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INVITED REVIEW

TGF-β as a driver of fibrosis: physiological roles and therapeutic opportunities

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Abstract

Many chronic diseases are marked by fibrosis, which is defined by an abundance of activated fibroblasts and excessive deposition of extracellular matrix, resulting in loss of normal function of the affected organs. The initiation and progression of fibrosis are elaborated by pro-fibrotic cytokines, the most critical of which is transforming growth factor-β1 (TGF-β1). This review focuses on the fibrogenic roles of increased TGF-β activities and underlying signaling mechanisms in the activated fibroblast population and other cell types that contribute to progression of fibrosis. Insight into these roles and mechanisms of TGF-β as a universal driver of fibrosis has stimulated the development of therapeutic interventions to attenuate fibrosis progression, based on interference with TGF-β signaling. Their promise in preclinical and clinical settings will be discussed.

Keywords: fibrosis; cytokines; transforming growth factor-β1; signaling

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Fibrosis in various organs and diseases

Fibrosis is defined by the progressive replacement of healthy parenchymal tissue with collagen-rich extracellular matrix (ECM) that is deposited by an excessive population of activated fibroblasts, resulting in loss of proper tissue function. Commonly originating as a reparative response to preserve tissue integrity following injury, fibrosis can develop in virtually any tissue that is exposed to repeated injury or persistent damage, as the consequence of an aberrant and imbalanced wound healing response. Chronic diseases associated with a fibrotic response include, but are not limited to, diabetes, hypertension, cardiomyopathy, interstitial lung disease, viral and nonviral hepatitis, non-alcoholic steatohepatitis (NASH), and autoimmune-related disorders such as scleroderma and inflammatory bowel disease [1–7]. With a high incidence of these and other chronic diseases linked to fibrosis-related organ failure, up to 45% of all deaths in the developed world can be attributed to fibrosis [8].

The severe clinical impact of fibrosis is well illustrated by its progressive roles in a wide array of diseases affecting vital organs. Renal fibrosis is a hallmark of end-stage chronic kidney disease, which affects an estimated 20 million adults in the United States and about 10% of the world population [3,9]. Chronic ECM deposition and fibroblast activity result in disruption of kidney architecture that functionally impacts the vasculature, glomeruli, and tubule-interstitium, thus reducing blood supply and organ function [3]. Myocardial fibrosis results in progressive stiffening of cardiac tissue following chronic heart failure, with the pattern and extent of fibrosis shown to decrease left ventricular ejection and lead to mortality [10]. Fibrosis also plays a significant role in the progression of other cardiovascular diseases, including aortic valve disease and atherosclerosis, each of which may increase the risk of myocardial infarct or stroke [11]. In the liver, the progressive fat deposition in hepatocytes in NASH is accompanied by excessive ECM deposition that reflects a response to functional liver damage [12]. Additionally, cirrhosis, the culmination of fibrotic remodeling of the liver and a major risk factor for hepatocellular carcinoma, is the 11th leading cause of death in the United States and accounts for more than one million deaths world-wide each year [13,14]. Fibrotic remodeling also associates with various respiratory diseases including asthma, chronic obstructive pulmonary disease, acute respiratory distress syndrome, and interstitial lung diseases, such as idiopathic pulmonary fibrosis (IPF) [15–19]. IPF, which involves fibrotic remodeling of the lung parenchyma, is characterized by

severe dyspnea and cough, and is particularly devastating, with a median survival time of 3.8 years for patients of 65 years or older in the United States [15]. These and other fibrosis-related diseases illustrate the severe clinical impact of fibrosis on human health.

Different types of fibrosis share common characteristics

Common characteristics in disease initiation and progression define fibrosis as a dysfunctional wound response in various organs. Fibrosis is initiated by cellular injury that is presumably sensed through impaired cell–cell or cell–ECM interactions (Figure 1). This damage at the cellular level promotes localized fibrin clot formation and release by damaged cells of pro-inflammatory factors that activate the innate immune response. Collectively called 'damage-associated molecular patterns' (DAMPs), these molecules, either of intracellular origin or released from the ECM, promote an inflammatory response that results in recruitment of neutrophils and macrophages to clear necrotic cells and cell debris [20–22]. During this acute inflammatory response, cells release cytokines, chemokines, and growth factors, including transforming growth factor-β1 (TGF-β1), that stimulate recruitment and proliferation of fibroblasts, and activate fibroblasts to enhance their protein synthesis and metabolic activity [22,23]. Activated fibroblasts and myofibroblasts that provide cell and tissue contractility show a markedly enhanced deposition of ECM proteins and stabilize the fibrotic tissue architecture [24,25]. In normal wound healing, their activation is transient, and these cells then undergo apoptosis or quiescence as the provisional ECM degrades and is replaced by parenchymal tissue architecture [26,27].

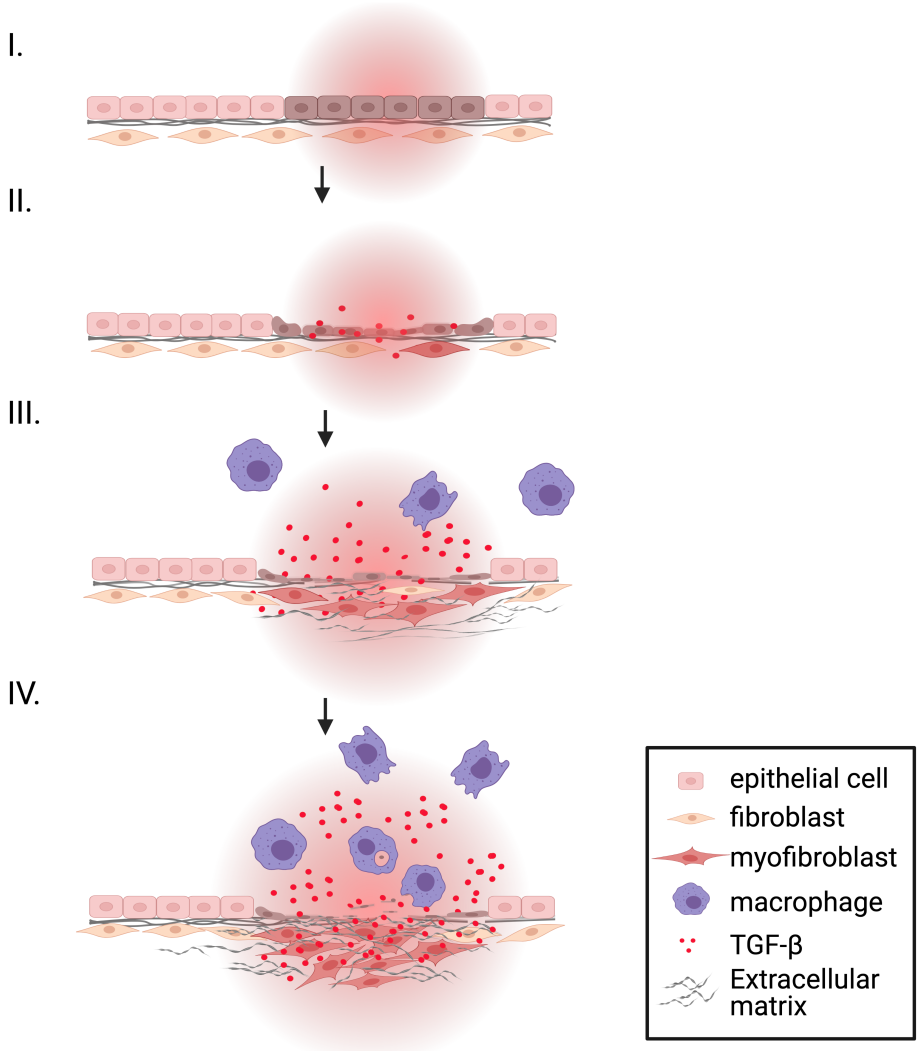

Figure 1. Fibrosis, a dysfunctional wound healing response. (I, II) Injured epithelial cells secrete signaling molecules, including TGF-β1, which promote fibroblast migration and proliferation, and differentiation into myofibroblasts. (II, III) TGF-β also recruits inflammatory cells to the site of injury. In normal wound healing, fibroblast-mediated contraction decreases the size of the wound while other cells, such as myofibroblasts, macrophages, and other inflammatory cells, undergo apoptosis, terminating collagen deposition and enabling the restoration of normal tissue architecture. (III) Repeated injury or persistent damage to the same site, as well as aberrant tissue-clearing responses, may result in loss of homeostasis of tissue integrity and stiffening of the tissue caused by increased collagen deposition. (IV) Higher tension or increased matrix stiffness can further activate fibroblasts, creating a positive feedback loop that promotes fibrosis.

However, in fibrosis, chronic injury prevents such resolution of the wound healing cascade, leaving a mixed cell population with pro-inflammatory and pro-fibrotic properties, largely consisting of perpetually activated fibroblasts and myofibroblasts that excessively deposit ECM proteins, including collagens [28,29]. The fibrotic microenvironment further amplifies the fibrotic response by inducing additional cell damage and oxidative stress due to hypoxia and mechanotransductive signaling in response to dysregulated and enhanced tissue stiffness [20,30] (Figure 1). The architectural and functional consequences of the fibrotic disease are variable and heterogeneous, depending largely on the tissue and organ affected.

The susceptibility of different tissues to inflammatory and fibrotic disease can also be affected by genetic factors, as seen in GWAS studies of links between primary sclerosing cholangitis and inflammatory bowel disease [31,32]. GWAS analyses of IPF additionally associate altered expression of *MUC5B*, *TERT*, *DSP*, *ATP11A*, *IVD*, *AKAP13*, *KANSL1*, *FAM13A*, *DPP9*, *KIF15*, *MAD1L1*, and *DEPTOR* with increased IPF susceptibility [33,34]. Environmental factors that induce or contribute to the fibrotic response also help to define the extent and progression of fibrotic disease. For example, occupational exposure to smoke and respirable dust or asbestos enhances the susceptibility to IPF [35]. Additionally, the diverse origins of liver fibrosis, including viral infection, genetic disorders, toxic injury, and metabolic dysfunction, help to define the disease [4]. For fibrotic disease known to result from environmental factors, removing the source of chronic injury that drives disease progression is often the most direct course of treatment. For example, antiviral drugs against hepatitis C have been shown to ameliorate liver fibrosis even in patients with advanced disease [36]. In most fibrotic indications, however, the source of injury is not well understood or is difficult to target directly.

The accumulation of fibrotic scar tissue resulting from activated fibroblasts and myofibroblasts depends on a dynamic balance between ECM protein deposition and degradation that defines ECM turnover. ECM synthesis and degradation, and activities of ECM stabilizing cross-linking enzymes, increase with accumulation of fibroblasts and myofibroblasts, and progression of fibrotic disease [37–39]. The increase in resident fibroblasts results not only from enhanced proliferation but also from fibroblast recruitment to the site of fibrotic injury. Furthermore, epithelial and endothelial cells can acquire mesenchymal characteristics and thus contribute to the fibrotic cell population through epithelial– or endothelial–mesenchymal transdifferentiation (EMT or EndMT) [40–42]. Regardless of their origin, the activation and functions of these fibroblasts and myofibroblasts are largely driven by increased expression and activities of pro-fibrotic cytokines and growth factors, among which TGF-β1 is most critical. Therapies that effectively target factors driving cell differentiation, ECM synthesis or ECM stabilization have the potential to repress fibrotic disease progression and even reverse it.

While resident fibroblasts are the major cell population that deposits collagen and ECM proteins in cardiac and liver fibrosis [43–45], other cell populations contribute to fibroblast activation and myofibroblast differentiation in lung and kidney fibrosis [46]. Inflammatory monocytes and resident tissue macrophages contribute to the state of fibrosis, consistent with their critical roles in normal wound healing from initiation to the resolution of the injury. In response to injury, these cells alter functionally and elaborate changes in the tissue microenvironment through secretion of growth factors and cytokines, such as TGF-β1, that promote fibrosis by recruiting more fibroblasts, enhancing their differentiation into myofibroblasts, or promoting ECM protein secretion [22].

Increased TGF–β signaling marks fibrosis

To understand the roles of TGF-β in fibrosis, one must appreciate some basics of TGF-β biology. The mammalian genome encodes three TGF-βs – TGF-β1, -β2, and -β3 – which act as disulfide-linked dimers. Each gene encodes a precursor protein with an amino-terminal signal peptide required for secretion, a long pro-segment, and a carboxy-terminal 112 amino acid, mature TGF-β polypeptide. During secretion, the pro-segments are cleaved from the mature polypeptides, yet remain associated with mature TGF-β as chaperones. Consequently, the mature TGF-β dimer is secreted as a 'latent' complex with two copies of the non-covalently associated pro-segment, often called 'latency-associated polypeptide' (LAP), that prevent TGF-β from binding to its cell surface receptors [47,48] (Figure 2). Hence, activation of latent TGF-β complexes is critically required for TGF-β to activate signaling in TGF-β-responsive cells [48,49]. Since TGF-β1, purified from platelets, primarily exists as a homodimer [50], cells are thought to express TGF-β1, TGF-β2, and TGF-β3 as homodimers, a notion reinforced by the commercial availability of TGF-β homodimers. Nevertheless, natural TGF-β1:β2 heterodimers have been isolated [51] and this raises the possibility of naturally expressed TGF-β heterodimers, e.g. TGF-β1:β3.

Fibrosis is consistently marked by increased TGF-β1 expression, although increased TGF-β2 and TGF-β3 mRNA expression has also been noted [52–54]. Activated fibroblasts and myofibroblasts represent a major cell population to express and respond to TGF-β1 [53,54], consistent with the notion of a self-perpetuating response to chronic injury and inflammation. Macrophages and epithelial cells also represent an important source of TGF-β1 expression. Impaired macrophage recruitment decreases the TGF-β1 levels in the fibrotic lesion in some models of fibrosis [54,55], while epithelial cells regulate TGF-β1 activity in others [56,57]. The contributions of TGF-β1 expression by other cell types in the fibrotic lesion are less defined, although platelets, epithelial cells, T-cells, and mast cells also express TGF-β1 [54]. Whether TGF-β2 and -β3 are

commonly and concomitantly expressed in different fibrosis types is unclear. Furthermore, the relative contributions of the different cell populations to increased TGF-β1 expression in fibrosis may differ between different types of fibrosis and disease states.

TGF-β1 expression is transcriptionally activated by AP1 transcription complexes, i.e. heterodimers of Jun and Fos proteins, as well as the transcription factors NF-AT or C/EBPβ, which bind *TGFB1* regulatory promoter sequences [58,59]. TGF-β1 expression is induced by growth factors and pro-inflammatory cytokines that activate MAPK pathways, and various other stimuli [47]. Additionally, TGF-β1 directs *TGFB1* expression [60], enabling auto-amplification beyond the initial paracrine TGF-β1 signaling.

The secreted latent TGF-β1 complexes often have one of the pro-segments linked covalently to a fibrillin-like latent TGF-β binding protein 1 (LTBP1) that enables their deposition to be localized within the ECM in proximity to TGF-β1-expressing cells (Figure 2B). Accordingly, ECM in fibrotic lesions is a major depot of latent TGF-β1, readily available for activation [47,49]. Alternatively, the pro-segments of latent TGF-β1 complexes also associate with a subset of LRRC transmembrane proteins, allowing cell-associated retention of latent TGF-β1 (Figure 2B). Latent TGF-β1 associates with LRRC32, also known as GARP, at the surface of Treg cells, activated B-cells, and platelets, enabling GARP to control retention of latent TGF-β complexes [61,62]. GARP is also expressed by endothelial cells and fibroblasts [63]. The GARP-related LRRC33 also associates with latent TGF-β1 and similarly controls TGF-β1 latency and activation in other cell types, including myeloid cells [64,65], while the related LRRC15, expressed by stromal fibroblasts of carcinomas [66], may similarly control cell-associated TGF-β1 retention. The expression

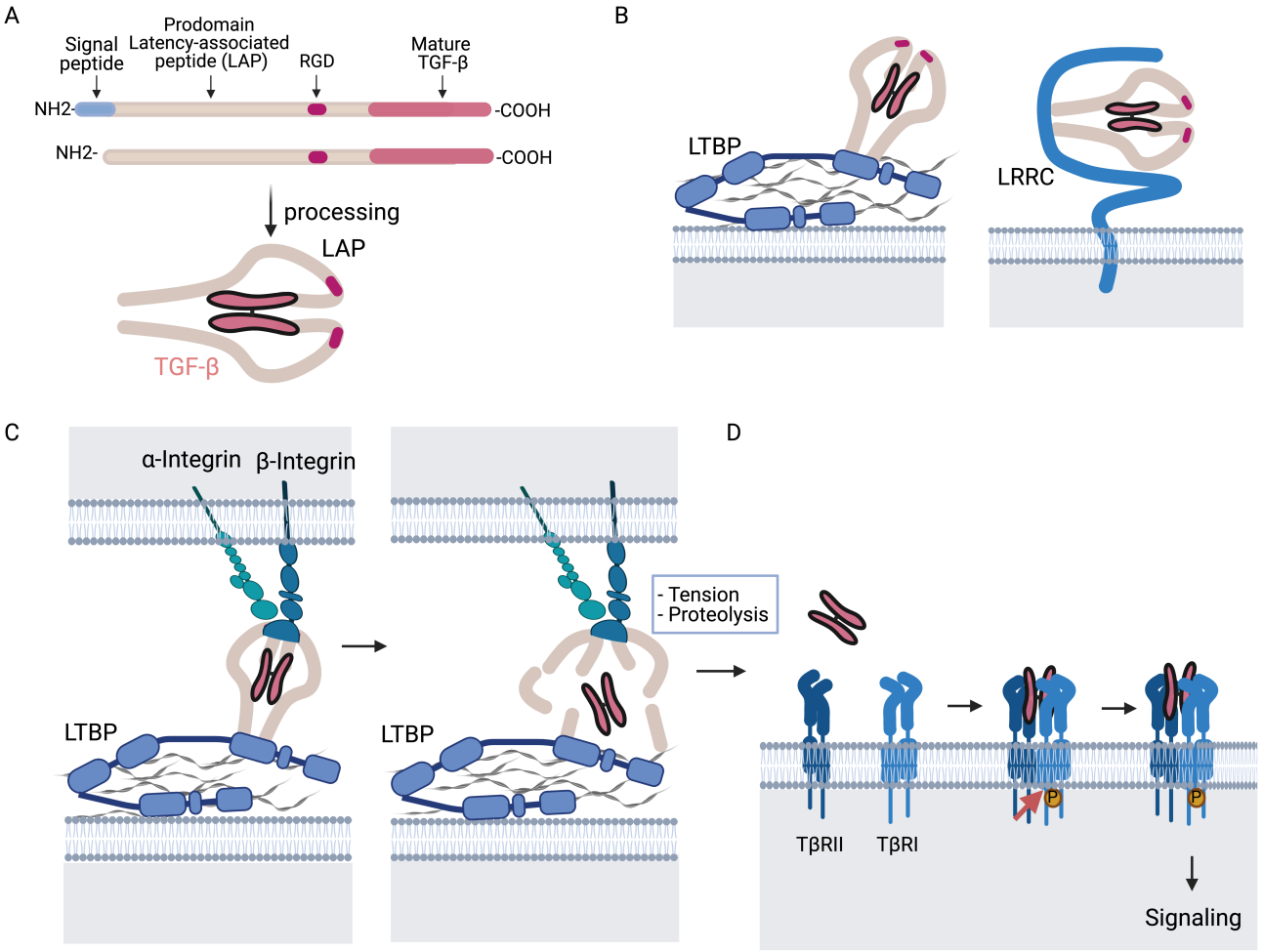

Figure 2. Latent TGF-β processing and initiation of TGF-β signaling. (A) TGF-β proteins are synthesized as precursor molecules consisting of a signal peptide, a pro-segment (termed latency-associated polypeptide or LAP), and the mature TGF-β monomer sequence. After signal peptide removal and proteolytic processing, the mature, disulfide-linked TGF-β dimer remains associated with two pro-segments. (B) Once released from cells, the TGF-β dimer that is kept latent by its associated pro-segments associates either with LTBP1, which targets latent TGF-β into the ECM, or with an LRRC molecule such as GARP at the surface of various cell types. (C) Select β integrins, in association with αv integrins, can bind the RGD sequence of the pro-segment in the latent TGF-β1 complex. The stoichiometry of this interaction is unclear. Physiological activation of latent TGF-β1 complexes, resulting in the release of biologically active TGF-β1, involves increased tension at the interface with the integrin and protease activities that confer degradation of the pro-segments. (D) Active TGF-β binds to heterotetrameric complexes of two types of TGF-β receptors, TβRI and TβRII receptors, at the cell surface. The ligand binding induces TβRII to phosphorylate and activate TβRI, leading to activation of TGF-β-induced Smad and non-Smad signaling pathways.

of these LRRC proteins in fibrosis has not been characterized.

The activation of latent TGF-β1 complexes has been studied extensively, while few studies have addressed the activation of TGF-β2 or -β3 homodimers or TGF-β heterodimers. Structural interactions of the TGF-β1 pro-segment that involve its Arg-Gly-Asp (RGD) sequence with selected integrins enable activation of latent TGF-β1 complexes [48,49,67] (Figure 2C). Integrins αvβ6 and αvβ1 mediate TGF-β1 activation at the surface of epithelial cells and fibroblasts in the fibrotic lesion, respectively [68,69], whereas αvβ8 is required for activation of GARP-bound TGF-β1 on Treg and endothelial cells [69,70]. Which integrin is involved in TGF-β1 latency and activation depends on cell type and context, and often involves heterotypic cell interactions. Thus, targeted interference of the integrin–pro-segment interface represents an approach to selectively inhibit TGF-β1 activation in defined cell populations. Contributions of proteases, often metalloproteases, in degrading the pro-segments, leading to release of active TGF-β1, have also been extensively documented [48,49,67]. Physiological TGF-β1 activation scenarios are likely to combine molecular deformation of the integrin–pro-segment interface with metalloprotease activities [49] (Figure 2C,D). The diversity of local TGF-β1 activation mechanisms may help to explain differences in susceptibility to TGF-β inhibition, depending on the cell composition and architectural organization of the lesion.

Active TGF-β binds tetrameric combinations of two different transmembrane kinases, type I and type II receptors, that are able to phosphorylate serine, threonine, and tyrosine (Figures 2D and 3). TGF-β binding to these complexes activates Smad2 and Smad3 through C-terminal serine phosphorylation by the type I receptor

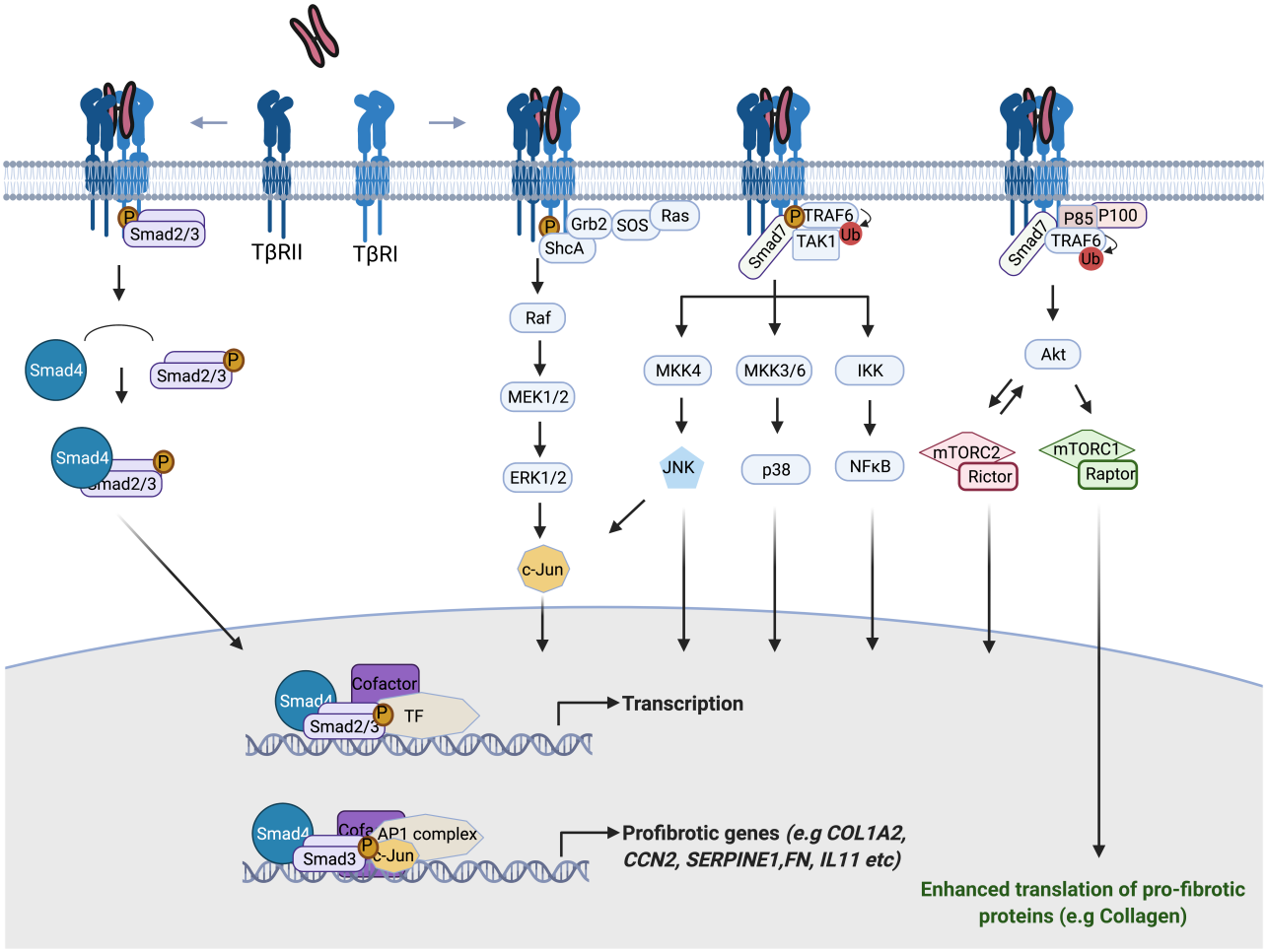

Figure 3. Contribution of Smad and non-Smad signaling pathways to fibrosis at the cellular level. TGF-β-induced activation of TβRI by TβRII leads to the recruitment and C-terminal phosphorylation of Smad2 and Smad3, which then associate with a Smad4. These activated heterotrimeric Smad complexes translocate into the nucleus, where they associate with high-affinity DNA binding transcription factors and transcription cofactors to activate or repress gene transcription. For example, Smad3/Smad4 complexes can cooperate with AP1 transcription complexes (Jun–Fos dimers) to promote pro-fibrotic gene transcription. TGF-β also activates non-Smad signaling, notably the MAPK pathways and Akt–mTOR signaling that also contribute to fibrogenic gene and cell differentiation responses. *COL1A2*, *CCN2* (*CTGF*), *SERPINE1* (*PAI-1*), *FN1*, and *IL11* encode collagen Iα2, connective tissue growth factor (CTGF), plasminogen activator inhibitor-1 (PAI-1), fibronectin, and interleukin-11, respectively. mTORC1 activation promotes protein translation and contributes to increased collagen synthesis. TF, transcription factor.

kinases [48,71]. These effector Smads, in combination with Smad4, then translocate into the nucleus, where they combine with DNA sequence-specific transcription factors and coregulators at regulatory gene sequences, and thus activate or repress target gene transcription in response to TGF-β [48,72,73] (Figure 3). Detection of C-terminally phosphorylated Smad2 and/or Smad3 is indicative of TGF-β/Smad signaling, yet may also result from activation in response to other TGF-β family members, such as activins [48,71]. Smad2 and Smad3 activation is elevated in activated fibroblasts and myofibroblasts in the fibrotic lesion [53,54], suggesting that these cells are primary targets of increased TGF-β1 signaling. While Smad signaling uniquely defines 'canonical' signaling for the TGF-β family proteins, TGF-β also activates non-Smad signaling pathways, including the PI3K–Akt–mTOR pathway, Erk MAPK and p38 MAPK signaling, and Src and Rho GTPases [48,74] (Figure 3). These pathways, however, are not diagnostic of TGF-β signaling, since they are strongly activated by receptor tyrosine kinases [75].

Increased TGF-β signaling promotes fibrosis

The increase in TGF-β signaling in fibrosis correlates etiologically with the initiation and progression of fibrosis. This notion is supported by the induction of fibrosis in response to topically administered or expressed TGF-β [76–78], and the decreased fibrosis in mice with inactivated TGF-β receptor or Smad3 expression [79–82]. The key role of TGF-β in driving fibrosis needs to be seen in the context of increased signaling by other cytokines and growth factors, many of which activate MAPK pathways and/or promote activation of transcription factors. This context is highly relevant since Smads control transcription through functional association and cooperation with high-affinity DNA-binding transcription factors, such as AP1 complexes that are activated by Erk and JNK MAPK pathways, or TCF/LEF and β-catenin that are activated by Wnt signaling [48,73,83]. Moreover, the stabilities and activities of effector Smads are controlled by (de)phosphorylation and (de)ubiquitylation in response to signaling [84]. Consequently, Smad signaling cooperates with, depends on, and is defined by functional interactions with other signaling pathways and transcription factors [48,73,85].

In fibrosis, increased TGF-β signaling prominently affects the behavior of the fibroblast population, which represents the majority of cells in fibrotic lesions. TGF-β is chemoattractive for fibroblasts at very low concentrations [86], resulting in their recruitment to sites of TGF-β1 activation. Furthermore, low TGF-β levels promote fibroblast proliferation [87–89]. These activities may explain the high number of fibroblasts in fibrotic lesions. Additionally, TGF-β-induced mesenchymal characteristics enable epithelial and endothelial cells to contribute to the fibroblast population in fibrotic diseases, as revealed in mouse models of fibrosis [41,42,90].

Activation of fibroblasts in response to TGF-β results in increased cell and nuclear sizes, enhanced protein synthesis capacity and ECM protein expression, and additional metabolic and gene expression changes. Among the upregulated ECM proteins expressed by fibroblasts, increased collagen production stands out as a hallmark of fibrosis [43]. The genes encoding collagen Iα1 and fibronectin are direct, transcriptional targets of TGF-β/Smad3 signaling [91,92]. Increased ECM protein expression in response to TGF-β is accompanied by attenuation of ECM protein degradation, e.g. through enhanced expression of protease inhibitors, thus facilitating ECM protein accumulation [53,93]. mTOR signaling, in response to TGF-β or growth factors, plays a key role in enhancing protein synthesis by activated fibroblasts and promotes both generally increased protein synthesis and selective proteomic alterations [94–96]. Selective metabolic responses activated by TGF-β are apparent with the increased expression of the glucose transporter GLUT1, thus enabling increased glucose import and glycolysis [97,98]. TGF-β also activates the expression of hexokinase 2, which catalyzes the first obligatory step of glucose metabolism, facilitates glucose entry, and is upregulated in IPF [99]. Additionally, TGF-β promotes the expression of glycolytic enzymes and regulators of enzyme metabolism, resulting in hyperglycolysis [98], and of the transcription factor ATF4, the master regulator of amino acid synthesis [100]. This enables increased protein synthesis, in particular of the serine–glycine biosynthetic pathway, to meet the demands of increased collagen and ECM protein synthesis [100]. This metabolic reprogramming requires cooperation of Smad and mTOR signaling and is essential for the pro-fibrotic effects of TGF-β and progression of fibrosis *in vivo* [100] (Figure 3).

An array of TGF-β-induced gene expression changes, besides those leading to increased ECM protein expression, contributes additionally to the establishment and progression of fibrotic lesions (Figure 3). Notably, TGF-β promotes differentiation of activated fibroblasts into contractile myofibroblasts [101]. Functional crosstalk with signaling induced by growth factor-activated tyrosine kinase receptors and integrins enables TGF-β signaling through Smad3 to induce a contractile protein expression program, including the expression of α-smooth muscle actin, that marks myofibroblast differentiation [101–103]. TGF-β/Smad3 signaling also directs the expression of connective tissue growth factor (CTGF/CCN2) in fibroblasts. CTGF contributes substantially to ECM protein expression and myofibroblast differentiation, and its expression greatly contributes to fibrosis in some mouse models [104,105]. Additionally, TGF-β directly induces the expression by fibroblasts and myofibroblasts of interleukin-11, a pro-fibrotic cytokine that is seen as an important regulator of fibrosis [106,107]. Interleukin-11 secretion by fibroblasts and epithelial cells contributes to fibroblast activation and myofibroblast differentiation, and consequently to ECM deposition. Its expression plays an essential role in some animal models

of fibrosis, such as the bleomycin-induced model of pulmonary fibrosis [108,109]. TGF-β/Smad signaling also directs the expression of the closely related c-Jun, JunB, and JunD transcription factors that heterodimerize with c-Fos or related proteins to form AP1 transcription complexes [110–112]. These are functionally activated in response to Erk or JNK MAPK signaling that is induced by various stimuli, including growth factors and cytokines, and TGF-β itself, and target an extensive variety of genes [112–114]. c-Jun expression is increased in fibrosis; increased c-Jun expression promotes fibrosis; and targeted inactivation of its gene attenuates fibrosis in mouse models, thus positioning c-Jun as a driver of fibrosis [115,116]. TGF-β-activated Smad3/4 complexes cooperate with AP1 complexes to direct TGF-β-induced expression of various target genes [117,118], including those encoding CTGF, interleukin-11, c-Jun, collagen Iα2, and fibronectin [106,112,119,120], all of which greatly contribute to fibrosis. While Smad3, in cooperation with Smad4, drives most TGF-β-induced gene expression responses in fibrosis and other physiological contexts, Smad2 often acts as a modifier of Smad3-mediated responses with additional and distinct roles [48,121].

The effects of increased TGF-β activation on cell populations in the fibrotic lesion other than fibroblasts have been studied less. Vascular endothelial and mural cells are expected to respond to TGF-β with gene expression changes that may resemble those of fibroblasts, thus also rendering these cells fibrogenic. Furthermore, TGF-β induces mesenchymal characteristics in both cell types, raising the possibility that they contribute to the fibroblast population [90,122]. In fact, mouse model studies strongly suggest that, in fibrosis, increased TGF-β signaling promotes EndMT to a variable extent, similarly to EMT, and that EndMT might substantially contribute to the fibrogenic fibroblast population [40,123,124]. Increased TGF-β signaling in epithelial cells, following αvβ6-mediated activation of latent TGF-β1, also plays significant roles in the initiation and progression of fibrosis, in part through acquisition of mesenchymal traits [56,68,78,125]. To what extent and how epithelial and endothelial cells following partial or complete EMT or EndMT contribute to fibrosis may depend on the type and model of fibrosis studied and approaches used to assess their contributions. However, single-cell analyses of human fibrotic lesions revealed cells with repressed epithelial or endothelial characteristics and acquired mesenchymal properties, strongly suggesting contributions of partial or complete EMT and EndMT to human fibrosis [126,127]. Finally, TGF-β signaling promotes angiogenesis, and increased TGF-β expression and signaling in the tumor micro-environment promote tumor angiogenesis in carcinomas [67,128]. Hence, increased TGF-β activity may contribute to neovascularization in some fibrotic settings.

Resident immune cells are also expected to respond to the increased TGF-β activation in fibrotic lesions. Increased TGF-β activation may be most relevant for macrophages, which play critical roles in fibrosis, in part through expression of cytokines that then act on fibroblasts [22,129]. Macrophages are highly responsive to locally activated TGF-β in normal physiological settings, as is apparent from their staining for activated Smad3 [55,130]. TGF-β1 is a potent chemotactic factor for macrophages, resulting in macrophage recruitment into the fibrotic lesion [131,132]. Additionally, TGF-β induces the expression of pro-fibrotic cytokines by macrophages, including TGF-β1 itself [132,133], thus amplifying the TGF-β activities. Furthermore, as in fibroblasts, TGF-β can stimulate ECM protein expression by macrophages [134,135]. These and other activities position TGF-β1 as a key regulator of macrophage recruitment and functions in the fibrotic lesions. Less is known about the extent to which TGF-β activation controls other immune cells, most notably T-cell populations, in fibrotic lesions. However, by analogy to the tumor microenvironment of carcinomas [67], we surmise that TGF-β affects many of the T-cell subpopulations, resulting in functional alterations and immunosuppressive activities.

TGF-β signaling is required for fibrosis

The role of TGF-β as a driver of fibrosis has been extensively documented using mouse models in which a TGF-β receptor or a TGF-β receptor-activated Smad was functionally or genetically inactivated. Pharmacological kinase inactivation of the type I TGF-β receptor TβRI, also known as ALK5 or TGFBR1, which initiates TGF-β-induced Smad2 and Smad3 activation and Erk MAPK signaling, prevents or strongly attenuates fibrosis in models of lung, liver, heart, kidney, and intestinal fibrosis, as well as injury-induced vascular fibrosis and myelofibrosis [136–140]. Downstream from the type I TGF-β receptor, Smad3 is essential in fibrosis [81,82], which is consistent with Smad3's role as a major mediator of TGF-β-induced, direct transcriptional responses [48,73]. Indeed, genetic inactivation of *Smad3* strongly inhibits fibrotic responses in various models of fibrosis, highlighting Smad3's role as a major pro-fibrotic mediator in fibroblasts [81,82]. These observations in a large variety of models highlight the roles of TGF-β-TβRI/ALK5–Smad3 signaling as a driver of fibrotic responses, independent of the type and site of fibrosis. In contrast, and consistent with Smad2's ability to attenuate TGF-β-induced Smad3 activities and its distinct activities [121], conditional *Smad2* inactivation was shown to have distinct, non-fibrogenic activities in models of kidney fibrosis [141] and liver fibrosis [142].

Upon stimulation, the TGF-β receptor complexes activate MAPK pathways and Akt–mTOR signaling that are known to cooperate with Smad signaling [74] (Figure 3). Various studies have addressed the roles of these pathways in fibrogenic responses and models of fibrosis. However, while these studies inform us about contributions of the individual pathways to fibrosis, they are not necessarily informative about their roles in direct responses to TGF-β, since diverse stimuli, most notably growth factors that activate receptor tyrosine kinases,

also upregulate these pathways in fibrosis [143–145]. Among the MAPK pathways, the MEK1/2–Erk MAPK pathway contributes to fibrosis in several mouse models [146–148], and p38 MAPK also contributes to renal and cardiac fibroses [149–153].

Consistent with mTOR's contributions to fibrogenic activities of fibroblasts [154–157], mTOR inhibition by rapamycin attenuates fibrosis in mouse models of kidney and lung fibrosis [158–160]. mTOR acts primarily through mTORC1 and mTORC2 complexes [161]. Often used to inhibit mTORC1, rapamycin also inhibits mTORC2, albeit less efficiently [162,163], and may only affect a subset of mTORC1-mediated responses [164,165]. Since mTORC1- and mTORC2-specific pharmacological inhibitors are not available, targeted inactivation of the genes encoding Raptor or Rictor has been used to dissect the differential contributions of mTORC1 and mTORC2, respectively, to fibrosis. Using this approach, mTORC1, acting through 4EBP1, was shown to be required for increased collagen synthesis by fibroblasts, albeit in a rapamycin-insensitive way, whereas mTORC2 was not required [96]. On the other hand, two lines of research support a role for mTORC2 in mouse models of kidney fibrosis. Fibroblast-specific inactivation of Rictor expression prevents kidney fibrosis [166], and mTORC2 is required in macrophages for cytokine release by macrophages, and fibroblast activation [155].

Among the immediate TGF-β target genes that are activated by Smad3/4 complexes are several with effector roles in promoting fibrosis, notably those encoding CTGF, IL-11 or c-Jun, that are expressed at high levels in fibrosis models and human fibrotic lesions [107,108,115,167]. Antibody-mediated inactivation of CTGF [168] or IL-11 [109] and targeted inactivation of c-Jun expression [115] are sufficient to attenuate or repress fibrosis in mouse models. Consequently, these mediators of fibrogenesis – two secreted proteins and a transcription factor – are seen as attractive targets for anti-fibrosis therapies. As mentioned, all three genes are transcriptionally activated through TGF-β-induced cooperation of Smad3/4 complexes with AP1 complexes at regulatory sequences [106,112,119], highlighting the cooperation of TGF-β/Smad3 with MAPK–AP1 signaling in fibrogenesis. Notably, attenuation of TGF-β activation and signaling in fibrotic tissues is expected to repress the expression of these key anti-fibrotic downstream targets.

Therapeutic approaches toward inhibition of TGF-β in fibrosis

With increasing appreciation of TGF-β's roles in fibrosis, much interest evolved toward TGF-β inhibition as a universal therapeutic approach to attenuate, halt or reverse fibrosis at diverse organ sites. The development of therapeutic anti-TGF-β approaches has, however, been mainly driven by approaches to inhibit cancer progression, recently in immuno-oncology, yet was tempered and delayed by concerns about potential adverse effects that were anticipated based on findings in mouse models [67]. Most anti-TGF-β therapeutic modalities fall into four groups: (1) small-molecule inhibitors of the TGF-β receptor kinases, (2) monoclonal antibodies that prevent TGF-β binding to its receptors, (3) ligand traps consisting of dimerized ectodomains of the type II TGF-β receptors (TβRII, TGFBR2) that sequester TGF-β and prevent its receptor binding, and (4) small molecules or antibodies that selectively interfere with TGF-β1 activation [67]. While the latter approaches aim to provide target selectivity, the former three classes are expected to systemically inhibit TGF-β, unless coupled to an antibody that directs target specificity.

Systemic TGF-β inhibition

Exemplified by galunisertib (LY2157299) [169] and vactosertib (EW-7197) [170], small-molecule inhibitors of TGF-β receptor kinases, primarily TβRI, block Smad2 and Smad3 activation in response to TGF-β. They do not target TGF-β receptors specifically and are equally effective against type I receptors for several other TGF-β-related proteins, such as activin, nodal, and myostatin, and possibly also other kinases, such as p38 MAPK [169]. They are simple to deliver orally but have poor pharmacokinetic and pharmacodynamic properties. In contrast, neutralizing TGF-β antibodies have exquisite ligand specificity, although they need to effectively interfere with the tight TGF-β binding affinity (in the 0.01 nM range) of the cell surface receptor complexes [48]. Among these, fresolimumab (1D11) [171] and SAR439459 [172] neutralize all three TGF-β homodimers, whereas NIS793 neutralizes TGF-β1 and TGF-β2 but not TGF-β3 [173]. Soluble, high-affinity ligand traps also prevent TGF-β from binding to its receptors. These comprise dimers of TβRII ectodomains (Fc-stabilized) that are designed to sequester TGF-β1 and TGF-β3, but not TGF-β2, and thus prevent them binding to receptors [174,175]. Distinct TGF-β traps differ in ligand-binding specificities or show enhanced ligand-binding efficacy [176]. Among those, AVID200 has been evaluated in mouse models and clinically [177] and was used as the basis for development of a bispecific trap, named M7824 or Bintrafusp α, that combines TGF-β binding to the TβRII ectodomains with a human anti-PD-L1 IgG1 toward immuno-oncological treatments [178].

Clinical studies with anti-TGF-β antibodies or TGF-β signaling inhibitors revealed adverse effects, consistent with anticipations based on mouse models [67]. A major fear was that TGF-β inhibition might induce metaplasia and tumor outgrowth, since TGF-β acts as a tumor suppressor in carcinoma development, and derepressed immune responses might lead to inflammation and autoimmune manifestations. Our current knowledge, primarily from clinical trials with galunisertib, fresolimumab, and the anti-TGF-β trap M7824, indicates that high doses of these agents infrequently lead to sporadic keratoacanthomas, which are low-grade cutaneous squamous

lesions that can be surgically managed [179,180]. Skin rashes have also been seen with fresolimumab and M7824 [179,180]. No dose-limiting immune-related adverse effects have been reported in clinical trials using small-molecule TβRI kinase inhibitors or anti-TGF-β antibodies. In early dose-escalation studies of galunisertib or other TβRI kinase inhibitors, cardiotoxicity was seen in some patients but was managed by alterations in treatment regimen [181,182]. Cardiovascular toxicity resulting in histopathological changes was also seen in mice and *Cynomolgus* monkeys treated with an antibody that neutralizes all three TGF-βs [183]. In contrast to treatment with the TβRI kinase inhibitor LY2109761 or a pan-TGF-β antibody, antibody-mediated repression of only TGF-β1 did not result in cardiac valvulopathies in a rat model [184]. Additionally, concerns over adverse effects on vascular integrity leading to hemorrhagic lesions need to be considered because TGF-β1 is required for endothelial integrity through effects on pericytes [185]. Toxicology studies with a potent, neutralizing pan-TGF-β antibody in mouse and monkey models resulted in persistent hemorrhagic bleeding and associated pathologies [183], whereas clinical trials using galunisertib or M7824 associate TGF-β inhibition with manageable mucosal bleeding [180,182]. These findings strongly suggest that systemic inhibition of all three TGF-βs might confer substantial toxicity that is attenuated with less efficient inhibition or by targeting TGF-β1 only. Nevertheless, using appropriate dosage and treatment regimens, small-molecule inhibitors and anti-TGF-β antibodies have manageable safety profiles, even though the therapeutic window is narrow [67].

Some anti-TGF-β approaches have been evaluated as monotherapies in animal models of fibrosis and limited clinical studies to target fibrosis-associated conditions. Multiple studies illustrate the abilities of neutralizing anti-TGF-β antibodies and TGF-β receptor kinase inhibitors to prevent fibrosis in preclinical models of lung, liver, heart, and kidney fibrosis, to the extent that they are often used as positive controls in the evaluation of novel approaches to inhibit fibrosis [186] or validation of novel research models [187,188]. For example, vactosertib prevents or attenuates fibrosis in liver fibrosis models upon carbon tetrachloride injury or bile duct ligation, bleomycin-induced lung fibrosis, and renal fibrosis following unilateral ureteral obstruction [189]. Treatment with vactosertib reduces Smad2 and Smad3 activation and collagen synthesis across all four models [189]. Additionally, in two models of ulcerative colitis, vactosertib reduces fibrotic gene expression and collagen accumulation in colon tissue, although this may be secondary to reduction in mucosal damage [190,191]. Consistent with these results, SB525334, an orally adsorbed, selective TGF-βRI kinase inhibitor, inhibits bleomycin-induced lung fibrosis in mice, reducing Smad2 and Smad3 activation, and collagen and fibronectin expression, and improving lung histology with reduced collagen accumulation in the lung parenchyma [192,193]. SB525334 was also effective in blocking lung fibrosis induced by adenoviral TGF-β1 expression, both prophylactically and after fibrosis was established [136]. Additionally, in a rat model of renal fibrosis, SB525334 reduced pro-fibrotic gene expression and improved renal function [194]. Among the neutralizing anti-TGF-β antibodies, a mouse version of fresolimumab has been well studied in preclinical models of fibrosis, notably renal fibrosis. This antibody, either as a single agent or with angiotensin II blockade of hypertension, significantly reduced fibrosis in the anti-Thy1 model of glomerulonephritis [195], a model of diabetic nephropathy-associated fibrosis [195], renal interstitial fibrosis following unilateral ureteral obstruction [196], and adriamycin-induced nephropathy [197].

Despite their robust efficacy in anti-mouse models, therapeutic approaches to attenuate fibrosis progression by systemically blocking TGF-β activity have disappointed in clinical studies. Treatment of patients with renal fibrosis with the pan-TGF-β neutralizing antibody fresolimumab or the TGF-β1-specific neutralizing antibody LY2382770 led to negative or inconclusive results. Fresolimumab was studied in focal segmental glomerulosclerosis patients, but while there was indication of efficacy without signs of toxicity, the study was concluded prior to completing enrollment due to expiration of the drug [198]. LY2382770 was studied in a large trial of advanced diabetic nephropathy patients, conclusively demonstrating no benefit of treatment in that cohort. This study also had significant limitations, potentially resulting from inadequate TGF-β inhibition under the treatment regimen used [199]. Fresolimumab and the anti-TGF-β trap AVID200 have been studied in small studies in systemic sclerosis patients. The reported results from both therapies are encouraging, with both agents leading to reductions in fibrosis biomarkers and improvements of skin stiffness [200,201]. Both studies were exploratory with limited patient populations, encouraging the initiation of larger clinical trials.

Selective TGF-β inhibition

Some antibodies and small molecules target the context-dependent TGF-β activation process and thus provide cell-type or tissue-type selective inhibition of TGF-β signaling. Latent TGF-β1 activation involves RGD sequence-mediated association of TGF-β1 pro-segments with selected β integrins in complexes with αv integrins at the cell surface (Figure 2). αvβ1, αvβ6, and αvβ8 integrins mediate TGF-β1 activation, with αvβ1 and αvβ6 primarily expressed on fibroblasts and epithelial cells, respectively [48,49,67–69], and αvβ8 receiving particular attention in the context of immuno-oncology [67,69,70]. Targeted interference with these interactions is expected to prevent TGF-β1 activation in a cell type-selective manner, without systemic TGF-β inhibition. However, the development of effective, selective, and in particular orally available αv integrin inhibitors comes with challenges [68,202,203].

Antibodies against integrins β1, β6 or β8 were shown to selectively impair TGF-β1 activation [70,204–207]. Among these, the best studied inhibitor for treatment of

fibrosis is the anti-αvβ6 antibody 3G9 [208], which led to the development of a clinical candidate, STX100/BG0011. Selective inhibition of αvβ6 integrin-mediated activation of TGF-β1 with this antibody effectively reduced fibrosis in multiple preclinical models of fibrosis, including bleomycin- and radiation-induced lung fibrosis [208,209], cholestatic liver disease [210,211], and renal fibrosis [212]. In these models, significant epithelial cell injury and, consequently, upregulation of αvβ6 expression on epithelial cells accompany the initiation of fibrosis [213]. However, in models where epithelial cell injury is not prominent, such as CCl_4-induced liver injury, αvβ6 appears to have a more limited role [213]. An antibody against αvβ8 integrin has shown anti-fibrotic activity against airway remodeling in a mouse model of chronic obstructive pulmonary disease [214]. Another antibody, SRK-181, developed to bind the TGF-β1 pro-segment, prevents dissociation of mature TGF-β1 from its pro-segment, consequently keeping TGF-β1 latent without affecting TGF-β2 or TGF-β3 activation [184]. In contrast to interference with integrin-mediated TGF-β activation, this antibody is expected to act systemically.

Among the small-molecule inhibitors of integrin-mediated TGF-β1 activation, inhibitors of αvβ1 were shown to effectively reduce liver, lung, and renal fibrosis in preclinical models [215]. Other studies demonstrate the ability of GSK3008348, an inhaled αvβ6 inhibitor of TGF-β1 activation, to inhibit TGF-β signaling and reduce the expression of fibrosis biomarkers and collagen expression in mouse models of fibrosis [186]. Additionally, PLN-74809, an oral small-molecule inhibitor of αvβ6- and αvβ1-mediated TGF-β1 activation, was shown to inhibit bleomycin-induced lung fibrosis in mice [216]. A conceptually different approach allows for fibroblast-specific inhibition of TGF-β signaling that leads to attenuation of lung and tumor fibrosis. Trihydroxyphenolic compounds, such as ellagic acid and corilagin, selectively inhibit the TβRI kinase, dependent on auto-oxidation and irreversible inhibition of the enzyme lysyl oxidase-like 2 (LOXL2). LOXL2 is expressed in fibroblasts and upregulated during fibrosis, suggesting that limiting TGF-β inhibition to these cells will avoid the safety issues associated with systemic inhibition. These compounds are able to repress collagen synthesis and fibrosis in a mouse model of bleomycin-induced lung fibrosis [217].

Several clinical studies have been evaluating these selective inhibitors of integrin-mediated TGF-β1 activation. The anti-integrin β6 antibody STX100/BG0011 that interferes with αvβ6-mediated TGF-β1 activation was studied in IPF patients. Although promising results in early studies demonstrated reduced TGF-β signaling in the lung [218], a subsequent phase IIb study was terminated early due to safety concerns [219] that may result from antibody-driven inflammation reportedly seen in mouse and primate studies [220]. Clinical trials of small-molecule inhibitors of integrin-mediated TGF-β1 activation have also been reported. IDL-2965, a multi-integrin inhibitor of αvβ1, αvβ3, and αvβ6 was studied in IPF patients [221], but this trial was halted prematurely with no additional data currently available. GSK3008348 was shown to reduce fibrosis marker expression and collagen deposition in precision-cut human lung tissue slices, and positron emission tomography (PET) imaging revealed target engagement in the lungs of IPF patients following a single dose of the inhaled drug [222]. PLN-74809 has been shown to reduce TGF-β signaling in alveolar macrophages in healthy volunteers [223] and inhibit fibrosis pathways in precision-cut human lung tissue slices [224]. PLN-74809 is currently in phase 2 trials in IPF patients [216] and primary sclerosing cholangitis patients [225], as well as in a PET tracer target engagement study in IPF patients [226]. Finally, the trihydrophenolic compound epigallocatechin gallate (EGCG), which acts as a dual inhibitor of LOXL2 and the TβRI kinase, was shown to inhibit TGF-β-induced Smad activation and pro-fibrotic gene expression in lung biopsies from IPF patients, demonstrating a strong correlation between Smad activation and soluble collagen in lung tissue [227]. Clearly, we are only at the very beginning of the development of selective agents that interfere with integrin-mediated TGF-β activation. Ongoing and future preclinical and clinical studies will reveal the feasibility of selective TGF-β inhibition strategies to attenuate progression of the different types of fibrosis.

In conclusion, since the early discovery of TGF-β's ability to induce fibrotic scar formation [76], substantial evidence has causally linked increased TGF-β activity to initiation and progression of fibrosis. Enhanced TGF-β signaling is now increasingly seen as a universal driver of fibrotic processes in diverse pathological contexts, tissues, and organs. Consequently, interference with TGF-β activation and signaling is now pursued as a therapeutic strategy to attenuate and reverse fibrosis and associated pathological consequences. With multiple normal physiological roles of TGF-β, selective approaches are being developed to pre-empt unwanted adverse effects of TGF-β-inhibition while achieving therapeutic efficacy in the fibrotic lesions.

Author contributions statement

All the authors contributed to the writing of the first draft, and throughout the many consecutive drafts. EHB designed and created the figures using BioRender.com.

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