

ASSOCIATE EDITOR: YOSHIHIRO ISHIKAWA

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

Rachel S. Knipe, Andrew M. Tager, and James K. Liao

Pulmonary and Critical Care Unit and Center for Immunology and Inflammatory Diseases, Massachusetts General Hospital and Harvard Medical School, Boston, Massachusetts (R.S.K., A.M.T.); and Section of Cardiology, Department of Medicine, University of Chicago, Chicago, Illinois (J.K.L.)

| | |
|---|-----|
| Abstract | 103 |
| I. Introduction | 104 |
| II. Rho-Associated Coiled-Coil Forming Protein Kinase Structure and Function | 104 |
| III. Upstream Activators and Downstream Targets of Rho-Associated Coiled-Coil Forming Protein Kinases in Pulmonary Fibrosis | 106 |
| A. Lysophosphatidic Acid | 106 |
| B. Thrombin | 108 |
| C. Transforming Growth Factor- β | 108 |
| D. Extracellular Matrix Stiffness | 109 |
| IV. Contributions of Rho-Associated Coiled-Coil Forming Protein Kinases to Profibrotic Cellular Responses to Lung Injury | 109 |
| A. Alveolar Epithelial Cells | 110 |
| B. Macrophages | 111 |
| C. Endothelial Cells | 111 |
| D. Fibroblasts and Myofibroblasts | 112 |
| V. Rho-Associated Coiled-Coil Forming Protein Kinase Inhibitors and Their Efficacy in Animal Models of Pulmonary Fibrosis | 112 |
| VI. Involvement of Rho-Associated Coiled-Coil Forming Protein Kinase Inhibitors in Fibrosis in Other Organs | 113 |
| VII. Potential Toxicities of Rho-Associated Coiled-Coil Forming Protein Kinase Inhibitors in Humans | 114 |
| VIII. Conclusions | 114 |
| References | 114 |

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

This research was supported by the National Institutes of Health National Heart, Lung, and Blood Institute [Grants T32-HL116275 (to R.S.K.), R01-HL095732 and R01-HL108975 (to A.M.T.), and R01-HL052233 and R01-HL091933 (to J.K.L.)]; the National Institutes of Health National Institute of Diabetes and Digestive and Kidney Diseases [Grant R01-DK085006 (to J.K.L.)]; the National Institutes of Health National Institute of Neurological Disorders and Stroke [Grant R01-NS070001 (to J.K.L.)]; and the Nirenberg Center for Advanced Lung Disease and the Scleroderma Research Foundation [(to A.M.T.)].

Address correspondence to: Dr. James K. Liao, Section of Cardiology, Department of Medicine, University of Chicago, 5841 S. Maryland Ave. MC 6080, Chicago, IL 60637. E-mail: jliao@medicine.bsd.uchicago.edu
dx.doi.org/10.1124/pr.114.009381.

the regression of already established fibrosis. Here we review ROCK structure and function, upstream activators and downstream targets of ROCKs in pulmonary fibrosis, contributions of ROCKs to profibrotic cellular responses to lung injury, ROCK inhibitors and their efficacy in animal models of pulmonary fibrosis, and potential toxicities of ROCK inhibitors in humans, as well as involvement of ROCKs in fibrosis in other organs. As we discuss,

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 effector 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 cells 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,

ABBREVIATIONS: AEC, alveolar epithelial cell; BAL, bronchoalveolar lavage; CAF, cancer-associated fibroblast; CCG-1423, *N*-[2-[4(4-chlorophenyl)amino]-1-methyl-2-oxoethoxy]-3,5-bis(trifluoromethyl)-benzamide; CPI-17, PKC-potentiated inhibitory protein of type 1 Ser/Thr phosphatase; CTGF, connective tissue growth factor; eNOS, endothelial nitric oxide synthase; ERM, ezrin-radixin-moesin; H-1152, (*S*)-(+)-2-methyl-1-[(4-methyl-5-isoquinolyl)sulfonyl]-hexahydro-1*H*-1,4-diazepine dihydrochloride; HA1077, 1-(5-isoquinolylsulfonyl)homopiperazine hydrochloride; IPF, idiopathic pulmonary fibrosis; LAP, latency-associated peptide; LPA, lysophosphatidic acid; MLC, myosin light chain; MLCP, myosin light chain phosphatase; MRTF, myocardin-related transcription factor; NO, nitric oxide; PAR, proteinase-activated receptor; PKC, protein kinase C; ROCK, Rho-associated coiled-coil forming protein kinase; S1P, sphingosine-1-phosphate; SRF, serum response factor; TAZ, transcriptional coactivator with PDZ-binding domain; TEAD, TEA domain; TGF- β , transforming growth factor- β ; T_H, T helper; Y27632, (+)-(*R*)-trans-4-(1-aminoethyl)-*N*-(4-pyridyl)cyclohexane carboxamide; YAP, Yes-associated protein.

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 Ca²⁺ since MLC kinase is Ca²⁺-sensitive (Amano et al., 1996). In addition, ROCKs regulate MLC phosphorylation indirectly through the inhibition of myosin light chain phosphatase (MLCP) activity (Fig. 2). MLCP holoenzyme is composed of three subunits: a catalytic subunit (PP1 δ), 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 Thr⁶⁹⁷, Ser⁸⁵⁴, and Thr⁸⁵⁵ (Kawano et al., 1999). Phosphorylation of Thr⁶⁹⁷ or Thr⁸⁵⁵ 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 Thr⁵⁶⁷ of ezrin, Thr⁵⁶⁴ of radixin, and Thr⁵⁵⁸ 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 Thr⁵⁰⁸ (Ohashi et al., 2000) and LIM kinase-2 at Thr⁵⁰⁵ (Sumi et al., 2001), which enhance the ability of LIM kinases to phosphorylate cofilin (Maekawa et al., 1999). Since cofilin is an actin-binding and -depolymerizing protein that regulates the turnover of actin filaments, the phosphorylation of LIM kinases by ROCKs inhibits cofilin-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

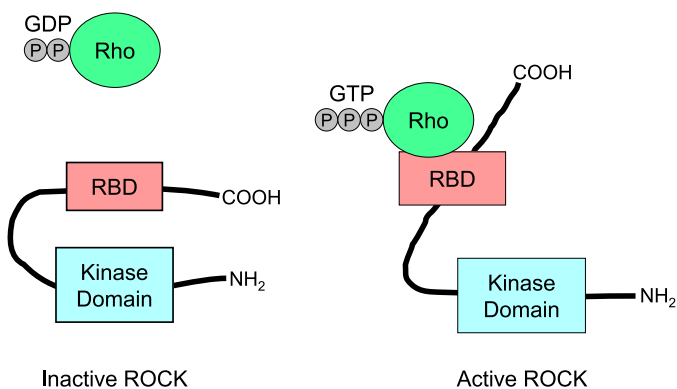


Fig. 1. Structure and activation of the two ROCK isoforms. In the closed inactive conformation, the RhoA-binding domains (RBDs) and the kinase domains of ROCK1 and ROCK2 fold over on each other (left). Activated GTP-bound RhoA is able to bind the RBDs, shifting the ROCKs to an open conformation (right), exposing and activating the kinase domains.

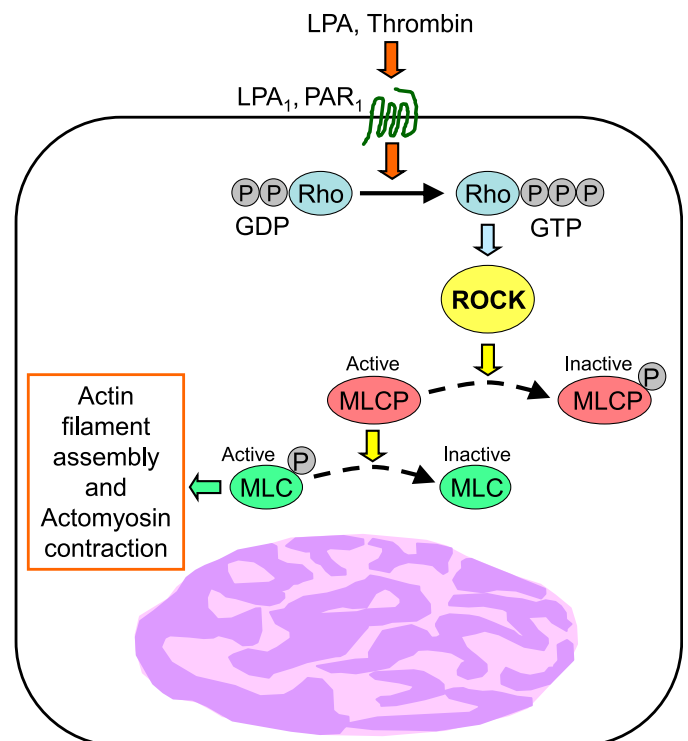


Fig. 2. ROCK control of cytoskeletal dynamics. ROCK isoforms are activated by GTP-bound RhoA (as in Fig. 1) downstream of G protein-coupled receptors, such as LPA₁ and PAR₁. Activated ROCKs phosphorylate MLC phosphatase, inhibiting its ability to dephosphorylate (and inactivate) MLC. Persistently phosphorylated active MLC is then able to induce stress fiber and focal adhesion formation, and cell contraction.

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 (Niino 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 knock-down 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 SM22a, 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-isoquinolinylnsulfonyl)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, G $\alpha_{12/13}$ -containing G proteins, RhoA, ROCKs, actin polymerization, MRTF-A and MRTF-B, and SRF (Fig. 3). In this pathway, LPA binding induces G $\alpha_{12/13}$ -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-bound MRTF-A and MRTF-B, allowing their translocation to the nucleus. Once in the nucleus, MRTF-A and MRTF-B transactivate SRF-dependent transcription of genes containing serum response elements in their promoters, including CTGF. In addition to peritoneal fibrosis (Sakai et al., 2013), CTGF has been hypothesized to play a central role in the development of pulmonary fibrosis (Lipson et al., 2012), and an anti-CTGF monoclonal antibody is currently being evaluated in IPF patients in a multicenter phase 2 clinical trial (ClinicalTrials.gov identifier NCT018902650). In the bleomycin model of pulmonary fibrosis, lung epithelial cells have been shown to be an important

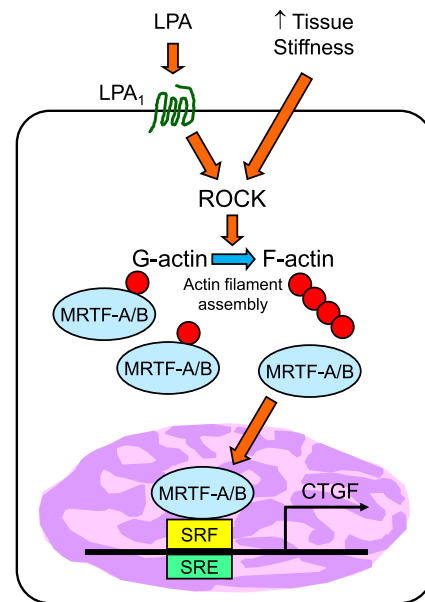


Fig. 3. ROCK activation of MRTF-SRF-directed profibrotic gene expression. ROCK activation has been shown to induce profibrotic gene expression through its ability to drive actin polymerization, which induces the nuclear translocation of G-actin-binding transcriptional coactivators, such as MRTF-A and 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.

source of CTGF, and CTGF expression by these cells has also been shown to be ROCK-dependent.

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 G $\alpha_{12/13}$, RhoA, ROCK, and actin polymerization (Fig. 4) (Yu et al., 2012).

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 $\alpha v\beta_6$ integrin expressed on epithelial

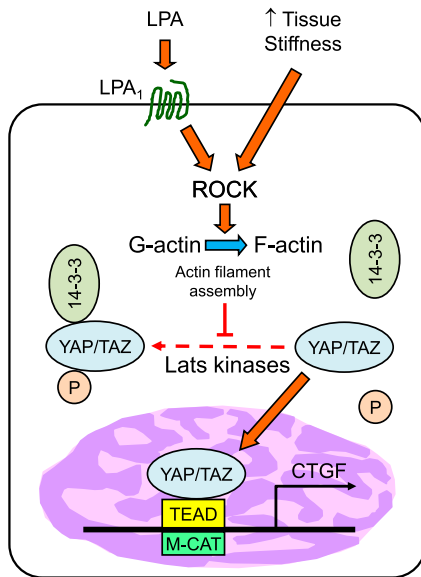


Fig. 4. ROCK activation of YAP/TAZ-TEAD-directed profibrotic gene expression. In addition to the MRTF-SRF pathway, ROCK has recently been shown to regulate gene expression through the downstream effectors of the Hippo pathway, the transcriptional coactivators YAP and TAZ. YAP and TAZ bind and activate the TEAD family of transcription factors, which directs the expression of genes that promote cell proliferation, including CTGF. 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; 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.

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 $\alpha\beta$ -containing integrins ($\alpha\nu\beta_1$, $\alpha\nu\beta_3$, $\alpha\nu\beta_5$, $\alpha\nu\beta_6$, and $\alpha\nu\beta_8$) (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 appears to be specifically $\alpha\nu\beta_6$ -dependent: Mice genetically deficient for the β_6 integrin subunit are completely protected from bleomycin-induced fibrosis (Munger et al., 1999), and mice treated with a blocking anti- $\alpha\nu\beta_6$ antibody are protected from radiation-induced fibrosis (Horan et al., 2008; Puthawala et al., 2008). An antibody targeting the $\alpha\nu\beta_6$ integrin is currently being evaluated in a phase 2 clinical trial in IPF (ClinicalTrials.gov identifier NCT01371305). Activation of $\alpha\nu\beta_6$ by LPA-LPA₂ signaling is ROCK-dependent, because $\alpha\nu\beta_6$ -mediated TGF- β activation was reduced in a concentration-dependent manner by the ROCK inhibitor H-1152 [(S)-(+)-2-methyl-1-[(4-methyl-5-

isoquinoliny]sulfonyl]-hexahydro-1H-1,4-diazepine dihydrochloride], as well as by the RhoA inhibitor botulinum toxin exoenzyme C3-transferase (Xu et al., 2009). These data indicate that TGF- β itself is a downstream target as well as an upstream mediator of ROCK activation.

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 $\alpha\nu\beta_6$ 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 $\alpha\nu\beta_6$ -mediated TGF- β activation in vitro by $\alpha\nu\beta_6$ -expressing mouse lung epithelial cells, and by $\alpha\nu\beta_6$ -transduced mouse embryonic fibroblasts. PAR₁ activation of $\alpha\nu\beta_6$ 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 $\alpha\nu\beta_6$ being mediated by ROCK effects on the actin cytoskeleton, $\alpha\nu\beta_6$ -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- β 1 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 $\alpha\nu\beta_6$ 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 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 triphasic 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.

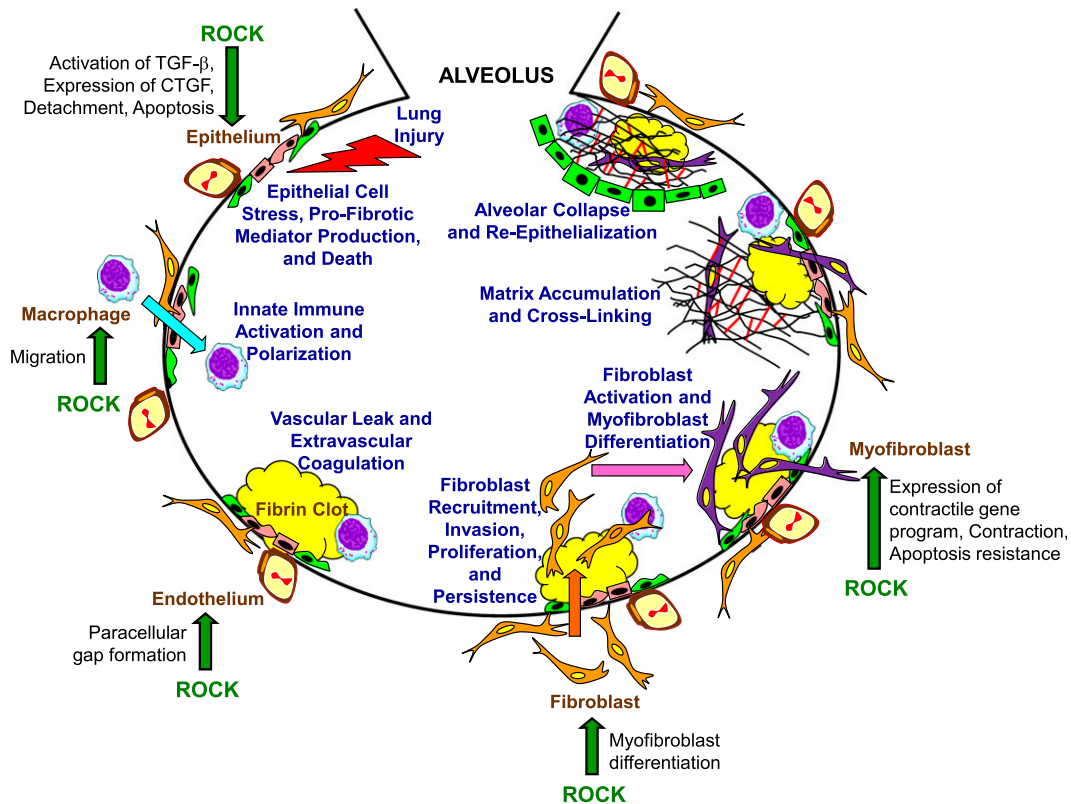


Fig. 5. Role of ROCK activation in aberrant responses to lung injury implicated in IPF pathogenesis. This schematic indicates the sequential profibrotic processes implicated in the currently prevailing paradigm of IPF pathogenesis, in which recurrent or persistent injury to the alveolar epithelium is 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 T_H2- and T_H17-type immunity cells have been shown to have profibrotic effects, whereas T_H1-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 apoptosis. 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 (Farkas 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

- Abraham DJ, Eckes B, Rajkumar V, and Krieg T (2007) New developments in fibroblast and myofibroblast biology: implications for fibrosis and scleroderma. *Curr Rheumatol Rep* **9**:136–143.
- Ahluwalia N, Shea BS, and Tager AM (2014) New therapeutic targets in idiopathic pulmonary fibrosis: aiming to rein in runaway wound healing responses. *Am J Respir Crit Care Med* **190**:867–878.
- Akhmetshina A, Dees C, Pilecky M, Szucs G, Spriewald BM, Zwerina J, Distler O, Schett G, and Distler JH (2008) Rho-associated kinases are crucial for myofibroblast differentiation and production of extracellular matrix in scleroderma fibroblasts. *Arthritis Rheum* **58**:2553–2564.
- Amano M, Ito M, Kimura K, Fukata Y, Chihara K, Nakano T, Matsuura Y, and Kaibuchi K (1996) Phosphorylation and activation of myosin by Rho-associated kinase (Rho-kinase). *J Biol Chem* **271**:20246–20249.
- Annes JP, Munger JS, and Rifkin DB (2003) Making sense of latent TGFbeta activation. *J Cell Sci* **116**:217–224.
- Balestrini JL, Chaudhry S, Sarrazy V, Koehler A, and Hinz B (2012) The mechanical memory of lung myofibroblasts. *Integr Biol (Camb)* **4**:410–421.
- Bei Y, Hua-Huy T, Duong-Quy S, Nguyen VH, Chen W, Nicco C, Batteux F, and Dinh-Xuan AT (2013) Long-term treatment with fasudil improves bleomycin-induced pulmonary fibrosis and pulmonary hypertension via inhibition of Smad2/3 phosphorylation. *Pulm Pharmacol Ther* **26**:635–643.
- Blackwell TS, Tager AM, Borok Z, Moore BB, Schwartz DA, Anstrom KJ, Bar-Joseph Z, Bitterman P, Blackburn MR, and Bradford W, et al. (2014) Future directions in idiopathic pulmonary fibrosis research. An NHLBI workshop report. *Am J Respir Crit Care Med* **189**:214–222.
- Bonnaud P, Margetts PJ, Kolb M, Schroeder JA, Kapoun AM, Damm D, Murphy A, Chakravarty S, Dugar S, and Higgins L, et al. (2005) Progressive transforming growth factor beta1-induced lung fibrosis is blocked by an orally active ALK5 kinase inhibitor. *Am J Respir Crit Care Med* **171**:889–898.
- Calvo F, Ege N, Grande-Garcia A, Hooper S, Jenkins RP, Chaudhry SI, Harrington K, Williamson P, Moeendarbary E, and Charras G, et al. (2013) Mechanotransduction and YAP-dependent matrix remodelling is required for the generation and maintenance of cancer-associated fibroblasts. *Nat Cell Biol* **15**:637–646.
- Carbajal JM, Gratrix ML, Yu CH, and Schaeffer RC Jr (2000) ROCK mediates thrombin's endothelial barrier dysfunction. *Am J Physiol Cell Physiol* **279**:C195–C204.
- Chevrier V, Piel M, Collomb N, Saoudi Y, Frank R, Paintrand M, Narumiya S, Bornens M, and Job D (2002) The Rho-associated protein kinase p160ROCK is required for centrosome positioning. *J Cell Biol* **157**:807–817.
- Coleman ML, Sahai EA, Yeo M, Bosch M, Dewar A, and Olson MF (2001) Membrane blebbing during apoptosis results from caspase-mediated activation of ROCK I. *Nat Cell Biol* **3**:339–345.

- Coward WR, Saini G, and Jenkins G (2010) The pathogenesis of idiopathic pulmonary fibrosis. *Ther Adv Respir Dis* 4:367–388.
- Davies SP, Reddy H, Caivano M, and Cohen P (2000) Specificity and mechanism of action of some commonly used protein kinase inhibitors. *Biochem J* 351:95–105.
- Desmoulière A, Chaponnier C, and Gabbiani G (2005) Tissue repair, contraction, and the myofibroblast. *Wound Repair Regen* 13:7–12.
- Dong J, Feldmann G, Huang J, Wu S, Zhang N, Comerford SA, Gayyed MF, Anders RA, Maitra A, and Pan D (2007) Elucidation of a universal size-control mechanism in *Drosophila* and mammals. *Cell* 130:1120–1133.
- Duffield JS, Lupher M, Thannickal VJ, and Wynn TA (2013) Host responses in tissue repair and fibrosis. *Annu Rev Pathol* 8:241–276.
- Dupont S, Morsut L, Aragona M, Enzo E, Giulitti S, Cordenonsi M, Zanconato F, Le Dıgabel J, Forcato M, and Bicciato S, et al. (2011) Role of YAP/TAZ in mechanotransduction. *Nature* 474:179–183.
- Dvorak HF (1986) Tumors: wounds that do not heal. Similarities between tumor stroma generation and wound healing. *N Engl J Med* 315:1650–1659.
- Eitzman DT, McCoy RD, Zheng X, Fay WP, Shen T, Ginsburg D, and Simon RH (1996) Bleomycin-induced pulmonary fibrosis in transgenic mice that either lack or overexpress the murine plasminogen activator inhibitor-1 gene. *J Clin Invest* 97:232–237.
- Eto M, Senba S, Morita F, and Yazawa M (1997) Molecular cloning of a novel phosphorylation-dependent inhibitory protein of protein phosphatase-1 (CPI17) in smooth muscle: its specific localization in smooth muscle. *FEBS Lett* 410:356–360.
- Farkas L, Gauldie J, Voelkel NF, and Kolb M (2011) Pulmonary hypertension and idiopathic pulmonary fibrosis: a tale of angiogenesis, apoptosis, and growth factors. *Am J Respir Cell Mol Biol* 45:1–15.
- Fattman CL (2008) Apoptosis in pulmonary fibrosis: too much or not enough? *Anti-oxid Redox Signal* 10:379–385.
- Feng J, Ito M, Ichikawa K, Isaka N, Nishikawa M, Hartshorne DJ, and Nakano T (1999) Inhibitory phosphorylation site for Rho-associated kinase on smooth muscle myosin phosphatase. *J Biol Chem* 274:37385–37390.
- Frisch SM and Francis H (1994) Disruption of epithelial cell-matrix interactions induces apoptosis. *J Cell Biol* 124:619–626.
- Fu P, Liu F, Su S, Wang W, Huang XR, Entman ML, Schwartz RJ, Wei L, and Lan HY (2006) Signaling mechanism of renal fibrosis in unilateral ureteral obstructive kidney disease in ROCK1 knockout mice. *J Am Soc Nephrol* 17:3105–3114.
- Funke M, Zhao Z, Xu Y, Chun J, and Tager AM (2012) The lysophosphatidic acid receptor LPA1 promotes epithelial cell apoptosis after lung injury. *Am J Respir Cell Mol Biol* 46:355–364.
- Gauthaman K, Fong CY, and Bongso A (2010) Effect of ROCK inhibitor Y-27632 on normal and variant human embryonic stem cells (hESCs) in vitro: its benefits in hESC expansion. *Stem Cell Rev* 6:86–95.
- Georges PC, Hui JJ, Gombos Z, McCormick ME, Wang AY, Uemura M, Mick R, Janney PA, Furth EE, and Wells RG (2007) Increased stiffness of the rat liver precedes matrix deposition: implications for fibrosis. *Am J Physiol Gastrointest Liver Physiol* 293:G1147–G1154.
- Giri SN, Hyde DM, and Hollinger MA (1993) Effect of antibody to transforming growth factor beta on bleomycin induced accumulation of lung collagen in mice. *Thorax* 48:959–966.
- Gross TJ and Hunninghake GW (2001) Idiopathic pulmonary fibrosis. *N Engl J Med* 345:517–525.
- Günther A, Lübke N, Ermert M, Schermuly RT, Weissmann N, Breithacker A, Markart P, Ruppert C, Quanz K, and Ermert L, et al. (2003) Prevention of bleomycin-induced lung fibrosis by aerosolization of heparin or urokinase in rabbits. *Am J Respir Crit Care Med* 168:1358–1365.
- Hamaguchi T, Ito M, Feng J, Seko T, Koyama M, Machida H, Takase K, Amano M, Kaibuchi K, and Hartshorne DJ, et al. (2000) Phosphorylation of CPI-17, an inhibitor of myosin phosphatase, by protein kinase N. *Biochem Biophys Res Commun* 274:825–830.
- Hao Y, Chun A, Cheung K, Rashidi B, and Yang X (2008) Tumor suppressor LATS1 is a negative regulator of oncogene YAP. *J Biol Chem* 283:5496–5509.
- Horan GS, Wood S, Ona V, Li DJ, Lukashew ME, Weinreb PH, Simon KJ, Hamm K, Allaire NE, and Rinaldi NJ, et al. (2008) Partial inhibition of integrin α (v) β 6 prevents pulmonary fibrosis without exacerbating inflammation. *Am J Respir Crit Care Med* 177:56–65.
- Horani A, Nath A, Wasserman MG, Huang T, and Brody SL (2013) Rho-associated protein kinase inhibition enhances airway epithelial Basal-cell proliferation and lentivirus transduction. *Am J Respir Cell Mol Biol* 49:341–347.
- Howell DC, Johns RH, Lasky JA, Shan B, Scotton CJ, Laurent GJ, and Chambers RC (2005) Absence of proteinase-activated receptor-1 signaling affords protection from bleomycin-induced lung inflammation and fibrosis. *Am J Pathol* 166:1353–1365.
- Huang LS, Fu P, Patel P, Harjith A, Sun T, Zhao Y, Garcia JG, Chun J, and Natarajan V (2013) Lysophosphatidic acid receptor-2 deficiency confers protection against bleomycin-induced lung injury and fibrosis in mice. *Am J Respir Cell Mol Biol* 49:912–922.
- Huang X, Yang N, Fiore VF, Barker TH, Sun Y, Morris SW, Ding Q, Thannickal VJ, and Zhou Y (2012) Matrix stiffness-induced myofibroblast differentiation is mediated by intrinsic mechanotransduction. *Am J Respir Cell Mol Biol* 47:340–348.
- Hunninghake GW and Schwarz MI (2007) Does current knowledge explain the pathogenesis of idiopathic pulmonary fibrosis? A perspective. *Proc Am Thorac Soc* 4:449–452.
- Ikenoya M, Hidaka H, Hosoya T, Suzuki M, Yamamoto N, and Sasaki Y (2002) Inhibition of rho-kinase-induced myristoylated alanine-rich C kinase substrate (MARCKS) phosphorylation in human neuronal cells by H-1152, a novel and specific Rho-kinase inhibitor. *J Neurochem* 81:9–16.
- Imokawa S, Sato A, Hayakawa H, Kotani M, Urano T, and Takada A (1997) Tissue factor expression and fibrin deposition in the lungs of patients with idiopathic pulmonary fibrosis and systemic sclerosis. *Am J Respir Crit Care Med* 156:631–636.
- Ishimaru K, Ueno H, Kagitani S, Takabayashi D, Takata M, and Inoue H (2007) Fasudil attenuates myocardial fibrosis in association with inhibition of monocyte/macrophage infiltration in the heart of DOCA/salt hypertensive rats. *J Cardiovasc Pharmacol* 50:187–194.
- Ishizaki T, Maekawa M, Fujisawa K, Okawa K, Iwamatsu A, Fujita A, Watanabe N, Saito Y, Kakizuka A, and Morii N, et al. (1996) The small GTP-binding protein Rho binds to and activates a 160 kDa Ser/Thr protein kinase homologous to myotonic dystrophy kinase. *EMBO J* 15:1885–1893.
- Ishizaki T, Uehata M, Tamechika I, Keel J, Nonomura K, Maekawa M, and Narumiya S (2000) Pharmacological properties of Y-27632, a specific inhibitor of rho-associated kinases. *Mol Pharmacol* 57:976–983.
- Iwasaki A and Medzhitov R (2004) Toll-like receptor control of the adaptive immune responses. *Nat Immunol* 5:987–995.
- Jenkins RG, Su X, Su G, Scotton CJ, Camerer E, Laurent GJ, Davis GE, Chambers RC, Matthay MA, and Sheppard D (2006) Ligation of protease-activated receptor 1 enhances α (v) β 6 integrin-dependent TGF- β activation and promotes acute lung injury. *J Clin Invest* 116:1606–1614.
- Jiang C, Huang H, Liu J, Wang Y, Lu Z, and Xu Z (2012) Fasudil, a rho-kinase inhibitor, attenuates bleomycin-induced pulmonary fibrosis in mice. *Int J Mol Sci* 13:8293–8307.
- Kaczorowski DJ, Mollen KP, Edmonds R, and Billiar TR (2008) Early events in the recognition of danger signals after tissue injury. *J Leukoc Biol* 83:546–552.
- Kawano Y, Fukata Y, Oshiro N, Amano M, Nakamura T, Ito M, Matsumura F, Inagaki M, and Kaibuchi K (1999) Phosphorylation of myosin-binding subunit (MBS) of myosin phosphatase by Rho-kinase in vivo. *J Cell Biol* 147:1023–1038.
- Kenyon NJ, Ward RW, McGrew G, and Last JA (2003) TGF- β 1 causes airway fibrosis and increased collagen I and III mRNA in mice. *Thorax* 58:772–777.
- Kikuchi Y, Yamada M, Imakiire T, Kushiya T, Higashi K, Hyodo N, Yamamoto K, Oda T, Suzuki S, and Miura S (2007) A Rho-kinase inhibitor, fasudil, prevents development of diabetes and nephropathy in insulin-resistant diabetic rats. *J Endocrinol* 192:595–603.
- Kitamura K, Tada S, Nakamoto N, Toda K, Horikawa H, Kurita S, Tsunematsu S, Kumagai N, Ishii H, and Saito H, et al. (2007) Rho/Rho kinase is a key enzyme system involved in the angiotensin II signaling pathway of liver fibrosis and steatosis. *J Gastroenterol Hepatol* 22:2022–2033.
- Kitazawa T, Takizawa N, Ikebe M, and Eto M (1999) Reconstitution of protein kinase C-induced contractile Ca²⁺ sensitization in triton X-100-demembrated rabbit arterial smooth muscle. *J Physiol* 520:139–152.
- Komers R, Oyama TT, Beard DR, Tikellis C, Xu B, Lotspeich DF, and Anderson S (2011) Rho kinase inhibition protects kidneys from diabetic nephropathy without reducing blood pressure. *Kidney Int* 79:432–442.
- Koyama M, Ito M, Feng J, Seko T, Shiraki K, Takase K, Hartshorne DJ, and Nakano T (2000) Phosphorylation of CPI-17, an inhibitory phosphoprotein of smooth muscle myosin phosphatase, by Rho-kinase. *FEBS Lett* 475:197–200.
- Koyanagi M, Takahashi J, Arakawa Y, Doi D, Fukuda H, Hayashi H, Narumiya S, and Hashimoto N (2008) Inhibition of the Rho/ROCK pathway reduces apoptosis during transplantation of embryonic stem cell-derived neural precursors. *J Neurosci Res* 86:270–280.
- Kuwano K, Kunitake R, Kawasaki M, Nomoto Y, Hagimoto N, Nakanishi Y, and Hara N (1996) P21Waf1/Cip1/Sd1 and p53 expression in association with DNA strand breaks in idiopathic pulmonary fibrosis. *Am J Respir Crit Care Med* 154:477–483.
- Lech M and Anders HJ (2013) Macrophages and fibrosis: How resident and infiltrating mononuclear phagocytes orchestrate all phases of tissue injury and repair. *Biochim Biophys Acta* 1832:989–997.
- Leung T, Manser E, Tan L, and Lim L (1995) A novel serine/threonine kinase binding the Ras-related RhoA GTPase which translocates the kinase to peripheral membranes. *J Biol Chem* 270:29051–29054.
- Li X, Meng G, Krawetz R, Liu S, and Rancourt DE (2008) The ROCK inhibitor Y-27632 enhances the survival rate of human embryonic stem cells following cryopreservation. *Stem Cells Dev* 17:1079–1085.
- Li Z, Dranoff JA, Chan EP, Uemura M, Sevigny J, and Wells RG (2007) Transforming growth factor-beta and substrate stiffness regulate portal fibroblast activation in culture. *Hepatology* 46:1246–1256.
- Liao JK, Seto M, and Noma K (2007) Rho kinase (ROCK) inhibitors. *J Cardiovasc Pharmacol* 50:17–24.
- Lipson KE, Wong C, Teng Y, and Spong S (2012) CTGF is a central mediator of tissue remodeling and fibrosis and its inhibition can reverse the process of fibrosis. *Fibrogenesis Tissue Repair* 5 (Suppl 1):S24.
- Liu F, Mih JD, Shea BS, Kho AT, Sharif AS, Tager AM, and Tschumperlin DJ (2010) Feedback amplification of fibrosis through matrix stiffening and COX-2 suppression. *J Cell Biol* 190:693–706.
- Liu GJ, Wang ZJ, Wang YF, Xu LL, Wang XL, Liu Y, Luo GJ, He GH, and Zeng YJ (2012) Systematic assessment and meta-analysis of the efficacy and safety of fasudil in the treatment of cerebral vasospasm in patients with subarachnoid hemorrhage. *Eur J Clin Pharmacol* 68:131–139.
- Maekawa M, Ishizaki T, Boku S, Watanabe N, Fujita A, Iwamatsu A, Obinata T, Ohashi K, Mizuno K, and Narumiya S (1999) Signaling from Rho to the actin cytoskeleton through protein kinases ROCK and LIM-kinase. *Science* 285:895–898.
- Matsui T, Maeda M, Doi Y, Yonemura S, Amano M, Kaibuchi K, Tsukita S, and Tsukita S (1998) Rho-kinase phosphorylates COOH-terminal threonines of ezrin/radixin/moesin (ERM) proteins and regulates their head-to-tail association. *J Cell Biol* 140:647–657.
- McKeown S, Richter AG, O’Kane C, McAuley DF, and Thickett DR (2009) MMP expression and abnormal lung permeability are important determinants of outcome in IPF. *Eur Respir J* 33:77–84.
- Mogulkoc N, Brutsche MH, Bishop PW, Murby B, Greaves MS, Horrocks AW, Wilson M, McCullough C, Prescott M, and Egan JJ; Greater Manchester Pulmonary Fibrosis Consortium (2001) Pulmonary (99m)Tc-DTPA aerosol clearance and survival in usual interstitial pneumonia (UIP). *Thorax* 56:916–923.

- Moodley YP, Caterina P, Scaffidi AK, Misso NL, Papadimitriou JM, McAnulty RJ, Laurent GJ, Thompson PJ, and Knight DA (2004) Comparison of the morphological and biochemical changes in normal human lung fibroblasts and fibroblasts derived from lungs of patients with idiopathic pulmonary fibrosis during FasL-induced apoptosis. *J Pathol* **202**:486–495.
- Moore BB, Lawson WE, Oury TD, Sisson TH, Raghavendran K, and Hogaboam CM (2013) Animal models of fibrotic lung disease. *Am J Respir Cell Mol Biol* **49**:167–179.
- Morrell NW, Adnot S, Archer SL, Dupuis J, Jones PL, MacLean MR, McMurtry IF, Stenmark KR, Thistlethwaite PA, and Weissmann N, et al. (2009) Cellular and molecular basis of pulmonary arterial hypertension. *J Am Coll Cardiol* **54**(1, Suppl) S20–S31.
- Munger JS, Huang X, Kawakatsu H, Griffiths MJ, Dalton SL, Wu J, Pittet JF, Kaminski N, Garat C, and Matthay MA, et al. (1999) The integrin alpha v beta 6 binds and activates latent TGF beta 1: a mechanism for regulating pulmonary inflammation and fibrosis. *Cell* **96**:319–328.
- Murray PJ and Wynn TA (2011) Protective and pathogenic functions of macrophage subsets. *Nat Rev Immunol* **11**:723–737.
- Nagatoya K, Moriyama T, Kawada N, Takeji M, Oseto S, Murozono T, Ando A, Imai E, and Hori M (2002) Y-27632 prevents tubulointerstitial fibrosis in mouse kidneys with unilateral ureteral obstruction. *Kidney Int* **61**:1684–1695.
- Nakagawa O, Fujisawa K, Ishizaki T, Saito Y, Nakao K, and Narumiya S (1996) ROCK-I and ROCK-II, two isoforms of Rho-associated coiled-coil forming protein serine/threonine kinase in mice. *FEBS Lett* **392**:189–193.
- Navaratnam V, Fogarty AW, McKeever T, Thompson N, Jenkins G, Johnson SR, Dolan G, Kumaran M, Pointon K, and Hubbard RB (2014) Presence of a prothrombotic state in people with idiopathic pulmonary fibrosis: a population-based case-control study. *Thorax* **69**:207–215.
- Niuro N, Koga Y, and Ikebe M (2003) Agonist-induced changes in the phosphorylation of the myosin-binding subunit of myosin light chain phosphatase and CPI17, two regulatory factors of myosin light chain phosphatase, in smooth muscle. *Biochem J* **369**:117–128.
- Nohria A, Prsic A, Liu PY, Okamoto R, Creager MA, Selwyn A, Liao JK, and Ganz P (2009) Statins inhibit Rho kinase activity in patients with atherosclerosis. *Atherosclerosis* **205**:517–521.
- Noma K, Oyama N, and Liao JK (2006) Physiological role of ROCKs in the cardiovascular system. *Am J Physiol Cell Physiol* **290**:C661–C668.
- Ohashi K, Nagata K, Maekawa M, Ishizaki T, Narumiya S, and Mizuno K (2000) Rho-associated kinase ROCK activates LIM-kinase 1 by phosphorylation at threonine 508 within the activation loop. *J Biol Chem* **275**:3577–3582.
- Olman MA, Mackman N, Gladson CL, Moser KM, and Loskutoff DJ (1995) Changes in procoagulant and fibrinolytic gene expression during bleomycin-induced lung injury in the mouse. *J Clin Invest* **96**:1621–1630.
- Olman MA, Simmons WL, Pollman DJ, Loftis AY, Bini A, Miller EJ, Fuller GM, and Rivera KE (1996) Polymerization of fibrinogen in murine bleomycin-induced lung injury. *Am J Physiol* **271**:L519–L526.
- Olson EN and Nordheim A (2010) Linking actin dynamics and gene transcription to drive cellular motile functions. *Nat Rev Mol Cell Biol* **11**:353–365.
- Peng W, Zhou Q, Ao X, Tang R, and Xiao Z (2013) Inhibition of Rho-kinase alleviates peritoneal fibrosis and angiogenesis in a rat model of peritoneal dialysis. *Ren Fail* **35**:958–966.
- Plataki M, Koutsopoulos AV, Darivaniaki K, Delides G, Siafakas NM, and Bouras D (2005) Expression of apoptotic and antiapoptotic markers in epithelial cells in idiopathic pulmonary fibrosis. *Chest* **127**:266–274.
- Poosti F, Yazdani S, Dolman ME, Kok RJ, Chen C, Ding G, Lacombe M, Prakash J, van den Born J, and Hillebrands JL, et al. (2012) Targeted inhibition of renal Rho kinase reduces macrophage infiltration and lymphangiogenesis in acute renal allograft rejection. *Eur J Pharmacol* **694**:111–119.
- Puthawala K, Hadjiangelis N, Jacoby SC, Bayongan E, Zhao Z, Yang Z, Devitt ML, Horan GS, Weinreb PH, and Lukashev ME, et al. (2008) Inhibition of integrin alpha(v)beta6, an activator of latent transforming growth factor-beta, prevents radiation-induced lung fibrosis. *Am J Respir Crit Care Med* **177**:82–90.
- Raghu G, Anstrom KJ, King TE Jr, Lasky JA, and Martinez FJ Idiopathic Pulmonary Fibrosis Clinical Research Network (2012) Prednisone, azathioprine, and N-acetylcysteine for pulmonary fibrosis. *N Engl J Med* **366**:1968–1977.
- Raghu G, Collard HR, Egan JJ, Martinez FJ, Behr J, Brown KK, Colby TV, Cordier JF, Flaherty KR, and Lasky JA, et al. ATS/ERS/JRS/ALAT Committee on Idiopathic Pulmonary Fibrosis (2011) An official ATS/ERS/JRS/ALAT statement: idiopathic pulmonary fibrosis: evidence-based guidelines for diagnosis and management. *Am J Respir Crit Care Med* **183**:788–824.
- Riento K and Ridley AJ (2003) Rocks: multifunctional kinases in cell behaviour. *Nat Rev Mol Cell Biol* **4**:446–456.
- Rikitake Y, Kim HH, Huang Z, Seto M, Yano K, Asano T, Moskowitz MA, and Liao JK (2005a) Inhibition of Rho kinase (ROCK) leads to increased cerebral blood flow and stroke protection. *Stroke* **36**:2251–2257.
- Rikitake Y and Liao JK (2005) Rho GTPases, statins, and nitric oxide. *Circ Res* **97**:1232–1235.
- Rikitake Y, Oyama N, Wang CY, Noma K, Satoh M, Kim HH, and Liao JK (2005b) Decreased perivascular fibrosis but not cardiac hypertrophy in ROCK1+/- haploinsufficient mice. *Circulation* **112**:2959–2965.
- Sakai N, Chun J, Duffield JS, Wada T, Luster AD, and Tager AM (2013) LPA1-induced cytoskeleton reorganization drives fibrosis through CTGF-dependent fibroblast proliferation. *FASEB J* **27**:1830–1846.
- Sakai N and Tager AM (2013) Fibrosis of two: Epithelial cell-fibroblast interactions in pulmonary fibrosis. *Biochim Biophys Acta* **1832**:911–921.
- Sandbo N, Kregel S, Taurin S, Bhorade S, and Dulin NO (2009) Critical role of serum response factor in pulmonary myofibroblast differentiation induced by TGF-beta. *Am J Respir Cell Mol Biol* **41**:332–338.
- Sandbo N, Lau A, Kach J, Ngam C, Yau D, and Dulin NO (2011) Delayed stress fiber formation mediates pulmonary myofibroblast differentiation in response to TGF-beta. *Am J Physiol Lung Cell Mol Physiol* **301**:L656–L666.
- Santibañez JF, Quintanilla M, and Bernabeu C (2011) TGF-beta/TGF-beta receptor system and its role in physiological and pathological conditions. *Clin Sci (Lond)* **121**:233–251.
- Satoh S, Yamaguchi T, Hitomi A, Sato N, Shiraiwa K, Ikegaki I, Asano T, and Shimokawa H (2002) Fasudil attenuates interstitial fibrosis in rat kidneys with unilateral ureteral obstruction. *Eur J Pharmacol* **455**:169–174.
- Scotton CJ and Chambers RC (2007) Molecular targets in pulmonary fibrosis: the myofibroblast in focus. *Chest* **132**:1311–1321.
- Scotton CJ, Krupiczko MA, Königshoff M, Mercer PF, Lee YC, Kaminski N, Morser J, Post JM, Maher TM, and Nicholson AG, et al. (2009) Increased local expression of coagulation factor X contributes to the fibrotic response in human and murine lung injury. *J Clin Invest* **119**:2550–2563.
- Sebbagh M, Renvoizé C, Hamelin J, Riché N, Bertoglio J, and Bréard J (2001) Caspase-3-mediated cleavage of ROCK I induces MLC phosphorylation and apoptotic membrane blebbing. *Nat Cell Biol* **3**:346–352.
- Selman M, King TE, and Pardo A; American Thoracic Society; European Respiratory Society; American College of Chest Physicians (2001) Idiopathic pulmonary fibrosis: prevailing and evolving hypotheses about its pathogenesis and implications for therapy. *Ann Intern Med* **134**:136–151.
- Selman M and Pardo A (2006) Role of epithelial cells in idiopathic pulmonary fibrosis: from innocent targets to serial killers. *Proc Am Thorac Soc* **3**:364–372.
- Shen Q, Wu MH, and Yuan SY (2009) Endothelial contractile cytoskeleton and microvascular permeability. *Cell Health Cytoskeleton* **2009**:43–50.
- Sheppard D (2006) Transforming growth factor beta: a central modulator of pulmonary and airway inflammation and fibrosis. *Proc Am Thorac Soc* **3**:413–417.
- Shi J, Zhang YW, Summers LJ, Dorn GW 2nd, and Wei L (2008) Disruption of ROCK1 gene attenuates cardiac dilation and improves contractile function in pathological cardiac hypertrophy. *J Mol Cell Cardiol* **44**:551–560.
- Shibuya M, Suzuki Y, Sugita K, Saito I, Sasaki T, Takakura K, Nagata I, Kikuchi H, Takemae T, and Hidaka H, et al. (1992) Effect of AT877 on cerebral vasospasm after aneurysmal subarachnoid hemorrhage. Results of a prospective placebo-controlled double-blind trial. *J Neurosurg* **76**:571–577.
- Shimizu Y, Dobashi K, Izuka K, Horie T, Suzuki K, Tugakoshi H, Nakazawa T, Nakazato Y, and Mori M (2001) Contribution of small GTPase Rho and its target protein rock in a murine model of lung fibrosis. *Am J Respir Crit Care Med* **163**:210–217.
- Shimizu Y, Thumkeo D, Keel J, Ishizaki T, Oshima H, Oshima M, Noda Y, Matsumura F, Taketo MM, and Narumiya S (2005) ROCK-I regulates closure of the eyelids and ventral body wall by inducing assembly of actomyosin bundles. *J Cell Biol* **168**:941–953.
- Shimokawa H, Hiramori K, Inuma H, Hosoda S, Kishida H, Osada H, Katagiri T, Yamauchi K, Yui Y, and Minamino T, et al. (2002) Anti-anginal effect of fasudil, a Rho-kinase inhibitor, in patients with stable effort angina: a multicenter study. *J Cardiovasc Pharmacol* **40**:751–761.
- Stefater JA 3rd, Ren S, Lang RA, and Duffield JS (2011) Metchnikoff's policemen: macrophages in development, homeostasis and regeneration. *Trends Mol Med* **17**:743–752.
- Sumi T, Matsumoto K, and Nakamura T (2001) Specific activation of LIM kinase 2 via phosphorylation of threonine 505 by ROCK, a Rho-dependent protein kinase. *J Biol Chem* **276**:670–676.
- Sun H, Breslin JW, Zhu J, Yuan SY, and Wu MH (2006) Rho and ROCK signaling in VEGF-induced microvascular endothelial hyperpermeability. *Microcirculation* **13**:237–247.
- Suzuki Y, Shibuya M, Satoh S, Sugimoto Y, and Takakura K (2007) A postmarketing surveillance study of fasudil treatment after aneurysmal subarachnoid hemorrhage. *Surg Neurol* **68**:126–131; discussion 131–132.
- Tada S, Iwamoto H, Nakamura M, Sugimoto R, Enjoji M, Nakashima Y, and Nawata H (2001) A selective ROCK inhibitor, Y27632, prevents dimethylnitrosamine-induced hepatic fibrosis in rats. *J Hepatol* **34**:529–536.
- Tager AM, LaCamera P, Shea BS, Campanella GS, Selman M, Zhao Z, Polosukhin V, Wain J, Karimi-Shah BA, and Kim ND, et al. (2008) The lysophosphatidic acid receptor LPA1 links pulmonary fibrosis to lung injury by mediating fibroblast recruitment and vascular leak. *Nat Med* **14**:45–54.
- Takemoto M, Sun J, Hiroki J, Shimokawa H, and Liao JK (2002) Rho-kinase mediates hypoxia-induced downregulation of endothelial nitric oxide synthase. *Circulation* **106**:57–62.
- Tasaka S, Koh H, Yamada W, Shimizu M, Ogawa Y, Hasegawa N, Yamaguchi K, Ishii Y, Richer SE, and Doerschuk CM, et al. (2005) Attenuation of endotoxin-induced acute lung injury by the Rho-associated kinase inhibitor, Y-27632. *Am J Respir Cell Mol Biol* **32**:504–510.
- Thannickal VJ and Horowitz JC (2006) Evolving concepts of apoptosis in idiopathic pulmonary fibrosis. *Proc Am Thorac Soc* **3**:350–356.
- Thomas SM, Morgan JT, Wood JA, Murphy CJ, and Russell P (2013) Substratum stiffness and latrunculin B modulate the gene expression of the mechanotransducers YAP and TAZ in human trabecular meshwork cells. *Exp Eye Res* **113**:66–73.
- Thumkeo D, Keel J, Ishizaki T, Hirose M, Nonomura K, Oshima H, Oshima M, Taketo MM, and Narumiya S (2003) Targeted disruption of the mouse rho-associated kinase 2 gene results in intrauterine growth retardation and fetal death. *Mol Cell Biol* **23**:5043–5055.
- Tschumperlin DJ, Liu F, and Tager AM (2013) Biomechanical regulation of mesenchymal cell function. *Curr Opin Rheumatol* **25**:92–100.
- van Nieuw Amerongen GP, van Delft S, Vermeer MA, Collard JG, and van Hinsbergh VW (2000a) Activation of RhoA by thrombin in endothelial hyperpermeability: role of Rho kinase and protein tyrosine kinases. *Circ Res* **87**:335–340.
- van Nieuw Amerongen GP, Vermeer MA, and van Hinsbergh VW (2000b) Role of RhoA and Rho kinase in lysophosphatidic acid-induced endothelial barrier dysfunction. *Arterioscler Thromb Vasc Biol* **20**:E127–E133.
- Velasco G, Armstrong C, Morrice N, Frame S, and Cohen P (2002) Phosphorylation of the regulatory subunit of smooth muscle protein phosphatase 1M at Thr850 induces its dissociation from myosin. *FEBS Lett* **527**:101–104.

- Vicari RM, Chaitman B, Keefe D, Smith WB, Chrysant SG, Tonkon MJ, Bittar N, Weiss RJ, Morales-Ballejo H, and Thadani U; Fasudil Study Group (2005) Efficacy and safety of fasudil in patients with stable angina: a double-blind, placebo-controlled, phase 2 trial. *J Am Coll Cardiol* **46**:1803–1811.
- Wang HW, Liu PY, Oyama N, Rikitake Y, Kitamoto S, Gitlin J, Liao JK, and Boisvert WA (2008) Deficiency of ROCK1 in bone marrow-derived cells protects against atherosclerosis in LDLR^{-/-} mice. *FASEB J* **22**:3561–3570.
- Wang Z, Wang DZ, Hockemeyer D, McAnally J, Nordheim A, and Olson EN (2004) Myocardin and ternary complex factors compete for SRF to control smooth muscle gene expression. *Nature* **428**:185–189.
- Washida N, Wakino S, Tonzuka Y, Homma K, Tokuyama H, Hara Y, Hasegawa K, Minakuchi H, Fujimura K, and Hosoya K, et al. (2011) Rho-kinase inhibition ameliorates peritoneal fibrosis and angiogenesis in a rat model of peritoneal sclerosis. *Nephrol Dial Transplant* **26**:2770–2779.
- Watanabe K, Ueno M, Kamiya D, Nishiyama A, Matsumura M, Wataya T, Takahashi JB, Nishikawa S, Nishikawa S, and Muguruma K, et al. (2007) A ROCK inhibitor permits survival of dissociated human embryonic stem cells. *Nat Biotechnol* **25**: 681–686.
- Wilhelm KR, Roan E, Ghosh MC, Parthasarathi K, and Waters CM (2014) Hyperoxia increases the elastic modulus of alveolar epithelial cells through Rho kinase. *FEBS J* **281**:957–969.
- Wipff PJ, Rifkin DB, Meister JJ, and Hinz B (2007) Myofibroblast contraction activates latent TGF-beta1 from the extracellular matrix. *J Cell Biol* **179**:1311–1323.
- Wojciak-Stothard B and Ridley AJ (2002) Rho GTPases and the regulation of endothelial permeability. *Vascul Pharmacol* **39**:187–199.
- Woodsome TP, Eto M, Everett A, Brautigan DL, and Kitazawa T (2001) Expression of CPI-17 and myosin phosphatase correlates with Ca(2+) sensitivity of protein kinase C-induced contraction in rabbit smooth muscle. *J Physiol* **535**:553–564.
- Wu DJ, Xu JZ, Wu YJ, Jean-Charles L, Xiao B, Gao PJ, and Zhu DL (2009) Effects of fasudil on early atherosclerotic plaque formation and established lesion progression in apolipoprotein E-knockout mice. *Atherosclerosis* **207**:68–73.
- Wynn TA and Ramalingam TR (2012) Mechanisms of fibrosis: therapeutic translation for fibrotic disease. *Nat Med* **18**:1028–1040.
- Xu MY, Porte J, Knox AJ, Weinreb PH, Maher TM, Violette SM, McAnulty RJ, Sheppard D, and Jenkins G (2009) Lysophosphatidic acid induces alpha6beta6 integrin-mediated TGF-beta activation via the LPA2 receptor and the small G protein G alpha(q). *Am J Pathol* **174**:1264–1279.
- Yu FX, Zhao B, Panupinthu N, Jewell JL, Lian I, Wang LH, Zhao J, Yuan H, Tumaneng K, and Li H, et al. (2012) Regulation of the Hippo-YAP pathway by G-protein-coupled receptor signaling. *Cell* **150**:780–791.
- Zhang YM, Bo J, Taffet GE, Chang J, Shi J, Reddy AK, Michael LH, Schneider MD, Entman ML, and Schwartz RJ, et al. (2006) Targeted deletion of ROCK1 protects the heart against pressure overload by inhibiting reactive fibrosis. *FASEB J* **20**: 916–925.
- Zhao B, Wei X, Li W, Udan RS, Yang Q, Kim J, Xie J, Ikenoue T, Yu J, and Li L, et al. (2007) Inactivation of YAP oncoprotein by the Hippo pathway is involved in cell contact inhibition and tissue growth control. *Genes Dev* **21**:2747–2761.
- Zhao B, Ye X, Yu J, Li L, Li W, Li S, Yu J, Lin JD, Wang CY, and Chinnaiyan AM, et al. (2008) TEAD mediates YAP-dependent gene induction and growth control. *Genes Dev* **22**:1962–1971.
- Zhao J, Zhou D, Guo J, Ren Z, Zhou L, Wang S, Zhang Y, Xu B, Zhao K, and Wang R, et al.; Fasudil Aneurysmal Subarachnoid Hemorrhage Study Group (2011) Efficacy and safety of fasudil in patients with subarachnoid hemorrhage: final results of a randomized trial of fasudil versus nimodipine. *Neurol Med Chir (Tokyo)* **51**: 679–683.
- Zhou H, Fang C, Zhang L, Deng Y, Wang M, and Meng F (2014) Fasudil hydrochloride hydrate, a Rho-kinase inhibitor, ameliorates hepatic fibrosis in rats with type 2 diabetes. *Chin Med J (Engl)* **127**:225–231.
- Zhou Y, Huang X, Hecker L, Kurundkar D, Kurundkar A, Liu H, Jin TH, Desai L, Bernard K, and Thannickal VJ (2013) Inhibition of mechanosensitive signaling in myofibroblasts ameliorates experimental pulmonary fibrosis. *J Clin Invest* **123**: 1096–1108.