Myofibroblasts Exhibit Enhanced Fibronectin Assembly That Is Intrinsic to Their Contractile Phenotype*

Received for publication, August 26, 2014, and in revised form, January 9, 2015. Published, JBC Papers in Press, January 27, 2015, DOI 10.1074/jbc.M114.606186

Elizabeth E. Torr‡, Caitlyn R. Ngam‡, Ksenija Bernau§, Bianca Tomasini-Johansson§, Benjamin Acton‡, and Nathan Sandbo‡

From the Departments of‡ Medicine and §Surgery, University of Wisconsin-Madison, School of Medicine and Public Health, Madison, Wisconsin 53792

Myofibroblasts have increased expression of contractile proteins and display augmented contractility. It is not known if the augmented contractile gene expression characterizing the myofibroblast phenotype impacts its intrinsic ability to assemble fibronectin (FN) and extracellular matrix. In this study we investigated whether myofibroblasts displayed increased rates of FN fibril assembly when compared with their undifferentiated counterparts. Freshly plated myofibroblasts assemble exogenous FN (488-FN) into a fibrillar matrix more rapidly than fibroblasts that have not undergone myofibroblast differentiation. The augmented rate of FN matrix formation by myofibroblasts was dependent on intact Rho/Rho kinase (ROCK) and myosin signals inasmuch as treatment with Y27632 or blebbistatin attenuated 488-FN assembly. Inhibiting contractile gene expression by pharmacologic disruption of the transcription factors megakaryoblastic leukemia-1 (MKL1)/serum response factor (SRF) during myofibroblast differentiation resulted in decreased contractile force generation and attenuated 488-FN incorporation although not FN expression. Furthermore, disruption of the MKL1/ SRF target gene, smooth muscle α-actin (α-SMA) via siRNA knockdown resulted in attenuation of 488-FN assembly. In conclusion, this study demonstrates a linkage between increased contractile gene expression, most importantly α-SMA, and the intrinsic capacity of myofibroblasts to assemble exogenous FN into fibrillar extracellular matrix.

Myofibroblasts, specially modified fibroblasts characterized by increased contractile gene expression (1–3). A key step in the formation of new ECM is the assembly of a fibrillar fibronectin (FN) matrix, which serves as a scaffold for the binding of collagen and other extracellular matrix proteins (4–6). The assembly of a fibrillar FN matrix by fibroblasts is a cell-mediated process (7, 8) that is dependent on the binding of FN dimers to α,β1 integrins and tension-mediated unfolding and assembly of FN molecules into fibrils (9). Individual cell types can demonstrate a differential intrinsic capacity to form fibrillar FN matrix (10). In response to transforming growth factor-β (TGF-β), fibroblasts differentiate to myofibroblasts and display increased expression and secretion of FN (11). In granulation tissue and fibrotic lung from patients with idiopathic pulmonary fibrosis, myofibroblasts are found in association with a dense fibronectin matrix (12, 13). Despite their association with abundant ECM in wound healing and fibrotic disorders, until now it has not been known if myofibroblasts exhibit an intrinsic capacity to more rapidly assemble a fibrillar FN matrix. In this study, we observed that human lung myofibroblasts assemble exogenous FN into a fibrillar FN matrix more rapidly than their undifferentiated counterparts independent of their expression of FN. We further observed that the increased contractile gene expression characterizing myofibroblasts is required for this effect. These findings establish a relationship between heightened contractile gene expression in myofibroblasts and ECM deposition and remodeling in fibrotic disorders of the lung.

EXPERIMENTAL PROCEDURES

Isolation and Primary Culture of Human Pulmonary Fibroblasts—Human lung fibroblasts (HLF) from normal lung were isolated as described previously (14–16). De-identified tissue samples were obtained from thoracic surgical resection specimens obtained by the Carbone Cancer Center Transla-

* This work was supported, in whole or in part, by National Institutes of Health Grants 5K88HL093367 (to N. S.) and P30 CA014520 (NCI; to the University of Wisconsin Comprehensive Cancer Center). This work was also supported by University of Wisconsin Development Funding (to N. S.).

1 Both authors contributed equally.

2 To whom correspondence should be addressed: Division of Allergy, Pulmonary, and Critical Care Medicine, Dept. of Medicine, University of Wisconsin, Madison, WI 53792. Tel.: 608-265-4576; Fax 608-263-3104; E-mail: nsandbo@medicine.wisc.edu.

3 The abbreviations used are: ECM, extracellular matrix; 488-FN, Alexa488-labeled fibronectin; α-SMA, smooth muscle α-actin; DOC, deoxycholate; FN, fibronectin; HLF, human lung fibroblasts; MKL1, megakaryoblastic leukemia-1; SRF, serum response factor; MLC, myosin light chain; FUD, functional upstream domain; RT, room temperature; ROCK, Rho kinase.
Fibronectin Assembly by Lung Myofibroblasts

Reagents—TGF-β, Y27632, and colchicine were from EMD Biosciences (616455, 688000, 234115; Gibbstown, NJ). CCG-1423 and Blebbistatin were from Cayman Chemical (10010350, 13186; Ann Arbor, MI). Cycloheximide was from Sigma (C7698). Mouse monoclonal antibodies against α-tubulin, β-actin, smooth muscle-α-actin (α-SMA), and vimentin was from Sigma (T6793, A1978, A5228, V6630). Rabbit polyclonal antibody against total FN was from Abcam (ab2413; Cambridge, MA). Mouse monoclonal antibody against the EDA (extra type III domain A) region of FN (IST-9) was from Abcam (ab6328). Rabbit polyclonal against collagen I was from Cedar Lane (CL50111AP, Burlington, NC). Mouse monoclonal antibody against β1-integrin was from BD Biosciences (610468). Mouse monoclonal antibody against GAPDH and rabbit polyclonal antibody against cofelin were from Santa Cruz Biotechnology (sc-32233, sc-33779, Santa Cruz, CA). Rabbit monoclonal antibodies against phospho-cofilin and myosin light chain 2 (MLC) as well as mouse monoclonal antibody against phospho-MLC were from Cell Signaling (3313, 8505, 3675; Beverly, MA). DAPI (4’,6-diamidino-2-phenylindole) was from Sigma (D9542). HiLyte Fluer 488 labeled FN (FN-488) was from Cytokeleton Inc. (FNRO2; Denver, CO). Secondary rhodamine- and fluorescein-conjugated secondary antibodies and developed by ECL reaction (Pierce). Digital chemiluminescent images were taken by a GE LAS4000 chemiluminescence imager. Densitometry of selected blots was performed using ImageGauge software (GE Healthcare) or ImageJ (20).

Deoxycholate (DOC) Extraction—Human lung fibroblasts (1 × 10⁵ per ml) were plated for 24 h in growth medium. Cells were then starved for 24 h in starvation medium and treated with TGF-β (1 ng/ml) for the desired times. Monolayers were washed twice with PBS and scraped into 2% DOC in TBS, 2 mM EDTA, 2 mM PMSF, and protease inhibitors and then treated with sample buffer as described above. All samples were boiled for 5 min before Western blot analysis.

Gel Contraction Assay—Fibroblasts were trypsinized and seeded in a 4-mg/ml rat-tail collagen solution (BD Biosciences) at a density of 3 × 10³ per ml in a 12-well plate (triplicates for each condition). Gels were allowed to solidify and were then released from the plate and allowed to contract in starvation medium for up to 48 h. Reduction of gel diameter was calculated in each condition using ImageJ.

siRNA Knockdown Assays—Cells were plated at 5 × 10⁴ cells/ml 24 h before transfection, reaching 70–80% confluency at the time of transfection. siRNA (Qiagen, Valencia, CA) was transfected using RNAiMAX transfection reagent (13778, Life Technologies) diluted in Opti-MEM (31985062, Gibco Life Technologies) with 1 μl of RNAiMAX per 10 pmol of siRNA according to the manufacturer’s instructions. 35 pmol of siRNA was used per well of a 12-well plate and 90 pmol per well for a 6-well plate. Cells were incubated for 24 h and then serum-starved, stimulated, or analyzed as required.

siRNA Sequences—Sequences were: Qiagen Allstars negative control (scrambled) siRNA (1027280), β-actin siRNA target sequence CGCGCGGCTGTGCTGACAA (SI03193946), and α-SMA siRNA target sequence TACGAGTTCCCTGATGGCGCA (SI00291340).

Reverse Transcription Quantitative Real Time PCR—Real time PCR was carried out as previously described (14). Briefly, total RNA was harvested, and 1 μg of total RNA was used as a template for random-primed reverse transcription using an iScript cDNA synthesis kit (1708891, Bio-Rad). Real time PCR
analysis was performed using iTaq SYBR Green supermix with ROX (1725120, Bio-Rad) in an ABI 7500 multicolor real time PCR detection system (Applied Biosystems). PCR primers for β-actin were CTCACCATGGATGATGATATCGC (forward) and AGGAATCCTTCTGACCCCATGC (reverse).

**Immunofluorescent Staining**—HLF treated with the desired conditions were plated into 8-well chamber μ-slides (Ibidi, Munich, Germany) at 2 × 10^5 per ml and allowed to adhere for 6 h. Cells were then washed twice with TBS, fixed with 4% paraformaldehyde, TBS for 30 min at room temperature (RT), and permeabilized with 0.2% Triton X-100, TBS for 5 min at RT. Cells were then blocked with 10% normal goat serum, 1% BSA in TBS for 1 h at RT and incubated overnight with the desired primary antibody at 4°C. Cells were washed with TBS and incubated with the corresponding rhodamine- or fluorescein (FITC)-conjugated secondary antibody for 75 min at 37°C, washed, incubated with DAPI/TBS (0.42 μg/ml) for 10 min at RT, washed, and mounted using Ibidi mounting media. Immunofluorescent images were obtained using an Olympus 1X71 fluorescent microscope and Q imaging Retiga 2000R camera.

**Fibronectin Assembly Assay**—We utilized an assay based upon those previously described (21–23). Briefly, pre-differentiated HLF were plated into 8-well chamber μ-slides at 2 × 10^5 cells/ml and allowed to adhere for 6 h 37°C 5% CO_2 in the presence of 10 μM cycloheximide. The medium was changed, and FN-488 was added to each well to achieve a final concentration of 4 μg/ml followed by incubation for the desired time points at 37°C 5% CO_2. The cells were then washed twice with TBS, fixed with 4% paraformaldehyde/TBS for 30 min at RT, and incubated with DAPI/TBS (as in immunofluorescent staining methods). Images were obtained using an Olympus 1X71 fluorescent microscope and Q imaging Retiga 2000R camera. For quantitation, 5 images at 10× magnification were taken per well using identical exposure times and thresholds. ImageJ was used to measure integrated density.

**DNA Transfection and Luciferase Reporter Assay**—HLF were plated at 5 × 10^4 cells/ml and incubated overnight in growth medium. Transient DNA transfections were performed using GenJet reagent (SL100488, SignaGen Laboratories, Gaithersburg, MD) following the standard manufacturer’s instructions. Cells were cotransfected with 300 ng of desired firefly luciferase reporter plasmid, 200 ng of constitutively active thymidine kinase promoter- Renilla luciferase reporter plasmid, and either 500 ng of pcDNA3.1 or 500 ng of p3xFLAG-MKL1. Cells were placed in growth media overnight and then serum-starved for 24 h followed by stimulation with the desired agonists for the desired time points as indicated in the figure legends. Cells were then washed with PBS and lysed in protein extraction reagent (78501, Thermo Fisher Scientific). The lysates were assayed for firefly and Renilla luciferase activity using the Dual-luciferase assay kit (E1960, Promega). To account for differences in transfection efficiency, firefly luciferase activity of each sample was normalized to Renilla luciferase activity.

**Statistical Analysis**—All the data represent the results of at least three independent experiments. Quantitative data were analyzed by Student’s t test when sole comparisons were made within each experiment. When multiple comparisons were made within the same data set, Bonferroni correction was utilized. A total false positive discovery rate of 0.05 was utilized for each experiment. Thus for each experiment, p ≤ 0.05/n, where n = number of planned comparisons within each experiment.

**RESULTS**

**Collagen Matrix Formation by Myofibroblasts Is Dependent on the Assembly of a FN Matrix**—HLF undergo myofibroblast differentiation and required fibrillar fibronectin assembly. HLF were treated with 1 ng/ml TGF-β for the times indicated. A, cells were extracted with a 2% DOC-containing buffer and reduced DOC-soluble FN and collagen increased. B, densitometry of Western blots of the indicated lysates and proteins. Data are normalized to the control condition for each protein to compare kinetics. C, HLF were treated with 1 ng/ml TGF-β for 24 h in the presence of the 56-residue FUD of the F1 adhesin protein of S. pyogenes (500 nM), D29 peptide control (500 nM), or vehicle alone. Cells were subjected to DOC extraction, with reduced samples subjected to PAGE and Western blotting with the indicated antibodies.

![FIGURE 1. Collagen deposition into the extracellular matrix accelerates during myofibroblast differentiation and requires fibrillar fibronectin assembly.](image-url)
Fibronectin Assembly by Lung Myofibroblasts

rate of FN assembly could impact ECM formation. Furthermore, the incorporation of collagen into the ECM requires the assembly of a FN matrix (4, 24) with the implication that changes in FN assembly rate may impact the formation of collagen matrix. Indeed, incubation with a 56-residue functional upstream domain of \textit{S. pyogenes} F1 adhesin (FUD), which interacts with FN and prevents its assembly into fibrils (18), inhibited the formation of DOC-insoluble FN matrix in both TGF-β-treated and untreated HLF (Fig. 1C). Importantly, FUD had no effect on the amount of soluble FN or collagen available for matrix deposition (Fig. 1C, left panel). This demonstrates the dependence of collagen matrix formation on assembly of fibrillar FN matrix by HLF (Fig. 1C), consistent with previous reports establishing the requirement of FN matrix assembly for collagen deposition (4). These data show that in HLF the expression of collagen is not sufficient to drive the formation of collagen matrix. Rather, deposition of collagen into the matrix can be limited by the cell-dependent assembly of a fibrillar FN matrix. In light of this we hypothesized that myofibroblasts may have intrinsic differences in their capacity to form a fibrillar FN matrix compared with undifferentiated fibroblasts, thereby impacting the formation of a mature collagen-containing ECM.

Myofibroblasts Assemble a Fibrillar FN Matrix More Rapidly Than Undifferentiated Fibroblasts, Independent of Endogenous FN Expression—We hypothesized that upon their differentiation to myofibroblasts, cells may be able to more rapidly assemble a fibrillar FN matrix, which could account for the accelerated formation of FN and collagen matrix over time in response to TGF-β shown in Fig. 1. To study the intrinsic ability of myofibroblasts to form a fibrillar FN matrix compared with their undifferentiated counterparts, we utilized an assay that allowed for direct assessment of the rate of FN assembly independent of cell-derived expression of FN. To do so, differentiated or undifferentiated cells were plated in the presence of cycloheximide to inhibit endogenous FN expression. After attachment and spreading, cells were incubated with Alexa488-labeled full-length FN (488-FN) for desired lengths of time followed by imaging, consistent with previously reported methodology (25, 26). Two separate models of myofibroblast differentiation were assessed. First, we utilized a prolonged exposure (72 h) of primary HLF to 1 ng/ml TGF-β to induce changes in morphology and gene expression that are consistent with a myofibroblast phenotype (15, 27), as in Fig. 1. Differentiated cells were then trypsinized and replated in the presence (or absence) of cycloheximide. Cells were then allowed to attach and spread for 6 h. After re-plating and attachment, cells previously differentiated with TGF-β retained increased expression of the myofibroblast marker, α-SMA, compared with unstimulated cells (Fig. 2, A and B) whether they were plated in cycloheximide or not. In contrast, after attachment and spreading in the presence of cycloheximide, neither fibroblasts nor myofibroblasts demonstrated any intracellular (Fig. 2A) or extracellular FN (Fig. 2C) consistent with previous observations in this model using cycloheximide to block FN synthesis (26). β1-Integrin expression did not appear to be significantly affected by re-plating cells in the presence of cycloheximide compared with vehicle control.

Next, to examine the cell-intrinsic potential of myofibroblasts to assemble soluble FN into a fibrillar matrix, we utilized a previously validated FN fibrillogenesis assay whereby 4 μg/ml Alexa488-labeled full-length FN (488-FN) was introduced into the cell culture media for the desired lengths of time followed by fixation and fluorescence microscopy (see “Experimental Procedures” and Refs. 21–23). Quantification of 488 intensity per cell revealed that myofibroblasts assembled significantly more 488-FN-containing fibrillar matrix than undifferentiated fibroblasts after 1.5 h of incubation with 488-FN. The differ-
enues between the amount of 488-FN incorporated into fibrils by myofibroblasts versus fibroblasts persisted at measured time points up to 24 h of incubation (Fig. 3, A–C). Representative images demonstrating increased 488-FN incorporation are shown for the 1.5- and 24-h time points (Fig. 3, B and C, respectively). By 6 h after the addition of 488-FN, a pool of DOC insoluble FN matrix had formed, as shown in Fig. 3D, which is consistent with previous reports (25). Together, these data reveal that TGF-β-induced myofibroblasts assemble a mature fibrillar FN matrix more rapidly than undifferentiated myofibroblasts, independent of the expression of endogenous FN.

To determine whether this finding was specific to TGF-β-induced myofibroblasts, we utilized colchicine, a microtubule disruptor that can also induce myofibroblast phenotype with prolonged exposure (16). We have previously shown that a 24-h exposure to 1 μM colchicine strongly induces both morphological and gene expression changes consistent with the myofibrobast phenotype (16). Here, we found that colchicine exposure stimulates increased expression of α-SMA compared with control cells (Fig. 4A). Furthermore, after inhibiting endogenous FN and adding 488-FN, significantly increased accumulation of 488-FN into fibrils was evident in myofibroblasts differentiated with colchicine compared with unstimulated controls (Fig. 4, B and C). Overall, these results demonstrate that myofibroblasts derived via either method display an increased cell-intrinsic ability to assemble exogenous FN into a fibrillar matrix compared with undifferentiated fibroblasts.

**FIGURE 3.** Myofibroblasts assemble exogenous fibronectin more rapidly than undifferentiated fibroblasts. Fibroblasts and myofibroblasts (treated with 1 ng/ml TGF-β for 72 h) were trypsinized and re-plated in the presence of 10 μM cycloheximide as in Fig. 2. After 6 h of attachment, they were then incubated with 4 μg/ml 488-FN for 0.5, 1.5, 6, or 24 h followed by fixation and counterstained with DAPI. A, integrated intensity of the 488 signal was quantified using ImageJ. Student’s t test with Bonferroni correction for multiple tests (total allowable false discovery rate = 0.05, thus *, p = 0.05/4 = 0.0125) was used for statistical analysis. NS, not significant. B, representative images from a 1.5-h incubation with 488-FN, with high power images showing the fibrillar matrix (scale bar = 250 μm for the top four panels and 50 μm for the bottom two panels). C, representative images from 24 h of incubation with 488-FN (scale bar = 250 μm). D, incubation with 4 μg/ml 488-FN for 6 h followed by DOC extraction and fixation showing residual DOC-insoluble matrix. Cells are absent due to DOC extraction (scale bar = 250 μm).

**FIGURE 4.** Colchicine-induced myofibroblasts assemble exogenous fibronectin more rapidly than undifferentiated fibroblasts. A, treatment of HLF with 1 μM colchicine (colch) for 24 h results in induction of α-SMA. B, fibroblasts and myofibroblasts (treated with 1 μM colchicine for 24 h) were trypsinized and re-plated in the presence of 10 μM cycloheximide, as in Fig. 2. After 6 h of attachment, they were then incubated with 4 μg/ml 488-FN for 1.5 h followed by fixation and counterstained with DAPI. Quantification of 488-FN incorporation using ImageJ was as in Fig. 3A. Student’s t test (*, p < 0.05) was used for statistical analysis. C, representative images after 1.5 h of incubation with 488-FN after fixation and DAPI co-stain (scale bar = 250 μm).

**FN Fibril Assembly by myofibroblasts Requires Rho Kinase (ROCK)/Myosin Signals—**FN assembly in fibroblasts requires intact actomyosin signaling via ROCK (28–30) and myosin (31). For this reason we explored the role ROCK played in mediating FN assembly by differentiated myofibroblasts. After the induction of myofibroblast differentiation by TGF-β (72 h), cells displayed increased levels of phospho-MLC, a downstream target of ROCK via regulation of MLC phosphatase, and phospho-cofilin, a downstream target of ROCK-activated LIM kinase (32) (Fig. 5, A and B). These results are consistent with our previous studies demonstrating a linear increase in cofilin phosphorylation over time in response to TGF-β stimulation (15). We have also previously shown that myofibroblast differentiation by TGF-β requires ROCK1/2, as incubation with the small molecule inhibitor of ROCK1/2, Y27632, just before prolonged TGF-β stimulation inhibits the expression of α-SMA (Ref. 15 and Fig. 5A). However, to determine the role of ROCK1/2 on the ability of myofibroblasts to assemble a fibrillar FN matrix, we added Y27632 to cells after they had been fully differentiated with TGF-β (72 h) but 0.5 h before incubation with 488-FN. As shown in Fig. 5A, short term incubation with Y27632 (0.5 or 2 h) after 72 h of TGF-β stimulation was able to decrease MLC and cofilin phosphorylation in myofibroblasts (Fig. 5, A and B) consistent with attenuation of ROCK1/2 activity. Importantly, short term treatment (0.5 or 2 h) did not alter the differentiation state of myofibroblasts, as α-SMA expression was unaffected. Incubation of Y27632 0.5 h before a 1.5-h incubation with 488-FN (2 h total) strongly inhibited the ability
of previously differentiated myofibroblasts to form FN fibrils (Fig. 5, C and D), confirming that differentiated myofibroblasts require intact ROCK signaling to assemble a fibrillar FN matrix. To investigate the contribution of myosin signaling to the augmented FN assembly exhibited by myofibroblasts, we utilized the myosin II inhibitor, blebbistatin. Pretreatment of previously differentiated myofibroblasts with blebbistatin 0.5 h before a 1.5-h 488-FN incubation (2 h total) prevented increased incorporation of 488-FN (Fig. 5, C and D), similar to the effects with Y27632. Importantly, treatment with blebbistatin did not affect ROCK activity (assessed by MLC and cofilin phosphorylation) (Fig. 5, A and B). In total, these observations demonstrate that the augmented assembly of fibrillar FN matrix by myofibroblasts depends on intact myosin signaling. Overall, our results reveal that although myofibroblasts have an increased intrinsic capacity to form a fibrillar FN matrix, both ROCK and myosin signaling remain critical to this process.

**FN Fibril Assembly by Myofibroblasts Requires Contractile Gene Expression**—Myofibroblasts exert increased cell-derived force on the ECM (33, 34), which could lead to increased transmission of cell-derived tension to the integrin-bound FN molecule with unfolding and exposure of cryptic FN binding sites (28), thereby increasing the assembly rate of FN. Our laboratory has previously shown that myofibroblast differentiation by TGF-β or colchicine is controlled by the transcription factors megakaryoblastic leukemia-1 (MKL1) and serum response factor (SRF) (14–16), which control the expression of smooth muscle contractile genes (35, 36). In light of these observations, we hypothesized that MKL1/SRF-dependent gene expression was critical for enabling a hypercontractile phenotype during myofibroblast differentiation and that loss of MKL1/SRF may impair the ability of myofibroblasts to efficiently assemble a fibrillar FN matrix. To investigate this possibility, we utilized the small molecule inhibitor of MKL1/SRF, CCG-1423, which acts via masking the nuclear localization signal of MKL1, disrupting its interaction with importins and inhibiting its ability to translocate to the nucleus and activate SRF (37). In HLF, CCG-1423 significantly attenuated the ability of exogenously expressed MKL1 to transactivate a luciferase reporter driven by the SRF binding element, CarG box (Fig. 6A). The degree of MKL1 inhibition by CCG-1423 in HLF was similar to the original report in HEK293 cells (38). In contrast, there was no effect of CCG-1423 on Smad-dependent signaling driven by TGF-β (Fig. 6B). HLF differentiated by TGF-β treatment for 72 h (myofibroblasts) demonstrate increased contractility of collagen gels compared with controls (Fig. 6, C and D). In contrast, cells treated with CCG-1423 for the duration of treatment with TGF-β showed attenuated collagen gel contraction compared with cells treated with TGF-β alone (Fig. 6, C, last well, and D). Overall, these results show that MKL1/SRF-dependent gene expression is required for the development of a contractile phenotype in myofibroblasts.

We then asked whether loss of MKL1/SRF affected FN assembly during myofibroblast differentiation. To assess the incorporation of unlabeled, endogenously expressed FN, we used extraction with DOC to isolate matrix-bound FN as pre-
MARCH 13, 2015 • VOLUME 290 • NUMBER 11

Fibronectin Assembly by Lung Myofibroblasts

Previously (10). Identical aliquots were reduced in the presence of dithiothreitol to dissociate multimerized FN and identify monomeric FN in subsequent Western blots without the worry of inefficient transfer of the multimers. These assays showed a decrease in TGF-β induction of DOC-insoluble monomeric FN by CCG-1423 (Fig. 7A, right panel) with no effect on TGF-β-induced increases in DOC-soluble FN (Fig. 7A, left panel). Again, this effect appeared to be independent of FN expression, as fibroblasts treated with TGF-β in the presence of CCG-1423 expressed the same amount of endogenous total FN or extratype III domain A containing FN (EDA-FN) as TGF-β treated fibroblasts alone (Fig. 7B). In contrast, CCG-1423 inhibited α-SMA induction compared with controls (Fig. 7B), consistent with our previous findings (14). To determine the effect of loss of MKL1-dependent gene expression on exogenous FN incorporation, we again utilized the 488-FN incorporation assay. HLF were treated with TGF-β for 72 h in the presence of CCG-1423 or vehicle control. HLF that were differentiated in the presence of CCG-1423 had attenuated levels of FN incorporation after 1.5 h (Fig. 7, C and D). This effect does not appear to be an off-target effect of CCG-1423, as short term treatment (2 h) of previously differentiated myofibroblasts with CCG-1423 did not result in an altered rate of FN assembly (Fig. 7, E and F). Altogether, these data suggest that although MKL1/SRF does not impact FN expression in response to TGF-β, efficient incorporation of FN into a fibrillar matrix by myofibroblasts requires that MKL1/SRF-dependent contractile genes be present.

α-SMA is a key MKL1/SRF-dependent contractile gene that is up-regulated in myofibroblasts (27). The significant induction in contractile force generation seen during myofibroblast differentiation parallels with, and is dependent upon, the expression of α-SMA (33). Thus, we hypothesized that the expression of α-SMA could be critical in facilitating the accelerated assembly of a FN matrix by myofibroblasts and partially account for the loss of FN matrix formation upon inhibition of MKL1/SRF gene expression with CCG-1423 (Fig. 7). Using an siRNA approach, we were able to efficiently inhibit the induction of α-SMA under conditions that promote myofibroblast differentiation (72 h TGF-β treatment) (Fig. 8A). By immunofluorescent staining, >60% of cells transfected with an siRNA targeted against α-SMA had no detectable staining (Fig. 8B), whereas the remainder had little staining, consistent with the near total loss of α-SMA expression seen by Western blotting (Fig. 8A). We utilized siRNA-mediated knockdown of the β isoform of actin as a control, which resulted in efficient knockdown of RNA levels (Fig. 8C). However, due to abundant basal β-actin expression in HLF and the stability of β-actin, protein levels were reduced by <50% compared with untransfected controls (Fig. 8A). After transfection, and upon re-plating, cells targeted with the siRNA against α-SMA maintained differences in α-SMA expression (Fig. 8D). Knockdown of α-SMA resulted in attenuated FN assembly compared with scrambled siRNA control or β-actin knockdown (Fig. 8, E and F). Overall, these findings allow us to conclude that α-SMA expression is required to facilitate the accelerated formation of FN matrix by myofibroblasts.

FIGURE 6. MKL1/SRF is required for maintenance of the contractile phenotype. A, HLF were co-transfected with cDNAs for the SRF firefly luciferase reporter, SREL, Renilla luciferase driven by thymidine kinase promoter, together with either empty vector (pcDNA3.1) or cDNA for full-length MKL1. After transfection, cells were incubated overnight in serum-free media in the presence or absence of 10 μM CCG-1423. Luciferase activity was measured as described under “Experimental Procedures.” Student’s t test (*, p < 0.05) was used for statistical comparison of vehicle and CCG-treated conditions. B, HLF were co-transfected with cDNAs for Smad-binding element (SBE)-driven firefly luciferase and Renilla luciferase driven by thymidine kinase promoter. After serum starvation, cells were incubated with 10 μM CCG-1423 (or vehicle) for 0.5 h followed by stimulation with 1 ng/ml TGF-β for 6 h. Cell lysates were used for luciferase activity. Student’s t test (p <0.05) was used for statistical comparison of vehicle and CCG-treated conditions. C, HLF were treated with 1 ng/ml TGF-β for 72 h in the presence of 10 μM CCG-1423 or vehicle control. Cells were seeded into collagen gels, released, and observed for contraction. Digital images were taken 48 h after gel release. D, gel areas were quantified from the digital images at each time point using ImageJ. Student’s t test (*, p <0.05) was used for statistical comparison of TGF-β and TGF-β/CCG groups 48 h post-release.
DISCUSSION

In our studies we have observed that myofibroblasts assemble a fibrillar FN matrix more rapidly than undifferentiated fibroblasts. This finding suggests that upon acquisition of a myofibroblast phenotype, cells obtain an intrinsic ability to increase FN matrix formation independent of their expression of FN. Given that FN assembly is a critical first step for the deposition of other ECM proteins, including collagen (4–6), it is possible that accelerated FN matrix formation may be a mechanism by which myofibroblasts can increase ECM formation during fibrosis.

During myofibroblast differentiation in response to TGF-β there is a marked increase in both the expression of proteins associated with the contractile apparatus (3) and cell contractility (39). The transcription factors MKL1/SRF regulate the expression of contractile genes (36) and are critical to mediating transition to the myofibroblast phenotype in response to TGF-β, matrix stiffness, and other profibrotic stimuli, (14–16, 40–43). Our studies demonstrate that although the expression of the TGF-β-responsive ECM protein FN is not regulated by MKL1/SRF, the assembly and deposition of a fibrillar FN matrix is nonetheless attenuated when MKL1/SRF-dependent gene expression is inhibited with CCG-1423. These results are consistent with the findings of Holtz and Misra, who noted decreased FN within the mesodermal ECM of the developing yolk sac upon conditional deletion of SRF (44).

The effect of MKL1/SRF inhibition is likely due, in part, to loss of α-SMA expression, as we observed that siRNA-mediated knockdown of α-SMA also resulted in significant attenuation of fibrillar FN assembly. Although it is also possible that MKL1/SRF inhibition could lead to loss of other components of the actomyosin filament system leading to attenuated force generation, our previous studies showed that TGF-β-induced actin stress fiber formation in HLF was preserved in the presence of...

![Image: Figure 7. Fibronectin incorporation in myofibroblasts requires intact MKL1 gene expression. A, HLF were stimulated with 1 ng/ml TGF-β1 in the presence of 10 μM CCG-1423 or vehicle control (added 30 min before stimulation) for 24 h. Cell lysates were separated into DOC-soluble and -insoluble fractions that were then treated with reducing sample buffers. Lysates were then subjected to PAGE on an 8% gel followed by immunoblotting with antibodies against total fibronectin, GAPDH, and vimentin. Fibronectin antibodies detect two predominant isoforms of fibronectin that are resolvable on 8% gels. B, cells were stimulated with 1 ng/ml TGF-β in the presence or absence of 10 μM CCG-1423 (added 30 min before stimulation) for 24 h. Whole cell lysates (in radioimmune precipitation assay buffer) were analyzed by PAGE on a 10% gel followed by immunoblotting with the indicated. C, HLF were stimulated with 1 ng/ml TGF-β for 72 h in the presence of 10 μM CCG-1423 or vehicle control (added 30 min before stimulation). After differentiation, cells were re-plated in the presence of cycloheximide and allowed to adhere for 6 h (as in Figs. 2 and 3). 488-FN at 4 μg/ml was then added for 1.5 h followed by fixation and co-staining with DAPI (scale bar = 125 μm). D, quantitation of 488-FN incorporation using ImageJ. Student’s t test (*, p < 0.05) was used for statistical analysis. E, HLF were differentiated with 1 ng/ml TGF-β for 72 h followed by re-plating and a 6-h attachment as above. After attachment, cells were then incubated with 10 μM CCG-1423 or vehicle control 30 min before the addition of 488-FN. Incubation with 4 μg/ml 488-FN for 1.5 h was followed by fixation and counterstained with DAPI (scale bar = 60 μm). F, quantification of 488-FN incorporation was done using ImageJ. Student’s t test (p < 0.05) was used for statistical analysis. NS, not significant.
CCG-1423 (15). Thus, the critical role that α-SMA plays in mediating increased force generation (33, 45) suggests that it is an indispensable part of the set of MKL1/SRF-dependent genes that potentiate myofibroblast contractile force generation and FN assembly by myofibroblasts. Overall, our findings suggest that increased contractile gene expression chronically modifies the fibroblasts’ potential for assembling FN, linking the hypercontractile features of the myofibroblast with accelerated ECM formation.

The mechanism by which increased α-SMA expression affects fibrillar FN assembly is likely via modulation of maximal force generation, as α-SMA has an essential role in facilitating
markedly increased contractile force generation and focal adhesion complex enlargement in myofibroblasts (33, 46). Transmission of cell-derived force to the FN molecule is thought to unwind the FN molecule, promoting fibrillogenesis via the unfolding and exposure of tension-sensitive FN binding sites (9). Activity of the small GTPase RhoA (28) and associated kinases, ROCK1 and ROCK2 (29), has been implicated in transient induction of force transmission to the FN molecule via the actin cytoskeleton and associated FN-binding focal adhesion complex. Increased α-SMA expression may lead to similar effects. Although this explanation may be the most straightforward, alternative mechanisms accounting for the increase in FN assembly could be at play. It is possible that the expression of α-SMA results in the reorganization of FN-binding focal adhesions, thereby increasing cell surface binding sites. Likewise, our studies have only tested two methods of inducing the myofibroblast phenotype, TGF-β and colchicine. It remains to be seen if this finding is preserved in myofibroblasts induced by matrix stiffness or via other agonists.

Given the role of FN matrix in serving as a template for subsequent deposition of other ECM proteins, it is tempting to see if this finding is preserved in myofibroblasts induced by matrix stiffness or via other agonists. Preliminary studies looking at interruption of Rho-dependent signaling have been performed, with evidence of a beneficial effect in a murine model of pulmonary fibrosis (47, 48). Additionally, recent reports have shown that inhibiting FN assembly can inhibit vascular remodeling and attenuate hepatic fibrosis (24, 49), suggesting that the regulation of this intrinsic cell function may hold promise for fibrosing disorders. Experiments assessing these possibilities are ongoing in our laboratory.

Acknowledgments—We thank Dr. Deane Mosher and Dr. Nickolai Dulin for review of the draft manuscript. We thank Dr. Deane Mosher for the gift of reagents. We thank the University of Wisconsin-Madison Carbone Cancer Center Biocore for collaboration in obtaining explanted lung tissue for the derivation of primary fibroblast lines.

REFERENCES

1. Phan, S. H. (1996) Role of the myofibroblast in pulmonary fibrosis. Kidney Int. Suppl. 54, S46–S48
2. Gabbiani, G., Ryan, G. B., and Majne, G. (1971) Presence of modified fibroblasts in granulation tissue and their possible role in wound contraction. Experientia 27, 549–550
3. Malmström, J., Lindberg, H., Lindberg, C., Bratt, C., Wieslander, E., Delander, E. L., Särnström, B., Burns, J. S., Mose-Larsen, P., Fey, S., and Marko-Varga, G. (2004) Transforming growth factor-β1 specifically induces proteins involved in the myofibroblast contractile apparatus. Mol. Cell. Proteomics 3, 466–477
4. Sottile, J., and Hocking, D. C. (2002) Fibronectin polymerization regulates the composition and stability of extracellular matrix fibrils and cell-matrix adhesions. Mol. Biol. Cell 13, 3546–3559
5. Sabatier, L., Chen, D., Fagotto-Kaufmann, C., Hubmacher, D., McKee, M. D., Annis, D. S., Mosher, D. F., and Reinhardt, D. P. (2009) Fibrillin assembly requires fibronectin. Mol. Biol. Cell 20, 846–858
6. Pereira, M., Rybarczyk, B. J., Oddrijn, T. M., Hocking, D. C., Sottile, J., and Simpson-Haidaris, P. J. (2002) The incorporation of fibrinogen into extracellular matrix is dependent on active assembly of a fibronectin matrix. J. Cell Sci. 115, 609–617
7. Peters, D. M., and Mosher, D. F. (1987) Localization of cell surface sites involved in fibronectin fibrillogenesis. J. Cell Biol. 104, 121–130
8. Singh, P., Carragher, C., and Schwarzbauer, J. E. (2010) Assembly of fibronectin extracellular matrix. Annu. Rev. Cell Dev. Biol. 26, 397–419
9. Baneyx, G., Baugh, L., and Vogel, V. (2002) Fibronectin extension and unfolding within cell matrix fibrils controlled by cytoskeletal tension. Proc. Natl. Acad. Sci. U.S.A. 99, 5139–5143
10. McKeown-Longo, P. J., and Mosher, D. F. (1983) Binding of plasma fibronectin to cell layers of human skin fibroblasts. J. Cell Biol. 97, 466–472
11. Ignoz, R. A., and Massagué, J. (1986) Transforming growth factor-β stimulates the expression of fibronectin and collagen and their incorporation into the extracellular matrix. J. Biol. Chem. 261, 4337–4345
12. Kuhn, C., and McDonald, J. A. (1991) The roles of the myofibroblast in idiopathic pulmonary fibrosis. Ultrastructural and immunohistochemical features of sites of active extracellular matrix synthesis. Am. J. Pathol. 138, 1257–1265
13. Singer, I. I., Kawka, D. W., Kazazis, D. M., and Clark, R. A. (1984) In vivo co-distribution of fibronectin and actin fibers in granulation tissue: immunofluorescence and electron microscope studies of the fibronexus at the myofibroblast surface. J. Cell Biol. 98, 2091–2106
14. Sandbo, N., Kregel, S., Taurin, S., Bhorade, S., and Dulin, N. O. (2009) Critical role of serum response factor in pulmonary myofibroblast differentiation induced by TGF-β. Am. J. Respir. Cell Mol. Biol. 41, 332–338
15. Sandbo, N., Lui, A., Kach, J., Ngam, C., Yau, D., and Dulin, N. O. (2011) Delayed stress fiber formation mediates pulmonary myofibroblast differentiation in response to TGF-β. Am. J. Physiol. Lung Cell Mol. Physiol. 301, L656–L666
16. Sandbo, N., Ngam, C., Torr, E., Kregel, S., Kach, J., and Dulin, N. (2013) Control of myofibroblast differentiation by microtubule dynamics through a regulated localization of mDia2. J. Biol. Chem. 288, 15466–15473
17. Tomasini-Johansson, B. R., Kaufman, N. R., Ensenberger, M. G., Ozeri, V., Hanksi, E., and Mosher, D. F. (2001) A 49-residue peptide from adhesin F1 of Streptococcus pyogenes inhibits fibronectin matrix assembly. J. Biol. Chem. 276, 23430–23439
18. Maurer, L. M., Tomasini-Johansson, B. R., Ma, W., Annis, D. S., Eicks-Taedt, N. L., Ensenberger, M. G., Sathyshur, K. A., and Mosher, D. F. (2010) Extended binding site on fibronectin for the functional upstream domain of protein F1 of Streptococcus pyogenes. J. Biol. Chem. 285, 41087–41099
19. Cen, B., Selvaraj, A., Burgess, R. C., Hitzler, J. K., Ma, Z., Morris, S. W., and Prywes, R. (2003) Megakaryoblastic leukemia 1, a potent transcriptional coactivator for serum response factor (SRF), is required for serum induc-
granulation tissue myofibroblasts and in quiescent and growing cultivated fibroblasts. J. Cell Biol. 122, 103–111
28. Zhong, C., Chranowska-Wodnicka, M., Brown, J., Shaub, A., Belkin, A. M., and Burridge, K. (1998) Rho-mediated contractility exerts a cryptic site in fibronectin and induces fibronectin matrix assembly. J. Cell Biol. 141, 539–551
29. Yoneda, A., Ushakov, D., Multhaupt, H. A., and Couchman, J. R. (2007) RPEL proteins are the molecular targets for CCG-1423, an inhibitor of RhoA transcriptional signaling. Mol. Biol. Cell 8, 1415–1425
30. Lemmon, C. A., Chen, C. S., and Romer, L. H. (2009) Cell traction forces direct fibronectin matrix assembly. Biophys. J. 96, 729–738
31. Maekawa, M., Ishizaki, T., Boku, S., Watanabe, N., Fujita, A., Iwamatsu, A., Obinata, T., Ohashi, K., Mizuno, K., and Narumiya, S. (1999) Signaling from Rho to the actin cytoskeleton through protein kinases ROCK and LIM kinase. Science 285, 895–898
32. Hinz, B., Celetta, G., Tomasek, J. J., Gabbiani, G., and Chaponnier, C. (2001) α-Smooth muscle actin expression up-regulates fibroblast contractile activity. Mol. Biol. Cell 12, 2730–2741
33. Hinz, B., Mastrangelo, D., Iselin, C. E., Chaponnier, C., and Gabbiani, G. (2001) Mechanical tension controls granulation tissue contractile activity and myofibroblast differentiation. Am. J. Pathol. 159, 1009–1020
34. Sobue, K., Hayashi, K., and Nishida, W. (1999) Expressional regulation of smooth muscle cell-specific genes in association with phenotypic modulation. Mol. Cell. Biochem. 190, 105–118
35. Miano, J. M., Long, X., and Fujiwara, K. (2007) Serum response factor: master regulator of the actin cytoskeleton and contractile apparatus. Am. J. Physiol. Cell Physiol. 292, C70–C81
36. Hayashi, K., Watanabe, B., Nakagawa, Y., Minami, S., and Morita, T. (2014) RPEL proteins are the molecular targets for CCG-1423, an inhibitor of Rho signaling. PLoS ONE 9, e89016
37. Evelyn, C. R., Wade, S. M., Wang, Q., Wu, M., Ihiughez-Lluhi, J. A., Merajver, S. D., and Neubig, R. R. (2007) CCG-1423: a small-molecule inhibitor of RhoA transcriptional signaling. Mol. Cancer Ther. 6, 2249–2260
38. Vaughan, M. B., Howard, E. W., and Tomasek, J. J. (2000) Transforming growth factor-β1 promotes the morphological and functional differentiation of the myofibroblast. Exp. Cell Res. 257, 180–189
39. Lockman, K., Hinson, J. S., Medlin, M. D., Morris, D., Taylor, J. M., and Mack, C. P. (2004) Sphingosine 1-phosphate stimulates smooth muscle cell differentiation and proliferation by activating separate serum response factor co-factors. J. Biol. Chem. 279, 42422–42430
40. Sandbo, N., Qin, Y., Taurin, S., Hogarth, D. K., Kreutz, B., and Dulin, N. O. (2005) Regulation of serum response factor-dependent gene expression by proteasome inhibitors. Mol. Pharmacol. 67, 789–797
41. Huang, X., Yang, N., Fiore, V. F., Barker, T. H., Desai, L., Bernard, K., and Thannickal, V. J. (2013) Inhibition of myofibroblasts genetic switch: inhibitors of myocardin-related transcription factor/serum response factor-regulated gene transcription prevent fibrosis in a murine model of skin injury. J. Pharmacol. Exp. Ther. 349, 480–486
42. Holtz, M. L., and Misra, R. P. (2011) Serum response factor is required for cell contact maintenance but dispensable for proliferation in visceral yolk sac endothelium. BMC Dev. Biol. 11, 18
43. Hinz, B., Gabbiani, G., and Chaponnier, C. (2002) The NH2-terminal peptide of α-smooth muscle actin inhibits force generation by the myofibroblast in vitro and in vivo. J. Cell Biol. 157, 657–663
44. Hinz, B., Dugina, V., Ballestrem, C., Wehrle-Haller, B., and Chaponnier, C. (2003) α-Smooth muscle actin is crucial for focal adhesion maturation in myofibroblasts. Mol. Biol. Cell 14, 2508–2519
45. Zhou, Y., Huang, X., Hecker, L., Kurundkar, D., Kurundkar, A., Liu, H., Jin, T. H., Desai, L., Bernard, K., and Thannickal, V. J. (2013) Inhibition of mechanosensitive signaling in myofibroblasts ameliorates experimental pulmonary fibrosis. J. Clin. Invest. 123, 1096–1108
46. Shimizu, Y., Dobashi, K., Iizuka, K., Horie, T., Suzuki, K., Takagoshi, H., Nakazawa, T., Nakazato, Y., and Mori, M. (2001) Contribution of small GTPase Rho and its target protein rock in a murine model of lung fibrosis. Am. J. Respir. Crit. Care Med. 163, 210–217
47. Altrock, E., Sens, C., Wuerfel, C., Vasel, M., Kawelke, N., Dooley, S., Sottile, J., and Nakchbandi, I. A. (2014) Inhibition of fibronectin deposition improves experimental liver fibrosis. J. Hepatol. 10.1016/j.jhep.2014.06.010