Mitogen-activated Protein Kinase-activated Protein Kinase 2 Inhibition Attenuates Fibroblast Invasion and Severe Lung Fibrosis

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Abstract

Severe pulmonary fibrosis such as idiopathic pulmonary fibrosis (IPF) is characterized by the accumulation of extracellular matrix and fibroblast activation. Targeting fibroblast activation has contributed to the development of antifibrotic therapeutics for patients with IPF. Mitogen-activated protein kinase-activated protein kinase 2 (MK2), downstream in the transforming growth factor- β /p38 mitogen-activated protein kinase pathway, has been implicated in inflammatory and fibrosing diseases. Increased concentrations of activated MK2 were expressed in IPF lung and in the mouse bleomycin model of lung fibrosis. The aim of the present study was to determine the role and the mechanisms of MK2 in fibroblast invasion and lung fibrosis. Our results showed that an MK2 inhibitor (MMI-0100) was able to inhibit the invasive capacity of lung fibroblasts isolated from patients with IPF, as well as fibroblasts isolated from both wild-type mice and mice with overexpressing hyaluronan synthase 2 (HAS2) in the myofibroblast compartment. We previously showed that hyaluronan and HAS2 regulate fibroblast invasion and lung fibrosis *in vivo*. The results of the present study showed that MMI-0100 reduced transforming growth factor- β -induced hyaluronan production in human and mouse fibroblasts *in vitro* and that HAS2

mediated MK2 activation, suggesting a feed-forward loop in fibroblast activation. More importantly, MK2 inhibition attenuated hyaluronan accumulation and reduced collagen content in bleomycin-injured mouse lungs *in vivo*. Conditional deletion of MK2 in fibroblasts attenuated bleomycin-induced lung fibrosis. These data provide evidence that MK2 has a role in fibroblast invasion and fibrosis and may be a novel therapeutic target in pulmonary fibrosis.

Keywords: lung fibrosis; idiopathic pulmonary fibrosis; fibroblast invasion; mitogen-activated protein kinase; hyaluronan synthase 2

Clinical Relevance

Progressive pulmonary fibrosis is an increasing cause of morbidity and mortality worldwide with limited therapeutic options. This study provides evidence that mitogen-activated protein kinase-activated protein kinase 2 has a role in fibroblast invasion and fibrosis and may be a novel therapeutic target in pulmonary fibrosis.

Progressive pulmonary fibrosis is an increasing cause of morbidity and mortality worldwide with limited therapeutic options. Idiopathic pulmonary fibrosis (IPF) is a particularly severe form of lung fibrosis with

no known etiology and a survival rate of 3 to 5 years without treatment (1–4). Histopathologically, IPF is characterized by fibroblastic foci in the lung interstitium resulting from excessive fibroblast proliferation and

myofibroblast activation (5). Hyaluronan (HA) is an extracellular glycosaminoglycan that has been reported to be elevated in the lungs of patients with IPF, and the concentrations of HA correlate with the

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severity of the disease (6). HA fragments that accumulate during inflammation stimulate inflammatory cells to release multiple proinflammatory and fibrogenic mediators, such as transforming growth factor (TGF)- β and IL-13, which in turn trigger myofibroblast proliferation and recruitment (7, 8). Fibroblasts are one of the major HA-producing cell types in the lung (9, 10). We showed that an invasive phenotype of fibroblasts is required for the development of severe lung fibrosis and that fibroblast invasion depends on expression of hyaluronan synthase 2 (HAS2) and CD44 (11) as well as β -arrestins (12). Fibroblasts isolated from IPF patient lung showed increased invasive capacity (11). Mice with overexpression of Has2 in fibroblasts had increased invasive capacity and worsened lung fibrosis, whereas mice with targeted deletion of Has2 in fibroblasts showed reduced fibroblast invasion and decreased lung fibrosis after bleomycin challenge (11). This phenotype was confirmed by our subsequent studies (13–15) as well as studies by other groups (16–21).

Pirfenidone (22, 23) and nintedanib (24) have been approved for patients with IPF, and they have been shown to reduce the loss of lung function in patients with IPF (22, 23). Pirfenidone showed an antifibrotic effect in bleomycin-induced lung fibrosis (25) and in other animal models (26). The antifibrotic effect of pirfenidone is attributed to its ability to block multiple fibrotic signaling pathways,

including TGF- β , platelet-derived growth factor (PDGF), and TNF- α (26). Therefore, it is plausible to target these signaling pathways to block fibroblast activation. Mitogen-activated protein kinase-activated protein kinase 2 (MK2) is an intracellular serine/threonine kinase and is activated by p38 mitogen-activated protein kinase (27). MK2 contains an N-terminus proline-rich region that interacts with SH3 (SRC homology 3) domain, a well-conserved catalytic domain, a nuclear export sequence, and a nuclear localization sequence. Thr222 and Thr334 are the main phosphorylation sites (28). MK2 targets the TGF- β pathway distal to p38 (27). MK2 was required for TGF- β -induced α -smooth muscle actin expression and myofibroblast differentiation (29). Elevated MK2 activation has been reported in lung tissues of patients with IPF and in bleomycin-challenged mouse lungs (30). In addition, MK2 deficiency in mice results in downregulation of inflammatory cytokine expression, including TNF- α , IL-1 β , and IL-6 (31, 32).

The MK2 inhibitor (MK2i), known as MMI-0100, is a synthetic, 22-amino acid, cell-permeant peptide. MMI-0100 inhibits MK2 activity once it is entered into the cells (33). Recent reports demonstrated promising antifibrotic effects of this peptide. MMI-0100 inhibited fibrosis after vascular graft and abdominal surgeries (34), prevented postoperative ileus in mice (35), reduced bleomycin-induced lung fibrosis (30), and reduced ischemic heart injury

and cardiac fibrosis (36–38). In the present study, we explored the role of MMI-0100 in regulating HAS2 expression and invasiveness of fibroblasts, and we further determined the molecular mechanism by which MMI-0100 inhibits pulmonary fibrosis. We identified a novel mechanism of a feed-forward loop between MK2 activation and HAS2 expression in fibrogenesis. We demonstrated that MK2 inhibition reduced the invasive capacity of lung fibroblasts from patients with IPF and bleomycin-treated fibrotic mice. *In vivo* administration of MMI-0100 was able to attenuate bleomycin-induced lung fibrosis.

Methods

Mice

α -Smooth muscle actin–human HAS2–transgenic mice (SMA-HAS2) were described previously (11, 39). Mice with MK2 deletion in collagen, type I, α 2 chain (Col1a2)-expressing fibroblasts (Col1a2-CreER; Rosa-Tomato; MK2^{fllox/fllox} or MK2 ^{Δ Col}) were generated by cross-breeding of Col1a2-CreER (40) and MK2^{fllox/fllox} (full name Mapkapk2^{tm1.1Yaff/J}; The Jackson Laboratory) mice (41) on a Rosa-Tomato background to facilitate cell sorting. All mice were housed in a pathogen-free facility at Cedars-Sinai Medical Center. All animal experiments were permitted by the institutional animal care and use committee at Cedars-Sinai Medical Center (protocols IACUC004722 and IACUC004751).

Figure 1. Mitogen-activated protein kinase-activated protein kinase 2 inhibitor (MK2i, MMI-0100) inhibition reduced hyaluronan (HA) production and invasion of idiopathic pulmonary fibrosis (IPF) fibroblasts. Fibroblasts were isolated from lung tissues of patients with IPF and healthy donors. (A) IPF fibroblasts were subjected to invasion assay with and without MMI-0100 treatment. The number of invasive cells per view was counted ($n = 3$ wells per treatment). * $P < 0.05$. (B) HAS2 (hyaluronan synthase 2) mRNA was determined with RT-PCR in invasive and noninvasive fibroblasts from 11 patients. * $P < 0.05$. (C) Invasive and noninvasive IPF fibroblasts were plated into multiple well plates. HA in conditioned media was measured ($n = 4$). * $P < 0.05$. (D) Equal numbers of IPF fibroblasts were plated into multiple-well plates and serum starved overnight. Cells were then treated with transforming growth factor (TGF)- β and with or without MMI-0100. HA concentrations were measured in the medium collected 24 hours after treatment ($n = 3$ or 4 wells per treatment). * $P < 0.05$; ** $P < 0.01$.

Figure 2. Knockdown of HAS2 reduced MK2 activation of IPF fibroblasts. IPF fibroblasts were serum starved after cultured to 80% confluence overnight. The cells were then transfected with HAS2 siRNA and control (Ctl) siRNA. (A) HAS2 siRNA interference reduced *HAS2* expression and (B) HA production. (C) Total cell lysates were harvested 48 hours after transfection and subjected to Western blotting for phospho-MK2 (pMK2) and total MK2. (D) Densitometric analyses of individual band intensities of expression of activated Thr334-MK2 when normalized to β -actin ($n = 3$). $^{**}P < 0.01$. hHAS2 = human HAS2. Complete images of uncropped gels are shown in Figure E4 in the data supplement.

Mouse Lung Fibrosis Model

Bleomycin (Hospira) at 2.5 U/kg was instilled as described previously (11, 42). For Col1a2-CreER;Rosa-Tomato; MK2^{flox/flox} mice and littermate control Col1a2-CreER;Rosa-Tomato mice, four doses of tamoxifen were injected 2 weeks before bleomycin administration. MMI-0100 at 37.5 μ g/kg was injected daily intraperitoneally at designated starting points after bleomycin injection.

Fibroblast Isolation and Culture

Human lung fibroblasts were isolated from lung transplant explants of patients with IPF and healthy donors as previously reported (43). Mouse lung fibroblasts were isolated from bleomycin-treated Day 10 mouse lungs (11). Cells from passages 4 to 7 were used for experiments. All experiments were approved by the Cedars-Sinai Institutional Review Board (IRB Pro00035396) and carried out in accordance with the guidelines outlined by the board.

Matrigel Invasion Assay

Fibroblast invasion assay was performed as described previously (11, 14). Equal numbers of fibroblasts were plated into the top chamber of Matrigel invasion Transwells, and Dulbecco's modified Eagle's medium containing 10 ng/ml PDGF (R&D Systems) for human fibroblasts or 10% FBS for mouse fibroblasts, respectively, was added to the bottom chamber. After 24 hours, the invading cells were counted, and invasive and noninvasive fibroblasts were harvested from the bottom and top of the Transwells, respectively.

RNA Interference Assay

Transfection with siRNA duplexes was performed as described previously (44). siRNA duplexes designed to target nucleotide sequences of the human

HAS2 gene (National Center for Biotechnology Information GenBank accession no. NM_005328) (*HAS2* small interfering RNA (si), CCAGCT AGTAGGTCTCATAAA), as well as a

Figure 3. MK2 activation was correlated with HA production and fibroblast invasion. Lung tissues were harvested from mice overexpressing HAS2 in the myofibroblast compartment (SMA-HAS2-transgenic mice) and littermate controls 10 days after bleomycin treatment, and lung fibroblasts were isolated. (A) HA concentration in 24-hour culture medium of fibroblasts treated with TGF- β for 24 hours ($n = 3$). $^{***}P < 0.001$. (B) Invasive capacity of fibroblasts from SMA-HAS2-transgenic mice and littermate controls ($n = 3$ or 4). $^{***}P < 0.001$. (C) Total lung tissue proteins were subjected to Western blot analysis for p-MK2 and total MK2. (D) Densitometric analyses of individual band intensities of activated Thr334-MK2 expression when normalized to GAPDH ($n = 4$). $^{**}P < 0.01$. Complete images of uncropped gels are shown in Figure E5 in the data supplement. (E) Total proteins of lung fibroblasts treated with and without MK2i were subjected to Western blot analysis for p-MK2 and total MK2. (F) Densitometric analyses of individual band intensities of activated Thr334-MK2 expression when normalized to GAPDH ($n = 4$). $^{***}P < 0.001$; $^{****}P < 0.0001$. Complete images of uncropped gels are shown in Figure E6 in the data supplement. WT = wild type.

Figure 4. MK2i reduced HA production and fibroblast invasion. Mouse lung fibroblasts were isolated from mice overexpressing HAS2 in the myofibroblast compartment (SMA-HAS2 mice) and WT mice 10 days after bleomycin treatment. Equal numbers of cells were plated into 24-well plates. The cells were starved overnight before being treated with TGF- β , and with or without MK2 inhibition with MMI-0100. HA concentrations were measured in 24-hour culture media of fibroblasts from (A) WT mice and (B) SMA-HAS2 mice ($n = 4$). $^{**}P < 0.01$; $^{****}P < 0.0001$. (C and D) Fibroblasts from bleomycin-treated SMA-HAS2 and control mice with and without MMI-0100 treatment at either (C) 4 μ M or (D) 6 μ M were subjected to invasion assay. Invasive capacity (invaded cells per view) of primary lung fibroblasts from (C) control mice and (D) SMA-HAS2 mice was compared ($n = 3$). $^{*}P < 0.05$; $^{**}P < 0.01$.

control siRNA (control small interfering RNA (si) with sense sequence UUCUCCGAACGU-GUCACGUdTdT) were obtained from Qiagen.

Quantification of mRNA Expression

Gene expression was examined with SYBR Green fluorescent dye, enabling real-time detection of PCR products. The primers used were as follows: human *HAS2* (National Center for Biotechnology Information GenBank accession no. NM_005328) forward: 5'-TCG CAA CAC GTA ACG CAA T; human *HAS2* reverse: 5'-ACT TCT CTT TTT CCA CCC CAT TT; human *GAPDH* (National Center for Biotechnology Information GenBank accession no. NM_002046) forward: 5'-CCC ATG TTC GTC ATG GGT GT; human *GAPDH* reverse: 5'-TGG TCA TGA GTC CTT CCA CGA TA. The fold change of the target genes was calculated by using the comparative cycle threshold method.

Western Blot Analysis

Proteins were assessed with Western blotting as previously described (44). The membranes were probed with antibodies against phospho-Thr³³⁴MK2 and total MK2 (Cell Signaling Technology). β -actin or GAPDH was used as a loading control. Quantitative densitometric analysis relative to β -actin or GAPDH was used to aid clarity.

HA Quantification

The HA contents in conditioned media from fibroblast culture and mouse BAL were measured as described previously (9).

Hydroxyproline Assay

Collagen content in the lung tissue was measured with the conventional hydroxyproline method as previously described (11).

Statistical Analysis

Data are presented as the mean $\pm$ SEM. Student's *t* tests were used for two-group comparisons. One-way or two-way ANOVA with the Bonferroni or Tukey posttest was performed for multiple comparisons. Results were considered statistically significant at $P \leq 0.05$. Statistical analysis was done with Prism software (GraphPad Software).

Results

MK2 Regulates Fibroblast Invasion and HA Production

We reported previously that fibroblasts isolated from IPF lungs were more invasive than fibroblasts from healthy human lungs (11). Because pirfenidone has been shown to be antifibrotic by inhibiting fibroblast biology, we tested if pirfenidone affects fibroblast invasion. Pirfenidone significantly reduced invasion of fibroblasts from three patients with IPF (see Figures E1A–E1C in the data supplement). The inhibitory effect of pirfenidone on fibroblast invasion was in a dose-dependent manner (Figure E1C). However, pirfenidone did not affect proliferation of IPF fibroblasts at these experimental conditions (Figure E1D). Therefore, pirfenidone inhibited the invasive capacity of IPF fibroblasts, and this

inhibitory effect was not due to inhibition of cell proliferation.

Pirfenidone has been shown to inhibit multiple kinase pathways, including PDGF and TGF- β . MK2 is a p38-associated kinase and has been shown to transmit multiple signals. MK2 activation was also elevated in the lung tissues of patients with IPF (30). We further demonstrated that MK2 activation was increased in IPF fibroblasts (Figure E2). To discern the role of MK2 in fibroblast activation and invasion, we

Figure 5. MK2i reduced HA accumulation in bleomycin-injured mouse lung *in vivo*. WT C57BL/6J mice were subjected to bleomycin-induced lung injury. From Day 3, mice started to receive intraperitoneal injections of MMI-0100 37.5 μ g/kg or PBS daily. (A) Mice were killed on Day 10, and their lung tissues were harvested. (B) HA contents in lung tissues were compared between the MMI-0100 treatment group and the PBS control group ($n = 4$). $^{**}P < 0.01$.

treated fibroblasts isolated from IPF lungs with MMI-0100 (an MK2i) *in vitro*. MMI-0100 treatment significantly reduced the invasive capacity of IPF fibroblasts (Figure 1A). We previously reported that HA accumulation was increased in IPF patient lungs (6) and that HA and HAS2 mediate fibroblast invasion (11). In the present study, we confirmed that IPF fibroblasts expressed high amounts of HAS2 mRNA (Figure E3A) and released more HA into culture media (Figure E3B). Furthermore, we confirmed that invasive fibroblasts isolated from IPF patient lungs had increased HAS2 expression (Figure 1B) and produced increased HA compared with noninvasive fibroblasts (Figure 1C). Importantly, MMI-0100 treatment reduced TGF- β -induced HA production of IPF fibroblasts (Figure 1D). These data indicated that elevated MK2 activation in IPF fibroblasts might cause an increase in invasion capacity by mediating HAS2 gene expression and HA

production. MK2 inhibition also blocked TGF- β -induced connective tissue growth factor (33). However, connective tissue growth factor had no effect on HA production or invasiveness of fibroblasts (Figures E3C and E3D).

HAS2 Regulates MK2 Activation in IPF Fibroblasts

To further explore the relationship between MK2 activation and HAS2 expression in IPF fibroblasts, we performed siRNA transfection to knock down HAS2 expression in lung fibroblasts isolated from patients with IPF. The efficiency of HAS2 siRNA interference was confirmed with a reduction in HAS2 expression (Figure 2A) and HA production of the transfected cells (Figure 2B). Knocking down HAS2 caused significant reduction of MK2 activation (Figures 2C and 2D), suggesting a feed-forward loop between MK2 activation and HAS2 expression.

Enhanced MK2 Activation in Mouse Lung Fibroblasts Overexpressing HAS2

To investigate the role of MK2 activation in lung fibrosis, we used a bleomycin-induced lung injury model with mice overexpressing HAS2 in myofibroblasts (SMA-HAS2 mice) (11). HA production of fibroblasts isolated from bleomycin-treated SMA-HAS2 mouse lungs were dramatically increased, both at baseline and after TGF- β treatment (Figure 3A). Consistent with our previous findings (11), fibroblasts isolated from SMA-HAS2 mouse lungs also had increased invasive capacity (Figure 3B). Next we looked at MK2 activation in SMA-HAS2 fibroblasts using Western blot analysis and found that phospho-MK2 was increased in the total protein of bleomycin-treated SMA-HAS2 lung tissue (Figures 3C and 3D) and fibroblasts (Figures 3E and 3F) compared with that of wild-type (WT) mice. These data indicated that HAS2 overexpression enhanced MK2 activation in fibroblasts.

Figure 6. MK2i attenuated mouse lung fibrosis *in vivo*. Mice overexpressing HAS2 in the myofibroblast compartment (SMA-HAS2-transgenic mice) and littermates were subjected to bleomycin-induced lung injury. From Day 7, the mice started to receive MMI-0100 at 37.5 μ g/kg or PBS daily. The mice were killed on Day 21, and BAL fluid and lung tissues were harvested (A). Hydroxyproline contents in lung tissues were compared between the MMI-0100 treatment group and the PBS control group in (B) WT mice and (C) SMA-HAS2-transgenic mice, respectively ($n = 7$ or 8). * $P < 0.05$. (D) Trichrome staining of lung slides from bleomycin Day 21 WT and SMA-HAS2-transgenic mice with and without MMI-0100 treatment (scale bars: 100 μ m). (E) Has2 mRNA expression ($n = 4$) and (F) hyaluronan concentrations in BAL fluid in four groups of mice were compared ($n = 5$ – 9). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

MK2 Inhibition Reduced HA Production and Invasive Capacity of Fibroblasts

To further study the role of MK2 activation in regulating matrix production and the invasive phenotype of fibroblasts, we isolated lung fibroblasts from bleomycin-injured SMA-HAS2 and WT mice. We treated the cells with MK2i together with TGF- β in the culture and measured HA concentration in the culture medium. MK2 inhibition reduced TGF- β -induced HA production of fibroblasts isolated from bleomycin-treated SMA-HAS2 mice as well as those from WT mice (Figures 4A and 4B). Next we looked at the effect of MK2 inhibition on fibroblast invasion and found that MMI-0100 treatment reduced the invasive capacity of the fibroblasts from bleomycin-injured SMA-HAS2 and WT mouse lungs (Figures 4C and 4D). A higher dose (6 μ M) of MK2i was required to reduce the invasive capacity of fibroblasts from bleomycin-treated SMA-HAS2 mice (Figure 4D), whereas a lower dose (4 μ M) was effective for inhibition of the invasive capacity of WT fibroblasts (Figure 4C).

MK2 Inhibition Reduced HA Production and Lung Fibrosis *In Vivo*

Next we tested to see if our observation on the role of MK2 activation on HA production and fibroblast invasive capacity had biological relevance *in vivo*. In a bleomycin model, HA accumulation starts at Day 3 and peaks at Day 7, and declines 10–14 days after injury (45). We treated WT mice with bleomycin and then started administration MMI-0100 by intraperitoneal injection daily from Day 3 after bleomycin treatment (Figure 5A). The mice were killed at Day 10, and HA concentrations were measured in the lung tissues. MMI-0100 treatment reduced HA accumulation in the lung compared with that of the control mice receiving PBS (Figure 5B). Next we tested the effect of MMI-0100 treatment on lung fibrosis. We treated SMA-HAS2 mice and littermate controls with bleomycin at Day 0 and started daily MMI-0100 treatment at Day 7 after bleomycin (Figure 6A). The mice were killed at Day 21, and their lungs were harvested for collagen deposition analysis. The results showed that MMI-0100 treatment was able to attenuate

bleomycin-induced lung fibrosis of SMA-HAS2 mice and WT controls, as evidenced by reduced collagen contents (Figures 6B and 6C) and reduced trichrome staining in the lungs

(Figure 6D). Has2 expression in lung tissues and HA concentrations in the BAL fluid of mice that received MMI-0100 treatment were also reduced (Figures 6E and 6F).

Figure 7. Deletion of MK2 in collagen, type I, α 2 chain (*Col1a2*)-expressing fibroblasts attenuated lung fibrosis. Mouse lung fibroblasts were isolated from *Col1a2*-CreER;*Rosa*-Tomato;*MK2*^{flx/flx} mice (*MK2* Δ Col) and littermate *Col1a2*-CreER;*Rosa*-Tomato mice 10 days after bleomycin treatment. (A) Fibroblast total cell protein lysis was subjected to Western blot analysis for p-MK2 and total MK2. (B) Densitometric analyses of individual band intensities of activated Thr334-MK2 expression when normalized to GAPDH knockdown ($n = 4$). *** $P < 0.001$. (C) Murine Has2 (*mHas2*) gene expression in fibroblasts with and without MK2 knockdown ($n = 4$). ** $P < 0.01$. (D) Invasive cells per view of fibroblasts isolated from *MK2* Δ Col mice and littermate control mice ($n = 3$). * $P < 0.05$. (E–G) *Col1a2*-CreER;*Rosa*-Tomato;*MK2*^{flx/flx} mice (*MK2* Δ Col) and littermate *MK2*^{flx/flx} mice were subjected to bleomycin for 21 days. (E) Hydroxyproline contents ($n = 7$ –10; * $P < 0.05$) and (F) trichrome staining (scale bars: 100 μ m), as well as (G) HA concentrations in BAL fluid ($n = 4$ –6; * $P < 0.05$) were determined. Complete images of uncropped gels in A are shown in Figure E7 in the data supplement.

Conditional Deletion MK2 in Fibroblasts Attenuated Lung Fibrosis in Mice

To further demonstrate the role of MK2 inhibition in pulmonary fibrosis, we generated a mouse line (Col1a2-CreER; Rosa-Tomato;MK2^{flox/flox} or MK2^{ΔCol}) with specific deletion of MK2 in collagen-expressing fibroblasts, in which Cre-mediated Tomato expression facilitated fibroblast isolation. Fibroblasts isolated from bleomycin-treated MK2^{ΔCol} mice showed reduced MK2 phosphorylation (Figures 7A and 7B), reduced Has2 expression (Figure 7C), and decreased invasion capacity (Figure 7D) compared with fibroblasts isolated from littermate control mice. Mice with MK2 deletion in fibroblasts developed less lung fibrosis, as shown by reduced hydroxyproline content in the lungs and reduced trichrome staining (Figures 7E and 7F) on Day 21 after bleomycin treatment. HA concentrations in BAL fluid of bleomycin-treated MK2^{ΔCol} mice were also reduced.

Discussion

In this study, we discovered that MK2 activation mediates HAS2 expression and HA production and that HAS2 in turn regulates MK2 activation, suggesting a feed-forward loop in fibroblast activation. MK2-blocking peptide inhibits fibroblast invasion *in vitro* and attenuates lung fibrosis *in vivo*. Thus, the study indicates that MK2 has a role in fibroblast invasion and fibrosis and that blocking MK2 may represent a feasible strategy for patients with severe pulmonary fibrosis.

We previously identified an invasive phenotype of fibroblasts from patients with IPF and from a bleomycin-induced fibrosis model (11). The invasive phenotype of fibroblasts is mediated by HAS2 (11). In the

present study, we provide several lines of evidence that the feed-forward regulation of MK2 and HAS2 may contribute to persistent fibroblast activation, augmented invasion, and relentless fibrosis in severe lung fibrosis:

1. MK2 activation mediated fibroblast invasion and HA release.
2. HAS2 activation and upregulation caused MK2 phosphorylation, and knockdown of HAS2 led to reduction in MK2 phosphorylation.
3. MK2 inhibition with a permeable peptide inhibited fibroblast invasion as well as HA production in lung fibroblasts from either patients with IPF or bleomycin-injured mice.
4. MK2 inhibition led to a decrease in HA production and lung fibrosis in a bleomycin-induced fibrosis model.
5. Conditional deletion of MK2 in collagen-expressing fibroblasts attenuated lung fibrosis.

Thus, the study identified a role for MK2 in lung fibrosis and provides additional insights into the mechanism by which MK2 mediates HAS2 activation to influence fibroblast invasion and lung fibrosis. These data are consistent with previous studies on MK2 in lung fibrosis. MK2 targets the TGF- β pathway distal to p38 (27) and is required for TGF- β -induced myofibroblast differentiation (29). Pirfenidone is able to block multiple fibrotic signaling pathways, including TGF- β (26), to elicit its antifibrotic effect. Although we do not know if the mode of action of MK2 inhibition is related to the mechanism of pirfenidone in limiting fibrosis, targeting these profibrotic pathways is a plausible strategy for patients with severe pulmonary fibrosis. Just as pirfenidone showed an antifibrotic effect in multiple-organ fibrosis (46, 47), MK2 inhibition seems to have a wide range of organ

fibrosis (30, 36–38). Because TGF- β is a main driver of tissue fibrosis (8), targeting TGF- β has been investigated in patients as well as in animal models. To avoid the potential adverse effect of blocking TGF- β directly, researchers in several studies reported modulation of TGF- β signaling. For example, partial inhibition of integrin $\alpha_v\beta_6$ or $\alpha_v\beta_1$ prevented pulmonary and other organ fibrosis without exacerbating inflammation (48, 49). We showed previously that mediating TGF- β signaling by targeting TGF- β /bone morphogenetic protein-associated molecules such as FSTL1 (follistatin-related protein 1) and BMPER (bone morphogenetic protein-binding endothelial regulator) can be used as a strategy for blocking lung fibrosis in cell culture *in vitro* and animal models *in vivo* (14, 50). MK2 has been shown to mediate TGF- β signaling and TGF- β -activated cellular events in multiple ways (30). We offer a new mechanism by which MK2 regulates fibroblast invasion through an MK2–HAS2 activation loop. Inhibition of MK2 would effectively block fibrogenesis.

Taken together, these data suggest that there is a feed-forward loop between MK2 activation and HAS2 expression in fibroblasts. MK2 inhibition with a permeable peptide inhibited fibroblast invasion and HA production in lung fibroblasts *in vitro*, and it also attenuated lung fibrosis in a bleomycin-induced fibrosis model *in vivo*. Thus, blocking MK2 may represent a feasible strategy for patients with severe pulmonary fibrosis. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

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