Metabolic Reprogramming Is Required for Myofibroblast Contractility and Differentiation*

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Background: Myofibroblasts, by virtue of their functions, are highly energy-dependent.

Results: TGF-β1-induced myofibroblast differentiation is associated with a metabolic reprogramming. This metabolic adaptation is essential to the expression of myofibroblast-related genes.

Conclusion: Metabolic reprogramming is a hallmark of myofibroblast differentiation and is critical for its contractile function.

Significance: This is the first report that links bioenergetics to myofibroblast activation.

Contraction is crucial in maintaining the differentiated phenotype of myofibroblasts. Contraction is an energy-dependent mechanism that relies on the production of ATP by mitochondria and/or glycolysis. Although the role of mitochondrial biogenesis in the adaptive responses of skeletal muscle to exercise is well appreciated, mechanisms governing energetic adaptation of myofibroblasts are not well understood. Our study demonstrates induction of mitochondrial biogenesis and aerobic glycolysis in response to the differentiation-inducing factor transforming growth factor β1 (TGF-β1). This metabolic reprogramming is linked to the activation of the p38 mitogen-activated protein kinase (MAPK) pathway. Inhibition of p38 MAPK decreased accumulation of active peroxisome proliferator-activated receptor γ coactivator 1α in the nucleus and altered the translocation of mitochondrial transcription factor A to the mitochondria. Genetic or pharmacologic approaches that block mitochondrial biogenesis or glycolysis resulted in decreased contraction and reduced expression of TGF-β1-induced α-smooth muscle actin and collagen α-2(I) but not of fibronectin or collagen α-1(I). These data indicate a critical role for TGF-β1-induced metabolic reprogramming in regulating myofibroblast-specific contractile signaling and support the concept of integrating bioenergetics with cellular differentiation.

Myofibroblasts are key effectors of normal wound healing, and their persistence contributes to the pathogenesis of fibrosis and cancer (1, 2). The myofibroblast is the primary cell that secretes provisional extracellular matrix proteins and contracts wound margins to facilitate re-epithelialization. Transforming growth factor β-1 (TGF-β1) is the prototypical repair cytokine that induces the expression of cytoskeletal proteins such as α-smooth muscle actin (α-SMA) and extracellular matrix proteins that are critical for its contractile/synthetic functions (3); hence, α-SMA expression is used as a marker of myofibroblast differentiation in association with the production of extracellular matrix components such as fibronectin and type I collagens (5, 6).

TGF-β1 signals via SMAD-dependent and -independent pathways including p38 MAPK, ERK, JNK, and c-Abl (7). In concert with TGF-β1-mediated myofibroblast differentiation, contraction of myofibroblasts maintains the differentiated state (8, 9). Indeed, it has been shown that force-induced polymerization of actin stress fibers promotes the translocation of the transcription factor MKL1 to the nucleus where it promotes the expression of α-SMA and collagen α-2(I) (col1A2) (9–11). This positive feedback loop is important for tissue repair because it sustains myofibroblast differentiation after the acute phase of cytokine-mediated myofibroblast activation.

Protein synthesis and contraction are highly energy-dependent processes that likely engage both oxidative phosphorylation and glycolysis. For example, the increased energetic demand encountered in the skeletal muscle cell during endurance training is achieved through an increase in mitochondrial mass (12–14). Both the renewal of damaged mitochondria and increase in mitochondrial mass rely on the stimulation of mitochondrial biogenesis (15). Mitochondrial biogenesis is controlled by the metabolic status of cells and/or external stimuli such as hormones. PGC-1α is a co-activator of transcription that promotes the expression of nuclearly encoded mitochondrial genes involved in mitochondrial biogenesis and/or function (16). PGC-1α mediates its action on gene expression by binding to NRF-1 and/or NRF-2 upon its phosphorylation (17, 18). The activity of PGC-1α is tightly regulated at both the transcriptional and post-translational levels (19–24). PGC-1α contains multiple phosphorylation sites that are targets of dif-
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TABLE 1

| Primer sequences | Gene, accession number | Primer sequence (5′–3′) | Product size (bp) |
|------------------|------------------------|-------------------------|------------------|
| Col1A1, NM_000088.3 | Forward, AGCGAGCAATCCACCACCATCACC | 129 |
| FN (all variants) | Reverse, AGGTAGTGCTTCGGACAGCC | 104 |
| α-SMA, NM_001498 | Forward, CTCCACAGCTCATCGAAG | 107 |
| TFAM, NM_003201.2 | Reverse, CAGGAGTGAGTCTGAGCAAG | 194 |
| 18S rRNA, NR_003286.2 | Forward, GTCTGGCTATAGGTTTCCTG | 111 |
| Col1A2, NM_000089.3 | Reverse, CAGGAGTAAGAAAGGTCTCAGT | 127 |

fert signaling pathways, thus conferring to PGC-1α its role as a signaling integrator (25, 26). Mitochondrial transcription factor A (TFAM) is a downstream target gene of PGC-1α (27, 28). TFAM controls the transcription of mitochondrial DNA-encoded genes as well as DNA replication during biogenesis (27). TFAM is synthesized as a 29-kDa precursor in the cytoplasm. Upon its import into the mitochondrion, TFAM is cleaved into a shorter polypeptide (25 kDa) to be activated (28–31).

TFAM is synthesized as a 29-kDa precursor in the cytoplasm. Upon its import into the mitochondrion, TFAM is cleaved into a shorter polypeptide (25 kDa) to be activated (28–31). Glycolysis is an alternate pathway to oxidative phosphorylation for ATP production. It converts glucose into pyruvate via a series of enzymatic activities located in the cytosol. Despite its relative inefficiency in producing ATP compared with oxidative phosphorylation (32, 33), glycolysis can respond rapidly to provide both ATP and other metabolites necessary for energetically demanding processes (34–36). Indeed, in response to increased energy demand, the stimulation of both oxidative phosphorylation and aerobic glycolysis is now emerging as a characteristic of many cells including those of the immune system and platelets (37–40).

In this study, we present evidence, for the first time, that TGF-β1 induces metabolic reprogramming via the stimulation of mitochondrial biogenesis and glycolysis in human lung fibroblasts in parallel with induction of profibrotic gene expression (41). We show that both TGF-β1-induced mitochondrial biogenesis and glycolysis are required for the expression of α-SMA and Col1A2 via energy-dependent contractile activity that is dependent on p38 MAPK. Our study supports the essential role for the integration of cellular bioenergetics with gene expression to sustain the differentiated phenotype of myofibroblasts.

Experimental Procedures

Reagents—Porcine platelet-derived TGF-β1 was purchased from R&D Systems (Minneapolis, MN), protease inhibitor mixture set III was from EMD Chemicals (San Diego, CA), p38 MAPK inhibitor SB202190 was from Tocris Bioscience (Minneapolis, MN), and MitoTracker® was from Life Technologies. We purchased antibodies to β-actin (clone AC-15), fibronectin (clone IST-4), and α-tubulin (clone B-5-1-2) from Sigma; α-SMA (clone ASM-1) from American Research Products (Belmont, MA); TFAM, total PGC-1α, hexokinase II (HKII), total MLC20, phospho-MLC20, GADPH, VDAC, p38 MAPK from Cell Signaling Technology (Boston, MA); TOM20 and lamin A/C from Santa Cruz Biotechnology (Dallas, TX); and phospho-PGC-1α from R&D Systems. All other reagents were purchased from Sigma unless otherwise specified.

Cell Culture—Human fetal lung fibroblasts (IMR-90 cells) at low population doubling (PDL 7) were purchased from Coriell Cell Repositories (Camden, NJ). All cells were cultured in DMEM (Life Technologies) supplemented with 10% fetal calf serum (HyClone Laboratories, Logan, UT), 100 units/ml penicillin, 100 μg/ml streptomycin, and 1.25 μg/ml amphotericin B at 37 °C in 5% CO2, 95% air. Cells treated with TGF-β1 were cultured in serum-free medium for 24 h prior to and during treatment.

Western Immunoblotting—We prepared cell lysates in radioimmune precipitation assay buffer (150 mM NaCl, 1.0% IGE-PAL® CA-630, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0; Sigma-Aldrich), and cytosolic and nuclear lysates were prepared using the Pierce NE-PER kit according to the manufacturer’s recommendations. Mitochondria were isolated using the Mitochondria Isolation kit for mammalian cells (Thermo Scientific). The total protein concentration of lysates was quantitated using a Micro BCA Protein Assay kit (Pierce) or the DC Protein Assay kit (Bio-Rad). Lysates were then subjected to SDS-PAGE under reducing conditions, and Western immunoblotting was performed as described previously (3). Immunoblots were imaged using an Amersham Biosciences 600 Imager (GE Healthcare). Signals were quantitated using ImageQuant TL software.

Quantification of Mitochondrial DNA Copies—The ratio of mitochondrial to nuclear DNA was assessed using the Human Mitochondrial DNA Monitoring Primer Set Ratio kit (Takara Bio, Mountain View, CA).

Real Time PCR—Total RNA was isolated from cells using the RNeasy® Mini kit (Qiagen, Valencia, CA) and reverse transcribed using iScript Reverse Transcription SuperMix for RT-quantitative PCR (Bio-Rad). Real time PCRs were performed using SYBR® Green PCR Master Mix (Life Technologies) and gene-specific primer pairs for collagen 1A1, collagen 1A2, fibronectin, α-SMA, TFAM, and 18S rRNA (for primer sequences, see Table 1). Reactions were carried out for 40 cycles (95 °C for 15 s and 60 °C for 1 min) in a StepOnePlus Real Time PCR System (Life Technologies). Real time PCR data are expressed for each target gene as 2−ΔΔCt versus endogenous 18S rRNA with error bars representing the standard error of the mean for three experiments. Two-tailed Student’s t tests were used for pairwise comparisons.

Plasmid Transfection—A plasmid encoding a dominant negative form of p38 MAPK (pCMV-p38DN) was transfected into
IMR-90 cells using Lipofectamine 2000 (Life Technologies) according to the manufacturer’s instructions (1:2 ratio of DNA (in µg) to Lipofectamine 2000 (in µl)). IMR-90 cells were incubated overnight in DNA-lipid complexes, and then cells were allowed to recover for 24 h in DMEM containing 10% FBS prior to serum starvation and TGF-β1 treatment.

**RNA Interference**—siRNA targeting TFAM as well as non-targeting control (Life Technologies; for siRNA sequences, see Table 2) was transfected into lung fibroblasts using Lipofectamine RNAiMAX (Life Technologies) at a final concentration of 100 nM.

**Assessment of Bioenergetics**—Cells were plated on Seahorse Extracellular Analyzer XF96 plates. Cells were washed in XF assay buffer (DMEM with 1 mM pyruvate, 5.5 mM d-glucose, 4 mM L-glutamine, pH 7.4) and brought to 180-µl final volume in this assay medium. Bioenergetic measurements were interpreted and performed as described (42). In short, the basal oxygen consumption rate (OCR) was measured for 24 min after which oligomycin (1 µg/ml), carbonyl cyanide p-trifluoromethoxyphenylhydrazone (0.6 µM), and antimycin A (10 µM) were injected sequentially. The extracellular acidification rate (ECAR) was measured simultaneously.

**Mitochondrial Assay**—This assay was performed on the Extracellular Analyzer as adapted from Salabei et al. (43). In short, cells were washed and brought to 180-µl final volume in mannitol and sucrose (MAS) buffer (70 mM sucrose, 220 mM mannitol, 10 mM K2HPO4, 5 mM MgCl2, 2 mM HEPES, 1 mM EGTA, pH 7.2). After three basal measurements were acquired, saponin (30 µg/ml) together with pyruvate (5 mM), malate (2.5 mM), and ADP (1 mM) were injected. Sequentially rotenone (1 µM), succinate (10 µM), ADP (1 mM), and then antimycin A (10 µM) were injected.

**Contractility Assay**—After cell harvesting, lung fibroblasts were mixed at a density of 2·10^5 cells/ml in a solution composed of 8 volumes of rat tail collagen type I (BD Biosciences), 1 volume of 10X Ham’s F-12 (Life Technologies), and 1 volume of reconstitution buffer (2% NaHCO3, 4.77% HEPES). The solution was poured into a 24-well plate (0.5 ml/well), and gelation was performed at 37 °C for 30 min. Gels were then overlaid with 1.5 ml of 1% serum-containing cell culture medium. The next day gels were treated with or without TGF-β1.

**Statistical Analysis**—Statistical analysis was performed using GraphPad software. The data are presented as mean ± S.E. Statistical comparisons were made by performing unpaired Student’s t tests unless otherwise indicated.

**Results**

**TGF-β1 Enhances Mitochondrial Oxygen Consumption without Affecting Electron Transport Activity**—To determine potential changes in cellular bioenergetics in TGF-β1-differentiated myofibroblasts, we analyzed OCRs and ECARs of normal human diploid lung fibroblasts (IMR-90) treated without or with TGF-β1 (2.5 ng/ml for 48 h) (3). TGF-β1 enhanced overall bioenergetics with a marked increase in basal OCR, ATP-linked respiration, maximal and reserve capacity, proton leak, and non-mitochondrial OCR (Fig. 1, A and C). Furthermore, TGF-β1 also increased ECAR from 16 ± 0.23 milli-pH units/min/10,000 cells in the unstimulated state to 30 ± 0.55 milli-pH units/min/10,000 cells in the TGF-β1-stimulated state (Fig. 1B). Interestingly, when mitochondrial ATP production was inhibited with oligomycin, the increase in ECAR was 63% higher in the TGF-β1-treated cells compared with the control, consistent with a greater capacity for glycolysis (Fig. 1B). Plotting OCR as a function of ECAR showed TGF-β1-induced metabolic reprogramming with induction of mitochondrial respiration and glycolysis resulting in a condition of aerobic glycolysis (Fig. 1D).

The increase in mitochondrial respiration could be due to an increase either in mitochondrial mass or in substrate supply to the electron transport chain. To delineate the mechanism of this increase in mitochondrial respiration, the functioning of complex I or II was measured using a mitochondrial assay where the plasma membrane of fibroblasts was permeabilized and exogenous respiratory substrates were delivered to the electron transport chain. In this assay, after basal OCR was measured, saponin, ADP, succinate (complex II-linked substrate), pyruvate, and malate (complex I-linked substrate) were injected to stimulate respiration (Fig. 1E). Next, rotenone was injected to quantify complex I-linked respiration followed by antimycin A to measure complex II-linked respiration. TGF-β1 induced a small, but significant, increase in complex I-linked respiration without a significant change in complex II-linked OCR at 48 h after treatment (Fig. 1E).

**TGF-β1 Stimulates Mitochondrial Biogenesis and Glycolysis in Lung Fibroblasts**—Next, we determined whether TGF-β1-induced mitochondrial biogenesis is associated with alterations in mitochondrial distribution and abundance. Staining of the mitochondrial compartment (with the fluorescent dye Mitotracker) demonstrated that the mitochondrial population was relatively homogeneous and mainly localized to the perinuclear region in unstimulated cells; in contrast, there was an apparent increase in mitochondrial abundance with a more diffuse cytoplasmic staining in TGF-β1-differentiated myofibroblasts (Fig. 2A). To determine whether these apparent changes in mitochondrial abundance are linked to mitochondrial biogenesis, we analyzed the effect of TGF-β1 on the expression of mitochondrial biogenesis markers. TGF-β1 induced a time-dependent increase in phosphorylated PGC-1α, a transcriptional co-activator that regulates nuclearly encoded mitochondrial genes (16) (Fig. 2B). Phosphorylation of PGC-1α occurred as early as 15 min after exposure to TGF-β1. Phosphorylation of PGC-1α was increased by 9.71-fold (p < 0.05) compared with baseline following 48-h treatment with TGF-β1. VDAC resides in the outer membrane of the mitochondria where it participates in metabolism and, under conditions of stress, in the formation of the mitochondrial permeability transition pore (44–47). We detected a time-dependent increase in VDAC expression following exposure to TGF-β1 (Fig. 2C), supporting increased mitochondrial mass in differentiated cells.
Mitochondria contain between two and 10 copies of mitochondrial DNA (48). Therefore, an increase in mitochondrial DNA copy number per cell correlates with an increase in mitochondrial mass. We tested the effect of TGF-β1 treatment on the mitochondrial DNA content in IMR-90 fibroblasts. TGF-β1 induced a significant increase of the mitochondrial DNA copy number:nuclear DNA ratio as determined by real-time PCR (Fig. 2D).

HKII catalyzes the first step of glycolysis (49) and showed a time-dependent increase in expression starting at 12 h after TGF-β1 exposure with maximal expression at 48 h (Fig. 2E). Together, these data suggest that TGF-β1 stimulates both mitochondrial biogenesis and glycolysis in human lung fibroblasts.

TGF-β1 Activates Metabolic Reprogramming via a p38 Mitogen-activated Protein Kinase-dependent Pathway—TGF-β1 signals via SMAD-dependent and -independent pathways (7). p38 MAPK is one of the effectors of the SMAD-independent actions of TGF-β1, and it is also a known activator of PGC-1α (50, 51). We have previously demonstrated the early activation of p38 MAPK in response to TGF-β1 in IMR-90 fibroblasts (52). We analyzed the effect of blocking p38 MAPK activity using the p38 MAPK inhibitor SB202190, which binds the ATP binding pocket of the active kinase (53, 54). IMR-90 cells were pretreated with SB202190 (5 μM) or vehicle 30 min prior to the addition of TGF-β1. In response to TGF-β1, we detected an increase in phosphorylated PGC-1α in the nuclear fraction (Fig. 3A) without an effect on total PGC-1α (Fig. 3B). This activation

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**FIGURE 1.** TGF-β1 enhances the oxygen consumption of human lung fibroblasts. A, IMR-90 cells were treated with or without TGF-β1 (2.5 ng/ml) for 48 h at 37 °C. The OCR was measured as a function of time. Oligomycin (0.5 μM), carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP) (1 μM), or antimycin A (AA) (10 μM) was added at the indicated time point. B, bar graph representing the effect of TGF-β1 treatment on the ECAR of IMR-90 cells. C, bar graph corresponding to the calculation of basal, ATP-linked, proton leak, maximal, reserve, and non-mitochondrial OCR. D, bioenergetic map of IMR-90 cells representing the shift in OCR and ECAR following TGF-β1 treatment. E, bar graph representing the effect of TGF-β1 on complex I- or complex II-linked OCR in saponin-permeabilized cells in the presence of ADP (State 3). Error bars represent S.E. *, p < 0.005 compared with control. mPH, milli-pH units.
of PGC-1α was inhibited by p38 MAPK inhibition (Fig. 3A). TGF-β1-induced PGC-1α phosphorylation was decreased by 54% after SB202190 treatment (n = 3, p < 0.01).

TFAM is a downstream target gene of PGC-1α and is involved in mitochondrial DNA transcription and replication. We analyzed TFAM expression in total and mitochondrial fractions isolated from cells treated with or without TGF-β1. In untreated IMR-90 fibroblasts, TFAM was predominantly detected at 29 kDa, corresponding to its precursor form; upon stimulation with TGF-β1, TFAM expression was increased in the mitochondrial fraction and was also processed to a 25-kDa form. This truncated form corresponds to the cleaved active product of TFAM following its import into the mitochondrial matrix. We detected increased expression of the 25-kDa active form of TFAM upon TGF-β1 stimulation; this effect and the associated increase in mitochondrial proteins VDAC and TOM20 (Fig. 3C) as well as the increase in mtDNA copy number (Fig. 3D) were inhibited by p38 MAPK blockade. In concert, the TGF-β1-induced expression of HKII was also suppressed by p38 MAPK inhibition (Fig. 3C). To confirm the role of p38 MAPK in mediating TGF-β1-induced mitochondrial biogenesis and glycolysis, we evaluated the effect of overexpressing a dominant negative mutant form of p38 MAPK (55). TGF-β1-induced HKII and VDAC expression was significantly decreased in cells expressing the p38 MAPK mutant (Fig. 4, A, B, C, and D). Together, these data indicate a key role for p38 MAPK in mediating mitochondrial biogenesis and glycolysis.

Mitochondrial Biogenesis Is Required for Expression of α-SMA in Response to TGF-β1—Next, we determined whether mitochondrial biogenesis during the process of myofibroblast differentiation was necessary to maintain the differentiated phenotype. To answer this question, we analyzed the effect of inhibiting TGF-β1-induced mitochondrial biogenesis by silencing TFAM and determined the effect on the expression of...
α-SMA, a key marker of myofibroblast differentiation. TFAM silencing decreased the protein expression of α-SMA in response to TGF-β1 at 48 h (Fig. 5A); densitometric analysis of α-SMA expression in non-targeting (NT) or TFAM siRNA-transfected cells showed that TFAM silencing reduced TGF-β1-induced α-SMA expression (by 55%; n = 6, p < 0.05) (Fig. 5B). TFAM silencing did not alter TGF-β1-induced fibronectin (FN) expression (-fold change in FN expression following 48-h TGF-β1 was 2-fold in controls and in TFAM-silenced cells; n = 3, p = 0.18). TFAM silencing significantly reduced VDAC expression (Fig. 5C; 50% decrease by densitometric analysis of n = 3 independent experiments; p < 0.05) in response to TGF-β1, thus confirming inhibition of mitochondrial biogenesis when TFAM is silenced.

To determine whether the effect of TFAM silencing on α-SMA down-regulation occurred at the mRNA level, we analyzed α-SMA mRNA expression under the same conditions. TFAM silencing reduced TGF-β1-induced expression of α-SMA mRNA by 70% compared with control cells (n = 3, p < 0.005) (Fig. 5E). Silencing of TFAM did not significantly alter TGF-β1-induced expression of fibronectin or collagen 1A1 (Col1A1) (Fig. 5, F and G) and modestly decreased the expression of Col1A2 (Fig. 5H). Together, these data indicate that induction of TFAM and mitochondrial biogenesis is critical for maintaining the differentiated state of myofibroblasts.

Myofibroblast Contraction and Differentiation Are Dependent on Mitochondrial Bioenergetics and Glycolysis—Contraction of myofibroblasts has been shown to promote myofibroblast differentiation (9, 10, 56). We determined whether the increase in mitochondrial biogenesis/bioenergetics is necessary to mediate contractility of differentiated myofibroblasts. In a three-dimensional type I collagen gel model, we treated fibroblasts with or without TGF-β1 in the absence or presence of rotenone, which inhibits complex I (57). TGF-β1-induced contraction was abolished in the presence of rotenone (Fig. 6A and B), suggesting that mitochondrial electron transport is required to mediate myofibroblast contractility.
edited the phosphorylation of MLC20 in response to TGF-β1 (Fig. 6C). This effect on reduced contractile activity correlated with a reduction in α-SMA protein expression, whereas fibronectin expression was not affected, similar to the observed effects with TFAM silencing (Fig. 5, A and B). Additionally, inhibition of mitochondrial biogenesis by silencing TFAM decreased MLC20 phosphorylation (Fig. 6, D and E). Together, these data support the requirement for mitochondrial biogenesis and oxidative phosphorylation for contractile signaling and myofibroblast differentiation.

Based on our findings of an increase in aerobic glycolysis following TGF-β1 treatment of lung fibroblasts (Figs. 1B and 2E), we investigated the contribution of TGF-β1-enhanced glycolysis in supporting myofibroblast differentiation. Treatment of lung fibroblasts with 2-deoxy-D-glucose (2-DG), a structural analogue of glucose that acts as a non-competitive inhibitor of hexokinase (59), markedly reduced the expression of TGF-β1-induced α-SMA expression without significantly affecting TGF-β1-induced fibronectin expression (Fig. 7, A, B, and C). Together, these data suggest that both mitochondrial biogenesis and aerobic glycolysis coordinate support myofibroblast differentiation and contractility induced by TGF-β1.

**Discussion**

Wound healing is a complex, bioenergetically intensive physiological process. Myofibroblasts are the key effectors of wound closure and deposition of a provisional matrix based on their contractile and synthetic properties (1, 60). The role of bioenergetics in this process has not been well defined. In this study, we demonstrate, for the first time, that the differentiation of fibroblasts to myofibroblasts is accompanied by robust metabolic reprogramming. This reprogramming induced by TGF-β1 is linked to the stimulation of both mitochondrial biogenesis and glycolysis, resulting in an aerobic glycolysis phenotype. Stimulation of mitochondrial biogenesis accounts for the observed TGF-β1-induced increase in OCR, whereas TGF-β1-induced stimulation of ECAR is most likely related to the coordinate induction of aerobic glycolysis. Inhibition of mitochondrial biogenesis/function by genetic and pharmacologic approaches attenuates myofibroblast contractility. Importantly, we show that blockade of energy-dependent contraction is sufficient to inhibit signals for myofibroblast differentiation, supporting a positive feedback loop to maintain the contractile and differentiated state of myofibroblasts.

Early evidence supporting a role for mitochondrial biogenesis in sustaining a contractile phenotype came from studies analyzing the response of skeletal muscle cells in response to exercise-induced stress (12–14). It was shown that nutrient deprivation and energy depletion due to an increase in exercise load triggered an adaptive response of the cell via an increase in mitochondrial mass (61, 62). Our studies are the first to demonstrate that this adaptive response to a high energy state occurs in myofibroblasts.

Our studies clearly demonstrate that inhibition of mitochondrial biogenesis (TFAM silencing) or electron transport (rotenone, complex I inhibitor), which suppresses mitochondrial function, is sufficient to block contractile signaling (as evi-
Phosphorylation and contractility require ATP, the most efficient source of which is mitochondrial respiration. This contractile activity can be transduced to nuclear signaling events by a process known as mechanotransduction (58). Indeed, our group has previously shown that the transcription factor MKL1 translocates to the nucleus under conditions of stress fiber formation and active contraction; a Rho kinase inhibitor that depolymerizes filamentous actin (F-actin) to globular actin (G-actin) inhibits the cytoplasmic-nuclear translocation of MKL1 and suppresses myofibroblast differentiation (9, 10). Whether the integration of cellular bioenergetics to signaling of myofibroblast differentiation in our system involves MKL1 requires further study.

It is also possible that bioenergetic “signaling” more directly regulates myofibroblast differentiation at the post-translational level. We noted effects on α-SMA protein levels after short periods (<2 h) of rotenone exposure. This suggests that the initial response to loss of energy (ATP) supply likely results in a more rapid adaptation (by decreasing contractility) to couple cellular energy consumption to energy production. The silencing of TFAM did decrease expression of α-SMA mRNA in response to TGF-β1. This effect was observed 24 h following TGF-β1 treatment. Therefore, mitochondrial biogenesis may be essential to the long term requirement for ATP in the highly

FIGURE 5. Silencing of TFAM down-regulates TGF-β1-induced expression of α-SMA. A, IMR-90 cells were transfected with NT or TFAM siRNA (100 nM). After a 24-h recovery period in 10% serum-containing medium, cells were serum-starved (1% serum) overnight and treated without or with TGF-β1 (2.5 ng/ml for 48 h at 37 °C). Cells were then lysed, and expression levels of TFAM, FN, α-SMA, and GAPDH were assessed by Western blotting. B, densitometry analysis of the Western blotting results described in A from six independent experiments. Values represent mean ± S.E. Error bars represent S.E. **, p < 0.005 compared with control; #, p < 0.05 compared with NT plus TGF-β1. C, expression levels of TFAM, VDAC, and GAPDH analyzed by Western blotting in total cell lysates from NT or TFAM siRNA-transfected cells. D, E, F, and H, analysis of TFAM, α-SMA, fibronectin, Col1A1, and Col1A2 mRNA levels by real time PCR in NT or TFAM siRNA-transfected cells treated with or without TGF-β1 for 24 h. Values represent mean ± S.E. (n = 3 per experimental condition). Error bars represent S.E. *, p < 0.05 and **, p < 0.005 compared with control; ††, p < 0.005 compared with NT without TGF-β1; #, p < 0.05 and ##, p < 0.005 compared with NT plus TGF-β1; n.s., non-significant difference between means (NT plus TGF-β1 compared with TFAM plus TGF-β1).
synthetic and contractile activities of myofibroblasts. In addition to the role of mitochondrial biogenesis, our studies show that glycolysis is also involved in sustaining the differentiated and contractile phenotype of myofibroblasts. Indeed, inhibition of glucose utilization by lung fibroblasts decreased TGF-β1-induced α-SMA expression. No significant effect of blocking glycolysis was observed on TGF-β1-induced fibronectin expression, which reinforces the concept that bioenergetics specifically regulates signaling associated with myofibroblast contractility.

Previous studies suggest that mitochondrial reactive oxygen species mediate myofibroblast differentiation independently of SMAD activation (63). Our studies are consistent with these findings; additionally, the current findings demonstrate that

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**FIGURE 6. Inhibition of mitochondrial function or biogenesis alters myofibroblast contractility.** A, IMR-90 cells were seeded in type I collagen gel. 22 h after treatment with TGF-β1 (2.5 ng/ml, 37 °C), vehicle (DMSO) or rotenone (0.5 or 5 μM) was added for 2 h. Gel contraction was assessed at the indicated time points after gel release. Images were captured with a FOTO/Analyst Express camera. B, gel surface area analysis from four independent experiments. Bar graphs represent mean ± S.E. Error bars represent S.E. **, p < 0.005 compared with control vehicle; ##, p < 0.005 compared with TGF-β1 plus vehicle. C, representative Western blot showing the expression levels of fibronectin, α-SMA, phosphorylated MLC20 (pMLC20), total MLC20, and GAPDH in IMR-90 cells treated with or without TGF-β1 in the presence of DMSO or 0.5 μM rotenone. D, representative Western blot showing the effect of TFAM silencing on phosphorylated MLC20 expression in response to TGF-β1 in IMR-90 cells. E, densitometry analysis of the Western blotting results described in D from three independent experiments. Values represent mean ± S.E. Error bars represent S.E. **, p < 0.005 compared with NT without TGF-β1; #, p < 0.05 compared with NT plus TGF-β1. A.U., arbitrary units.
TGF-β1/p38 MAPK signaling induces mitochondrial biogenesis and that this may serve to keep myofibroblasts in an activated state by providing a source of reactive oxygen species for profibrogenic signaling. In addition, mitochondrial reactive oxygen species have been shown to modulate the SMAD3-dependent pathway of TGF-β1-induced myofibroblast differentiation (64, 65).

An important implication of our studies is that TGF-β1-induced metabolic reprogramming is p38 MAPK-dependent. This may allow for selective targeting of this pathway in pathological conditions in which mitochondrial signaling may be excessive or unremitting (66, 67) without affecting the homeostatic functions of TGF-β1/SMAD signaling in immune suppression and tumor suppression (68, 69). p38 MAPK is a well-known activator of PGC-1α. As expected, the inhibition of p38 MAPK decreased the phosphorylation of PGC-1α and its translocation to the nucleus in our cell system. Thus, blocking p38 MAPK activity is expected to impair the availability of active PGC-1α to promote the transcription of nuclearily encoded mitochondrial genes in concert with NRF-1/NRF-2. TFAM is a mitochondrial transcription factor, and its expression is regulated by PGC-1α (17, 27, 28). Interestingly, we did not observe a decrease in TFAM levels when p38 MAPK was inhibited in total cell lysates; rather, we detected deficient import and processing of TFAM to its active form. This suggests that TGF-β1/p38 MAPK signaling regulates a critical step in TFAM activation and transduction of mitochondrial genes.

In conclusion, our study highlights the requirement for metabolic reprogramming in TGF-β1-induced myofibroblast differentiation. Stimulation of mitochondrial biogenesis and glycolysis allows the cell to increase its oxidative capacity for ATP production. This increase in oxidative phosphorylation is crucial to support the energetic demand of myofibroblasts. In physiological conditions, this pathway may serve an adaptive function to promote normal wound healing; however, TGF-β1/p38 MAPK/mitochondrial signaling may be perpetuated by self-amplifying loops that result in fibrocontractive diseases (70). The elucidation of mechanisms controlling these responses is crucial in identifying targets that may prove beneficial in the treatment of pathological conditions such as fibrosis and cancer.

Author Contributions—K. B. and V. J. T. conceived and coordinated the study, designed experiments, analyzed and interpreted data, and wrote the manuscript. N. J. L., S. R., N. X., and B. P. P. performed experiments and analyzed the data. S. R., J. W. Z., K. M., G. L., and V. M. D.-U. assisted with data analysis, data interpretation, and manuscript preparation.
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