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COMMENTARY

ROCK and Rho

Promising Therapeutic Targets to Ameliorate Pulmonary Fibrosis

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Idiopathic pulmonary fibrosis (IPF) is a progressive and usually fatal fibrotic disease of the distal lung alveolar gas exchange units.[1] IPF is typically diagnosed in older individuals, after the disease is well established. IPF patients experience progressive shortness of breath, reduced quality of life, and a median survival of only 2 to 3 years after diagnosis.[2] The disease burden is significant considering that the prevalence of disease in the United States is estimated to be between 14 and 42.7 cases per 100,000 individuals[3] and rising.[4] Until the recent US Food and Drug Administration approval of pirfenidone and nintedanib, there were no effective therapies.[5,6] However, despite these recent breakthroughs, the outlook for IPF patients remains bleak, and there continues to be an urgent need for new therapies to impede disease progression and, more desirably, reverse established fibrosis.

IPF is thought to develop in response to extensive and repetitive injury to the alveolar epithelial cells, resulting in apoptosis and the development of an abnormal repair response.[7] As is typical of wound repair in other sites, such as the skin, resident fibroblasts and myofibroblast progenitors proliferate and migrate to the injured lung parenchyma. In the transforming growth factor (TGF)-β–rich profibrotic microenvironment of the alveoli and interstitium, fibroblasts and myofibroblast progenitors express contractile proteins, such as α smooth muscle actin (α-SMA), ramp up production of extracellular matrix (ECM) proteins,[8,9] and acquire a canonical myofibroblast phenotype. However, unlike normal resolving wound repair, in which myofibroblasts contract the newly synthesized provisional matrix, undergo apoptosis, and are cleared from the wound once repair is complete, lung myofibroblasts develop resistance to apoptosis induction, continue to produce excessive amounts of fibrotic ECM, and maintain their contractile properties in the lungs of IPF patients.[10] Thus, a full understanding of the molecular mechanisms that control fibroblast–myofibroblast differentiation and their acquired resistance to apoptosis is essential to develop therapeutic approaches to reduce lung myofibroblast numbers, excessive ECM production, and tissue contraction in IPF patients.

Activation of Myofibroblasts in Fibrosis and the Role of ROCK Signaling

Studies reported by Sisson et al[11] in this issue of *The American Journal of Pathology* expand current insights into the mechanisms that control fibroblast–myofibroblast differentiation and their resistance to apoptosis induction in IPF. As illustrated in Figure 1, fibroblast–myofibroblast differentiation can be initiated by a cadre of quintessential

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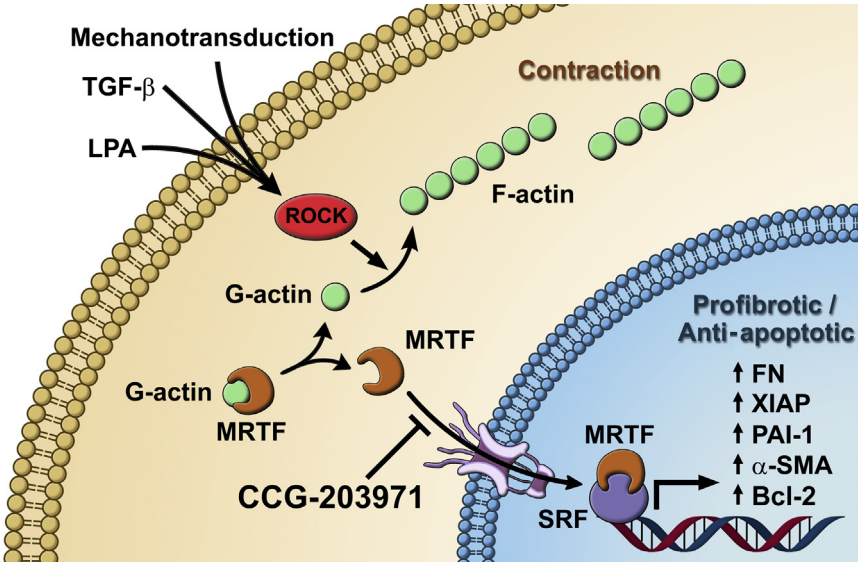

Figure 1 Fibroblast—myofibroblast differentiation is initiated by environmental factors, such as transforming growth factor (TGF)-β, lysophosphatidic acid (LPA), and mechanotransduction, and is mediated via the Rho-ROCK—myocardin-related transcription factor (MRTF) signaling pathway. These initial signaling events result in the polymerization of G-actin to F-actin and the formation of contractile fibers, a characteristic of myofibroblasts. Polymerization of F-actin frees the transcription factor MRTF, normally sequestered in the cytoplasm by association with G-actin, to translocate to the nucleus, initiating transcription of several profibrotic and antiapoptotic genes. CCG-203971 exerts its antifibrotic effect by inhibiting MRTF nuclear translocation, thereby preventing the up-regulation of gene expression. α-SMA, α smooth muscle actin; Bcl-2, B cell lymphoma 2; FN, fibronectin; PAI-1, plasminogen activator inhibitor-1; SRF, serum response factor; XIAP, X-linked inhibitor of apotosis.

profibrotic molecules, including TGF-β, lysophosphatidic acid, thrombin, plasminogen activator inhibitor-1, and mechanotransductive sensing of the increasing stiffness of fibrotic ECM.[12,13] Many of these molecules initiate fibroblast—myofibroblast differentiation by stimulating GTP loading of Rho family GTPases, especially RhoA, which activates the downstream Rho-associated coiled-coil-containing protein kinases (ROCK)-1 and -2.[14] These homologous serine/threonine kinases phosphorylate multiple downstream substrates, including myosin light-chain phosphatase, that permit myosin light-chain phosphorylation, facilitating the polymerization of globular G-actin into filamentous F-actin and initiating the assembly of the actomyosin contractile machinery.

Myocardin-related transcription factors (MRTFs) A and B (alias megakaryoblastic leukemia factors 1 and 2) are transcriptional coactivators that, when constitutively bound to G-actin, remain sequestered in the cytoplasm and transcriptionally inactive. During ROCK-1/2—dependent F-actin assembly, MRTFs dissociate from G-actin, translocate to the nucleus, and coactivate serum response factor—mediated transcription of fibronectin, procollagen 1, and other ECM genes.[15] Nuclear translocation of MRTF-A also promotes the expression of α-SMA and anti-apoptotic proteins B cell lymphoma 2 (Bcl-2) and X-linked inhibitor of apotosis (XIAP), thereby enhancing contractile potential and promoting myofibroblast resistance to apoptosis.[16] *In vivo* studies in a mouse model of pulmonary fibrosis have validated the importance of these collective findings by showing that the development of pulmonary fibrosis is impaired in mice that have been prophylactically treated with the ROCK inhibitors Y27632 or fasudil before bleomycin instillation.[16–18] The development of pulmonary fibrosis is also impaired in MRTF-deficient mice.[16] Thus, components of the Rho-ROCK-MRTF signaling pathway are legitimate therapeutic targets that, when blocked, inhibit the development of pulmonary fibrosis and accelerate its resolution.

In Vivo Blockade of MRTF-A Signaling Reduces Pulmonary Fibrosis

Sisson et al[11] build on the concept of therapeutically targeting the Rho-ROCK-MRTF signaling pathway to treat fibrotic lung disease. After its release from G-actin during ROCK-1/2—dependent F-actin assembly, MRTF-A is translocated from the cytoplasm into the nucleus through the activity of the importin nuclear translocator.[19] Sisson et al[11] hypothesized that reducing nuclear import of MRTF-A would impair the expression of several profibrotic and antiapoptotic genes that are involved in the development and maintenance of fibrosis. Using a high-throughput screen to discover candidate small molecule inhibitors of serum response factor/MRTF transcriptional activity, they identified CCG-203971. Their initial studies showed that CCG-203971 reduced TGF-β—induced MRTF-A nuclear translocation and suppressed TGF-β—induced expression of α-SMA and fibronectin in human fibroblast cell lines. These findings provided impetus to determine the effect of CCG-203971 in experimental models of pulmonary fibrosis in mice.

Intratracheal instillation of bleomycin has been widely used to study the mechanisms involved in the development of pulmonary fibrosis. The bleomycin model is characterized by an initial inflammatory acute lung injury peaking at day 7 and associated with extensive alveolar epithelial cell apoptosis. This phase is then replaced by a postinflammatory fibrotic phase, peaking at 14 to 21 days, and associated with fibroblast—myofibroblast differentiation and the expression of increased profibrotic gene signatures. Sisson et al[11] investigated the effect of CCG-203971 on the fibrotic phase by initiating treatment

with CCG-203971 at day 11. The treatment with CCG-203971 reduced the amount of lung fibrosis at day 21 and resulted in an improvement in lung architecture, indicating resolution of pre-existing fibrosis. They also tested CCG-203971 in a second fibrotic model involving targeted induction of apoptosis in alveolar type II cells that results in mild fibrosis lacking the characteristic inflammatory response of the bleomycin model. CCG-203971 reduced the fibrotic response in this alternative model as well, suggesting that the initial findings in bleomycin-instilled mice were not model specific.

To gain further mechanistic insight into the antifibrotic action of CCG-203971, the number of apoptotic myofibroblasts were quantified by evaluating terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL)/α-SMA double-positive cells in the bleomycin-induced fibrotic lesions. Consistent with the literature, the fibrotic lungs of bleomycin-instilled mice contained predictably few apoptotic myofibroblasts. However, inhibition of MRTF-A nuclear translocation in CCG-203971–treated fibrotic mice resulted in increased numbers of TUNEL/α-SMA–positive myofibroblasts, suggesting that the compound may also be promoting myofibroblast apoptosis. This result is similar to observations made after ROCK inhibition with fasudil,[16] suggesting a similar mechanism of action *in vivo* after the upstream blockade of the Rho-ROCK-MRTF pathway. Taken together, the *in vivo* studies reported by Sisson et al[11] suggest that inhibition of the nuclear import of the MRTF-A by CCG-203971 promotes the resolution of established pulmonary fibrosis, at least in part, by reducing the profibrotic phenotype of myofibroblasts and by promoting their apoptosis.

Rho-ROCK-MRTF Signaling during Myofibroblast Apoptosis

Although little is known about the receptors, ligands, and other processes that promote fibroblast apoptosis during the resolution of wound repair *in vivo*, there are strong implications that the death receptor Fas may be involved. Mice with inactivating mutations in FasL have been observed to develop a mild fibroproliferative interstitial pneumonia that is similar to some forms of rheumatoid arthritis–associated interstitial lung disease.[20] Furthermore, lung fibroblasts isolated from the fibrotic lungs of bleomycin-induced wild-type mice and primary cultures of lung fibroblasts from IPF patients have uniformly been found to be resistant to Fas-induced apoptosis.[21,22] With these findings in mind, Sisson et al[11] next investigated the effect of CCG-203971 on Fas-induced lung fibroblast apoptosis. The authors had previously shown that fibroblasts incubated in the presence of TGF-β were protected from Fas-induced apoptosis through a mechanism that involved increased expression of the broad-acting caspase-inhibitor protein, XIAP.[23] In their current studies, Sisson et al[11] found that while treatment with CCG-203971 alone had no effect on lung fibroblast apoptosis, it sensitized the cells to Fas-induced apoptosis even in the presence of TGF-β. The ability of CCG-203971 to sensitize fibroblasts to Fas-induced apoptosis was similar in normal fibroblasts and IPF myofibroblasts. Thus, these *in vitro* studies identify a potential mechanism to account for the increased number of apoptotic myofibroblasts in the previously fibrotic lungs of CCG-203971–treated mice.

In the setting of IPF, the data reported by Sisson et al[11] reveal important new insights into the mechanisms controlling lung myofibroblast function and sensitivity to apoptosis. Inevitably, however, they also raise additional questions. For example, Sisson et al[11] found that blocking MRTF nuclear translocation with CCG-203971 sensitized myofibroblasts to apoptosis by reducing cytoplasmic levels of the intrinsic pan-caspase inhibitor, XIAP. In contrast, Zhou et al[16] found that the mechanism of apoptosis induced by the ROCK inhibitor fasudil involved down-regulation of the antiapoptotic protein Bcl-2 in a Fas-independent manner. Golan-Gerstl et al[22] suggested that lung myofibroblast resistance to apoptosis is mediated by an additional antiapoptotic mechanism involving increased c-FLIP expression. Although it is conceivable that multiple antiapoptotic mechanisms may contribute to the resistance of fibrotic lung myofibroblast to apoptosis, a comprehensive analysis of these mechanisms remains to be conducted. An additional issue that deserves attention is the role of the death receptor Fas in myofibroblast apoptosis. *In vitro* studies, including the study by Sisson et al,[11] have clearly shown that Fas ligation plays an important role in promoting the apoptosis of appropriately sensitized primary lung myofibroblasts. However, little is known about the physiological mechanisms that promote myofibroblast apoptosis *in vivo* or the role of Fas ligand-dependent Fas ligation in this process. Indeed, although our understanding of the mechanisms involved in myofibroblast accumulation and persistence in IPF has greatly improved over the past decade, much remains to be determined about how these cells die and are removed in physiological repair processes and in the setting of fibrotic diseases of the lung and other organs.

Therapeutic Potential of the Rho-ROCK-MRTF Pathway

In summary, the findings reported in this issue of the *AJP* by Sisson et al[11] highlight the importance of the Rho-ROCK-MRTF signaling pathway in maintaining myofibroblast function in the setting of fibrotic lung disease. Importantly, they identify CCG-203971, a novel small molecule therapeutic inhibitor of MRTF-A nuclear translocation, and show that it blocks fibroblast-myofibroblast differentiation, sensitizes myofibroblasts to apoptosis, and accelerates the resolution of pulmonary fibrosis in mice, thus revealing an additional and innovative approach to therapeutically target

Rho-ROCK-MRTF signaling in IPF. Lastly, and of broader significance, their findings may be of considerable relevance to the control of fibrosis in other organs since MRTF-A has also been implicated in the development of cardiac and renal fibrosis.[24,25]

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