The Rho Kinases: Critical Mediators of Multiple Profibrotic Processes and Rational Targets for New Therapies for Pulmonary Fibrosis

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Abstract

Idiopathic pulmonary fibrosis (IPF) is characterized by progressive lung scarring, short median survival, and limited therapeutic options, creating great need for new pharmacologic therapies. IPF is thought to result from repetitive environmental injury to the lung epithelium, in the context of aberrant host wound healing responses. Tissue responses to injury fundamentally involve reorganization of the actin cytoskeleton of participating cells, including epithelial cells, fibroblasts, endothelial cells, and macrophages. Actin filament assembly and actomyosin contraction are directed by the Rho-associated coiled-coil forming protein kinase (ROCK) family of serine/threonine kinases (ROCK1 and ROCK2). As would therefore be expected, lung ROCK activation has been demonstrated in humans with IPF and in animal models of this disease. ROCK inhibitors can prevent fibrosis in these models, and more importantly, induce

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ROCK activation is required for multiple profibrotic responses, in the lung and multiple other organs, suggesting ROCK participation in fundamental pathways that contribute to the pathogenesis of a broad array of fibrotic diseases. Multiple lines of evidence therefore indicate that ROCK inhibition has great potential to be a powerful therapeutic tool in the treatment of fibrosis, both in the lung and beyond.

## I. Introduction

Idiopathic pulmonary fibrosis (IPF) is a devastating disease that causes breathlessness in affected persons, and has a short median survival of only approximately 3 years from the time of diagnosis (Raghu et al., 2011). Effective therapeutic options for this disease are limited, creating a large unmet need for new IPF treatment. In the current paradigm of IPF pathogenesis, fibrosis develops as a consequence of aberrant wound healing responses to repetitive lung injury (Selman et al., 2001; Thannickal and Horowitz, 2006). Wound healing responses to tissue injury, such as vascular leak, fibroblast recruitment, myofibroblast differentiation, and re-epithelialization, all fundamentally involve reorganization of the actin cytoskeleton of participating cells, including epithelial cells, fibroblasts, and endothelial cells. Actin filament assembly and actomyosin contraction are directed by the Rho-associated coiled-coil forming protein kinase (ROCK) family of serine/threonine kinases, including ROCK1 and ROCK2. These kinases are activated by Rho GTPases downstream of multiple ligand–receptor pairs that have been implicated in pulmonary fibrosis, including ligand–G protein–coupled receptor pairs, such as lysophosphatidic acid LPA–LPA1, sphingosine-1-phosphate S1P–S1P1, and thrombin–proteinase-activated receptor PAR1, and ligand–receptor tyrosine kinase (RTKs), such as transforming growth factor-β (TGF-β) and its receptors.

It is therefore not surprising that ROCK activation has been demonstrated in the lungs of humans with IPF and mice in models of this disease. ROCK activity assessed in situ is increased specifically in areas of the lungs developing fibrosis in mice and humans (Zhou et al., 2013) activated fibroblasts isolated from these lungs demonstrate increased ROCK activity compared with quiescent fibroblasts isolated from normal lungs. Activated fibroblasts are central effectors cells in pulmonary fibrosis, and ROCK signaling is required for their activation in response to both biochemical and biomechanical signals present in the fibrosis lung. ROCK signaling also appears to be involved in profibrotic responses of epithelial and endothelial cells to tissue injury. Their involvement in the profibrotic responses of multiple cell types suggests that the Rho kinases are focal points in pulmonary fibrosis, through which many upstream signals induce profibrotic downstream responses. ROCK inhibition may therefore be a particularly potent therapeutic strategy for pulmonary fibrosis. Pharmacologic ROCK inhibitors have been shown to prevent the development of pulmonary fibrosis in mice when administered prior to lung injury, and were more recently shown to reverse already established pulmonary fibrosis (Shimizu et al., 2001; Jiang et al., 2012; Bei et al., 2013; Zhou et al., 2013). ROCK inhibition may also be able to selectively target profibrotic cells and processes in involved tissues, without affecting normal cells and processes in uninvolved tissues; activated lung fibroblasts isolated from persons with IPF appear to be sensitive to ROCK inhibitor-induced apoptosis, whereas quiescent lung fibroblasts from persons without fibrosis are not (Zhou et al., 2013).

Here we review the studies that have demonstrated important roles from the Rho kinases in pulmonary fibrosis, as well as the studies demonstrating efficacy of available ROCK inhibitors in animal models of this disease.

## II. Rho-Associated Coiled-Coil Forming Protein Kinase Structure and Function

ROCKs are protein serine/threonine kinases that regulate cell shape and function by modulating the actin cytoskeleton. They share 40%–50% homology with other actin cytoskeleton kinases, such as myotonic dystrophy kinase, myotonic dystrophy–related cdc42-binding kinase, and citron kinase (Riento and Ridley, 2003). There are two known human ROCK isoforms,
ROCK1 and ROCK2, which have 65% overall sequence identity and 92% identity in their kinase domains. This homology is the same for the two mouse ROCK isoforms (Nakagawa et al., 1996; Liao et al., 2007). ROCK1 is ubiquitously expressed, whereas ROCK2 appears to be more selectively expressed in brain and muscle, particularly smooth muscle. Each isoform has a C-terminal RhoA-binding domain and an N-terminal kinase domain that fold over on each other in the inactive closed conformation (Fig. 1). The carboxy terminus consequently serves as an autoregulatory inhibitor. When activated, GTP-bound RhoA binds to the RhoA-binding domain, ROCK changes into an open conformation, freeing the amino terminus and exposing and activating the kinase domain (Fig. 1).

In response to activators of Rho, such as LPA or S1P, which stimulate RhoGEF and lead to the formation of active GTP-bound Rho, ROCKs mediate a broad range of cellular responses that involve cellular contraction and actin cytoskeleton remodeling. For example, they control assembly of the actin cytoskeleton and cell contractility by phosphorylating a variety of proteins, such as myosin light chain (MLC) phosphatase, LIM kinases, adducin, and ezrin-radixin-moesin (ERM) proteins. The consensus amino acid sequences for phosphorylation are R/KXS/T or R/KXXS/T (R, arginine; K, lysine; X, any amino acid; S, serine; T, threonine) (Kawano et al., 1999; Sumi et al., 2001). ROCKs can also be autophosphorylated, which might modulate their function (Leung et al., 1995; Ishizaki et al., 1996). Specifically, ROCKs phosphorylate Ser19 of MLC, the same residue that is phosphorylated by MLC kinase. Thus, ROCKs can alter the sensitivity of smooth muscle cell contraction to Ca^{2+} since MLC kinase is Ca^{2+}-sensitive (Amano et al., 1996). In addition, ROCKs regulate MLC phosphatase indirectly through the inhibition of myosin light chain phosphatase (MLCP) activity (Fig. 2). MLCP holoenzyme is composed of three subunits: a catalytic subunit (PP1), a myosin-binding subunit composed of a 58-kDa head and 32-kDa tail region, and a small noncatalytic subunit, M21. Depending upon the species, ROCKs phosphorylate the myosin-binding subunit at Thr^{697}, Ser^{854}, and Thr^{855} (Kawano et al., 1999). Phosphorylation of Thr^{697} or Thr^{855} attenuates MLCP activity (Feng et al., 1999) and, in some instances, the dissociation of MLCP from myosin (Velasco et al., 2002). ROCKs also phosphorylate ERM proteins, namely Thr^{567} of ezrin, Thr^{564} of radixin, and Thr^{558} of moesin (Matsui et al., 1998). ROCK-mediated phosphorylation leads to the disruption of the head-to-tail association of ERM proteins and actin cytoskeletal reorganization. In addition, ROCK1 phosphorylates LIM kinase-1 at Thr^{565} (Ohashi et al., 2000) and LIM kinase-2 at Thr^{565} (Sumi et al., 2001), which enhance the ability of LIM kinases to phosphorylate coflin (Maekawa et al., 1999). Since coflin is an actin-binding and -depolymerizing protein that regulates the turnover of actin filaments, the phosphorylation of LIM kinases by ROCKs inhibits coflin-mediated actin filament disassembly and leads to an increase in the number of actin filaments. A specific MLCP inhibitor, protein kinase C (PKC)–potentiated inhibitory protein of type 1 Ser/Thr phosphatase (CPI-17), was recently found to be expressed in arterial smooth muscle (Eto et al., 1997). The expression of CPI-17 is higher in arterial than gastrointestinal smooth muscle (Woodsome et al., 2001). A critical finding is that the
inhibitory activity of CPI-17 requires the phosphorylation at Thr38 (Eto et al., 1997). Originally, PKC was thought to be the kinase responsible for the phosphorylation of CPI-17 (Eto et al., 1997; Kitazawa et al., 1999). However, it was subsequently shown that other protein kinases such as ROCK (Koyama et al., 2000) and protein kinase N (Hamaguchi et al., 2000) can phosphorylate CPI-17 in vitro. However, the identity of the physiologic CPI-17 kinase has not been determined, but could be ROCK. Indeed, ROCK can phosphorylate CPI-17 in vitro and the kinetics of increase in CPI-17 phosphorylation correlates with MLC phosphorylation (Niir et al., 2003), suggesting that CPI-17 phosphorylation could regulate MLCP activity in vascular smooth muscle.

Despite substantial similarities in structure and molecular action, the two ROCK isoforms are not redundant, because global genetic deletion of either ROCK1 or ROCK2 leads to nonviability of most offspring. The causes of nonviability and phenotypes of the rare survivors differ between isoform deletions, further indicating that ROCK1 and ROCK2 have unique functions. Most ROCK1-deficient mice die soon after birth from omphalocele development caused by failure of umbilical ring closure due to impairment of filamentous actin assembly (Shimizu et al., 2005). By contrast, most ROCK2-deficient mice develop lethal intrauterine growth retardation from placental dysfunction caused by extensive placental thrombus formation (Thumkeo et al., 2003). The rare ROCK1-deficient mice that survive to adulthood appear to have normal development (Shimizu et al., 2005), whereas most of the rare ROCK2-deficient mice that survive after birth are runts.

ROCK1 and ROCK2 are ubiquitously expressed in mouse tissues from early embryonic development to adulthood (Noma et al., 2006). In particular, ROCK2 mRNA is highly expressed in cardiac muscle and vascular tissues, which indicates that the ROCK2 isoform may have a specialized role in these cell types. By contrast, ROCK1 is more abundantly expressed in immunologic cells and has been shown to colocalize to centrosomes (Chevrier et al., 2002). From gene knockdown or knockout studies, ROCK1 appears to be more important for mediating fibrosis, although ROCK2 is also involved (Rikitake et al., 2005b; Fu et al., 2006; Zhang et al., 2006; Kitamura et al., 2007; Shi et al., 2008). Furthermore, recent studies suggest an important role of serum response factor (SRF), myocardin, and Id-2/3 in Rho/ROCK-mediated actin polymerization and expression of smooth muscle differentiation marker genes, such as SM22α, calponin, and α-actin (Wang et al., 2004). Whether these are downstream effectors of ROCK1 or ROCK2 remains to be determined.

Pharmacologic inhibitors of ROCKs, such as Y27632 [(+)-(R)-trans-4-(1-aminoethyl)-N-(4-pyridyl)cyclohexane carboxamide] and fasudil/hydroxyfasudil (HA1077 [1-(5-isoquinolinylsulfonyl)homopiperazine hydrochloride]), which target their ATP-dependent kinase domains, inhibit ROCK1 and ROCK2 at equimolar concentrations (Rikitake et al., 2005a). Furthermore, at higher concentrations, Y27632 can also inhibit PKC-related kinase-2, protein kinase N, and citron kinase (Ishizaki et al., 2000), whereas fasudil can inhibit protein kinase A and PKC (Ikenoya et al., 2002). Therefore, functions that are ascribed to ROCKs using these ROCK inhibitors may be misleading because they are nonselective for ROCK and can nonspecifically inhibit other protein kinases. Whereas fasudil is relatively safe for human use since 1995 and has an indication for cerebral vasospasm in Japan, Y27632 was abandoned for use clinically because of toxicity.

III. Upstream Activators and Downstream Targets of Rho-Associated Coiled-Coil Forming Protein Kinases in Pulmonary Fibrosis

Profibrotic signals are delivered to cells after lung injury by both biochemical mediators and mechanical forces, and ROCK activation is central to many cellular responses to both types of signals. ROCK activation is induced by the increased mechanical forces that act on cells when extracellular matrix stiffness is pathologically increased in fibrotic tissues. ROCK activation is also induced by multiple biochemical mediators that are thought to be important in pulmonary fibrosis, including LPA, thrombin, and TGF-β. The profibrotic activities of these mediators appear to be due at least in part to their ability to activate ROCK.

A. Lysophosphatidic Acid

LPA signaling through two of its receptors, LPA1 and LPA2, is shown to be required for the development of pulmonary fibrosis in mouse models (Tager et al., 2008; Huang et al., 2013). We found that LPA levels are increased in the bronchoalveolar lavage (BAL) fluid acquired from persons with IPF compared with healthy controls. LPA levels were also increased in BAL of mice after intratracheal challenge with the chemotherapeutic agent bleomycin (Tager et al., 2008). Bleomycin-induced pulmonary fibrosis is the most widely studied mouse model of pulmonary fibrosis (Moore et al., 2013) and may also develop in humans treated with bleomycin for a variety of cancers. Mice genetically deficient for LPA1 or LPA2 are significantly protected from fibrosis and mortality in this model (Tager et al., 2008; Huang et al., 2013). We also found that LPA1 was highly expressed by fibroblasts recovered from BAL fluid of persons with IPF, and inhibition of LPA1 markedly reduced fibroblast responses to the chemotactic activity present in their BAL fluid (Tager et al., 2008), demonstrating the potential relevance of LPA1 to human pulmonary fibrosis. A small molecule
LPA₁-selective antagonist is currently being evaluated in IPF patients in a multicenter phase 2 clinical trial (ClinicalTrials.gov identifier NCT01766817).

In studies with LPA₁-deficient mice, we found that LPA₁ expression was required for epithelial apoptosis, loss of endothelial barrier function, and fibroblast migration and persistence induced in the bleomycin model of pulmonary fibrosis (Tager et al., 2008; Funke et al., 2012). ROCK activation, by regulating actin skeleton dynamics in epithelial cells, endothelial cells, and fibroblasts, may contribute to each of these profibrotic effects of LPA–LPA₁ signaling, as discussed in the section below on the potential contributions of ROCKs to profibrotic cellular responses to lung injury. In addition to these profibrotic cellular effects, the cytoskeletal effects of LPA-induced ROCK activation can potently induce profibrotic gene expression. ROCK regulation of actin cytoskeletal dynamics has been demonstrated to regulate gene expression by governing the subcellular localization of, and consequently the activity of, two sets of transcriptional coactivators: 1) myocardin-related transcription factor (MRTF)-A and MRTF-B, which bind to and augment the transcriptional activity of SRF (Olson and Nordheim, 2010); and 2) Yes-associated protein (YAP) and transcriptional coactivator with PDZ-binding motif (TAZ), which bind to and activate members of the TEA domain (TEAD) family of transcription factors (Yu et al., 2012).

Profibrotic target genes of the MRTF-A/B-SRF pathway include α-smooth muscle actin and connective tissue growth factor (CTGF). Working with peritoneal mesothelial cells, an important source of profibrotic mediators in the peritoneal fibrosis that can complicate peritoneal dialysis (Sakai et al., 2013), we found that CTGF expression is primarily driven through a pathway sequentially involving LPA₁, Gα₁₂/1₃-containing G proteins, RhoA, ROCKs, actin polymerization, MRTF-A and MRTF-B, and SRF (Fig. 3). In this pathway, LPA binding induces Gα₁₂/1₃-containing G proteins that are coupled to LPA₁ to sequentially activate RhoA and ROCKs, which in turn drives G-actin polymerization into F-actin. Whereas G-actin binds MRTF-A and MRTF-B and sequesters them in the cytoplasm. Actin polymerization into F-actin liberates G-actin–binding transcriptional coactivators, such as MRTF-A and MRTF-B. In this pathway, activation of G protein-coupled receptors, such as LPA₂, or matrix stiffening, induces ROCK activation, which, in turn, drives G-actin polymerization into F-actin. G-Actin binds MRTF-A and MRTF-B and sequesters them in the cytoplasm. Actin polymerization into F-actin liberates G-actin–bound MRTF-A and MRTF-B, allowing their translocation to the nucleus where they transactivate SRF-dependent transcription of important profibrotic genes containing serum response elements (SREs) in their promoters, such as CTGF.

Profibrotic target genes of the YAP/TAZ-TEAD pathway include CTGF and plasminogen activator inhibitor-1 (Zhao et al., 2008; Thomasy et al., 2013). The subcellular localization of YAP and TAZ is determined by their phosphorylation state. Phosphorylation of YAP and TAZ by Lats 1/2 kinases results in their sequestration in the cytoplasm due to binding by 14-3-3 regulatory proteins (Dong et al., 2007; Zhao et al., 2007; Hao et al., 2008); inhibition of Lats 1/2 kinases results in YAP/TAZ dephosphorylation and nuclear translocation (Dupont et al., 2011; Yu et al., 2012). In mouse embryonic fibroblasts, LPA has been shown to inhibit Lats 1/2 kinases, thus promoting YAP/TAZ dephosphorylation and nuclear translocation, through a signaling pathway that also involves Gα₁₂/1₃. RhoA, ROCK, and actin polymerization (Fig. 4) (Yu et al., 2012).

Fig. 3. ROCK activation of MRTF-SRF-directed profibrotic gene expression. ROCK activation has been shown to induce profibrotic gene expression through its ability to drive actin polymerization, which induces the nuclear translocation of G-actin–binding transcriptional coactivators, such as MRTF-A and MRTF-B. In this pathway, activation of G protein-coupled receptors, such as LPA₁, or matrix stiffening, induces ROCK activation, which, in turn, drives G-actin polymerization into F-actin. G-Actin binds MRTF-A and MRTF-B and sequesters them in the cytoplasm. Actin polymerization into F-actin liberates G-actin–bound MRTF-A and MRTF-B, allowing their translocation to the nucleus where they transactivate SRF-dependent transcription of important profibrotic genes containing serum response elements (SREs) in their promoters, such as CTGF.

Studies with LPA₂-deficient mice have revealed a requirement for this receptor as well as LPA₁ for epithelial apoptosis, loss of endothelial barrier function, and profibrotic gene expression induced in the bleomycin model of pulmonary fibrosis (Huang et al., 2013). LPA signaling specifically through LPA₂ has also been shown to induce TGF-β activation, by activating the αvβ₆ integrin expressed on epithelial
cells (Xu et al., 2009). For TGF-β to bind its receptors and exert its biologic effects, this cytokine must be extricated from latent complexes that it forms with its latency-associated peptide (LAP) and latent TGF-β binding proteins (Annes et al., 2003). The LAP contains an arginine-glycine-aspartate (RGD) motif, which allows the binding of several integrins, including all five αβ-containing integrins (αβ₁, αβ₃, αβ₅, αβ₆, and αβ₈) (Coward et al., 2010). When these integrins bind the LAP, their activation induces LAP conformational changes that release active TGF-β. In mouse models of pulmonary fibrosis induced by bleomycin or radiation, initial activation of TGF-β is determined by their phosphorylation state. Phosphorylation of YAP and TAZ by Lats 1/2 kinases results in their sequestration in the cytoplasm due to binding by 14-3-3 regulatory proteins; inhibition of Lats 1/2 kinases results in YAP/TAZ dephosphorylation and nuclear translocation. LPA activation of its G protein-coupled receptors has been shown to inhibit Lats 1/2 kinases, and promote YAP/TAZ dephosphorylation, as has matrix stiffening, through a signaling pathway that also involves ROCK activation and actin polymerization.

B. Thrombin

Extravascular coagulation is a hallmark of tissue injury, and intra-alveolar activation of the coagulation cascade is an important component of the fibrotic response to lung injury (Olman et al., 1995, 1996; Eitzman et al., 1996; Imokawa et al., 1997; Günther et al., 2003; Scotton et al., 2009). Activation of PARs by coagulation proteases such as thrombin and Factor Xa appears to critically link activation of the coagulation cascade and fibrosis. Mice genetically deficient specifically for PAR₁ are protected from bleomycin-induced pulmonary fibrosis (Howell et al., 2005). PAR₁ was required for induction of monocyte chemoattractant protein-1, CTGF, and TGF-β expression in this model (Howell et al., 2005), as well as for TGF-β activation induced by the αβ₆ integrin (Jenkins et al., 2006). Using nuclear phosphorylated Smad2 immunoreactivity as an in situ indicator of lung TGF-β activation, the increased immunoreactivity induced by bleomycin challenge of wild-type mice was significantly reduced in PAR₁-deficient mice. The PAR₁-activating peptide SFLLRN directly induces αβ₆-mediated TGF-β activation in vitro by αβ₆-expressing mouse lung epithelial cells, and by αβ₆-transduced mouse embryonic fibroblasts. PAR₁ activation of αβ₆ in these cells was also shown to be ROCK-dependent, as SFLLRN-induced TGF-β activation was reduced in a concentration-dependent manner by the ROCK inhibitor Y27632. Consistent with ROCK activation of αβ₆ being mediated by ROCK effects on the actin cytoskeleton, αβ₆-mediated TGF-β activity is completely abolished by inhibition of actin polymerization with cytochalasin D (Munger et al., 1999). ROCK inhibition did not affect Smad2 phosphorylation in mouse lung epithelial cells or embryonic fibroblasts induced by treatment with recombinant active TGF-β, indicating that ROCK is required for activation of TGF-β, but not for its canonical downstream Smad signaling. ROCK activation induced by TGF-β, however, in addition to Smad signaling, is critically required for TGF-β–induced myofibroblast differentiation, as described below.

C. Transforming Growth Factor-β

TGF-β is a major profibrotic cytokine in pulmonary fibrosis that drives fibroblast activation and differentiation into myofibroblasts, increasing the ability of these cells to produce and contract extracellular matrix (Sheppard, 2006). Delivery of an adenoviral vector producing active TGF-β1 to the rodent lung is sufficient to induce pulmonary myofibroblast accumulation and fibrosis (Kenyon et al., 2003). Conversely,
inhibiting TGF-β with neutralizing antibodies or a type I receptor inhibitor suppresses experimental pulmonary fibrosis (Giri et al., 1993; Bonniaud et al., 2005). As noted above, activation of endogenous latent TGF-β during the development of pulmonary fibrosis is initiated by activated \(\alpha_v\beta_6\) integrin, and activation of this integrin requires ROCK-dependent actin cytoskeletal reorganization. Once freed from its latent complexes, active TGF-β binds to TGF-β receptor type II, which phosphorylates and heterodimerizes with TGF-β receptor type I. In the canonical TGF-β signaling pathway, activated TGF-β receptor type I binds to TGF-βs which phosphorylates Smad2 and Smad3, which heterodimerize with Smad4 to form Smad2/Smad4 or Smad3/Smad4 complexes (Santibañez et al., 2011). These complexes translocate to the nucleus, where they bind to promoter Smad response elements to drive TGF-β-induced gene expression. Although Smad phosphorylation in TGF-β’s canonical signaling pathway occurs independently of ROCK, TGF-β induction of myofibroblast differentiation, its quintessential profibrotic activity, requires ROCK activation as well.

Based on a series of elegant studies by Sandbo et al. (2009, 2011), these investigators have proposed a tripartite model of myofibroblast differentiation in response to TGF-β that involves both Smad signaling and ROCK activation. In the first phase, canonical Smad signaling transcription downstream of TGF-β leads to the expression of intermediate signaling molecules that drive ROCK activation and actin polymerization into stress fibers. Consistent with requirements for Smad signaling, de novo protein synthesis, and ROCK activation, TGF-β–induced stress fiber formation is blocked by an inhibitor of TGF-β receptor kinase activity, which blocks TGF-β–induced phosphorylation of Smad2 and Smad3, an inhibitor of new protein synthesis, cycloheximide, and the ROCK inhibitor Y27632 (Sandbo et al., 2011). In the second phase, ROCK-induced actin polymerization drives nuclear translocation of MRTF-A, leading to SRF activation. Consistent with a requirement for the MRTF-A/B-SRF transcriptional pathway, TGF-β–induced myofibroblast differentiation is blocked by a pharmacologic inhibitor of this pathway, CCG-1423 (N-[2-[4(4-chlorophenyl)amino]-1-methyl-2-oxoethoxy]-3,5-bis trifluoromethyl)-benzamide), or by knockdown of SRF expression with RNA interference (Sandbo et al., 2011). In the third phase, SRF-dependent increases in the expression of MRTF-A and of SRF itself further drive myofibroblast differentiation (Sandbo et al., 2011).

D. Extracellular Matrix Stiffness

In addition to increased collagen accumulation, pathologic fibrosis is characterized by increased collagen cross-linking (Tschumperlin et al., 2013), which has been shown to increase the rigidity, or stiffness, of fibrotic tissues (Georges et al., 2007). Rather than simply being a consequence of tissue fibrosis, this increased tissue stiffness in turn amplifies myofibroblast differentiation and matrix production (Li et al., 2007; Wipff et al., 2007; Liu et al., 2010; Balestrini et al., 2012; Huang et al., 2012), creating a feed-forward loop that could by itself drive fibrosis progression (Liu et al., 2010). Myofibroblast differentiation driven by matrix stiffness is also ROCK-dependent (Huang et al., 2012). In response to matrix stiffening, normal human lung fibroblasts demonstrate increased production and activation of RhoA, increased ROCK activity, increased actin polymerization, MRTF-A nuclear translocation, and MRTF-A/B-SRF–dependent expression of proteins of the myofibroblast contractile program (Fig. 3). Consistent with stiffness-induced myofibroblast differentiation requiring ROCK and MRTF-A activation, this differentiation is abrogated by the ROCK inhibitor Y27632, and absent in mouse lung fibroblasts deficient for MRTF-A (Huang et al., 2012).

A similar feed-forward loop between matrix stiffness and fibroblast activation has been described in cancer-associated fibroblasts (CAFs), involving ROCK activation, and the YAP-TEAD transcriptional pathway (Calvo et al., 2013). Matrix stiffening enhances YAP activation in CAFs through a ROCK- and actomyosin-dependent pathway (Fig. 4), and YAP activation maintains the CAF phenotype, which includes the ability to promote matrix stiffening. Matrix stiffening during the development of pulmonary fibrosis would be expected to increase YAP activation in a ROCK-dependent manner in lung fibroblasts as well.

IV. Contributions of Rho-Associated Coiled-Coil Forming Protein Kinases to Profibrotic Cellular Responses to Lung Injury

According to the prevailing paradigm of IPF, fibrosis develops as a consequence of aberrant wound healing responses to repetitive lung injury. Tissue responses to injury involve coordinated activities of multiple cell types that, when appropriate in duration and magnitude, restore normal tissue structure and function. When dysregulated or overexuberant, however, these injury responses can result in progressive tissue fibrosis and loss of function. Lung injury in IPF appears to primarily target alveolar epithelial cells (AECs), and their death triggers wound healing responses including vascular leak and extravascular coagulation; innate immune activation; fibroblast recruitment, proliferation, and activation; extracellular matrix synthesis and cross-linking; and alveolar collapse and re-epithelialization (Fig. 5). The behaviors of the cells involved in these wound healing responses, particularly epithelial cells, endothelial cells and fibroblasts, are fundamentally regulated by ROCK signaling (Fig. 5). The critical role of the ROCKs in these responses further underscores the therapeutic potential of ROCK inhibition for pulmonary fibrosis.
A. Alveolar Epithelial Cells

The causes of AEC injury in IPF remain to be identified, but inhaled particulates, viral infections, or gastroesophageal reflux may contribute (Gross and Hunninghake, 2001; Selman et al., 2001; Hunninghake and Schwarz, 2007). Whatever the cause, this injury results in AEC death, and phenotypic alterations of surviving cells are thought to reflect a “reprogramming” that predisposes these cells to further injury and promotes abnormal repair (Selman and Pardo, 2006; Sakai and Tager, 2013; Blackwell et al., 2014). ROCK signaling can contribute to multiple profibrotic behaviors of epithelial cells after lung injury, including their increased susceptibility to apoptosis, their increased production of profibrotic mediators, and their impaired capacity to re-epithelialize denuded tissues. AECs appear to be particularly sensitive to apoptosis after lung injury in IPF (Kuwano et al., 1996; Günther et al., 2003; Plataki et al., 2005). As noted above, we have found that LPA signaling through LPA1, a prototypical pathway of ROCK activation, induces lung epithelial cell apoptosis in the bleomycin pulmonary fibrosis model, further suggesting a potential role for ROCK signaling in profibrotic AEC death after lung injury. In this study, we found that LPA–LPA1 signaling promoted lung epithelial detachment, which then promoted anoikis (i.e., the detachment-induced apoptosis of anchorage-dependent cells) (Frisch and Francis, 1994). Exposure of lung epithelial cells to hyperoxia and cyclic stretch, done to simulate injury to AECs when persons with respiratory failure are mechanically ventilated and experience ventilator-induced lung injury, similarly promoted epithelial cell detachment. Detachment in this model was prevented by ROCK inhibition (Wilhelm et al., 2014), further supporting a role for ROCK signaling in AEC anoikis induced by lung injury. A role for ROCK signaling in apoptotic pathways more broadly has been suggested by the finding that ROCK1 activation by caspase cleavage is responsible for plasma membrane blebbing in the execution phase of apoptosis (Coleman et al., 2001; Sebbagh et al., 2001).

Lung epithelial cells surviving after lung injury appear to be a major source of the mediators that drive fibroblast activation, and this profibrotic epithelial cell–fibroblast cross-talk appears to be centrally involved in the development of pulmonary fibrosis, as we previously reviewed (Sakai and Tager, 2013; Sakai et al., 2013). TGF-β and CTGF are two critical mediators of this cross-talk, and ROCK activation is required for the epithelial cell activation of the former, and epithelial cell expression of the latter.

Fig. 5. Role of ROCK activation in aberrant responses to lung injury implicated in IPF pathogenesis. This schematic indicates the sequential profibrotic processes implicated in the currently prevailing paradigm of IPF pathogenesis, in which recurrent or persistent injury to the alveolar epithelium is thought to drive aberrant wound healing responses, resulting in fibrosis rather than repair. (Figure was adapted from Ahluwalia et al., 2014, and inspired by Selman et al., 2001.) Proposed roles of ROCK activation in cells participating in IPF pathogenesis are placed in the context of the profibrotic process(es) they are thought to mediate.
Re-epithelialization is an essential part of repair that both reconstitutes normal tissue structure and signals for the cessation of the wound healing responses that drive fibrosis when left unchecked (Günther et al., 2003). This process requires the proliferation of epithelial precursors, which may be inhibited by ROCK signaling. ROCK signaling impairs the survival of embryonic or induced pluripotent stem cells (Watanabe et al., 2007; Koyanagi et al., 2008; Li et al., 2008; Gauthaman et al., 2010; Horani et al., 2013), and this effect appears to extend to epithelial cell progenitors in the lung as well. ROCK inhibition has been shown to enhance basal cell proliferation in cultured human tracheobronchial and mouse tracheal epithelial cells (Horani et al., 2013). ROCK signaling thus could impair postinjury re-epithelialization by impairing basal cell proliferation.

B. Macrophages

Danger-associated molecular patterns produced by tissue injury are well recognized to activate innate immune responses (Kaczorowski et al., 2008), and cells of this arm of the immune system such as macrophages have well established roles in wound healing (Stefater et al., 2011). Different classes of macrophages are now recognized, with different functions that would be expected to be profibrotic or antifibrotic in IPF. M1 and M2a-like macrophages can both secrete cytokines that promote fibrosis progression. In addition, reactive oxygen species produced by M1 macrophages can promote fibrosis by extending tissue injury, and arginase expressed by M2a-like macrophages promotes the production of hydroxyproline, enabling fibroblasts to increase collagen synthesis. By contrast, regulatory macrophages (Mreg/M2c-like macrophages) can promote the resolution of fibrosis through multiple mechanisms, including the production of suppressive cytokines. The course of IPF therefore may be strongly influenced by the prevailing macrophage phenotype(s) that infiltrate patients’ lungs in this disease (Murray and Wynn, 2011; Duffield et al., 2013; Lech and Anders, 2013).

Macrophage infiltration of injured tissues has been shown to be ROCK-dependent in mouse models of multiple fibrotic diseases, including renal tubulointerstitial fibrosis (Nagatoya et al., 2002; Satoh et al., 2002), diabetic nephropathy (Kikuchi et al., 2007), renal allograft rejection (Poosti et al., 2012), peritoneal fibrosis (Washida et al., 2011), atherosclerosis (Wu et al., 2009), and myocardial fibrosis (Ishimaru et al., 2007). In the bleomycin mouse models of pulmonary fibrosis, treatment with the ROCK inhibitor Y27632 reduced fibrosis and blunted bleomycin-induced increases in BAL macrophages (Shimizu et al., 2001). Y27632 also inhibited, in a concentration-dependent manner, the chemotaxis of MH-S mouse alveolar macrophages induced in vitro by lipopolysaccharide. We previously showed that ROCK1-deficient mouse macrophages have reduced migration to monocyte chemoattractant protein-1 (Wang et al., 2008), suggesting that ROCK activation is required for macrophage migration induced by multiple stimuli.

Innate immune activation induces adaptive immune responses (Iwasaki and Medzhitov, 2004), which also can have both profibrotic and antifibrotic roles in pulmonary fibrosis. T helper Th2- and Th17-type immunity cells have been shown to have profibrotic effects, whereas Th1-type immunity and regulatory T cells may have antifibrotic effects (Wynn and Ramalingam, 2012). Treatment with prednisone and azathioprine was recently demonstrated to worsen IPF outcomes (Raghu et al., 2012), but these drugs do not target adaptive immunity specifically. More specific targeting of the adaptive immune system, to selectively inhibit its profibrotic components, and/or augment its antifibrotic components, may lead to future beneficial therapies.

C. Endothelial Cells

Vascular permeability is characteristically increased in the early phase of repair after tissue injury (Dvorak, 1986). Alveolar-capillary permeability is increased in the lungs of persons with IPF, and the extent of this increase predicts disease progression and mortality (Mogulkoc et al., 2001; McKeown et al., 2009). This increased permeability allows plasma proteins to enter the airspaces, including coagulation factors. As noted above, activation of coagulation proteases such as thrombin in the airspaces promotes fibrosis, in a ROCK-dependent manner, by signaling through the PARs. Patients with IPF are also more likely to have a prothrombotic state than matched controls, and the presence of a prothrombotic state adversely affects survival (Navaratnam et al., 2014).

Endothelial barrier function is maintained by adherens and tight junctions that mediate endothelial cell–cell adhesions, and focal adhesions that tether endothelial cells to the extracellular matrix (Shen et al., 2009). These cell–cell and cell–matrix adhesions maintain the integrity of vascular endothelium by counteracting the actomyosin contractile tension of the endothelial cell cytoskeleton. Mediators of increased vascular permeability during wound repair increase cytoskeletal contractile tension and/or cause disorganization of interendothelial junction structures, resulting in intercellular gap formation and paracellular hyperpermeability (Shen et al., 2009). ROCK activation is centrally involved in increasing endothelial cell cytoskeletal contractile tension that leads to paracellular gap formation (Wojciak-Stothard and Ridley, 2002). Several of the mediators of ROCK activation that have been prominently implicated in IPF pathogenesis, including LPA and thrombin, induce vascular hyperpermeability by increasing ROCK-dependent actomyosin contractile tension in endothelial cells.
The increases in vascular permeability induced by LPA and thrombin, as well as those induced by other important edemagenic mediators such as vascular endothelial growth factor and tumor necrosis factor-α, are abrogated by ROCK inhibition (Carbajal et al., 2000; van Nieuw Amerongen et al., 2000a,b; Tasaka et al., 2005; Sun et al., 2006).

In addition to increased permeability, pulmonary endothelial cell ROCK activation may also contribute to the development of pulmonary hypertension, which is a common vascular complication of IPF that increases mortality in this disease (Farkas et al., 2011). In health, diffusion of nitric oxide (NO) produced by endothelial cells to vascular smooth muscle cells mediates vascular smooth muscle cell relaxation and vasodilatation. Reduced NO production by dysfunctional endothelial cells is implicated in the pathogenesis of pulmonary hypertension (Morrell et al., 2009). We have shown that ROCK activation by hypoxia or thrombin inhibits endothelial nitric oxide synthase (eNOS) expression and activity in endothelial cells in vitro (Takemoto et al., 2002), and that ROCK inhibition with fasudil or Y27632 in a middle cerebral artery occlusion mouse stroke model increased brain eNOS expression and activity, increased cerebral blood flow to both ischemic and nonischemic brain areas, and reduced cerebral infarct size and neurologic deficits (Rikitake et al., 2005a). ROCK inhibition therefore has the potential to treat pulmonary hypertension as well as pulmonary fibrosis in IPF patients.

**D. Fibroblasts and Myofibroblasts**

In wound healing, fibroblast recruitment, proliferation, and activation transform cellular, edematous “granulation tissue” into paucicellular scar tissue composed largely of dense collagen (Dvorak, 1986). Similar to macrophage migration as described above, fibroblast migration is dependent on ROCK activation: migration of mouse lung fibroblasts induced by platelet-derived growth factor in vitro was abrogated by the ROCK inhibitor Y27632 (Shimizu et al., 2001). Once fibroblasts enter sites of tissue injury, these cells are the principle source of collagens and other extracellular matrix components generated in pathologic fibrosis. Fibroblasts in both wound healing and fibrotic diseases characteristically differentiate into myofibroblasts (Scotton and Chambers, 2007), which are distinguished by their acquisition of contractile features of smooth muscle cells, such as the expression of α-smooth muscle actin (Abraham et al., 2007), and are marked by the ability to secrete increased levels of matrix components (Desmoulière et al., 2005). Targeting the pathways responsible for myofibroblast differentiation has great potential as a therapeutic strategy in IPF. As described above, myofibroblast differentiation in pulmonary fibrosis is driven by biochemical and biomechanical signals, typified by TGF-β and matrix stiffness, respectively. Since myofibroblast differentiation induced by TGF-β and matrix stiffness both require ROCK activation, ROCK inhibition has the potential to prevent myofibroblast differentiation induced by either chemical or mechanical stimuli. By inhibiting myofibroblast differentiation so broadly, ROCK inhibition has the potential to be an extremely potent therapeutic strategy to prevent fibrosis progression.

In normal wound healing, myofibroblasts are cleared through apoptosis (Desmoulière et al., 2005). Myofibroblasts in IPF resist apoptosis (Moodley et al., 2004), and their abnormal persistence contributes to IPF progression (Thannickal and Horowitz, 2006; Fattman, 2008). In addition to preventing fibrosis progression, however, therapeutic strategies targeting the pathways responsible for myofibroblast persistence would have the potential to induce regression of established fibrosis. Zhou et al. (2013) recently demonstrated that ROCK inhibition with fasudil is able to specifically induce apoptosis of myofibroblasts isolated from the lungs of persons with IPF, but not fibroblasts isolated from the lungs of control subjects without IPF. In an elegant series of experiments, these investigators found that IPF myofibroblast resistance to apoptosis is mediated by their overexpression of antiapoptotic gene B-cell CLL/lymphoma 2 (BCL-2), and that ROCK inhibition induces the apoptosis of these cells by reducing their Bcl-2 expression. Increased IPF myofibroblast Bcl-2 expression is driven by increased binding of MRTF-A-SRF complexes to the BCL-2 promoter. As described above, MRTF-A/B-SRF–directed transcription is induced by ROCK activation by both mechanical (e.g., matrix stiffening) and chemical (e.g., TGF-β) stimuli. Therapeutic ROCK inhibition by administration of fasudil to mice with already established fibrosis in the bleomycin model markedly reduced lung collagen content (Zhou et al., 2013), providing proof of concept that ROCK inhibitors could induce regression of established fibrosis by inducing myofibroblast fibrosis.

By targeting MRTF-A/B-SRF–induced Bcl-2 overexpression specifically in myofibroblasts, this mechanism of action of ROCK inhibitors would have no effect on fibroblasts in normal tissues, potentially providing such agents with specificity for fibrotic tissues.

**V. Rho-Associated Coiled-Coil Forming Protein Kinase Inhibitors and Their Efficacy in Animal Models of Pulmonary Fibrosis**

Pharmacologic ROCK inhibitors have previously been developed for treatment of cardiovascular and cerebrovascular diseases, because ROCK activation is thought to contribute to pathologic smooth muscle contraction in cerebral and coronary vasospasm as well as systemic and pulmonary hypertension (Liao et al., 2007). We previously showed that ROCK inhibition increases cerebral blood flow and decreases cerebral infarct size...
via upregulation of eNOS in a mouse stroke model (Rikitake et al., 2005a). Many of the studies of pharmacologic ROCK inhibition as a therapeutic strategy in animal models of human diseases, including pulmonary fibrosis, have used two small molecule inhibitors, fasudil and/or Y27632. Fasudil has been approved for prevention and treatment of cerebral vasospasm after surgery for subarachnoid hemorrhage in Japan and China since 1995 (Liao et al., 2007). Y27632 and fasudil both competitively inhibit the ROCK ATP-dependent kinase domain. Since the kinase domains of ROCK1 and ROCK2 are very highly homologous, these two inhibitors are both isoform nonselective. Although they have been widely used in research, both Y27632 and fasudil are relatively weak ROCK inhibitors (Davies et al., 2000). In addition to these specific ROCK inhibitors, 3-hydroxy-3-methylglutaryl-CoA reductase inhibitors, or statins, inhibit ROCK as well. Statins appear to have clinical benefits in atherosclerosis that are not dependent on their cholesterol-lowering effects. Experimental and clinical studies suggest that at least some of these benefits are mediated by upregulation of eNOS activity in response to statins’ inhibition of ROCK, as we previously reviewed (Rikitake and Liao, 2005).

Despite fasudil and Y27632 being relatively weak ROCK inhibitors, both have shown good efficacy in animal models of pulmonary fibrosis. Both inhibitors have been shown to significantly reduce the extent of pulmonary fibrosis that develops in the bleomycin mouse model of this disease when administered to mice in a “preventive” regimen at the time of bleomycin challenge (Shimizu et al., 2001; Jiang et al., 2012). In the studies with both of these inhibitors, reductions in fibrosis correlated with reductions in lung ROCK activity as measured by lung MLCP phosphorylation. The development of pulmonary hypertension, a common vascular complication of IPF that increases its mortality as noted above (Parkas et al., 2011), is recapitulated in the bleomycin model (Bei et al., 2013). Reduced endothelial NO production by ROCK inhibition of eNOS may contribute to the pathogenesis of pulmonary hypertension (Takemoto et al., 2002), as also noted above. In an additional study of fasudil administered in a preventive regimen in the bleomycin model, this ROCK inhibitor significantly reduced the increase in right ventricular systolic pressure, the right ventricular hypertrophy, and the pulmonary vascular remodeling produced in this model, in addition to significantly reducing the pulmonary fibrosis produced (Bei et al., 2013). More recently, fasudil administered in a “therapeutic” regimen, initiated 14 days after bleomycin challenge at a time when lung fibrosis has already been established, induced highly significant regression of fibrosis (Zhou et al., 2013). Mechanistically, ROCK inhibition with fasudil has been demonstrated to reduce expression of CTGF (Jiang et al., 2012), to reduce expression of TGF-β (Jiang et al., 2012) or its canonical SMAD signaling (Bei et al., 2013), to reduce myofibroblast differentiation (Jiang et al., 2012; Zhou et al., 2013) and to induce myofibroblast apoptosis, by inhibiting the MRTF-A–directed expression of the antiapoptotic protein BCL-2 that is induced by ROCK activation in these cells (Zhou et al., 2013).

### VI. Involvement of Rho-Associated Coiled-Coil Forming Protein Kinase Inhibitors in Fibrosis in Other Organs

ROCK activation has been implicated in the development of fibrosis in multiple organs in addition to the lungs, including the heart, liver, kidneys, peritoneum, and skin. We previously demonstrated that genetic haploinsufficiency of ROCK1 prevents the development of perivascular fibrosis in four different models of cardiac fibrosis, including fibrosis induced by angiotensin II infusion, N-nitro-L-arginine methylester treatment, transaortic constriction, or myocardial infarction (Rikitake et al., 2005b). The first two of these models, angiotensin II infusion and N-nitro-L-arginine methylester treatment, also induce cardiac hypertrophy, which interestingly was not affected by ROCK1 haploinsufficiency. Mechanistically, ROCK1 haploinsufficiency reduced CTGF and TGF-β expression in the cardiac fibrosis models we investigated (Rikitake et al., 2005b). In the liver, ROCK inhibition with fasudil has demonstrated antifibrotic efficacy in a rat model of type 2 diabetes–induced hepatic fibrosis, reducing TGF-β expression and fibroblast activation (Zhou et al., 2014), and Y27632 has demonstrated antifibrotic efficacy in a rat model of hepatic fibrosis induced by dimethylnitrosamine (Tada et al., 2001). In the kidney, fasudil and Y27632 have both been shown to attenuate tubulointerstitial fibrosis in rodent unilateral ureteral obstruction models (Nagatoya et al., 2002; Satoh et al., 2002), and fasudil has been shown to reduce renal fibrosis in a rat model of diabetic nephropathy as well (Komers et al., 2011). ROCK inhibition has also been shown to be effective in a rat model of peritoneal fibrosis, a common and potentially life-threatening complication of long-term peritoneal dialysis (Washida et al., 2011; Peng et al., 2013). In this model induced by intraperitoneal injections of chlorhexidine, Y27632 reduced peritoneal ROCK activity, fibrosis, and markers of angiogenesis (Washida et al., 2011), reducing expression of both TGF-β and vascular endothelial growth factor. Finally, ROCK activity has also been implicated in dermal fibrosis, in ex vivo experiments with fibroblasts isolated from the affected skin of persons with scleroderma. ROCK inhibition of these fibroblasts prevented TGF-β–induced myofibroblast differentiation and extracellular matrix production (Akhmetshina et al., 2008). The antifibrotic effects of ROCK genetic
targeting or pharmacologic inhibition in so many different models suggests that ROCK activation is centrally involved in the development of fibrosis in most organs.

VII. Potential Toxicities of Rho-Associated Coiled-Coil Forming Protein Kinase Inhibitors in Humans

Fasudil has been approved for human use in China and Japan since 1995, and has been generally well tolerated. It was shown to reduce cerebral vasospasm and subsequent ischemic injury in patients undergoing surgery for subarachnoid hemorrhage (Shibuya et al., 1992), and subsequently was approved for prevention or treatment of cerebral vasospasm in these patients. Fasudil’s ability to mediate cerebral vasodilation in this setting initially raised concerns that it might induce systemic hypotension, although clinical trials for various indications in humans have not borne out these concerns. Postmarketing surveillance data collected from 1426 persons who received Fasudil in Japan revealed that only 3.8% experienced adverse effects; hemorrhage was the most common, occurring in 1.7%, with hypotension observed in only one person (0.07%) (Suzuki et al., 2007). In a trial comparing fasudil with the calcium channel blocker nimodipine for treatment of cerebral vasospasm, fasudil was associated with significantly better clinical outcomes and no serious adverse events, including no episodes of hypotension requiring drug cessation (Zhao et al., 2011). A recent meta-analysis of the efficacy and safety of fasudil for the treatment of cerebral vasospasm in persons with subarachnoid hemorrhage found no increase in adverse events with fasudil use (Liu et al., 2012). Fasudil has also been evaluated in persons with stable angina. In two trials, fasudil was found to have beneficial effects on exercise tolerance, with no adverse effects on blood pressure or heart rate (Shimokawa et al., 2002; Vicari et al., 2005). In this population, fasudil appeared to induce vasodilation selectively in constricted coronary vessels, without having systemic vasodilator effects (Shimokawa et al., 2002). ROCK activity in persons with stable atherosclerosis has also been shown to be significantly reduced by treatment with statins, and ROCK inhibition by statins has also not been shown to induce hypotension (Nohria et al., 2009). In summary, ROCK inhibition in humans with fasudil, albeit a relatively weak ROCK inhibitor, or statins has been well tolerated, without adverse hemodynamic effects.

VIII. Conclusions

Patients with IPF are in desperate need of new and effective therapies to halt progression and reverse the relentless scarring in their lungs. ROCK activation is required for the development of fibrosis in animal models of lung fibrosis, as well as fibrosis of multiple other organs, suggesting involvement of these enzymes in pathogenetic pathways common to a broad array of fibrotic diseases. With respect to lung fibrosis in particular, targeting the ROCKs even with relatively weak inhibitors is effective in not only preventing the development of fibrosis, but also in reversing established fibrosis, a long-sought-after goal for IPF therapy. Since the inhibitors used in these studies have not been isoform selective, the relative contributions of ROCK1 versus ROCK2 to the development of fibrosis in the lungs and other organs are not known. Additional animal studies, either using isoform-selective inhibitors or taking a genetic approach to specifically delete ROCK1 or ROCK2, will be necessary to elucidate specific roles of ROCK1 and ROCK2 in the development of fibrosis. Such studies will be needed to determine whether an inhibitor for either ROCK1 or ROCK2 may be as effective for pulmonary fibrosis as a nonselective inhibitor, and may be better tolerated, even if substantially more potent than fasudil or Y27632. However, studies to date have already demonstrated that ROCK inhibition has great potential to be a powerful therapeutic tool in the treatment of pulmonary fibrosis in the future.

Authorship Contributions

Wrote or contributed to the writing of the manuscript: Knipe, Tager, Liao.

References


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