The Rho Kinase Isoforms ROCK1 and ROCK2 Each Contribute to the Development of Experimental Pulmonary Fibrosis

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Abstract

Pulmonary fibrosis is thought to result from dysregulated wound repair after repetitive lung injury. Many cellular responses to injury involve rearrangements of the actin cytoskeleton mediated by the two isoforms of the Rho-associated coiled-coil-forming protein kinase (ROCK), ROCK1 and ROCK2. In addition, profibrotic mediators such as transforming growth factor- β , thrombin, and lysophosphatidic acid act through receptors that activate ROCK. Inhibition of ROCK activation may be a potent therapeutic strategy for human pulmonary fibrosis. Pharmacological inhibition of ROCK using nonselective ROCK inhibitors has been shown to prevent fibrosis in animal models; however, the specific roles of each ROCK isoform are poorly understood. Furthermore, the pleiotropic effects of this kinase have raised concerns about on-target adverse effects of ROCK inhibition such as hypotension. Selective inhibition of one isoform might be a better-tolerated strategy. In the present study, we

used a genetic approach to determine the roles of ROCK1 and ROCK2 in a mouse model of bleomycin-induced pulmonary fibrosis. Using ROCK1- or ROCK2-haploinsufficient mice, we found that reduced expression of either ROCK1 or ROCK2 was sufficient to protect them from bleomycin-induced pulmonary fibrosis. In addition, we found that both isoforms contribute to the profibrotic responses of epithelial cells, endothelial cells, and fibroblasts. Interestingly, ROCK1- and ROCK2-haploinsufficient mice exhibited similar protection from bleomycin-induced vascular leak, myofibroblast differentiation, and fibrosis; however, ROCK1-haploinsufficient mice demonstrated greater attenuation of epithelial cell apoptosis. These findings suggest that selective inhibition of either ROCK isoform has the potential to be an effective therapeutic strategy for pulmonary fibrosis.

Keywords: pulmonary fibrosis; Rho kinase; apoptosis; vascular leak; myofibroblast

Idiopathic pulmonary fibrosis (IPF) is a devastating disease of progressive respiratory decline associated with high morbidity and mortality and a median time

to organ failure and death of only 3 years from the time of diagnosis (1). In 2014, nintedanib and pirfenidone received Food and Drug Administration approval for

patients with IPF after clinical trials demonstrated that each drug slowed the rate of lung function decline (2, 3). Though this was a critical advance, a

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Clinical Relevance

The development of pulmonary fibrosis is thought to be driven by aberrant or overexuberant wound-healing responses to recurrent lung injury, including exaggerated alveolar epithelial cell apoptosis, pulmonary vascular permeability, and fibroblast activation. We demonstrate that both isoforms of Rho-associated coiled-coil-forming protein kinase (ROCK), ROCK1 and ROCK2, contribute to all three of these processes, as well as to the development of pulmonary fibrosis, in the bleomycin model. Partial inhibition of either ROCK isoform or both together thus has the potential to be an effective therapeutic strategy for pulmonary fibrosis. Importantly, complete elimination of both isoforms is not required for an antifibrotic phenotype.

great unmet need remains for more effective IPF treatments capable of stopping or even reversing the progression of pulmonary fibrosis. The development of novel therapeutic strategies will require a better understanding of the biological processes that drive pulmonary fibrosis.

Multiple mediators thought to contribute to pulmonary fibrosis, such as lysophosphatidic acid (LPA), thrombin, and transforming growth factor- β (TGF- β), have receptors that activate Rho-associated coiled-coil-forming protein kinase (ROCK) as part of their signaling pathways (4). ROCK activation in the lungs has been demonstrated in the bleomycin mouse model of pulmonary fibrosis, as well as in the lungs of patients with IPF (5). There are two human and mouse isoforms of ROCK, ROCK1 and ROCK2 (6). In mice, inhibition of both ROCK isoforms with nonselective pharmacologic inhibitors, such as fasudil and Y-27632, has been shown to prevent the development of pulmonary fibrosis when administered before bleomycin challenge, as well as to induce regression when administered after fibrosis has already been established (5, 7–9). Taken together, these data suggest that ROCK inhibition may be a particularly promising therapeutic strategy for pulmonary fibrosis.

Although inhibition of ROCK has great potential to mitigate pulmonary fibrosis,

the pleiotropic effects of this kinase have raised concerns about on-target adverse effects of nonselectively inhibiting both ROCK isoforms together, such as hypotension (10). It has been reported that at least one ROCK inhibitor, Y-27632, inhibits smooth muscle contraction and corrects the blood pressure in hypertensive rat models (11).

In the present study, we sought to evaluate the individual contributions of the two ROCK isoforms to pulmonary fibrogenesis to determine whether selective inhibition of either ROCK1 or ROCK2 may provide similar antifibrotic effects while minimizing untoward consequences. The two ROCK isoforms are structurally similar, particularly in their kinase domains, where they are 90% homologous (6). Both fasudil and Y-27632 target these kinase domains and inhibit ROCK1 and ROCK2 with equal potency (12). Consequently, the effects of these inhibitors *in vivo* cannot be attributed to the inhibition of one ROCK isoform versus the other. We therefore took a genetic approach to evaluate ROCK1 and ROCK2 individually. Prior genetic targeting experiments have indicated that ROCK1 and ROCK2 have nonredundant functions *in vivo* (13, 14). Homozygous deletion of either ROCK1 or ROCK2 leads to nonviability of most offspring, indicating that the ability of these isoforms to compensate for each other is not complete. Furthermore, the causes of nonviability, as well as the phenotypes of the rare survivors, differ between isoform deletions, which are consistent with these isoforms having nonredundant functions (13, 14). To investigate the contributions of the individual ROCK isoforms to the development of pulmonary fibrosis in adult mice, we induced fibrosis through injection of intratracheal bleomycin in mice that were haploinsufficient for ROCK1, ROCK2, or both isoforms. Previous investigations have shown that these mice are viable into adulthood, exhibit an approximately 50% reduction in protein expression of each ROCK isoform, and have informative phenotypes in several other disease models (15–17).

In this study, we found that haploinsufficiency of either ROCK isoform protects from the development of pulmonary fibrosis in the bleomycin model. Given the pleiotropic effects of ROCK signaling, we evaluated whether ROCK1 and/or ROCK2 haploinsufficiency mitigated several different essential profibrotic responses to bleomycin-induced lung injury, involving three different cell types: 1) alveolar epithelial cell apoptosis,

2) loss of endothelial cell barrier function and vascular leak, and 3) myofibroblast differentiation. We found differing contributions of the two ROCK isoforms in these three different profibrotic responses, further indicating that ROCK1 and ROCK2 serve nonredundant functions during the development of pulmonary fibrosis. Our findings demonstrate that even partial inhibition of a single ROCK isoform could have therapeutic efficacy, which is encouraging because we know that some degree of ROCK activity is important for maintaining normal homeostasis.

Methods

Animals

ROCK1-haploinsufficient (ROCK1^{+/-}) and ROCK2-haploinsufficient (ROCK2^{+/-}) mice were generated as described previously (16). Mice haploinsufficient for both isoforms (ROCK1^{+/-}2^{+/-} mice) were generated by crossing ROCK1^{+/-} and ROCK2^{+/-} mice. ROCK1^{+/-}2^{+/-} mice were underrepresented in the litters produced, representing approximately 10% of pups rather than the expected 25%, but these mice appeared to be phenotypically normal. DNA was extracted from tail snips of all mice used in experiments to identify their ROCK1 and ROCK2 genotypes. The control mice used are described in the METHODS section of the data supplement. The mice were maintained in a specific pathogen-free environment certified by the American Association for Accreditation of Laboratory Animal Care. All protocols performed were approved by the Massachusetts General Hospital Institutional Animal Care and Use Committee. For experiments, we used mice at 8–12 weeks of age, and whenever required, mice were anesthetized with ketamine (80 mg/kg) and xylazine (10 mg/kg).

Western Blotting

Whole-lung lysates were separated by SDS-PAGE and then transferred to polyvinylidene difluoride membranes using the NuPAGE electrophoresis and transfer systems (Life Technologies) under reducing conditions. After blocking with 5% milk, membranes were incubated with anti-ROCK1 or anti-ROCK2 primary antibodies (both from BD Transduction Laboratories) followed by horseradish peroxidase-conjugated secondary antibodies (Cell Signaling Technology), and then they were visualized with Amersham enhanced chemiluminescence substrate (GE

Healthcare). Membranes were then stripped and similarly reprobed for GAPDH as a loading control. Densitometry of the immunoblots was performed using ImageJ software (NIH).

Bleomycin-induced Pulmonary Fibrosis

Fibrosis was induced by single-dose intratracheal injection of bleomycin (Fresenius Kabi) at 1.2 U/kg and quantified by measuring hydroxyproline content, as previously described (18).

Histological Analysis and Immunohistochemistry

Lungs were inflated with 10% formalin at 25 cm H₂O for 15 minutes after the right ventricle was flushed with PBS. Lungs were subsequently excised and fixed in 10% formalin before being embedded in paraffin. Paraffin-embedded 5- μ m sections were stained with hematoxylin and eosin (H&E) or Masson's trichrome stain. For immunohistochemical analyses of epithelial apoptosis and myofibroblast differentiation, TUNEL staining or staining with an anti- α -smooth muscle actin (anti- α -SMA) antibody, respectively, was performed on deparaffinized lung sections as previously described (19, 20). TUNEL quantification was averaged by blinded counting of 10 high-powered fields per sample. α -SMA

immunohistochemistry was quantified as mean expression over each entire slide using WinROOF imaging software (Mitani Shoji Co.).

Assessment of Vascular Permeability

Lung vascular permeability was assessed by measuring BAL albumin concentration using ELISA kits specific for mouse albumin (Bethyl Laboratories) according to the manufacturer's instructions. To measure vascular permeability *in vivo*, Evans blue (EB) dye assays were performed as described previously (18, 21).

Human Fibroblast Experiments

Human lung fibroblasts were collected from discarded excess tissue from surgical lung resections. Additional details are provided in the METHODS section of the data supplement.

Statistical Analysis

Differences between groups were analyzed for statistical significance with two-tailed Student's *t* tests, except for hydroxyproline assays, which were analyzed with one-tailed tests, using Microsoft Excel software (Microsoft). $P \leq 0.05$ was considered significant for all comparisons.

Results

Characterization of ROCK1 and ROCK2 Expression in ROCK-Haploinsufficient Mice

We confirmed the genotypes of ROCK1^{+/-}, ROCK2^{+/-}, and ROCK1^{+/-}2^{+/-} mice by PCR analysis of genomic DNA (Figure 1A). To assess the protein expression levels of both ROCK isoforms in the lungs of ROCK1^{+/-}, ROCK2^{+/-}, ROCK1^{+/-}2^{+/-}, and wild-type (WT) control mice, we performed Western blot analysis for ROCK1 and ROCK2 on lung homogenates from each genotype (Figure 1B). Densitometric analyses of the blots confirmed reduced ROCK1 expression in ROCK1^{+/-} and ROCK1^{+/-}2^{+/-} mice (reduced 44% and 60%, respectively, in these mice) (Figure 1B). Densitometry similarly showed reduced ROCK2 expression in ROCK2^{+/-} and ROCK1^{+/-}2^{+/-} mice (reduced 33% and 30%, respectively, in these mice) (Figure 1B and expanded images in Figure E1 in the data supplement). There was a small compensatory increase in the alternate isoform in the single-haploinsufficient mice. ROCK1 expression was significantly increased in ROCK2^{+/-} mice as compared with WT mice ($P = 0.04$), but ROCK2 expression was not

Figure 1. Rho-associated coiled-coil-forming protein kinase (ROCK) isoform ROCK1 and/or ROCK2 expression is reduced in ROCK1-haploinsufficient (ROCK1^{+/-}), ROCK2-haploinsufficient (ROCK2^{+/-}), and double-haploinsufficient (ROCK1^{+/-}2^{+/-}) mice. (A) The genotype of pups in litters produced by mating ROCK1^{+/-} and ROCK2^{+/-} mice were distinguished by PCR amplification of tail DNA from each mouse using allele-specific primers. The representative gels demonstrate the PCR products generated from DNA from wild-type (WT), ROCK1^{+/-} (R1^{+/-}), ROCK2^{+/-} (R2^{+/-}), and ROCK1^{+/-}2^{+/-} (R1^{+/-}2^{+/-}) mice. PCR products generated from DNA from a control WT mouse (positive control) and from no DNA (negative control) are also shown. (B) Immunoblotting of lung homogenates of representative WT, ROCK1^{+/-}, ROCK2^{+/-}, and ROCK1^{+/-}2^{+/-} mice using ROCK1- and ROCK2-specific antibodies. Densitometry performed on immunoblots with three mice per genotype demonstrated an approximately 50% reduction in ROCK1 protein expression in the ROCK1^{+/-} and ROCK1^{+/-}2^{+/-} mice and an approximately 50% reduction in ROCK2 protein expression in the ROCK2^{+/-} and ROCK1^{+/-}2^{+/-} mice. $P < 0.05$ comparing expression of designated ROCK isoforms in haploinsufficient versus WT mice. $P = 0.04$ comparing expression of ROCK1 in ROCK2^{+/-} mice as compared with WT mice.

significantly increased in $ROCK1^{+/-}$ mice as compared with WT mice. FACS was performed to quantify ROCK isoform expression in relevant cell populations (Figure E2 and METHODS section in the data supplement). Protein obtained from epithelial cells (epithelial cell adhesion molecule-positive [$EpCAM^{+}$], $CD31^{-}$, $CD45^{-}$, platelet-derived growth factor receptor- α -negative [$PDGFR\alpha^{-}$]), hematopoietic cells ($CD45^{+}$, $CD31^{-}$, $EpCAM^{-}$, $PDGFR\alpha^{-}$), endothelial cells ($CD31^{+}$, $EpCAM^{-}$, $CD45^{-}$, $PDGFR\alpha^{-}$),

and fibroblasts ($PDGFR\alpha^{+}$, $EpCAM^{-}$, $CD31^{-}$, $CD45^{-}$) all demonstrated reductions in ROCK1 and ROCK2 expression in the respective haploinsufficient mice.

Mice Haploinsufficient for Either ROCK1 or ROCK2 Alone or Both Isoforms Together Were Protected from Bleomycin-induced Lung Fibrosis

To determine the contribution of each isoform individually or together to the development of pulmonary fibrosis, we

subjected $ROCK1^{+/-}$, $ROCK2^{+/-}$, and $ROCK1^{+/-}2^{+/-}$ mice, as well as their WT control littermates, to the bleomycin model of pulmonary fibrosis. Lungs at Day 14 post-bleomycin challenge typically demonstrate pathological changes consistent with peribronchiolar and parenchymal fibrosis. The extent of these changes demonstrated by H&E staining of lung sections from WT control mice was decreased in $ROCK1^{+/-}$, $ROCK2^{+/-}$, and $ROCK1^{+/-}2^{+/-}$ mice (Figure 2A). The amount of lung collagen visualized by

Figure 2. ROCK-haploinsufficient mice are protected from bleomycin-induced pulmonary fibrosis. (A) Representative lung sections stained with hematoxylin and eosin (H&E; left panels) or Masson's trichrome (right panels) from WT, $ROCK1^{+/-}$, $ROCK2^{+/-}$, and $ROCK1^{+/-}2^{+/-}$ mice at Day 14 post-bleomycin challenge (magnification, $\times 4$). (B) Biochemical analysis of bleomycin-induced fibrosis. Hydroxyproline content was measured in the lungs of WT, $R1^{+/-}$, $R2^{+/-}$, and $R1^{+/-}2^{+/-}$ mice at Day 14 post-bleomycin challenge or at Day 14 post-PBS control (Day 14 post-bleomycin, $n = 7$ –12 mice/group; Day 14 post-PBS, $n = 4$ –7 mice/group). Data shown are a combination of three separate experiments with all data included. Data are expressed as mean $\pm$ SEM hydroxyproline content per lung set. * $P \leq 0.05$ comparing $ROCK1^{+/-}$, $ROCK2^{+/-}$, and $ROCK1^{+/-}2^{+/-}$ mice 14 days post-bleomycin versus WT mice 14 days post-bleomycin. (C) Weight changes compared with Day 0 baseline in WT, $ROCK1^{+/-}$, $ROCK2^{+/-}$, and $ROCK1^{+/-}2^{+/-}$ mice at Day 7 and Day 14 post-bleomycin challenge (Day 7, $n = 3$ –17 mice/group; Day 14, $n = 5$ –10 mice/group). Data are expressed as mean $\pm$ SEM percentage loss of weight from baseline. * $P < 0.05$ comparing $ROCK1^{+/-}$, $ROCK2^{+/-}$, and $ROCK1^{+/-}2^{+/-}$ mice 14 days post-bleomycin versus WT mice 14 days post-bleomycin. Scale bars: 100 μ m. Bleo = bleomycin.

Masson's trichrome staining of lung sections from WT control mice 14 days after bleomycin was also decreased in ROCK1^{+/-}, ROCK2^{+/-}, and ROCK1^{+/-}2^{+/-} mice (Figure 2A). H&E- and trichrome-stained lung sections of naive ROCK1^{+/-}, ROCK2^{+/-}, and ROCK1^{+/-}2^{+/-} mice demonstrated no obvious abnormalities in the lung architecture of these mice at baseline (Figure E3).

Biochemical analysis of lung collagen, as defined by hydroxyproline content, confirmed the protection of ROCK1^{+/-}, ROCK2^{+/-}, and ROCK1^{+/-}2^{+/-} mice from lung fibrosis. There were no differences in total lung hydroxyproline between WT control mice and ROCK1^{+/-}, ROCK2^{+/-}, and ROCK1^{+/-}2^{+/-} mice after PBS challenge. At Day 14 post-bleomycin challenge, total lung hydroxyproline content was increased by 154% over baseline in WT control mice, but it was increased by a smaller extent over baseline in ROCK-haploinsufficient mice: by 91% in ROCK1^{+/-} mice, by 96% in ROCK2^{+/-} mice, and by 58% in ROCK1^{+/-}2^{+/-} mice (Figure 2B). These results indicate that genetic reduction in either or both ROCK isoforms affords protection from bleomycin-induced pulmonary fibrosis in this model. Although there appeared to be some additional

protection afforded to the ROCK1^{+/-}2^{+/-} mice, the difference between single- and double-isoform haploinsufficiency did not meet significance.

ROCK-haploinsufficient mice also demonstrated protection from the weight loss produced in the bleomycin model. At Day 7 post-bleomycin challenge, all groups of laboratory mice had lost between 5 and 15% of their body weight. At Day 14 post-challenge, however, the ROCK1^{+/-}2^{+/-} mice had regained weight to slightly above their prechallenge baseline, and all three ROCK-haploinsufficient genotypes were significantly closer to their baseline weights than were WT control mice (Figure 2C).

ROCK1 Haploinsufficiency Protects against Bleomycin-induced Alveolar Epithelial Cell Apoptosis to a Greater Extent than ROCK2 Haploinsufficiency

Apoptosis of alveolar epithelial cells (AECs) is a postinjury event observed in IPF lungs and shown to contribute to the development of pulmonary fibrosis in mouse models (22–25). ROCK activation has been reported to promote AEC detachment induced by cyclic stretch and hyperoxia, and ROCK1 specifically has been implicated in driving the cellular machinery involved in the execution phase of apoptosis (26, 27).

We have previously demonstrated that AEC apoptosis peaks early after bleomycin challenge at Days 1 and 3 and then wanes by Day 5 (19). We therefore compared AEC apoptosis in naive and Day 3 post-bleomycin challenge WT control mice in addition to ROCK1^{+/-}, ROCK2^{+/-}, and ROCK1^{+/-}2^{+/-} mice. TUNEL staining of lung sections of naive mice demonstrated minimal numbers of apoptotic cells in all experimental groups (Figure E3), with no significant differences between groups. At Day 3 post-bleomycin challenge, however, there were significantly fewer apoptotic cells in ROCK1^{+/-}, ROCK2^{+/-}, and ROCK1^{+/-}2^{+/-} mice than in bleomycin-treated WT control mice (Figure 3A). Although ROCK1^{+/-} and ROCK2^{+/-} mice both had significantly fewer apoptotic AECs than WT control mice post-bleomycin challenge (Figure 3B), protection from AEC apoptosis was significantly greater in ROCK1^{+/-} than in ROCK2^{+/-} mice. ROCK1^{+/-}2^{+/-} mice also had significantly reduced numbers of apoptotic AECs compared with WT control mice (Figure 3B), but this reduction was not significantly different from that seen in mice haploinsufficient for ROCK1 alone. These results indicate that both ROCK1 and ROCK2 contribute to bleomycin-induced AEC, but that ROCK1 plays a

Figure 3. Lung epithelial cell apoptosis induced by bleomycin injury is diminished in ROCK-haploinsufficient mice. (A) Assessment of lung epithelial cell apoptosis by TUNEL staining. Representative TUNEL-stained lung sections from WT, ROCK1^{+/-}, ROCK2^{+/-}, and ROCK1^{+/-}2^{+/-} mice at Day 3 post-bleomycin challenge (magnification, $\times 20$). Arrows indicate positive nuclear staining consistent with cell apoptosis. (B) Numbers of TUNEL⁺ cells in lungs of WT, R1^{+/-}, R2^{+/-}, and R1^{+/-}2^{+/-} mice at Day 0 and Day 3 post-bleomycin challenge ($n = 3$ mice per genotype at each time point). * $P < 0.05$; ** $P < 0.01$; and *** $P < 0.001$ WT mice at Day 3 or for indicated pairwise comparison. Scale bars: 50 μm .

larger role than ROCK2 in this profibrotic response to lung injury.

To further validate these findings, we performed immunofluorescent TUNEL staining in addition to costaining for alveolar type 1 cells (T1 α^+) or alveolar type 2 cells (surfactant protein C-positive [SPC $^+$]). We found a clear decrease in TUNEL $^+$ cells in ROCK1 $^{+/-}$ mice and ROCK1 $^{+/-}$ 2 $^{+/-}$ mice at Day 3 after bleomycin injury (Figures E4 and E5) and approximately 50% colocalization of TUNEL $^+$ and T1 α^+ cells. We only observed rare colocalization of TUNEL $^+$ and SPC $^+$ staining; in fact, there was a notable paucity of SPC staining in regions with high numbers of TUNEL $^+$ cells. This could be due to a lack of apoptotic type 2 alveolar cells in the lungs at Day 3 post-bleomycin, or more likely, these injured cells no longer expressed SPC protein. We previously reported similar results in our paper looking at TUNEL $^+$ SPC $^+$ cells in LPA $_1$ -knockout mice (19). In naive mice that did not receive bleomycin, there were only rare TUNEL $^+$ cells (Figures E6 and E7).

ROCK1 and ROCK2 Haploinsufficiency Protect against Bleomycin-induced Vascular Permeability

Increased pulmonary vascular permeability and subsequent leak of profibrotic proteins such as thrombin have been shown to contribute to the development of pulmonary fibrosis by promoting thrombin-dependent $\alpha_v\beta_6$ integrin-mediated TGF- β activation (28). Nonselective pharmacologic inhibition of both ROCK isoforms has been shown to protect against vascular leak after lung injury (29). These results implicate ROCK as a critical mediator of lung injury-induced vascular permeability (29). To characterize the contributions of both ROCK1 and ROCK2 isoforms to the increase in lung vascular permeability induced by bleomycin, we performed EB dye extravasation assays and measured albumin extravasation from the plasma into the airspaces. To characterize the time course of bleomycin-induced vascular permeability, we assessed BAL albumin concentrations in naive WT mice (Day 0) and in WT mice at Days 3, 7, and 14 post-bleomycin challenge (Figure 4A). BAL albumin concentrations significantly increased after bleomycin challenge in WT mice, rising progressively from Day 0

to Day 14. We measured hydroxyproline content in the lungs of these same mice at each time point and found that lung collagen was not significantly increased from baseline values until Day 14 post-challenge (Figure 4B). We therefore selected Day 7 as the time point at which to evaluate ROCK contributions to vascular permeability, because it represents the time of maximum vascular leak before the establishment of fibrosis in this model. At this time point, we saw significant reductions in BAL albumin concentrations of ROCK1 $^{+/-}$, ROCK2 $^{+/-}$, and ROCK1 $^{+/-}$ 2 $^{+/-}$ mice compared with WT control mice, demonstrating that reduction of either isoform was associated with a reduction in lung vascular permeability (Figure 4C).

We performed EB dye assays on naive and Day 7 post-bleomycin WT control mice, in addition to ROCK1 $^{+/-}$ and ROCK2 $^{+/-}$ mice, to evaluate the contribution of each ROCK isoform to the loss of lung endothelial barrier function *in vivo*. At Day 7 post-bleomycin challenge, vascular permeability was significantly reduced in the lungs of both ROCK1 $^{+/-}$ and ROCK2 $^{+/-}$ mice compared with WT control mice (Figure 4D). EB dye indices, calculated as the ratio of lung to plasma dye concentrations ([EB] lung/[EB] plasma), were significantly reduced in both ROCK1 $^{+/-}$ and ROCK2 $^{+/-}$ mice compared with WT control mice (Figure 4E). These results indicate that both ROCK1 and ROCK2 contribute to vascular permeability induced by bleomycin lung injury.

Both ROCK1 and ROCK2 Contribute to Bleomycin-induced Fibroblast Differentiation to Myofibroblasts *In Vivo*

ROCK activation in fibroblasts has previously been shown to be required for their differentiation into myofibroblasts in response to either biochemical stimuli such as TGF- β or biomechanical stimuli such as increased stiffness of the extracellular matrix (ECM) (30–32). To investigate the role of ROCK isoforms in the activation of myofibroblasts in the bleomycin-induced pulmonary fibrosis model, we performed immunofluorescence (Figure 5A, left panels) and immunohistochemical (Figure 5A, right panels) staining on lung sections from mice of all experimental groups at Day 14 post-bleomycin with an

antibody that recognizes α -SMA, a marker of myofibroblast differentiation. α -SMA is expressed at baseline in smooth muscle cells that line both blood vessels and airways in the lungs. We performed immunohistochemical staining of lung sections from WT control mice in addition to ROCK1 $^{+/-}$, ROCK2 $^{+/-}$, and ROCK1 $^{+/-}$ 2 $^{+/-}$ mice at Day 0 (naive) and demonstrated that baseline α -SMA expression was maintained despite reductions in ROCK isoform expression (Figure E3). However, at Day 14 post-bleomycin, WT control mice demonstrated substantial staining of lung parenchymal cells consistent with myofibroblast accumulation. These observed changes were largely absent from ROCK1 $^{+/-}$, ROCK2 $^{+/-}$, and ROCK1 $^{+/-}$ 2 $^{+/-}$ mouse lungs after bleomycin as demonstrated by significant reductions in α -SMA staining at Day 14 post-bleomycin in ROCK1 $^{+/-}$, ROCK2 $^{+/-}$, and ROCK1 $^{+/-}$ 2 $^{+/-}$ mice at Day 14 post-bleomycin compared with WT control mice (Figure 5B).

***In Vitro* Either ROCK Isoform Is Sufficient for Human Lung Fibroblast-to-Myofibroblast Differentiation in Response to TGF- β**

We performed *in vitro* experiments inducing myofibroblast differentiation of normal human lung fibroblasts in response to TGF- β after knocking down expression of either isoform ROCK1 or ROCK2 or both using siRNA transfection. ROCK1 and ROCK2 mRNA expression were each reduced by 80% with siRNA transfection compared with nontargeting control siRNA (Figures 6A and 6B). Although we did not observe a significant decrease in α -SMA expression with individual ROCK1 or ROCK2 knockdown as compared with nontargeting siRNA in response to TGF- β , we found that siRNA knockdown of both ROCK1 and ROCK2 simultaneously was required to reduce α -SMA expression in response to TGF- β (Figure 6C). We did observe a significant reduction in collagen expression with ROCK1 knockdown or with knockdown of both isoforms in response to TGF- β (Figure 6D).

Discussion

We have shown that each of the two ROCK isoforms, ROCK1 and ROCK2, contributes to the development of pulmonary fibrosis in

Figure 4. Vascular permeability induced by bleomycin injury is diminished in ROCK-haploinsufficient mice. (A) Assessment of time course of increased lung vascular permeability induced by bleomycin injury as measured by BAL albumin concentration. BAL samples were obtained from three WT mice at each time point. Data are expressed as mean $\pm$ SEM BAL albumin concentration. * $P < 0.005$; ** $P < 0.0005$; and *** $P < 0.0001$ compared with Day 0 post-bleomycin. (B) Assessment of time course of increased lung collagen induced by bleomycin injury as measured by lung hydroxyproline content. Hydroxyproline content was measured in lungs from three WT mice at each time point. Data are expressed as mean $\pm$ SEM hydroxyproline content per lung set. * $P < 0.05$ comparing hydroxyproline content at Day 14 versus Day 0 post-bleomycin. (C) Assessment of lung vascular permeability in ROCK-haploinsufficient mice by BAL albumin concentration at Day 7 post-bleomycin challenge. BAL samples were collected from five WT mice at Day 7 post-PBS or bleomycin challenge and from five ROCK1-haploinsufficient (ROCK1^{+/-}, R1^{+/-}), five ROCK2-haploinsufficient (ROCK2^{+/-}, R2^{+/-}), and three double-haploinsufficient (ROCK1^{+/-}2^{+/-}, R1^{+/-}2^{+/-}) mice at Day 7 post-bleomycin challenge. Data are expressed as mean $\pm$ SEM BAL albumin concentration. * $P < 0.05$ compared with WT mice at Day 7 (D7) post-bleomycin. (D) Assessment of lung vascular permeability by extravasation of Evans blue dye. Gross appearance of lungs from representative WT, ROCK1^{+/-}, and ROCK2^{+/-} mice 7 days post-bleomycin challenge and 3 hours post-Evans blue dye injection. (E) Evans blue dye (EB) indices of WT, ROCK1^{+/-}, and ROCK2^{+/-} mice before and 7 days after bleomycin challenge. Lung and plasma samples were collected from three naive mice/group and three WT, four ROCK1^{+/-}, and four ROCK2^{+/-} mice at Day 7 post-bleomycin challenge. Experiments with naive and bleomycin-challenged mice were performed separately. The experiment with bleomycin-challenged mice was repeated twice, and data shown are from one experiment that was representative of results in both. Data are expressed as mean $\pm$ SEM EB index. * $P < 0.05$ compared with WT mice at D7.

the bleomycin mouse model, as well as that reduction in each isoform is protective against bleomycin-induced pulmonary fibrosis. Our use of mice that were genetically haploinsufficient for ROCK1 or ROCK2 allowed us to evaluate the contributions of each of these isoforms individually. Despite their kinase domains being 90% homologous, we found that only a 50% reduction of either ROCK1 or ROCK2 was sufficient to significantly protect mice from bleomycin-induced

pulmonary fibrosis, as well as that ROCK1 and ROCK2 are unable to compensate for each other's haploinsufficiency in this model of lung fibrosis.

We also examined whether both ROCK isoforms contribute to several key profibrotic responses to lung injury that are induced by bleomycin challenge: AEC apoptosis, vascular permeability, and myofibroblast differentiation. We found that although both ROCK isoforms contribute to bleomycin-induced AEC

apoptosis, ROCK1 made a greater contribution to this profibrotic response to bleomycin injury, as indicated by a greater reduction in AEC apoptosis post-bleomycin challenge in ROCK1-haploinsufficient than in ROCK2-haploinsufficient mice. In contrast, we found that both ROCK isoforms contribute to bleomycin-induced vascular permeability without significant differences between ROCK1^{+/-} and ROCK2^{+/-} mice. We also demonstrated a similar reduction in α -SMA expression in

Figure 5. Both ROCK isoforms contribute to fibroblast activation induced by bleomycin injury. (A) Assessment of bleomycin-induced fibroblast differentiation into myofibroblasts as indicated by α -smooth muscle actin (α -SMA) immunostaining. Representative lung sections from WT, ROCK1^{+/-} (R1^{+/-}), ROCK2^{+/-} (R2^{+/-}), and ROCK1^{+/-}R2^{+/-} (R1^{+/-}R2^{+/-}) mice at Day 14 post-bleomycin challenge with immunofluorescent (left panels) or immunohistochemical (right panels) α -SMA staining (magnification, $\times 20$). (B) Lung area comprised of α -SMA⁺ cells. Data are expressed as mean $\pm$ SEM α -SMA⁺ staining area ($n = 3$ mice/genotype for Day 0; $n = 5$ –8 mice/genotype for Day 14). Data shown are combined from two separate experiments. * $P < 0.05$ comparing ROCK1^{+/-}, ROCK2^{+/-}, and ROCK1^{+/-}R2^{+/-} mice 14 days post-bleomycin versus WT mice 14 days post-bleomycin. Scale bars: 100 μ m.

fibroblasts *in vivo* at Day 14 after bleomycin in both ROCK1- and ROCK2-haploinsufficient mice; *in vitro*, however, we saw a reduction in α -SMA only with siRNA knockdown of both ROCK1 and ROCK2 together. We thus found differing contributions of the two ROCK isoforms for the different profibrotic responses to lung injury that we investigated, further indicating that ROCK1 and ROCK2 serve nonredundant functions during the development of pulmonary fibrosis.

In the prevailing paradigm of IPF pathogenesis, fibrosis results from aberrant wound-healing responses to repetitive injury that primarily targets AECs (33). In response, AECs undergo apoptosis at an increased rate. Increased AEC apoptosis is

sufficient by itself to drive pulmonary fibrosis in mouse models when it is induced by anti-Fas antibody (23), overexpression of TGF- β (24), or diphtheria toxin-induced injury targeted directly to AECs (25). Patients with IPF have been observed to have increased numbers of apoptotic AECs as well (22).

Both ROCK1 and ROCK2 are downstream mediators of cell surface receptors such as LPA₁. We previously demonstrated that LPA signaling through LPA₁ also contributes to the development of AEC apoptosis after bleomycin challenge: LPA₁-deficient mice also had reduced numbers of apoptotic AECs at 1 and 3 days post-bleomycin challenge compared with WT mice (19). We found

evidence that LPA–LPA₁ signaling mediates lung epithelial cell anoikis, the apoptosis of anchorage-dependent cells induced by their detachment. We found that LPA specifically induced lung epithelial cell detachment but did not influence the rate at which cells became apoptotic once they were detached. ROCK activation has been shown to promote AEC detachment induced by injurious stimuli other than bleomycin challenge. Exposure of AECs to cyclic stretch and hyperoxia induced their detachment, which was prevented by pharmacologic ROCK inhibition with the non-isoform-selective inhibitor Y-27632 (26). ROCK1 more than ROCK2 may therefore promote AEC apoptosis after bleomycin challenge by inducing AEC

Figure 6. *In vitro* transforming growth factor- β -induced fibroblast activation requires both ROCK isoforms together. (A) RT-PCR data confirming 80% reduction of ROCK1 (R1) expression in fibroblasts treated with ROCK1 siRNA and in fibroblasts treated with ROCK1 and ROCK2 (R2) siRNA. (B) RT-PCR data confirming 80% reduction of ROCK2 expression in fibroblasts treated with ROCK2 siRNA and in fibroblasts treated with ROCK1 and ROCK2 siRNA. (C) siRNA knockdown of both ROCK1 and ROCK2 was required *in vitro* for a significant reduction in α -SMA expression. RT-PCR performed to quantify α -SMA expression using RNA extracted from fibroblasts isolated from healthy patient volunteers. Data are expressed as fold change α -SMA relative to β_2 -microglobulin ($\beta 2m$). * $P < 0.05$ compared with nontargeting siRNA-treated mice. (D) RT-PCR performed to quantify collagen expression using RNA extracted from fibroblasts isolated from discarded excess normal lung tissue from surgical lung resections. Data are expressed as fold change of collagen relative to $\beta 2m$. * $P < 0.05$ compared with nontargeting siRNA. NT = nontargeting control siRNA.

detachment and anoikis, a hypothesis we will explore in future studies. In addition to promoting AEC apoptosis by promoting cell detachment, ROCK1 specifically has also been implicated in driving the cellular machinery involved in the execution phase of apoptosis (27). ROCK1 but not ROCK2 is cleaved to a truncated active form by caspases during apoptosis. This constitutively active form of ROCK1 promotes the actin-myosin contractility that drives the characteristic membrane bleb formation of the final stages of apoptosis.

In addition to AEC apoptosis, ROCK1 and/or ROCK2 promote other profibrotic activities of AECs after lung injury. Activation of the prototypical profibrotic cytokine TGF- β after lung injury is initially dependent on activation of the epithelial cell-restricted integrin $\alpha_v\beta_6$, which occurs through a ROCK-dependent pathway (34). AECs are also responsible for the production of other cytokines that activate fibroblasts after lung injury, including connective tissue growth factor (CTGF) (35). AEC production of

CTGF is dramatically suppressed by pharmacologic ROCK inhibition with the non-isoform-selective inhibitor Y-27632 (36). Determining which ROCK isoform(s) mediate these other profibrotic AEC activities will be the subject of our future studies.

Exaggerated pulmonary vascular permeability is also one of the aberrant wound-healing responses to repetitive lung injury that can contribute to the development of pulmonary fibrosis. Increased vascular permeability is one of the cardinal responses to tissue injury in general (37), and we have previously shown that increased vascular leak after lung injury promotes the development of pulmonary fibrosis in the bleomycin mouse model (21). In patients with IPF, lung vascular permeability is increased and predicts outcome. Clearance rates of an inhaled radiolabeled macromolecule, ^{99m}Tc -diethylenetriaminepentaacetic acid, were faster in patients with IPF than in control subjects, and among patients with IPF, faster clearance rates were associated with more rapid declines in pulmonary function and increased

mortality (38, 39). Similarly, BAL/plasma protein ratios were elevated in patients with IPF compared with control subjects, and they were greatest in those patients who died within 3 years of follow-up (40).

Endothelial barrier function is maintained by adherens and tight junctions between adjacent endothelial cells that mediate cell-cell adhesion. ROCK activation within endothelial cells promotes actin-myosin contractility, which provides the physical force that pulls adjacent cell membranes apart from one another, opening paracellular gaps through which plasma and plasma proteins leak into the airspaces (41). Paracellular gap formation induced by multiple edemagenic mediators, including thrombin, LPA, vascular endothelial growth factor, and TNF- α , has been shown to be suppressed by pharmacologic ROCK inhibition and consequently to be ROCK dependent (29, 42–46). We found that pulmonary vascular leak induced in the bleomycin model of lung fibrosis was markedly reduced in mice that were haploinsufficient for ROCK1 or ROCK2, suggesting that activation of both ROCK isoforms is required to promote the endothelial cell actin-myosin contractility that drives paracellular gap formation and vascular leak after lung injury.

Exaggerated fibroblast recruitment and fibroblast differentiation into myofibroblasts are also among the aberrant wound-healing responses to repetitive lung injury that contribute to the development of pulmonary fibrosis. Fibroblast activation and differentiation into myofibroblasts are responsible for ECM production and contraction in pulmonary fibrosis. Fibroblast differentiation into myofibroblasts can be directed by biochemical stimuli, such as TGF- β , and/or by biomechanical stimuli, such as increased stiffness of the ECM. ROCK activation in fibroblasts has previously been shown to be required for their differentiation into myofibroblasts in response to either class of stimulus: ROCK inhibition suppresses myofibroblast differentiation induced by TGF- β (30, 31) and myofibroblast differentiation induced by increased matrix stiffness (32). ROCK inhibition has also been shown to suppress fibroblast migration (7), indicating that this profibrotic fibroblast function is ROCK dependent as well. We found that myofibroblast accumulation induced in the lung in the bleomycin model was markedly reduced in mice that were haploinsufficient

for ROCK1 or ROCK2, suggesting that activation of both ROCK isoforms is required to promote myofibroblast accumulation.

In vitro reduction of α -SMA was only significant with knockdown of both isoforms. This finding matches the results of another recent publication in which researchers reported that loss of α -SMA fiber assembly was seen on stiff matrix only in the absence of both ROCK isoforms (47). The interesting difference we found between *in vivo* and *in vitro* α -SMA expression in activated fibroblasts could be due to several variables. In our haploinsufficient mouse model, there is reduction in ROCK expression in all cell types, so the ultimate effect on myofibroblast differentiation could be due to reduction in ROCK in more than just fibroblasts. *In vivo* there are also additional mediators other than TGF- β present, so although our *in vitro* data suggest that single-isoform reduction is insufficient to block TGF- β -induced myofibroblast differentiation, it may be sufficient to block differentiation induced by other mediators, and this could be explored in future studies.

Our findings that mice that were haploinsufficient for either ROCK1 or ROCK2 were protected from bleomycin-induced pulmonary fibrosis, as well as from bleomycin-induced AEC apoptosis, vascular leak, and myofibroblast differentiation, suggest that specifically targeting one ROCK isoform could be an effective therapeutic strategy for pulmonary fibrosis. Whether there is increased benefit from reduction of both ROCK isoforms is unclear on the basis of our current data. Other than underrepresentation in litters, we did not observe adverse effects of a genetic reduction in both isoforms. Reductions in bleomycin-induced increases in apoptotic AECs observed in WT mice were greater in

ROCK1-haploinsufficient mice, although the differences between ROCK1- and ROCK2-haploinsufficient mice with respect to quantification of fibrosis by lung hydroxyproline did not reach statistical significance. ROCK signaling is centrally involved in the vascular smooth muscle cell contraction that regulates blood pressure (48), and its inhibition consequently may precipitate hypotension (10). It has been reported that at least one ROCK inhibitor, Y-27632, inhibits smooth muscle contraction and corrects the blood pressure in hypertensive rat models (11). Expression and activity of ROCK2 is higher than ROCK1 in vascular smooth muscle cells (49), suggesting that ROCK2 may be more involved in regulation of systemic blood pressure than ROCK1 and that ROCK2 inhibition may be more likely to induce hypotension than ROCK1 inhibition. The nonselective ROCK inhibitor fasudil has been used in Japan to treat cerebral vasospasm after intracerebral hemorrhage. Although postmarketing studies did not show high rates of hypotension with this treatment (50), fasudil is a relatively weak ROCK inhibitor, and concerns about hypotension remain for the possible development of more potent ROCK inhibitors for the treatment of pulmonary fibrosis. Therefore, our results do support consideration of selective inhibition of ROCK1 as a targeting strategy that both maximizes antifibrotic efficacy and minimizes potential unwanted on-target effects.

One potential limitation in our data is that in the ROCK2-haploinsufficient mice, there was a statistically significant upregulation of ROCK1 (seen by Western blotting in Figure 1). This could suggest that reduction of overall ROCK activity is not reduced as much as it is in the ROCK1-haploinsufficient mice.

In summary, our study provides evidence derived from specific gene-targeting experiments that ROCK is critically involved in the development of pulmonary fibrosis, consistent with the results of prior studies using pharmacologic ROCK inhibitors. Our results build on these previous studies by demonstrating that both ROCK isoforms, ROCK1 and ROCK2, contribute to multiple profibrotic responses to lung injury, including AEC apoptosis, vascular leak, and myofibroblast differentiation. Why both isoforms are so important for these processes will be the subject of future mechanistic studies. Our results suggest that selective targeting of individual ROCK isoforms may have some benefit as a therapeutic strategy for pulmonary fibrosis without additional evidence for mitigation of unwanted on-target adverse effects. We believe that inhibition of ROCK has great potential as an antifibrotic therapy based on its significant roles in many key fibrotic mechanisms. Partial reduction of ROCK activity, perhaps even normalization of the increased activity reported in IPF, has the potential to be an effective therapeutic strategy for pulmonary fibrosis because it permits the remaining ROCK activity to participate in nonfibrotic functions necessary for homeostasis. ■

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