ORIGINAL RESEARCH

Myocardin Is Involved in Mesothelial–Mesenchymal Transition of Human Pleural Mesothelial Cells

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Abstract

Pleural fibrosis is characterized by severe inflammation of the pleural space and pleural reorganization. Subsequent thickening of the visceral pleura contributes to lung stiffness and impaired lung function. Pleural mesothelial cells (PMCs) can become myofibroblasts *via* mesothelial–mesenchymal transition (MesoMT) and contribute to pleural organization, fibrosis, and rind formation. However, the mechanisms that underlie MesoMT remain unclear. Here, we investigated the role of myocardin in the induction of MesoMT. Transforming growth factor β (TGF-β) and thrombin induced MesoMT and markedly upregulated the expression of myocardin, but not myocardin-related transcription factor A (MRTF-A) or MRTF-B, in human PMCs (HPMCs). TGF-β stimulation notably induced the nuclear translocation of myocardin in HPMCs, whereas nuclear translocation of MRTF-A and MRTF-B was not observed. Several genes under the control of myocardin were upregulated in cells undergoing MesoMT, an effect that was accompanied by a dramatic cytoskeletal reorganization of HPMCs consistent with a migratory phenotype. Myocardin gene silencing blocked TGF-β– and thrombin-induced MesoMT. Although myocardin upregulation was blocked, MRTF-A and MRTF-B were unchanged. Myocardin, α-SMA, calponin, and smooth muscle myosin were notably upregulated in the thickened pleura of carbon black/bleomycin and empyema mouse models of fibrosing pleural injury. Similar results were observed in human nonspecific pleuritis. In a TGF-β mouse model of pleural fibrosis, PMC-specific knockout of myocardin protected against decrements in lung function. Further, TGF-β–induced pleural thickening was abolished by PMC-specific myocardin knockout, which was accompanied by a marked reduction of myocardin, calponin, and α-SMA expression compared with floxed-myocardin controls. These novel results show that myocardin participates in the development of MesoMT in HPMCs and contributes to the pathogenesis of pleural organization and fibrosis.

Keywords: myocardin; α-smooth muscle actin; smooth muscle; mesothelial–mesenchymal transition; pleural fibrosis

Injury to the pleural surfaces activates pleural mesothelial cells (PMCs), which are resident cells that line the visceral and parietal pleural surfaces. These activated cells play a critical role in the progression of pleural fibrosis and contribute to pleural rind formation and lung restriction, as occurs in fibrothorax (1). Under such circumstances, the cells characteristically change their phenotype with the acquisition of mesenchymal properties in response to the stimulation of inflammatory cytokines and procoagulants (2), a process called mesothelial–mesenchymal transition (MesoMT). It has been demonstrated that this transition characterizes the pathogenesis of pleural organization and fibrosis *in vivo* (3–8).

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However, the mechanisms underlying the pathogenesis of MesoMT (2) remain poorly understood.

During the development of MesoMT, mesothelial cells begin expressing α-smooth muscle actin (α-SMA) and demonstrate phenotypic changes resembling those that occur in myofibroblasts. Cells undergoing MesoMT also increase the expression and secretion of extracellular matrix proteins such as collagen 1, which contributes to the development of pleural fibrosis. Because α-SMA is a marker of MesoMT (3, 5, 8) and is under the control of myocardin (9–11), we hypothesize that myocardin or myocardin-related transcription factors (MRTFs) contribute to MesoMT and induce a range of gene products, including smooth muscle contractile and regulatory proteins that contribute to the process. We posit that this regulatory system plays an important role in phenotypic control of PMCs, which in turn contributes to pleural organization and fibrosis.

Myocardin is a smooth muscle cell (SMC)– and cardiac muscle cell–specific transcriptional coactivator of serum response factor (10, 11), and is recognized as a master gene for a number of genes expressed in SMCs and cardiac muscle cells. In SMCs, myocardin regulates a number of SMC marker proteins, including α-SMA, smooth muscle myosin heavy chain (sm-myosin), calponin, h-caldesmon, and SM22. In addition to myocardin, MRTFs have been found to play a role in cardiovascular growth and differentiation (12, 13). Therefore, pathological processes linked to myocardin are best understood in heart failure and vascular diseases, although the involvement of myocardin family proteins in other diseases, such as cancer, Alzheimer's disease, and ocular muscle fibrosis, has been suggested (14–17).

Because genes that regulate cellular phenotype and α-SMA are under the control of myocardin, we hypothesized that myocardin activation is a critical step in the mesenchymal transition of PMCs that characterizes fibrosing pleural injury. In the present study, we interrogated the role of myocardin in MesoMT and obtained evidence supporting its participation in the development of this process, thus addressing potentially important gaps in our understanding of the pathogenesis of organizing pleural injury and fibrosis (2).

Methods

Human Nonspecific Pleuritis Tissues

Deidentified human pleural tissues were obtained from the National Disease Research Interchange from surgical biopsies or autopsy specimens from patients with pneumonia and a clinical diagnosis of nonspecific pleuritis (ages 64–97 yr) (4) or from patients with histologically near-normal pleural tissues who died of causes otherwise unrelated to any pleural pathologic process (ages 35–82 yr) (4). Lung histology and immunostaining were performed as previously described (8, 18, 19). For further details regarding the materials and methods used in this work, *see* the data supplement.

Human PMC Isolation and Culture

Permission to collect and use human PMCs (HPMCs) was given by the Institutional Human Subjects Review Board of the University of Texas Health Science Center at Tyler (UTHSCT). All experiments relating to human subjects were performed in accordance with relevant guidelines and regulations. Cells were isolated from pleural fluids collected from patients with congestive heart failure or post-coronary-bypass pleural effusions (20) and maintained on CellBIND dishes (Corning) using LHC-8 culture medium (Gibco) containing 3% FBS (Life Technologies), 2% antibiotic-antimycotic (Life Technologies), and GlutaMAX (Gibco) in a humidified incubator at 37°C and 5% CO_2/95% air. Before use in experiments, all primary HPMC and mouse PMC (MPMC) cultures were assayed for expression of the mesothelial cell marker calretinin as previously described (21). Only cultures with a >95% calretinin positivity were used.

Cell Treatment Conditions

Cells were incubated in serum-free medium (RPMI 1640; Hyclone) supplemented with GlutaMAX (Gibco) for 8–16 hours before treatment. The serum-starved cells were treated with PBS or transforming growth factor β (TGF-β; 5 ng/ml; R&D Systems) or thrombin (13 nM) in serum-free media. The cells were then allowed to incubate for 24 hours (qPCR analysis) or 48 hours (Western blotting and immunostaining analysis) at 37°C in 5% CO_2/95% air.

Immunofluorescence Staining

Cells were washed with PBS, placed in fixation solution (4% formaldehyde, 2 mM $MgCl_2$, and 1 mM EGTA in PBS), washed with PBS, and permeabilized with 0.1% Triton X-100 in PBS for 10 minutes. The cells were then washed and blocked with 1% BSA including 0.02% azide in PBS for 30 minutes. Myocardin (sc-21561; Santa Cruz Biotechnology), collagen (1310-01; SouthernBiotech), α-SMA (MAB1420; R&D Systems), sm-myosin (T4026; Sigma-Aldrich), calponin-1 (ab46794; Abcam), MRTF-A (ab115319; Abcam), and MRTF-B (sc-47282; Santa Cruz Biotechnology) antibodies were diluted in 1% BSA. Primary antibodies were visualized with Alexa Fluor 488, 568, and 647 secondary antibodies (Life Technologies), and nuclei were stained with DAPI (ThermoFisher Scientific). Cells were mounted onto slides with ProLong Gold Antifade Reagent (Life Technologies). Fluorescence images were taken with the use of Leica TCS SP8 systems (Leica Microsystems) for confocal microscopy. Tissue staining was done as previously described (22, 23). For these analyses, a separate myocardin antibody (MAB4028; R&D Systems) was used. All of the detector settings were identical for all images.

Animal Models of Pleural Fibrosis

All experiments involving animals were approved by the Institutional Animal Care and Use Committee of the UTHSCT. All experiments relating to animals were performed in accordance with relevant guidelines and regulations. Wild-type C57BL/6j mice were intrapleurally treated with saline, carbon black/bleomycin (CBB) for 14 days, or Streptococcus *pneumoniae* for 7 days as previously described (8, 23). A TGF-β adenoviral (AdTGF) model of pleural fibrosis was generated as previously described with minor modifications (5). Briefly, mesothelial-specific myocardin knockout (KO) ($myocd^{-/-}$) and floxed myocardin ($myocd^{fl/fl}$) mice (10–12 wk old, ≈20 g) were first lightly anesthetized with isoflurane. The mice were then intrapleurally injected with 3×10^8 plaque-forming units of the constitutively active TGF-$\beta^{C223S/C225S}$ adenoviral construct (AdTGF) or an enhanced GFP (EGFP) construct (AdGFP). Over the 7-day course, mice were monitored for signs of respiratory distress or moribund condition. On day 7, lung functions were determined as previously described (8, 19, 23). Tissue staining for α-SMA, myocardin, and

calponin was also performed as described in the previous section.

Production of Myocardin Conditional KO Mice

Mice were housed and bred under specific pathogen-free conditions at the UTHSCT and all experiments conformed to ethical principles and guidelines approved by the UTHSCT Institutional Animal Care and Use Committee. Calb2-Cre mice on the C57Bl/6J background, which have Cre recombinase expression directed to calretinin-expressing cells, such as PMCs, were purchased from the Jackson Laboratory. The myocd$^{fl/fl}$ mice were obtained through a material transfer agreement from the University of Pennsylvania (24). Homozygous myocd$^{fl/fl}$ mice were crossed with homozygous Calb2-Cre mice to create pleural mesothelium–specific myocardin KO mice. Genotyping was performed to identify double homozygous mice (MyocdFl/Fl/Calb2-Cre$^{+/+}$).

Statistics

All statistical analyses were performed using the Mann-Whitney *U* test or Student's *t* test. A *P* value of less than 0.05 was considered significant.

Results

Myocardin and Related Downstream Gene Expression Are Enhanced during MesoMT of HPMCs Induced by TGF-β or Thrombin

We and others have shown that external stimuli such as profibrotic mediators and procoagulants, including TGF-β and thrombin, induce phenotypic changes of HPMCs and the upregulation of expression of α-SMA that is characteristic of MesoMT (3, 8, 25). Figure 1A shows the effect of TGF-β and thrombin on the mRNA expression of various genes during MesoMT induced by TGF-β and thrombin, respectively. In addition to α-SMA, the mRNA levels of other smooth muscle marker genes (9), calponin and sm22, were likewise significantly upregulated. Calponin, an actin-binding protein that is highly expressed in smooth muscle, was increased by >50-fold with TGF-β treatment. Conversely, sm-myosin and h-caldesmon were only moderately upregulated. Western blot analysis confirmed that α-SMA was notably increased after TGF-β or thrombin stimulation. Sm-myosin was likewise increased by TGF-β and thrombin treatment (Figure 1B). Calretinin, a mesothelial cell marker, was unchanged by TGF-β or thrombin treatment, as confirmed by quantitative (qPCR) analyses (data not shown). Because the expression of calponin, sm22, sm-myosin, and α-SMA is reported to be controlled by myocardin (26–31), we next examined the effect of TGF-β and thrombin on myocardin expression (Figure 1C). TGF-β and thrombin stimulation of HPMCs markedly increased myocardin mRNA. Because the myocardin family factors MRTF-A and MRTF-B also influence the expression of α-SMA, we also examined the effect of TGF-β and thrombin on their expression. In contrast to myocardin, the mRNA expression of MRTF-A and MRTF-B was not significantly upregulated by TGF-β or thrombin (Figure 1C).

Changes in the Cytoskeletal Structure of HPMCs by TGF-β and Thrombin

Because the proteins that are upregulated by TGF-β and thrombin stimulation are actin modulatory proteins (32), we next studied changes in the cytoskeletal structure of HPMCs. As shown in Figure 1D, both TGF-β and thrombin increased the expression of α-SMA and sm-myosin. Furthermore, these same mediators increased stress fiber formation (middle and bottom panels). The HPMCs also showed leading edges that are indicative of a migratory cellular phenotype, and sm-myosin and α-SMA were localized to the leading edges of the cells. This was more evident in thrombin-treated HPMCs. It should be noted that sm-myosin colocalized more with α-SMA than with F-actin. α-SMA does not show filamentous localization of F-actin because of the presence of G-actin, in contrast to phalloidin staining, which only detects F-actin.

TGF-β Induced Nuclear Translocation of Myocardin in HPMCs

It is known that nuclear translocation is required for the transcriptional activity of myocardin and MRTFs (10, 31). Because TGF-β more potently induces myocardin, we next assayed the ability of TGF-β to induce and mobilize myocardin in HPMCs. As shown in Figure 2, the myocardin signal was weak before TGF-β stimulation. Moreover, myocardin was predominantly localized in the cytoplasm, and nuclear localization was not evident before TGF-β stimulation. Conversely, myocardin showed notable nuclear localization after TGF-β stimulation, indicating the activation of myocardin (Figure 2A). These results suggest that TGF-β not only upregulates myocardin gene expression but also activates its ability as a transcription coactivator. MRTF-A and MRTF-B did not localize to the nucleus, even after TGF-β stimulation, suggesting that TGF-β does not activate the activity of MRTF-A and MRTF-B in HPMCs (Figures 2B and 2C).

Effect of Myocardin Gene Silencing by Specific siRNA on MesoMT

We next examined whether myocardin downregulation diminishes TGF-β– or thrombin-induced MesoMT. We first examined the effect of myocardin downregulation on the induction of α-SMA by TGF-β and thrombin. Figure 3A shows the effect of myocardin siRNA on α-SMA protein expression of HPMCs. Although α-SMA protein expression was markedly increased by TGF-β and thrombin, myocardin siRNA significantly diminished α-SMA protein induction (Figure 3A).

We also examined the effect of myocardin downregulation on other genes known to be controlled by myocardin. Myocardin siRNA, as expected, significantly diminished myocardin mRNA in TGF-β–stimulated HPMCs (Figure 3B). TGF-β–mediated induction of the myocardin downstream actin-binding proteins calponin and sm22 was likewise significantly reduced by myocardin downregulation (Figure 3B). These results indicate that myocardin siRNA treatment downregulates myocardin, which in turn reduces the expression of its downstream genes, including α-SMA, calponin, and sm22. Neither MRTF-A nor MRTF-B expression was affected by myocardin downregulation (Figure 3C).

We next studied the effect of myocardin gene silencing on the HPMC cytoskeletal structure and phenotype. Myocardin knockdown (Figure 4) abolished the TGF-β–induced mesenchymal morphology observed in Figure 1D. α-SMA and sm-myosin were barely detectable, as were actin stress fibers. The actin structure showed a cortical actin form, i.e., the actin cytoskeleton that lies just beneath the plasma membrane, and no actin structures were found at the leading

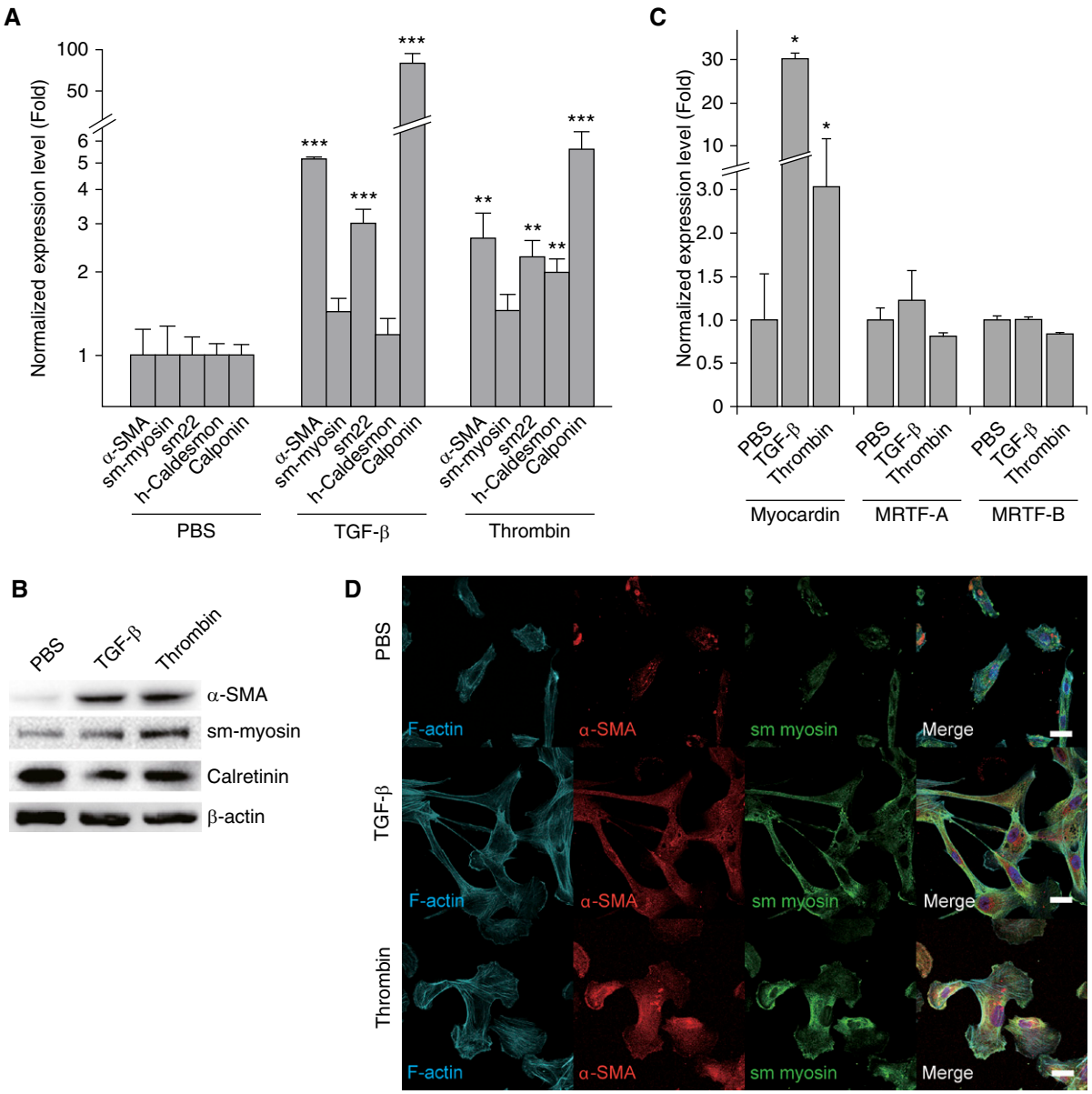

Figure 1. Effects of transforming growth factor β (TGF-β) and thrombin on the expression of myocardin and genes under the control of myocardin during mesothelial–mesenchymal transition (MesoMT). (*A*) Upregulation of smooth muscle marker genes in cells undergoing MesoMT. mRNA levels of smooth muscle marker genes (α-smooth muscle actin [α-SMA], smooth muscle myosin heavy chain [sm-myosin], high-molecular-weight caldesmon [h-caldesmon], and calponin) during MesoMT induced by TGF-β (5 ng/ml) and thrombin (13 nM) stimulation were determined by quantitative PCR (qPCR) analysis. Expression levels were normalized to the control (PBS). $n = 3$, $^{**}P < 0.01$ and $^{***}P < 0.001$ versus PBS. (*B*) Human pleural mesothelial cells (HPMCs) were treated with TGF-β (5 ng/ml) and thrombin (13 nM) to induce MesoMT. Cell lysates were resolved by SDS-PAGE and immunoblotted for α-SMA, calretinin, and sm-myosin. β-actin was the loading control. (*C*) TGF-β (5 ng/ml) and thrombin (13 nM) induced myocardin but not myocardin-related transcription factor A (MRTF-A) or MRTF-B mRNA expression in HPMCs. mRNA levels were determined by qPCR. $n = 3$, $^{*}P < 0.05$ versus PBS. (*D*) TGF-β and thrombin induced cytoskeletal changes in HPMCs. Confocal images of HPMCs after TGF-β (5 ng/ml, middle panel) and thrombin (13 nM, bottom panel) stimulation. Upper panel (PBS) shows control cells without stimulation. F-actin (cyan), α-SMA (red), and sm-myosin (green) expression is enhanced in TGF-β– and thrombin-treated cells. Merged images include nuclei (DAPI, blue). Scale bars: 10 μm; 30 fields per slide; three to four slides per treatment.

edge of the cells. These findings suggest that the cells are not motile and instead are relatively quiescent. These results further demonstrate that myocardin gene silencing reverses TGF-β– or thrombin-induced MesoMT of HPMCs.

TGF-β–induced Increments in the Expression of Smooth Muscle Marker Genes Are Abrogated in MPMCs Obtained from Myocardin KO Mice

To further elucidate the role of myocardin in the expression of smooth muscle–associated genes in PMCs, we isolated MPMCs from myocardin conditional knockout (KO) mice and wild-type mice as previously described (8, 23), and examined these cells for myocardin and related gene expression profiles. Figure 3D shows the effect of

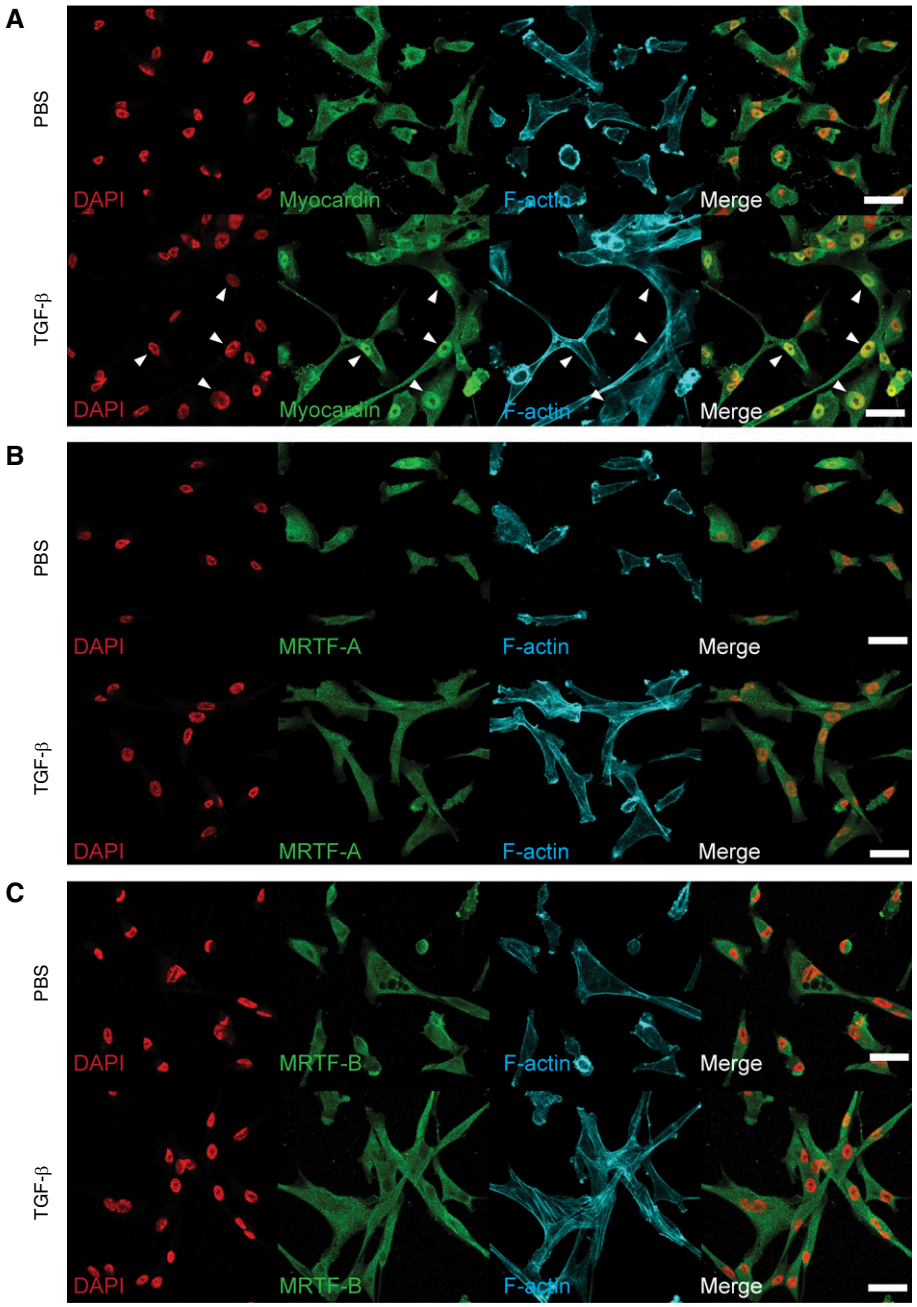

Figure 2. TGF-β induces nuclear translocation of myocardin during MesoMT. (*A*) Confocal images of myocardin localization in HPMCs in the presence and absence of TGF-β (5 ng/ml). Merged images show nucleus (DAPI, red) and myocardin (green). Arrowheads indicate areas of nuclear localization of myocardin (yellow). (*B*) Confocal images of MRTF-A (green) localization in HPMCs with or without TGF-β stimulation. Merged images show nucleus (DAPI, red) and MRTF-A. (*C*) Confocal images of MRTF-B (green) localization in HPMCs with or without TGF-β stimulation. Merged images show nucleus (DAPI, red) and MRTF-B signal. Scale bars: 20 μm.

TGF-β on the mRNA expression of various smooth muscle marker genes in MPMCs obtained from control and myocardin KO mice. Although TGF-β stimulation markedly upregulated α-SMA, calponin, and myocardin in control MPMCs, the upregulation of these genes was significantly abolished in the MPMCs of myocardin KO mice. This result further supports our hypothesis that myocardin contributes to the upregulation of smooth muscle marker genes, including α-SMA, during MesoMT.

Increased Expression of Myocardin and Its Downstream Genes Occurs in Human Pleural Tissue from Patients with Nonspecific Pleuritis

Because the above results support our postulate that myocardin upregulation is critical for the induction of MesoMT by TGF-β and thrombin, we next asked whether myocardin expression is upregulated in pleural tissues of patients who have received a diagnosis of nonspecific pleuritis. Figure 5 illustrates representative Trichrome and immunofluorescent analyses of histologically normal human lung tissues and lung tissues from patients with nonspecific pleuritis. The nonspecific pleuritis tissues showed deposition of collagen in the pleura, which is common in fibrosing lung and pleural injuries (Figure 5A). Further, α-SMA expression was increased in the nonspecific pleuritis tissues compared with normal pleural tissues, consistent with our previous report (Figure 5C) (8). Increased α-SMA in the nonspecific pleuritis tissues coincided with an increase in the expression of sm-myosin. Immunofluorescence analyses also revealed an apparent increase in collagen, myocardin, and calponin expression in the thickened pleura of patients with nonspecific pleuritis (Figure 5B). These results indicate that myocardin and its downstream genes are upregulated in close proximity to organization with fibrotic repair in the pleural tissues of human subjects with nonspecific pleuritis.

Myocardin Expression Is Increased in the Visceral Pleura of Mice with CBB-induced Pleural Fibrosis and *S. pneumoniae*–induced Empyema

We next sought to determine whether myocardin and its downstream genes are upregulated in disparate forms of pleural injury that resolve with pleural organization and fibrosis. To address this issue, we used two murine models of fibrosing pleural injury: one induced by CBB (8) and one induced by *S. pneumoniae* empyema (19). First, we performed immunohistochemistry on the pleural tissues of CBB- and empyema-injured mice. Trichrome staining of these tissues showed pleural thickening and collagen deposition (blue) in the submesothelial regions of the pleura (Figure 6A). We next performed immunofluorescent analyses of α-SMA, a marker of MesoMT (Figure 6C). α-SMA expression was

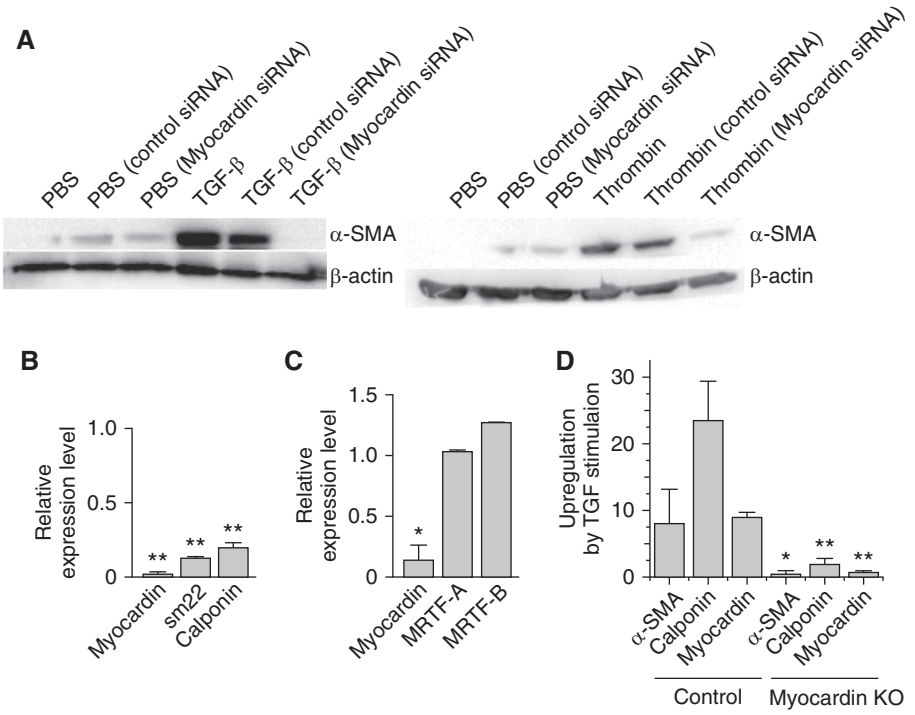

Figure 3. Effect of myocardin knockdown on myocardin and myocardin-related genes during MesoMT. Untransfected, control siRNA–, and myocardin siRNA–transfected cells were serum starved for 24 hours. The cells were then treated with PBS, TGF-β (5 ng/ml), or thrombin (13 nM) for 48 hours. (*A*) Cell lysates were resolved by SDS-PAGE and immunoblotted for α-SMA. β-actin was the loading control. A representative image of three independent experiments is shown. (*B* and *C*) Total RNA was isolated from control and myocardin siRNA–transfected cells in the presence of TGF-β. (*B*) Myocardin, sm22, and calponin mRNA was measured by qPCR analyses. Expression was normalized to the mRNA level of control siRNA–treated cells to be 1.0. $^{*}P < 0.05$ and $^{**}P < 0.01$ versus control siRNA. (*C*) Myocardin, MRTF-A, and MRTF-B mRNA was measured by qPCR analyses. Expression was normalized to the mRNA level of TGF-β–treated control siRNA cells, with the value set to 1.0. $^{*}P < 0.05$ and $^{**}P < 0.01$ versus control siRNA. Data are expressed as ± SEM, $n = 3$–4 independent experiments. (*D*) Mouse PMCs (MPMCs) were isolated from myocardin tissue–specific knockout (myocardin KO) and floxed myocardin mice (control). Serum-starved cells were then treated with TGF-β (5 ng/ml) and mRNA expression was determined by qPCR analysis. The mRNA level of PBS-treated MPMCs was set to a value of 1.0. $^{*}P < 0.05$ and $^{**}P < 0.01$ versus control. Data are expressed as ± SEM, $n = 3$ independent experiments.

markedly increased in the injured pleura (Figure 6C), consistent with previous reports (8, 19, 23). Calponin expression was concurrently increased in the thickened pleura of CBB- and *S. pneumoniae*–injured mice (Figure 6C). The results are consistent with *in vitro* data, which showed marked upregulation of calponin induced by external stimuli such as TGF-β and thrombin (Figure 1). Immunofluorescence staining of the tissues from the models also revealed a marked increase in myocardin expression within the visceral pleural tissues of the injured mice compared with saline-treated control mice (Figure 6B). Increased expression of myocardin coincided with the expression of sm-myosin (Figure 6B), one of the genes whose expression is controlled by myocardin (9). These results support our inference that myocardin activation is involved in fibrosing, chemically induced, or infectious pleural injuries through the upregulation of numerous genes that influence the cytoskeletal reorganization of PMCs during MesoMT.

Myocardin KO Mice Are Protected from TGF-β–mediated Pleural Fibrosis

To determine the role of myocardin in the progression of pleural fibrosis *in vivo*, we induced pleural fibrosis in myocd$^{-/-}$ and myocd$^{fl/fl}$ mice by intrapleural administration of AdTGF. No significant differences in pulmonary functions were observed between the AdGFP-treated groups (Figure 7A). However, the pulmonary functions of the AdTGF-treated floxed mice were significantly worsened compared with the AdEGFP controls. Conversely, the lung compliance of TGF-β–treated myocd$^{-/-}$ mice was not significantly different from that of the AdEGFP-treated controls (Figure 7A). Trichrome staining of the tissues revealed notable pleural thickening and collagen deposition (blue) in the submesothelial regions of the pleura of AdTGF-treated floxed mice. On the other hand, these AdTGF-induced changes were markedly reduced in the pleura of myocd$^{-/-}$ mice (Figure 7B). Immunofluorescent analyses also revealed that expression of α-SMA, a marker of MesoMT, was markedly increased in the TGF-β–injured pleura of myocd$^{fl/fl}$ mice. Similar to what was observed in the CBB- and *S. pneumoniae* empyema–induced fibrosis models (Figure 6), myocardin expression was markedly increased in the TGF-β–injured pleura of myocd$^{fl/fl}$ mice (Figure 7C). Consistent with myocardin upregulation, the expression of calponin, another gene product under the control of myocardin, was notably increased (Figure 7C). Conversely, expression of α-SMA, calponin, and myocardin in the pleural mesothelium of TGF-β–treated myocd$^{-/-}$ mice was reduced compared with similarly treated floxed control mice (Figure 7C). The AdEGFP-treated mice showed no pleural thickening or detectable increments of α-SMA expression (data not shown). These findings strongly support our hypothesis that myocardin is a critical determinant of MesoMT and subsequent pleural fibrosis *in vivo*.

Discussion

In this study, we found, for the first time, that myocardin plays a regulatory role in the progression of MesoMT, which contributes to pleural fibrosis. Both TGF-β and thrombin stimulation significantly increased the expression of myocardin during MesoMT. In addition, myocardin translocated to the nucleus during MesoMT, which enabled myocardin to serve as a transcriptional coactivator. Furthermore, myocardin knockdown blocked thrombin and TGF-β–mediated induction of α-SMA and phenotypic

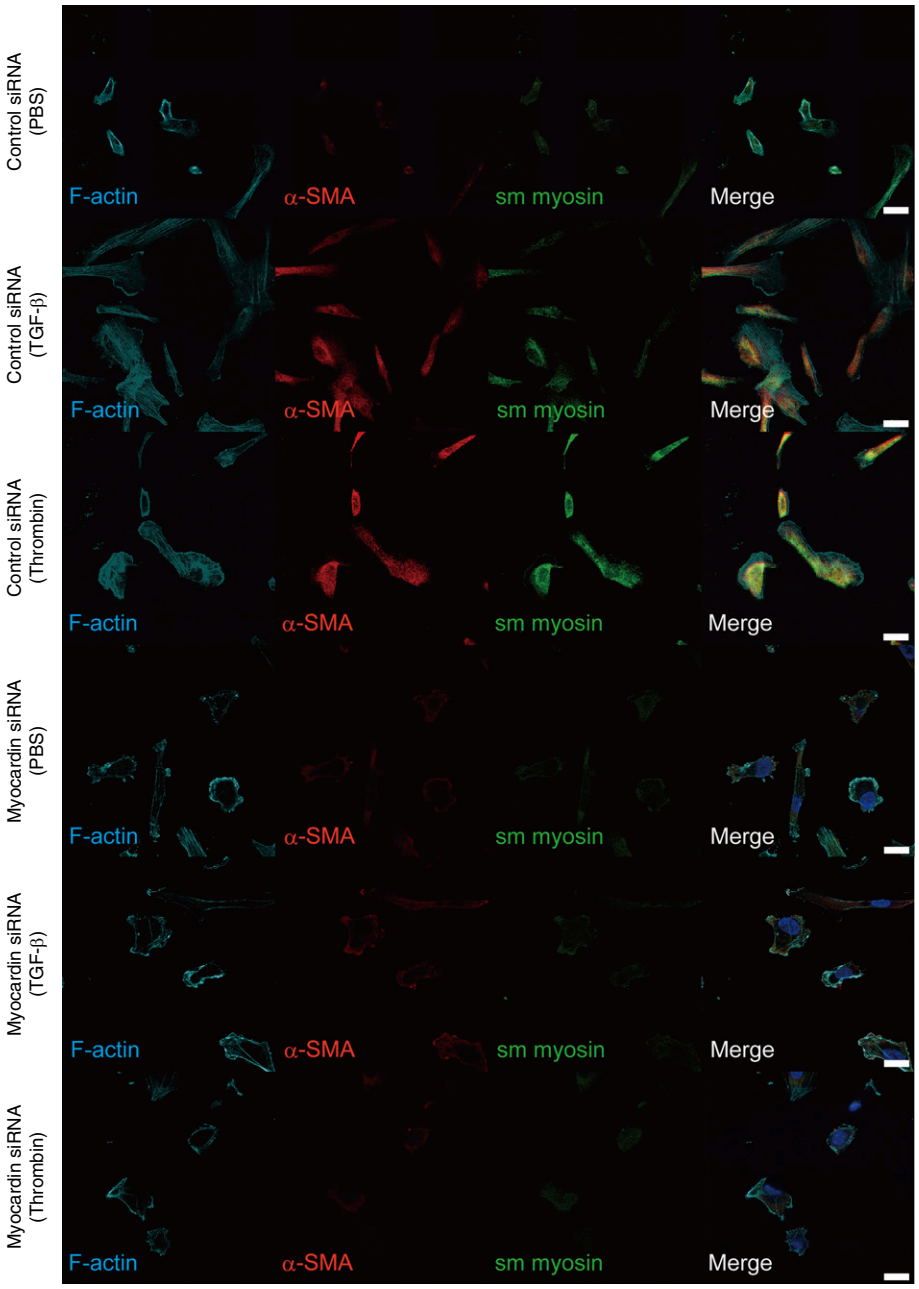

Figure 4. Effect of myocardin gene silencing on cell morphology and actin cytoskeletal structure of TGF-β– and thrombin-treated HPMCs. HPMCs were cultured on glass coverslips. The cells were transfected with control or myocardin siRNA and then treated with PBS, TGF-β, or thrombin. F-actin (cyan), α-SMA (red), and sm-myosin (green) expression was then detected by immunofluorescent imaging. Merged images include nuclei (DAPI, blue). Scale bars: 10 μm; 30 fields per slide; three to four slides per treatment.

changes associated with MesoMT. Induction of α-SMA, calponin, and sm22 was likewise abolished in MPMCs of myocardin KO mice. Expression of myocardin was upregulated in the pleural mesothelium of patients with pleuritis and in the thickened pleura of mice with CBB-induced injury and with empyema, suggesting that it participates in the regulation of MesoMT within the context of evolving pleural organization and fibrosis.

Several studies have shown that MRTF-A, a myocardin family transcription factor, is involved in epithelial–mesenchymal transition, a process that shares biomarker and phenotypic correlates with MesoMT, including induction of α-SMA. Korol and colleagues reported that MRTF-A is involved in TGF-β–induced epithelial–mesenchymal transition of lens epithelial cells (33). Shiwen and colleagues found that MRTF-A translocated to the nucleus in scleroderma tissues, and that knockdown of MRTF-A diminished the systemic sclerosis myofibroblast-enhanced contractility (34). Recently, it was reported that an MRTF inhibitor, CCG-203971, prevented nuclear localization of MRTF-A in lung fibroblasts and blocked TGF-β–induced lung myofibroblast differentiation (35). In the present study, we found that TGF-β induced a marked upregulation of myocardin in MesoMT, but only marginally affected MRTF-A and MRTF-B expression. Moreover, although TGF-β induced nuclear translocation of myocardin, localization of MRTF-A and MRTF-B was unaffected. In parallel studies, MPMCs obtained from myocardin KO mice did not show TGF-β–induced upregulation of α-SMA and other smooth muscle marker genes. These results extend prior studies in the field and show that myocardin is involved in MesoMT, which is a completely novel observation. Our results also show that myocardin contributes to MesoMT and fibrosing pleural injury. Further study is required to determine whether the MRTFs substantially contribute to MesoMT.

In contrast to the MRTFs, myocardin expression was believed to be restricted to cardiac and smooth muscle (11, 26, 36, 37) and constitutively located in the nucleus (26). We found that basal myocardin expression in HPMCs was low but detectable. However, TGF-β treatment markedly increased myocardin expression in these cells. These results suggest that the expression of myocardin can increase in HPMCs after exposure to stimuli linked to the pathogenesis of MesoMT and pleural fibrosis, including TGF-β and thrombin. The upregulation of myocardin in these cells subsequently increases the expression of downstream genes to induce cytoskeletal reorganization and phenotypic changes.

We also found that myocardin was exclusively localized to the cytoplasm before stimulation, and that TGF-β stimulation induced nuclear translocation of myocardin. In contrast to the MRTFs, it has been believed that myocardin is constitutively

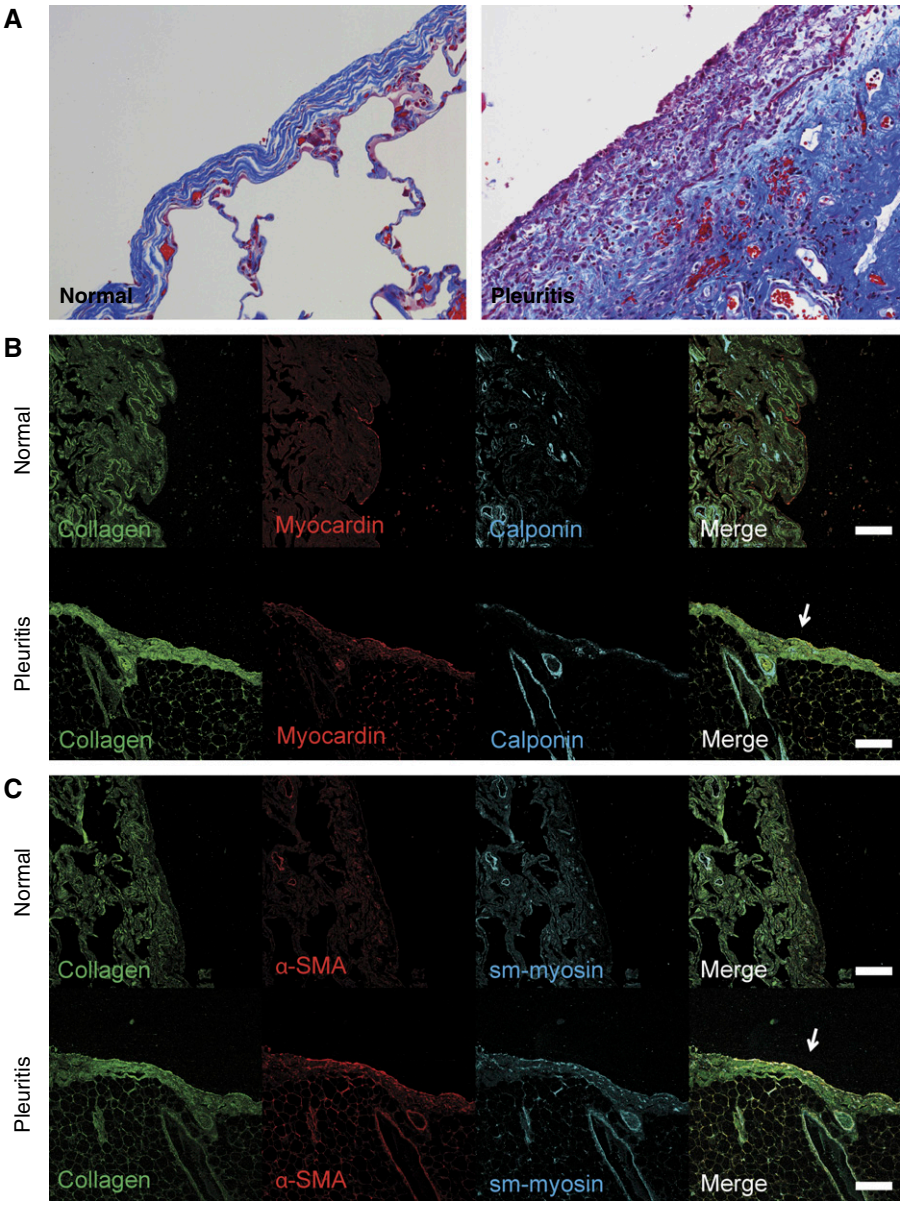

Figure 5. Upregulation of myocardin and its downstream genes in human nonspecific pleuritis tissues. (*A*) Lung tissue sections from normal subjects (left) and patients with pleuritis (right) were Trichrome stained to show collagen deposition (blue) and changes in pleural architecture. (*B*) Human pleural tissue sections were immunofluorescently stained for collagen (green), myocardin (red), and calponin (cyan). Myocardin and calponin colocalized in the pleuritis tissue. Collagen expression was enhanced in pleuritis tissues compared with normal lung tissues. (*C*) Human pleural tissue sections were immunofluorescently stained for collagen (green), α-SMA (red), and sm-myosin (cyan). Collagen, α-SMA, and sm-myosin were colocalized at the pleura. White arrows indicate pleura; 30 fields per slide; four slides per group. Scale bars: 200 μm.

located in the nucleus (26, 36, 37). In the case of MRTFs, the RPEL motifs have a high affinity for G-actin, and the binding of G-actin to MRTFs prevents their translocation into the nucleus (38). This mechanism was not believed to function for myocardin, as the affinity of RPEL motifs of myocardin to G-actin is very weak (38). At present, it is unclear how TGF-β promotes the translocation of myocardin to the nucleus. Further investigation is required to elucidate the nuclear translocation mechanism and function of myocardin in various cell types in pathophysiological conditions.

Critical questions raised by our results include how external stimuli—in particular, TGF-β —activate myocardin, and how myocardin activation facilitates the progression of MesoMT. At present, the responsible mechanisms remain unclear, although the literature provides important clues. TGF-β promotes RhoA activity and signaling, which promotes actin reorganization and polymerization (39). This actin polymerization has been suggested to regulate myocardin and related myocardin family members (31, 40). Furthermore, the RhoA/ROCK pathway is reported to regulate myocardin expression (41, 42). Smooth muscle differentiation marker proteins such as α-SMA, calponin, and sm22 have also been shown to be regulated by RhoA (39). Taken together, these observations suggest that the RhoA pathway is likely involved in TGF-β–induced upregulation of myocardin in HPMCs. Conversely, our study shows that myocardin activity regulates the gene expression of several effector proteins. TGF-β–mediated induction of calponin was dramatically attenuated by myocardin downregulation. Because calponin is an actin-binding protein that is believed to regulate contractile actin-myosin filaments as well as the noncontractile actin cytoskeletal structure (43), the increase in calponin expression might influence the actin cytoskeletal structure, thus promoting MesoMT via myocardin activation and localization to the nucleus.

The pleural rind tissues from patients with nonspecific pleuritis characteristically showed increased collagen deposition and increased pleural thickening, as previously reported (8). We also found that myocardin and myocardin-regulated proteins, such as calponin, were likewise increased in human pleuritis compared with normal tissues. Furthermore, these proteins colocalized in the thickened pleural mesothelium. Although we found that myocardin is involved in the induction of MesoMT and occurs in pleural tissues undergoing fibrotic repair, its role in evolving pleural fibrosis remains to be more clearly defined. For example, myocardin acts as a transcription factor and serves as the master regulator for the expression of smooth muscle genes, such as calponin. As such, these other related genes may serve as effectors for myocardin and may likewise be capable of attenuating the induction of MesoMT. Additional studies, which lie outside the

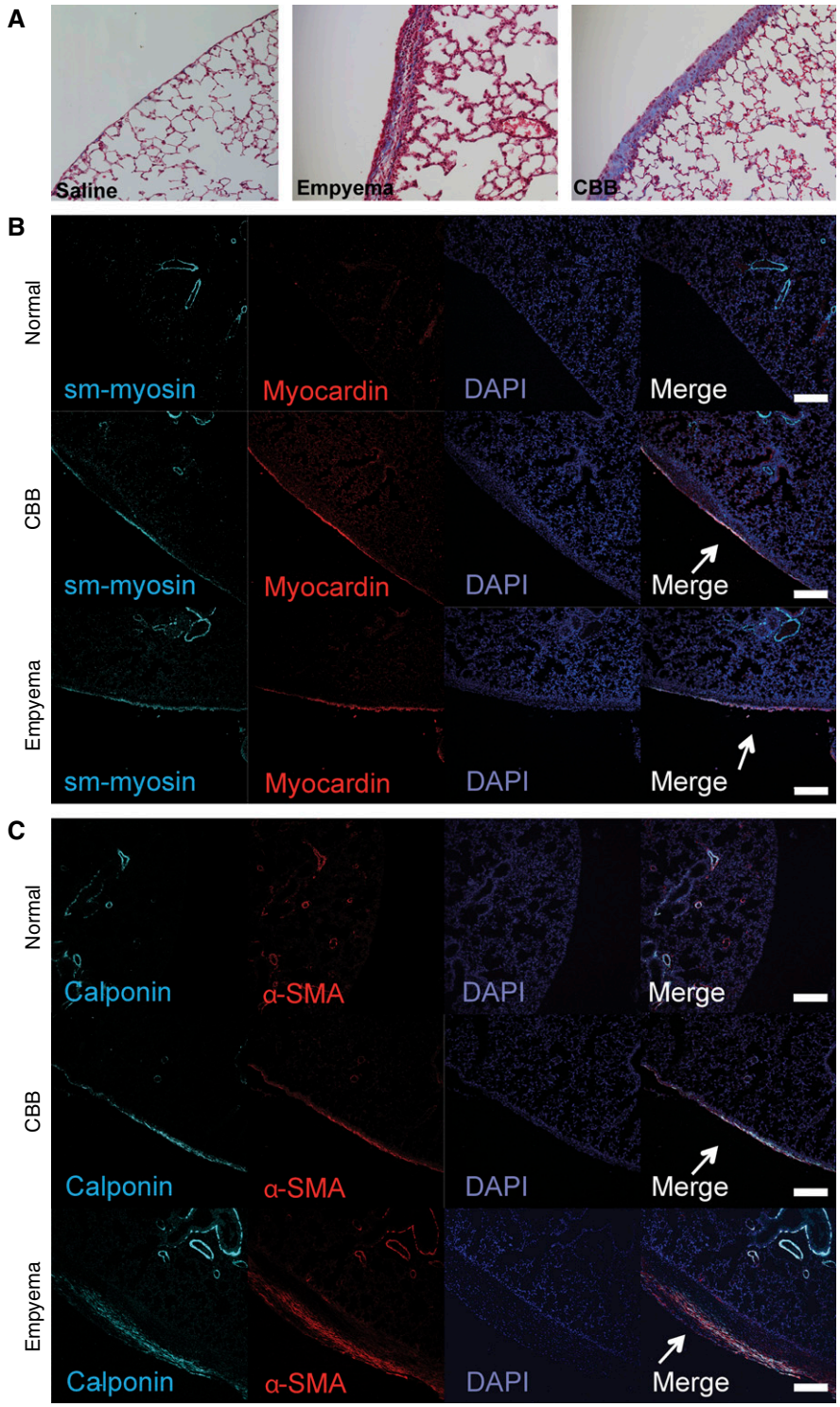

Figure 6. Upregulation of myocardin and its downstream genes in two fibrosing pleural injury models in mice (carbon black/bleomycin [CBB] and empyema). (*A*) Lung tissue sections from saline-, CBB-, and empyema-treated mice were Trichrome stained to show collagen deposition (blue) and changes in pleural architecture. (*B*) Mouse pleural tissue sections were immunofluorescently stained for sm-myosin (green), myocardin (red), and nuclei (DAPI, blue). Myocardin and sm-myosin colocalized in the CBB- and empyema-treated tissues. (*C*) Mouse pleural tissue sections were immunofluorescently stained for calponin (green), α-SMA (red), and nuclei (DAPI, blue). Calponin and α-SMA were colocalized at the pleura in CBB- and empyema-treated lung tissues. Solid white arrows indicate pleura; 30 fields per slide; three to four slides per group. Scale bars: 200 μm.

scope of this work, are required to confirm this hypothesis.

The relative overexpression of myocardin and related genes observed in patients with nonspecific pleuritis raises other considerations. It may be that sustained expression of myocardin or myocardin-related genes is required for the development of clinical fibrothoraces. It may also be that myocardin is critical for pleural rind formation or loculation in patients with empyema; however, resolving this issue would require dedicated analyses of pleural tissues from such patients. The preclinical data from mice with CBB- or empyema-induced pleural injury suggest that myocardin could play a role in the pathogenesis of a range of clinical conditions characterized by pleural organization and fibrosis. At present, the role of myocardin in these conditions is unknown and will require additional study.

Our results also showed that myocardin and related proteins were commonly upregulated *in vivo* in both chemically induced and empyema pleural injury models. Although bleomycin has long been used to induce lung fibrosis in mice, carbon black nanoparticles in combination with bleomycin (the CBB model) have been used to induce robust pleural fibrosis in mice (4, 8). CBB-injured mice demonstrate reduced lung function and increased pleural thickening (4, 8). Although the CBB model is an excellent pleural injury model in which to study pleural fibrosis, we also employed an infectious disease model using the clinically relevant pathogen *S. pneumoniae* as described by Boren and colleagues (23). As previously reported, α-SMA was notably increased in both pleural injury models, suggesting the increasing presence of myofibroblasts within organizing, fibrosis-prone pleural tissues. Here, we show for the first time that myocardin expression was likewise increased in the thickened pleural rind in both models. Consistent with the *in vitro* findings, calponin expression was likewise markedly upregulated in pleural tissue from the injured mice.

To evaluate the role of myocardin in the progression of pleural fibrosis, we used the AdTGF murine model. Previous studies by Decologne and colleagues (5) using similar AdTGF constructs were limited to rats and much higher doses. We found that tissue-specific knockdown of myocardin in the pleural mesothelium attenuated the

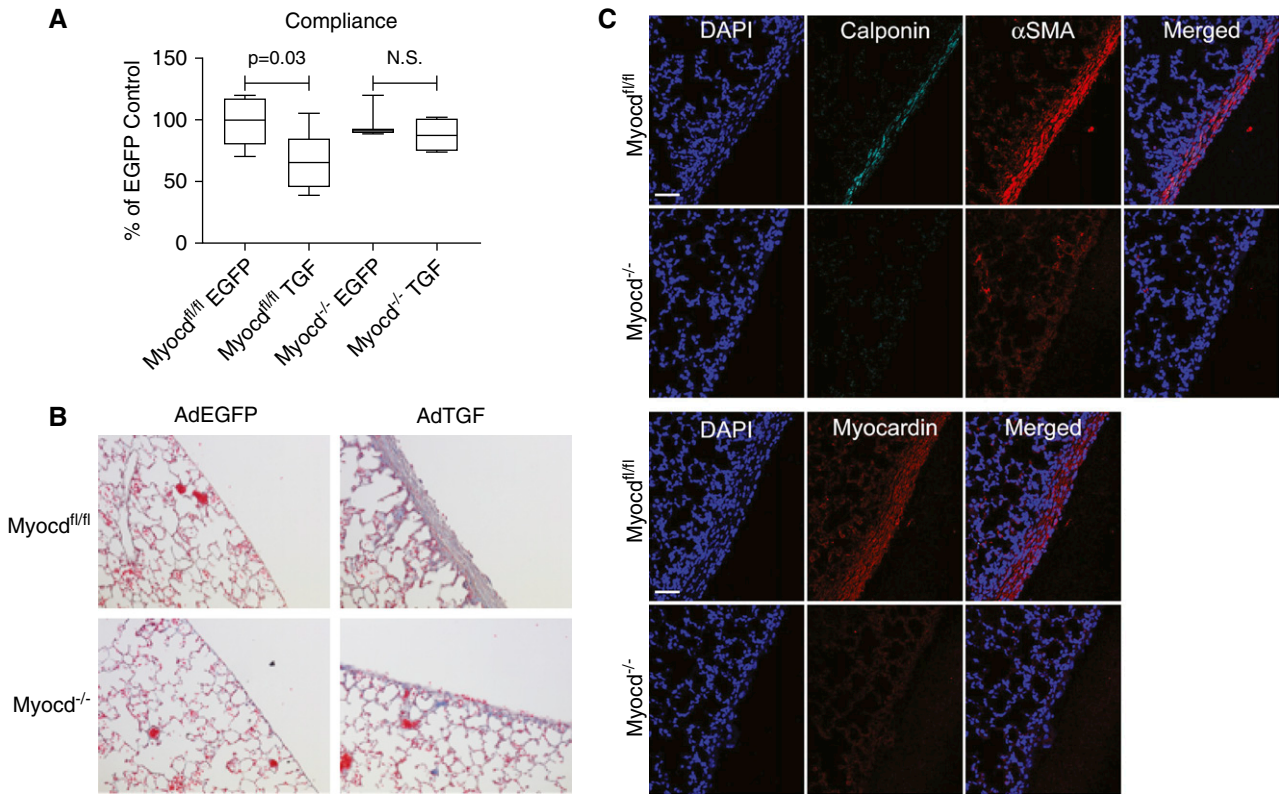

Figure 7. Myocardin KO mice are protected from TGF-β–induced pleural fibrosis. Myocd$^{fl/fl}$ and tissue–specific myocardin knockout (myocd KO) mice were treated with adenoviral vectors encoding enhanced GFP (EGFP) or TGF-β. (*A*) After 7 days, pulmonary function (compliance) was determined. Data are expressed as ± SEM, $n = 3$–7 mice per treatment. (*B*) After 7 days, lung tissue sections from myocd$^{fl/fl}$ or myocd KO mice treated with adenoviral vectors encoding EGFP or TGF-β were Trichrome stained to show collagen deposition (blue) and changes in pleural architecture. (*C*) Lung tissue was collected from TGF-β adenoviral-treated mice. Mouse pleural tissue sections were immunofluorescently stained for nuclei (DAPI, blue), calponin (green), and α-SMA (red). Top two panels: calponin and α-SMA colocalized in the pleura of TGF-β–treated myocardin floxed mice. Bottom two panels: mouse pleural tissue sections were immunofluorescently stained for nuclei (DAPI, blue) and myocardin (red). TGF-β adenoviral treatment of myocd$^{fl/fl}$ mice showed increased pleural thickening and α-SMA, myocardin, and calponin expression compared with myocd KO mice; 30 fields per slide; three to seven slides per group. Representative images are shown. Scale bars: 50 μm. N.S. = not significant.

progression of pleural fibrosis in a model of TGF-β–mediated pleural fibrosis. Although α-SMA and calponin were induced in this model, their expression was reduced in myocardin KO mice. Furthermore, the decrements in lung function induced by TGF-β were blocked by myocardin knockdown. These results further support our hypothesis that myocardin upregulation plays a critical role in the progression of MesoMT and may strongly contribute to pleural fibrosis. In contrast to our *in vitro* findings, sm-myosin was notably upregulated in the injured pleura of both animal models. In the *in vivo* mouse models, pleural tissue is exposed to multiple types of stimuli, including inflammatory, profibrotic, fibrinolytic, and coagulation mediators, for extended periods of time. Combinations of stimuli could have contributed to the differential response of sm-myosin that we observed in our *in vitro* and *in vivo* analyses. These results collectively support the idea that activation of the genes controlled by myocardin plays an important role in pleural remodeling during the development of pleural organization and fibrosis.

Among the myocardin family proteins, MRTFs, but not myocardin, have been fully investigated for the upregulation of α-SMA during TGF-β–induced mesenchymal transition of the cells. This may be in part because it has been believed that myocardin function is rather restricted in cardiac and smooth muscle development. The present study indicates that myocardin participates in the development of MesoMT in HPMCs and may thereby contribute to the pathogenesis of pleural organization and fibrosis. The role of myocardin in pathological processes, including various types of fibrosis, has not been thoroughly investigated. However, myocardin expression is markedly upregulated during MesoMT, suggesting that myocardin may play an important role in pathogenic conditions in various types of cells, whereas physiological myocardin expression might be limited to cardiac and smooth muscle tissues. The present study sheds light on the possible function of myocardin in various pathological conditions, including fibrosis and diseases involving mesenchymal transition. ■

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

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