TGF-β signaling and the fibrotic response

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ABSTRACT **The cause of fibrotic diseases, pathologies characterized by excessive production, deposition, and contraction of extracellular matrix, is unknown. To understand the molecular basis of fibrotic disease, it is essential to appreciate how matrix deposition is normally controlled and how this process is dysregulated in fibrogenesis. This review discusses the current state of knowledge concerning interactions among the profibrotic proteins transforming growth factor-β (TGF-β), connective tissue growth factor (CTGF, CCN2), and ED-A fibronectin (ED-A FN) and the antifibrotic proteins tumor necrosis factor-α (TNF-α) and γ-interferon (IFN-γ).—Leask, A., Abraham, D. J. TGF-β signaling and the fibrotic response. *FASEB J.* 18, 816–827 (2004)**

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FIBROTIC DISEASES are characterized by excessive scarring due to excessive production, deposition, and contraction of extracellular matrix. This process usually occurs over many months and years, and can lead to organ dysfunction or death. Examples of fibrotic diseases include diabetic nephropathy, liver cirrhosis, idiopathic pulmonary fibrosis, rheumatoid arthritis, fibrosarcomas, arteriosclerosis, and scleroderma (systemic sclerosis; SSc). Fibrotic disease represents one of the largest groups of disorders for which there is no effective therapy and thus represents a major unmet medical need. Often the only redress for patients with fibrosis is organ transplantation; since the supply of organs is insufficient to meet the demand, patients often die while waiting to receive suitable organs. Rheumatoid arthritis and other connective tissue disorders often have associated lung pathologies. Lung fibrosis alone can be a major cause of death in scleroderma lung disease, idiopathic pulmonary fibrosis, radiation- and chemotherapy-induced lung fibrosis and in conditions caused by occupational inhalation of dust particles. The lack of appropriate antifibrotic therapies arises primarily because the etiology of fibrotic disease is unknown.

Tissue fibrosis is generally considered to arise due to a failure of the normal wound healing response to terminate (1, 2). After injury, new connective tissue needs to be synthesized. During this process, mesenchymal fibroblasts become "activated" in that they proliferate and migrate into the wound and synthesize elevated levels of matrix proteins, including collagen and fibronectin. The mesenchymal cells activated during tissue repair and wound healing in kidney and liver are called mesangial cells and stellate cells, respectively (3, 4). The fibroblasts present in a wound are a specialized form of fibroblasts termed myofibroblasts as they express elevated levels of α-smooth muscle actin (α-SMA) and consequently display a markedly enhanced ability to contract extracellular matrix (2). This aspect of fibroblast function is necessary for wound closure (2). Myofibroblasts are present in abundance within fibrotic lesions and thus contribute to the excessive scarring observed in lesions of fibrotic disease (2).

To understand how the wound healing process develops into fibrotic disease, it is essential to appreciate how normal tissue repair is controlled and how this process goes awry in fibrotic disease. Normal wound healing is regulated by a complex set of interactions within a network of profibrotic and antifibrotic cytokines and secreted proteins. These proteins include profibrotic proteins transforming growth factor-β (TGF-β) and connective tissue growth factor (CTGF) and the antifibrotic proteins tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ). As TGF-β induces fibroblasts to synthesize and contract ECM, this cytokine has long been believed to be a central mediator of the fibrotic response (5). CTGF, discovered more than a decade ago as a protein secreted by human endothelial cells (6), is induced by TGF-β and is considered a downstream mediator of the effects of TGF-β on fibroblasts (7, 8). Similarly, TGF-β induces expression of the ED-A form of the matrix protein fibronectin (ED-A FN), a variant of fibronectin that occurs through alternative splicing of the fibronectin transcript (9). This induction of ED-A FN is required for TGF-β1-triggered enhancement of α-SMA and collagen type I expression (10). Conversely, the proinflammatory cytokine TNF-α, which is expressed by macrophages during the wound healing response (11), is antifibrotic in that this cytokine suppresses the expression of matrix genes (12). The proinflammatory cytokine IFN-γ released by T cells immediately after injury suppresses collagen synthesis (13, 14). Much information has recently been obtained concerning the signal transduction pathways through which the action of TGF-β on cells is controlled and

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how the action of this protein is enhanced by CTGF and fibronectin but suppressed by TNF-α and IFN-γ. This review focuses on these recent advances.

The TGF-β superfamily

The TGF-β superfamily consists of a diverse range of proteins that regulate many different physiological processes, including embryonic development, homeostasis, wound healing, chemotaxis, and cell cycle control (15, 16). Cytokines of the TGF-β superfamily are dimeric proteins with conserved structures and have pleiotropic functions in vitro and in vivo (15, 16). The TGF-β superfamily includes nearly 30 proteins in mammals including TGF-βs, activins and inhibins, nodal, myostatin, bone morphogenetic proteins (BMPs), growth/differentiation factors (GDFs), and anti-Müllerian hormone (AMH, also called Müllerian-inhibiting substance or MIS) (15, 16). TGF-β/BMP-like proteins are found in various species, including *Xenopus, Caenorhabditis elegans,* and *Drosophila melanogaster.*

TGF-β1, 2, and 3 are the prototype of the TGF-β superfamily (for review, see refs 2, 15, 16). TGF-β1, 2, and 3 inhibit proliferation in most types of cells and induce the apoptosis of epithelial cells. Conversely, TGF-β1, 2, and 3 stimulate mesenchymal cells to proliferate and produce extracellular matrix and induce a fibrotic response in various tissues in vivo. Inhibins were originally identified as cytokines that inhibit the secretion of follicle-stimulating hormone (FSH) from the pituitary gland. Inhibins are composed of disulfide-bonded dimers of inhibin-α and -β chains. In contrast, dimers composed of inhibin-β chains (also called activin-β chains) are activins that stimulate the production of FSH by the pituitary gland. Activins induce dorsal mesoderm during early embryogenesis, and regulate growth, differentiation, and apoptosis of epithelial cells and hematopoietic cells. Nodal plays critical roles in the induction of dorsal mesoderm, anterior patterning, and formation of left-right asymmetry during embryogenesis. Myostatin, also known as GDF-8, is produced by cells of the skeletal muscle lineage and inhibits their growth. BMPs, originally identified as cytokines that induce bone and cartilage tissues in vivo (17), induce ventral mesoderm during early embryogenesis. GDF-5, also known as cartilage-derived morphogenetic protein 1 (CDMP-1), is structurally related to BMPs and induces cartilage-like tissue in vivo. AMH induces Müllerian duct regression during embryogenesis and inhibits the transcription of gonadal steroidogenic enzymes. Thus, the TGF-β superfamily regulates wide-ranging and diverse roles in development, differentiation, and homeostasis.

TGF-β ACTIVATION

The TGF-β isoforms (TGF-β1, TGF-β2, and TGF-β3) are synthesized as latent precursors complexed with latent TGF-β binding proteins (LTBP-1, -3, and -4: **Fig. 1**). TGF-β is activated when LTBP is removed extracellularly via proteolytic cleavage (18; Fig. 1). This process has been extensively reviewed elsewhere (18). Early in the assembly of the latent TGF-β complex, disulfide linkages are formed between cysteine residues of latent TGF-β via its propeptide latency-associated peptide (LAP), and LTBP (19, 20). Due to this interaction, latent TGF-β is incapable of binding to its receptors (21, 22). The LTBP/TGF-β complex is found primarily in the matrix; the amino-terminal region of LTBP-1 is covalently cross-linked to ECM proteins by transglutaminase (23). Activation of TGF-β activators requires cleavage of the carboxyl-terminal proregion,

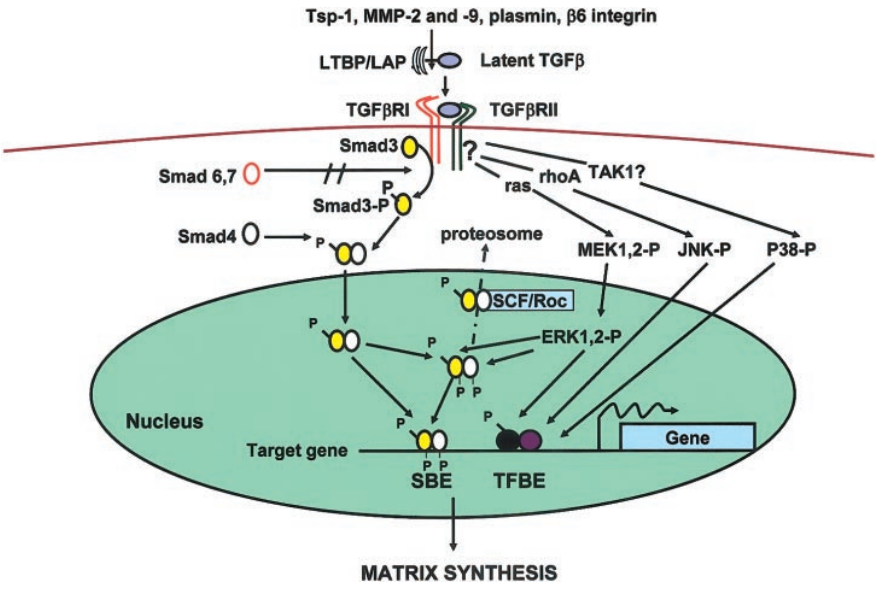

Figure 1. Model showing positive regulation of immediate-early TGF-β signaling resulting in the induction of ECM. LTBP/LAP = latent TGF-β binding protein; SBE = Smad binding element; TGF-βR = TGF-β receptor; TFBE = transcription factor binding element, whose identity depends on the identity of the TGF-β-responsive promoter, P = phosphorylated protein. LTBP/LAPs are cleaved from latent TGF-β, releasing active TGF-β. TGF-β then binds to TGF-βR I and II. TGF-βRI phosphorylates Smad3, which binds to Smad4. The resultant complex migrates into the nucleus to interact with the SBE and basal transcription factors binding to the TFBE. Activity of the Smad/basal transcription factor complex is modified by other signaling cascades depending on the promoter of interest. The net result is the induction of gene expression, including the elevated expression of genes encoding extracellular matrix. For details, see text.

to which LTBP is bound, from the amino-terminal portion of the protein (18). The TGF-β activators found thus far are proteins intimately associated with the wound healing response. For example, the plasmin proteases MMP-2 and -9, which promote matrix degradation (24), activate TGF-β (25, 26). Another activator of TGF-β, thrombospondin-1 (TSP-1; ref 27), modulates cell adhesion, promotion of angiogenesis, and reconstruction of the matrix (28). A final activator of TGF-β, the integrin $\alpha_v\beta_6$, which is normally expressed at low levels only in epithelia (29), is induced during wounding or inflammation (29). Studies using knockout animals have supported the notion that TSP-1 and $\alpha_v\beta_6$ integrin are involved with TGF-β activation. TSP-1 null mice demonstrate a partial phenotypic overlap with TGF-β1 null animals (30), and integrin $\beta6^{-/-}$ are highly resistant to lung fibrosis induced by the profibrotic drug bleomycin (31). Thus, TGF-β activation occurs in response to agents induced during the wound healing response in vivo.

TGF-β RECEPTORS AND SMADS

Once activated, TGF-βs bind to a heteromeric receptor complex consisting of one TGF-β type I and one TGF-β type II receptor (32). Both of these receptors possess tyrosine kinase activity (32; Fig. 1). TGF-β may also be presented to the type II receptor by the accessory proteins betaglycan and endoglin (33, 34), which may modify the cellular response to TGF-β (35–37); for example, overexpression of endoglin in fibroblasts suppresses Smad3-dependent activity (37). After binding to the TGF-β receptors, TGF-β signals within the cell through the Smad family of transcriptional activators. The Smad family has been extensively reviewed elsewhere (see refs 32, 38, 39). In the presence of TGF-β ligand, the receptor-activated Smads (R-Smads), Smad2 and 3, are phosphorylated directly by the TGF-β receptor I kinase, bind to the common mediator Smad, Smad4, and translocate into the nucleus (Fig. 1, **Fig. 2**). Maximal affinity of recombinant Smad3 and Smad4 to DNA is observed with the 5 bp sequence CAGAC (40).TGF-β induction of gene expression often requires the transcriptional cofactor p300; the TGF-β-mediated phosphorylation of Smad3 potentiates the association between Smad3 and p300 (41). Smad2, on the other hand, is not believed to bind DNA directly, but rather requires a nuclear DNA binding protein of the family Fast (Fast-1) to bind DNA, in association with Smad4, and activate transcription in response to TGF-β and activin (42). In a recent experiment using microarrays to compare gene expression profiles of fibroblasts taken from adult $Smad3^{-/-}$ and $Smad3^{+/+}$ mice, TGF-β was unable to induce transcription in $Smad3^{-/-}$ fibroblasts (43). Thus, in adult fibroblasts Smad3 is required for TGF-β-induced gene expression. Intriguingly, mice homozygous for a deletion in Smad2 die during embryogenesis (44) whereas mice homozygous for a deletion of Smad3 are viable and fertile (45). These observations suggest that Smad2 is required in normal development. These data also suggest there are fundamental differences in the processes mediated by Smad2 and Smad3 in vivo. A third group of Smad proteins, the inhibitory Smad (I-Smad), such as Smad6 or Smad7, prevents R-Smad phosphorylation and subsequent nuclear translocation of R-Smad/Smad4 heterocomplexes; it appears that Smad7 competes for binding for Smad 2 and Smad 3 to the TGF-βR1 (46; **Fig. 3**). TGF-β also induces Smad7 through a Smad3- and 4-dependent mechanism, suggesting that TGF-β can suppress its action via the induction of Smad7 (47).

An additional method of controlling the Smad signaling system has been revealed by recent studies showing that the level of Smad proteins can be controlled by interactions with various components of the 26S proteasome system. These data have been reviewed elsewhere (for example, see ref 48). After completion of its transcriptional role, carboxyl-terminally phosphorylated nuclear Smad3 is ubiquitinated by the SCF/Roc1 E3 ligase complex (49; Fig. 3). Recruitment of the Smad transcriptional coactivator p300 to nuclear Smad3 not only potentiates the activation of gene expression but also facilitates interaction with the E3 ligase complex, and so triggers the degradation process of Smad3 (49). Smad3 bound to ROC1-SCF(Fbw1a) is then exported from the nucleus to the cytoplasm for degradation by the 26S proteosome (49). TGF-β/Smad3 signaling is thus irreversibly terminated by the ubiquitin-proteasome pathway.

Consistent with the notion that TGF-β signaling is involved with a wide variety of cellular events, the above data

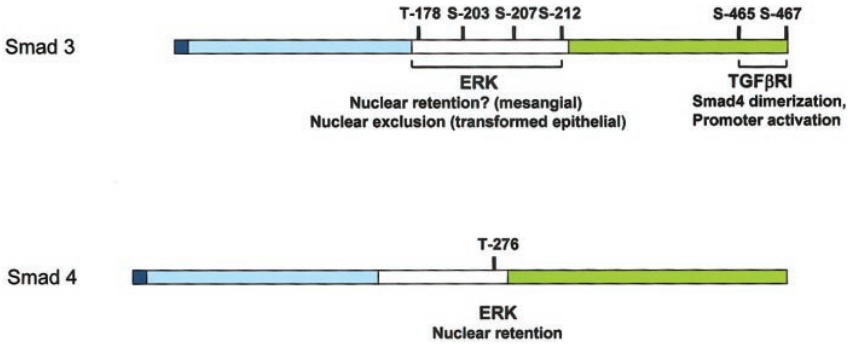

Figure 2. Phosphorylation sites in Smad3 and Smad4. Serine (S) and threonine (T) sites phosphorylated by TGF-βRI and ERK are shown, as well as known and putative functions of these phosphorylations.

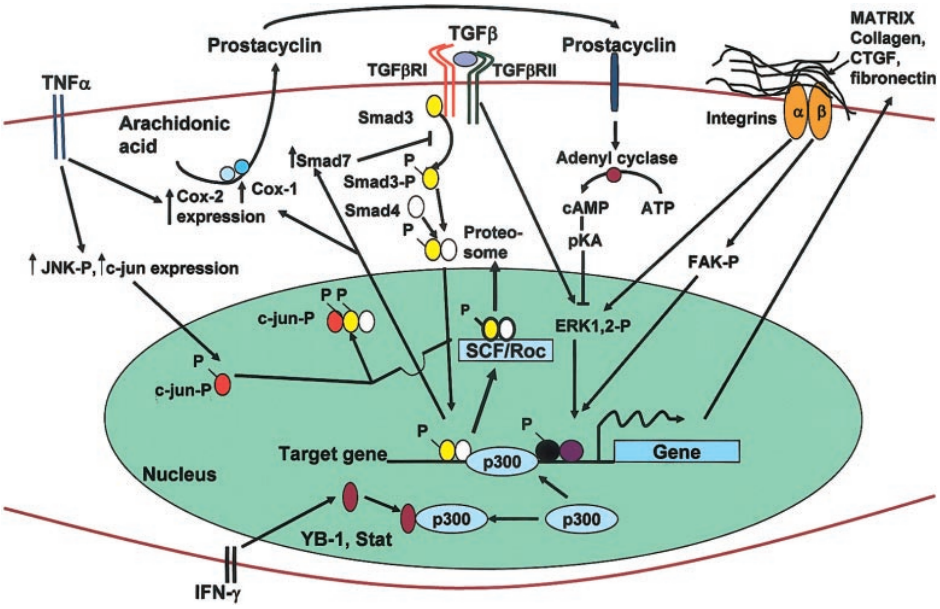

Figure 3. Model showing positive and negative controls on TGF-β signaling that can occur after immediate-early signaling events. Smad-dependent activation of profibrotic genes is controlled by several methods. TGF-β terminates the induction of its own target genes by the induction of Smad7 and prostacyclin. Induction of CTGF and ED-A fibronectin enhances the profibrotic effects of TGF-β. TNF-α suppresses TGF-β-dependent transcriptional responses by increasing prostacyclin synthesis, elevating c-jun levels, and inducing JNK. IFN-γ suppresses the action of the Smad coactivator p300 by inducing YB-1 and Stat, which sequester free p300. Prostacyclin suppresses induction of profibrotic genes by suppressing MEK/ERK. See text for details.

suggest that cells normally exhibit very tight, complex controls over the TGF-β/Smad signaling cascade.

TGF-β SIGNALING AND THE RAS/MEK/ERK CASCADE

TGF-β/Smad signaling is tightly controlled by MAP kinase signaling cascades. Recent data have shown that ras/MEK/ERK MAP kinase cascade phosphorylates and modifies Smad activity (50; Figs. 1, 2). ERK phosphorylation sites exist in the linker region of Smad3 (Fig. 2). In epithelial cells, overexpression of constitutively active members of the ras/MEK/ERK cascade blocks nuclear accumulation of Smads; this may explain why transformed epithelial cells that show constitutive activation of ras are not responsive to TGF-β (51). Conversely, in kidney mesangial cells overexpression of constitutively active members of the ras/MEK/ERK cascade promotes Smad3-dependent processes (52). The basis for the differences between responses in epithelial and mesangial cells to sustained activation of the ERK cascade is unknown; it is possible that adaptor proteins linking the ERK cascade to the Smad signaling pathway may differ between the two cell types. ERK phosphorylation site also exists in the linker region of Smad4. In epithelial cells this site is required for a maximal transcriptional response to Smad4; Smad4 lacking this phosphorylation site do not efficiently accumulate in the nuclei of transfected cells (53; Fig. 2).

In untransformed epithelial cells, mesangial cells, and fibroblasts, addition of TGF-β results in a transient activation of the ras/MEK/ERK cascade (54, 55). In all three cell types, ras/MEK/ERK is required for the ability of TGF-β to induce expression of some proteins (56, 57). In both mesangial cells and fibroblasts, TGF-β induction of a generic Smad3-responsive promoter occurs in the presence of either dominant negative ras or the MAP kinase inhibitor U0126; instead, the absolute requirement for the ras/MEK/ERK cascade in the induction of TGF-β-responsive genes seems to be restricted in a promoter-specific fashion (43, 58–60). These observations are consistent with the notion that Smads do not themselves activate transcription (38). Rather, Smads are believed to assist in the formation of a functional transcriptional complex on target promoters with other transcription factors; the activity of these transcriptional complexes is then adjusted by other signal transduction cascades whose identity varies depending on the promoter or cell type of interest (38). Collectively, these data suggest that, depending on the cell type, the ras/MEK/ERK MAP kinase cascade might enhance or suppress Smad-dependent responses.

TGF-β INDUCTION OF EXTRACELLULAR MATRIX GENES

TGF-β causes matrix deposition in mesenchymal cells in culture by promoting expression of ECM genes and suppressing the activity of genes such as matrix metalloproteinases, which degrade ECM (61, 62). For several years is has been known that TGF-β induction of collagen occurs in an immediate-early fashion requiring the action of the Smads (63). Indeed, it has been shown that Smad 3 is essential for the induction of matrix, as induction of profibrotic genes such as type I collagen and connective tissue growth factor (CTGF) is prevented in fibroblasts lacking the Smad3 gene (64, 65). However, as discussed above, Smad3 appears to be generally required in fibroblasts for the transcriptional response to TGF-β (43). In mesangial cells, TGF-β

induction of type I collagen promoter also requires the ras/MEK/ERK MAP kinase cascade but not the p38 MAP kinase cascade; conversely, in dermal fibroblasts this response requires p38 and not ras/MEK/ERK (66, 67; Fig. 3). However, the induction of CTGF in both mesangial cells and fibroblasts requires ras/MEK/ERK (58-60; Fig. 3). Induction of fibronectin by TGF-β in fibroblasts is Smad independent, requiring the JNK MAP kinase cascade and c-jun (68). However, TGF-β-mediated induction of genes that do not require JNK/c-jun, including a generic Smad3-responsive promoter, is antagonized by activation of JNK and overexpression of c-jun owing to the ability of c-jun and Smad3 to bind to each other, reducing the availability of free Smad3 (59, 69). Thus, p38 and ERK MAP kinase cascades appear to be profibrotic whereas the JNK MAP kinase cascade may be generally antifibrotic (Fig. 3). Overall, these studies point to the potential complexity of the interplay among MAP kinase and Smad signaling pathways in the control of matrix expression.

TGF-β SIGNALING IN WOUND HEALING AND FIBROTIC RESPONSES

Upon cutaneous injury, TGF-β is rapidly induced (70). This release assists in the attraction of neutrophils, macrophages, and fibroblasts, which in turn release more TGF-β (71); accordingly, TGF-β is consistently present in wound fluid throughout the repair process. Expression of TGF-β and TGF-β receptors are elevated in fibroblasts of human postburn hypertrophic scars, in keloids (benign, limited fibrotic skin lesions that result from an excessive wound healing response), and in keloid-derived fibroblasts (72, 73). In dermal fibrotic lesions of scleroderma patients, elevated TGF-β levels are found at the leading edge of the forming scar tissue but not in the established lesions (74); similarly, fibrotic mesenchymal cells overexpress the TGF-β ancillary endoglin that suppresses Smad3-dependent responses in cell culture (37, 75, 76). These results suggest that TGF-β is involved with the initiation of the fibrotic response in vivo.

Supporting the notion that TGF-β promotes fibrosis in vivo, treatment of fetal wounds with TGF-β promotes wound closure and scarring (77, 78); TGF-β induces excess matrix synthesis when injected subcutaneously in mice or into metal chambers implanted in the back of rats (78, 79). Incisional rat wounds, if treated with anti-TGF-β antibodies or antisense oligonucleotides, suppress ECM synthesis and scarring (80, 81). Intriguingly, TGF-β3 added exogenously has been shown to inhibit scarring in some systems or enhance scarring in others (82, 83). The role of endogenous TGF-β3 in the normal wound response and postsurgical scarring leading to fibrosis remains to be clarified.

Additional evidence supporting the profibrotic role of TGF-β in vivo are data showing that TGF-β1-deficient mice display severely impaired late-stage wound repair, including decreased re-epithelialization and collagen deposition, compared with control mice; however, mice lacking TGF-β1 also show a severe wasting syndrome accompanied by a pronounced, generalized inflammatory response and tissue necrosis resulting in organ failure and death (84, 85). Collectively, the above results stress the potential challenges regarding the use of TGF-β as an antifibrotic target. The use of TGF-β as a target for antifibrotic intervention may be limited to acute situations, such as immediately after surgery (86), which would require application of the antifibrotic for a limited period. Conversely, anti-TGF-β strategies might not be appropriate in treating chronic fibrotic disease, such as in the prevention of diabetic nephropathy, which develops over many years, owing to the long period of compound dosing necessary for such diseases. Thus further detailed characterization of the responses downstream of TGF-β and the TGF-β receptors may be necessary to develop more selective, clinically appropriate antifibrotic therapies.

After incisional wounding, animals lacking Smad3 show accelerated wound healing, reduced granulation tissue formation, increased epithelization, and reduced inflammation possibly due to an impaired chemotactic response (87) and resistance to cutaneous fibrosis caused by radiation injury (88). Giving further support to the role of Smad3 in the fibrotic response are observations that elevated levels of activated nuclear Smad3 exist in several models of fibroblast acquisition, including bleomycin-induced fibrosis, hepatic stellate cell activation, and the leading edge of scleroderma lesions (89–91). However, models examining stages of established fibrosis do not show such activation, which suggests that overexpression of profibrotic markers is Smad independent (65, 92, 93). Thus, although Smad3 plays a key role in the initiation of the fibrotic response, Smad3 per se might not be required for the maintenance of the fibrotic phenotype. Taken together, as for TGF-β, results examining the function of Smad3 tend to suggest that using Smad3 as an antifibrotic target may be limited to acute postsurgical applications or early onset disease and further emphasize the importance of identifying more selective strategies to modify the fibrotic response that might be more useful for the treatment of chronic disease. Although one report showed decreased levels of the inhibitory Smad7 in scleroderma fibroblasts (94), two other studies showed no difference in Smad7 levels between normal and scleroderma fibroblasts (65, 91); thus, Smad7 deficiency does not seem to be a general feature of scleroderma fibroblasts. As Smad3 might not be required for the maintenance of the fibrotic response, overexpressing Smad7 might not be expected to possess an antifibrotic effect in established lesions.

ENHANCEMENT OF THE TGF-β RESPONSE

Downstream of TGF-β, several proteins are required for the enhancement of a cellular response to TGF-β and hence for prolongation of the wound healing and

fibrotic response. These either *1)* enhance the contractile phenotype of the fibroblast or *2)* prolong the induction of ECM in fibroblasts. These proteins include connective tissue growth factor and the ED-A form of fibronectin.

Connective tissue growth factor (CTGF)

In adult skin, CTGF normally is not expressed unless induced—for example, during the normal wound repair process (95). TGF-β induces CTGF expression in dermal fibroblasts, but not in keratinocytes, via consensus Smad and transcription enhancer factor (TEF) elements in the CTGF promoter (59, 65). TEF family members have also been shown to be involved with tissue-specific expression of muscle proteins (96). In contrast to the situation in normal fibroblasts, CTGF is constitutively overexpressed in dermal fibrotic lesions such as in scleroderma (95, 97, 98), liver (99), renal (100, 101), lung (102), and pancreatic fibrosis (103). These observations suggest that CTGF may act as a downstream effecter of at least some of the profibrotic effects of TGF-β (8).

CTGF, a member of the CCN family of matricellular proteins (104–106), promotes fibroblast proliferation, matrix production, and granulation tissue formation (6, 98, 107). CTGF promotes cell adhesion and migration in a wide variety of cell types (108–110) as well as collagen matrix contraction in fibroblasts (98). An expression vector encoding CTGF transfected into fibroblasts can activate a cotransfected reporter construct driven by the type I collagen promoter, suggesting that a CTGF response element exists in the type I collagen promoter (98). Experiments using recombinant CTGF and neutralizing antibodies targeting CTGF have suggested that CTGF mediates at least some of the effects of TGF-β on fibroblast proliferation, adhesion, and ECM production, including collagen and fibronectin (8, 101, 110–113). Mice heterozygous for a deletion for the CTGF gene die immediately after birth due at least in part to defects on matrix organization and synthesis during osteogenesis, resulting in a major defect in the development of the skeletal component of the rib cage (114). Although a specific CTGF receptor has yet to be identified, CTGF appears to perform many of its functions through integrins, heparin sulfate-containing proteoglycans, and the low density lipoprotein receptor-related protein (109, 112, 115–118). CTGF may enhance the ability of TGF-β to bind to its receptors at low TGF-β concentrations (119). In vivo, whereas application of CTGF or TGF-β causes a transient fibrotic response (120, 121), simultaneous subcutaneous coinjection of TGF-β; CTGF causes a sustained fibrotic response persisting at least a week after cessation of ligand injection (121). Collectively, these results suggest that the constitutive overexpression of CTGF observed in fibrotic lesions enhances the profibrotic response to TGF-β. However, the signal transduction cascades by which this process occurs remain to be elucidated.

ED-A fibronectin

The fibroblast present in the wound is a specialized, highly contractile collagen-producing cell, termed the myofibroblast, and it is the persistence of the myofibroblast in the wound that is a feature of fibrosis. This cell type is responsible for the excessive contraction of matrix characteristic of scar tissue. The mechanical tension exerted by the myofibroblast is mediated through integrin-dependent signaling events including focal adhesion kinase, tyrosine kinase, and ERK activation (122, 123). TGF-β, a major promoter of myofibroblast differentiation, induces α-SMA, modulates the expression of adhesive receptors, and enhances the synthesis of extracellular matrix (ECM) molecules including ED-A fibronectin (ED-A FN), an isoform of fibronectin arising from alternative splicing of the fibronectin mRNA (2, 10, 68). ED-A FN is expressed during embryogenesis and is de novo expressed during wound healing and fibrosis (2, 10, 68). ED-A FN deposition precedes α-SMA expression by fibroblasts during granulation tissue evolution in vivo and after TGF-β1 stimulation in vitro. However, incubation of fibroblasts with the anti-ED-A monoclonal antibody IST-9 or recombinant ED-A domain specifically blocked the TGF-β1-triggered enhancement of α-SM actin and collagen type I, but not that of plasminogen activator inhibitor-1 mRNA (10). TGF-β also increases the size of focal adhesions in an ED-A-dependent fashion (124). ED-A domain-containing fibronectin is present in abundance in fibrotic lesions (125–128), suggesting that ED-A might contribute to the excessive scarring observed in chronic fibrosis, and thus suggests that soluble ED-A domain might represent a novel antifibrotic therapeutic.

Control of the profibrotic response to TGF-β

Given that fibrosis can be considered as resulting from a failure to suppress the normal wound healing response, clues as to how fibrosis develops might emerge form the identification of methods that mammals have of suppressing the normal wound healing process and how these might be inoperative in fibrosis.

Tumor necrosis factor-α (TNF-α)

The proinflammatory cytokine TNF-α expressed by macrophages during the wound healing response (97) has long been known to possess antifibrotic ability in that it suppresses the expression of matrix genes (129) and the TGF-β induction of collagen and CTGF (97, 130). Mice lacking the TNF-α receptor p55 show, after wounding, increased angiogenesis, collagen content, and re-epithelialization (129). TNF-α has been proposed to suppress TGF-β signaling via the NF-κB induction of Smad7 (131), but this appears to be a cell type-specific effect and is not operative in dermal fibroblasts as TNF-α does not induce Smad7 in this cell

type (132). On the other hand, TNF-α suppresses Smad3-dependent signaling in this cell type by elevating c-jun expression and activating JNK (132), which results in suppression of the ability of Smad3 to activate gene expression due to the ability of c-jun to interact with Smad3 resulting in the off-DNA sequestering of Smad3 (59,69; Fig. 3). Intriguingly, although TNF-α suppresses the TGF-β induction of CTGF, the overexpression of CTGF in scleroderma lesions, which occurs by a Smad-independent and Sp1-dependent mechanism (65, 92), is not suppressed by TNF-α (97), suggesting that the chronic fibrosis observed in scleroderma may be a consequence of the ability of lesional scleroderma fibroblasts to escape of the negative, antifibrotic effects of this cytokine.

Both TNF-α and TGF-β induce prostaglandin production in fibroblasts (133, 134). Prostaglandins, which are metabolites of arachidonic acid produced by the action of cyclooxygenase 1 and 2 (COX1 and 2), are lipids used as signaling mediators by several pathways (for review, see ref 135). COX-1 expression is induced by TGF-β whereas COX-2 is induced by TNF-α (134). Prostaglandin (PGE2) and prostacyclin (PGI2) elevate cellular cAMP levels and activate protein kinase A (PKA; 136). Some of the earliest known agents that suppressed collagen synthesis in cell culture and in vivo were those that activated protein kinase A, including prostacyclin (PGI2), prostaglandin (PGE2), and stable cAMP analogs (137–139). Recently, 8-Br-cAMP was shown to block the TGF-β induction of granulation tissue in wound chambers, metal cylinders placed in the back of rats (79). The stable prostacyclin analog Iloprost was shown to alleviate symptoms of fibrosis, including CTGF and collagen levels, in scleroderma patients and to reduce TGF-β induction of these proteins in vitro and in vivo (60, 140). The effect of Iloprost was dependent on an elevation of cellular cAMP levels and resulted in antagonism of the ras/MEK/ERK signaling cascade necessary for induction of the profibrotic protein CTGF (59, 60). The potential utility of synthetic prostacyclins as antifibrotic therapies is heightened by observations that these compounds are well tolerated, already being used clinically to treat hypertension associated with fibrosis owing to their vasodilatory capabilities (141) and successful at reducing the skin score of scleroderma patients in a clinical trial (142). Prostaglandin levels appear to be reduced in idiopathic pulmonary fibrosis (IPF) due in part to a reduced expression of COX2 by fibroblasts in this disorder (143, 144), suggesting the fibrosis may be exacerbated in IPF due to a reduction in the ability of fibroblasts to synthesize this normally antifibrotic agent, and illustrating that the utility of exogenously added prostaglandins to suppress the fibrotic response may result from a rescue of this deficiency. Thus, it is tempting so speculate that fibrosis may develop in part due to a failure of the normal precise control that suppresses and terminates the wound healing response to operate.

Interferon-γ (IFN-γ)

IFN-γ, a pleiotropic cytokine produced by T cells and NK cells, plays fundamental roles in innate and acquired immune responses (145). Transcriptional responses induced by IFN-γ in most cells are mediated through the Jak-Stat pathway (146). Upon stimulation by IFN-γ, tyrosine-phosphorylated cytoplasmic Stat1α forms homodimeric complexes that can translocate into the nucleus and bind directly to IFN-γ-responsive target gene promoters. Stat1α thus serves as an essential mediator of IFN-γ-induced transcriptional responses. Stat1α physically associates with p300 near its amino-terminal domain; this interaction plays an important functional role in positive regulation of IFN-γ-induced transcriptional responses (146, 147). In addition to transcriptional stimulation, IFN-γ can negatively regulate the transcription of matrix (13), but no common IFN-γ-specific inhibitory elements have yet been identified. Recently it was shown that IFN-γ induces the Smad inhibitor Smad7 in the fibrosarcoma-derived U4A cell line (148); however, this mechanism does not seem to be generally applicable to normal fibroblasts (14). Rather, in this cell type IFN-γ seems to suppress ECM synthesis by the ability of nuclear Stat1α to compete with the transcriptional cofactor p300 (14). More recently, an alternative IFN-γ signal transduction was shown to lead to transcriptional repression of *COL1A2*, namely, the Jak1/CK2/YB-1 pathway (149). Experiments using the double-stranded specific RNAs indicated that both YB-1 and STAT1 were necessary for transcriptional repression of *COL1A2* by IFN-γ through competition with the common Smad3 for the cellular p300 (149) Thus, TGF-β and IFN-γ exert opposite effects on collagen synthesis. Because these two cytokines are secreted by inflammatory cells at sites of tissue injury, their antagonistic interactions regulating collagen synthesis are likely to be of great importance in the maintenance of connective tissue homeostasis.

FUTURE PROSPECTS

Although TGF-β has long been known to induce matrix synthesis and contraction by fibroblasts, the precise contribution of this protein to fibrotic disease is still unclear. In part, this issue is due to the difficulty of translating what have been primarily studies involving immediate-early events of a growth factor in cell culture, which occur in a few minutes or a few hours, into meaningful insights into the pathogenesis of fibrotic disease, which occurs over months or years. However, these studies have expanded into a growing appreciation of the complexity of the cellular controls operating in enhancing or suppressing the profibrotic response to TGF-β that might be operative during normal wound healing. Given that fibrotic disease might arise from a failure to suppress the normal wound healing response,

information gathered from these experiments and those identifying proteins and signaling pathways involved with the pathogenesis of fibrosis is likely to suggest new targets for drug intervention to combat chronic fibrosis. FJ

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