The Smad pathway in transforming growth factor-β signaling

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Abstract The Smad pathway is involved in transforming growth factor-β (TGF-β) signal transduction. The Smad complex binds with the promoter of target gene to modulate gene transcription. Various transcriptional coactivators and corepressors associate directly with Smads for appropriate binding of Smads to target promoters and regulation of Smads transcriptional activities. The ultimate degradation of Smads mediated by the ubiquitin-proteasome pathway (UPP) has been established as a mechanism to shut off the Smad pathway. In addition to the Smad pathway, TGF-β can also activate other signaling pathway such as the MAPK pathway. The cross-talk of the Smad pathway with other signaling pathways constitutes an important mechanism for the regulatory network of TGF-β signaling.

Keywords: **TGF-β signaling, Smad, Smad pathway, MAPK pathway, UPP.**

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The transforming growth factor β (TGF-β) superfamily comprises a great number of structurally related polypeptide growth factors, such as TGF-βs, activins, inhibins, bone morphogenic proteins (BMPs), growth differentiation factors (GDFs), Müllerian inhibitory substance, and glial cell-derived neurotrophic factor (GDNF), etc[1]. The TGF-β superfamily members are multifunctional agonists involved in a broad spectrum of biological processes such as cell proliferation and differentiation, embryogenesis, angiogenesis, tumorigenesis, extracellular matrix (ECM) formation, and bone formation and remodeling[2—4]. Accumulated evidence has advanced our understanding of the molecular mechanisms of TGF-β signaling, which mediates such a diverse range of biological processes.

1 TGF-β receptors

A milestone in the research of TGF-β signaling was the cloning of type II receptor for activin and TGF-β by Mathews and Lin et al. [5,6]. The discoveries of these serine/threonine kinase receptors present a novel signaling strategy based on previously characterized tyrosine kinase signaling. The TGF-β signals are transmitted by two types of transmembrane serine/threonine kinase receptors, the type II receptor and the downstream type I receptor. The type II receptor determines the

ligand binding specificity. TGF-β ligand first binds to the type II receptor, leading to the recruitment of the type I receptor to the cell membrane and its subsequent phosphorylation. In some cases, TGF-β ligand can also bind directly to the type I receptor[7]. The type II receptor phosphorylates the type I receptor on its serine residues at the highly conserved GS domain, a glycine and serine-rich domain between the transmembrane domain and the kinase domain[8]. The type II and type I receptors found to date which are involved in the signaling of TGF-βs, activins, and BMPs are summarized in table 1.

In addition to the type I and type II receptors, there are other TGF-β binding proteins on the cell surface, such as the type III, IV, V, and VI receptors, glycosylphosphatidylinositol (GPI)-anchored proteins, and endoglin, functioning to facilitate the binding of TGF-β with type I and type II receptors[9]. It has been reported that endoglin shows some sequence similarity with TβR III (the type III TGFβ receptor), thus promoting the binding of TGF-β1 and TGF-β3 with TβR II and TβR I[10]. Furthermore, endoglin has also been shown to be an accessory receptor for activins and BMPs[11].

Internalization of TGF-β receptor complex occurred after ligand binding by a mechanism distinct from normal endocytosis via clathrin-coated vesicles[12]. It was also reported that the majority of TGFβR I was intracellularly immunolocalized and accumulated in the nucleus after TGF-β1 treatment[13]. Therefore, the ligand-induced receptor down regulation and reduced receptor affinity are probably results of receptor complex internalization.

Table 1 Receptors involved in the signaling of TGF-β superfamily members[14]

	TGF-βs	Activins	BMPs
Type II receptor	TβR II	ActR-II ActR-IIB	BMPR-II ActR-II ActR-IIB
Type I receptor	TβR I (Alk5) Alk1	ActR-I B (Alk4) ActR-I (Alk2)	BMPR-1A (Alk3) BMPR-1B (Alk6) ActR- I (Alk2)

TβRII, TGF-β type II receptor; ActR-II, activin type II receptor; BMPR-II, BMP type II receptor; Alk, activin receptor-like kinase; BMPR-1A, BMP type I receptor A; BMPR-1B, BMP type I receptor B.

It is well recognized that TGF-β members act by binding to the transmembrane receptor complex, which subsequently activate the intracellular signaling molecules, known as Smads, by phosphorylation. The phosphorylation of the type I receptor activates the Smad pathway, which involves the binding of Smads with the receptor complex, the activation and the oligomerization of Smads, and the translocation of the Smad complexes to the nucleus where they modulate the transcription of target genes by binding with the *cis*-acting elements in the promoter regions[15]. TGF-β receptors also activate other signaling pathway such as the mitogen-activated protein kinase (MAPK) pathway, the protein phosphatase 2A (PP2A)/$p70^{s6K}$ pathway, and the phosphoinositol-3-OH kinase (PI3K)-Akt pathway[16]. The cooperation and counteraction between different pathways are responsible for their balanced activation in response to TGF-β and the ultimate fulfillment of different TGF-β biological effects.

2 Activation of the Smad pathway by TGF-β receptors

2.1 The classification and structure of Smads

Two signal transducers of *dpp*, the BMP homologue in *Drosophila melanogaster*, were first discovered and named *Mothers against dpp (Mad)*[17] and *Medea*[18] by Raftery and Sekelsky et al. in 1995. Savage and co-workers[19] cloned *Sma-2*, *-3*, and-*4* recognized by the type II receptor *daf-4* in *Caenorhabditis elegans*. As *Mad*, *Sma*, and other homologues were essential for TGF-β signaling, and they shared specific conserved N-terminal and C-terminal domains, a unified nomenclature was soon adopted as Smad proteins, originated from the combination of gene names from *Sma* and *Mad* by Derynck et al. in 1996[20].

Smad proteins constitute a family of nine members serving as intracellular mediators of TGF-β signals. They are divided into three classes based on their structural and functional features. The first class is receptor-regulated Smads (R-Smads), of which Smad1, Smad5, and Smad8 respond to BMPs whereas Smad2 and Smad3 mediate TGF-βs/activins signals. R-Smads are phosphorylated by the type I receptor and are pathway-restricted. Common-mediator Smads (Co-Smads), as a second class of Smads, form heteromeric complexes with R-Smads, followed by the translocation into the nucleus to regulate gene transcription. Inhibitory Smads (I-Smads), including Smad6 and Smad7, are members of the third class which counteract the activities of Smad complexes. Until recently, only a single Co-Smad in mammals, Smad4, has been found, whereas in *Xenopus* two members of Co-Smads have been identified, known as Smad4 and Smad4β (also called Smad10)[7,21].

Smad family members have two conserved domains, the N-terminal Mad-homology domain 1 (MH1) and the C-terminal Mad-homology domain 2 (MH2), separated by the linker region of variable length and sequence.

The MH1 domain, highly conserved among R-Smads and Co-Smads, regulates binding to DNA in the promoter region of target gene and interacting with Smad partners such as calmodulin, vitamin D receptor (VDR), Jun, transcription factor E3 (TFE3), and activating transcription factor 2 (ATF2), etc.[7, 22]. The MH1 domain itself can form a compact globular fold, a secondary structure composed of four α-helices, six short β strands, and five loops. The β-hairpin structure functions to contact the specific DNA in the major groove[7].

The MH2 domain is highly conserved among all Smads. It serves functions in the binding of R-Smads with the type I receptor and Smad anchor for receptor activation (SARA), the oligomerization of R-Smads and Co-Smad, as well as the binding of Smads with a number of transcriptional co-modulators, such as forkhead activin signal transducer (FAST), c-Fos, polyoma virus enhancer binding protein 2/core binding factor (PEBP2/CBF), CREB (cAMP response element binding protein)-binding protein (CBP)/p300, homeodomain protein TG-interacting factor (TGIF), and Ski/SnoN, etc. The specificity of R-Smads phosphorylation by the type I receptor is determined by the L45 loop in the type I receptor and the L3 loop in the MH2 domain of R-Smads[22, 23].

The secondary structure formed in the MH2 domain consists of five α-helices (H1—H5) and three loops (L1, L2, L3), which constitute a β-sandwich structure[24]. C-terminal truncated Smad4 could not form homo-oligomers by itself, nor form hetero-oligomers with phosphorylated Smad2[25], indicating the essential role of the MH2 domain in Smads oligomerization.

The linker region is associated with binding to c-Jun[26] and the ubiquitination of Smads[22]. The linker region contains important peptide motifs, despite that is not well conserved among all Smads. For instance, the linker regions of Smad1, Smad2, and Smad3 contain serine residues phosphorylation sites for ERK (extracellular stimulus responsive kinase)-family MAPK. The phosphorylation of these sites could inhibit the activities of Smads, and the mutation of these serine residues inhibited nuclear translocation of Smads[27].

The two most C-terminal phosphorylated serine residues and a third non-phosphorylated serine residue form a specific and conserved SSXS motif in all R-Smads[28]. The SSXS motif is the site where R-Smads are phosphorylated by the type I receptor kinases[29], and it is also demonstrated that ERK phosphorylates Smads at this motif[30].

In addition, there is a conserved proline and tyrosine-rich PY motif in the linker region of R-Smads or I-Smads which has been verified to mediate association of Smad1 with Smad ubiquitination regulatory factor 1 (Smurf1), a E3 ubiquitin ligase[31]. A specific proline-rich domain, called Smad4 activation domain (SAD), exists in the linker region of Smad4[32].

The domains of three Smad classes and their associated functions are diagrammatically illustrated in fig. 1.

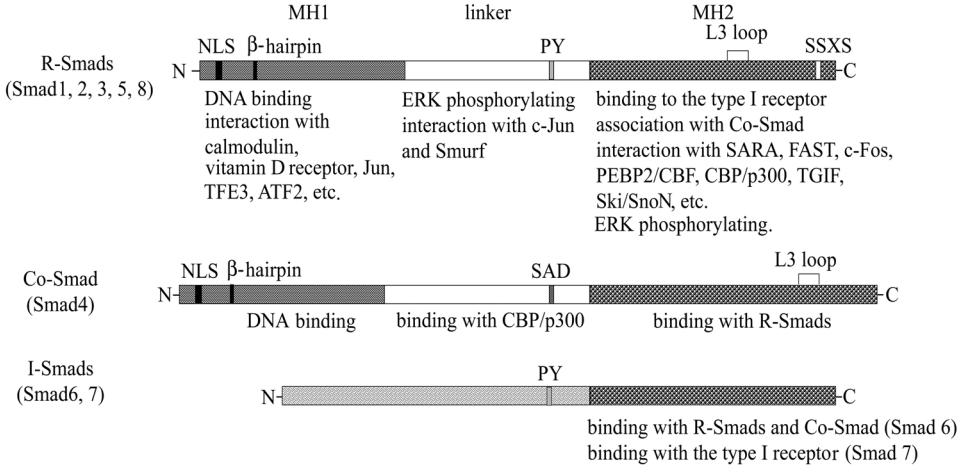

Fig. 1. Diagrammatic representation of Smads domains and associated functions. R-Smads, Receptor-regulated Smads; Co-Smad, Common-mediator Smad; I-Smads, Inhibitory Smads; MH1, Mad-homology domain 1; MH2, Mad-homology domain 2; TFE3, transcription factor E3; ATF2, activating transcription factor 2; ERK, extracellular stimulus responsive kinase; Smurf, Smad ubiquitination regulatory factor; SARA, Smad anchor for receptor activation; FAST, forkhead activin signal transducer; PEBP2/CBF, polyoma virus enhancer binding protein 2/core binding factor; CBP, CREB (cAMP response element binding protein)-binding protein; TGIF, homeodomain protein TG-interacting factor; NLS, nuclear localization signal; SAD, Smad4 activation domain.

2.2 The process of the Smad pathway

2.2.1 The presentation of cytoplasmic R-Smads to the activated type I receptor. A cell membrane-localized Smad/receptor anchor protein, known as SARA, recruits cytoplasmic unphosphorylated R-Smads to the cell membrane where the activated receptor kinase is located[33]. Human SARA and its *Xenopus* homolog share an overall identity of 62%, with 85% identity in the C-terminal two-thirds of the proteins[34]. SARA can bind to the MH2 domain of Smad2 and Smad3, but not bind to Smad1 and Smad4. Phosphorylations of R-Smads free them from SARA, and SARA can then recruit additional unphosphorylated R-Smads from the cytoplasmic pool to the membrane. SARA resides at the cell membrane via a double zinc finger domain known as FYVE domain[35]. Downstream of the FYVE domain is a 45-amino acid Smad binding domain (SBD), through which unphosphorylated Smad2 is recognized and concentrated at the membrane in proximity to the receptor complex[26].

2.2.2 The dissociation of phosphorylated R-Smads from the receptor complex and the concomitant heteromerization with Co-Smad. The heteromers of R-Smads and Smad 4 are formed through the association between the MH2 domains of the two Smads. Unphosphorylated R-Smads exist mainly as monomers. Upon phosphorylation, R-Smads form homo-oligomers with themselves, and subsequently, quickly form hetero-oligomers with Co-Smad[36]. Unphosphorylated cytoplasmic R-Smads are auto-inhibited through the intramolecular interaction between the MH1 domain and the MH2 domain[29]. A unique loop in the MH2 domain of Smad4 prevents its self-oligomerization[37].

2.2.3 The translocation of Smad heteromers into the nucleus. A lysine-rich motif in the MH1 domains of all Smads has been verified as a nuclear localization signal (NLS) in Smad1 and Smad3. The C-terminal phosphorylation of Smad3 results in the changes of its conformation and the exposure of the NLS, followed by nuclear translocation with the assistance of importin β1and Ran[38]. Nuclear import of R-Smads is independent of the presence of Smad4, while Co-Smad does not accumulate in the nucleus without a phosphorylated R-Smad[39].

2.2.4 Binding of Smad heteromers to specific DNA in target gene promoter. In the promoter regions of various TGF-β-responsive genes, such as plasminogen activator inhibitor 1 (*PAI-1*), type I collagen α strand, *Smad7*[40], *c-myc*[41], $p15^{INK4b}$[42], $p21^{CIP1}$[43], and matrix metalloproteinases (*MMPs*)[44], Smad-binding element (SBE)[45] and TGF-β inhibitory element (TIE)[41] exist as functional *cis*-acting elements for genes transcription. Smad3 and Smad4 bind directly but with low affinity to SBE via the conserved β-hairpin loops in their MH1 domains. The α-helices in the MH1 domain of Smad3 can enhance the binding activity of Smad3 to SBE[46]. The presence of the unique exon-3-encoded sequence in the MH1 domain of Smad2 is the reason why Smad2 cannot bind to SBE directly[47]. The MH1 domains of Smad3 and Smad4 also bind to GC-rich motifs in promoters of target genes with a relaxed DNA-binding specificity[48].

2.3 The regulation of the Smad pathway

Smad heteromers bind to DNA directly after their entry into the nucleus. The DNA binding mediated by the MH1 domain is of low affinity and low specificity; the formation of a Smads-DNA complex with high affinity and high specificity involves the participation of other DNA binding proteins, known as Smad partners. Thus Smads rely on interactions with Smad partners to activate transcription of target genes.

2.3.1 Transcriptional coactivators. Most transcriptional coactivators associate with the MH2 domains of Smads and participate in the transactivation function of Smads. The first identified Smad transcriptional partner is FAST[49]. FAST can bind to specific DNA of target genes with high affinity and high specificity, but only plays an accessory role in the transactivation function of Smads in that it cannot activate gene transcription by itself. In *Xenopus*, a transcriptional factor complex containing FAST1, Smad2, and Smad4 assembles on the promoter of *Mix.2* thus enhancing its activin-responsive gene transactivation. Analysis of the complex showed that Smad2 associated directly with FAST1 via its MH2 domain. Smad4 did not interact directly with and functioned to stabilize DNA binding activity of the Smad-FAST complex[49]. Similar to the FAST1 complex on the promoter of *Mix.2*, FAST2-Smad2 complex binds to and activates transcription from the *goosecoid (gsc)* promoter, whereas FAST2-Smad3 complex inhibits gene transcription of *gsc*[48].

p300, also known as CREB (cAMP response element-binding protein)-binding protein (CBP), has intrinsic histone acetylase activity which can modify chromatin structure thus directly affects the structure of the promoter region. Numerous investigations have demonstrated that CBP/p300 could bind directly to the MH2 domain of Smad2 and Smad3, and the linker region of Smad4[14]. Furthermore, exogenous CBP/p300 could augment TGF-β-induced gene transactivation in a Smad4-dependent fashion[50]. The unique SAD domain of Smad4 functions to induce stronger association of Smad complex with CBP/p300[32].

Activating protein-1 (AP-1) is a transcriptional factor composed of members of the Jun and Fos families. AP-1 binding sites exist in the promoters of several TGF-β-responsive genes, either overlapping or adjacent to SBE. c-Fos binds to the MH2 domain of Smad3, while c-Jun binds to the linker region of Smad3. The complex consisting of c-Jun, c-Fos, Smad3, and Smad4 binds to the AP-1 binding site or SBE in target gene promoter and activates gene transcription[51].

PEBP2/CBF[52] and Olf-1/EBF associated zinc finger (OAZ)[53] interact with the MH2 domain of R-Smads, resulting in the cooperation with the R-Smads/Co-Smad complexes to induce transcriptional activation of target genes.

Several transcriptional coactivators bind to the MH1 domain of Smads, such as vitamin D receptor[54], and transcriptional factor TFE3[55] and ATF2[56].

New data indicate that loss of tumor suppressor genes, such as nuclear protein Menin (*MEN1* gene product), could also block the Smad pathway. Moreover, Smad3 could interact directly with

Menin[57]. Whether some products of tumor suppressor genes function as transcriptional corepressors remains to be uncovered.

2.3.2 Transcriptional corepressors. TGIF, as a homodomain DNA-binding protein, can associate with the MH2 domain of Smad2 resulting in the block of Smad target genes transcription. Histone deacetylases (HDACs) was shown to interact with TGIF-Smad complex by binding to the MH1 domain of Smad3 thus reducing the TGIF-dependent transcriptional repression[58].

Calmodulin binds to two different α-helices in the MH1 domains of R-Smads in a calcium-dependent manner. Exogenous calmodulin could inhibit Smads-mediated transactivation of target genes[59].

Ski and SnoN are two members of the Ski family of proto-oncogene products. Ski/SnoN was found to interact with the MH2 domain of Smad2, Smad3, and Smad4 and to inhibit their transactivation functions. Ski/SnoN regulates the Smad pathway via a negative feedback mechanism: the extracellular stimulation of TGF-β leads to the rapid degradation of Ski/SnoN, thus promising the proceeding of the signaling pathway; the subsequent nuclear accumulation of Smad complexes in turn enhances the expression of Ski/SnoN, resulting in the timely shut-off of TGF-β signaling[60]. Ski-interacting protein (SKIP) could reverse the transcriptional inhibiting activity of Ski/SnoN through interacting with the MH2 domain of Smad2 and Smad3[61].

2.3.3 The functions of I-Smads in the Smad pathway. I-Smads are distinct from R-Smads and Co-Smads in that these Smads contain specific MH1 domains and lack C-terminal phosphorylation sites. It has been generally recognized that I-Smads compete with R-Smads for binding to the receptor complex, resulting in the block of the formation of R-Smad-Smad4 heteromers[22]. Further investigations by Hata and Kimura et al. demonstrated that Smad6 could interfere with the formation of Smad complex by competing with Smad4 for binding to phosphorylated Smad1 (but not to Smad2)[62], or by binding directly to Smad4[63]. Smad7 can bind directly to the phosphorylated type I receptor, and this in turn inhibits the phosphorylation of R-Smads and induces the degradation of the receptor[64].

2.3.4 The shut-off of the Smad pathway by the ubiquitin-proteasome pathway (UPP). The UPP-mediated proteolysis functions to control the activities of numerous cellular proteins in many physiological events such as signal transduction and cell cycle regulation. In 1999 the finding of a novel member of Hect domain E3 ubiquitin ligases that degrades R-Smads, designated as Smurf1[31], provides the first evidence that the shut-off of the Smad pathway originates from the UPP-mediated proteolysis of Smads.

A two-hybrid screen identified Smurf1. Coexpression of Smurf1 and Smad1 in two mammalian cell lines, 293T and COS-1, led to a significant and dose-dependent decrease in the level of Smad1 protein, thus supporting the concept that Smurf1 could target Smad1 for ubiquitination. In addition to its Hect domain, Smurf1 has WW domains, which mediate the interaction of Smurf1

with the PY motif of Smad1 and Smad5 (but not of Smad2 and Smad3), and induce the ubiquitination and subsequent degradation of both Smads[31]. Smurf1 mediates the ubiquitination of Smad7 and its subsequent export to the cytoplasm. Furthermore, Smurf1 associates with TβRI through Smad7, and targets TβRI and Smad7 that become degraded[65].

Smurf2 is implicated in the ubiquitination of Smad1, Smad2, and Smad3, but cannot associate with Smad4[66]. Moreover, Smurf2 can interact with TGF-β receptor complex via Smad7, thus inducing the degradation of the receptor complex and Smad7[64]. Smad2-Smurf2 complex, together with anaphase-promoting complex (APC) and a member of UbcH5 family of E2 ubiquitin conjugating enzymes, can induce the degradation of SnoN[67].

The SCF/Roc1 E3 ubiquitin ligase is found to be involved in the ubiquitination of phosphorylated nuclear Smad3 and its proteasomal degradation in the cytoplasm[68].

Despite that UPP-mediated Smads proteolysis is a mechanism of the shut-off of the Smad pathway, the E1 ubiquitin activating enzymes and the E2 ubiquitin conjugating enzymes mediating Smads ubiquitination remain to be systematically uncovered.

3 The activation of other signaling cascades by TGF-β receptors

The TGF-β receptors can activate three different MAPK signaling cascades: the ERK1/2 pathway (Raf/MEK1,2/ERK1,2 cascade), the JNK/SAPK pathway (MEKK1-4/MKK4,7/JNK1-3 cascade), and the p38 MAPK pathway (MAPKKK/MKK3,6/p38 cascade), and the specific pathway utilized varies with cell type[16, 69, 70]. In HaCat cells, TGF-β induction of $p15^{INK4b}$ and $p21^{CIP1}$ occurred through Smad-independent activation of the MAPK pathway[71]. In kidney epithelial cells, the activation of Raf antagonized TGF-β-induced apoptosis. Overexpression of Raf did not affect the phosphorylation and nuclear entry of Smad[72]. In human fibrosarcoma cells, TGF-β induced fibronectin synthesis through the JNK/SAPK signaling cascade in a Smad4-independent manner[73]. These results suggest that TGF-β receptors can activate the MAPK pathway via other downstream mediators instead of Smads. TGF-β-activating kinase 1 (TAK1), as a novel identified MAPKKK, has been reported to function as a mediator of TGF-β receptors to activate p38 MAPK pathway and subsequently lead to the phosphorylation of ATF2, which associates with Smad4 as a transcriptional coactivator in activating gene transcription[56]. However, the mediators of TGF-β receptors involved in the activation of TAK1 remain to be elucidated. Protein kinase C (PKC)[74] and an apoptosis inhibitor protein XIAP[16] were found to couple the TGF-β receptors and the BMP receptors to the MAPK pathway, respectively, thus providing us a new horizon on the finding of downstream mediators coupling receptors to the MAPK pathway.

The PP2A/$p70^{s6K}$ pathway involves activation of the phosphatase PP2A through the release of its Bα subunit from the TGF-β receptors, with resultant $p70^{s6K}$ activity inhibition and G1 arrest. In some cases, the PP2A/$p70^{s6K}$ pathway can mediate Smad-independent growth-inhibitory response to TGF-β, and coexists in parallel with the functional Smad pathway in some epithelial

cells. This indicates that the Smad pathway is dispensable for certain TGF-β inhibitory responses[75].

In addition, TGF-β can activate PI3K-Akt (also known as protein kinase B, PKB) pathway. Upon activation of PI3K by the tyrosine kinase receptors, the catalytic subunit of PI3K generates phosphoinositide phosphate 2 (PIP2) and PIP3, followed by the activation of serine/threonine kinase Akt, which functions to inhibit the activities of the Forkhead box transcription factor class O (FOXO), p53, and nuclear factor-κB (NF-κB)[76]. TGF-β1 promoted *in vitro* angiogenesis partially via activation of the PI3K-Akt pathway[77]. TGF-β1 was involved in epithelial to mesenchymal transition (EMT) through inducing phosphorylation of Akt at serine-473 and Akt *in vitro* kinase activity[78].

4 The cross-talk between the Smad pathway and other signaling pathways

4.1 The cross-talk between the Smad pathway and MAPK pathway

The biological effects of TGF-β involve the cooperation and counteraction between the Smad pathway and the MAPK pathway. Smads can interact directly with transcriptional factors activated by MAPK such as AP-1 and ATF2. SBE is often found in proximity to AP-1 binding site in promoter region, resulting in Smads competition with AP-1 for binding the specific DNA[16]. In addition, loss of Smad4 led to the hyperactivation of the ERK1/2 pathway and overexpression of Ras[79]. These indicate the regulatory roles of Smads in the MAPK pathway.

The MAPK pathway is implicated in the regulation of the Smad pathway at the level of Smad. Activated Ras could enhance UPP-mediated proteolysis of Smad4, suggesting a changed activation balance between the Smad pathway and the MAPK pathway results from the suppression of the Smad pathway in carcinomas expressing activated Ras. Inhibition of the Smad signal transduction by Ras constitutes an important mechanism for blocking TGF-β biological effect in transformed cells[80]. In addition, the phosphorylation of R-Smads in the linker region by ERK can block the entry of Smad complexes into the nucleus[27]. The ERK pathway can block Smads activities by stabilizing the formation of TGIF-Smad complex[81]. In contrast, there is also compelling evidence that the MAPK pathway can facilitate flux through the Smad pathway. Smad3 phosphorylation outside its SSXS motif by JNK facilitates both its activation by the type I receptor and its nuclear accumulation[82]. Four phosphorylation sites for ERK in the linker region of rat Smad1 are essential for its transactivation function[83]. Furthermore, the phosphorylation of Smad1 by ERK can enhance its heteromerization with Smad4 and subsequent nuclear accumulation[84]. Therefore, the molecular basis for the dual effect of MAPK on the Smad pathway deserves further investigations.

4.2 The cross-talk between the Smad pathway and the JAK/STAT pathway

IFN-γ signaling involves the JAK/STAT pathway. The Smad pathway has been found to be inhibited by the STAT-induced expression of Smad7[85]. In primary fetal neural progenitor cells,

LIF activated STAT3, and the cooperation between STAT3 and Smad7 affected the differentiation of these cells into astrocytes[86]. In dermal fibroblast cells, the stimulation of IFN-γ induced the activation of STAT1α, resulting in competition of STAT1α with Smad3 for binding to p300/CBP and subsequent block of transcriptional activation of TGF-β-responsive genes[87]. These results demonstrate the negative regulation of the Smad pathway by the JAK/STAT pathway.

The mechanisms of TGF-β signaling are summarized in fig. 2.

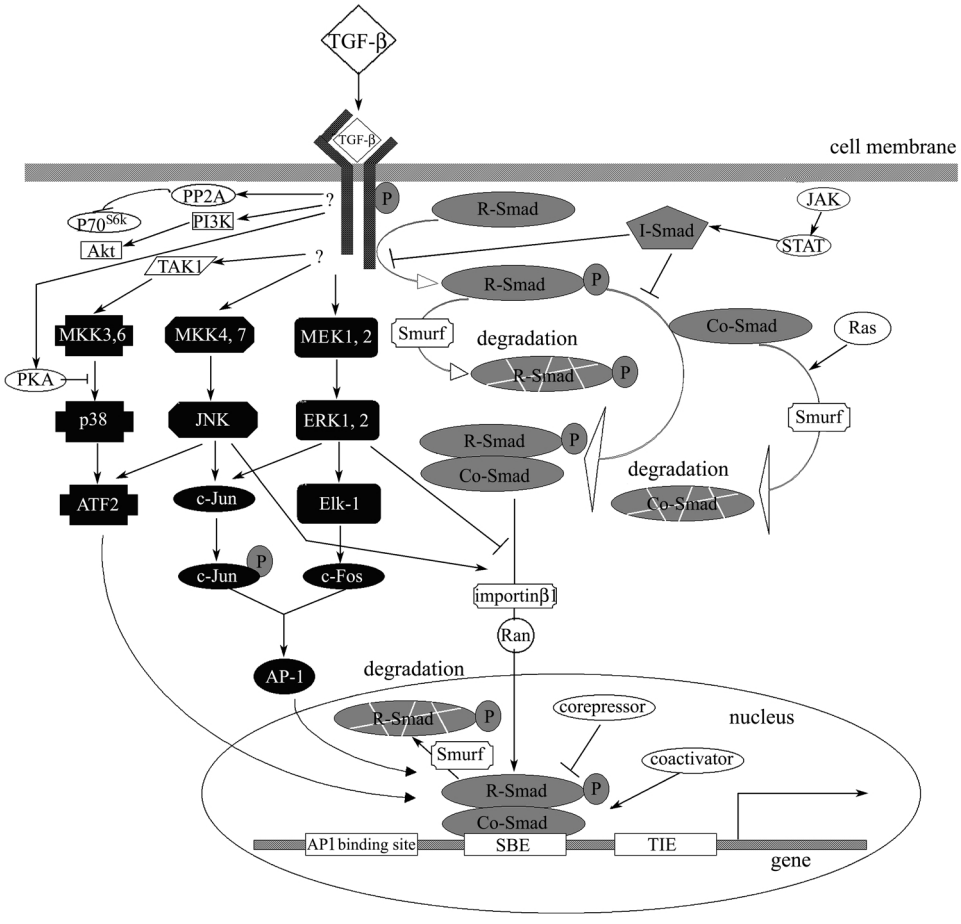

Fig. 2. TGF-β signaling pathways. TGF-β, Transforming growth factor-β; R-Smad, receptor-regulated Smad; Co-Smad, common-mediator Smad; I-Smad, inhibitory Smad; MKK, MAPK (mitogen-activated protein kinase) kinase; JNK, c-Jun NH2-terminal kinase; ERK, extracellular stimulus responsive kinase; MEK, MAPK or ERK kinase; ATF2, activating transcription factor 2; AP-1, activating protein-1; PP2A, protein phosphatase 2A; PI3K, phosphatidylinositol-3-OH kinase; Akt, protein kinase identified in the Akt virus; TAK1, TGF-β-activating kinase 1; PKA, protein kinase A; JAK, Janus kinase; STAT, signal transducer and activator of transcription; Smurf, Smad ubiquitination regulatory factor; SBE, Smad-binding element; TIE, TGF-β inhibitory element.

5 Perspectives

The specificity of TGF-β signaling lies in its transmembrane serine/threonine kinase recep-

tors, and its complexity lies in that TGF-β receptors could activate other signaling pathways in addition to the Smad pathway, and that there are cross-talk between the Smad pathway and other signaling pathways. Clearly, important aspects of the molecular mechanisms of TGF-β signaling remain to be clarified. Among those, the primary future tasks are the elucidation of: the mechanism of various TGF-β binding proteins in helping TGF-β bind with its receptors; the mechanism of endocytosis of TGF-β-receptor complex; the relative importance of various R-Smads in the signaling; the mechanisms of oligomerization and nucleocytoplasmic shuttling of Smads; cofactors for Smad complex and their functioning mechanisms; the *cis*-acting elements in target genes to which coactivators- or corepressors-Smads complexes bind; new members of E1, E2, and E3 mediating the ubiquitination of different Smads. Further research on the mutation of transcriptional corepressors could promise to provide more effective drug targets for gene therapy of genetic disease and cancer.

A major quest of TGF-β signaling is to elaborate the cross-talk mechanisms of the Smad pathway with other signaling pathways. A key problem remaining, however, is the identity of the downstream mediator coupling TGF-β receptors to the activation of MAPK pathway. Thus far, only serine residues phosphorylation sites for ERK were found in the linker regions of R-Smads, but it has not been reported whether phosphorylation sites for p38 and JNK exist in Smads. In addition, since the phosphorylation of R-Smads by ERK can lead to stimulatory or inhibitory effect on the nuclear entry of Smad complex, whether there are specific molecules mediating this dual effect or how they function deserves future clarification. Although it has been shown that TGF-β could activate the PP2A/$p70^{s6K}$ pathway and the PI3K-Akt pathway, the process of the activation remains unexplored. The research on the cross-talk between the Smad pathway and the JAK/STAT pathway is preliminary. Whether Smads could modulate the activation of the JAK/STAT pathway, and whether there are cross-talk of the Smad pathway with the PP2A/$p70^{s6K}$ pathway and the PI3K-Akt pathway, are additional future goals of importance. Furthermore, other growth factors and cytokines also appear to be involved in TGF-β signaling. Therefore, the specificity of TGF-β signaling should be integrated into the whole signaling network during physiological and pathological processes.

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References

1. Massague, J., TGF-β signal transduction, Annu. Rev. Biochem., 1998, 67: 753—791.
2. Balemans, W., Van Hul, W., Extracellular regulation of BMP signaling in vertebrates: A cocktail of modulators, Dev. Biol., 2002, 250(2): 231.
3. Iwamoto, T., Oshima, K., Seng, T. et al., STAT and SMAD signaling in cancer, Histol. Histopathol., 2002, 17(3): 887—895.
4. Ten Dijke, P., Goumans, M. J., Itoh, F. et al., Regulation of cell proliferation by Smad proteins, J. Cell. Physiol., 2002, 191(1): 1—16.

5. Mathews, L. S., Vale, W. W., Expression cloning of an activin receptor, a predicted transmembrane serine kinase, Cell, 1991, 65(6): 973—982.
6. Lin, H. Y., Wang, X. F., Ng-Eaton, E. et al., Expression cloning of the TGF-β type II receptor, a functional transmembrane serine/threonine kinase, Cell, 1992, 68(4): 775—785.
7. Wrana, J. L., Attisano, L., The Smad pathway, Cytokine Growth Factor Rev., 2000, 11(1-2): 5—13.
8. Wrana, J. L., Attisano, L., Wieser, R. et al., Mechanism of activation of the TGF-β receptor, Nature, 1994, 370(6488): 341—347.
9. Zhu, H. J., Burgess, A. W., Regulation of transforming growth factor-β signaling, Mol. Cell. Biol. Res. Commun., 2001, 4(6): 321—330.
10. Lux, A., Attisano, L., Marchuk, D. A., Assignment of transforming growth factor β1 and β3 and a third new ligand to the type I receptor ALK-1, J. Biol. Chem., 1999, 274(15): 9984—9992.
11. Barbara, N. P., Wrana, J. L., Letarte, M., Endoglin is an accessory protein that interacts with the signaling receptor complex of multiple members of the transforming growth factor-β superfamily, J. Biol. Chem., 1999, 274(2): 584—594.
12. Zwaagstra, J. C., El-Alfy, M., O'Connor-McCourt, M. D., Transforming growth factor (TGF)-β 1 internalization: Modulation by ligand interaction with TGF-β receptors types I and II and a mechanism that is distinct from clathrin-mediated endocytosis, J. Biol. Chem., 2001, 276(29): 27237—27245.
13. Zwaagstra, J. C., Guimond, A., O'Connor-McCourt, M. D., Predominant intracellular localization of the type I transforming growth factor-β receptor and increased nuclear accumulation after growth arrest, Exp. Cell Res., 2000, 258(1): 121—134.
14. Wakefield, L. M., Piek, E., Bottinger, E. P., TGF-β signaling in mammary gland development and tumorigenesis, J. Mammary Gland Biol. Neoplasia, 2001, 6(1): 67—82.
15. Dennler, S., Goumans, M. J., ten Dijke, P., Transforming growth factor β signal transduction, J. Leukoc. Biol., 2002, 71(5): 731—740.
16. de Caestecker, M. P., Piek, E., Roberts, A. B., Role of transforming growth factor-β signaling in cancer, J. Natl. Cancer Inst., 2000, 92(17): 1388—1402.
17. Raftery, L. A., Twombly, V., Wharton, K. et al., Genetic screens to identify elements of the decapentaplegic signaling pathway in *Drosophila*, Genetics, 1995, 139(1): 241—254.
18. Sekelsky, J. J., Newfeld, S. J., Raftery, L. A. et al., Genetic characterization and cloning of mothers against dpp, a gene required for decapentaplegic function in *Drosophila melanogaster*, Genetics, 1995, 139(3): 1347—1358.
19. Savage, C., Das, P., Finelli, A. L. et al., *Caenorhabditis elegans* genes sma-2, sma-3, and sma-4 define a conserved family of transforming growth factor β pathway components, Proc. Natl. Acad. Sci. U. S. A., 1996, 93(2): 790—794.
20. Derynck, R., Gelbart, W. M., Harland, R. M. et al., Nomenclature: Vertebrate mediators of TGFβ family signals, Cell, 1996, 87(2): 173.
21. Chen, W., Fu, X., Sheng, Z., Review of current progress in the structure and function of Smad proteins, Chin. Med. J., 2002, 115(3): 446—450.
22. Moustakas, A., Souchelnytskyi, S., Heldin, C. H., Smad regulation in TGF-β signal transduction, J. Cell Sci., 2001, 114(Pt 24): 4359—4369.
23. Lo, R. S., Chen, Y. G., Shi, Y. et al., The L3 loop: A structural motif determining specific interactions between SMAD proteins and TGF-β receptors, EMBO. J., 1998, 17(4): 996—1005.
24. Shi, Y., Hata, A., Lo, R. S. et al., A structural basis for mutational inactivation of the tumour suppressor Smad4, Nature, 1997, 388(6637): 87—93.
25. Maurice, D., Pierreux, C. E., Howell, M. et al., Loss of Smad4 function in pancreatic tumors: C-terminal truncation leads to decreased stability, J. Biol. Chem., 2001, 276(46): 43175—43181.
26. Zimmerman, C. M., Padgett, R. W., Transforming growth factor β signaling mediators and modulators, Gene, 2000, 249(1-2): 17—30.
27. Kretzschmar, M., Doody, J., Timokhina, I. et al., A mechanism of repression of TGFβ/Smad signaling by oncogenic Ras,

Genes Dev., 1999, 13(7): 804—816.

28. Souchelnytskyi, S., Tamaki, K., Engstrom, U. et al., Phosphorylation of Ser465 and Ser467 in the C terminus of Smad2 mediates interaction with Smad4 and is required for transforming growth factor-β signaling, J. Biol. Chem., 1997, 272(44): 28107—28115.
29. Hata, A., Lo, R. S., Wotton, D. et al., Mutations increasing autoinhibition inactivate tumour suppressors Smad2 and Smad4, Nature, 1997, 388(6637): 82—87.
30. de Caestecker, M. P., Parks, W. T., Frank, C. J. et al., Smad2 transduces common signals from receptor serine-threonine and tyrosine kinases, Genes Dev., 1998, 12(11): 1587—1592.
31. Zhu, H., Kavsak, P., Abdollah, S. et al., A SMAD ubiquitin ligase targets the BMP pathway and affects embryonic pattern formation, Nature, 1999, 400(6745): 687—693.
32. de Caestecker, M. P., Yahata, T., Wang, D. et al., The Smad4 activation domain (SAD) is a proline-rich, p300-dependent transcriptional activation domain, J. Biol. Chem., 2000, 275(3): 2115—2122.
33. Bauer, M., Schuppan, D., TGFβ1 in liver fibrosis: Time to change paradigms? FEBS. Lett., 2001, 502(1-2): 1—3.
34. Tsukazaki, T., Chiang, T. A., Davison, A. F. et al., SARA, a FYVE domain protein that recruits Smad2 to the TGFβ receptor, Cell, 1998, 95(6): 779—791.
35. Burd, C. G., Emr, S. D., Phosphatidylinositol(3)-phosphate signaling mediated by specific binding to RING FYVE domains, Mol. Cell, 1998, 2(1): 157—162.
36. Correia, J. J., Chacko, B. M., Lam, S. S. et al., Sedimentation studies reveal a direct role of phosphorylation in Smad3:Smad4 homo- and hetero-trimerization, Biochemistry, 2001, 40(5): 1473—1482.
37. Tada, K., Inoue, H., Ebisawa, T. et al., Region between α-helices 3 and 4 of the mad homology 2 domain of Smad4: Functional roles in oligomer formation and transcriptional activation, Genes Cells, 1999, 4(12): 731—741.
38. Xiao, Z., Liu, X., Henis, Y. I. et al., A distinct nuclear localization signal in the N terminus of Smad 3 determines its ligand-induced nuclear translocation, Proc. Natl. Acad. Sci. U. S. A., 2000, 97(14): 7853—7858.
39. Liu, F., Pouponnot, C., Massague, J., Dual role of the Smad4/DPC4 tumor suppressor in TGFβ-inducible transcriptional complexes, Genes Dev., 1997, 11(23): 3157—3167.
40. Wells, R. G., Fibrogenesis, V., TGF-β signaling pathways, Am. J. Physiol. Gastrointest. Liver Physiol., 2000, 279(5): G845—850.
41. Chen, C. R., Kang, Y., Massague, J., Defective repression of c-myc in breast cancer cells: A loss at the core of the transforming growth factor β growth arrest program, Proc. Natl. Acad. Sci. U. S. A., 2001, 98(3): 992—999.
42. Feng, X. H., Lin, X., Derynck, R., Smad2, Smad3 and Smad4 cooperate with Sp1 to induce p15 (Ink4B) transcription in response to TGF-β, EMBO. J., 2000, 19(19): 5178—5193.
43. Pardali, K., Kurisaki, A., Moren, A. et al., Role of Smad proteins and transcription factor Sp1 in p21(Waf1/Cip1) regulation by transforming growth factor-β, J. Biol. Chem., 2000, 275(38): 29244—29256.
44. White, L. A., Mitchell, T. I., Brinckerhoff, C. E., Transforming growth factor β inhibitory element in the rabbit matrix metalloproteinase-1 (collagenase-1) gene functions as a repressor of constitutive transcription, Biochim. Biophys. Acta, 2000, 1490(3): 259—268.
45. Zawel, L., Dai, J. L., Buckhaults, P. et al., Human Smad3 and Smad4 are sequence-specific transcription activators, Mol. Cell, 1998, 1(4): 611—617.
46. Kusanagi, K., Kawabata, M., Mishima, H. K. et al., α-helix 2 in the amino-terminal mad homology 1 domain is responsible for specific DNA binding of Smad3, J. Biol. Chem., 2001, 276(30): 28155—28163.
47. Yagi, K., Goto, D., Hamamoto, T. et al., Alternatively spliced variant of Smad2 lacking exon 3. Comparison with wild-type Smad2 and Smad3, J. Biol. Chem., 1999, 274(2): 703—709.
48. Labbe, E., Silvestri, C., Hoodless, P. A. et al., Smad2 and Smad3 positively and negatively regulate TGF β-dependent transcription through the forkhead DNA-binding protein FAST2, Mol. Cell, 1998, 2(1): 109—120.
49. Chen, X., Weisberg, E., Fridmacher, V. et al., Smad4 and FAST-1 in the assembly of activin-responsive factor, Nature, 1997, 389(6646): 85—89.

50. Itoh, S., Itoh, F., Goumans, M. J. et al., Signaling of transforming growth factor-β family members through Smad proteins, Eur. J. Biochem., 2000, 267(24): 6954—6967.
51. Wong, C., Rougier-Chapman, E. M., Frederick, J. P. et al., Smad3-Smad4 and AP-1 complexes synergize in transcriptional activation of the c-Jun promoter by transforming growth factor β, Mol. Cell. Biol., 1999, 19(3): 1821—1830.
52. Hanai, J., Chen, L. F., Kanno, T. et al., Interaction and functional cooperation of PEBP2/CBF with Smads. Synergistic induction of the immunoglobulin germline Cα promoter, J. Biol. Chem., 1999, 274(44): 31577—31582.
53. Hata, A., Seoane, J., Lagna, G. et al., OAZ uses distinct DNA- and protein-binding zinc fingers in separate BMP-Smad and Olf signaling pathways, Cell, 2000, 100(2): 229—240.
54. Yanagisawa, J., Yanagi, Y., Masuhiro, Y. et al., Convergence of transforming growth factor-β and vitamin D signaling pathways on SMAD transcriptional coactivators, Science, 1999, 283(5406): 1317—1321.
55. Hua, X., Liu, X., Ansari, D. O. et al., Synergistic cooperation of TFE3 and smad proteins in TGF-β-induced transcription of the plasminogen activator inhibitor-1 gene, Genes Dev., 1998, 12(19): 3084—3095.
56. Zhang, W., Liu, H. T., MAPK signal pathways in the regulation of cell proliferation in mammalian cells, Cell Res., 2002, 12(1): 9—18.
57. Kaji, H., Canaff, L., Lebrun, J. J. et al., Inactivation of menin, a Smad3-interacting protein, blocks transforming growth factor type β signaling, Proc. Natl. Acad. Sci. U. S. A., 2001, 98(7): 3837—3842.
58. Liberati, N. T., Moniwa, M., Borton, A. J. et al., An essential role for Mad homology domain 1 in the association of Smad3 with histone deacetylase activity, J. Biol. Chem., 2001, 276(25): 22595—22603.
59. Zimmerman, C. M., Kariapper, M. S., Mathews, L. S., Smad proteins physically interact with calmodulin, J. Biol. Chem., 1998, 273(2): 677—680.
60. Stroschein, S. L., Wang, W., Zhou, S. et al., Negative feedback regulation of TGF-β signaling by the SnoN oncoprotein, Science, 1999, 286(5440): 771—774.
61. Leong, G. M., Subramaniam, N., Figueroa, J. et al., Ski-interacting protein interacts with Smad proteins to augment transforming growth factor-β-dependent transcription, J. Biol. Chem., 2001, 276(21): 18243—18248.
62. Hata, A., Lagna, G., Massague, J. et al., Smad6 inhibits BMP/Smad1 signaling by specifically competing with the Smad4 tumor suppressor, Genes Dev., 1998, 12(2): 186—197.
63. Kimura, N., Matsuo, R., Shibuya, H. et al., BMP2-induced apoptosis is mediated by activation of the TAK1-p38 kinase pathway that is negatively regulated by Smad6, J. Biol. Chem., 2000, 275(23): 17647—17652.
64. Kavsak, P., Rasmussen, R. K., Causing, C. G. et al., Smad7 binds to Smurf2 to form an E3 ubiquitin ligase that targets the TGF β receptor for degradation, Mol. Cell, 2000, 6(6): 1365—1375.
65. Ebisawa, T., Fukuchi, M., Murakami, G. et al., Smurf1 interacts with transforming growth factor-β type I receptor through Smad7 and induces receptor degradation, J. Biol. Chem., 2001, 276(16): 12477—12480.
66. Lin, X., Liang, M., Feng, X. H., Smurf2 is a ubiquitin E3 ligase mediating proteasome-dependent degradation of Smad2 in transforming growth factor-β signaling, J. Biol. Chem., 2000, 275(47): 36818—36822.
67. Bonni, S., Wang, H. R., Causing, C. G. et al., TGF-β induces assembly of a Smad2-Smurf2 ubiquitin ligase complex that targets SnoN for degradation, Nat. Cell. Biol., 2001, 3(6): 587—595.
68. Fukuchi, M., Imamura, T., Chiba, T. et al., Ligand-dependent degradation of Smad3 by a ubiquitin ligase complex of ROC1 and associated proteins, Mol. Biol. Cell., 2001, 12(5): 1431—1443.
69. Mulder, K. M., Role of Ras and Mapks in TGFβ signaling, Cytokine Growth Factor Rev., 2000, 11(1-2): 23—35
70. Attisano, L., Wrana, J. L., Signal transduction by the TGF-β superfamily, Science, 2002, 296(5573): 1646—1647.
71. Hu, P. P., Shen, X., Huang, D. et al., The MEK pathway is required for stimulation of p21(WAF1/CIP1) by transforming growth factor-β, J. Biol. Chem., 1999, 274(50): 35381—35387.
72. Lehmann, K., Janda, E., Pierreux, C. E. et al., Raf induces TGFβ production while blocking its apoptotic but not invasive responses: A mechanism leading to increased malignancy in epithelial cells, Genes Dev., 2000, 14(20): 2610—2622.
73. Hocevar, B. A., Brown, T. L., Howe, P. H., TGF-β induces fibronectin synthesis through a c-Jun N-terminal kinase-dependent, Smad4-independent pathway, EMBO. J., 1999, 18(5): 1345—1356.

74. Axmann, A., Seidel, D., Reimann, T. et al., Transforming growth factor-β1-induced activation of the Raf-MEK-MAPK signaling pathway in rat lung fibroblasts via a PKC-dependent mechanism, Biochem. Biophys. Res. Commun., 1998, 249(2): 456—460.
75. Wakefield, L. M., Roberts, A. B., TGF-β signaling: Positive and negative effects on tumorigenesis, Curr. Opin. Genet. Dev., 2002, 12(1): 22—29.
76. Brunet, A., Datta, S. R., Greenberg, M. E., Transcription-dependent and -independent control of neuronal survival by the PI3K-Akt signaling pathway, Curr. Opin. Neurobiol., 2001, 11(3): 297—305.
77. Vinals, F., Pouyssegur, J., Transforming growth factor β1 (TGF-β1) promotes endothelial cell survival during *in vitro* angiogenesis via an autocrine mechanism implicating TGF-α signaling, Mol. Cell. Biol., 2001, 21(21): 7218—7230.
78. Bakin, A. V., Tomlinson, A. K., Bhowmick, N. A. et al., Phosphatidylinositol 3-kinase function is required for transforming growth factor β-mediated epithelial to mesenchymal transition and cell migration, J. Biol. Chem., 2000, 275(47): 36803—36810.
79. Iglesias, M., Frontelo, P., Gamallo, C. et al., Blockade of Smad4 in transformed keratinocytes containing a Ras oncogene leads to hyperactivation of the Ras-dependent Erk signalling pathway associated with progression to undifferentiated carcinomas, Oncogene, 2000, 19(36): 4134—4145.
80. Saha, D., Datta, P. K., Beauchamp, R. D., Oncogenic ras represses transforming growth factor-β /Smad signaling by degrading tumor suppressor Smad4, J. Biol. Chem., 2001, 276(31): 29531—29537.
81. Lo, R. S., Wotton, D., Massague, J., Epidermal growth factor signaling via Ras controls the Smad transcriptional co-repressor TGIF, EMBO. J., 2001, 20(1-2): 128—136.
82. Engel, M. E., McDonnell, M. A., Law, B. K. et al., Interdependent SMAD and JNK signaling in transforming growth factor-β-mediated transcription, J. Biol. Chem., 1999, 274(52): 37413—37420.
83. Liu, X., Yue, J., Frey, R. S. et al., Transforming growth factor β signaling through Smad1 in human breast cancer cells, Cancer Res., 1998, 58(20): 4752—4757.
84. Yue, J., Frey, R. S., Mulder, K. M., Cross-talk between the Smad1 and Ras/MEK signaling pathways for TGFβ, Oncogene, 1999, 18(11): 2033—2037.
85. Ulloa, L., Doody, J., Massague, J., Inhibition of transforming growth factor-β/SMAD signalling by the interferon-γ/STAT pathway, Nature, 1999, 397(6721): 710—713.
86. Nakashima, K., Yanagisawa, M., Arakawa, H. et al., Synergistic signaling in fetal brain by STAT3-Smad1 complex bridged by p300, Science, 1999, 284(5413): 479—482.
87. Ghosh, A. K., Yuan, W., Mori, Y. et al., Antagonistic regulation of type I collagen gene expression by interferon-γ and transforming growth factor-β. Integration at the level of p300/CBP transcriptional coactivators, J. Biol. Chem., 2001, 276(14): 11041—11048.