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Fibrosis is defined by excess deposition of the extracellular matrix (ECM), for which the most commonly identified components are collagen and fibronectin. This progressive injury often results in organ failure and death, targeting nearly any tissue in the body. The recognized cellular conductor of fibrosis is the myofibroblast. This activated form of a fibroblast is induced by local conditions, including mechanical stress, growth factors, adhesion proteins, and cytokines. These highly contractile cells classically express α-smooth muscle actin (α-SMA) and display increased migration and proliferation. Under normal physiological events, fibroblasts are activated and become myofibroblasts to promote wound healing; after epithelialization has occurred, they are lost through apoptosis.

Over the past decades, specific receptor systems that, in combination, induce myofibroblast transition have been identified (154). These include lysophosphatidic acid (LPA), endothelin (ET) 1 (ET-1), connective tissue growth factor (CTGF), transforming growth factor-β (TGFβ), and others. However, only more recently has a downstream genetic program that modulates the actin cytoskeleton. Their GTPases are a subfamily of small GTP-binding proteins within the Ras superfamily that modulate the actin cytoskeleton. Their activation is regulated through Rho guanine nucleotide exchange factors (GEFs), which directly bind Rho proteins, allowing for exchange of GDP for GTP (124). In the GTP-bound, active state, they are able to interact with downstream effector proteins. Two main effector proteins for Rho signaling are Rho-associated, coiled-coil-containing protein kinase (ROCK) (53, 75) and mouse diaphanous-related formin-1 (mDia1) (140). Mechanistically, mDia1 is thought to induce nucleation of F-actin filaments, while ROCK phosphorylation modulates F-actin stabilization through multiple downstream targets, including myosin light chain phosphatase (MYPT1).

Recent analyses of the Rho GTPase signaling cascade have appreciated downstream changes in gene expression induced by Rho activity and the serum response factor (SRF) transcription factor. A key regulatory mechanism of SRF-mediated gene transcription includes the myocardin-related transcription factors (MRTFs) A and B. The NH2-terminal region of MRTFs contains a unique nuclear localization sequence that is enveloped by G-actin-binding motifs (RPEL) (93, 97). When there is a surplus of G-actin monomers within the cytoplasm, the RPEL motifs bind to MRTF, sequestering it from the nucleus. Rho activation results in F-actin stress fiber formation, reducing the abundance of G-actin and exposing the nuclear localization sequence of MRTF. This allows for nuclear accumulation of MRTF, where it can cooperate with SRF and induce gene expression. Importantly, multiple target genes for MRTF/SRF are known drivers of fibrosis (Table 1) (19, 43, 81, 84, 126). Additionally, SRF-mediated gene transcription has been shown to be essential for myofibroblast differentiation (Fig. 1) (20, 120, 160). The remainder of this review focuses on the signaling pathways known to drive cellular fibrosis and highlights how each of these receptor systems feeds into the Rho GTPase pathway, activating MRTF/SRF gene transcription leading to myofibroblast activation.

Lysophosphatidic Acid

By acting through specific G protein-coupled receptors, LPA, a phospholipid that is produced by the enzyme autotaxin, mediates many diverse cellular responses. There are six recognized receptors that signal in response to LPA, designated LPA1–6 (15). Among them, a key role for LPA1 has been demonstrated in the development of tissue fibrosis in a variety of organ systems. LPA1 acts through three distinct families of G proteins: Goαs, Goαq11, and Goα12/13 (22, 35). Activation of LPA1 leads to inhibition of the adenyl cyclase pathway and activation of the MAP kinase, phospholipase C, Akt, and Rho pathways. These pathways drive cytoskeletal changes, SRF-mediated gene transcription, cell proliferation, migration, and collagen synthesis (22).
LPA-LPA1 pathway involvement has been demonstrated in various animal models of skin (18), lung (138), kidney (106), peritoneal fibrosis (118), and liver (149). Bleomycin-induced dermal fibrosis, characterized by increased dermal thickness and accumulation of collagen, is absent in LPA1 knockout mice (18). In contrast, LPA2 knockout mice show fibrosis similar to that of wild-type mice. In addition, pharmacological antagonism of LPA1 by AM095 significantly attenuates bleomycin-induced skin fibrosis in both prevention and therapeutic treatment regimens. Similar results are seen in bleomycin-induced lung fibrosis in LPA1 knockout animals; they show decreased fibroblast and collagen deposition, as well as attenuation of vascular leakage (138). The orally active LPA1-specific antagonist AM966 prevents bleomycin-induced pulmonary fibrosis (137). Both released LPA and LPA1 receptor expression increase significantly in a renal fibrosis model induced by unilateral ureteral obstruction in mice (106). Furthermore, unilateral ureteral obstruction-induced fibrosis is significantly attenuated in LPA1 knockout mice compared with wild-type animals. Inactivation of LPA1 by the LPA1/LPA3 antagonist Ki16425 similarly attenuates fibrosis by decreasing the expression of CTGF and TGFβ (106). Genetic depletion or pharmacological inhibition of LPA1 protects mice from chlorhexidine gluconate-induced peritoneal fibrosis through decreasing CTGF expression, fibroblast proliferation, and myofibroblast accumulation (118). As mentioned above, LPA1 couples with three types of G proteins. In the peritoneal fibrosis model, LPA-LPA1-induced CTGF expression is mediated by Goα12/13 signaling, RhoA/ROCK activation, cytoskeletal reorganization, nuclear translocation of MRTF, and SRF-induced transcription (118). LPA signaling may also play a role in liver fibrosis. In various liver injury models, plasma LPA and autotaxin levels are increased and correlate with disease severity parameters (149).

Other LPA receptors also contribute to fibrosis. Xu et al. (156) showed that LPA activates TGFβ via α,β,γ-integrins in human epithelial cells by activating LPA2 and signaling through Goαi and RhoA/Rho kinases. In addition, both LPA2 and α,β,γ-integrin are upregulated in areas of fibrosis in animals with bleomycin-induced lung injury, as well as in patients with usual interstitial pneumonia. The LPA-induced LPA2/Goαi/α,β,γ-integrin/Rho signaling to transactivate latent TGFβ is similarly seen in cultured proximal tubular cells (37). In the rat renal fibrosis model induced by ischemia-reperfusion injury, increased TGFβ, LPA2, and β6-integrin expression is observed, as is expression of the profibrotic factors platelet-derived growth factor (PDGF)-B and CTGF. In another study, changes in LPA-induced barrier function of pulmonary artery and microvascular endothelial cells suggest that LPA2 and, possibly, LPA6, but not LPA1, are involved (112). Most recently, using LPA2-deficient mice, Huang et al. (51) demonstrated the role of LPA2 in lung fibrosis. These animals are protected against bleomycin-induced lung injury compared with their wild-type controls. They show attenuation of fibronectin, α-SMA, and collagen in lung tissue, as well as reduced levels of IL-6 and TGF-β in bronchoalveolar lavage fluids. Huang et al. speculate that the contrasting results from Castelino et al. (18) are due to the genetic background differences of the animals.

![Fig. 1. Rho GTPase signaling pathway represents a convergent approach to targeting fibrosis. Many current drugs being developed for fibrotic diseases are targeting specific receptors known to be involved in stimulating fibroblasts into myofibroblasts. Interestingly, many of these specific receptor systems converge onto Rho small GTPase signaling. GTP-bound active Rho can interact with downstream effector proteins, most notably Rho-associated, coiled-coil containing protein kinase (ROCK) kinase and mouse diaphanous-related formin-1 (mDia1), which together initiate and stabilize actin stress fibers. This increase in F-actin and resulting decrease in G-actin monomers frees myocardin-related transcription factor (MRTF) to translocate into the nucleus where it cooperates with serum response factor (SRF) to induce gene transcription. Many MRTF/SRF target genes are known drivers of fibrosis including connective tissue growth factor (CTGF), α-smooth muscle actin (α-SMA), and collagen; together this activation of gene transcription induces and maintains the activation of fibroblasts to myofibroblasts, GPCR, G protein-coupled receptor; TGFβ, transforming growth factor-β.](http://ajp-cell.physiology.org/)

Table 1. MRTF/SRF-regulated genes involved in fibrosis

<table>
<thead>
<tr>
<th>Gene</th>
<th>Protein</th>
<th>Reference No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACTA2</td>
<td>α-Smooth muscle actin</td>
<td>67</td>
</tr>
<tr>
<td>CTGF</td>
<td>Connective tissue growth factor/CCN2</td>
<td>14, 34</td>
</tr>
<tr>
<td>COL1A2</td>
<td>Collagen I</td>
<td>65</td>
</tr>
<tr>
<td>CYR61</td>
<td>Cysteine-rich angiogenic inducer 61/CCN1</td>
<td>14, 34</td>
</tr>
<tr>
<td>SRF</td>
<td>Serum response factor</td>
<td>14, 103</td>
</tr>
<tr>
<td>TIEG1</td>
<td>Transforming growth factor-β-inducible</td>
<td>103</td>
</tr>
<tr>
<td>VCL</td>
<td>Vinculin</td>
<td>103</td>
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MRTF, myocardin-related transcription factor; SRF, serum response factor.
The molecular mechanisms governing the activation of Rho following LPA receptor-ligand binding are well understood. The RhoGEFs p115-RhoGEF, PDZ-RhoGEF, leukemia-associated RhoGEF, and, possibly, lymphoid blast crisis-RhoGEF have been shown to be directly regulated by G_{12/13} proteins (133). These GEFs can then directly interact with members of the Rho GTPase family, most notably RhoA and RhoC, allowing exchange of GDP for GTP and interaction with downstream effector proteins, which are essential for profibrotic phenotypes.

Clinically, the role of LPA and its receptors has been studied in idiopathic pulmonary fibrosis (IPF). Elevated levels of LPA are observed in bronchoalveolar lavage fluid of IPF patients (138). In addition, elevated levels of autotaxin have been found in hyperplastic bronchiolar epithelium and in the alveolar epithelium surrounding areas of fibroblastic foci (103). The increased expression of the enzyme responsible for LPA production might explain higher LPA levels in IPF lungs. LPA and autotaxin are also elevated in patients with chronic hepatitis C, and their levels correlated with the histological stage of liver fibrosis (148). The involvement of the LPA/autotaxin pathway is also emerging in systemic sclerosis (SSc) and diabetic retinopathy (3, 139).

**Endothelin-1**

There are three isoforms of ETs: ET-1, ET-2, and ET-3. Among them, ET-1 is the predominant isoform in humans, and it acts as a potent vasconstrictor. It also participates in angiogenesis, cell survival, epithelial-to-mesenchymal transition (EMT), and tumor-related activities (76). ET-1 is produced as a 212-amino acid precursor and subsequently cleaved twice, first by endopeptidase to form the big ET-1 and then by the membrane-bound metalloproteinase ET-converting enzyme-1 to form a biologically active 21-amino acid peptide (104a). It is produced by a number of cell types, including endothelial cells, epithelial cells, smooth muscle cells, fibroblasts, macrophages, and cardiomyocytes, and its expression can be induced by various stimuli such as angiotensin II and TGFβ (92). ETs mediate their physiological effects by binding to two seven-transmembrane G protein-coupled receptors, ETA and ETB. The ETA receptor is ET-1-selective, while the ETB receptor has equal affinity for all three ET isoforms. While the ETA receptor appears to be profibrotic, the ETB receptor has been shown to be antiproliferative for myofibroblasts (41, 85, 86).

The role of ET-1 in fibrogenesis has been studied extensively. In healthy lung fibroblasts, ET-1 is able to induce ECM and α-SMA synthesis, as well as contractile activity (131, 157). It induces expression of α-SMA in lung fibroblasts through the ETA receptor and subsequent activation of the Rac/phosphoinositide-3-kinase (PI3K)/Akt pathway (131). The ETA receptor is also responsible for ECM contraction (131, 157). In contrast, production of ECM is mediated by both ETA and ETB receptors. The role of ET-1 in promoting fibrogenesis is further demonstrated using transgenic mice with overexpression of human ET-1 (48). These animals spontaneously develop progressive pulmonary fibrosis and lung inflammation. In addition, ET-1 acts synergistically with TGFβ to induce fibrosis (49, 67, 130). Blockade of both ETA and ETB receptors suppresses TGFβ- or bleomycin-induced fibrosis in vivo (67).

In addition, ET-1 acts through TGFβ to mediate EMT in the lung via the ETA receptor (56).

Similar to LPA receptors, the G protein-mediated mechanisms leading to activation of Rho have been well mapped. Coupling of the two main types of ET receptors, ETA and ETB, to both G_{12/13} and G_{q/11} has been identified (132). The formation of stress fibers and activation of ROCK following ET-1 stimulation in vitro are likely mediated partially through G_{12/13} and G_{q/11} family mechanisms (61). Although G_{12/13} subunits have classically been identified to activate Rho through the GEFs listed above, Goq_{11} can also directly activate RhoA/C through stimulation of p63 RhoGGEF (83, 132).

ET-1 plays significant roles in the pathophysiology of various fibrosis diseases. ET-1 is overexpressed in SSc dermal fibroblasts (60), and the elevation of ET-1 correlates with disease severity in diffuse SSC patients compared with those with limited SSC (159). Enhanced ET-1 expression and binding are observed in lung fibroblasts obtained from SSC-Interstitial lung disease (ILD) patients, and blocking the ET-1 pathway significantly reduces α-SMA levels in these cells (131). In IPF, ET-1 is elevated in patient serum (145) and bronchoalveolar lavage fluid (111), as well as in lung biopsies (119). Big ET-1, along with ET-converting enzyme-1, are found colocalized in the lung in a manner that correlates with disease severity. In a diabetes-induced cardiac fibrosis model, elevated plasma ET-1 levels are associated with cardiac fibrosis (152). Increased ET-1, which is produced by endothelial cells, promotes endothelial-to-mesenchymal transition. Therefore, targeting ET-1 may be beneficial in the prevention of diabetic cardiomyopathy. In experimental hepatic fibrosis, ET in the injured liver not only induces stellate cell contraction but also contributes to stellate cell activation and, therefore, the fibrogenic response, all of which can be altered by blockade of the ET receptors (115).

**Transforming Growth Factor-β**

TGFβ is the central profibrogenic cytokine that is an indispensable player in connective tissue homeostasis and considered the “master cytokine” in fibrosis (166). It attracts neutrophils and induces migration of fibroblasts in the wound-healing process. It also promotes differentiation of myofibroblasts, which are the major cell type for ECM protein synthesis in the healing of damaged tissue. In addition, TGFβ promotes fibroblast proliferation and differentiation and induces cytokine production. It also stimulates the expression of ET-1 and CTGF, both of which induce fibrosis (61, 97). Abnormal sustained activation of myofibroblasts by TGFβ or inflammation results in pathological conditions, including fibrosis, cancer, inflammation, and immune system defects.

The TGFβ superfamily comprises TGFβ1, TGFβ2, and TGFβ3, which are synthesized by a wide variety of cell types. Among them, TGFβ1 is the most abundant and is the prototype of this family. It is secreted in its latent form, a complex including the latency-associated peptide (LAP) and the active cytokine itself. LAP, in turn, forms disulfide bonds to members of the latent TGFβ-binding proteins that are cross-linked to ECM proteins. This interaction allows the cytokine to be stored and tethered in its latent form in the extracellular space. TGFβ can be activated by various mechanisms, such as proteolysis and physiochemical processes, and by interaction with throm-
b checkpoints. Once activated, it binds to a TGFβ receptor and transduces signals from the cell surface to the nucleus via the canonical Smad signaling pathway and via noncanonical pathways that include MAP kinase and PI3K pathways.

The classical pathway of TGFβ signaling occurs when TGFβ receptor II, which is constitutively active, trans-phosphorylates and forms a complex with the TGFβ-bound TGFβ receptor I. This, in turn, phosphorylates serine residues of cytoplasmic R-Smad, a complex of Smad2 and Smad3. The two heterodimerize and bind to the co-Smad Smad4, and the whole complex translocates across the nuclear membrane to interact with Smad binding elements, recruiting coactivators, corepressors, or transcription factors to modulate gene expression, such as that of collagen and CTGF. Inhibitory Smad7, a negative regulator of the TGFβ receptor and transduces signals from the cell surface to the nucleus, such as that of collagen and CTGF. Inhibitory Smad7 in fibrogenesis was demonstrated by Schultz et al. (123), who showed that angiotensin II induced cardiac fibrosis in wild-type mice, but not in TGFβ1-null animals. Similar results are obtained in Smad3 knockout mice, which are protected from bleomycin-induced skin and lung fibrosis and dimethylnitrosamine-induced liver fibrosis (68, 71, 167). In addition, Smad4-deficient mice are protected from unilateral ureteral obstruction-induced renal fibrosis (91). On the other hand, inhibitory Smads, such as Smad7, counteract TGFβ-mediated fibrosis. The role of Smad7 as a negative regulator of collagen synthesis is shown by adenovirus-mediated overexpression of Smad7 in animal models. Overexpression of Smad7 stops bleomycin-induced lung fibrosis and bile duct ligation-induced liver fibrosis (28, 102).

TGFβ can also induce fibrosis through noncanonical pathways. It has been suggested that TGFβ activates MAP kinases such as JNK, Erk, and p38 (29, 105, 164), as well as PI3K (161). TGFβ induces phosphorylation on TGFβ receptors I and II and/or Shc, which recruit Grb2/Sos to activate Erk through membrane-anchored Ras and downstream MAP kinase cascades (74). Together with the canonical Smad pathway, the TGFβ-Erk axis regulates gene transcription to control EMT, as well as CTGF and type I collagen synthesis (23, 72, 80). Erk also inhibits R-Smad activity through phosphorylation (65). In addition to Erk, JNK and p38 can be rapidly activated by TGFβ through MAP kinase kinases (MKK) (29, 164). TGFβ activates TGFβ receptors I and II, promoting interaction with TNF receptor-associated factor (TRAF6), which forms polyubiquitin chains. This complex then recruits TGFβ-activated kinase 1 to activate MKK4 or MKK3/6 and, subsequently, activate JNK and p38, respectively (147, 150). In conjunction with Smads, the activated JNK/p38 pathways regulate TGFβ-mediated fibroblast differentiation into α-SMA-expressing myofibroblasts (164). The PI3K pathway is another non-Smad pathway contributing to TGFβ-induced fibrosis. It induces two profibrotic pathways: Akt-mammalian target of rapamycin and p21-activated kinase 2/Abelson kinase (c-Abl). TGFβ activates PI3K/Akt and, subsequently, mammalian target of rapamycin/S6 kinase to control myofibroblast differentiation (69). On the other hand, c-Abl, which acts downstream of PI3K, plays a key role in TGFβ-mediated fibroblast proliferation (153). It promotes fibrosis through its downstream mediators, including PKCδ/Fli-1 (16) and early growth response (Egr)-1, -2, and -3 (12, 33).

It has also been observed that TGFβ stimulation leads to Rho activation; however, unlike GPCR mechanisms such as LPA and ET-1, which have been well studied, the pathway leading from TGFβ receptor activation to Rho signaling remains mostly unresolved. The formation of stress fibers following LPA or ET-1 ligand stimulation occurs very rapidly, while TGFβ-induced stress fiber formation is delayed to ~18–24 h following stimulation. This suggests an indirect mechanism such as Smad2/3-dependent transcriptional regulation of other Rho activators (121). Known TGFβ/Smad target genes that could induce this activation of Rho include ET-1 (116), sphingosine kinase-1 (158), and the RhoGEFs NET1 and H1/Lfc (73, 129, 142).

Connective Tissue Growth Factor

CTGF, also known as CCN2, is a matricellular protein that belongs to the ECM-associated signaling CCN family. It is involved in angiogenesis, cell migration, adhesion, proliferation, tissue wound repair, and ECM regulation (17). Under physiological conditions, CTGF is minimally expressed, but in fibrotic conditions, it is significantly elevated. CTGF functions by binding to various cell surface receptors, including integrins (10, 36), cell surface heparan sulfate proteoglycans (36), low-density lipoprotein receptor-related protein/α-1-macroglobulin receptor (125), and tyrosine kinase receptor TrkA (146). It also binds growth factors and ECM proteins such as VEGF (44), TGFβ/bone morphogenetic proteins (2), and fibronectin (50). CTGF expression is induced by a variety of extracellular stimuli such as TGFβ, PDGF, ET-1, and IL-1β, likely through a Rho/MRTF mechanism (59), as it is a direct MRTF/SRF transcription target. CTGF appears to act as the downstream mediator of several of these profibrogenic factors (59). On the other hand, it reciprocally induces a variety of cytokines such as TGFβ and VEGF, forming a positive-feedback loop that induces more expression of CTGF. It aids TGFβ in fibrogenesis and helps sustain fibrosis (96). Overexpression of CTGF in fibroblasts promotes fibrosis in multiple organs, while deletion of CTGF in fibroblasts or smooth muscle cells greatly reduces bleomycin-induced skin fibrosis (79, 136). In addition, inhibition of CTGF alleviates fibrosis in animal models of cardiac, liver, and kidney fibrosis (70, 104, 144). CTGF may also function as a positive-feedback mechanism in Rho signaling. In addition to being a transcriptional target of MRTF, CTGF has been shown to induce focal adhesion complexes and stress fibers through binding of α,β-integrin (21).

Integrins

Members of the integrin family of cell adhesion molecules are key players in fibrosis through modulation of cell-cell and cell-matrix interactions. They are heterodimeric transmembrane glycoproteins that govern the initiation, maintenance, and resolution of fibrosis by providing communication between cells (e.g., fibroblasts and inflammatory cells) and the ECM. These cellular receptors consist of noncovalently associated α- and β-subunits. There are 18 α-subunits and 8 β-subunits, with a total of 24 heterodimers with distinct specificities for different ECM components.
α5-integrins, specifically α5β3, α5β5, α5β6, and α5β8, are crucial in the activation of latent TGFβ1 and TGFβ3 by binding to the arginine-glycine-aspartic acid (RGD) motif in the LAP (5, 8, 9, 98, 99). Their importance in tissue fibrosis has been elucidated in a recent study using a PDGF-B receptor (pdgfrb)-Cre model to deplete the α5-integrin gene selectively in myofibroblasts (46). Henderson et al. (46) show that the α5-integrin gene deletion protects animals from COLα1-induced hepatic fibrosis and bleomycin-induced fibrosis in the lung, as well as the unilateral ureteric obstruction model of kidney fibrosis. This is not observed in β1-, β5-, or β6-integrin knock-out mice or a conditional hepatic stellate cell β5-integrin knockout model. A synthetic, small-molecule RGD peptidomimetic antagonist (CWHM 12) that potently inhibits α5-integrin-containing intern attenuates COLα1-induced hepatic fibrosis (in prevention and treatment models) (46). Similar results are seen in a treatment model of bleomycin-induced lung fibrosis (46).

In addition to the α5-integrins, other integrins also participate in promoting tissue fibrosis. Mice bearing specific depletion of fibroblast β1-integrin are resistant to bleomycin-induced skin fibrosis and have reduced collagen and α-SMA expression (78). In mouse models that have a mutation in the fibrillin-1 gene that mimics the stiff skin syndrome, fibroblast is observed, with increased collagen deposition, disorganized microfibrillar aggregates in the dermis, increased plasmacytoid dendritic cells and T helper cells, and autoantibody production. This is prevented by a β1-integrin-activating antibody and by depletion of β5-integrin. It is also reversed by a pan-specific TGFβ-neutralizing antibody (38). These results show that integrin modulation has therapeutic potential for fibrotic diseases. Similar to TGFβ, integrin activation of Rho signaling has been identified, but the exact mechanism has not been established. It is clear, however, that fibronectin-stimulated integrin signaling results in a rapid formation of actin stress fibers and stimulation of the MAP kinase cascade, both of which require Rho activity (113).

**Recent Clinical Trials Targeting Profibrotic Pathways**

Ultimately, one goal of understanding the signaling pathways involved in fibrosis is development of novel therapies to address this tremendous unmet medical need. The general approach has been to target individual receptors or pathway components (Fig. 2). Active trials of this sort are underway, as described below, but no highly efficacious agents have been identified.

**LPA.** Several LPA receptor antagonists have entered clinical trials. The LPA1 antagonist AM152 (now termed BMS-986020) was safe and well tolerated in healthy subjects (108) and is now in phase II trials to evaluate its safety and efficacy in IPF patients (ClinicalTrials.gov NCT01766817). A LPA1/3 dual antagonist, SAR100842, is in phase II clinical trials to evaluate its safety and tolerability in patients with early diffuse cutaneous SSC (ClinicalTrials.gov NCT01651143).

**ET-1.** An early trial (BUILD-1) of the nonselective ET receptor antagonist bosentan showed a trend toward improved symptoms and delayed time to death in IPF (63). However, subsequent trials (BUILD-2 in SSC-ILD and BUILD-3 in biopsy-proven IPF) did not meet their primary objectives, which in BUILD-3 was delay in the time to IPF worsening or death (64, 134). Similarly, a phase III study of ambrisentan (ARTEMIS-IPF trial), a selective ETA receptor blocker, in subjects with IPF and pulmonary arterial hypertension was also terminated due to limited efficacy (109). This highlights the difficulty in translating preclinical work into successful clinical trials.

**TGFβ.** Since aberrant TGFβ expression and signaling have been implicated in several conditions, such as SSC (7, 27, 66), renal diseases (90), and liver and pulmonary fibrosis (34, 52, 87), neutralizing antibodies to TGFβ have been evaluated in several clinical trials. Despite promising early studies, trials of two anti-TGFβ antibodies [CAT-192 (TGFβ1) and CAT-152 (TGFβ2>TGFβ3)] did not show clinical improvements in early diffuse cutaneous SSC (25) and glaucoma (135), respec-

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**Fig. 2.** Schematic model of current therapeutic approaches. Several receptor mechanisms being targeted in fibrosis are illustrated. All can activate Rho GTPase and the downstream MRTF/SRF gene transcription mechanism. By blocking MRTF-regulated gene transcription and myofibroblasts, our compounds (e.g., CCG-203971) represent a novel approach targeting a key convergence point (genetic switch) in the intrinsic fibroblast-myofibroblast conversion. This may prove more effective than attempting to disrupt each individual profibrotic input. LPA: lysophosphatidic acid; LARG: Leukemia-associated RhoGEF; GEF: guanine nucleotide exchange factor.
tively. In a phase I/II double-blind, proof-of-concept, placebo-controlled trial of a human anti-TGFβ1 monoclonal antibody (CAT-192) in patients with early diffuse cutaneous SSc, more adverse events affecting gastrointestinal, musculoskeletal, respiratory, and skin systems were seen in the CAT-192-treated group, with no differences in efficacy parameters such as the modified Rodnan skin thickness score (24). GC1008, a pan-specific antibody for all TGFβ isoforms, was well tolerated in patients with focal segmental glomerulosclerosis in a phase I study (141) and is now in phase I clinical trials in SSC and IPF (ClinicalTrials.gov NCT01284322 and NCT00125385). A synthetic peptide (P144) derived from the ligand-binding domain of betaglycan is able to block TGFβ activity and suppress bleomycin-induced skin fibrosis in mice (122). A single-center phase II trial in SSC (ClinicalTrials.gov NCT00574613) was recently completed; its results are pending.

Nonreceptor targets. Pirfenidone, a pyridone with anti-inflammatory and antifibrotic effects, is the only approved antifibrotic. Although its exact mechanism of action is not known, it is believed to be related to inhibition of TGFβ production or activity. In pulmonary fibrosis models, pirfenidone suppresses TGFβ gene expression in tissue and lavage fluid (54). Pirfenidone treatment decreases ECM accumulation in experimental lung fibrosis (55), cardiac and renal fibrosis in streptozotocin-diabetic rats (94), and liver fibrosis (168). In a pilot study to examine the antifibrotic effect of pirfenidone in liver, fibrosis was reduced in 30% of the patients in a 12-mo treatment period (6). However, it showed limited success in treatment of stage 3 diabetic chronic kidney disease patients (17). This agent is approved in Europe and Japan for IPF treatment and yielded favorable results in a recently completed phase III study in IPF in the United States (ClinicalTrials.gov NCT01504334) and is being evaluated in an open-label trial in SSC-ILD (LOTUSS, NCT01933334).

Tyrosine kinase inhibitors are another option to block TGFβ signaling. Imatinib mesylate is approved by the US Food and Drug Administration for treatment of chronic myelogenous leukemia through targeting BCR-ABL. Studies in SSC are inconclusive, and patient tolerability appears to be an issue. Of six clinical trials, five were completed, with three showing promising results and one terminated for safety reasons (13, 107). Tolerability was also an issue in a proof-of-concept trial of imatinib in SSc-ILD (62). In bronchoalveolar lavage of a subset of patients at baseline and 1 yr, imatinib resulted in reduced IL-4-producing T cells but increased CD4+ T cells, suggesting that it may be effective in a properly designed trial (26). An open-label study was conducted to evaluate the safety of dasatinib, a second-generation tyrosine kinase inhibitor, in treatment of SSc-ILD and IPF (ClinicalTrials.gov NCT00764309). Adverse events have been reported in all participants, but efficacy data are pending. In addition, there are reports of spontaneous pulmonary arterial hypertension in patients treated with dasatinib (95). BIBF 1120 (nimotardenib) is a tyrosine kinase inhibitor acting on PDGF receptors-α and -β, VEGF receptors 1–3, and fibroblast growth factor receptors 1–3 (47). Since these pathways play critical roles in IPF and liver fibrosis (1, 42, 163), it is a compelling candidate. A phase II study showed an acceptable safety profile and potential clinical benefits of this drug in patients with IPF (114).

CTGF and integrins. A humanized anti-CTGF antibody, FG-3019, has been evaluated in several clinical trials for diabetes and kidney disease and is well tolerated in these studies. Phase II trials for IPF (ClinicalTrials.gov NCT01890265) and liver fibrosis (ClinicalTrials.gov NCT01217632) are pending. STX-100, a monoclonal antibody targeting αvβ3- and αvβ5-integrin, is also being evaluated in a phase II clinical trial for treatment of patients with IPF (ClinicalTrials.gov NCT01371305). Cilengitide is a cyclic RGD pentapeptide that inhibits αvβ3- and αvβ5-integrin, with less potency toward αvβ6-integrin (88), and is now in clinical trials to treat cancer (39), but, to our knowledge, no trials are underway in fibrosis.

Future Perspective: Targeting Rho GTPase Signaling and MRTF/SRF-Mediated Gene Transcription

Although individual receptor systems such as TGFβ and LPA are important to drive fibrosis, treatment of complex diseases such as SSc and IPF may demand a multifaceted approach. Here we discuss the targeting of a gene transcription mechanism (MRTF/SRF) downstream of Rho as an alternative target. The convergent role of Rho signaling in pathways downstream of LPA, ET-1, TGFβ, and integrins in fibrosis (Figs. 1 and 3) suggests that blocking this mechanism may provide greater efficacy. Despite the substantial body of work on Rho GTPases in cancer (117), its importance in fibrosis has only recently been recognized (4, 120). At first glance, the GTPase itself appears to be the most logical target; however, development of potent, selective inhibitors of small GTPases offers multiple challenges. The most successful attempts have used virtual screening against the structural interface between RhoA and its GEFs. This approach has identified Rhosin and Y16, small-molecule inhibitors that display high binding affin-
ity and cellular activity in cancer models (127, 128). These compounds have not been tested in fibrosis.

Instead of targeting the GTPase, most pharmaceutical development on this pathway has targeted the downstream effector kinase ROCK. ROCK is a ~160-kDa serine/threonine kinase that is regulated by phosphorylation and interaction with Rho GTPase. The two best-studied inhibitors, fasudil and Y-27632, bind the kinase enzymatic domain, essentially inhibiting ROCK activity, and have shown actions in vitro and in vivo, in primary human and cancer cells (77). Y-27632 treatment inhibits myofibroblast differentiation of primary human dermal fibroblasts from healthy volunteers. The elevation of CTGF expression is blocked by CCG-203971 blocks TGF-β-induced collagen expression (151). Further, CCG-1423 blocks TGF-β-induced SRF and SMA expression in dermal fibroblasts obtained from patients with diffuse SSc (4). In vivo treatment with fasudil prevented bleomycin-induced fibrosis (169). Interestingly, fasudil has been used in Japan to treat cerebral vasospasm for nearly 20 years and has a safe clinical profile (162). Fasudil is being tested in clinical trials for pulmonary hypertension (110) and Raynaud’s phenomenon (NCT00498615) associated with SSc. While ROCK inhibitors may have greater efficacy than blocking individual receptors, the critical gene-transcription signals downstream of Rho are only partially blocked by Y-27632 (32), perhaps because mDia or other mechanisms can bypass ROCK to induce actin stress fibers and nuclear translocation of MRTF.

Cholesterol-lowering statin drugs can indirectly inhibit ROCK signaling. Rho GTPases have a prenyl group lipid modification on their COOH terminus that is essential for membrane localization and activity (30). Statin drugs, such as atorvastatin, inhibit 3-hydroxy-3-methylglutaryl-CoA reductase, a critical enzyme in the formation of cholesterol in the liver. 3-Hydroxy-3-methylglutaryl-CoA reductase is responsible for the production of mevalonate, a molecular precursor in the cascade that produces cholesterol, as well as prenyl groups required for geranylation of RhoA (143). Treatment of cells with statins results in inhibition of ROCK signaling in multiple cell types (14, 143). Simvastatin modulates the profibrotic marker CTGF and blocks TGFβ-induced collagen expression (151). However, statin use enhances pulmonary fibrosis in smokers, and, in retrospective studies, statin use showed no benefit in patients with IPF (101, 155). It is unclear whether clinically used doses of statins actually reduce ROCK signaling. Also, pleiotropic actions of statins may be a complicating factor.

In our laboratory, we identified CCG-1423, the first small-molecule inhibitor of Rho/MRTF/SRF-regulated gene transcription, by use of a serum response element-luciferase reporter high-throughput screen. CCG-1423 was shown to inhibit LPA receptor-stimulated DNA synthesis, cell growth, cell survival, and Matrigel invasion in multiple cancer cell lines (32). CCG-1423, as well as other structurally related analogs, inhibits Rho/MRTF signaling downstream of Rho/ROCK and blocks MRTF nuclear accumulation, possibly through modulation of an intranuclear actin-binding protein, MICAL-2 (11, 31, 57, 82), although it was recently reported that CCG-1423 may directly inhibit MRTF (45). In pulmonary fibroblasts, CCG-1423 blocked TGFβ-induced SRF and α-SMA expression independent of SMAD-mediated signaling (120). In a chlorhexidine model of peritoneal fibrosis, CCG-1423 reduced collagen synthesis, CTGF expression, and fibrosis in vivo (118). A new analog from this compound series, CCG-203971, blocked TGFβ and stiff-matrix-induced intestinal fibrosis in human colonic fibroblasts (58). As shown in Fig. 4, CCG-203971 blocks the TGFβ-induced myofibroblast transformation of dermal fibroblasts from healthy volunteers. The elevated α-SMA mRNA and protein expression in dermal fibroblasts obtained from patients with diffuse SSc was blocked by CCG-203971, as is mRNA expression of CTGF (40). Furthermore, CCG-203971 also prevented bleomycin-induced fibrosis in vivo, as assessed by skin thickness and hydroxyproline content. Taken together, these data suggest that further exploration of targeting of the Rho/MRTF/SRF transcriptional pathway is warranted as a potential new therapy for diseases of fibrosis.

Fig. 4. CCG-203971 modulates myofibroblast transition of dermal fibroblasts. A: primary human dermal fibroblasts from normal donors were treated with or without 10 ng/ml TGFβ for 3 days to induce a myofibroblast transition; during stimulation, cells were also treated with 10 μM CCG-203971 or DMSO. CCG-203971 markedly decreased α-SMA levels induced by TGFβ. Two representative individual samples are shown. B: elevated α-SMA expression in dermal fibroblasts from patients with diffuse systemic sclerosis (SSc) was blocked by CCG-203971. Two representative individual samples are shown. DAPI, 4',6-diamidino-2-phenylindole. [From Haak et al. (40). Adapted with permission from the American Society for Pharmacology and Experimental Therapeutics.]
Summary

Fibrosis is a complex process that involves a confluence of inflammatory signals and intrinsic mechanisms of fibroblast differentiation. Signal transduction plays key roles and suggests many novel therapeutic targets. Here, we emphasize cellular and molecular mechanisms that control intrinsic fibroblast differentiation and ECM production. We propose that MRTF/SRF-regulated gene transcription is a key genetic switch in this process, and we outline progress toward targeting this mechanism as a novel therapeutic approach.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

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