2001; Mazars et al. 2001), probably because of increased TRAF6 pathway activity.

Regulation of Other Signaling Pathways by I-Smads

TGF- β has anti-inflammatory activity that is mediated through the induction of I-Smads. Specifically, Smad7 aids in the inhibition of proinflammatory TNF- α signaling (Lallemand et al. 2001). The TNF receptor 1 signals through TRAF2, which activates TAK1 in the presence of TAB2 and TAB3, but Smad7 sequesters TAB2 and TAB3, inhibiting the activation of TAK1 and downstream NF- κ B (Hong et al. 2007). Accordingly, Smad7 induces apoptosis in podocytes through inhibition of NF- κ B signaling (Schiffer et al. 2001).

Smad6 also suppresses innate immunity responses. Toll-like receptors (TLRs) are involved in innate immunity by excluding invading pathogens. Among the TLRs, TLR4 and TLR2 signal through MyD88 to induce the activation of NF- κ B. TGF- β inhibits the MyD88-dependent pathway by inducing Smad6 expression. Smad6 then recruits Smurfs to MyD88 and triggers its polyubiquitylation and proteasomal degrada-

tion (Lee et al. 2011). In addition, Smad6 sequesters Pellino, an adaptor protein that interacts with interleukin-1 receptor-associated kinase 1 (IRAK1) and interferes with the formation of the IRAK1-mediated signaling complex downstream of MyD88 in the TLR4 signaling pathway (Choi et al. 2006). A subregion of the Smad6 MH2 domain spanning amino acid residues 422–441 is responsible for the association with Pellino. Smaducin-6, a palmitic acid-conjugated peptide containing the Pellino-binding sequence, is therapeutically effective in cecal ligation puncture-induced sepsis, a mouse model of TLR4-mediated inflammatory disease (Lee et al. 2015).

Wnt proteins transmit signals through glycogen synthase kinase 3 β (GSK-3 β) and stabilize β -catenin. The expression of c-myc and other genes is induced by complexes of β -catenin with one of the related transcription factors, lymphoid enhancer-binding factor 1 (LEF1) or T-cell factor (TCF). Smad7 has also been shown to interact with β -catenin and LEF1 or TCF in response to TGF- β stimulation, and to induce β -catenin accumulation in a p38 MAPK-dependent manner (Edlund et al. 2005). Thus, the induction of c-myc expression by β -catenin may contribute, at least in part, to the induction of apoptosis by Smad7. Smad7 is also required for the TGF- β -induced phosphorylation of Akt and GSK-3 β (Edlund et al. 2005). Thus, Smad7 appears to function as a scaffold protein for direct activation of p38 MAPK and other signaling pathways. However, Smad7 inhibits apoptosis by suppressing TGF- β signaling in some cells (Yamamura et al. 2000; Arnold et al. 2004), suggesting that these signaling pathways may be activated by Smad7 in cell-type-specific and context-dependent fashion. Smad6 plays an important role in promoting the exit of neuronal progenitor cells from the cell cycle and inducing neuronal differentiation in the developing chick dorsal spinal cord, where Smad6 recruits CtBP to the β -catenin/TCF complex, suppressing the Wnt/ β -catenin pathway (Xie et al. 2011).

Smad7, but not Smad6, has been shown to interact through its MH2 domain with c-Cbl in keratinocytes (Ha Thi et al. 2015). Smad7

destabilizes epidermal growth factor (EGF)-induced complex formation between c-Cbl and the epidermal growth factor receptor (EGFR), inhibiting ligand-induced ubiquitylation and degradation of EGFR (Ha Thi et al. 2015). These observations are in contrast to the enhanced EGFR signaling in *Smad7* ^{Δ ex1} mice (Krampert et al. 2010), suggesting multiple and complex functions of Smad7 in the regulation of EGFR signaling.

Smad7 can also directly interact with other transcription factors and regulate their stability and/or function. Smad7 promotes skeletal muscle differentiation through association with MyoD, a master regulator of myogenic differentiation, and enhances its transcriptional activity (Kollias et al. 2006) by protecting it from repression by MEK (Miyake et al. 2010). In turn, MyoD binds to the *Smad7* proximal promoter region and induces its expression. Thus, Smad7 and MyoD form a positive feedback loop to drive myogenic differentiation (Kollias et al. 2006). When Smad7 interacts with interferon regulatory factor 1 (IRF1), it increases the affinity of this transcription factor for the interferon-stimulated response element (ISRE) DNA sequence, regulating the cell death pathway by enhancing the expression of target genes, including the gene encoding caspase 8 (Hong et al. 2013). Smad7 also interacts with c-Myc, but it induces the down-regulation of c-Myc protein via ubiquitylation-mediated proteolysis on recruitment of the F-box protein Skp2, leading to cytostasis (Kim et al. 2014).

Smad6 interacts with the glucocorticoid receptor and represses the transactivation induced by glucocorticoid receptor through recruitment of HDAC-3 (Ichijo et al. 2005). Smad6 also cooperates with Smurf1 in the degradation of Runx2 (Shen et al. 2006). Although Smad6 functions in many cases as a transcriptional repressor, it appears to enhance the expression of osteopontin, Hex, and Id2 during macrophage differentiation by binding to their promoter regions either directly or indirectly (Glesne and Huberman 2006). During macrophage differentiation, Smad6 is phosphorylated at Ser435 by protein kinase X, which may regulate the nuclear function of Smad6 (Glesne and Huberman 2006).

CONTROL OF I-SMAD FUNCTIONS BY POSTTRANSLATIONAL MODIFICATIONS AND PROTEIN INTERACTIONS

The functions of I-Smads are regulated by posttranslational modifications and interactions with other proteins, which in turn control their stability and association with receptors (Fig. 3).

Regulation of I-Smad Protein Stability

Multiple proteins, including ubiquitin ligases, promote or enhance TGF- β family signaling by inducing degradation of I-Smads in cooperation with accessory molecules. Smad7 is ubiquitylated at Lys64 and Lys70. These lysines are also acetylated by the acetyltransferase p300 (Grönroos et al. 2002), conferring resistance to ubiquitylation. In addition, Smad7 interacts with HDACs, associating with class I HDACs (HDAC-1 and -3) and class II HDACs (HDAC-5 and -6) through its MH2 domain (Simonsson et al. 2005), and with the class III HDAC sirtuin 1 (SIRT1) through its N domain (Kume et al. 2007). HDAC-1 and SIRT1 deacetylate Smad7 and facilitate its ubiquitylation. Thus, modification of Smad7 by acetylation, deacetylation, and ubiquitylation determines its stability. TGF- β signaling inhibits the acetylation of Smad7, although the interaction between Smad7 and HDACs occurs independently of TGF- β signaling (Grönroos et al. 2002; Simonsson et al. 2005). The regulatory mechanisms for these modifications to Smad7 remain to be elucidated.

Arkadia (also known as RNF111), a RING type E3 ubiquitin ligase, was first identified as a protein that enhances signaling by Nodal, inducing the Spemann's organizer during early embryogenesis (Episkopou et al. 2001; Niederlander et al. 2001). Arkadia controls the amplification of TGF- β family signaling through interactions with I-Smads. In contrast to Smurfs, Arkadia induces ubiquitin-dependent degradation of Smad6 and Smad7 but not of the type I receptors, leading to enhanced TGF- β family signaling (Koinuma et al. 2003; Tsubakihara et al. 2015). The enhancement of Smad signal-

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Figure 3. Molecules regulating the function of inhibitory Smads (I-Smads). In addition to homologous to the E6-accessory protein (HECT)-type E3 ligases (Smurf1, Smurf2, WWP1/Tiul1, and NEDD4-2), other proteins promote the association of I-Smads with activated type I receptors, although they do not directly induce type I receptor degradation. In contrast, PRMT1, UBE2O, AMSH, and TSC-22 inhibit the interaction of I-Smads with receptors and receptor-regulated Smads (R-Smads). Arkadia, RNF12, Jab1/CSN5, and Cbl-b induce the degradation of Smad7. Transcription of *Smad7* is regulated by various transcription factors and repressors. microRNAs (miRNAs) inhibit translation or induce the decay of *Smad7* messenger RNA (mRNA). Molecules that inhibit transforming growth factor β (TGF- β) family signaling by supporting the function of I-Smads are shown in red, and those that enhance TGF- β signaling by inhibiting I-Smad function are shown in blue.

ing by Arkadia is also attributed to its ability to down-regulate c-Ski and SnoN, which suppresses Smad signaling in the nucleus (Levy et al. 2007; Nagano et al. 2007; Le Scolan et al. 2008). The association of Arkadia with Smad7, but not c-Ski or SnoN, is enhanced in the presence of Axin (Liu et al. 2006; Koinuma et al. 2011), a scaffold protein that assembles APC, GSK-3 β , and casein kinase I α , and regulates Wnt signaling through degradation of β -catenin. Axin forms a ternary complex with Smad7 and Arkadia to facilitate the degradation of Smad7 (Liu et al. 2006). Similarly, the association of Arkadia with c-Ski, but not Smad7 nor SnoN, is enhanced in the presence of RB1CC1 (RB1-inducible coiled-coil 1, also known as FIP200) (Koinuma et al. 2011). Thus, Arkadia appears to require accessory proteins to preferentially target regulators of Smad signaling.

Screening ubiquitin ligases that regulate TGF- β signaling using a small interfering RNA (siRNA) library showed that, in addition to Arkadia (Levy et al. 2007), RING-H2 finger protein 12 (RNF12) also enhances TGF- β family signaling through ubiquitin-dependent degradation of Smad7 (Zhang et al. 2012). Furthermore, the nuclear receptor NR4A1 interacts with Axin2 and Smad7, facilitating ubiquitylation of Smad7 in cooperation with Arkadia or RNF12, and strongly enhancing TGF- β signaling (Zhou et al. 2014).

Jab1/CSN5 is a component of the COP9 signalosome complex involved in protein degradation through the ubiquitin-proteasome pathway. Jab1/CSN5 interacts with and translocates Smad7 from the nucleus to the cytoplasm, facilitating its degradation (Kim et al. 2004a). However, Jab1/CSN5 also inhibits

TGF- β -induced Smad signaling by inducing the degradation of Smad4 (Wan et al. 2002), suggesting that Jab1/CSN5 may positively or negatively regulate TGF- β family signaling through the degradation of Smad7 and/or Smad4.

Cbl-b is a RING type E3 ubiquitin ligase highly expressed in T cells that inhibits T-cell activation. *Cblb*^{-/-} mice develop spontaneous autoimmunity (Bachmaier et al. 2000), and CD4⁺CD25⁻Foxp3⁻ effector T cells from *Cblb*^{-/-} mice do not efficiently convert into Foxp3⁺ regulatory T cells in response to TGF- β in vitro (Wohlfert et al. 2006). Subsequently, Cbl-b was shown to interact with and ubiquitylate Smad7, leading to decreased Smad7 levels and efficient TGF- β signaling in T cells (Gruber et al. 2013). Consistent with these findings, the repression of interleukin-2 and interferon- γ expression by TGF- β was attenuated in T cells prepared from *Cblb*^{-/-} mice, but restored in those prepared from *Cblb*^{-/-}/CD4Cre-Smad7^{fl/fl} mice (Gruber et al. 2013).

Hydrogen peroxide-inducible clone-5 (Hic-5), an adaptor protein containing LIM domains, interacts with Smad7 and induces its down-regulation (Wang et al. 2008), which is not inhibited by proteasomal inhibitors. Because Hic-5 also associates with Smad3 and inhibits Smad3-dependent transcription (Wang et al. 2005), it may preferentially enhance Smad2-dependent and non-Smad signaling through targeted degradation of Smad7 (Wang et al. 2008).

Regulation of the Interaction between I-Smads and Type I Receptors

Smad6 is methylated at Arg38 by protein arginine *N*-methyltransferase 1 (PRMT1) (Inamitsu et al. 2006; Xu et al. 2013). The methylation is facilitated in the BMP-induced receptor complex; PRMT1 associates with the type II receptor while Smad6 interacts with the type I receptor (Xu et al. 2013). The resultant methylation of Smad6 in response to BMP leads to its dissociation from the type I receptor, permitting efficient signal transduction through phosphorylation of Smad1, -5, and -8. This reaction,

a prerequisite for BMP signaling, partly explains the slow kinetics of Smad phosphorylation after BMP stimulation.

The UBE2O (ubiquitin-conjugating enzyme E2O, also known as E2-230K), which functions as an E2-E3 hybrid ubiquitin ligase, was identified as an I-Smad-binding protein in a proteomics screening (Zhang et al. 2013b). UBE2O interacts with and monoubiquitylates Smad6 at Lys174, reducing the interaction between Smad6 and BMP type I receptors. Thus, UBE2O promotes BMP signaling by suppressing Smad6 function. UBE2O also interacts with Arkadia and RNF12, recruiting these proteins to Smad7 and facilitating the polyubiquitylation of Smad7 (Zhang et al. 2013b). The contribution of this mechanism to TGF- β family signaling is yet to be examined.

Smad7 is phosphorylated at Thr96 by the murine protein serine/threonine kinase 38 (MPK38), which results in the translocation of Smad7 from the nucleus to the cytoplasm, enhancing the inhibitory activity of Smad7 at the T β RI receptor (Seong et al. 2010). Similarly, Smad6 is phosphorylated at Thr176 by MPK38, enhancing the inhibition of BMP signaling by Smad6 (Seong et al. 2010). In contrast, phosphorylation of Smad7 at Ser249 by other unknown kinase(s) does not significantly affect the inhibitory activity of Smad7 on TGF- β signaling (Pulaski et al. 2001).

Another protein, AMSH (associated molecule with the SH3 domain of STAM), binds Smad6 on BMP stimulation and antagonizes the inhibitory effects of Smad6 by preventing the interaction of Smad6 with BMP type I receptors and Smad1 (Itoh et al. 2001). In response to BMP-7 stimulation, Smad6 is exported to the cytoplasm and colocalizes with AMSH. BMP signaling induces the phosphorylation of AMSH by JNK and/or p38 MAPK, leading to attenuation of the antagonistic effects of AMSH on Smad6 function (Itoh et al. 2001). AMSH2, an AMSH-related protein, also interacts with Smad7 and suppresses its inhibitory activity (Ibarrola et al. 2004). The activity of AMSH is regulated by RNF11, a small RING finger protein that interacts with Smurf2 and AMSH (Li and Seth 2004). The RNF11-Smurf2

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complex induces ubiquitin-dependent degradation of AMSH, resulting in the inhibition of TGF- β family signaling in RNF11- and Smurf2-expressing cells. AMSH belongs to a family of deubiquitylating enzymes, but whether the deubiquitylation activity is required for its inhibitory action on I-Smads is unclear.

Tob (transducer of ErbB2) is a member of the “antiproliferative protein” family, which also includes Tob2, BTG1, BTG2/PC3/TIS21, and BTG3. Tob associates with Smad1, -5, and -8, and represses BMP-dependent transcription in osteoblasts (Yoshida et al. 2000). In addition, Tob and Tob2 interact with I-Smads and attenuate BMP signaling by enhancing the interaction of I-Smads with activated BMP type I receptors at the plasma membrane (Yoshida et al. 2003). Similarly, YAP65 and STRAP interact with Smad7, facilitate its association with activated type I receptors, and augment its inhibitory activity (Datta and Moses 2000; Ferrigno et al. 2002).

TGF- β -stimulated clone 22 (TSC-22), a protein with a conserved TSC box and a leucine zipper motif, interferes with the association of the Smad7–Smurf complex with T β RI, preventing ubiquitylation-dependent receptor degradation (Yan et al. 2011). Because TSC-22 is posttranscriptionally up-regulated by TGF- β , it is a positive feedback regulator of TGF- β signaling.

CONTROL OF I-SMAD EXPRESSION

The expression of I-Smads is regulated in response to a variety of stimuli, including TGF- β , BMP, interferon- γ , cytokines that activate NF- κ B signaling, laminar shear stress, and UV irradiation. In response to TGF- β , the Smad3–Smad4 complex associates with the *Smad7* promoter and activates its transcription, whereas BMP induces Smad1 to bind and activate *Smad7* transcription through distinct regulatory elements (Nagarajan et al. 1999; Denissova et al. 2000; Benchabane and Wrana 2003). The transcription factors AP-1, TFE3, and Sp1 also bind to the *Smad7* promoter and regulate its transcription (Brodin et al. 2000; Hua et al. 2000), and GATA transcription factors cooper-

ate with Smad1 in BMP-induced *Smad7* expression (Benchabane and Wrana 2003). Conceptually similarly, Smad1 and -5 associate with the *Smad6* promoter in response to BMP and induce its transcription (Ishida et al. 2000). OAZ, a transcription factor with 30 Krüppel-like zinc fingers (Hata et al. 2000), interacts with Smad1 and induces BMP-dependent transcription of *Smad6* by binding to its promoter (Ku et al. 2006).

Under certain conditions, I-Smads enable signaling cross talk. In some cells, interferon- γ and interleukin-6 repress TGF- β family signaling through the induction of Smad7 expression by the Jak-STAT pathway (Ulloa et al. 1999; Jenkins et al. 2005). Interleukin-7 activates Smad7 expression and inhibits TGF- β signaling in fibroblasts derived from pulmonary fibrosis induced by bleomycin (Huang et al. 2002). Norepinephrine and the proinflammatory cytokines TNF- α and interleukin-1 induce Smad7 expression in an NF- κ B-dependent manner (Bitzer et al. 2000; Kanamaru et al. 2001). The transmembrane protein CD40, which is structurally related to the TNF receptor family, also induces the expression of Smad7 through the NF- κ B pathway (Patil et al. 2000).

CD4⁺CD25⁺ regulatory T cells play a crucial role in maintaining immunological self-tolerance. TGF- β induces a regulatory phenotype in human and mouse CD4⁺CD25⁺ T cells through the expression of the winged-helix/forkhead transcription factor Foxp3. Foxp3 efficiently suppresses the expression of Smad7 (Fig. 3), leading to enhanced TGF- β signaling and acquisition of regulatory properties by these cells, including an antiproliferative effect on CD4⁺ T cells (Fantini et al. 2004). Thus, a positive autoregulatory loop of TGF- β signaling is formed in CD4⁺CD25⁺ T cells through Foxp3-mediated attenuation of Smad7 expression.

TGF- β signaling rapidly induces the expression of TIEG1 (TGF- β -inducible early gene-1), a Krüppel-like transcription factor. TIEG1 directly associates with the *Smad7* promoter and represses its expression, thereby enhancing TGF- β signaling (Johnsen et al. 2002a). Thus, TIEG1 forms a positive feedback loop of TGF- β signaling similar to Foxp3. The E3 ubiquitin

ligase SIAH1 (seven in absentia homolog 1A) associates with TIEG1 and induces its ubiquitylation and degradation, relieving the suppression of Smad7 expression (Johnsen et al. 2002b).

c-Ski and SnoN repress the basal transcription of *Smad7* by binding to the Smad-binding sequence of the *Smad7* promoter through Smad4 (Denissova and Liu 2004; Briones-Orta et al. 2006). However, on TGF- β stimulation, these repressive complexes dissociate from the *Smad7* promoter region, permitting induction of *Smad7* expression by the activated Smad3–Smad4 complex.

During osteoblast differentiation, the expression of I-Smads is strongly induced by BMP signaling in a biphasic manner; Smad6 and Smad7 expression are transiently induced by BMP within a few hours, and then later induced during osteoblast maturation (Maeda et al. 2004). Endogenous TGF- β also promotes the expression of I-Smads during osteoblast maturation, and the ALK-5/T β RI kinase inhibitor SB431542 represses endogenous TGF- β signaling and, thus, the induction of I-Smad expression. Consequently, inhibition of TGF- β signaling by SB431542 facilitates mesenchymal stem cell differentiation into osteoblasts in the maturation phase (Maeda et al. 2004).

Smad6 and Smad7 expression is detected in human vascular endothelium *in vivo*. Because Smad6 and Smad7 are induced by laminar shear stress, they may modulate the gene expression induced by TGF- β and BMPs in response to humoral and mechanical stimulation, respectively, in the vasculature in homeostasis and disease (Topper et al. 1997).

MicroRNA-21 (miR-21) is a TGF- β - and BMP-regulated miRNA. Activated Smad proteins promote the maturation of miR-21 independent of Smad4 by associating with the stem region of pri-miR-21 (Davis et al. 2008). Interestingly, miR-21 suppresses the expression of Smad7 protein in pulmonary fibroblasts (Liu et al. 2010), and appears to inhibit Smad7 messenger RNA (mRNA) translation while not affecting the Smad7 mRNA level (Li et al. 2013). In contrast, many microRNAs, including miR-181 and the miR-106b-25 and miR-216a/217 clus-

ters, suppress Smad7 expression by inducing Smad7 mRNA degradation (Smith et al. 2012; Xia et al. 2013; Parikh et al. 2014), enhancing TGF- β signaling. miRNAs that control the expression of Smad6 have not yet been reported.

Several low molecular weight compounds regulate the expression of I-Smads, affecting TGF- β signaling. Simvastatin inhibits Smad6 and Smad7 expression in CD4⁺Foxp3⁺ T cells to promote the induction of Foxp3⁺ cells by TGF- β (Kim et al. 2010). In contrast, halofuginone induces Smad7 mRNA expression (Xavier et al. 2004). How these low molecular weight compounds affect the expression of I-Smads remains to be elucidated.

IN VIVO FUNCTIONS OF I-SMADS

Smad6

Smad6 is expressed in the heart and blood vessels, and *Smad6*^{-/-} mice show multiple cardiovascular abnormalities (Galvin et al. 2000), including defects in outflow tract septation and hyperplasia of the cardiac valves. These findings indicate important roles of Smad6 in the regulation of endocardial cushion transformation. Ossification of the outflow tracts of the heart and elevated blood pressure have also been observed in these mice. These findings are consistent with the role of Smad6 in repressing BMP signaling during normal development of the heart valves and outflow tract (Kruithof et al. 2012). Intriguingly, two nonsynonymous mutations that affect the inhibitory activity of Smad6 have been found in patients with cardiovascular malformation accompanying aortic stenosis (Tan et al. 2012). These mutations are located at evolutionarily conserved positions in the MH2 domain. The Smad6 C484F mutant has minimal inhibitory activity in BMP signaling, whereas the P415L mutant is hypomorphic. These findings reveal a critical role of Smad6 in cardiovascular organogenesis.

BMP signaling is also involved in the regulation of endochondral bone formation at multiple stages; it stimulates the proliferation of chondrocytes, but slows their hypertrophic differentiation (Minina et al. 2001; Valcourt

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et al. 2002). *Smad6*^{-/-} mice show craniofacial, axial, and appendicular skeletal abnormalities because of stage-specific defects in endochondral bone formation (Estrada et al. 2011) that can be attributed to enhanced BMP signaling in chondrocytes. Consistent with these findings, transgenic mice that express *Smad6* in chondrocytes show dwarfism with osteopenia, delayed chondrocyte hypertrophy, and thin trabecular bone (Horiki et al. 2004).

Smad7

The phenotypes of *Smad7* mutant mice have been reported by several groups (reviewed in Beppu 2013). Some of the phenotypes can be explained by increased TGF- β or BMP activity, but others suggest that *Smad7* functions independent of TGF- β family signaling. The first reported *Smad7* mutant mice lack the coding region in exon 1 and only partially lose *Smad7* functions (Li et al. 2006). These *Smad7* ^{Δ ex1} mice with a hypomorphic allele are smaller than wild-type mice, but are viable and fertile. The smaller body size of *Smad7* ^{Δ ex1} mice appears to be due largely to attenuated differentiation of bone and skeletal muscle (Estrada et al. 2013; Cohen et al. 2015).

As with *Smad6*^{-/-} mice, *Smad7* ^{Δ ex1} mice show abnormalities in axial and appendicular skeletal development (Estrada et al. 2013). Low *Smad7* activity results in impaired cell cycle progression in chondrocytes and defects in terminal maturation, which can be attributed to enhanced BMP and TGF- β signaling in the growth plates. Both *Smad6* mutant mice and *Smad7* mutant mice show anterior and posterior transformations, indicating that they have overlapping functions. However, *Smad7* mutant mice have defects in lumbar patterning, whereas *Smad6*^{-/-} mice do not, suggesting a unique function of *Smad7*.

The decreased muscle mass observed in *Smad7* ^{Δ ex1} mice can be attributed to enhanced myostatin (also known as GDF-8) signaling (Cohen et al. 2015). Myostatin is a member of the TGF- β family that potently suppresses skeletal muscle growth (Lee 2004) and decreases the transcriptional activity of MyoD in the absence

of *Smad7* (Kollias et al. 2006). *Smad7* ^{Δ ex1} mice also show altered myofiber type composition toward oxidative types, impaired skeletal muscle regeneration, and decreased satellite cell proliferation, probably as a result of enhanced myostatin signaling (Cohen et al. 2015).

TGF- β is a potent inducer of tissue fibrosis. In the CCl₄-induced chronic liver damage model, *Smad7* ^{Δ ex1} mice show more severe liver injury, elevated collagen deposition, and increased numbers of activated hepatic stellate cells (Hamzavi et al. 2008) compared with control mice. The mutant mice also show enhanced tissue injury, more progressive fibrosis, inflammation in a unilateral ureteral obstruction (UUO) model of renal fibrosis (Chung et al. 2009), streptozotocin-induced model of diabetic kidney injury (Chen et al. 2011), and angiotensin II-induced hypertensive nephropathy and cardiac remodeling (Liu et al. 2013; Wei et al. 2013). Enhanced inflammation in *Smad7* mutant mice may be attributed, at least in part, to the inhibitory effect of *Smad7* on NF- κ B signaling. Mutant B lymphocytes also show phenotypes with enhanced TGF- β signaling: facilitated class switch recombination to IgA, enhanced spontaneous apoptosis, and attenuated proliferation after stimulation with lipopolysaccharide (Li et al. 2006).

Intriguingly, *Smad7* ^{Δ ex1} mice are more susceptible to diethylnitrosoamine-induced hepatocarcinogenesis, suggesting that *Smad7* has a tumor suppressor function in the liver (Wang et al. 2013). In addition, enhanced cell proliferation and suppressed apoptosis have been observed in the mutant mice. The phenotypes may be caused by derepression of *c-Myc* expression, enhanced NF- κ B signaling, and/or attenuation of the TRAF6 pathway of TGF- β signaling.

Smad7 ^{Δ/Δ} mutant mice, which lack expression of the entire *Smad7* protein, are embryonic lethal (Kleiter et al. 2010). Furthermore, *Smad7* ^{Δ MH2} mice on a C57BL/6 background, which lack the MH2 domain required for the inhibitory activity of *Smad7*, die before weaning (Chen et al. 2009; Tojo et al. 2012). *Smad7* ^{Δ MH2} mice that die in utero have cardiac defects, including ventricular septal defects, noncompaction, and outflow tract malformation (Chen

et al. 2009). These phenotypes do not overlap with those of *Smad6*^{-/-} mice (Galvin et al. 2000), suggesting functional specificity of each I-Smad in the cardiac system. Unexpectedly, the induction of TGF- β or BMP target genes, such as the genes encoding plasminogen activator inhibitor-1 or Id1, respectively, is not enhanced in mouse embryonic fibroblasts prepared from *Smad7* ^{Δ MH2} embryos on the C57BL/6 background (Tojo et al. 2012) compared with cells from different organs in other reports (Kleiter et al. 2010; Zhu et al. 2011; Estrada et al. 2013). In addition, *Smad7* ^{Δ MH2} mice with an internal control region (ICR) genetic background develop to adulthood, although their body size is smaller (Tojo et al. 2012). Thus, the phenotypic effects of *Smad7* inactivation are largely dependent on the context of the cells or organs.

High expression of *Smad7* is observed in peripheral CD4⁺ cells from multiple sclerosis patients during relapse (Kleiter et al. 2010). The expression of *Smad7* correlates positively with that of T-bet, a transcription factor involved in T helper cell type 1 (T_H1) responses. Consistent with these observations, transgenic mice with increased *Smad7* expression in T cells (*CD2-Smad7*) show enhanced experimental autoimmune encephalomyelitis, a model of multiple sclerosis in which the infiltration of inflammatory cells and T_H1 responses are facilitated in the central nervous system. In contrast, T-cell-specific *Smad7* knockout in *CD4Cre-Smad7*^{f/f} mice results in immunosuppression and reduced T_H1 responses, with unaltered T_H17 responses. The *Smad7* expression level in T cells has been shown to be a determinant of T_H1 differentiation (Kleiter et al. 2010).

Smad7 is expressed in the lens and retina during embryonic eye development in mice, where BMP signaling has been shown to play important roles in lens induction, optic vesicle invagination, and retinal spatial patterning (Zhang et al. 2013a). *Smad7* ^{Δ MH2} mice have multiple defects in eye development, including coloboma and microphthalmia. The effects of *Smad7* inactivation during eye development depend on the cell type, developmental stage, and genetic background of the mice. These phenotypes observed in mutant mice with the

C57BL/6 background are rarely seen in mice with the 129/FVB hybrid background. In addition, enhanced apoptosis in the retina of *Smad7* ^{Δ MH2} mice has been observed at E10.5 (Zhang et al. 2013a), whereas conditional inactivation of *Smad7* in developing neural retina (*α Cre-Smad7*^{f/f}) has been shown to attenuate apoptosis at E16.5 and later, in which TGF- β signaling protects against developmental cell death through the induction of nerve growth factor (NGF) expression (Braunger et al. 2013).

Hepatocyte-specific deletion of the *Smad7* MH2 domain using *albumin-Cre* transgenic mice results in spontaneous liver dysfunction with the apoptosis of hepatocytes and aggravation of alcohol-induced liver injury because of the down-regulation of alcohol dehydrogenase 1 (ADH1) expression (Zhu et al. 2011). Spontaneous liver damage is not observed in hypomorphic *Smad7* ^{Δ ex1} mice (Hamzavi et al. 2008), probably because of incomplete inactivation of *Smad7* activity. Conditional silencing of *Smad7* in the pancreas at E10.5 (*PdxCre-ERT-Smad7*^{fx/fx}) results in a diminished number of hormone-producing cells, whereas genetic ablation of both *Smad2* and *Smad3* expression has effects that are opposite to those of *Smad7* (El-Gohary et al. 2013). These phenotypes are explained by increased TGF- β signaling.

Smad7 may function independently from TGF- β and BMP signaling in certain situations. The proliferation of adult neural stem/progenitor cells is normally inhibited by TGF- β in vitro and in vivo (Wachs et al. 2006). However, the cells derived from *Smad7* ^{Δ ex1} mice have higher potential to proliferate, form spheres, and self-renew compared with cells from wild-type mice as a result of enhanced EGF signaling (Krampert et al. 2010). Another example is promotion of pancreas β -cell proliferation during inflammation induced by pancreatic duct ligation (PDL) (Xiao et al. 2014). After PDL, infiltrated M2 macrophages secrete TGF- β 1, which induces *Smad7* in β cells. *Smad7* then promotes the proliferation of β cells by inducing the expression of cyclin D1 and D2 and excluding p27^{Kip1} from the nucleus. Increased *Smad7* expression is required and sufficient for β -cell proliferation. The effect of *Smad7* does not appear to

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be caused by inhibition of TGF- β signaling, as conditional deletion of both TGF- β type I and II receptors in β cells substantially inhibits β -cell proliferation after PDL (El-Gohary et al. 2014).

DYSREGULATION OF I-SMADS IN DISEASE

Fibrosis

Decreased expression or deficient function of Smad7 leading to the acceleration of TGF- β -induced fibrosis has been reported in various diseases, including those of the skin, kidney, and lung. Smad7 expression is higher in scleroderma fibroblasts than in normal fibroblasts, but the inhibitory effect of Smad7 on TGF- β signaling is impaired in scleroderma fibroblasts (Asano et al. 2004). The expression levels of Smurfs do not differ significantly between normal and scleroderma cells (Asano et al. 2004), and the mechanisms underlying the impaired negative regulation of TGF- β signaling by the Smad7-Smurf system remain to be elucidated.

Although the Smad7 mRNA level is increased, a significant decrease in Smad7 protein has been observed in obstructive nephropathy in mice with UUO as a result of increased ubiquitination and degradation of Smad7 (Fukasawa et al. 2004). Although the expression of Smurf1 and Smurf2 is increased in UUO kidneys, how Smad7 protein expression is reduced is unknown.

Consistent with the finding that TGF- β signaling plays important roles in the development of tissue fibrosis, adenovirus-mediated expression of *Smad7* attenuates TGF- β -induced fibrosis in various tissues, including the lung and kidney (Nakao et al. 1999; Terada et al. 2002). In addition, adenoviral expression of *Smad7* prevents the epithelial-mesenchymal transition (EMT) of lens epithelial cells and accelerates the healing of corneal tissue after ocular burns (Saika et al. 2005). Transgenic mice expressing Smad7 in the skin show severe epithelial abnormalities, including epidermal hyperplasia and aberrant morphogenesis of hair follicles (He et al. 2002).

Inflammation and Wound Healing

Analysis of transgenic mice that selectively express Smad7 in mature T cells from a distal *lck* promoter segment revealed the increased production of T_H1 and T_H2 cytokines and enhanced antigen-induced airway inflammation and reactivity, suggesting that the regulation of T cells by TGF- β plays an important role in regulating the inflammatory response (Nakao et al. 2000).

TGF- β is produced by various cells to prevent intestinal inflammation. Increased TGF- β expression is observed in the guts of patients with chronic inflammatory bowel diseases (IBDs), including Crohn's disease, and is expected to inhibit the production of inflammatory cytokines by mononuclear cells of the lamina propria. TGF- β can also attenuate the activation of NF- κ B by TNF- α through the induction of I κ B α expression (Monteleone et al. 2004a). However, even in the presence of TGF- β , reduced levels of activated Smad3 and high NF- κ B activity have been observed in samples from patients with active IBD because Smad7 protein is highly expressed in these patients (Monteleone et al. 2001). Smad7 inhibits TGF- β signaling, sustains high NF- κ B activity, and expands the inflammatory response in IBD. In patients with IBD, Smad7 mRNA levels are not elevated above normal and, in contrast to some other types of cells, Smad7 expression is not induced by NF- κ B (Monteleone et al. 2004b). These findings suggest that the Smad7 levels in patients with IBD may be regulated posttranscriptionally, including through ubiquitin-dependent degradation. Remarkably, an oral *SMAD7* antisense oligonucleotide (Mongersen) was effective in the treatment of patients with active Crohn's disease in a phase II study (Monteleone et al. 2015), supporting a role of Smad7 in the pathogenesis of the disease.

Helicobacter pylori infection induces chronic gastric inflammation, leading to peptic ulcer and gastric cancer in some patients. The inflammatory response is characterized by overactive T_H1 cytokines, and TGF- β suppresses the development of T_H1 cells. In *H. pylori*-infected gastric mucosa, interferon- γ secreted by T_H1

cells induces Smad7 expression in a STAT1-dependent manner, leading to sustained T_H1-induced tissue injury (Monteleone et al. 2004c).

High-dose radiation often induces oral mucositis, accompanied by excessive inflammation and epithelial ablation. Transgenic mice overexpressing Smad7 in keratinocytes are resistant to radiation-induced oral mucositis (Han et al. 2013). Smad7 suppresses inflammation through inhibition of the NF- κ B pathway and enhances keratinocyte migration through derepression of Rac1 expression. Rac1 expression is down-regulated by TGF- β signaling in the presence of CtBP. Notably, Smad7 protein fused to a cell-permeable tag (Tat) shows therapeutic, as well as prophylactic effects on radiation-induced oral mucositis when topically applied to oral mucosa in mice (Han et al. 2013). In wounded skin, Smad7 is up-regulated in migrating keratinocytes and proliferative fibroblasts, accelerating wound healing and remodeling (Han et al. 2011).

Cancer

Decreased sensitivity to TGF- β -induced growth inhibition because of Smad7 overexpression may aid in the progression of some tumors. Both Smad7 and Smad6 have been shown to be overexpressed in pancreatic cancer cells and to confer resistance to TGF- β family signaling in these cells (Kleeff et al. 1999a,b). Pancreas-specific expression of Smad7 in mice results in the development of premalignant ductal lesions characterized by pancreatic intraepithelial neoplasia and increased fibrosis (Kuang et al. 2006), suggesting a critical role of up-regulated Smad7 expression in pancreas carcinogenesis. Increased expression of Smad7 correlates with poor prognosis for certain types of cancer, including hepatocellular carcinoma and gastric cancer (Kim et al. 2004b; Park et al. 2004). In addition, *SMAD7* copy number and patient survival correlate inversely in colorectal cancer (Boulay et al. 2003). Furthermore, single nucleotide polymorphisms in *SMAD7* are associated with increased risk of colorectal cancer (Broderick et al. 2007; Slattery et al. 2010). Although these polymorphisms do not affect the coding

sequence of Smad7, they may result in decreased mRNA expression (Pittman et al. 2009).

UV irradiation may contribute to the progression of squamous cell carcinomas as a result of Smad7-mediated suppression of TGF- β signaling (Quan et al. 2005). The expression of Smad7 and GADD34 is up-regulated by UV irradiation, and the GADD34–PP1c complex cooperates with Smad7 in the inhibition of TGF- β signaling by promoting receptor dephosphorylation (Shi et al. 2004). Thus, the Smad7–GADD34–PP1c complex may induce the proliferation and hyperplasia of keratinocytes and reduce extracellular matrix deposition and premature skin aging, leading to the progression of cancer.

Smad7 has been showed to play a role in the transformation of keratinocytes. Overexpression of Smad7 alone results in facilitated proliferation and the prevention of differentiation of mouse keratinocytes, but fails to induce tumor formation. Cells manipulated to express *v-ras^{Ha}* or *v-ras^{Ha}* and *Smad6* form benign papilloma in vivo, but those coexpressing *v-ras^{Ha}* and *Smad7* rapidly progress to squamous cell carcinoma (Liu et al. 2003). In addition, TGF- β signaling is suppressed and the production of EGF family growth factors, including TGF- α , heparin binding (HB)-EGF, or amphiregulin, is induced in keratinocytes expressing *v-ras^{Ha}* and *Smad7*. Thus, Smad7 may cooperate with *v-ras^{Ha}* in the rapid progression of keratinocytes from benign papilloma to malignant squamous carcinoma by preventing TGF- β signaling and enhancing EGFR signaling.

Although perturbations in TGF- β signaling result in the transformation of normal cells, TGF- β signaling also facilitates the invasion by and metastasis of some advanced cancers. Using the JygMC(A) mouse breast cancer cell line, which spontaneously metastasizes to the lung and liver after subcutaneous inoculation in nude mice, systemic administration of an adenovirus expressing Smad7 was shown to prevent metastasis to the lung and liver and prolonged mean survival (Azuma et al. 2005). Smad7 directly affects the cancer cells to inhibit EMT and prevent metastasis. Thus, the blockade of TGF- β signaling by Smad7 may provide new

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therapeutic strategies for preventing metastasis in patients with advanced cancers.

CONCLUSION AND PERSPECTIVES

I-Smads are now known to regulate both Smad and non-Smad pathways of TGF- β family signaling through multiple mechanisms. In addition, recent studies have suggested that I-Smads are also involved in the regulation of other signaling pathways. Although it is well established that Smad7, but not Smad6, effectively inhibits TGF- β signaling, how these functional differences arise remains unclear. Because I-Smads are implicated in the development of certain clinical diseases, it is important to elucidate the molecular mechanisms by which I-Smads inhibit TGF- β family signaling. Recent progress in the field have revealed that the functions of I-Smads are tightly regulated by various enzymes, including ubiquitin ligases, acetyltransferases, deacetylase, and methyl transferase. Elucidating the functional roles of these regulators in vivo will be important. As aberrant I-Smad expression has been suggested to play roles in various diseases, it will also be important to determine how the expression levels of I-Smad proteins are controlled in these diseases. Moreover, I-Smads play crucial roles in fine-tuning the magnitude of TGF- β family signaling. Thus, defining the regulatory mechanisms of I-Smad function may aid in understanding how TGF- β family signaling coordinates the growth, differentiation, and morphogenesis of various cells and tissues in physiological and pathological conditions.

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