AMP-activated Protein Kinase Inhibits Transforming Growth Factor-β-induced Smad3-dependent Transcription and Myofibroblast Transdifferentiation*

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In wound healing, myofibroblast transdifferentiation (MFT) is a metaplastic change in phenotype producing profibrotic effector cells that secrete and remodel the extracellular matrix. Unlike pathways that induce MFT, the molecular mechanisms that negatively regulate MFT are poorly understood. Here, we report that AMP-activated protein kinase (AMPK) blocks MFT in response to transforming growth factor-β (TGFβ). Pharmacological activation of AMPK inhibited TGFβ-induced secretion of extracellular matrix proteins collagen types I and IV and fibronectin. AMPK activation also prevented induction of the myofibroblast phenotype markers α-smooth muscle actin and the ED-A fibronectin splice variant. AMPK activators did not prevent MFT in cells transduced with an adenovirus expressing dominant negative, kinase-dead AMPKα2. Moreover, AMPK activators did not inhibit MFT induction in AMPKα1,2−/− fibroblasts, demonstrating a requirement for AMPKα expression. Adenoviral transduction of constitutively active AMPKα2 was sufficient to prevent TGFβ-induced collagen I, α-smooth muscle actin, and ED-A fibronectin. AMPK did not reduce TGFβ-stimulated Smad3 COOH-terminal phosphorylation and nuclear translocation, which are necessary for MFT. However, AMPK activation inhibited TGFβ-induced transcription driven by Smad3- binding cis-elements. Consistent with a role for AMPK in transcriptional regulation, nuclear translocation of AMPKα2 correlated with the appearance of active AMPKα in the nucleus. Collectively, these results demonstrate that AMPK inhibits TGFβ-induced transcription downstream of Smad3 COOH-terminal phosphorylation and nuclear translocation. Furthermore, activation of AMPK is sufficient to negatively regulate MFT in vitro.

Myofibroblast transdifferentiation (MFT)2 is a fundamental cellular program activated in embryonic development, tumor-stroma interactions, wound repair, and fibrosis in the lung, heart, liver, and kidney (1–4). Myofibroblasts are mesenchymal cells that display phenotypic markers of both muscle and non-muscle cells. At sites of tissue injury, MFT produces profibrotic effector cells that assemble a fibrotic lesion (1, 3, 4). MFT is induced in an autocrine or paracrine fashion by cytokines, growth factors, and mechanical forces such as shear stress (3, 5).

The temporal sequence of MFT in wound healing is not entirely clear, but it involves early de novo expression of the ED-A fibronectin (ED-A FN) splice variant and formation of a proto-myofibroblast (1, 6). The transition of proto-myofibroblasts into myofibroblasts is marked by abundant de novo expression of α-smooth muscle actin (αSMA) (7). Stress fibers rich in αSMA develop and link to focal adhesions at the plasma membrane (1, 2). Finally, the mature myofibroblasts secrete a fibrillar collagen- and fibronectin-rich extracellular matrix that is remodelled into a fibrotic lesion (3, 8).

AMP-activated protein kinase (AMPK) was initially characterized as a protein activated by nutrient and bioenergetic stress that raises intracellular AMP and lowers ATP (9–11). AMPK belongs to the evolutionarily conserved SNF1/sucrose non-fermentor family of serine/threonine kinases that mediate the energy sensor (15, 16). AMPK also regulates the AMP/ATP ratio by phosphorylating a variety of metabolic enzymes that are necessary for ATP production (9). AMPK is highly sensitive to AMP/ATP ratio, which is known to activate AMPK in response to the energy stress that raises AMP and lowers ATP (11, 12). AMPK activation is a critical feature of MFT (1–14). Stimulation of AMPK also requires phospho-rylation of a critical threonine residue (Thr-172) in the activation loop of the α-subunit. At least two upstream AMPK kinases have been identified, including the tumor suppressor LKB1 and the Ca2+–calmodulin-dependent protein kinase kinases α and β (11, 15, 16). In cells, AMPK activation slows metabolic reactions that consume ATP and stimulates reactions that produce ATP, thereby restoring the AMP/ATP ratio and the normal cellular energy state (12). Recently it

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2 The abbreviations used are: MFT, myofibroblast transdifferentiation; AMPK, AMP-activated protein kinase; TGFβ, transforming growth factor β; β-catenin, 5-aminoimidazole-4-carboxamidé riboside; αSMA, α-smooth muscle actin; PAI-1, plasminogen activator inhibitor-1; FN, fibronectin; ELISA, enzyme-linked immunosorbent assay; dn, dominant negative; Ad, adenovirus; GFP, green fluorescent protein; ca, constitutively active.

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has become clear that AMPK can be activated independent of changes in the AMP/ATP ratio (15–17), implying that AMPK might have cellular functions unrelated to its role as a nutrient stress defense pathway.

In this study we asked whether MFT is negatively regulated by AMPK. Numerous stimuli of MFT have been identified, such as endothelin-1 and transforming growth factor-β (TGFβ), but negative regulation of MFT has not been extensively characterized. Here, we report that AMPK activation blocks Smad3-dependent transcription and MFT in response to TGFβ.

EXPERIMENTAL PROCEDURES

Experimental Reagents—We purified and characterized recombinant human adiponectin from the supernatant of 293-T cells (generous gift from Philipp Scherer, Albert Einstein College of Medicine) stably transfected with the pFM1 vector containing an Acrp30 gene exactly as described (18). Adiponectin from a mammalian tissue culture system was used because bacteria cannot properly process the hydroxyl-prolineated collagenous domain of adiponectin (18). Recombinant human TGFβ1 was from R&D, and AICAR was from Sigma. A-769662 has been previously described (19). Antibodies were as follows: affinity-purified rabbit anti-human collagen type I (Biodesign International); collagen IV and fibronectin (Rockland); αSMA (Epitomics); mouse monoclonal ED-A FN and β-actin (Sigma). All other antibodies were from Cell Signaling.

Cell Culture and Induction of MFT—Human primary mesangial cells (HMC) were from Cambrex Bioscience Inc. (Walkersville, MD) and were characterized and cultured as previously reported (20, 21). Human primary mesangial cells were used in passages 4–10. Where indicated we also studied MFT in murine embryonic fibroblasts null for AMPKα2 (22). To induce MFT, cells in 60 mm plates at ~80% confluence were made quiescent for 24 h in DMEM with 0.5% FBS, then stimulated with 0.5 ng/ml TGFβ1. The media contained 50 μg/ml β-aminopropionitrile to minimize cross-linking. Twelve, 24 or 48 h after adding TGFβ, the supernatant was collected and the monolayer was solubilized in a 5 M guanidine-0.1 M Tris buffer (pH 8.6) with protease inhibitors as described (23).

Adenoviral Transduction of Human Primary Mesangial Cells with Dominant Negative and Constitutively Active AMPKα2—Replication-defective adenoviral vectors encoding dominant negative AMPKα2 (Ad.dnAMPKα2), constitutively active AMPKα2 (Ad.caAMPKα2) and control adenovirus (Ad.trGFP) have been described (24–26). Adenovirus expressing a dominant negative mutant of AMPK α2 was provided by Dr. Ken Walsh (25, 27). All viral constructs were amplified in 293 cells and purified by using an Adeno-X™ virus mini-purification kit from Clontech Inc. (Mountain View, CA). Viral titers were determined as plaque forming units. For transduction, human primary mesangial cells were incubated with adenovirus at a multiplicity of infection of 50 for 12 h. The optimal multiplicity of adenoviral infection was determined using a GFP-encoding adenovirus. Under these conditions, the transduction efficiency was >90%.

Direct ELISA for Collagens, Fibronectins, and αSMA—ELISA for secreted collagen I, collagen IV, and FN in the medium was as described (24, 28). We also used ELISA to measure cell-associated ED-A FN and αSMA (29). 100 μl of media or cell extracts were absorbed to Nunc Maxisorp 96-well plates overnight at 4 °C. After washing three times with phosphate-buffered saline/0.1% Tween, nonspecific binding was blocked in 1% bovine serum albumin, 100 mM phosphate buffer, pH 8.2. The wells were then incubated with a primary antibody, the appropriate affinity-purified and biotin-conjugated goat anti-IgG, and a horseradish peroxidase-conjugated streptavidin. A solution of 3,3’,5’,5’-tetramethylbenzidine was added followed by quenching with acid and an absorbance reading at 450 nm in a SpectraMax 190 (Molecular Devices). All values were in the linear range and were normalized for cell number.

Immunocytochemical and Immunofluorescent Analysis of ED-A FN and PSMAD3—For immunocytochemistry, cells were fixed in 2% paraformaldehyde for 20 min, incubated with 0.6% hydrogen peroxide for 30 min at room temperature, and permeabilized with 0.1% Triton X-100 in TBS for 15 min. After

FIGURE 1. The AMPK activators AICAR and adiponectin block TGFβ-stimulated collagen I secretion. A, time course of TGFβ-induced collagen I secretion with and without AMPK activation by AICAR and adiponectin (Adipo). Quiescent cells were preincubated for 30 min with 0.5 mM AICAR or 20 μg/ml adiponectin before adding 0.5 ng/ml TGFβ for 12, 24, and 48 h. Collagen I secretion in the supernatant was measured by ELISA. *, p < 0.05 versus time 0. B, cells were preincubated with 100 μM A-769662 or vehicle (0.01% Me_SO) for 30 min before addition of TGFβ for 48 h and measurement of secreted collagen I by ELISA. *, p < 0.05 versus untreated with vehicle. Data are mean ± S.D. for three experiments in duplicate.
blocking with 3.0% bovine serum albumin in TBS-T for 1 h, primary antibody (1:500) was added overnight at 4 °C. The primary antibody was detected using the ImmPress Universal Antibody detection system (Vector Laboratories) exactly as described by the manufacturer. The monolayer was mounted in Vectamount (Vector Laboratories). A similar protocol without hydrogen peroxide pretreatment was used for immunofluorescent staining with detection by a fluorescein isothiocyanate-conjugated secondary antibody, counterstaining with 4′,6-diamidino-2-phenylindole and mounting in Vectashield. The ALPSmad3 antibody recognizes Smad3 phosphorylated at Ser-423/425. Images were acquired with a SPOT RT camera (Diagnostic Instruments).

**Measurement of Smad3-directed Transcriptional Activity**—We employed mink lung cells stably transfected with a minimal promoter from the plasminogen activator inhibitor-1 (PAI-1) gene linked to luciferase (30). Cells were also transiently transfected (31) with pSBE4-Luc (46), kindly provided by Dr. Bert Vogelstein, to measure Smad3 transactivation. After treatment with TGFβ and AMPK activators for 24 h, cytosolic luciferase activity was measured as described (31).

**Subcellular Fractionation**—Cells were homogenized at 4 °C in 0.25 M sucrose containing 10 mM Tris-Cl (pH 8.0), 1 mM KCl, 1.5 mM MgCl2, 1 mM dithiothreitol with protease inhibitors (Roche Applied Science). The cell lysate was centrifuged at 3000 × g for 10 min at 4 °C, and the resulting pellet and supernatant were collected as the nuclear and cytoplasmic (post nuclear supernatant) fractions.

**AMPK Activators Inhibit MFT in Cells Treated with TGFβ**—To study MFT in response to TGFβ, we used human renal mesangial cells, an in vitro model of MFT (32–34). In cul-

**FIGURE 2. AMPK activators prevent collagen IV and fibronectin secretion in cells stimulated with TGFβ.** Quiescent cells were stimulated for 48 h with 0.5 ng/ml TGFβ plus or minus 0.5 mM AICAR, 20 μg/ml adiponectin, or 100 μM A-769662. Media were collected and analyzed by ELISA for collagen IV (A) and fibronectin (B) secretion. In A and B, *, p < 0.05 versus untreated control. Data are mean ± S.D. for three independent experiments in duplicate.

**FIGURE 3. In cells treated with TGFβ, AMPK activators inhibit induction of the MFT markers αSMA and ED-A FN.** A, TGFβ was added to mesangial cells for 48 h with and without the AMPK activators (preincubated for 30 min) AICAR (0.5 mM), adiponectin (20 μg/ml), or A-769662 (100 μM). αSMA was measured in cell lysates by ELISA. B, the ED-A FN splice variant was assessed qualitatively by immunocytochemistry in quiescent cells and in cells stimulated with TGFβ (C) or TGFβ plus A-769662 (D). E, to control for nonspecific binding, cells stimulated with TGFβ were stained with a monoclonal isotype control antibody. F, cells were treated as described, and secreted ED-A FN was measured by ELISA in the cell media. Data in A and F are mean ± S.D. for three experiments. In A and F, *, p < 0.05 versus untreated control.
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![Diagram of AMPKα2](image)

**Figure 4.** Dominant negative AMPKα2 reverses inhibition of TGFβ-stimulated MFT marker proteins by AMPK activators. A, schematic illustration of the AMPKα2 dominant negative mutant with an inactive mutation in the kinase domain. Quiescent cells were transfected for 24 h with adenoviral vectors expressing dnAMPKα2 (Ad.dnAMPK) or GFP (Ad.trGFP). Transduced cells were treated with 0.5 ng/ml TGFβ after a 30-min preincubation with the AMPK activators A-769662 (100 μm) and AICAR (0.5 mm). After 48 h collagen I in the media (B), αSMA in cell lysates (C), and secreted ED-A FN (D) were measured, and the results were normalized to the untreated cells transfected with Ad.trGFP. *, p < 0.05 versus untreated with Ad.trGFP. Data are mean ± S.D. of three experiments.

AMPK also induces collagen IV and fibronectin (39). We observed that the AMPK activators AICAR, adiponectin, and A-769662 blocked the 2.3-fold induction of collagen IV secretion, demonstrating that the effect of dnAMPKα2 was not a nonspecific consequence of adenoviral gene transfer. Moreover, AICAR blocked expression of collagen I in cells transduced with Ad.trGFP. In cells transduced with Ad.dnAMPKα2, the 2.3-fold induction of αSMA by TGFβ was not inhibited by AICAR, adiponectin, and A-769662 (Fig. 4C). Similarly, AMPK activators did not block the 2.7-fold elevated secretion of ED-A FN in cells transduced with Ad.dnAMPKα2 (Fig. 4D). Basal and stimulated levels of αSMA or ED-A FN were not altered by adenoviral transduction with Ad.trGFP. These results with dnAMPKα2 support the hypothesis that AMPK signaling blocks MFT.

Experiments in murine embryonic fibroblasts lacking AMPKα1 and AMPKα2 also demonstrated that inhibition of TGFβ-stimulated MFT by the activators specifically requires AMPK. We confirmed that basal or activated AMPKα protein was absent in AMPKα1−/− fibroblasts (data not shown). TGFβ-induced collagen I secretion was similar in AMPKα1−/− compared with AMPKα1+/+ fibroblasts (1.9-fold compared with 1.8-fold stimulation, Fig. 5A), demonstrating intact TGFβ signaling in the null fibroblasts. A-769662 blocked TGFβ-stimulated collagen I secretion in AMPKα1+/+ cells but not in AMPKα1−/− cells (Fig. 5A), suggesting a requirement for AMPK. AICAR was similarly ineffective in the AMPKα1−/− fibroblasts but was inhibitory in AMPKα1+/+ cells (Fig. 5A). These results demonstrate that AMPK negatively regulates MFT.

**Nuclear Localization of dnAMPKα2**

![Image of nuclear localization](image)

**Figure 5.** Nuclear localization of dnAMPKα2. A, immunofluorescence staining with Alexa Fluor 546- and 488-conjugated secondary antibodies. DAPI staining shows nuclear localization of dnAMPKα2. Bar, 25 μm.
AMPK is required for inhibition of TGFβ-stimulated collagen I by A-769662 and AICAR.

Similarly, in AMPKα−/− null fibroblasts A-769662 and AICAR did not inhibit expression of the MFT markers αSMA and ED-A FN. Induction of αSMA by TGFβ was similar in AMPKα+/+ or AMPKα−/− cells, demonstrating that the signals linking TGFβ receptor activation to induction of αSMA are unaffected in the null fibroblasts (Fig. 5B). However, the 2.5-fold induction of αSMA in AMPKα+/+ cells treated with TGFβ was not inhibited by A-769662 or AICAR in AMPKα−/− null fibroblasts (Fig. 5B). A-769662 and AICAR also failed to block ED-A FN secretion in AMPKα−/− fibroblasts exposed to TGFβ (Fig. 5C). Collectively, these experiments in AMPKα−/− fibroblasts provide strong evidence that AMPK negatively regulates TGFβ-stimulated MFT.

We next asked whether AMPK activity is sufficient to block TGFβ-stimulated MFT. An AMPKα2 mutant consisting of only the kinase domain and lacking the auto-inhibitory and β/γ interaction domains (Fig. 6A) is constitutively active (caAMPKα2), even in the absence of AMPK activators (26). We expressed caAMPKα2 by means of an adenoviral gene transfer vector. Transduction of Ad.caAMPKα2 had no effect on basal collagen I secretion, but it blocked TGFβ-stimulated expression (Fig. 6B). Neither basal nor TGFβ-stimulated collagen I secretion were affected by transduction with Ad.trGFP (Fig. 6B). caAMPKα2 also blocked induction of αSMA and ED-A FN protein by TGFβ (Fig. 6, C and D). Transduction with Ad.trGFP had no effect on induction of αSMA and ED-A FN. Taken together, these results with dn.AMPKα2, AMPKα−/− null fibroblasts, and caAMPKα2 demonstrate that AMPKα is sufficient to prevent MFT in cells exposed to TGFβ.

**AMPK Blocks Smad3-mediated Transcription without Inhibiting Smad3 Phosphorylation or Nuclear Translocation**—To determine the mechanism underlying inhibition of MFT by AMPK, we studied signals downstream of TGFβ receptor activation. Because Smad3 is essential for TGFβ-stimulated MFT (41–44), we asked whether AMPK blocks Smad3 phosphorylation or nuclear translocation.

Immunofluorescent analysis showed rapid TGFβ-stimulated COOH-terminal activating phosphorylation of Smad3 and nuclear localization. PSmad3 was nearly undetectable in the cytosol or nucleus of unstimulated cells, but after 30 min of TGFβ 83% of cell nuclei were positive for PSmad3 (Fig. 7, A and B). Two hours after TGFβ, nuclear levels of PSmad3 started to modestly decline. The AMPK activators AICAR and A-769662 did not reduce the staining intensity or nuclear localization of PSmad3 in response to TGFβ (Fig. 7, A and B). An unexpected finding was that AMPK activators alone transiently increased PSmad3 levels and translocation, albeit much less intensely than with TGFβ alone or TGFβ plus the AMPK activators (Fig. 7B). These results demonstrate that AMPK does not inhibit Smad3 phosphorylation at the activating epitope. Moreover, AMPK does not inhibit nuclear translocation of PSmad3.

Because AMPK did not block phosphorylation or nuclear translocation of Smad3, we investigated whether AMPK inhibited Smad3-mediated transcription downstream of the nuclear localization step. To that end we employed reporter cells with three Smad3-sensitissise cis-elements in the context of a heterologous human PAI-1 promoter driving transcription of luciferase (30, 45). TGFβ increased luciferase reporter activity 24.7-fold (Fig. 8A), and the increase in Smad3-dependent transcription was blocked by a TGFβ-neutralizing antiserum, demonstrating specificity for TGFβ (data not shown). Both AICAR and A-769662 blocked the increase in luciferase activity in cells exposed to TGFβ (Fig. 8A). Activation of Smad3-dependent transcription was not inhibited

**FIGURE 5.** AMPK activators do not inhibit MFT in murine embryonic fibroblasts null for AMPKα. Quiescent AMPKα−/− fibroblasts lacking both α1 and α2 subunits and AMPKα+/+ wild-type controls were stimulated for 48 h with 0.5 ng/ml TGFβ with and without 100 μM A-769662 or 0.5 mM AICAR. ELISA was then used to measure collagen I secretion (A), αSMA accumulation (B), and ED-A FN in the media (C). *, p < 0.05 versus untreated control in respective cell type. Data are mean ± S.D. for three experiments in duplicate.
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A schematic diagram of the truncated AMPKα2 mutant with constitutively active (caAMPKα2).

Quiescent cells were transduced for 24 h with adenoviral vectors expressing constitutively active AMPKα2 (Ad.caAMPK) or GFP (Ad.trGFP). TGFβ (0.5 ng/ml) or medium alone was added for an additional 48 h and collagen I, αSMA, and ED-A FN were assessed by ELISA. *p < 0.05 versus untreated with Ad.trGFP. Data are mean ± S.D. from n = 3 experiments.

FIGURE 6. A constitutively active AMPKα2 mutant suffices to prevent induction of MFT marker proteins by TGFβ. A, schematic diagram of the truncated AMPKα2 mutant with constitutively active (caAMPKα2). Quiescent cells were transduced for 24 h with adenoviral vectors expressing constitutively active AMPKα2 (Ad.caAMPK) or GFP (Ad.trGFP). TGFβ (0.5 ng/ml) or medium alone was added for an additional 48 h and collagen I (B), αSMA (C), and ED-A FN (D) were assessed by ELISA. *p < 0.05 versus untreated with Ad.trGFP. Data are mean ± S.D. from n = 3 experiments.

Our experiments show that two mechanistically different pharmacological activators of AMPK block induction of myofibroblast marker proteins in response to TGFβ. A caveat for interpreting results with AICAR is that 5-aminoimidazole-4-carboxamide-1-d-ribofuranosyl 5′-monophosphate can weakly stimulate glycogen phosphorylase (55, 56) and inhibit fructose-1,6-bisphosphatase (57). Unlike AICAR, however, A-769662 does not affect glycogen phosphorylase or fructose-1,6-bisphosphatase activity (19).

The notion that AMPK negatively regulates TGFβ-stimulated Smad3-dependent transcription by a mechanism downstream of TGFβ-stimulated Smad3 phosphorylation and nuclear translocation.

We also asked whether active AMPK was present in mesangial cell nuclei, consistent with a role for AMPK in transcriptional regulation. Because it is well established that AMPKα1 does not translocate to the nucleus (47, 50), we assessed translocation of AMPKα2 in subcellular fractions.

Nuclear translocation of AMPKα2 correlated with the appearance of P172ThrAMPKα (active kinase) in the nucleus. AICAR-stimulated nuclear localization and activation of AMPKα2 was unaffected by addition of TGFβ, and addition of TGFβ alone weakly elevated P172ThrAMPKα at 1 h, which presumably reflected phosphorylation of AMPKα by the TGFβ-activated protein kinase (49, 50). However, we note that the level of AMPKα activation by TGFβ was of insufficient magnitude and duration to inhibit MFT. This result demonstrates that AMPKα2 can translocate to the nucleus in an activated form in mesangial cells and support a role for AMPKα in regulation of Smad3-dependent transcription.

DISCUSSION

MFT, a metaplastic change in cell phenotype, is essential for the development of profibrotic effector cells in the wound healing response and is induced by extracellular cues such as growth factors, cytokines, and mechanical stress. Yet the molecular mechanisms that negatively regulate MFT are poorly understood. In this study, we demonstrate that AMPK is sufficient to repress TGFβ induction of MFT of mesangial cells in vitro.

AMPK as a Negative Regulator of MFT—In eukaryotes AMPK has an evolutionarily conserved role regulating the catabolic and anabolic metabolism of glucose and fatty acids at the cellular and whole body levels (10–12). Recent studies have revealed novel functions for AMPK, including regulation of a cell cycle checkpoint (51), the decision to enter autophagy or apoptosis (52, 53), and cell fate decisions in development (54).
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FIGURE 7. AMPK activation does not alter the activating phosphorylation and nuclear translocation of Smad3. A, quiescent mesangial cells on glass coverslips were stimulated for 30 min with 0.5 ng/ml TGFβ alone or TGFβ plus 0.5 mM AICAR or 100 μM A-769662. PhosphoSer-423/425 Smad3 (top row) and 4',6-diamidino-2-phenylindole nuclear staining (bottom row) in fixed cells were detected by immunofluorescence. B, in the time course studies cells were treated for the times indicated and analyzed for PSmad3 by immunofluorescence, and cells with nuclear PSmad3 were counted and expressed as a percentage of the 4',6-diamidino-2-phenylindole-stained cells. Data are mean ± S.D. from two experiments.

the AMPKα2 kinase domain results in a dominant negative mutant that antagonizes AMPK signaling (40). Adenoviral transduction of this AMPKα2 mutant prevented repression of collagen I, αSMA, and ED-A FN in mesangial cells stimulated with TGFβ. Second, a constitutively active mutant of AMPKα2, which stimulates AMPK signaling (26), prevented induction of the MFT marker proteins. These findings also suggest that AMPK activity is sufficient in mesangial cells to block the MFT transition in response to TGFβ and concurs with our results with AICAR and A-769662. Finally, AMPK activators were unable to block MFT in murine embryonic fibroblasts null for AMPKα subunits. Double knockouts of AMPKα1 and AMPKα2 are embryonic lethal (22), thus mesangial cells lacking both AMPKα subunits could not be prepared. These results in murine embryonic fibroblasts are also important, because they suggest that the negative effect of AMPK on MFT is not restricted to glomerular mesangial cells and that AMPK might be a more general inhibitor of MFT in other cellular contexts.

A Smad3-based Mechanism for Inhibition of MFT by AMPK—As a window into potential mechanisms that underlie negative regulation of MFT by AMPK, we asked whether AMPK inhibits transcriptional regulation by Smad3. Numerous signal transduction pathways have been associated with MFT in vitro and in vivo, but most studies have shown that Smad3 is required for MFT in response to TGFβ (see Refs. 41–44 for review). The activated TGFβ receptor kinase phosphorylates the COOH-terminal domain of regulatory Smads (Smad2/3), which then form heterodimers or trimers with Smad4 and translocate to the nucleus. Smad complexes in the nucleus bind to Smad-binding elements and activate or repress gene transcription. In our experiments COOH-terminal stimulatory phosphorylation of Smad3 was unaffected by the AMPK activators. Moreover, nuclear translocation of PSmad3 was not altered by AMPK, suggesting that AMPK does not block the early steps in Smad3 signaling.

Inhibition of Smad3-stimulated signaling by AMPK occurs downstream of nuclear translocation and involves transcriptional regulation. AMPK blocked TGFβ-stimulated transactivation of a luciferase reporter construct (pSBE4-Luc) that is highly selective for Smad3. AMPK also inhibited activation of the human PAI-1 promoter, an informative model of Smad3-dependent transcription (45, 58–60). Stimulation of the human PAI-1 promoter by TGFβ has been previously shown to require Smad3 and three Smad3-binding cis-elements (45, 58–60). In addition, PAI-1 gene induction is relevant to the study of MFT and glomerular fibrosis: several laboratories have documented that PAI-1 is potently induced in MFT and that it contributes to the increase in fibrosis that results from MFT (61–63). TGFβ also induces transcription and expression of PAI-1 in human mesangial cells (64), and genetic depletion of PAI-1 augments mesangial matrix protein accumulation and glomerular fibrosis in an experimental model of chronic kidney disease (65). Our results demonstrate that AMPK prevents transcriptional activation of the PAI-1 promoter by TGFβ. Inhibition of Smad3-dependent activation of the PAI-1 promoter by the AMPK activators was antagonized by adenovirus transduction of the dnAMPKα2 mutant, confirming that repression by AICAR and A-769662 required AMPK activity. Translocation of active AMPKα2 to the mesangial cell nucleus, which has previously been demonstrated in other cells types (47, 48), provides further support for the notion that AMPK regulates Smad3-directed gene expression at the level of transcription. Whether
AMPK prevents regulated recruitment of Smad3 to the PAI-1 promoter, or instead regulates Smad3-binding co-activators or co-repressors, remains to be determined.

In the wound healing response of kidney, heart, liver, and lung, MFT is a common phenotypic transition associated with reorganization of the extracellular matrix, development of fibrosis and loss of functional tissue. Thus regulators of MFT represent a potential approach for limiting fibrotic tissue injury in vivo (3, 4, 66). With the present observations, we define AMPK as a new negative regulator of MFT and speculate that AMPK activation in vivo might modulate the wound healing response and fibrosis.

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