Matrix Stiffness–Induced Myofibroblast Differentiation Is Mediated by Intrinsic Mechanotransduction

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The mechanical properties of the extracellular matrix have recently been shown to promote myofibroblast differentiation and lung fibrosis. Mechanisms by which matrix stiffness regulates myofibroblast differentiation are not fully understood. The goal of this study was to determine the intrinsic mechanisms of mechanotransduction in the regulation of matrix stiffness–induced myofibroblast differentiation. A well established polyacrylamide gel system with tunable substrate stiffness was used in this study. Megakaryoblastic leukemia factor-1 (MKL1) nuclear translocation was imaged by confocal immunofluorescent microscopy. The binding of MKL1 to the α-smooth muscle actin (α-SMA) gene promoter was quantified by quantitative chromatin immunoprecipitation assay. Normal human lung fibroblasts responded to matrix stiffening with changes in actin dynamics that favor filamentous actin polymerization. Actin polymerization resulted in nuclear translocation of MKL1, a serum response factor coactivator that plays a central role in regulating the expression of fibrotic genes, including α-SMA, a marker for myofibroblast differentiation. Mouse lung fibroblasts deficient in *Mkl1* did not respond to matrix stiffening with increased α-SMA expression, whereas ectopic expression of human *MKL1* cDNA restored the ability of *Mkl1* null lung fibroblasts to express α-SMA. Furthermore, matrix stiffening promoted production and activation of the small GTPase RhoA, increased Rho kinase (ROCK) activity, and enhanced fibroblast contractility. Inhibition of RhoA/ROCK abrogated stiff matrix–induced actin cytoskeletal reorganization, MKL1 nuclear translocation, and myofibroblast differentiation. This study indicates that actin cytoskeletal remodeling–mediated activation of MKL1 transduces mechanical stimuli from the extracellular matrix to a fibrogenic program that promotes myofibroblast differentiation, suggesting an intrinsic mechanotransduction mechanism.

Keywords: lung fibrosis; transcription factor; α-smooth muscle actin

Myofibroblasts are a key effector cell type that manifests connective tissue remodeling after lung injury (1, 2). These cells are responsible

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for excessive extracellular matrix (ECM) deposition in idiopathic pulmonary fibrosis (IPF). Fibroblasts and mesenchymal cells are a major cellular source of myofibroblasts (2). Acquisition of α smooth muscle actin (α-SMA) expression characterizes fibroblast-to-myofibroblast differentiation. Recent studies suggest that matrix stiffness, a measure of matrix resistance to mechanical deformation, regulates myofibroblast differentiation (3). Stiff matrix–induced myofibroblast differentiation has been extensively reported in fibroblasts isolated from heart (4), aortic valves (5), lung (6–8), liver (9, 10), and gingiva (11). Despite this, the molecular mechanisms by which matrix stiffness regulates myofibroblast differentiation are not well understood. A previous study demonstrates that myofibroblast contraction–induced matrix latent TGF-β1 activation requires stiffened matrix (7). Because activated TGF-β1 is a potent fibrogenic cytokine that promotes myofibroblast differentiation, this finding suggests that stiff matrix may regulate myofibroblast differentiation through an extrinsic mechanotransduction pathway in which stress fiber–generated contractile forces in response to matrix stiffening are transduced across the cell membrane and converted into the fibrogenic signal by activation of latent TGF-β1, resulting in myofibroblast differentiation (12).

Actin cytoskeletal filaments are physically connected to the ECM and neighboring cells through the focal adhesion complex and cadherins. Actin cytoskeletal components are crucial for mediating mechanical effects (13, 14). Actin microfilaments exist in a dynamic equilibrium. This dynamic structure monitors the external and internal tension on the cell and plays an important role in regulating diverse cellular functions, including proliferation, differentiation, migration, and gene expression (15–19). The small GTPase RhoA regulates the dynamic process of actin polymerization and depolymerization between monomeric (G-) and polymerized (F-) actin (20–22). Molecular mechanisms by which Rho affects the dynamics of actin filaments are complex and involve at least two major pathways: the mDia-mediated actin polymerization and the LIM kinase/cofilin-mediated actin depolymerization inhibition (23–25). Blocking RhoA and its effector Rho kinase (ROCK) signal has been shown to inhibit matrix stiffening–induced α-SMA expression and stress fiber formation in lung fibroblasts (8).
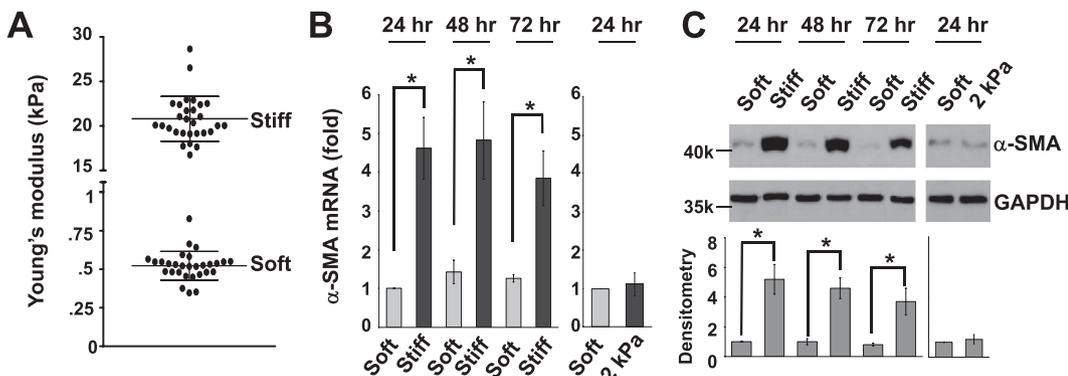
Figure 1. Stiff matrix promotes α-smooth muscle actin (α-SMA) expression by normal human lung fibroblasts. (*A*) Stiffness/elasticity of soft and stiff polyacrylamide (PA) hydrogels were determined by atomic force microscope indentation over multiple locations. Young's modulus of each individual location (*dots*) on gels and the mean values of soft and stiff gel stiffness (*long horizontal lines*) ± SD (*short horizontal lines*) are shown. (*B*) Normal human lung fibroblasts (CCL-210) were cultured on collagen-coated soft, stiff, and 2-kPa PA gels for 24 to 72 hours. Levels of α-SMA mRNA were determined by real-time PCR. 18S rRNA was used as reference control. The level of α-SMA mRNA from cells cultured on soft gels for 24 hours was set at 1. (*C*) Levels of α-SMA protein were determined by immunoblot. Relative levels of α-SMA protein were determined by scanning densitometry of the blots and normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression. The level of α-SMA protein from cells cultured on soft gels for 24 hours was set at 1. Results are the means of three separate experiments ± SD. *$P < 0.05$ for comparisons as indicated.

MKL1 (also known as myocardin-related transcription factor-A or MAL) is an actin dynamics sensor and a serum response factor (SRF) coactivator that plays a central role in activating fibrotic gene program (18, 26–31). Mice deficient in *Mkl1* show reduced myofibroblast differentiation and attenuated scar formation in response to myocardial infarction and angiotensin II treatment (26). MKL1 binds to G-actin in the cytoplasm, and this binding prevents MKL1 from nuclear import. When G-actin is polymerized into F-actin, MKL1 dissociates from G-actin. The release of MKL1 from G-actin allows its nuclear entry. In the nucleus, MKL1 interacts with SRF and transactivates fibrotic gene programming, such as α-SMA gene expression, which specifies myofibroblast differentiation (18, 26).

In this study, we demonstrated that actin cytoskeletal dynamics and MKL1 constitute an intrinsic mechanotransduction pathway that transduces and converts mechanical stimuli from the ECM into a fibrogenic signal that promotes fibroblast-to-myofibroblast differentiation.

MATERIALS AND METHODS

Preparation of Soft and Stiff Polyacrylamide Hydrogels and Mechanical Testing

Polyacrylamide (PA) gels with tunable stiffness were fabricated using a published protocol (32). Gel surfaces were coated with 0.1 mg/ml rat tail collagen I (BD Biosciences, San Jose, CA).
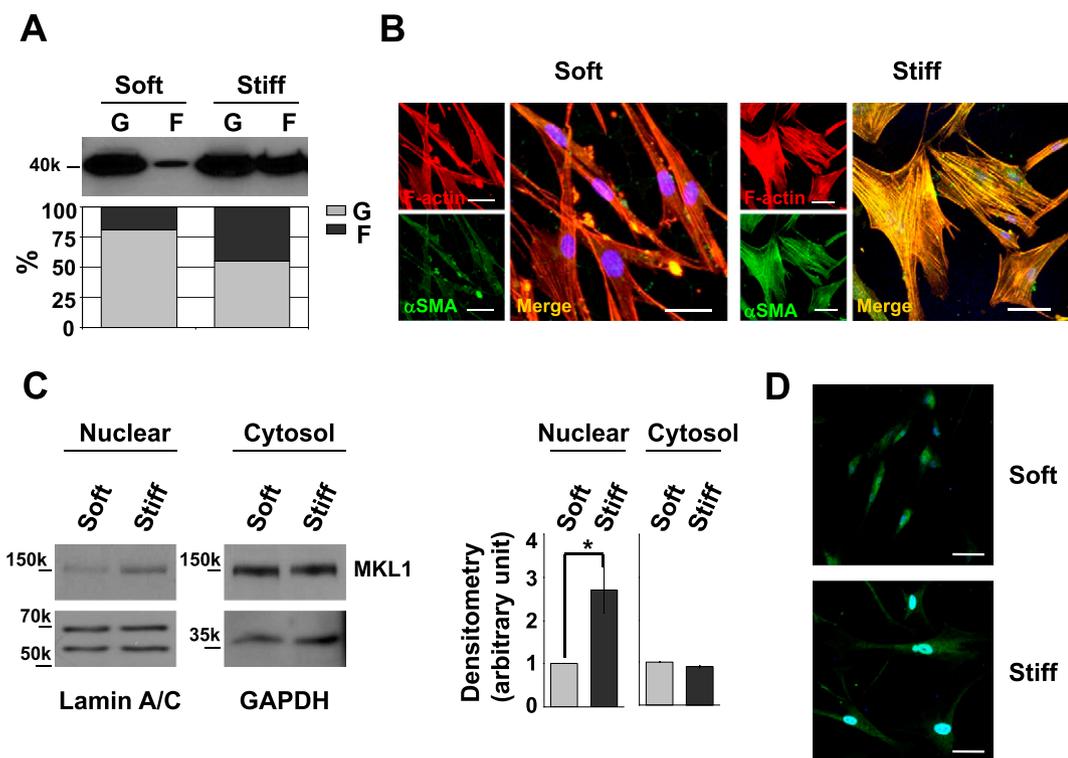
Figure 2. Stiff matrix induces changes in actin dynamics in favor of F-actin polymerization and α-SMA–containing stress fiber formation and promotes nuclear translocation of MKL1. CCL-210 cells were cultured on soft and stiff PA gels for 48 hours. (*A*) Changes in actin dynamics were determined by measurements of F/G-actin content using immunoblot and densitometric analyses. (*B*) Cells were fixed and stained for α-SMA with FITC-conjugated anti–α-SMA antibody (*green*) and F-actin with rhodamine-conjugated phalloidin (*red*). Confocal immunofluorescent images were taken and overlaid to colocalize α-SMA expression and F-actin (*yellow*). Nuclei were stained with DAPI. *Scale bars*: 50 μm. (*C*) Subcellular localization of MKL1 was determined by protein levels of MKL1 in the cytosolic fraction (Cytosol) and the nuclear fraction (nuclear). Relative levels of MKL1 protein were determined by scanning densitometry of the blots and normalized to GAPDH (for cytosolic MKL1) or lamin A/C (for nuclear MKL1). The level of MKL1 protein from cells cultured on soft gels was set at 1. Results are the means of three separate experiments ± SD. *$P < 0.05$. (*D*) Immunofluorescent staining and confocal microscopy were performed to visualize subcellular localization of MKL1 (*green*). Nuclei were stained with DAPI (*blue*). *Scale bars*: 50 μm.

PA gel mechanical properties were measured using an MFP-3D-BIO atomic force microscope (Asylum Research, Santa Barbara, CA) in contact mode. Samples were probed with a 4.74-μm-diameter beaded-tip (Bruker, Camarillo, CA), and cantilever spring constants were measured before sample analysis using the thermal fluctuation method (33), with nominal values of 20 to 30 mN/m. Young's modulus was obtained from force-indentation profiles using a Hertzian model and a sample Poisson's ratio of 0.5. A minimum of 20 independent measurements were obtained and analyzed for soft and stiff PA gels.

Isolation of Mouse Lung Fibroblasts

$Mkl1^{+/+}$ and $Mkl1^{-/-}$ lung fibroblasts were isolated from $Mkl1$ knockout mice and wild-type (WT) littermates (34) using an established protocol (35). Cells were passaged at a ratio of 1:3 and were used between passages 6 and 10.

F/G-actin Content

Relative proportions of F-actin and G-actin were determined using a kit from Cytoskeleton (Denver, CO). Blot images were scanned. Densitometry was performed using Scanalytic's One-Dscan version 1.31.

Quantitative Chromatin Immunoprecipitation Assay

A total of 1×10^6 cells were treated with 1% formaldehyde for 10 minutes at 37°C to cross-link histones to DNA. The cross-linked chromatin was sonicated to shear chromatin fragments of 200 to 1,000 base pairs. A portion of sheared chromatin was reversed at 65°C for 4 hours, and crosslinked DNA was purified by phenol/chloroform extraction. The DNA was saved and used for internal reference control in the after real-time PCR reactions. The rest of sonicated chromatin was immunoprecipitated with anti-SRF antibody at a concentration recommended by the manufacturer, while negative control was immunoprecipitated with no antibody. Immunocomplexes were recovered with Protein A agarose beads. Crosslinks were reversed and treated by Proteinase K to remove protein from the DNA. DNA was purified by phenol/chloroform extraction. Real-time PCR was performed to quantify SRF-binding α-SMA promoter fragments using the following primers: forward, 5'-CCTCCCACTTGCTTCCCAAACAAGGAGC-3'; reverse, 5'-AGCAGACCAGTGGAATGCA GTGGAAGAGAC-3'.

Immunofluorescence and Confocal Laser Scanning Microscopy

Immunofluorescent staining and confocal laser scanning microscopy were performed as described in our previous study (36).

Statistical Analysis

Statistical differences among treatment conditions were determined using one-way ANOVA (Newman-Keuls method for multiple comparisons). The analysis was performed with SigmaStat 3.0 software (SPSS Inc., Chicago, IL). Values of $P < 0.05$ were considered significant.

Additional details are provided in an online supplement.

RESULTS

Stiff Matrix Promotes α-SMA Gene Expression by Normal Human Lung Fibroblasts

PA hydrogel is a well established matrix substrate system that has been widely used for matrix stiffness studies (8, 37–40). Here, we used a recently published protocol (32) to fabricate soft and stiff PA gels that mimic the physical properties of normal and fibrotic lung microenvironments. Atomic force microscope indentation measurements showed that the soft PA gels had a mean stiffness value of 0.52 ± 0.09 kPa (Young's modulus) and the stiff PA gels had a mean
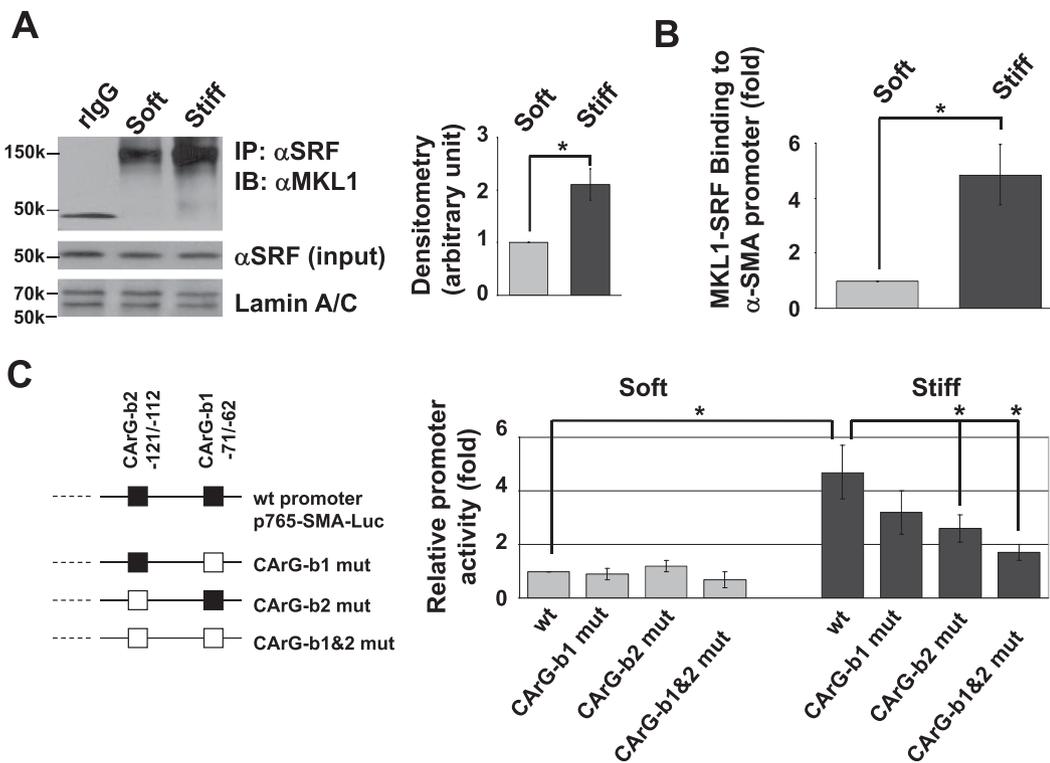

Figure 3. Stiff matrix promotes the formation of nuclear MKL1-SRF transactivator complex and the binding of the complex to α-SMA promoter, resulting in enhanced α-SMA promoter activity. (A) CCL-210 cells were cultured on soft and stiff PA gels for 48 hours. Nuclear proteins were extracted and subjected to immunoprecipitation with anti-SRF antibody (αSRF). Immunoprecipitates were blotted with anti-MKL1 antibody (αMKL1). Relative levels of MKL1 protein were determined by scanning densitometry of the blots and normalized to lamin A/C in the input. The level of MKL1 from cells on soft gels was set at 1. (B) Chromatin isolated from cells cultured on soft and stiff PA gels was immunoprecipitated with anti-SRF antibody. DNA in immunoprecipitated chromatin was purified and subjected to real-time PCR to quantify the amounts of α-SMA promoter fragment. Amplification of the same α-SMA promoter fragment using DNA extracted from preimmunoprecipitated chromatin as templates were used as reference control. (C) Schematic drawing of a 765 nt wild-type (WT) rat proximal α-SMA promoter fragment and three mutated promoter fragments harboring CArG box 1 mutations (CArG-b1 mut), CArG box2 mutations (CArG-b2 mut), and CArG box1 and box2 mutations (CArG-b1and2 mut). WT and mutated promoter reporters were transfected into CCL-210 cells. Transfected cells were cultured on soft and stiff PA gels for 24 hours. Promoter activity was determined by luciferase assay. Results are the means of three separate experiments ± SD; each experiment was performed in triplicate. *$P < 0.05$.

stiffness value of 20.80 ± 2.52 kPa (Figure 1A). These values were within the range of normal and fibrotic lung rigidities (8).

Normal human lung fibroblasts (CCL-210) cultured on stiff PA gels showed increases in the α-SMA mRNA level at 24 (4.6-fold), 48 (3.4-fold), and 72 hours (3.0-fold) compared with cells cultured on soft PA gels (Figure 1B). Consistent with this finding, stiff matrix promoted increases in the level of α-SMA protein at 24 (5.2-fold), 48 (4.6-fold), and 72 hours (4.2-fold) (Figure 1C). We also compared α-SMA expression by CCL-210 cells between 0.5 kPa stiffness condition and 2 kPa stiffness condition, a value on the higher end but within the stiffness range of normal lung (8). Lung fibroblasts expressed equal levels of α-SMA mRNA and protein under 0.5 and 2 kPa conditions (Figures 1B and 1C). Together, these data suggest that stiff matrix promotes myofibroblast differentiation of human lung fibroblasts.

Transmembrane integrins are important mechanotransducers. The α_v, α_{11}, β_1, β_3, and β_5 integrin subunits have been linked to the regulation of fibroblast/mesenchymal cell-to-myofibroblast differentiation (7, 41–46). To determine whether gene expression of these integrins was modulated by matrix stiffening, we performed real-time PCR–based array analysis. The data showed that these integrin gene transcriptions were not significantly altered by matrix stiffening (*see* Figure E1 in the online supplement).

Stiff Matrix Induces Changes in Actin Dynamics in Favor of α-SMA–Containing Stress Fiber Formation and Promotes Nuclear Translocation of MKL1

Actin microfilaments physically link to the ECM and are highly sensitive to the mechanical cues from the ECM (47). Meanwhile, changes in actin cytoskeletal dynamics (i.e., the F-actin/G-actin ratio) are associated with cell signaling events that regulate important cell functions, including differentiation and gene expression (15–19). In this study, we determined whether stiff matrix induced changes in actin dynamics. The F/G-actin ratio measurement showed that human lung fibroblasts cultured on soft matrix contained 19% F-actin and 81% G-actin, whereas the actin pool of cells derived from stiff matrix consisted of 45% F-actin and 55% G-actin (Figure 2A). These data suggest that stiff matrix changes actin dynamics in favor of filamentous actin polymerization. Immunofluorescent analysis showed that human lung fibroblasts cultured on stiff matrix had more intense staining of F-actin than did fibroblasts cultured on soft matrix (Figure 2B), consistent with the F/G-actin ratio result. Furthermore, stiff matrix enhanced the α-SMA staining signal and promoted α-SMA incorporation into stress fibers (Figure 2B).

MKL1 is an actin dynamics sensor and a SRF coactivator that has been shown to play a key role in activation of fibrotic gene programming (26, 27, 29, 30). To determine whether stiff matrix–induced changes in actin dynamics altered the subcellular localization of MKL1, we isolated nuclear and cytosolic proteins from human lung fibroblasts cultured on soft and stiff PA gels. Immunoblot analysis showed that stiff matrix induced a significant increase (2.7-fold) in the level of nuclear MKL1 protein (Figure 2C). Immunofluorescent analysis demonstrated increased nuclear localization of MKL1 in lung fibroblasts cultured on stiff matrix as opposed to cells cultured on soft matrix (Figure 2D). These data suggest that stiff matrix promotes actin cytoskeletal remodeling, resulting in nuclear translocation of MKL1.
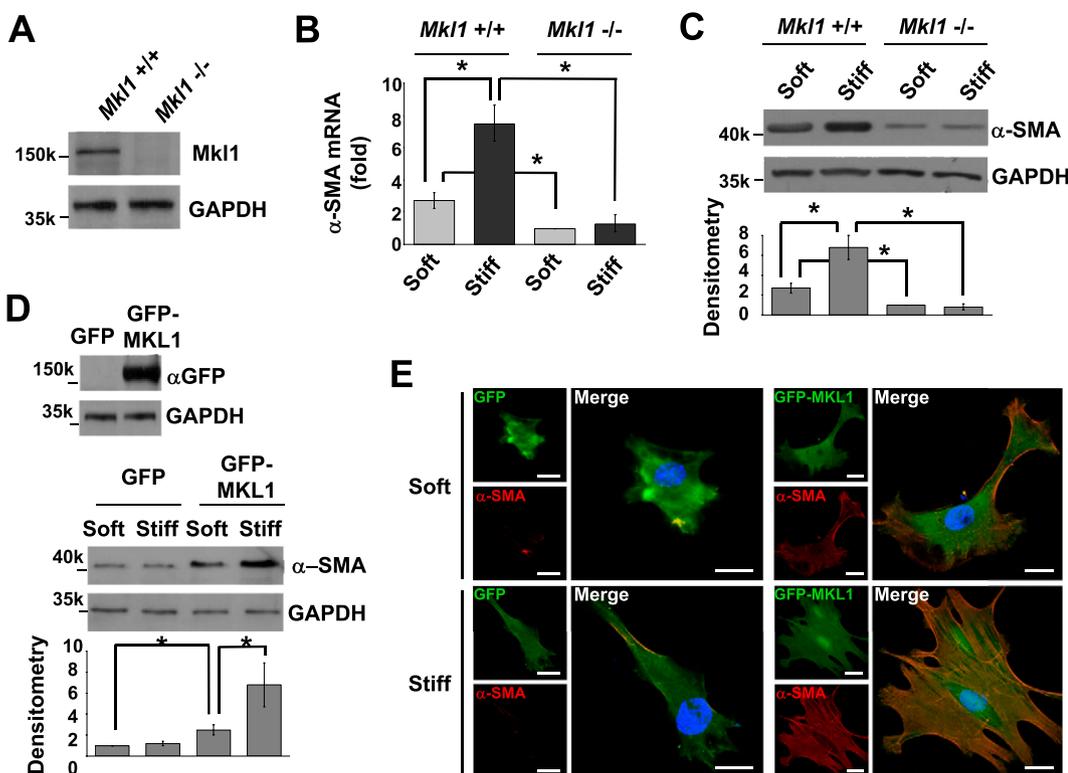

Figure 4. *Mkl1* deficiency renders mouse lung fibroblasts resistant to stiff matrix–induced myofibroblast differentiation. (*A*) Cell lysates from *Mkl1*$^{+/+}$ and *Mkl1*$^{-/-}$ lung fibroblasts were analyzed for Mkl1 expression with immunoblot. GAPDH was used as loading control. (*B*) *Mkl1*$^{+/+}$ and *Mkl1*$^{-/-}$ lung fibroblasts were cultured on stiff and soft PA gels for 48 hours. Levels of α-SMA mRNA were determined by real-time PCR. 18S rRNA was used as reference control. The level of α-SMA mRNA from cells cultured on soft gels was set at 1. (*C*) Levels of α-SMA protein were determined by immunoblot. Relative levels of α-SMA protein were determined by scanning densitometry of the blots and normalized to GAPDH expression. The level of α-SMA protein from cells cultured on soft gels was set at 1. (*D*) *Mkl1*$^{-/-}$ lung fibroblasts were transfected with green fluorescent protein (GFP)-human MKL1 cDNA expression vector or GFP vector alone (control). Expression of GFP-MKL1 fusion protein was determined by immunoblot with anti-GFP antibody. Transfected cells were cultured on stiff and soft PA gels for 48 hours. α-SMA protein levels were determined as described in C. Results are the means of at least three separate experiments ± SD. (*E*) *Mkl1*$^{-/-}$ lung fibroblasts transfected with GFP-MKL1 and empty vectors were cultured on soft and stiff PA gels. α-SMA stress fiber formation was visualized by immunofluorescent staining and confocal microscopy. GFP-MKL1 translocated into nuclear under a stiff matrix condition. *Scale bars*: 20 μm. *$P < 0.05$ for comparisons as indicated.

Stiff Matrix Increases Nuclear MKL1-SRF Transactivator Complex Formation and the Binding of MKL1-SRF Complex to the α-SMA Promoter, Resulting in Enhanced α-SMA Promoter Activity

Next, we determined whether stiff matrix–induced nuclear translocation of MKL1 promoted α-SMA transcriptional activation. To determine whether MKL1 nuclear accumulation increased the formation of the MKL1-SRF transactivator complex, we immunoprecipitated nuclear proteins isolated from human lung fibroblasts cultured on soft and stiff matrix with anti-SRF antibody. Immunoprecipitates were subjected to immunoblot analysis with anti-MKL1 antibody. Results showed that fibroblasts cultured on stiff matrix had a significant increase in the amount of SRF-binding MKL1 in nuclear compared with cells cultured on soft matrix (Figure 3A), suggesting that stiff matrix promotes nuclear MKL1-SRF complex formation.

To determine whether increased MKL1-SRF complex formation resulted in more MKL1-SRF transactivator binding to the α-SMA promoter, we performed quantitative chromatin immunoprecipitation assay. Results showed that SRF antibody–immunoprecipitated chromatin isolated from cells cultured on stiff matrix contained a significantly higher amount of α-SMA promoter sequence than chromatin isolated from soft matrix (Figure 3B), indicative of increased binding of the MKL1-SRF complex to the α-SMA promoter.

To determine whether stiff matrix–induced increases in the binding of MKL1-SRF to the α-SMA promoter promoted the promoter activity, we transfected a 765-bp section of WT rat proximal α-SMA promoter reporter (48) and three mutated promoter reporters that harbor mutations at the specific MKL1-SRF–binding DNA sequences—CArG box1 (CArG-b1), CArG box2 (CArG-b2), or both CArG box1 and box2 (CArG-b1and2)—into human lung fibroblasts (Figure 3C). For cells transfected with WT promoter reporter, stiff matrix significantly increased luciferase expression under α-SMA promoter control (Figure 3C), suggesting that matrix stiffening promotes the α-SMA promoter activity. Mutations at both CArG box1 and box2 or CArG box2 alone significantly inhibited stiff matrix–induced α-SMA promoter activation (Figure 3C), suggesting that increased promoter activity is MKL1-SRF dependent. Although mutations at CArG box1 also decreased stiff matrix–induced α-SMA promoter activity compared with WT, the inhibitory effect was not statistically significant (Figure 3C). Taken together, these results suggest that stiff matrix–induced nuclear translocation of MKL1 promotes nuclear MKL1-SRF complex formation and the binding of MKL1-SRF complex to the α-SMA promoter, resulting in enhanced α-SMA promoter activity.

Mkl1 Deficiency Renders Mouse Lung Fibroblasts Resistant to Stiff Matrix–Induced Myofibroblast Differentiation

To further determine whether MKL1 mediated stiff matrix–induced α-SMA expression and lung myofibroblast differentiation, we isolated primary lung fibroblasts from *Mkl1* knockout mice and the WT littermates. Immunoblot analysis confirmed that *Mkl1*$^{+/+}$ lung fibroblasts did, whereas *Mkl1*$^{-/-}$ lung fibroblasts did not, express Mkl1 (Figure 4A). *Mkl1*$^{+/+}$ lung fibroblasts cultured on stiff matrix had a 2.8-fold increase in the level of α-SMA mRNA
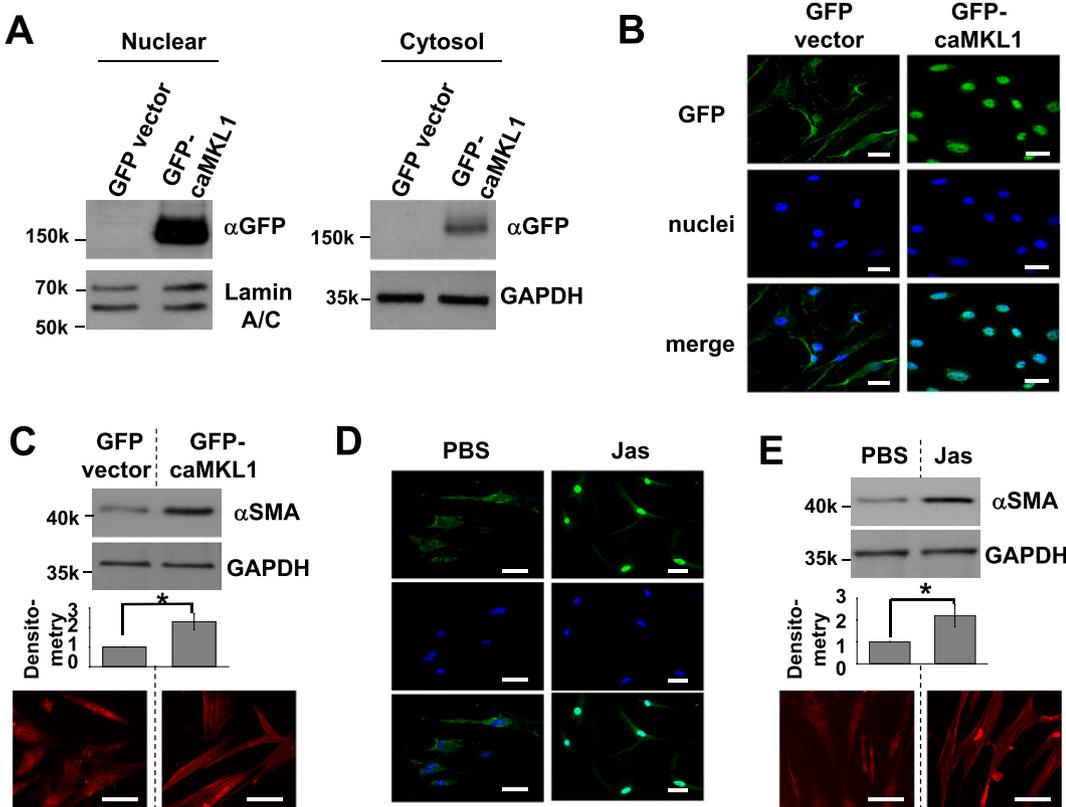

Figure 5. Overexpression of constitutively active MKL1 (caMKL1) and promotion of actin polymerization enable human lung fibroblasts to differentiate into myofibroblasts on soft matrix. (*A*) GFP-caMKL1 expression vector or GFP vector (control) was transfected into CCL-210 cells. GFP-caMKL1 expression in the nuclear fraction and the cytosolic fraction was determined by immunoblot with anti-GFP antibody (αGFP). GAPDH and lamin A/C were used as loading control. (*B*) Subcellular localization of GFP and GFP-caMKL1 in transfected cells were imaged with confocal immunofluorescent microscopy. Nuclei were stained by DAPI. *Scale bars*: 50 μm. (*C*) Lung fibroblasts expressing GFP-caMKL1 or GFP alone were cultured on soft PA gels for 48 hours. Levels of α-SMA protein were determined by immunoblot. Relative levels of α-SMA protein were determined by scanning densitometry of the blots and normalized to GAPDH expression. The level of α-SMA protein from control GFP-expressing cells was set at 1. Results are the means of three separate experiments ± SD. α-SMA stress fibers were stained using anti–α-SMA antibody followed by rhodamine-conjugated secondary antibody and imaged by confocal microscopy. (*D*) CCL-210 cells were cultured on soft PA gels in the presence of 200 nM jasplakinolide (Jas) or an equal volume of PBS for 24 hours. Subcellular localization of MKL1 was determined by immunofluorescent analysis followed by confocal microscopy. Nuclei were stained by DAPI. (*E*) Cells were treated as described in *D*. Levels of α-SMA protein and α-SMA stress fiber formation were determined as described in *C*. *$P < 0.05$. *Scale bars*: 50 μm.

(Figure 4B) and a 2.5-fold increase in the level of α-SMA protein (Figure 4C) compared with cells cultured on soft matrix, suggesting that stiff matrix promotes myofibroblast differentiation of WT mouse lung fibroblasts. In contrast, $Mkl1^{-/-}$ lung fibroblasts cultured on stiff matrix expressed α-SMA mRNA and protein at the levels equivalent to cells cultured on soft matrix (Figures 4B and 4C). These data suggest that $Mkl1$ deficiency renders mouse lung fibroblasts resistant to stiff matrix–induced lung myofibroblast differentiation. In addition, our data showed that $Mkl1^{-/-}$ mouse lung fibroblasts expressed significantly lower levels of baseline α-SMA mRNA and protein, both on soft and stiff matrix, than $Mkl1^{+/+}$ mouse lung fibroblasts (Figures 4B and 4C). This result was consistent with the previous report that MKL1 is a key factor that regulates smooth muscle gene expression (18).

To determine whether exogenous expression of human $MKL1$ cDNA restored the ability of $Mkl1^{-/-}$ mouse lung fibroblasts to respond to stiff matrix with increased α-SMA expression, we transfected green fluorescent protein (GFP)–full-length $MKL1$ cDNA expression vector and GFP control vector into $MKL1^{-/-}$ mouse lung fibroblasts. Ectopic expression of $MKL1$ rescued the ability of $MKL1^{-/-}$ lung to stiff matrix–induced myofibroblast differentiation, as evidenced by α-SMA protein expression (Figure 4D) and the formation of functional α-SMA stress fibers (Figure 4E), whereas cells transfected with GFP control vector did not show stiff matrix–induced myofibroblastic phenotype. Together, these data suggest that MKL1 mediates the transduction and conversion of mechanical stimuli from the matrix into the nuclear transactivation signal that promotes lung myofibroblast differentiation.

Overexpression of Constitutively Active MKL1 (Nuclear MKL1) and Promotion of Actin Polymerization Enable Lung Fibroblast-to-Myofibroblast Differentiation on Soft Matrix

To determine whether nuclear accumulation of MKL1 was sufficient to induce lung fibroblast-to-myofibroblast differentiation, we transfected an expression vector encoding GFP-tagged mutated $MKL1$, in which the N-terminal, G-actin–binding RPEL motifs has been deleted (18), into human lung fibroblasts. The mutated $MKL1$ autonomously locates in the nuclear due to the lack of G-actin binding and functions as a constitutively active MKL1 (caMKL1) (18). Consistent with the previous studies (18, 29, 49), transfected lung fibroblasts expressed GFP-ca MKL1 primarily in the nucleus (Figures 5A and 5B), whereas control cells transfected with vector alone expressed GFP in the cytoplasm (Figure 5B). On soft matrix, lung fibroblasts expressing GFP-caMKL1 had a significant increase in the level of α-SMA protein and formed α-SMA stress fibers compared with fibroblasts expressing GFP alone (Figure 5C). In a second approach, we induced forced actin polymerization in lung fibroblasts cultured on soft matrix by treating the cells with 200 nM jasplakinolide (Jas), a cell-permeable actin polymerization agonist (18). Compared with PBS treatment (control), Jas treatment induced MKL1 nuclear translocation (Figure 5D), α-SMA expression and the formation of functional α-SMA stress fibers (Figure 5E) in normal lung fibroblasts on soft matrix. Because a previous study showed that myofibroblast differentiation requires the formation of large focal adhesions (6), we determined whether forced nuclear translocation of MKL1 by overexpression of caMKL1 induces large focal adhesion formation on soft matrix. Overexpression of caMKL1 indeed promoted the formation of large focal adhesion complexes on soft PA gels (Figure E2). Together, these data suggest that actin polymerization-dependent MKL1 nuclear accumulation is sufficient to override matrix stiffness–regulated lung myofibroblast differentiation.

Stiff Matrix Promotes RhoA Production and Activation, Increases ROCK Activity, and Elevates Phosphorylation of the 20-kD Myosin Light Chain

It has been shown that Y-27632, a ROCK inhibitor, attenuates stiff matrix–induced actin stress fiber formation in lung
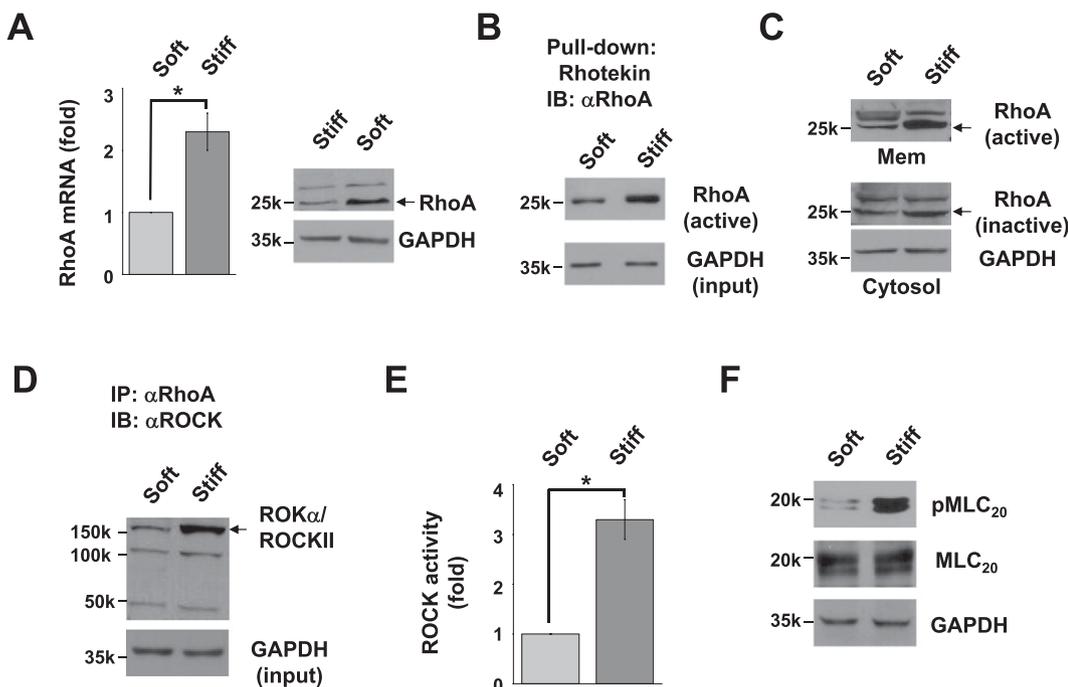

Figure 6. Stiff matrix promotes RhoA production and activation and increases ROCK activity. CCL-210 cells were cultured on soft and stiff PA gels for 48 hours. (*A*) Levels of RhoA mRNA and protein (*arrow*) were determined by real-time PCR and immunoblot, respectively. 18S rRNA was used as reference control (for real-time PCR). GAPDH was used as loading control (for immunoblot). (*B*) RhoA activity was determined by Rhotekin pull-down assay. Levels of Rhotekin-binding active RhoA were determined by immunoblot with anti-RhoA antibody (αRhoA). GAPDH in the input was used as loading control. (*C*) Subcellular localization of RhoA was determined by RhoA levels in the cytosolic faction (Cytosol) (*arrow*) and the membrane fraction (Mem) (*arrow*). GAPDH was used as loading control. (*D*) RhoA–ROCK interaction. Cell lysates were immunoprecipitated with anti-RhoA antibody (αRhoA). Immunoprecipitated proteins were blotted with anti-ROKα/ROCK II antibody (αROCK). GAPDH in the input was used as loading control. (*E*) ROCK activity was determined by incubation of cell lysates with ROCK-specific substrates and MYPT1 followed by colorimetric immunoassay. The level of ROCK activity from cells cultured on soft gels was set at 1. Results are the means of three separate experiments ± SD. (*F*) Levels of phosphorylated 20-kD myosin light chain (pMLC$_{20}$) and total 20-kD myosin light chain (MLC$_{20}$) were determined by immunoblot analysis. GAPDH was used as loading control. *$P < 0.05$ for comparisons as indicated.

fibroblasts (8), suggesting that RhoA/ROCK signal may be involved in the regulation of stiff matrix–induced actin cytoskeleton remodeling and lung myofibroblast differentiation. In this study, we examined effects of stiffened matrix on RhoA/ROCK production and/or activity. We showed that stiff matrix promoted RhoA expression at the mRNA level and at the protein level (Figure 6A). Rhotekin pull-down–based RhoA activity assay demonstrated increased RhoA activity in response to matrix stiffening (Figure 6B). Consistent with this, stiff matrix increased the level of membrane-bound active RhoA (Figure 6C). RhoA-ROCK interaction analysis revealed that increased RhoA activation promoted the binding of RhoA to ROCK (Figure 6D). Measurement of ROCK activity demonstrated increased ROCK activity in response to matrix stiffening (Figure 6E).

To determine the effect of matrix stiffening on fibroblast contractility, we analyzed phosphorylation of 20-kD myosin light chain (MLC_{20}), a key process involved in the contraction of smooth muscle cells and nonmuscle cells (50). Matrix stiffening increased the level of phosphorylated MLC_{20} (Figure 6F), suggestive of increased contractility. Collectively, these data provide evidence that stiff matrix directly promotes RhoA production and activation, increases ROCK activity, and promotes lung fibroblast contraction.

RhoA/ROCK Inhibition Abrogates Stiff Matrix–Induced Changes in Actin Dynamics and Actin Cytoskeletal Reorganization, MKL1 Nuclear Translocation, and Lung Myofibroblast Differentiation

We cultured human lung fibroblasts on stiff PA gels in the presence of RhoA/ROCK inhibitor (Y-27632 at 10 μM) or an equal volume of PBS (vehicle control). Compared with PBS treatment, Y-27632 treatment decreased F-actin content from 49 to

16% (Figure 7A) and attenuated α-SMA–containing stress fiber formation (Figure 7B). Y-27632 treatment inhibited nuclear translocation of MKL1 in lung fibroblasts cultured on stiff matrix (Figures 7C and 7D), abrogated stiff matrix–induced MLC_{20} phosphorylation (Figure 7E), and blocked stiff matrix–induced α-SMA expression at the mRNA level and at the protein level (Figures 7F and 7G). These data suggest that stiff matrix activates RhoA/ROCK and blocking RhoA/ROCK signal inhibits stiff matrix–induced lung myofibroblast differentiation and contractile activity by modulating actin cytoskeleton dynamics, MKL1 nuclear translocation, and myosin light chain phosphorylation.

DISCUSSION

In this study, we identified that actin dynamics and MKL1 mediate an intrinsic mechanotransduction pathway that regulates matrix stiffening–induced lung fibroblast-to-myofibroblast differentiation. This finding, along with previous findings that myofibroblast contraction promotes latent TGF-β1 activation on stiffened matrix (7, 12), suggests that matrix stiffening promotes myofibroblast differentiation via contraction/latent TGF-β1 activation–mediated extrinsic mechanotransduction pathway and actin dynamics/MKL1–mediated intrinsic mechanotransduction pathway (Figure E3).

Mechanical force–induced latent TGF-β1 activation requires the crosslinking of the large latent TGF-β1 complex into the ECM through the formation of covalent bonds between the latent TGF-β binding proteins and matrix fibronectin and fibrillin 1 (51, 52). It takes a relatively longer period of time (5–7 days) for in vitro cultured lung (myo)fibroblasts to form a latent TGF-β1–enriched ECM (7, 12). In the current study, we observed that matrix stiffening induces MKL1 nuclear translocation and
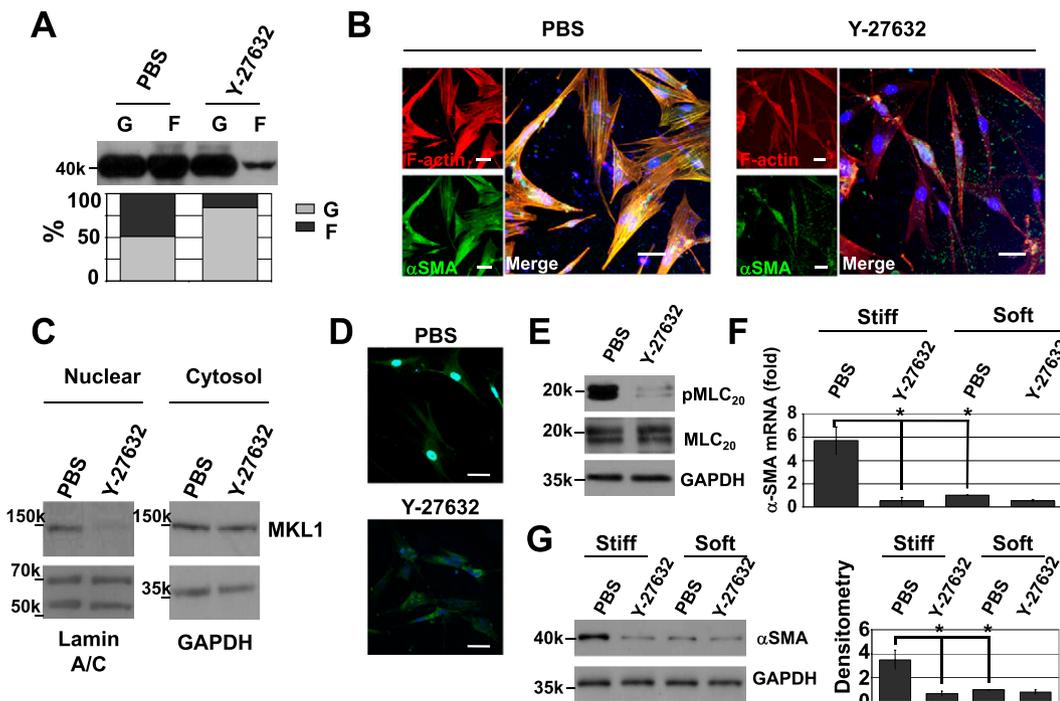

Figure 7. RhoA/ROCK inhibition blocks stiff matrix–induced actin cytoskeletal reorganization, MKL1 nuclear translocation, and lung myofibroblast differentiation. CCL-210 cells were cultured on stiff PA gels (*A–D*) or compliant and stiff PA gels (*E* and *F*) in the presence of 10 μM Y-27632 or an equal volume of PBS (vehicle control) for 48 hours. (*A*) Changes in actin dynamics were determined by immunoblot and densitometric analyses as described previously. (*B*) α-SMA–containing stress fibers were determined by costaining α-SMA (*green*) and F-actin (*red*). Images were taken under a confocal microscope. Nuclei were stained with DAPI. *Scale bars*: 50 μm. (*C* and *D*) Subcellular localization of MKL1 were determined by cell fractionation followed by immunoblot analysis. Confocal immunofluorescent microscopy was used to visualize MKL1 (*green*) subcellular localization. Nuclei were stained with DAPI (*blue*). *Scale bars*: 50 μm. (*E*) Levels of $pMLC_{20}$ and total MLC_{20} were determined by immunoblot analysis. GAPDH was used as loading control. (*F*) Levels of α-SMA mRNA were determined by real-time PCR. 18S rRNA was used as reference control. (*G*) Levels α-SMA protein were determined by immunoblot. GAPDH was used as loading control. Relative levels of α-SMA mRNA and protein were determined by scanning densitometry and normalized to 18S rRNA or GAPDH expression. The levels of α-SMA mRNA or protein from cells cultured on soft gels in the presence of PBS were set at 1. Results are the means of three separate experiments ± SD. *$P < 0.05$.

α-SMA expression in cultured lung fibroblasts as early as 24 hours, a time period in which latent TGF-β1–enriched ECM has not been formed. In addition, we precoated PA gels with collagen I (not fibronectin) to facilitate cell adhesion, excluding the possibility of predeposition of exogenous fibronectin in the PA substrates. These experimental designs ruled out that matrix stiffening–induced α-SMA gene expression in normal lung fibroblasts was a function of contractile force–induced latent TGF-β1 activation. This is particularly important because recent studies suggest that MKL1 also mediates TGF-β1–induced α-SMA expression and myofibroblast differentiation (26, 28, 53, 54). To confirm that TGF-β1 signal is not involved in matrix stiffening–induced myofibroblast differentiation in our system, we cultured human lung fibroblasts on stiff and soft matrix in the presence or absence of TGF-β neutralizing antibody for 24 hours. TGF-β neutralizing antibody did not block matrix stiffening–induced α-SMA expression (Figure E4). Together, our findings suggest that actin cytoskeletal remodeling–mediated activation of MKL1 represents a novel, intrinsic mechanism for matrix stiffening–induced lung fibroblast-to-myofibroblast differentiation. This mechanism is independent of the previously identified extrinsic mechanotransduction mechanism involved in mechanical tension–induced latent TGF-β1 activation.

Matrix stiffness, cell-generated forces (e.g., contractile forces), and externally applied forces are different mechanical stimuli. It is not known whether or not varying mechanical stimuli exert changes in cell behavior and function through common mechanotransduction pathways (13). A previous study has shown that externally applied forces promote α-SMA expression in cardiac fibroblasts through a MKL1-dependent mechanism (55). More recently, Dupont and colleagues (19) demonstrated that YAP and TAZ transcription factors translocate into nucleus in response to matrix stiffening and cytoskeletal tension, where they activate the gene program that promotes mesenchymal stem cell differentiation and endothelial cell survival. These studies, together with the current report, suggest that actin cytoskeleton and actin cytoskeleton–associated transcription factors constitute common mechanotransduction pathways that transduce mechanical stimuli into biochemical signals that activate gene expression. Our finding that matrix stiffening promotes fibroblast contractility, as evidenced by increased MLC_{20} phosphorylation, also suggests a correlation between stiffness sensing and cell-generated contractile forces.

RhoA cycles between an active GTP-bound form and an inactive GDP-bound form and its activity is regulated by different types of factors (25, 56). Although the link between matrix stiffening and RhoA activation has not been determined in the current study, several mediators, including G protein–coupled receptors (57–59) and integrins (60–62), could potentially regulate the process.

In addition to regulating α-SMA transcriptional activation, MKL1-SRF complex has been shown to bind the CArG consensus sequences in type I collagen α2 chain (Col1α2) gene promoter and promotes Col1α2 gene expression (63). Furthermore, MKL1 regulates miR-486, which represses phosphatase and tensin homolog, a negative regulator of the PI3K/Akt proliferative and survival signal (64). Phosphatase and tensin homolog downregulation has been linked to IPF lung myofibroblasts (46). Taken together, these findings indicate that stiff matrix–induced nuclear translocation of MKL1 may regulate not only the contractile characteristics (i.e., α-SMA expression) but also the high synthetic capacity for ECM proteins (i.e., Col1α2) and the antiapoptotic nature of myofibroblasts that are seen in persistent and progressive fibrosis such as IPF (65).

In summary, this study identified that actin dynamics/MKL1 constitute an intrinsic mechanotransduction pathway that mediates stiff matrix–induced lung myofibroblast differentiation. It suggests that blockade of stiff matrix–induced mechanotransduction by targeting key mechanotransducers, such as actin cytoskeleton and MKL1, may be an effective strategy for the treatment of persistent and progressive fibrosis.

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