

Aortic Carboxypeptidase-like Protein (ACLP) Enhances Lung Myofibroblast Differentiation through Transforming Growth Factor β Receptor-dependent and -independent Pathways*

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Background: Dysregulated fibroblast-to-myofibroblast transitions cause fibrotic diseases.

Results: Aortic carboxypeptidase-like protein (ACLP) stimulates myofibroblast differentiation through activation of transforming growth factor β receptor signaling.

Conclusion: Eliminating ACLP activity reduces SMA expression and myofibroblast formation.

Significance: Reducing ACLP function in fibrotic tissue may provide a novel strategy to reduce the rate of fibrotic disease progression.

Idiopathic pulmonary fibrosis (IPF) is a chronic and fatal lung disease characterized by the overgrowth, hardening, and scarring of lung tissue. The exact mechanisms of how IPF develops and progresses are unknown. IPF is characterized by extracellular matrix remodeling and accumulation of active TGF β , which promotes collagen expression and the differentiation of smooth muscle α -actin (SMA)-positive myofibroblasts. Aortic carboxypeptidase-like protein (ACLP) is an extracellular matrix protein secreted by fibroblasts and myofibroblasts and is expressed in fibrotic human lung tissue and in mice with bleomycin-induced fibrosis. Importantly, ACLP knockout mice are significantly protected from bleomycin-induced fibrosis. The goal of this study was to identify the mechanisms of ACLP action on fibroblast differentiation. As primary lung fibroblasts differentiated into myofibroblasts, ACLP expression preceded SMA and collagen expression. Recombinant ACLP induced SMA and collagen expression in mouse and human lung fibroblasts. Knockdown of ACLP slowed the fibroblast-to-myofibroblast transition and partially reverted differentiated myofibroblasts by reducing SMA expression. We hypothesized that ACLP stimulates myofibroblast formation partly through activating TGF β signaling. Treatment of fibroblasts with recombinant ACLP induced phosphorylation and nuclear translocation of Smad3. This phosphorylation and induction of SMA was dependent on TGF β receptor binding and kinase activity. ACLP-induced collagen expression was independent of interaction with the TGF β receptor. These findings indicate that ACLP stimulates the fibroblast-to-myofibroblast transition by promoting SMA expression via TGF β signaling and promoting collagen expression through a TGF β receptor-independent pathway.

Idiopathic pulmonary fibrosis (IPF)³ is a chronic lung disease with no effective cure (1). It is characterized by sequential lung injury that results in epithelial and endothelial cell damage, inflammation, and progressive deposition of ECM molecules, including collagen. Fibrotic lungs contain large numbers of contractile, filament-laden parenchymal cells known as myofibroblasts and an increase in overall tissue contractility (2). These myofibroblasts characteristically express smooth muscle α -actin (SMA) and are known to be critical components of wound healing (2, 3). Myofibroblasts originate from different sources, including fibroblasts, epithelial cells, and bone marrow-derived cells (4–8). Myofibroblasts are spindle- or stellate-shaped cells that are similar to smooth muscle cells in that they are both contractile and contain SMA (2). Differentiated myofibroblasts are also responsible for increased collagen synthesis in the lung in IPF (2).

In the fibrotic lung, normal fibroblastic cells become activated by cytokines released from local inflammatory and resident cells after tissue injury to promote ECM component synthesis (9, 10). Mechanical challenges in the extracellular environment also stimulate the fibroblast-to-myofibroblast transition. In response to mechanical challenges, these cells develop stress fibers that connect the cell to ECM proteins (11). Along with extracellular remodeling, accumulation of active TGF β and the presence of ECM proteins like the extra domain A (ED-A) splice variant of fibronectin are required events for the production of SMA-positive myofibroblasts (9). TGF β is found at high levels in human IPF lungs (12) as well as in the lungs of mice and hamsters with bleomycin-induced fibrosis (13, 14). TGF β overexpression is sufficient to induce fibrosis in rat lungs (15). Additionally, TGF β promotes the formation of granulation tissue with abundant SMA-expressing myofibroblasts in rats and induces SMA expression in cultured fibroblasts (16). TGF β has also been shown to activate the transcriptional regulator myocardin-related transcription factor A

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³ The abbreviations used are: IPF, idiopathic pulmonary fibrosis; ECM, extracellular matrix; SMA, α -smooth muscle actin; MRTFA, myocardin-related transcription factor A; ACLP, aortic carboxypeptidase-like protein; rACLP, recombinant aortic carboxypeptidase-like protein.

(MRTFA), which stimulates both SMA and collagen expression (17, 18).

Canonical TGF β signaling is initiated when the latent TGF β complex is secreted by the cell and is dissociated and activated in the extracellular environment (19, 20). TGF β forms a dimer that binds to TGF β receptor II (T β RII), a serine/threonine kinase that activates TGF β receptor I (T β RI) (21). T β RI phosphorylates Smad1, Smad2, Smad3, or Smad5 (20, 22). The phosphorylated Smad forms a complex with Smad4 and translocates into the nucleus and acts as a transcription factor or DNA binding factor to promote the transcription of ECM genes such as fibrillar collagens (23).

Because ECM proteins and growth factors regulate the fibroblast-to-myofibroblast transition, our laboratory investigated the role of aortic carboxypeptidase-like protein (ACLP) in myofibroblast differentiation. ACLP is a secreted protein with more than 1100 amino acids that is associated with the ECM (24, 25). It contains an N-terminal signal sequence; a charged lysine, proline, and glutamic acid-rich domain; a discoidin domain; and a catalytically inactive metallocarboxypeptidase domain (24, 26, 27). ACLP is secreted by fibroblasts, myofibroblasts, and smooth muscle cells and is expressed in collagen-rich tissues (28). It contributes to both vascular smooth muscle cell proliferation and the wound healing process (29). Human lung tissue from patients with IPF and mouse lung tissue from mice with bleomycin-induced fibrosis exhibit abundant ACLP expression (25). Importantly, ACLP knockout mice are protected from bleomycin-induced fibrosis and have a decreased accumulation of lung myofibroblasts while exhibiting a normal inflammatory response. These findings indicate that ACLP potentially acts downstream of the inflammatory response (25). Additionally, the ACLP discoidin domain is at least partially responsible for mediating collagen matrix contraction (25).

The goal of this work is to elucidate the mechanisms by which ACLP influences myofibroblast formation. We found that ACLP promotes myofibroblast differentiation, in part, by stimulating the TGF β signaling pathway. These studies uncovered a novel pathway that may serve as a therapeutic target to combat IPF and other types of fibrosis.

EXPERIMENTAL PROCEDURES

Cell Culture—Primary lung fibroblasts were isolated from wild-type C57Bl6 mice or from MRTFA^{+/+} and MRTF^{-/-} mice (provided by Dr. Eric Olson) (30) harboring the Col1a1 promoter driving GFP (col3.6GFPtpz, provided by Dr. David Rowe) (31) as described (18). The Boston University School of Medicine Institutional Animal Care and Use Committee approved all animal experiments. Briefly, mice between 6 and 15 weeks of age were euthanized with CO₂, and the lungs were perfused through the right ventricle with cold PBS containing calcium and magnesium. The lungs were minced finely, and the tissue was digested with 1 \times dispase (BD Falcon), 1 mg/ml type 1 filtered collagenase (Worthington), and 4.5 μ g/ml DNase (Worthington). Lung fibroblasts were cultured in DMEM supplemented with 10% fetal bovine serum (Hyclone) and 1% penicillin/streptomycin and incubated in a 5% CO₂ atmosphere at 37 °C. Cells were analyzed between passages 0 and 4. IMR90 fetal human lung fibroblasts (ATCC) were grown in DMEM

supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin and incubated in a 5% CO₂ atmosphere at 37 °C. Where indicated, IMR90 cells were serum-starved and treated in low-serum medium containing 0.5% fetal bovine serum and 1% penicillin/streptomycin. Mink lung epithelial cells stably transfected with a plasminogen activator inhibitor 1 promoter-luciferase reporter construct (MLEC-TGF β) were cultured as described (25, 32). Luciferase activity was measured using luciferase assay substrate (Promega) on a BioTek Synergy HT plate reader.

SDS-PAGE and Western Blotting—Total protein lysates were harvested and analyzed by Western blot analysis as described previously (24). Nuclear and cytoplasmic protein fractionation was performed using a NE-PER kit (Thermo). Briefly, equal amounts of protein were diluted in SDS-PAGE sample buffer, boiled, run on 4–20% Novex SDS-polyacrylamide gels (Invitrogen) or 4–20% Mini-PROTEAN TGX gels (Bio-Rad), and transferred onto a nitrocellulose membrane according to standard procedures. Antibodies used include ACLP (24) (1:4000); SMA (Sigma, 1:4000); collagen α 1(I) (Rockland, 1:4000); pan-actin (Sigma, 1:4000); phospho-Smad3, phospho-Smad1/5/8, total Smad2/3, total Smad1 (Cell Signaling Technology, 1:1000); TGF β (Cell Signaling Technology, 1:1000); HDAC2 (Santa Cruz Biotechnology, 1:1000); smooth muscle myosin heavy chain (provided by Robert S. Adelstein, 1:1000); vimentin (BD Biosciences, 1:5000); and GAPDH (Sigma, 1:10000). Blots were imaged using either film or a Bio-Rad Chemidoc imaging system. Protein quantification relative to pan-actin or GAPDH was measured using Image Lab software.

Expression and Purification of Recombinant ACLP—To generate recombinant ACLP (rACLP), 293 cells were stably transfected with an ACLP construct with a BM40 (Sparc) signal sequence replacing the signal peptide and a C-terminal myc-His tag for detection and purification (Fig. 2A) (33). These cells were cultured in suspension in serum-free conditions. The conditioned medium was collected and dialyzed using 100,000 molecular weight cut off (MWCO) dialysis tubing into 300 mM KCl, 50 mM KH₂PO₄ (pH 8). The protein was bound to an immobilized metal affinity chromatography column (Bio-Rad Duo Flow) and washed exhaustively. During the final wash, the column was briefly washed with a sodium carbonate buffer (pH 11) as described (34) to remove remaining contaminating proteins, including, potentially, TGF β , which was not detected in the final rACLP preparations (Fig. 2B). Protein was eluted from the column with 250 mM imidazole and concentrated and dialyzed into 1 \times PBS with calcium and magnesium. Protein purity was determined by SDS-PAGE followed by Coomassie staining (Fig. 2C). Protein concentration was determined via a Bradford assay, and accuracy was calibrated using amino acid analysis (Molecular Biology Core, Dana Farber Cancer Institute). The authenticity of the recombinant protein was also determined by mass spectrometry (Taplin Mass Spectrometry Facility, Harvard Medical School).

Quantitative Real-time PCR—RNA was isolated from cells using RNeasy (Qiagen) according to the instructions of the manufacturer. cDNA was generated from 0.2–0.5 μ g of mRNA using a Superscript III reverse transcript kit (Invitrogen). mRNA was quantified using an ABI7300 instrument with SYBR probes (Invitrogen). Intron-spanning primers were used

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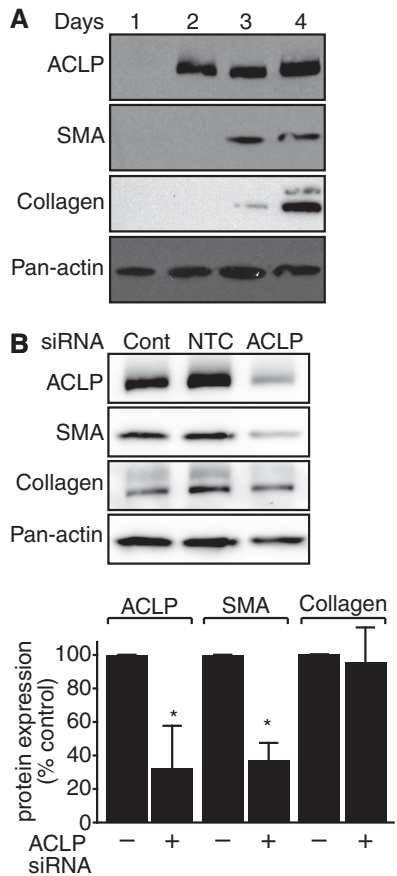


FIGURE 1. ACLP expression drives SMA and collagen expression in primary differentiating mouse lung fibroblasts. *A*, primary lung fibroblasts were isolated from wild-type mice and plated onto tissue culture plastic. Protein was harvested 1, 2, 3, and 4 days post-isolation and analyzed using SDS-PAGE and Western blot analysis with antibodies against ACLP, SMA, collagen, and pan-actin. *B*, differentiating mouse lung fibroblasts were transfected with siRNA targeting ACLP 1 day after isolation. Protein was harvested 2 days after transfection and analyzed by SDS-PAGE and Western blot analysis with antibodies against ACLP, SMA, collagen I, and pan-actin. Protein levels were quantified relative to pan-actin levels. Data presented are representative of three separate experiments. *Cont.*, control. *NTC.*, non-targeting control. *, $p < 0.05$ versus control treated cells.

as follows. 18 S, 5'-CGGCTACCACATCCAAGGAA-3' (forward) and 5'-TTTTTCGTCACCTCCCCG-3' (reverse); Acta2, 5'-TGACGCTGAAGTATCCGATAGA-3' (forward) and 5'-GTACGTCCAGAGGCATAGAGG-3' (reverse); and Col1a2, 5'-GTAACCTCGTGCCTAGCAACA-3' (forward) and 5'-CCTTTGTCAGAATACTGAGCAGC-3' (reverse).

siRNA—Primary lung myofibroblasts were transfected with siRNA targeting ACLP (Dharmacon) using RNAiMAX (Invitrogen) either on day 1 post-isolation or between passages 2 and 4. IMR90 fibroblasts and day 2 primary lung fibroblasts were transfected with siRNA targeting Smad2 and Smad3 (siGenome SMARTpool Thermo) using RNAiMAX. Knockdown was measured by Western blot analysis. Non-targeting control siRNA was used as a control in all siRNA experiments (Dharmacon). Sequences are available upon request.

Immunofluorescence Staining—IMR90 cells were plated onto glass chamber slides and treated as described in the figure legends. At the time of harvest, cells were washed, fixed in 4% paraformaldehyde, permeabilized with 0.1% Triton X-100, and incubated with anti-Smad2/3 antibodies (Cell Signaling Tech-

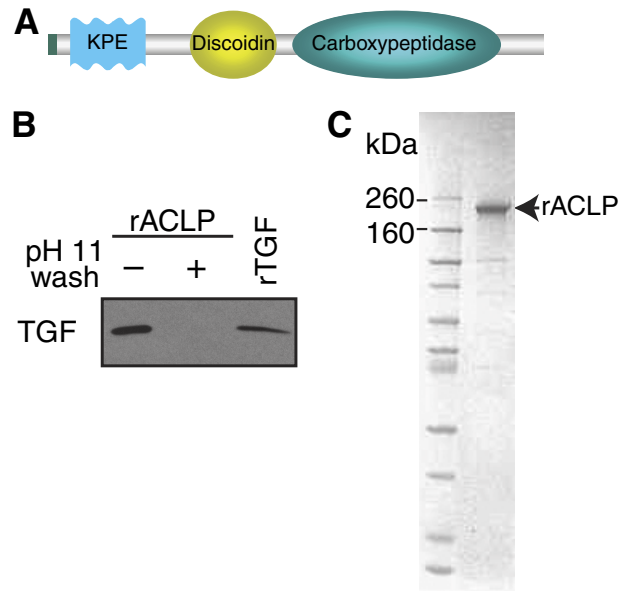


FIGURE 2. Purification of recombinant ACLP. Recombinant ACLP was purified from the conditioned medium of AD293 cells stably transfected with an ACLP construct containing a BM40 signal sequence and a His tag. *A*, schematic of the ACLP construct. *B*, ACLP (1 μ g) prior to a sodium carbonate (pH 11) wash, 1 μ g of recombinant ACLP after a sodium carbonate (pH 11) wash, and 0.4 ng of TGF β were analyzed by Western blot analysis with anti-TGF β . *C*, Protein gels were stained with Coomassie Blue.

nology, catalog no. 8685), followed by Alexa Fluor 488- or 568-conjugated secondary antibodies (Invitrogen). Samples were counterstained with DAPI and mounted. Images were taken at identical exposures using an Axiovert 200 M microscope (Carl Zeiss) and an ORCA-ER camera (Hamamatsu) or an Eclipse TE2000e microscope and Digital Sight DSQi1Mc camera (Nikon). Passaged primary lung myofibroblasts isolated from MRTEA^{+/+} and MRTEA^{-/-} mice were plated onto glass chamber slides and treated with 3.75 μ g/ml rACLP or 1 nM TGF β . At the time of harvest, Cells were then fixed in 1 \times PIPES-HEPES-EGTA-magnesium (PHEM) buffer, 3.7% paraformaldehyde and mounted with DAPI counterstain (35). Images were taken on a Zeiss LSM confocal microscope with constant exposure times.

TBR-I HA K232R Transfection—IMR90 cells were plated onto chamber slides coated with 0.1% gelatin and transfected with 500 ng of pCMV TBR-I HA K232R using Lipofectamine 2000 (Invitrogen) (21). The cells were serum-starved, treated as described, fixed, permeabilized, and stained with anti-HA (Roche) and anti-Smad2/3, followed by Alexa Fluor 568 goat anti-rabbit IgG and Alexa Fluor 488 goat anti-rat IgG (Invitrogen).

ACLP Binding Assays—rACLP or TGF β (R&D Systems) were biotinylated using an EZ-Link Sulfo-NHS-LC-Biotin kit (Thermo). Approximately 1 μ g of biotinylated rACLP or 0.5 μ g of TGF β was immobilized on streptavidin high binding capacity coated plates (Thermo) overnight at 4 $^{\circ}$ C in binding buffer (PBS containing magnesium, calcium, 0.1% BSA, and 0.05% Tween 20). The next day, the plates were washed with binding buffer and blocked with the same buffer containing 1% BSA at room temperature for 1 h, followed by incubation with increasing amounts of TBR-II Fc chimera (R&D Systems) at room temperature for 1 h. Wells were washed, and binding was detected with anti-human-IgG-HRP diluted 1:800, followed by

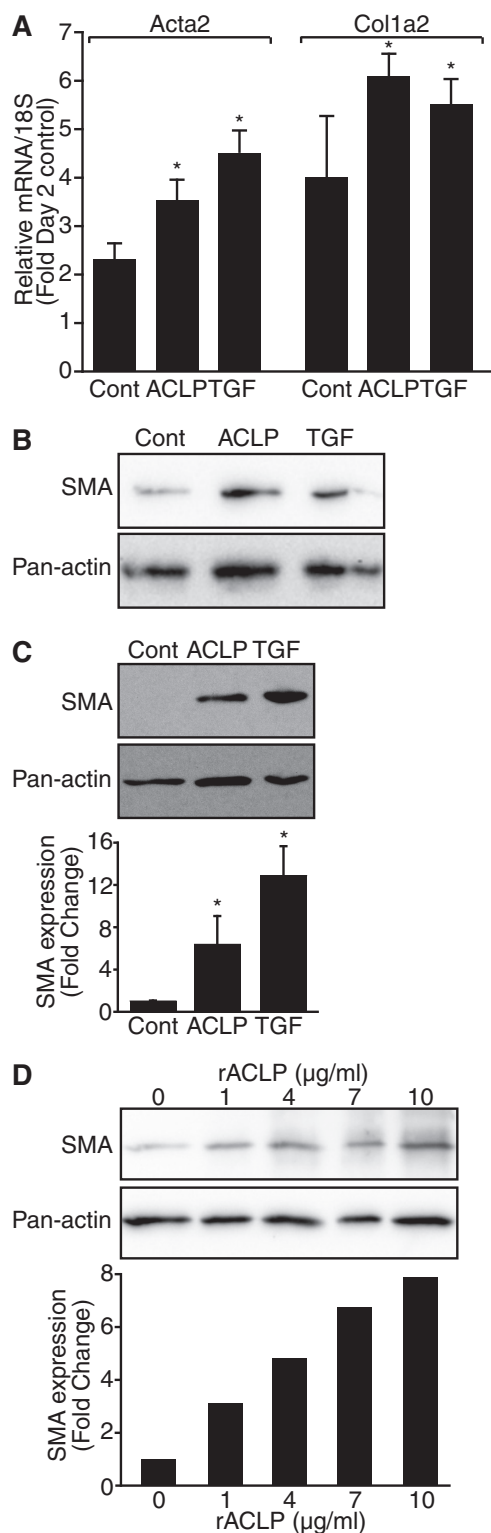


FIGURE 3. Recombinant ACLP promotes myofibroblast formation. *A*, mouse primary lung fibroblasts were treated at the time of isolation with 3.75 μg/ml (~30 nM) rACLP or 1 nM TGFβ. RNA was harvested from treatments in triplicate after 3 days. RT quantitative PCR was used to measure gene expression of Acta2 (SMA) and Col1a2. Gene expression was normalized to 18 S expression of control-treated (Cont.) cells harvested 2 days after isolation. *, *p* < 0.05 versus control-treated cells. *B*, mouse primary lung fibroblasts were treated at the time of isolation with 3.75 μg/ml rACLP or 1 nM TGFβ. Protein lysate was harvested on day 3 post-isolation and analyzed by Western blot analysis with antibodies against SMA and pan-actin. *C*, IMR90 human lung fibroblast cells were treated in low-serum medium with 3.75 μg/ml rACLP or 1 nM TGFβ for 48 h and analyzed by SDS-PAGE and Western blot analysis with

incubation with 3,3',5,5'-tetramethylbenzidine (eBioscience). The reaction was quenched with 2 M H₂SO₄, and absorbance at 450 nm was measured.

Statistical Analysis—Data are presented as mean ± S.E. Statistical significance was determined by Student's *t* test for comparisons between two groups or analysis of variance for groups of more than two and defined as *p* < 0.05.

RESULTS

ACLP Promotes the Fibroblast-to-Myofibroblast Transition in Differentiating Primary Lung Cells—Passaged primary lung fibroblasts express high levels of myofibroblast markers, including collagen I, SMA, and ACLP (data not shown), which suggests that these cells quickly differentiate into myofibroblasts when grown on plastic. To characterize the kinetics of the differentiation process and elucidate the role of ACLP in myofibroblast differentiation, protein was harvested from freshly isolated mouse lung fibroblasts 1, 2, 3, and 4 days post-isolation and analyzed by Western blot analysis (Fig. 1*A*). On day 1, the cells did not express detectable ACLP, SMA, or collagen. ACLP protein expression was first detected on day 2 after isolation, whereas SMA and collagen were first detected on day 3 post-isolation. By day 4, the cells expressed high levels of ACLP, SMA, and collagen. These data suggest that ACLP protein expression precedes SMA and collagen I protein expression as fibroblasts differentiate into myofibroblasts. On the basis of these results and our prior *in vivo* studies correlating a loss of ACLP with a reduction in lung fibrosis (25), we hypothesized that ACLP promotes the fibroblast-to-myofibroblast transition.

To examine the role of ACLP in promoting fibroblast-to-myofibroblast differentiation, we used siRNA-mediated knock-down of ACLP during myofibroblast differentiation. Freshly isolated wild-type primary lung fibroblasts were transfected on day 1 post-isolation with siRNA targeting ACLP (Fig. 1*B*). Cells transfected with ACLP siRNA showed a 68% reduction in ACLP protein expression and exhibited a statistically significant 63% reduction in SMA protein expression as compared with cells transfected with control siRNA. Collagen levels were reduced by only 5%, suggesting that ACLP knockdown alone is insufficient to significantly regulate collagen at this time point.

Recombinant ACLP Enhances the Fibroblast-to-Myofibroblast Transition—We generated and purified recombinant ACLP from mammalian cells to perform gain of function studies in fibroblasts (Fig. 2). Trace amounts of TGFβ were removed from recombinant ACLP preparations by adding a sodium carbonate buffer (pH 11) wash as described (34) (Fig. 2*B*). Recombinant ACLP was glycosylated in a pattern consistent with native ACLP (not shown). Primary lung fibroblasts were isolated from wild-type mice and plated into medium containing rACLP or recombinant TGFβ (R&D Systems) as a positive control because TGFβ is a well known mediator of the fibroblast-

antibodies against SMA and pan-actin. SMA protein levels were determined relative to pan-actin levels. *, *p* < 0.05 versus control-treated cells. *D*, IMR90 cells were treated in low-serum medium with 0, 1, 4, 7, or 10 μg/ml rACLP for 48 h and analyzed by SDS-PAGE and Western blot analysis with antibodies against SMA and pan-actin. SMA protein levels were determined relative to levels of pan-actin. Data presented are representative of three separate experiments.

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to-myofibroblast transition (9, 16). RNA was harvested after 3 days and analyzed by RT quantitative PCR (Fig. 3A). Importantly, rACLP induced a statistically significant increase in *Acta2* (SMA) and *Coll1a2* gene expression in differentiating myofibroblasts. Treatment of primary differentiating fibroblasts with rACLP also induced an increase in SMA protein levels on day 3 post-isolation as compared with untreated control cells (Fig. 3B). These data indicate that exogenous ACLP can enhance myofibroblast formation in differentiating primary lung fibroblasts.

To further define the role of ACLP in the fibroblast-to-myofibroblast differentiation process, IMR90 human lung fibroblast cells were used. IMR90 cells do not express endogenous ACLP and are, therefore, a good system to examine the effects of ACLP in gain of function studies. IMR90 cells were grown in medium containing 3.75 $\mu\text{g/ml}$ (30 nM) rACLP or 1 nM TGF β as a positive control (Fig. 3C). Dose-response experiments revealed that 3.75 $\mu\text{g/ml}$ or ~ 30 nM was optimal to induce myofibroblast differentiation (Fig. 3D). Compared with untreated control cells, rACLP induced a 6-fold increase in SMA protein expression in IMR90 cells after a 48-h treatment. Taken together, these data suggest that ACLP promotes the fibroblast-to-myofibroblast differentiation by significantly stimulating expression of SMA and collagen I.

ACLP Stimulates TGF β Signaling Pathways—Because ACLP is similar to TGF β in its ability to stimulate SMA and collagen I expression (16), we investigated whether the mechanism of ACLP action might be related to TGF β signaling pathways. TGF β signaling is regulated by a complex process involving a number of extracellular factors, including activation of latent TGF β by thrombospondin (36), integrin signaling (37), and receptor activity (21). Additionally, collagen can interact with the T β R complex independently of the TGF β ligand (38, 39). TGF β signaling in fibroblasts induces the phosphorylation of Smad3, which then binds to Smad4 and translocates into the nucleus to promote transcription of myofibroblast markers (22, 40). On the basis of this knowledge, serum-starved IMR90 cells were treated with rACLP for 15, 30, 45, or 60 min or 24 h (Fig. 4A). Acute rACLP treatment stimulated Smad3 phosphorylation, first detected after 15 min, and dramatically increased Smad1 phosphorylation after 30 min. This phosphorylation was transient and was not detectable after 24 h. Interestingly, Smad2/3 protein expression was reduced after long term rACLP treatment. On the basis of these findings, we examined the dose response of rACLP treatment at 30 min. As little as 1 $\mu\text{g/ml}$ rACLP induced Smad3 phosphorylation in IMR90 fibroblasts, whereas higher doses also increased Smad1 phosphorylation (Fig. 4B).

Smad2/3 Knockdown Inhibits ACLP-Induced Fibroblast-to-Myofibroblast Transition—Short-term rACLP treatment of IMR90 cells induced Smad3 phosphorylation, whereas long-term rACLP treatment of IMR90 cells induced SMA protein expression. TGF β is known to mediate SMA protein expression in lung myofibroblasts via several pathways, including Smad3 phosphorylation (41). Therefore, we hypothesized that ACLP increases SMA protein expression by inducing Smad3 phosphorylation. IMR90 cells were transfected with siRNA targeting Smad2 and Smad3 and treated with rACLP for 48 h (Fig.

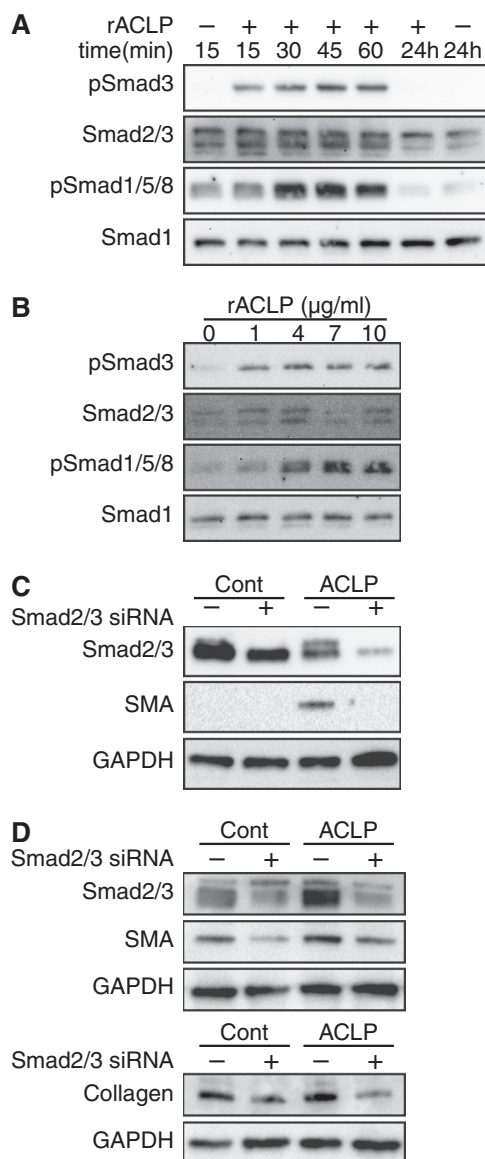


FIGURE 4. ACLP stimulates Smad3 phosphorylation and enhances SMA protein expression. A, IMR90 human lung fibroblasts were serum-starved overnight and treated with 3.75 $\mu\text{g/ml}$ rACLP for 15, 30, 45, or 60 min or 24 h. Protein lysate was harvested and analyzed by SDS-PAGE and Western blot analysis with antibodies against phospho-Smad3, total Smad2/3, phospho-Smad1/5/8, and total Smad1. B, serum-starved IMR90 cells were treated with 0, 4, 7, or 10 $\mu\text{g/ml}$ rACLP for 30 min. Protein lysate was harvested and analyzed by SDS-PAGE and Western blot analysis with antibodies against phospho-Smad3, total Smad2/3, phospho-Smad1/5/8, and total Smad1. C, IMR90 cells were transfected with siRNA targeting Smad2 and Smad3 and then treated with 3.75 $\mu\text{g/ml}$ rACLP for 48 h. Protein lysates were analyzed by Western blot analysis with antibodies against SMA, total Smad2/3, and GAPDH. Cont., control. D, primary mouse lung fibroblasts were transfected with siRNA targeting Smad2 and Smad3 2 days post-isolation and then treated with 3.75 $\mu\text{g/ml}$ rACLP for 48 h. Protein lysates were analyzed by Western blot analysis with antibodies against SMA, total Smad2/3, and GAPDH. A second gel was run and analyzed by antibodies against collagen and GAPDH. Data presented are representative of three separate experiments.

4C). A knockdown of ~ 40 – 50% of Smad2/3 resulted in a dramatic reduction in SMA protein expression. Although the transfection efficiency of IMR90 cells is relatively low, ACLP-induced SMA protein expression was dependent on Smad2/3. These data suggest that ACLP stimulates Smad3 phosphorylation to promote myofibroblast differentiation. Additionally,

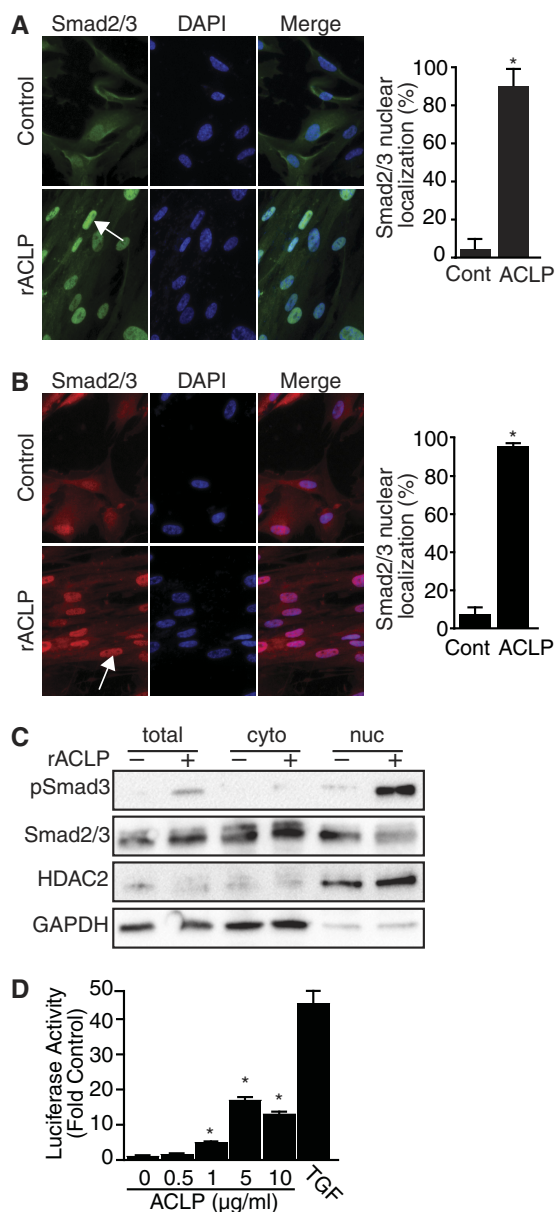


FIGURE 5. ACLP induces Smad3 nuclear translocation and transcriptional reporter activity. *A*, IMR90 fibroblasts were treated for 30 min in low-serum medium containing 3.75 $\mu\text{g/ml}$ rACLP. Cells were fixed and stained for Smad2/3 using Alexa Fluor 488 goat anti-rabbit IgG as a secondary antibody. Nuclei were counterstained with DAPI. Images were captured at identical exposures. *Cont.*, control. *B*, IMR90 fibroblasts were treated for 48 h in low-serum medium containing 3.75 $\mu\text{g/ml}$ rACLP. Cells were fixed and stained for Smad2/3 using Alexa Fluor 568 goat anti-rabbit IgG as a secondary antibody. Nuclei were counterstained with DAPI. Images were captured at identical exposures. *C*, IMR90 human lung fibroblasts were serum-starved overnight and treated with 3.75 $\mu\text{g/ml}$ rACLP for 30 min. Cytoplasmic (*cyto*) and nuclear (*nuc*) extracts were isolated and analyzed analyzed by SDS-PAGE and Western blot analysis with antibodies against phospho-Smad3, total Smad2/3, HDAC2, and GAPDH. *D*, mink lung epithelial cells stably transfected with the PAI-1 promoter driving luciferase activity (MLEC-TGF β) were treated for 24 h in low-serum medium containing 0, 0.5, 1, 5, or 10 $\mu\text{g/ml}$ rACLP. Cell lysates were analyzed for luciferase activity. Data are presented as relative to untreated control cells. *, $p < 0.05$ versus control-treated cells. Data presented are representative of three separate experiments.

primary lung fibroblasts were transfected with siRNA targeting Smad2 and Smad3 on day 2 post-isolation, followed by treatment with rACLP (Fig. 4D). Smad2/3 siRNA reduced Smad2/3 expression in these primary cells by 60–70%. rACLP increased

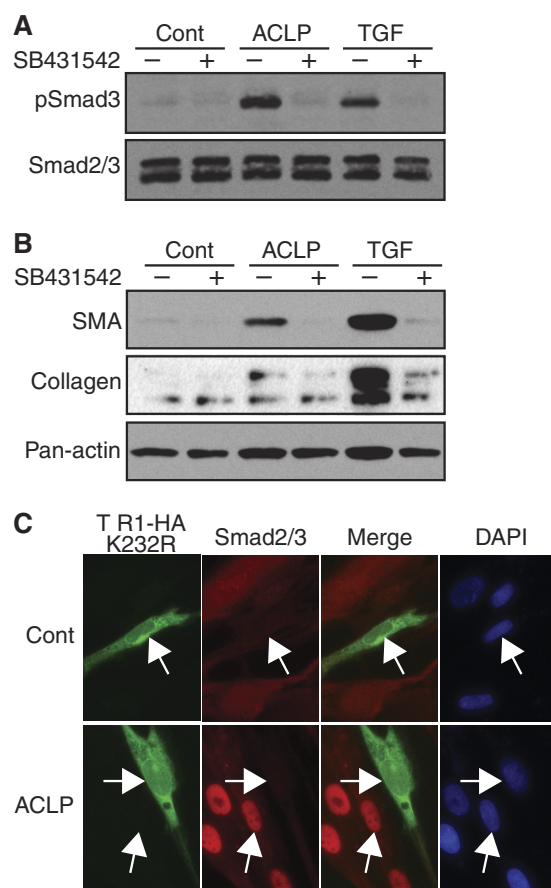


FIGURE 6. ACLP induction of TGF β signaling pathways and myofibroblast protein expression is dependent on TGF β receptor activity. IMR90 lung fibroblasts were treated in low-serum medium with 3.75 $\mu\text{g/ml}$ rACLP or 1 nM TGF β with either dimethyl sulfoxide as a vehicle control or 5 μM SB431542, a TGF β receptor inhibitor. *A*, cell lysates were analyzed by Western blot analysis after 30-min treatment following serum starvation with antibodies against phospho-Smad3 and total Smad2/3. *Cont.*, control. *B*, cell lysates were analyzed by Western blot analysis after 48 h with antibodies against SMA and pan-actin. *C*, IMR90 cells plated onto 0.1% gelatin-coated chamber slides were transfected with pCMV T β R1 HA K232R using Lipofectamine 2000, serum-starved overnight, and then treated with 3.75 $\mu\text{g/ml}$ rACLP for 30 min. Cells were fixed and stained for HA and Smad2/3 using Alexa Fluor 488 goat anti-rat IgG and Alexa Fluor 568 goat anti-rabbit IgG as secondary antibodies. Nuclei were counterstained with DAPI. Data presented are representative of three separate experiments.

SMA protein expression in non-targeting control transfected cells, and this increase was blunted in the cells with Smad2/3 knocked down. Similarly, rACLP increased collagen protein expression in non-targeting control transfected cells, and this increase was reduced in cells transfected with Smad2/3 siRNA. Together, these findings indicate that Smad2/3 participates in ACLP-mediated myofibroblast differentiation.

ACLP Stimulates Smad2/3 Nuclear Translocation—Because Smad3 phosphorylation is followed by its translocation into the nucleus (22), the effects of ACLP on Smad3 localization were investigated. Serum-starved IMR90 cells plated onto chamber slides were treated with rACLP for 30 min and stained for Smad2/3 (Fig. 5A). rACLP treatment resulted in a clear nuclear localization of Smad2/3 as compared with untreated control cells. 90% of cells treated with rACLP showed Smad2/3 nuclear translocation. Interestingly, although ACLP-induced Smad3 phosphorylation was transient by Western blot analysis (Fig.

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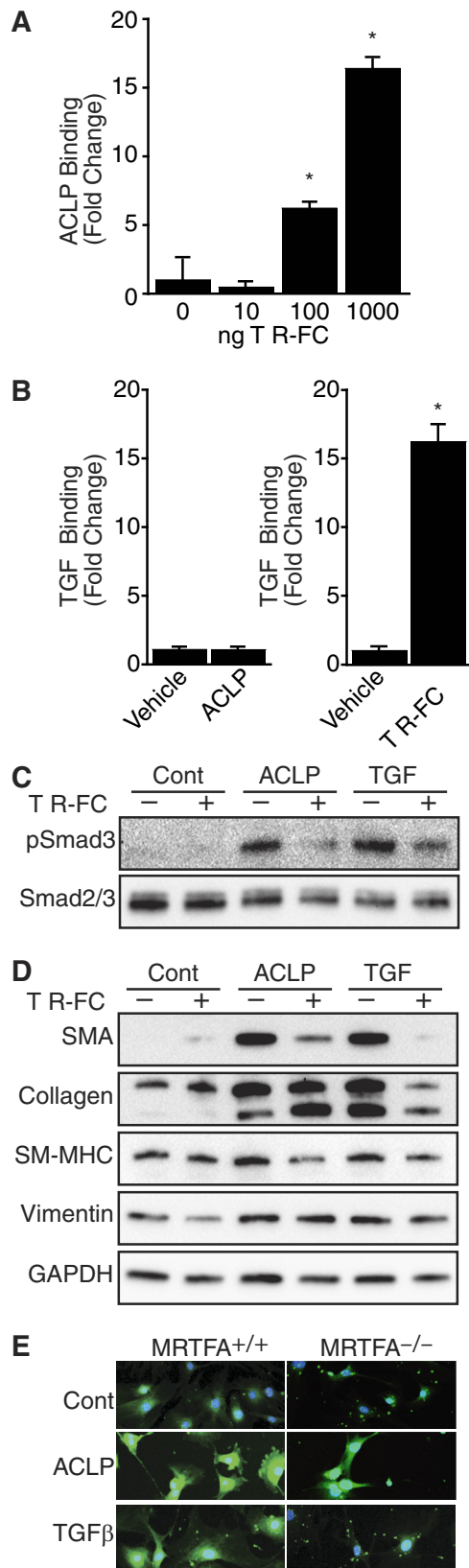


FIGURE 7. ACLP promotes myofibroblast protein expression via direct interaction with the TGF β receptor. *A*, rACLP was biotinylated and immobilized to streptavidin-coated plates. Binding buffer alone was used as a control. The wells were blocked, incubated with increasing amounts of T β R-II Fc chimera (0, 10, 100, 1000 ng), followed by human IgG-HRP and 3,3',5,5'-tetramethylbenzidine substrate. The reaction was quenched with 2 M H₂SO₄, and binding was measured by reading the absorbance at 450 nm. Data are

presented as binding minus T β R-II Fc background binding. *, $p < 0.05$ versus control-treated cells. *B*, TGF β was biotinylated and immobilized to streptavidin-coated plates overnight. Binding buffer alone was used as a control. The wells were blocked and incubated with either rACLP or T β R-II Fc chimera, followed by anti-ACLP and rabbit IgG-HRP or human IgG-HRP and 3,3',5,5'-tetramethylbenzidine substrate. The reaction was quenched with 2 M H₂SO₄, and binding was measured by reading the absorbance at 450 nm. Results are presented as binding minus ACLP (left panel) or T β R-II Fc (right panel) background binding. *, $p < 0.05$ versus control-treated cells. *C*, serum-starved IMR90 cells were treated in low-serum medium containing 100 ng/ml of a T β R-Fc chimera (R&D Systems) with 3.75 μ g/ml rACLP or 100 pM TGF β for 30 min. Protein lysates were analyzed by Western blot analysis with antibodies against phospho-Smad3 or total Smad2/3. *Cont.*, control. *D*, IMR90 cells were treated in low-serum medium containing 100 ng/ml of a T β R-Fc chimera (R&D Systems) with 3.75 μ g/ml rACLP or 100 pM TGF β for 48 h. Protein lysates were analyzed by Western blot analysis with antibodies against SMA, collagen I, smooth myosin heavy chain (SM-MHC), vimentin, and GAPDH. *E*, primary lung myofibroblasts isolated from MRTFA^{+/+} and MRTFA^{-/-} mice were treated with 3.75 μ g/ml rACLP or 1 nM TGF β for 48 h. Cells were then fixed in 1 \times PHEM buffer, 3.7% paraformaldehyde and mounted with DAPI counterstain. Pictures were taken on a Zeiss LSM confocal microscope with constant exposure times. Data presented are representative of three separate experiments.

4A), there was a persistent increase in nuclear localization of Smad2/3 in cells stained after 48-h treatment with rACLP (Fig. 5B). 96% of IMR90 cells treated with rACLP for 48 h exhibited Smad2/3 nuclear translocation. To further explore rACLP-induced Smad2/3 nuclear translocation, nuclear and cytoplasmic extracts were harvested from IMR90 cells treated with rACLP for 30 min (Fig. 5C). The nuclear fraction of cells treated with rACLP for 30 min had an increase in phospho-Smad3 expression as compared with control-treated cells. There were very low levels of phospho-Smad3 found in the cytoplasm of ACLP-treated cells, confirming nuclear translocation. Taken together, these results indicate that ACLP stimulates Smad signaling pathways important to the fibroblast-to-myofibroblast transition.

ACLP Stimulates TGF β Reporter Activity—To identify the mechanism of action of ACLP, MLEC-TGF β cells were treated with increasing amounts of rACLP for 24 h (Fig. 5D). Protein was harvested, and luciferase activity was measured. Cells treated with as little as 1 μ g/ml rACLP had a statistically significant increase in luciferase activity as compared with control-treated cells.

ACLP Stimulates the Fibroblast-to-Myofibroblast Transition, in Part, through the TGF β Receptors—Smad3 phosphorylation is induced upon T β R-II activation (22). Given the induction of Smad3 phosphorylation of ACLP, we examined the relationship between ACLP and the T β Rs. Serum-starved IMR90 cells were treated with rACLP in the presence of a TGF β -receptor kinase inhibitor (SB431542, Sigma) for 30 min (Fig. 6A) or 48 h (B). SB431542 inhibited rACLP-induced Smad3 phosphorylation at 30 min and SMA and collagen protein expression after 48 h. These data suggest that ACLP promotion of the fibroblast-to-myofibroblast transition is, at least in part, dependent on TGF β receptor kinase activity. To further confirm the role of TGF β receptor kinase activity on ACLP-induced Smad3 signaling, IMR90 cells were transfected with pCMV T β R-I HA K232R, a kinase-dead (dominant negative) mutant of T β R-I (21), serum-starved, treated with rACLP for 30 min, and stained for the HA tag and Smad2/3 localization (Fig. 6C). Compared with cells negative for HA, cells positive for T β R-I

HA K232R exhibited a strong reduction (~82%) in Smad2/3 nuclear translocation.

ACLP Binds to the TGF β Receptor II—Several proteins, including bone morphogenic proteins, growth and differentiation factors, Müllerian inhibiting substance, nodals, activins, and inhibins, as well as collagen peptides, can modulate TGF β receptor activity and Smad signaling (38, 42). Because ACLP can stimulate downstream Smad signaling, we examined whether ACLP binds directly to T β R-II. rACLP was biotinylated, immobilized on a streptavidin-coated plate, and incubated with increasing amounts of T β R-II Fc chimera (R&D Systems), and then binding was detected with human IgG-HRP (Fig. 7A). The T β R-II Fc chimera contains the extracellular domain of mouse T β R-II fused to the Fc domain of human IgG and has been used as a global inhibitor of TGF β (43). In these assays, T β R-II Fc did not bind to streptavidin wells without rACLP. Wells with immobilized rACLP had statistically significantly more binding to the T β R-II Fc chimera as compared with control-coated wells.

ACLP and TGF β Do Not Bind Directly—To determine whether ACLP might bind directly to TGF β , biotinylated TGF β was immobilized on a streptavidin-coated plate and incubated with either rACLP or the T β R-II Fc chimera, and binding was detected with either anti-ACLP and rabbit IgG-HRP or human IgG-HRP. Wells incubated with ACLP showed no binding above background (Fig. 7B, left panel). In contrast, wells incubated with the T β R-II Fc chimera had binding significantly above background (Fig. 7B, right panel).

ACLP Interacts with the TGF β Receptor to Promote Fibroblast-to-Myofibroblast Differentiation—Smad3 phosphorylation is known to be induced when the TGF β ligand binds to T β R-II (22). Because ACLP stimulates Smad3 phosphorylation in fibroblasts and binds to T β R-II *in vitro*, we hypothesized that the effects of ACLP might be inhibited by the T β R-II Fc chimera. IMR90 fibroblasts were treated with rACLP or TGF β in the presence of 100 ng/ml of the T β R-II Fc chimera. Cotreatment of fibroblasts with rACLP and T β R-II Fc for 30 min resulted in a reduction of Smad3 phosphorylation as compared with cells treated with rACLP alone (Fig. 7C). Similarly, fibroblasts treated with rACLP and T β R-II for 48 h had lower levels of SMA as compared with cells treated with rACLP alone (Fig. 7D). Similar effects were observed with smooth myosin heavy chain and vimentin. Although the T β R-II Fc treatment blunted TGF β -induced collagen expression, it did not alter ACLP-dependent collagen changes. These data suggest that ACLP interacts with T β R-II to promote Smad3 phosphorylation and nuclear translocation to promote SMA protein expression and myofibroblast differentiation. These studies also indicated that ACLP regulates collagen expression through a pathway that is distinct from that used by TGF β . Furthermore, these studies also uncovered a differential regulation of SMA and collagen in lung fibroblasts.

To explore the mechanism by which ACLP stimulates collagen expression (Fig. 3A) and to probe the differences in pathways stimulated by ACLP and TGF β , we examined the role of MRTFA. Our recent work established an important role for MRTFA in stimulating type I collagen expression through complexes with both serum response factor and Sp1 (18). We exam-

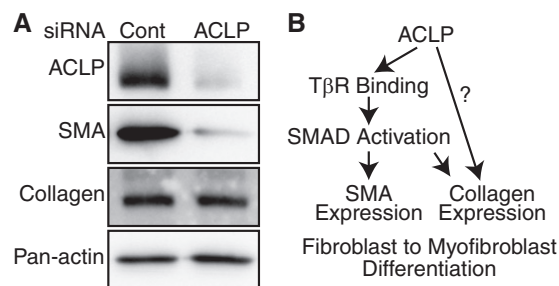


FIGURE 8. ACLP knockdown partially reverses the myofibroblast SMA expression. *A*, primary lung myofibroblasts were transfected at low passage number with 2 nM ACLP or non-targeting control siRNA (*Cont.*) using RNAiMax (Invitrogen). Protein was harvested 48 h later and analyzed with antibodies against ACLP, SMA, collagen I, and pan-actin. Data presented are representative of three separate experiments. *B*, proposed scheme for ACLP induction of the fibroblast-to-myofibroblast transition.

ined lung fibroblasts isolated from MRTFA^{+/+} and MRTFA^{-/-} mice harboring the Col1a1 promoter driving GFP expression (col3.6GFPtpz). Unlike TGF β , ACLP stimulated collagen promoter activity in the absence of MRTFA (Fig. 7E). Together, these results indicate that ACLP stimulation of collagen expression does not absolutely require the TGF β receptor or the serum response factor coactivator MRTFA.

ACLP Knockdown Slows and Partially Reverts the Fibroblast-to-Myofibroblast Transition—We have shown previously that ACLP knockout mice are protected from bleomycin-induced fibrosis (25) and that ACLP knockdown slows myofibroblast differentiation in primary mouse lung fibroblasts (Fig. 1B). Therefore, we examined whether knockdown of ACLP in primary lung myofibroblasts would cause them to revert back into fibroblasts. Passaged primary lung myofibroblasts were transfected with siRNA targeting ACLP (Fig. 8A). ACLP knockdown resulted in a decrease in SMA protein expression as compared with non-targeting control-transfected cells. As seen in differentiating myofibroblasts, ACLP knockdown alone is not sufficient to significantly decrease collagen expression. These results suggest that ACLP plays an integral role in the differentiation of fibroblasts to myofibroblasts by signaling through T β R-II to promote Smad3 phosphorylation and nuclear translocation to stimulate SMA. Additionally, ACLP signals through an MRTFA-independent pathway to promote collagen expression (Fig. 8B).

DISCUSSION

This study identified a novel mechanism by which ACLP controls lung myofibroblast differentiation. Specifically, we discovered that ACLP interacts with and activates TGF β receptors and stimulates downstream Smad3 signal transduction pathways to enhance SMA expression and signals through an MRTFA-independent pathway to promote collagen expression. Furthermore, we identified two strategies to interfere with ACLP-mediated myofibroblast differentiation. Together with the known roles of TGF β signaling in fibrotic lung disease, these studies indicate that ACLP is a novel regulator that stimulates and maintains SMA, collagen, and myofibroblast gene expression.

Idiopathic pulmonary fibrosis is a rapidly progressive and fatal disease with no effective therapies (1). It is characterized

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by an increased deposition of collagen and other ECM proteins as well as increased expression of SMA (2), leading to scarring and thickening of tissue and a progressive decline of lung function (44). One key effector cell of IPF is the myofibroblast. It is responsible for increased ECM protein deposition and increased expression of SMA (2). Our laboratory has previously shown an increase in ACLP in human fibrotic lungs and early up-regulation of ACLP in the bleomycin model of fibrosis in mice (25). Using a rapidly differentiating lung fibroblast model, we have shown that ACLP precedes SMA and collagen expression in differentiating myofibroblasts (Fig. 1A) and that exogenous ACLP is sufficient to replicate this process (Fig. 3).

ACLP knockdown slows the fibroblast-to-myofibroblast transition, as evidenced by a subsequent reduction in SMA protein expression. However, collagen protein expression was not reduced significantly. This could be due to the stability of collagen or to other factors playing a role in regulating its expression. Collagen and SMA can both be regulated by TGF β signaling through Smad phosphorylation and MRTFA localization (17, 18). However, our results suggest that there is a differential regulation of SMA and collagen by ACLP. Additionally, the promotion of collagen expression of ACLP is not inhibited by the T β R-II Fc chimera, whereas TGF β -induced collagen expression is blocked by the T β R-II Fc chimera. This indicates that ACLP and TGF β promote collagen expression via distinct pathways. This is also shown when ACLP knockdown in either differentiating myofibroblasts or fully differentiated myofibroblasts results in only a small reduction in collagen expression. Differential regulation of the myofibroblast differentiation by ACLP and TGF β was confirmed in our MRTFA knockout system. It will be interesting to study the effects of ACLP on SMA expression and stress fiber formation in the MRTFA-null system. TGF β requires the presence of MRTFA to promote collagen promoter activity, whereas ACLP promotes collagen promoter activity in an MRTFA-independent manner. Collagen transcription can be positively regulated by several transcription factors, including Sp1, AP1, Ets1, Sp3, and CBF (45). These factors represent several possible targets of ACLP signaling.

Our results suggest that ACLP is a novel regulator of T β R signaling. ACLP binds to T β R-II without binding directly to TGF β . The activity of ACLP is inhibited by the soluble T β R-II, is dependent on T β R kinase activity, is blocked by a dominant negative form of the T β R-I, and is dependent on the presence of Smad2/3. On the basis of the findings reported here, ACLP may act to stabilize the TGF β -T β R complex, or ACLP may bind to T β R-II to promote its recruitment of T β R-I in a TGF β -independent manner. T β R signaling may be activated independently of the TGF β ligand. For example, collagen fragments activate Smad signaling to promote cross-talk between integrins and the T β R complex (38). Collagen I also stimulates the epithelial-mesenchymal transition by signaling through T β R-I independently of TGF β ligand (39). Consistent with these findings, ACLP may interact with collagen at the cell surface via its discoidin domain and promote collagen expression as it facilitates the fibroblast-to-myofibroblast transition. Therefore, it is possible that ACLP mediates Smad signaling by facilitating the interaction between collagen and the T β R complex.

Previous studies have identified roles for ACLP in cell differentiation processes. For example, ACLP is down-regulated during adipogenesis (46), and retroviral ACLP overexpression inhibits adipogenesis while promoting a smooth muscle-like phenotype with increased contractile protein expression and enhanced stress fiber formation (47). One significant advance of this work over previous studies is the use of rACLP, which allowed us to examine ACLP function in signaling assays as well as to identify ACLP as playing a role in regulating collagen expression. Sorisky and colleagues (48) demonstrated that the effects of ACLP on adipogenesis may be mediated by its interaction with collagen. This collagen interaction is consistent with our previous results showing that ACLP is expressed in collagen-rich tissues (28) and enhances the contractility of lung fibroblasts via its collagen-binding discoidin domain (25).

Myofibroblasts are mechanically active cells that generate a feed-forward mechanism, promoting their differentiation (49). Work from Hinz and colleagues and others (50–52) has shown that myofibroblast contractility is an independent driver of fibrosis, including activation of latent forms of TGF β and Rho-Rho kinase pathways. The increased stiffness of fibrotic lungs has been attributed to the presence of myofibroblasts (53). A recent study suggests that targeting the ability of a myofibroblast to contract the matrix could reduce fibrosis by blocking myofibroblast differentiation from precursor cells and by inducing myofibroblast apoptosis (52).

In this study, we have shown that removing ACLP leads to a decrease in SMA expression in differentiated myofibroblasts. Our study and the Zhou study (52) suggest the possibility of targeting different components of the TGF β signaling pathway as disease progression occurs. TGF β itself is not considered to be a good IPF drug target because of the chronic nature of the disease (54) and because blocking TGF β long-term could lead to many detrimental side effects. Our findings that ACLP functions through both TGF β receptor-dependent and -independent pathways may lead to future therapies for fibrotic conditions.

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