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▶ **To cite this version:**

Alain Mauviel. Transforming growth factor-beta: a key mediator of fibrosis.. Methods in molecular medicine, 2005, 117, pp.69-80. 10.1385/1-59259-940-0:069 . inserm-00147400

HAL Id: inserm-00147400

https://inserm.hal.science/inserm-00147400

Submitted on 21 May 2007

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Methods in molecular medicine 2005; 117 69-80

Transforming growth factor-β: the Smad pathway and its implications in fibrosis

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Abstract

Transforming growth factor (TGF)-β is a prototypic multifunctional cytokine whose broad modulatory affect numerous biological functions both at the cell and organism levels. These include, but are not limited to, control of immune functions, embryogenesis, carcinogenesis, tissue responses to injury, cell proliferation, extracellular matrix (ECM) synthesis and degradation, and cell migration. The identification of Smad proteins, TGF-β receptor kinase substrates that translocate into the cell nucleus to act as transcription factors, has increased our understanding of the molecular mechanisms underlying TGF-β action. This introductory chapter will outline the current knowledge on how specific signals initiated by the TGF-β receptors are brought to the nucleus to regulate gene expression, with a specific emphasis on how such signaling relates to connective tissue remodeling, repair and fibrosis.

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1. Introduction

The Transforming Growth Factor-β (TGF-β) family comprises over 30 members throughout the animal reign that include TGF-βs *stricto sensu*, Activins and Bone Morphogenic Proteins (BMPs), critical in governing cell fate determination and patterning in the developing embryo. They regulate a broad spectrum of biological responses in the adult [1]. Deregulation of TGF-β signaling during embryonic development leads to developmental abnormalities and, when occurring after birth, participates in several human diseases, the most prominent ones being cancer, autoimmune disorders, and tissue fibrosis [2-4].

2. TGF-β biosynthesis

The three mammalian TGF-β □□□□□□□□, TGF-β1, TGF-β2 and TGF-β3, are encoded by three distinct genes, then secreted as latent precursor molecules (LTGF-β) requiring activation into a mature form for receptor binding and subsequent activation of signal transduction pathways [5]. The LTGF-β molecules consist of 390 to 414 amino acids with three specialized regions: an amino-terminal hydrophobic signal peptide region, the latent-associated peptide (LAP) region of 249 residues, and the C-terminal, potentially bioactive, region consisting of 112 amino acids per monomer. Each TGF-β molecule is characterized by a cysteine knot composed of six cysteine residues involved in intrachain disulfide bonds that stabilize several β-sheet bands. A seventh cysteine allows an interchain disulfide bond with an identical monomeric

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TGF-β chain to generate a mature TGF-β dimer. LTGF-β is secreted as a large latent complex (LLC) covalently bound via the LAP region to LTGF-β-binding protein (LTBP), or as a small latent complex (SLC) without LTBP. The LAP confers latency to the complex whereas LTBP binds TGF-β to the extracellular matrix and enables its proteolytic activation. Conformational changes of the latent TGF-β complex induced by either cleavage of the LAP by various proteases such as plasmin, thrombin, plasma transglutaminases or endoglycosylases, or by physical interactions of the LAP with other proteins, such as thrombospondin-1, releases bioactive, mature TGF-β (Figure 1).

3. TGF-β Receptors

Following activation, TGF-βt□□□□□□□□sits □□□□□□ across the plasma membrane via specific serine/threonine kinase receptors named type I and type II receptors (TβRI and TβRII) [6, 7]. Structurally, they both consist in a small cysteine-rich extracellular region and an intracellular portion bearing the kinase domain. TβRII exhibits intrinsic kinase activity whereas TβRI requires activation by TβRII to exert its kinase function. In unstimulated cells, TβRI is stabilized in an inactive conformation by its association with FKBP12 (FK506 binding protein 12) [8]. Following TGF-β binding to TβRII, recruitment and subsequent activation of TβRI occurs by phosphorylation of serine and threonine residues within a region rich in glycine and serine residues, the GS domain, preceding the kinase domain of TβRI. Receptors are then rapidly internalized into both caveolin- and EEA1-positive endosome depending on their cell membrane association with either lipid raft or non-raft membrane domains [9].

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Of note, a third type of TGF-β receptor, betaglycan, a transmembrane proteoglycan also known as TβRIII, allows high-affinity binding of TGF-β to TβRII but does not transduce signal [10].

4. From cell membrane receptors to the nucleus

Specific signal transduction from the TGF-β receptors to the nucleus is mediated by phosphorylation of evolutionarily conserved cytoplasmic proteins of the Smad family [6, 7], divided into three functional groups: Receptor-associated Smads (R-Smads), directly interact with activated TβRI and are ligand-specific, co-Smads, such as Smad4, a common mediator of all TGF-β family members, and inhibitory Smads (I-Smads), including Smad7.

R-Smads are characterized by two highly conserved proline-rich globular domains called Mad-homology (MH) domains, held together by a variable linker region. Mad is the Drosophila homolog of Smads. The MH1 domain is located in their N-terminal end and the MH2 domain in their C-terminus. In response to TGF-β, TβRI phosphorylates Smad2 and Smad3 on two serine residues within a conserved, R-Smad-specific, –SSXS motif at the extreme C-terminus of the MH2 domain. Microtubules play an important role in guiding R-Smads to the plasma membrane, where a FYVE-domain protein, Smad-Anchored for Receptor Activation (SARA), presents the Smad2 and Smad3 to the receptor [11]. Upon phosphorylation by TβRI, R-Smads partner with Smad4, and translocate into the nucleus where they activate downstream transcriptional responses. Active nuclear import processes regulated by Ran and importins allow for rapid, efficient, and controlled, nuclear import of Smad complexes [12]. Smad4 only translocates to the

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nucleus when complexed with R-Smads, whereas ligand-activated Smad2 and Smad3 may translocate into the nucleus in a Smad4-independent fashion [13]. In the absence of Smad4, however, neither Smad2 nor Smad3 are capable of transcriptional activity, suggesting that the principal function of Smad4 is to regulate transcription rather than to transmit signals from the cytoplasm to the nucleus.

R-Smad/Smad4 complexes may then function as transcription factors, binding DNA either directly or in association with other DNA binding proteins. Maximal affinity of recombinant Smad3 and Smad4 is observed with the CAGAC nucleotidic sequence, via their MH1 domain [14]. The transactivating role of Smad proteins has been ascribed to their MH2 domain. A 30-aa insertion within the Smad2 MH1 domain, as compared to the Smad3 MH1 domain, prevents direct DNA binding of Smad2 [15]. The latter requires a nuclear DNA-binding protein of the FAST/FoxH1 family to bind DNA in association with Smad4, to activate transcription in response to TGF-β [16].

Smad7, an inhibitory Smad specific for the TGF-β receptors, binds activated TβRI, thereby preventing phosphorylation of Smad2/3 [17]. It also recruits the E3 ubiquitin-ligases Smurf1 and Smurf2, to the activated TβRI, leading to proteasomal degradation of the receptor complexes. Of note, Smad7 may function as a negative feedback loop, as its expression is induced by TGF-β itself, in a Smad-dependent manner. Finally, several proteins, such as STRAP or YAP-65, have been shown to stabilize the Smad7-TβRI association, thereby exerting either additive or synergistic activities with Smad7 to prevent R-Smad phosphorylation by TβRI and subsequent intracellular signaling [18, 19]

Once in the nucleus, activated SMAD complexes may either activate or repress gene expression, depending on the recruitment of transcriptional co-activators or co-repressors into

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DNA-bound complexes. An essential role for the histone acetyl-transferases CREB Binding Protein (CBP) and p300 as coactivators of SMAD-driven gene expression is well documented [20-24]. Inversely, competition for p300/CBP has been suggested to mediate some examples of signal-induced transcriptional repression. For example, 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-driven gene transcription [25, 26]. Similarly, agents that activate PKA, e.g. PGE_2 and cAMP-elevating agents, antagonize SMAD signaling via PKA-dependent, CREB-mediated, disruption of SMAD-p300/CBP complexes [27].

SMADs may also recruit transcriptional corepressors such as TG3-interacting factor (TGIF), Sloan-Kettering Institute proto-oncogene (SKI), Ski-related novel gene N (SnoN), or SNIP1 [28-31]. Generally, these proteins bind and recruit chromatin-condensating histone deacetylases, which oppose the histone acetyl-transferase activity associated with p300 and CBP. The level of expression of these co-repressors may thus have important physiological consequences, by either setting a threshold for TGF-β-induced transcriptional activation involving p300/CBP, or by helping to terminate TGF-β signal.

3. Role of TGF-β and the Smad pathway in extracellular matrix gene expression

A key function for TGF-β in wound healing and fibrosis is to regulate the expression of proteins of the extracellular matrix (ECM), including fibrillar collagens and fibronectin. TGF-β also represses ECM degradation, by inhibiting the expression of metalloproteinases and serine proteases, and by enhancing the expression of protease inhibitors such as the tissue inhibitors of metalloproteinases (TIMPs) and plasminogen activator inhibitors (PAIs). TGF-β is therefore

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considered a potent anabolic factor that enhances connective tissue deposition and repair, and whose sustained signaling likely leads to the development of tissue fibrosis [32].

Knockout experiments in mice have revealed that certain functions are specific for one isoform. For example, TGF-β3, which is mostly expressed during embryonic life, exhibits unique anti-scarring properties, as exemplified by the fact that TGF-β3 knockout animals in which embryonic wound healing is not scarless, as opposed to embryos expressing TGF-β3 [33]. On the other hand, TGF-β1 is also critical for maintenance of proper immune functions and knockout mice lacking TGF-β1 die a few weeks after birth from aberrant regulation of the immune response, which culminates in lethal cardiopulmonary inflammation. In this context, TGF-β has been shown to play pivotal role in multiple stages of T cell apoptosis, selection, activation and clearance [3].

4. Modulation of collagen gene regulation by TGF-β, antagonistic activities exerted by inflammatory TNF-α and IFN-γ

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 have been suggested to block SMAD signaling via mechanisms that implicate either c-Jun [25] or nuclear factor-κB (NF-κB) [34]. The RelA subunit of NF-κB mediates TNF-α-induced expression of the inhibitory SMAD, SMAD7, which, in turn, blocks TGF-β signaling. Alternatively, c-Jun and JunB, both activated

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by TNF-α via the Jun-N-terminal kinase (JNK) pathway, are also capable of interrupting SMAD3-mediated transcription: Jun/SMAD3 complexes promoted by JNK activity may form off-DNA, not compatible with SMAD3 binding to cognate DNA sequences [35]. The outcome of such mechanism in terms of ECM gene expression is best illustrated in $JNK_1^{-/-}$-$JNK_2^{-/-}$ ($JNK^{-/-}$) fibroblasts, where TNF-α has no effect on TGF-β-induced fibrillar collagen gene transactivation unless ectopic expression of *jnk1* is allowed [36]. In contrast, in NF-κB Essential Modulator knockout ($NEMO^{-/-}$) fibroblasts, which lack NF-κB activity, TNF-α is able to act as an antagonist of TGF-β, ruling out a possible role for NF-κB in mediating TNF-α effect against TGF-β[36]. 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 [37]. 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. Firstly, it has been shown that IFN-γ induces the expression of SMAD7 via the activation of the JAK1/STAT1 pathway, SMAD7 then preventing the interaction of SMAD3 with the TβRI [38]. Secondly, the JAK/STAT pathway may alter SMAD-driven transcription because activated STAT1 may compete against SMAD3 for limiting amounts of cellular

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coactivators such as p300/CBP, a mechanism that may explain the antagonistic activity of IFN-γ against TGF-β-induced *COL1A2* gene transcription [26]. Thirdly, IFN-γ may inhibit *COL1A2* gene transcription, both basal and induced by TGF-β by activating the transcription factor YB-1 [39, 40]. The latter not only binds a proximal region of the *COL1A2* promoter identified as a negative IFN-γ-responsive element, but also physically interacts with SMAD3 to prevent its binding to the TGF-β-response element of the *COL1A2* promoter.

4. Experimental targeting of SMAD signaling in fibrosis

Inactivation of the Smad3 gene in mice has allowed to explore the contribution of the Smad pathway to tissue repair [41]. In contrast to predictions made on the basis of the ability of exogenous TGF-β to improve wound healing, Smad3-null ($Smad3^{ex8/ex8}$) mice paradoxically show accelerated cutaneous wound healing compared with wild-type mice, characterized by an increased rate of re-epithelialization and significantly reduced local infiltration of monocytes. Interestingly, these Smad3-null mice exhibit resistance to ionizing radiation-induced tissue fibrosis[42], and are protected from renal fibrosis induced by unilateral ureteral obstruction [43]. Together, these results unequivocally demonstrate the role of the Smad pathway in the pathogenesis of fibrosis.

It has been suggested that defects in SMAD7 may, in certain instances, lead to fibrotic conditions, as reported in the heart and in scleroderma [44, 45]. 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 [46].

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The therapeutic potential of targeted delivery of this inhibitory SMAD family member to prevent TGF-β-mediated fibrosis *in vivo* has been investigated in various experimental models of kidney, lung, or liver fibrosis have been developed and confirm this initial hypothesis. Thus, intratracheal injection of a recombinant adenovirus expressing SMAD7 prevents bleomycin-induced lung fibrosis in mice [47]. Similar promising results with SMAD7 gene transfer have been achieved in *in vivo* models of liver or renal fibrosis [48, 49]. In systemic sclerosis, deficient SMAD7 expression is regarded as a putative molecular defect leading to disease development [45]. Furthermore, it was shown in the same study that *in vitro* adenoviral gene transfer of SMAD7 re-establishes normal, not pathologically intensified, TGF-β signaling in SSc fibroblasts.

Conclusions

Several hurdles remain before TGF-β targeting can be considered a therapeutic alternative for the treatment of fibrosis, as significant problems may arise with regard to overall tolerance or biological outcome. For example, transgenic mice expressing a soluble form of TβRII are protected against metastasis without adverse side effects [50]. However, 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 [51], which could suggest that mesenchyme-specific targeting of the SMAD pathway may be required. It gets even more complicated, as 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 [52].

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Figure 1. TGF-β1 maturation. TGF-β1 is synthesized as pre-pro-TGF-β, that is cleaved by endopeptidases in the Golgi apparatus to form a small latent complex (SLC) containing a mature 24 kDa TGF-β1 homodimer non covalently associated with two 80 kDa latency-associated peptides (LAP). It is usually secreted as a large latent complex (LLC), covalently bound with latent TGF-β binding protein (LTBP). Final activation involves the release of mature TGF-β1 from the LLC. (from Javelaud and Mauviel, The International Journal of Biochemistry & Cell Biology 36 (2004) 1161–1165, copyright to Elsevier Publishing Co.).

Figure 2. The Smad pathway. TβRI is stabilized in an inactive conformation by its association with FKBP12. Upon TGF-β ligation to TβRII, the latter phosphorylates TβRI, which in turn phosphorylates Smad2/3. R-Smads are presented to the TβRI by a membrane-bound protein, SARA. Activated R-Smads bind Smad4 and translocate to the nucleus to act as transcription factors, controlled by a balance between transcriptional co-activators (co-A) or co-repressors (co-R). Inhibitory Smad7 binds activated TβRI, thereby preventing phosphorylation of R-Smads, or recruits the ubiquitine ligases Smurf1 and Smurf2 to induce proteasomal degradation of the receptor complexes. (from Javelaud and Mauviel, The International Journal of Biochemistry & Cell Biology 36 (2004) 1161–1165, copyright to Elsevier Publishing Co.).