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Summary Members of the transforming growth factor-β (TGF-β) superfamily are pleiotropic cytokines that have the ability to regulate numerous cell functions, including proliferation, differentiation, apoptosis, epithelial-mesenchymal transition, and production of extracellular matrix, allowing them to play an important role during embryonic development and for maintenance of tissue homeostasis. Three TGF-β isoforms have been identified in mammals. They propagate their signal via a signal transduction network involving receptor serine/threonine kinases at the cell surface and their substrates, the SMAD proteins. Upon phosphorylation and oligomerization, the latter move into the nucleus to regulate transcription of target genes. This review will summarize recent advances in the understanding of the mechanisms underlying SMAD modulation of extracellular matrix gene expression in the context of wound healing and tissue fibrosis.

Abbreviations

BMP	bone morphogenic proteins
CBP	CREB Binding Protein
ECM	extracellular matrix
FKBP12	FK506-binding protein
IFN-γ	interferon-γ
JNK	Jun-N-terminal kinase
MH	MAD Homology domain
NF-κB	nuclear factor κB
NEMO	NF-κB Essential Modulator
PAI-1	plasminogen activator inhibitor-1
SARA	SMAD anchor for receptor activation
SKI	Sloan-Kettering Institute proto-oncogene
SMAD	for SMA/MAD related
SnoN	Ski-related novel gene N
TβRI/II	TGF-β type I/II receptor protein
TGF-β	transforming growth factor-β
TGIF	TG3-interacting factor
TNF-α	tumor necrosis factor-α

1. The TGF-β super-family

The transforming growth factor-β (TGF-β) superfamily, a large group of pleiotropic cytokines, consists of more than 30 structurally related factors found in vertebrates, insects, and nematodes. Phylogenically, members of the TGF-β family are classified into several subfamilies including the bone morphogenic proteins (BMP), the activins, various intermediate members, and the TGF-β subfamily (for review see [1; 2]). With their specific receptors being ubiquitously expressed, they are implicated in various cell functions, including apoptosis, proliferation, differentiation, motility, and adhesion. Accordingly, these factors play a pivotal role in physiological circumstances, including embryonic development and wound healing, and in pathological conditions such as cancer and tissue fibrosis.

Three structurally almost identical isoforms of TGF-β (TGF-β1-3), encoded by three distinct genes, have been identified in mammals. Their isoforms share a common structural knot motif consisting of six cysteine residues joined together by three intrachain disulfide bonds that stabilize the β-sheet bands. One free cysteine forms an interchain disulfide bond with an identical monomeric chain to permit formation of mature TGF-β dimers and their secretion as large latent complex, covalently bound with latent TGF-β binding protein. Activation of the LTGF-β by cleavage of the latency associated peptide region by various proteases such as plasmin, thrombin or plasma transglutaminase, allows the bioactive TGF-β to interact with specific receptors at the cell surface [3].

Even if their *in vitro* effects seem to be indistinguishable, knockout experiments in mice have revealed that each TGF-β isoform plays an independent and non-redundant role during the course of embryonic development. For instance, targeted disruption of the mouse TGF-β1 gene results to a wasting syndrome accompanied by a multifocal,

mixed inflammatory cell reaction and tissue necrosis, leading ultimately to organ failure and death, indicating a critical role of TGF-β1 in immune responses [4]. TGF-β2 knockout mice have multiple severe developmental defects including cardiac, lung, craniofacial, limb, spinal column, eye, inner ear and urogenital defects [5]. Finally, mice lacking TGF-β3 exhibit an incompletely penetrant phenotype characterized by failure of the palatal shelves to fuse, resulting in cleft palate. This defect results from impaired adhesion of the apposing medial edge epithelia of the palatal shelves and subsequent elimination of the mid-line epithelial seam, suggesting that TGF-β3 is crucial for epithelial differentiation [6]. Of note, exogenous addition of TGF-β3 to cutaneous experimental wounds reduces scarring in adult rats and leads to a final result that is almost indistinguishable from that of embryonic scarless wound healing [7; 8]. Together, their phenotypes emphasize the numerous non-compensatory functions of these three TGF-β isoforms.

2. TGF-β receptors

The TGF-β superfamily members transduce their signal from the membrane to the nucleus through distinct combinations of transmembrane type I and type II serine/threonine receptors and their downstream effectors, known as SMAD proteins (Fig. 1) [2; 9]. So far, only one TGF-β type II receptor protein (TβRII) has been isolated that binds TGF-βs [10].

From the structural point of view, TGF-β type I (TβRI) and II receptors are very similar glycoproteins, characterized by a cysteine-rich extracellular domain, a single hydrophobic transmembrane domain, and a C-terminal cytoplasmic serine/threonine kinase domain. The TβRIs have a highly conserved regulatory segment rich in glycine and serine residues, known as the GS region, upstream of the serine/threonine kinase

domain in the cytoplasmic portion of the receptor [1; 11]. Upon binding of its ligands, TβRII recruits the TβRI into an activated heterotetrameric receptor complex. TβRII via its intrinsic kinase activity phosphorylates the TβRI regulatory GS region. In turn the cytoplasmic TβRI serine/threonine kinase domain is activated, leading to subsequent phosphorylation of its intracellular downstream effectors, SMAD proteins [1; 9; 12]. The nine amino acid sequence between kinase subdomains IV and V in TβRIs, termed the L45 loop, is a critical determinant in specifying activation of different SMAD proteins, thereby guaranteeing specificity in transcriptional responses [12]. Unphosphorylated TβRI forms complexes with the FK506-binding protein (FKBP12). Binding of the latter to the GS region of TβRI caps the TβRII phosphorylation sites, thus further stabilizing the inactive conformation of TβRI [12].

3. SMADs as mediators of TGF-β signaling

Proteins of the SMAD family are the first identified substrates of the TGF-β type I receptor kinases. They play a central role in the transduction of receptor signals to specific target genes in the nucleus. So far, eight SMAD family members have been identified which, based on structural and functional differences, have been subdivided into three groups: (1) receptor-associated SMADs (R-SMADs) are direct substrates of TGF-β family receptor kinases (SMAD1, 2, 3, 5, 8), (2) co-SMADs that associate during signaling with these receptor-regulated SMADs (SMAD4), and (3) inhibitory SMADs (SMAD6, 7) that antagonize the signaling function of the former groups. Among the R-SMADs, SMAD1, SMAD5 and SMAD8 are substrates for BMP type I receptors [13] and accordingly mediate BMP signals, while SMAD2 and SMAD3 are TβRI substrates and mediate both TGF-β and activin signals [14]. R-SMADs interact directly with ligand-activated TβRI and are subsequently phosphorylated on two serine residues within a

conserved –SSXS-motif at their C-terminus [2]. SARA (SMAD Anchor for Receptor Activation), a FYVE domain membrane-bound protein that interacts directly with SMAD2 and SMAD3, facilitates their recruitment to the activated receptor complexes by controlling the subcellular localization of the two R-SMADs [15]. R-SMAD recruitment and activation by TβRI may also be regulated by the microtubule network [16]. Once phosphorylated, R-SMADs associate as heterodimeric complexes with SMAD4 and move into the nucleus where they bind DNA and initiate target gene transactivation, either alone or in association with other transcriptional partners (Fig. 1) [2].

Inhibitory SMADs have the ability to form stable associations with TβRI, and to interfere with the phoshorylation of R-SMADs and their heteromerization with SMAD4 [17; 18]. SMAD6 may also compete specifically with SMAD4 for binding to the BMP receptor-activated SMAD1, by forming an inactive SMAD1/SMAD6 complex in the cytoplasm [19]. Most recently, SMAD7 has been shown to interact with the E3 ubiquitin ligases Smurf 1 and Smurf 2, recruiting them to TβR complexes and inducing the degradation of activated TβRI [20; 21].

R-SMADs and co-SMADs are characterized by two highly conserved globular N-terminal and C-terminal domains, MH1 and MH2 (MAD Homology domain), respectively, and a linker region of variable length and sequence [22]. The MH2 domain from R-SMADs contains the –SSXS– receptor phosphorylation site. Once phosphorylated by activated receptor kinases, the MH2 domain dissociates from the MH1 domain through conformational changes, allowing the signaling cascade to proceed [23]. In the basal state the MH2 domain mediates the association of R-SMADs with type I receptors [24], while in the activated state the MH2 domain is involved in protein/protein interactions with co-SMADs, various DNA-binding cofactors, and transcriptional coactivators and corepressors. Once in the nucleus, activated R-SMADs

contact DNA, which, in the case of SMAD3, is the DNA sequence CAGAC, through their MH1 domain [22; 25]. The presence of a 30 amino-acid insertion within the MH1 domain of SMAD2 as compared to that of SMAD3 prevents its direct interaction with specific DNA-binding motifs. Accordingly, SMAD2 dependent gene transcription requires the recruitment of putative transcription factors like FAST1 and FAST2, which allows the binding of the SMAD2/SMAD4/FAST-1 complexes to activin response elements [26; 27; 28].

4. Coactivators and corepressors of SMAD-dependent gene transcription

In the nucleus, activated SMAD complexes may either activate or repress gene expression, depending on the recruitment of coactivators or corepressors into transcriptional complexes. The role for CREB Binding Protein (CBP) and p300 as essential coactivators for SMAD-driven gene expression has been well documented [29; 30; 31; 32; 33], and competition for p300/CBP has been suggested to mediate some examples of signal-induced transcriptional repression. Indeed, in the context of SMAD signaling, p300/CBP squelching by c-Jun or STAT1 may explain, at least in part, the antagonism exerted by tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ) against SMAD signaling [34; 35]. In addition, we have recently established that agents that activate PKA, e.g. PGE_2 and cAMP increasing agents, are able to antagonize SMAD signaling via PKA-dependent, CREB-mediated, disruption of SMAD-p300/CBP complexes (Fig. 1) [36].

SMADs may also recruit transcriptional corepressors such as TG3-interacting factor (TGIF) [37], Sloan-Kettering Institute proto-oncogene (SKI), Ski-related novel gene N (SnoN) [38; 39], or SNIP1 [40]. Generally, these proteins bind chromatin-condensating histone deacetylases, which oppose the function of the histone acetyltransferase

activity associated with the coactivators p300 and CBP. For example, overexpression of SNIP1 inhibits multiple gene responses to TGF-β and p300/CBP, as well as the formation of SMAD4/p300 complexes [40]. Even a slight reduction in the activity of TGIF has been shown to have devastating developmental consequences [37]. Thus, the level of expression of these corepressors has important physiological consequences, by either setting a threshold for TGF-β-induced transcriptional activation involving p300/CBP or helping to terminate TGF-β signal.

5. Transcriptional regulation of SMAD target genes

Once in the nucleus, activated SMAD complexes become transcriptional factors binding directly to SMAD-specific DNA-binding motifs. One of the first described genuine SMAD binding sequences, which binds an activated SMAD3 complex in response to a TGF-β signal, was identified within the human *COL7A1* promoter [41; 42]: TGF-β upregulation of *COL7A1* gene expression is mediated by rapid and transient binding of a SMAD-containing complex to a bipartite element consisting of two CAGA repeats in its 5' end, and a Medea-like (Drosophilia SMAD4 homologue) binding site in 3', framing an AP-1 binding site whose integrity is not necessary for either SMAD-driven transactivation or SMAD/DNA complex formation [43]. Subsequently, CAGA elements have been characterized as optimal for recombinant SMAD3 and SMAD4 binding [25] and this sequence has been identified within the promoter regions of numerous TGF-β-regulated genes, including plasminogen activator inhibitor 1 (PAI-1) [44], JunB [45], COL1A2 [46], c-Jun [45], SMAD7 [47], platelet-derived growth factor B-chain [48], integrin β5 [49], cyclin-dependent kinase inhibitor $p15^{INK4}$ [50], or connective tissue growth factor [51]. Moreover, several extracellular matrix (ECM) encoding genes are direct SMAD targets, including COL1A2, COL3A1, COL5A2, COL6A1, COL6A3,

and TIMP-1, illustrating the key role of the SMAD signaling pathway for the simultaneous activation of fibrillar collagen genes by TGF-β [52].

6. The role of TGF-β in tissue fibrosis and wound healing

TGF-β is a crucial regulator of ECM deposition, as it control both the expression of components of ECM network, such as the fibrillar collagens and fibronectin, and the expression of protease inhibitors, including PAI-1 or TIMPs [53]. These combined anabolic and anti-catabolic effects of TGF-β make it a key growth factor in the development of tissue fibrosis [54]. Indeed, type I collagen and ECM deposition is one of the unifying histopathologic hallmarks of fibrotic disorders such as liver cirrhosis, renal sclerosis, systemic sclerosis and keloid scars. Mechanistically, collagen deposition in the skin could result from either enhanced biosynthetic activity or from a reduced rate of degradation, both phenomena leading to an imbalance in the collagen turnover in affected tissues.

Various studies have provided evidence that transcriptional activation of collagen gene expression is probably the most important regulatory mechanism leading to fibrosis [55; 56]. SMADs, the only downstream substrates of TβRI known so far, are central in most actions of the TGF-β family regarding ECM gene expression [52; 53; 57]. However, other factors such as connective tissue growth factor, may contribute to some of the TGF-β effects on extracellular matrix gene expression (reviewed in [58]). In addition TGF-β has been reported to activate members of the stressed activated MAP kinase cascade in various cell types [59; 60; 61; 62]. The relevance of these signaling pathways in the context of tissue fibrosis is still not fully understood, but *in vitro* studies suggest that TGF-β-activated p38 phosphorylation may be implicated in *COL1A1* gene expression in dermal fibroblasts [63; 64]. In addition, in keloid fibroblasts the

synchronous activation of the ERK and phosphatidylinositol 3-kinase pathway has been demonstrated to be critical for increased collagen type I and III production [65].

In contrast to predictions made on the basis of the ability of exogenous TGF-β to improve wound healing, SMAD3-null mice paradoxically show accelerated cutaneous wound healing compared to wild-type mice, characterized by an increased rate of re-epithelialization and significantly reduced local infiltration of monocytes [66]. From these data, it may therefore be concluded that SMAD3 may mediate *in vivo* signaling that is inhibitory to epithelial wound healing and that during the physiological process of wound healing, suppression of SMAD3 levels may engage. Thus, complete loss of this signaling intermediate in SMAD3-null mice results in further acceleration of the wound closure. Of note, reduced deposition of ECM in these mice could be abolished by exogenous TGF-β1, implicating that ECM gene regulation by TGF-β may also occur via SMAD3-independent mechanisms. Together, this report provides support for therapeutic intervention aimed at disrupting the SMAD3 pathway *in vivo*, in the context of impaired wound healing.

7. Interfering with the fibrotic process at the transcriptional level: mechanisms of antagonism between pro-inflammatory cytokines and TGF-β

It is reasonable to hypothesize that a better understanding of the mechanisms of TGF-β-mediated upregulation of ECM gene expression in fibrotic tissue will provide novel approaches to the therapy of these essentially incurable diseases. Accordingly, a better understanding of the mechanisms by which pro-inflammatory cytokines, such as TNF-α, are able to interfere with the TGF-β-induced SMAD signaling are of utmost importance. TNF-α has been suggested to block SMAD signaling via mechanisms that implicate either c-Jun [34] or nuclear factor κB (NF-κB) [67]. The RelA subunit of NF-κB

mediates TNF-α-induced expression of the inhibitory SMAD, SMAD7 (Fig. 1 and 3), which, in turn, blocks TGF-β signaling [67]. Data from our own laboratory indicate that c-Jun and JunB, both activated by TNF-α via the Jun-N-terminal kinase (JNK) pathway, are also capable of interrupting SMAD3-mediated transcription: Jun/SMAD3 complexes may form off-DNA, preventing SMAD3 binding to cognate DNA sequences [34]. JNK activity promotes such Jun/SMAD3 association [68]. Accordingly, in $JNK_1^{-/-}$-$JNK_2^{-/-}$ ($JNK^{-/-}$) fibroblasts, TNF-α inhibited basal *COL1A2* expression but had no effect on TGF-β-driven gene transactivation unless *jnk1* was introduced exogenously (Fig. 1 and 2) [69]. On the contrary lack of NF-κB activity in NF-κB Essential Modulator knockout ($NEMO^{-/-}$) fibroblasts did not influence the antagonism exerted by TNF-α against TGF-β but prevented repression of basal *COL1A2* gene expression [69]. These results defined an alternate usage of distinct signaling pathways by TNF-α to inhibit the expression of *COL1A2*, depending on the activation state of this target gene. With regard to JNK function in the context of ECM turnover, these findings are complementary to a recent study indicating that a synthetic inhibitor of JNK, SP600125, suppresses interleukin-1-induced phospho-Jun accumulation, Jun–DNA interactions and interstitial collagenase (MMP-1) gene expression in synovial fibroblasts [70]. Thus the benefit of JNK targeting in degenerative inflammatory diseases such as rheumatoid arthritis may result not only from blocking degradative events induced by interleukin-1 or TNF-α, but also from preventing cytokines to antagonize the anabolic functions of TGF-β on ECM deposition. Conversely, means to activate the JNK pathway may be of interest in pathological situations where interfering with TGF-β signaling and subsequent ECM deposition is critical, such as in fibrosis.

The pleiotropic cytokine IFN-γ exerts opposite effects on diverse cellular functions modulated by TGF-β [71; 72; 73]. It has been shown that in cells of epithelial origin,

IFN-γ induces the expression of SMAD7 via the activation of the JAK1 and STAT1, SMAD7 preventing the interaction of SMAD3 with the TGF-β receptor [74]. Another mechanism by which the JAK/STAT pathway may alter SMAD-driven transcription, involves competition between activated STAT1 and SMAD3 for limiting amounts of cellular p300/CBP coactivators, a mechanism which may explain the antagonistic modulation of IFN-γ against TGF-β-induced *COL1A2* gene transcription (Fig. 3) [35]. Alternatively, IFN-γ may inhibit *COL1A2* gene transcription, both basal and induced by TGF-β [72; 75]. IFN-γ activates the transcription factor YB-1, which not only binds a proximal region of the *COL1A2* promoter [75], but also physically interacts with SMAD3 to prevent its binding to the TGF-β-response element of the *COL1A2* promoter [76]. Because TGF-β and IFN-γ are secreted by inflammatory cells at sites of tissue injury, one may assume that their antagonistic interactions regulating collagen synthesis are likely to be important for the maintenance of connective tissue homeostasis.

8. Experimental targeting of SMAD signaling in fibrosis

SMAD7, a natural antagonist of TGF-β signaling, acts both by preventing SMAD2/3-receptor interactions and subsequent SMAD phosphorylation, and by recruiting E3 ubiquitin ligases called Smurf1 and Smurf2 to the receptor complexes, leading to the degradation of the latter. It has been suggested that defects in SMAD7 may, in certain instances, lead to fibrotic conditions, as reported in the heart and in scleroderma. However, ligand-independent constitutive activation of the intracellular TGF-β/SMAD signaling axis in scleroderma fibroblasts, without profound differences of SMAD7 mRNA levels between control and scleroderma fibroblasts, has also been reported [77].

The therapeutic potential of targeted delivery of this inhibitory SMAD family member to prevent TGF-β-mediated fibrosis *in vivo* has been confirmed in various experimental

models of kidney, lung and liver fibrosis. Thus, intratracheal injection of a recombinant adenovirus carrying mice SMAD7 cDNA prevents bleomycin-induced lung fibrosis in mice [78]. Similar promising results with SMAD7 gene transfer have been achieved in *in vivo* models of liver or renal fibrosis [79; 80]. In systemic sclerosis, deficient SMAD7 expression is regarded as a putative molecular defect leading to disease development [81]. Thus, it is not surprising that *in vitro* adenoviral gene transfer with SMAD7 re-establishes normal, not pathologically intensified, TGF-β signaling in systemic sclerosis fibroblasts [81]. Several hurdles remain before TGF-β targeting can be considered a realistic therapeutic alternative for the treatment of fibrosis, as significant problems may arise with regard to overall tolerance or biological outcome. Even though transgenic mice expressing a soluble form of TβRII have been shown to be protected against metastasis without adverse side effects [82], it has also been shown that fibroblast-specific expression of a kinase-deficient TβRII in mice leads to paradoxical activation of TGF-β signaling pathways with dermal and pulmonary fibrosis [83]. Furthermore, epithelial overexpression of SMAD7, or that of a dominant-negative TGF-β type II receptor, results in severe pathological alterations of epithelial tissues in transgenic mice [84; 85], which could suggest that mesenchyme-specific targeting of the SMAD pathway may be required.

9. Conclusions

There have been major advances in our understanding of the TGF-β-induced intracellular signaling since the identification of SMAD proteins as direct links between the cell surface and the nucleus. The recent development of several SMAD pathway specific knockout mice and transgenic animals has confirmed the pivotal nature of the SMAD pathway in fibrogenesis and tumorigenesis. Still, several difficulties remain

before the TGF-β/SMAD pathway can be efficiently targeted in situations such as tissue fibrosis or impaired wound healing. In particular, the precise spatio-temporal role of each TGF-β/SMAD pathway component during the development of excessive ECM deposition leading to tissue fibrosis remains to be ascertained.

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Figure legends

Fig. 1. The TGF-β/SMAD signaling pathway. This schematic diagram summarizes the various aspects of SMAD signaling, from receptor activation to gene transcription. Interference mechanisms, e.g. SMAD7 or cytokine-induced pathways, are depicted. For further detail, refer to the corresponding text within the manuscript.

Fig. 2. The stress-activated MAP Kinase cascade downstream the TNF-α receptors. Binding of TNF-α to its receptors induces the activation of MAP kinase kinase kinases (MAPKKK) MEKK1 and/or ASK1 via the adaptor protein TRAF2 (TNF-R associated factor 2). These activated MAPKKKs then activate the MAPKK MKK4 and MKK7 which, in turn, phosphorylate the two tyrosine/threonine residues of JNK. MKK4 may also phosphorylate the p38 MAP kinase, which itself is predominantly activated by MKK3 and MKK6 (downstream of ASK1 and TAK1). Activated JNK binds to the N-terminal region of c-Jun and phosphorylates it at Ser63 and Ser73. ATF-2 may be phosphorylated and activated by both JNK and p38, whereas c-Jun is phosphorylated only by JNK.

Fig. 3. The NF-κB pathway. NF-κB transcription factors are normally found in the cytoplasm, retained by interaction with the inhibitory molecule IκB (Inhibitor of κB). Receptor-mediated activation of the NF-κB signaling cascade results in the phosphorylation, ubiquitination and degradation of IκB by 26S proteasome, allowing nuclear translocation of the transcriptional active NF-κB complex. Responsible for phosphorylation of IκB, it is a high molecular weight complex containing two kinases (IKKα and IKKβ) and a regulatory subunit (NEMO).