AMP-activated protein kinase/myocardin-related transcription factor-A signaling regulates fibroblast activation and renal fibrosis

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Chronic kidney disease is a major cause of death, and renal fibrosis is a common pathway leading to the progression of this disease. Although activated fibroblasts are responsible for the production of the extracellular matrix and the development of renal fibrosis, the molecular mechanisms underlying fibroblast activation are not fully defined. Here we examined the functional role of AMP-activated protein kinase (AMPK) in the activation of fibroblasts and the development of renal fibrosis. AMPKα1 was induced in the kidney during the development of renal fibrosis. Mice with global or fibroblast-specific knockout of AMPKα1 exhibited fewer myofibroblasts, developed less fibrosis, and produced less extracellular matrix protein in the kidneys following unilateral ureteral obstruction or ischemia-reperfusion injury. Mechanistically, AMPKα1 directly phosphorylated cofilin leading to cytoskeleton remodeling and myocardin-related transcription factor-A nuclear translocation resulting in fibroblast activation and extracellular matrix protein production. Thus, AMPK may be a critical regulator of fibroblast activation through regulation of cytoskeleton dynamics and myocardin-related transcription factor-A nuclear translocation. Hence, AMPK signaling may represent a novel therapeutic target for fibrotic kidney disease.

Disease-related injury in organs triggers cellular and molecular responses that can culminate in tissue fibrosis. The dynamic deposition and insufficient resorption of extracellular matrix (ECM) promotes fibrosis and chronic loss of organ function, which account for an estimated one-third of natural deaths worldwide. As a major fibrotic disorder, renal fibrosis is a hallmark of chronic kidney disease. Renal interstitial fibrosis is characterized by fibroblast activation and excessive production and deposition of ECM, which leads to the destruction of renal parenchyma and progressive loss of kidney function to end-stage renal disease.

Activated fibroblasts are the principal cells responsible for ECM production, and their activation is regarded as a key event in the pathogenesis of renal fibrosis. These cells express α-smooth muscle actin (α-SMA), and are referred to as myofibroblasts. However, the molecular mechanisms underlying fibroblast activation are not fully understood.

Adenosine monophosphate-activated protein kinase (AMPK) is a conserved sensor of cellular energy status and environmental stress, and regulates the activities of a number of enzymes through phosphorylation. Mammalian AMPKs are heterotrimeric complexes containing a catalytic (z) subunit and 2 regulatory (β and γ) subunits. In addition to regulating energy homeostasis and metabolism, AMPK plays important roles in protein synthesis, cell growth, apoptosis, cell differentiation, cell cycle regulation, actin cytoskeleton reorganization, and gene transcription. Recent studies have shown that AMPKα1 participates in the development of renal fibrosis. However, how AMPK regulates the development of renal fibrosis is unknown.

To examine the functional role of AMPKα1 in the activation of fibroblasts and the development of renal fibrosis, we generated mice expressing tamoxifen-inducible Cre-recombinase (Cre/ESR1) under the control of the chicken beta actin promoter/enhancer coupled with the cytomegalovirus immediate-early enhancer (CAG-Cre) or proα2(I) collagen promoter (Coll1-Cre), thus permitting temporally controlled deletion of floxed AMPKα1 gene in a global or fibroblast-specific manner. These transgenic mice were subjected to unilateral ureteral obstruction (UUO) or ischemia-reperfusion injury (IRI) to induce renal fibrosis. We show that AMPKα1 is induced in the kidney during the...
development of renal fibrosis, and targeted disruption of AMPKα1 inhibits fibroblast activation and attenuates the development of renal fibrosis. Furthermore, we demonstrate that AMPKα1 directly phosphorylates coflin, which results in actin cytoskeleton reorganization and nuclear translocation of myocardin-related transcription factor-A (MRTF-A; also known as MKL1, MAL, BSAC) leading to fibroblast activation and fibrogenesis.

RESULTS
AMPKα1 is induced during the development of renal fibrosis

We first determined whether AMPKα1 is induced in the kidneys in response to obstructive injury. Western blot analysis showed that the protein levels of AMPKα1 were markedly increased in kidneys after 10 days of UUO compared with the contralateral kidneys (Supplementary Figure S1). To characterize the cell types responsible for AMPKα1 induction in the kidney, serial kidney sections were stained with an anti-AMPKα1 antibody by immunohistochemistry. The results revealed that AMPKα1-positive cells increased in the tubulointerstitium of the fibrotic kidneys (Supplementary Figure S2). To examine whether AMPKα1 is activated in myofibroblasts of the kidney, kidney sections were stained for p-AMPKα and α-SMA, a myofibroblast marker, by immunofluorescence (Supplementary Figure S3). The immunofluorescence results demonstrated that p-AMPKα-positive cells are positive for α-SMA, indicating that AMPKα1 is mainly activated in myofibroblasts of the kidney in response to obstructive injury.

Global deletion of AMPKα1 reduces renal fibrosis

To examine whether AMPKα1 regulates renal fibrosis in vivo, mice with loxP-flanked alleles of AMPKα1 were bred with mice harboring a hemizygous CAG-Cre-ER transgene. CAG-Cre+AMPKα1fl/fl mice were referred to as GAKO mice, and littermate CAG-Cre+AMPKα1fl/fl mice served as controls (Supplementary Figure S4). These mice were treated with tamoxifen and then subjected to UUO for 10 days. Western blot analysis showed that protein levels of AMPKα1 and p-AMPKα were markedly increased in the obstructed kidneys of control mice, and p-AMPKαz were significantly increased in the kidneys of control mice with IRI, and substantially reduced in the kidneys of GAKO mice with IRI (Figure 2a). Global deletion of AMPKα1 markedly reduced interstitial collagen deposition in the IRI-injured kidneys (Figure 2b). Furthermore, global deletion of AMPKα1 significantly inhibited the expression of α-SMA, collagen I, and fibronectin in kidneys following IRI (Figure 2c–f). These data indicate that global deletion of AMPKα1 attenuates IRI-induced fibroblast activation and interstitial fibrosis in the kidney.

Fibroblast-specific deletion of AMPKα1 reduces renal fibrosis

To determine whether the observed effect of AMPKα1 on renal fibrosis was mediated by AMPKα1 in fibroblasts, we generated mice with fibroblast-specific deletion of AMPKα1 by crossing AMPKα1fl/fl mice with tamoxifen-inducible collagen type I promoter/enhancer-driven Cre-ER recombinase transgenic mice (Coll1-Cre+). AMPKα1fl/fl littermate controls and Coll1-Cre+AMPKα1fl/fl mice (referred to as FAKO) were treated with tamoxifen to induce fibroblast-specific deletion of AMPKα1 (Supplementary Figure S4). These mice were then subjected to UUO or IRI to induce renal fibrosis. Western blot analysis showed that AMPKα1 protein levels were markedly reduced in the kidneys of FAKO mice compared with those of littermate controls (Figures 3a and 4a). Immunofluorescence staining revealed that AMPKα1 abundance was considerably reduced in the PDGFRβ-positive fibroblasts (Figure 3c). We then examined the effect of AMPKα1 deficiency in fibroblasts on the development of renal fibrosis. Total collagen accumulation and deposition were significantly attenuated in the injured kidneys of FAKO mice compared with their littermate controls (Figures 3d and 4b). Furthermore, both Western blot analysis and immunofluorescence staining demonstrated that fibroblast-specific AMPKα1 deficiency attenuated the upregulation of α-SMA, collagen I, and fibronectin in the kidneys with UUO (Figure 3e–h) or IRI (Figure 4c–f). Collectively, these data indicate that fibroblast-specific deletion of AMPKα1 attenuates renal fibrosis in UUO and IRI models by inhibiting fibroblast activation and ECM protein production.

AMPKα1 is required for fibroblast activation

To examine the role of AMPKα1 in activating kidney fibroblasts, the normal rat kidney fibroblasts (NRK-49F), primary kidney fibroblasts, and bone marrow-derived monocytes were treated with a direct activator of AMPK, A-769662, for 24 hours. A-769662 induced fibroblast activation identified as increased production of α-SMA, fibronectin, and collagen I (Figure 5a–c). Compound C, a selective AMPK inhibitor, blocked A-769662-induced fibronectin, collagen I, and α-SMA production in NRK-49F cells (Figure 5a). Furthermore, AMPKα1 deficiency abolished A-769662-induced fibronectin, collagen I, and α-SMA protein production in primary kidney fibroblasts and bone marrow–derived monocytes (Figure 5b and c, and Supplementary Figure S5). Bone marrow–derived monocytes were also treated with...
adiponectin, a cytokine that activates AMPK pathway.\textsuperscript{15,20,21} Western blot analysis showed that adiponectin activated AMPK\textsubscript{1} through phosphorylation and stimulated the expression of \(\alpha\)-SMA, fibronectin, and collagen I, which was blocked by compound C (Figure 5d). In addition, ectopic expression of a constitutively active AMPK\textsubscript{122,23} in the human embryonic kidney cells resulted in increased protein levels of \(\alpha\)-SMA, fibronectin, and collagen I (Figure 5e).
Collectively, these results indicate that activation of AMPK promotes fibroblast activation and ECM protein production.

**MRTF-A mediates AMPKα1-induced fibroblast activation**

We then explored the molecular mechanism underlying AMPKα1-induced fibroblast activation. Recent studies have shown that the transcriptional coactivator MRTF-A regulates fibroblast activation and ECM protein production. To determine whether MRTF-A mediates the effects of AMPKα1 on fibroblast activation, NRK-49F cells and bone marrow–derived monocytes were pre-treated with CCG-203971, a selective inhibitor of MRTF-A nuclear localization, and...
then stimulated with A-769662. AMPK-induced fibroblast activation was blocked by CCG-203971 (Figure 6a and b). These data indicate that MRTF-A mediates the effect of AMPK on fibroblast activation. Immunofluorescence staining was then performed to determine whether AMPK affects MRTF-A nuclear translocation. Activation of AMPK resulted in nuclear accumulation of MRTF-A, which was associated with F-actin formation (Figure 6c). Western blot analysis confirmed that MRTF-A levels increased in the nuclei following AMPK activation (Figure 6d). These results indicate that AMPK promotes nuclear translocation and accumulation of MRTF-A leading to fibroblast activation and ECM protein expression.

AMPK phosphorylates coflin

We next investigated how AMPK activation resulted in F-actin formation and MRTF-A nuclear accumulation. Recent studies have shown that MRTF-A nuclear localization is regulated by relative amounts of free monomeric globular actin (G-actin) and polymeric filamentous actin (F-actin), which are modulated by coflin. To determine whether AMPK acts on coflin, we first examined whether AMPK activation affects phosphorylation of coflin (Ser3). NRK-49F cells and bone marrow–derived monocytes were incubated with A762669 for different periods of time, or different concentrations. Activation of AMPK with A-769662 increased coflin phosphorylation in a dose- and time-dependent manner (Figure 7a–d). Furthermore, ectopic expression of constitutively active AMPK increased the levels of coflin phosphorylation (Figure 7e). These data indicate that AMPK activation leads to the phosphorylation of coflin.

To examine the effect of AMPKz1 on the phosphorylation of coflin in the fibrotic kidneys in vivo, kidney sections were stained for phospho-coflin (Ser3). The positive staining for phosphorylated coflin increased significantly in the obstructed kidneys after 5 days (Supplementary Figure S6) and 10 days (Figure 7f and Supplementary Figure S7) of UUO. AMPKz1 deficiency reduced the numbers of phospho-coflin-positive cells in the fibrotic kidneys (Figure 7f and Supplementary Figure S7). Similar results were obtained in the IRI model (Supplementary Figure S8). Moreover, kidney sections were stained for AMPKz1 and phospho-coflin (Ser3). AMPKz1-positive cells were also positive for phospho-coflin in the tubulointerstitium of the injured kidneys (Supplementary Figure S9). Finally, kidney sections were stained for phospho-coflin (Ser3) and α-SMA. AMPK deficiency significantly reduced the number of p-coflin and α-SMA dual-positive cells in the obstructed kidneys (Supplementary Figure S10). These results indicate that AMPKz1 induces coflin phosphorylation in myofibroblasts in the kidney in response to obstructive injury in vivo.

To further determine whether AMPKz1 interacts with coflin, we performed immunoprecipitation (IP) assay using an anti-AMPKz1 antibody to pull down endogenous proteins in NRK-49F cells. The results showed that the anti-AMPKz1 antibody pulled down coflin, indicating a direct interaction between AMPKz1 and coflin (Figure 7g).

Finally, we tested whether AMPKz1 can directly phosphorylate coflin. We used purified recombinant AMPK kinase containing active human AMPKz1+β1+γ1 proteins as the whole enzyme, and recombinant human coflin as the substrate. Our results showed that AMPK phosphorylated coflin in a dose-dependent manner (Figure 7h). These results indicate that AMPK can directly phosphorylate coflin.

DISCUSSION

Chronic fibrotic diseases account for nearly 45% of deaths in developed countries. However, there is no effective therapy for these devastating disorders. To develop effective therapeutic strategies, it is essential to understand the cellular and molecular mechanisms underlying fibroblast activation and fibrotic development. In the preclinical animal models, we discovered that AMPKz1 was significantly induced and activated in the kidney during the development of renal fibrosis. We hypothesize that AMPKz1 may modulate fibroblast activation and fibrogenesis in the kidney. Using the genetic approach, our study identifies AMPKz1 as a critical regulator in fibroblast activation and the development of renal fibrosis. Some studies have reported that pharmacological activators of AMPK may have a protective effect on acute and chronic kidney diseases including possible beneficial effects of metformin in diabetic kidney disease. However, these pharmacological agents may have AMPK-independent effects. Indeed, it has been reported that the inhibitory effect of metformin gluconeogenesis is independent of AMPK and the renal protective effect of metformin is not dependent on AMPK. Utilizing UUO and IRI kidney fibrotic models and conditional AMPKz1-KO mice, we provide compelling experimental evidence that AMPKz1 contributes to the development of renal fibrosis through activation of fibroblasts (Figures 1–4). We have further demonstrated that either global or fibroblast-specific deletion of AMPKz1 reduces

Figure 3 | Fibroblast-specific depletion of AMPKz1 reduces unilateral ureteral obstruction (UUO)-induced renal fibrosis. (a) Representative Western blots show the protein levels of AMPKz1 in the contralateral control (CON) and UUO kidneys of Col1-CreAMPKz1loxP/lox mouse (Fako) and their littermate controls (CTRL). (b) Representative photomicrographs of kidney sections stained for AMPKz1 (brown) and counterstained with hematoxylin (blue). Bar = 20 μm. (c) Representative photomicrographs of kidney sections stained for AMPKz1 (red), PDGFR-β (green), and 4,6-diamidino-2-phenylindole (DAPI) (blue). Bar = 30 μm. (d) Representative photomicrographs show kidney sections stained for total collagen deposition. Bar = 20 μm. (e) Representative Western blots show the protein levels of fibronectin (FN), collagen I (Col I), and α-smooth muscle actin (α-SMA) in the kidneys. Representative photomicrographs of the kidney sections stained for (f) collagen I, (g) fibronectin, and (h) α-SMA, and counterstained with DAPI (blue). Bar = 30 μm. **P < 0.01 compared with the CTRL-CON group, #P < 0.05 compared with the CTRL-UUO group, and +P < 0.05 compared with the Fako-UUO group; n = 6 mice per group. To optimize viewing of this image, please see the online version of this article at www.kidney-international.org.
renal fibrosis and the expression of ECM proteins. These results indicated that AMPKα1 has a key role in fibroblast activation and the development of renal fibrosis, which provides a novel link between metabolism and fibroblast activation. It should be emphasized that these 2 experimental models of renal fibrosis differ considerably in terms of etiology and metabolic consequence, suggesting that AMPK may play a more general role in fibroblast activation and fibrogenesis. Recently, we have demonstrated that adiponectin, a cytokine that activates AMPK signaling pathway,15,20,21 is induced in the kidney following ureteral obstruction or ischemia-reperfusion injury; genetic

Figure 4 | Fibroblast-specific depletion of AMPKα1 inhibits ischemia-reperfusion injury (IRI)-induced renal fibrosis. (a) Representative Western blots show the protein levels of AMPKα1 in the contralateral (CON) and IRI kidneys of Col1-Cre+/AMPKα1fl/fl (FAKO) mice and their littermate controls (CTRL). (b) Representative photomicrographs show kidney sections stained with Sirius red for total collagen deposition. Bar = 20 μm. (c) Representative Western blots show the protein levels of fibronectin (FN), collagen I (Col I), and α-smooth muscle actin (α-SMA) in the kidneys. Representative photomicrographs of the kidney sections stained for (d) collagen I, (e) fibronectin, and (f) α-SMA, and counterstained with 4',6-diamidino-2-phenylindole (DAPI) (blue). Bar = 30 μm. **P < 0.01 compared with the CTRL-CON group, #P < 0.05 compared with the CTRL-IRI group, and +P < 0.05 compared with the FAKO-IRI group; n = 6 mice per group. To optimize viewing of this image, please see the online version of this article at www.kidney-international.org.
Figure 5 | AMPK activation results in fibroblast activation and extracellular matrix (ECM) protein production in the cultured cells. (a) Western blots show A-769662 (A-7, 100 μM for 24 hours) induced the expression of fibronectin (FN), collagen I (Col I), and α-smooth muscle actin (α-SMA) in NRK-49F cells, which was abolished by compound C (Com C, 10 μM). **P < 0.01 versus vehicle controls and #P < 0.05 versus A-769662 alone; n = 3 per group. Western blots show A-769662 (100 μM for 24 hours) induced fibronectin, collagen I, and α-SMA expression in (b) primary kidney fibroblasts and (c) bone marrow–derived monocytes from mice, and deletion of AMPKα1 blocked A-769662-induced fibronectin, collagen I, and α-SMA expression in these cells. AMPKα1 was deleted in these cells from CAG-Cre+/AMPKα1fl/fl mice by treating with 1 μM 4-hydroxytamoxifen (4-OHT) for 3 days. **P < 0.01 versus vehicle controls and #P < 0.05 versus A-769662 alone; n = 3 per group. (d) Western blotting shows adiponectin (APN, 10 μg/ml for 24 hours) induced the expression of fibronectin (FN), collagen I, and α-SMA in bone marrow–derived monocytes, which was blocked by compound C. **P < 0.01 versus vehicle controls and #P < 0.05 versus adiponectin alone; n = 3 per group. (e) Ectopic expression of constitutively active AMPKα1 increased fibronectin, collagen I, and α-SMA in HEK-293T cells. The cells were transfected with control (pEBG-GST) or pEBG-GST-AMPKα1 (1-312) plasmids for 24 hours. **P < 0.01 versus pEBG plasmid control; n = 3 per group. p-ACC, phospho-Acetyl-CoA Carboxylase; ACC, Acetyl-CoA Carboxylase. To optimize viewing of this image, please see the online version of this article at www.kidney-international.org.
disruption of adiponectin prevents kidney injury and fibrosis.\textsuperscript{15,41} The current study suggests that AMPK is at an important nexus for adverse fibrotic remodeling in the kidney.

Our study demonstrates that AMPKa1 deficiency reduces collagen I and fibronectin protein accumulation and deposition in the kidneys 10 days after UUO or 14 days after IRI. Mia \textit{et al.} reported that global deletion of AMPKa1 reduced considerably collagen I mRNA expression and, slightly but significantly, collagen I protein production in the obstructed kidneys 7 days after UUO.\textsuperscript{16} Furthermore, they did not detect a significant difference in collagen I deposition by immunostaining of the kidneys 7 days after obstructive injury. The discrepancy can be explained in that the semiquantitative method of scoring collagen staining is insensitive and may have failed to detect a reduction in fibrosis that is indicated by other parameters.

Myofibroblasts are generally considered to be the main source of increased ECM deposition in renal fibrosis.\textsuperscript{6–8} Their number correlates closely with the severity of fibrosis and the progression of chronic kidney disease.\textsuperscript{8,42,43} These activated myofibroblasts predominantly originate from resident renal fibroblasts and bone marrow.\textsuperscript{44–46} We have shown that both accumulation of myofibroblasts identified as \(\alpha\)-SMA-positive cells and expression of \(\alpha\)-SMA protein are increased in the fibrotic kidneys of WT mice after UUO or IRI, whereas both myofibroblast accumulation and \(\alpha\)-SMA expression are significantly reduced in the fibrotic kidneys of Cre-mediated

\textbf{Figure 6 | AMPKa1 activation induces MRTF-A nuclear translocation leading to fibroblast activation and extracellular matrix protein production.} Western blot analyses show inhibition of the MRTF signaling pathway with CCG-203971 (CCG) prevents expression of fibronectin (FN), collagen I (Col I), and \(\alpha\)-smooth muscle actin (\(\alpha\)-SMA) in (a) NRK-49F cells and (b) bone marrow-derived monocytes. The cells were pre-treated with vehicle or 10 \(\mu\)M CCG-203971 for 30 minutes and then incubated with vehicle or 100 \(\mu\)M A-769662 (A-7) for 24 hours. *\(P < 0.05\) versus vehicle controls and \#\(P < 0.05\) versus A-769662 without CCG-203971; \(n = 3\) per group. (c) NRK-49F cells were serum-starved (0.5% fetal bovine serum) for 48 hours, and then stimulated with 200 \(\mu\)M A-769662 for 30 minutes. MRTF-A subcellular localization was determined by immunofluorescence staining for MRTF-A (red), 4',6-diamidino-2-phenylindole (DAPI) (blue) and F-actin (green). Bar = 30 \(\mu\)m. (d) NRK-49F cells were treated with 200 \(\mu\)M A-769662, harvested at indicated time points (hour). Nuclear translocation of MRTF-A was determined by nuclear extraction and Western blotting. *\(P < 0.05\) compared with controls and **\(P < 0.01\) compared with controls; \(n = 3\) per group. p-ACC, phospho-Acetyl-CoA Carboxylase; ACC, Acetyl-CoA Carboxylase. To optimize viewing of this image, please see the online version of this article at www.kidney-international.org.
AMPKα1 KO mice. Furthermore, our in vitro studies have shown that AMPK activation induced α-SMA and ECM protein expression, whereas AMPK inhibition or AMPKα1 deletion attenuates expression of α-SMA and ECM proteins (Figure 5). These results strongly indicate that AMPK plays a pivotal role in fibroblast activation and fibrogenesis.

MRTF-A, a member of the MRTF family of transcriptional coactivators, plays a central role in fibroblast activation.26,27,31,47–49 However, MRTF-A is sequestered in the cytoplasm, where it binds to G-actin, and its cytoplasm-nucleus translocation is under the dynamic control of G-actin and F-actin. Polymerization of G-actin into F-actin releases MRTF-A, and enables it to translocate into the nucleus, functioning as a coactivator of SRF-mediated transcriptions of CArG box-containing genes.25–27,29,30,49 In the present study, we have shown that AMPK activation results in F-actin formation and MRTF-A nuclear accumulation; inhibition of MRTF-A nuclear translocation with a small molecule inhibitor, CCG-203971, reduced AMPK-induced fibroblast activation and ECM production (Figure 6). These results suggest that MRTF-A signaling mediates AMPK-induced fibroblast activation and ECM protein production.

Members of the actin-depolymerizing factor (ADF) or coflin family are conserved small actin-binding protein, and play a pivotal role in actin dynamics. Coflin binds to F-actin, and severs and depolymerizes F-actin into G-actin. Coflin phosphorylation was determined by immunoblotting with a phospho-cofilin (Ser3) antibody. To optimize viewing of this image, please see the online version of this article at www.kidney-international.org.
inactivated by phosphorylation at Ser 3, leading to stabilization of F-actin and depletion of G-actin pools, which induces G-actin dissociation from MRTF-A and the latter's nuclear translocation. Phosphorylation of coflin is catalyzed by kinases. Previous study has shown that coflin can be phosphorylated at Ser 3 by LIM kinases and testicular protein kinase. In this study, the most important and unexpected discovery is that AMPK directly phosphorylates coflin (Figure 7). For the first time, we identify coflin-1 protein as a substrate of AMPK. In an in vitro kinase assay, we have demonstrated that AMPK directly phosphorylates coflin. Furthermore, we have shown that AMPKα1 and coflin directly interact within cells, and AMPK activation increases coflin phosphorylation. In addition, the immunofluorescence staining of mouse fibrotic kidneys reveals that AMPKα1 induces coflin phosphorylation in vivo. Taken together, our study provides compelling experimental evidence that phosphorylation of coflin by AMPK is the primary trigger for AMPK-induced fibroblast activation and fibrogenesis.

In summary, our study identifies AMPKα1 as a critical regulator of fibroblast activation and renal fibrogenesis. In response to obstructive or ischemia-reperfusion injury, activated AMPK phosphorylates coflin resulting in F-actin polymerization, MRTF-A nuclear accumulation, which activates fibroblasts and promotes fibrogenesis (Figure 8). Our findings yield novel insights into the molecular mechanisms of fibroblast activation and fibrogenesis and provide new therapeutic approaches for chronic fibrotic kidney disease.

MATERIALS AND METHODS

Mice

The mice were handled according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All experiments were approved by the Institutional Animal Care and Use Committee of Baylor College of Medicine. The Prkaa1 mutant mice (AMP-Kα1fl/fox) possess loxP sites flanking exon 3 of the protein kinase, AMP-activated, α1 catalytic subunit (Prkaa1) gene. The CAG-Cre+ mice have a tamoxifen-inducible Cre-mediated recombination system driven by the chicken beta actin promoter/enhancer coupled with the cytomegalovirus immediate-early enhancer. The Cre/ESR1 protein produced by these transgenic mice was restricted to the cytoplasm, and can only gain access to the nuclear compartment after exposure to tamoxifen. These mice were purchased from the Jackson Laboratory (Bar Harbor, ME). The Coll1-Cre+ mice carrying the tamoxifen-inducible Cre-recombinase (Cre/ESR1) under the control of fibroblast-specific regulatory sequence from the proα2(I) collagen gene were kindly gifted from Benoit de Crombrugghe. The AMPKα1fl/fox mice mated with CAG-Cre+ mice and

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Figure 8 | A proposed model depicting the molecular mechanism by which AMPK regulates actin dynamics, MRTF-A nuclear translocation, and fibroblast activation. Under normal condition, coflin severs and depolymerizes F-actin into G-actin, and cytoplasmic G-actin associates and retains MRTF-A in the cytoplasm. In response to injury, activated AMPK directly phosphorylates coflin, which promotes G-actin to F-actin polymerization. Lower G-actin concentration results in releases of MRTF-A from binding to G-actin, which enters into the nucleus and induces fibroblast activation.
Col1-Cre$^+$ mice, respectively. The offspring were used for generating tamoxifen-induced, Cre-mediated $AMPK_\alpha_1^{\text{fl}x/\text{fl}x}$ gene deletions. Genotyping was performed by polymerase chain reaction of tail biopsies (see Supplementary Figure S4C for primer sequences).

Male mice homozygous for $AMPK_\alpha_1^{\text{fl}x/\text{fl}x}$ with hemizygous Cre (Cre$^+$AMPK$\alpha_1^{\text{fl}x/\text{fl}x}$) were administered tamoxifen to delete the $AMPK_\alpha_1$ gene. Male littermate mice homozygous for $AMPK_\alpha_1^{\text{fl}x/\text{fl}x}$ without Cre (Cre$^-$$AMPK_\alpha_1^{\text{fl}x/\text{fl}x}$) were used as controls. Both cre and control mice aged 8 to 10 weeks (weighing 20–30 g) were given daily i.p. injections of 0.1 ml of 10 mg/ml tamoxifen (Sigma-Aldrich, St. Louis, MO) in corn oil for 10 days before surgeries. Daily tamoxifen treatment was continued until the day before killing because fibroblasts continue to be activated during the development of renal fibrosis.

**Mouse models**

Male mice were subjected to UUO or IRI surgery after 10 days' tamoxifen treatment. The UUO procedure was performed as described previously. To induce unilateral IRI, left kidneys were exposed through flank incision and were subjected to ischemia by clamping renal pedicles with nontraumatic microaneurysm clamps, as described previously, for 60 minutes. Kidneys were harvested 2 weeks after IRI. Unless otherwise specified, 6 mice per group were used in each experiment.

**Cell culture and treatment**

All cells were cultured in a humid incubator at 37°C and 5% CO$_2$. The normal rat kidney fibroblasts NRK-49F (ATCC CRL1570) and the human embryonic kidney cells HEK-293T (ATCC CRL-3216) were obtained from the American Type Culture Collection and cultured according to manufacturer’s protocols. NRK-49F cells were starved overnight and stimulated in Dulbecco’s modifed Eagle’s medium (DMEM) with 1 g/l glucose containing 0.5% fetal bovine serum (FBS) and 1% penicillin and streptomycin.

Primary kidney fibroblasts were isolated from mice as described previously. Briefly, mouse kidney was decapsulated, minced, and digested with Liberase (Roche, Indianapolis, IN) for 30 minutes at 37°C. Cells were filtered through a 40-μm strainer, centrifuged, and cultured in DMEM containing 10% FBS and 1% penicillin and streptomycin. After subculture, more than 98% of adherent cells were kidney fibroblasts as identified by positive staining for type I collagen and vimentin.

Mouse bone marrow–derived monocytes were isolated and cultured as described previously. Briefly, bone marrow–derived monocytes were isolated from 8- to 10-week-old mice and cultured in RPMI-1640 medium containing 10% FBS, 10% L929-conditioned medium, and 1% penicillin and streptomycin. After 5 days, cells were serum-starved and stimulated in RPMI-1640 medium containing 2% FBS and 2% L929-conditioned medium.

4-hydroxytamoxifen (Sigma-Aldrich) at 1 μM was used to delete AMPK$\alpha_1$ in primary kidney fibroblasts and bone marrow–derived monocytes from CAG-Cre$^+$AMPK$\alpha_1^{\text{fl}x/\text{fl}x}$ mice.

The pEBG-AMPK$\alpha_1$ (amino acids 1-312) and pEBG (empty backbone of GST fusion vector) plasmids were obtained from Addgene (plasmid numbers 27632 and 22227). Cells were transfected with plasmid DNAs using the Neon Transfection System (Thermo Fisher Scientific, Rockford, IL) according to the manufacturer’s protocol, or using X-tremeGENE HP DNA transfection reagent (Sigma-Aldrich).

**In vitro AMPK kinase assay**

Active human AMPK$\alpha_1$ + AMPK$\beta_1$ + AMPK$\gamma_1$ full-length protein (ab79803; Abcam, Cambridge, MA) was used as AMPK kinase, and recombinant human collagen full-length protein (ab62958, Abcam) was used as the substrate. Various concentrations of AMPK kinase and 1 μg of collagen were incubated for 1 hour at 37°C in a kinase buffer containing 20 mM HEPES (pH 7.4), 1 mM EGTA, 0.4 mM EDTA, 5 mM MgCl$_2$, 0.2 mM AMP, 0.1 mM ATP, 0.05 mM DTT, and 50 ng/μl BSA. Reactions were terminated with SDS sample buffer, and subjected to SDS-PAGE. Phosphorylation of collagen was determined by Western blot.

**Renal morphology**

Mice were killed and perfused with phosphate-buffered saline (PBS) to remove blood. One portion of the kidney tissue was fixed in 10% buffered formalin and embedded in paraffin, cut at 4-μm thickness, and stained with Sirius red to identify collagen fibers. The Sirius red-stained sections were scanned using a microscope equipped with a digital camera (Nikon Instruments, Melville, NY), and quantitative evaluation was performed using the NIS-Elements Br 3.0 software (Nikon Instruments).

**Immunohistochemistry**

Immunohistochemical staining was performed on paraffin sections, which were processed as described previously. Antigen retrieval was performed with antigen unmasking solution (Vector Laboratories, Burlingame, CA). Endogenous peroxidase activity was quenched with 3% H$_2$O$_2$. Vectastain Elite ABC kits (Vector Laboratories) were used for the staining according to manufacturer’s instructions. The reaction was visualized by incubation with diaminobenzidine solution (ImmPACT DAB, Vector Laboratories). Slides were then counterstained with hematoxylin. The images were acquired with a microscope equipped with a digital camera.

**Immunofluorescence**

Immunofluorescence staining of kidney sections was performed on paraffin sections as described previously. After antigen retrieval, nonspecific binding was blocked with protein block (Dako, Carpinteria, CA). Kidney sections were incubated with primary antibodies overnight, followed by appropriate Alexa Fluor 488- or 594-conjugated secondary antibodies (Life Technologies). Cell staining was performed according to the immunocytochemistry (ICC) protocol. Cells were grown on glass cover slips. After washing with PBS, the samples were fixed in 4% paraformaldehyde (Alfa Aesar, Ward Hill, MA), and permeabilized with 0.5% Triton X-100 in PBS. Cells were stained with anti-MRTF-A (Santa Cruz Biotechnology) and Alexa Fluor 594-conjugated phalloidin (Cell Signaling Technology) was used to stain F-actin. Slides were mounted with medium containing 4',6-diamidino-2-phenylindole (Vector Laboratories). Fluorescence intensity was visualized using a Nikon microscope image system. Quantitative analysis of sections was performed using NIS-Elements Br 3.0 software.

**Coimmunoprecipitation**

Cells were harvested in Pierce IP Lysis Buffer (Thermo Fisher Scientific) containing cocktail proteinase and phosphatase inhibitors after washing in PBS. Protein A/G PLUS-agarose (Santa Cruz Biotechnology) was used for immunoprecipitation, according to the manufacturers’ protocols. Beads were washed 5 times with lysis buffer.
buffer. Eluted proteins were analyzed by Western blotting. In this study, goat IgG was used as a negative control.

**Western blot analysis**

Tissue samples or cells were homogenized and lysed in radioimmunoprecipitation (RIPA) buffer containing cocktail proteinase and phosphatase inhibitors (Thermo Fisher Scientific). Protein concentration was determined using the BCA Protein Assay Kit (Thermo Fisher Scientific). Nuclear extracts were prepared as described previously. Equal amounts of protein were separated on SDS-polyacrylamide gels in a Tris/glycine buffer system, transferred onto nitrocellulose membranes, and blotted according to standard procedures. The specific bands of target proteins were analyzed using an Odyssey IR scanner (LI-COR Bioscience, Lincoln, NE), and signal intensities were quantified using NIH ImageJ software.

**Statistical analysis**

Data were expressed as mean ± SEM. Multiple group comparisons were performed by analysis of variance followed by the Bonferroni procedure for comparison of means. Comparisons between 2 groups were analyzed by the 2-tailed t-test. P values of less than 0.05 were considered statistically significant.

**DISCLOSURE**

All the authors declared no competing interests.

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**SUPPLEMENTARY MATERIAL**

Reagents.

**Figure S1.** AMPKα1 is upregulated in the kidney following UUO. Representative Western blots show the protein levels of AMPKα1 in the kidneys from wild-type mice after 10 days of unilateral ureteral obstruction (UUO). AMPKα1 protein expression was normalized with glyceraldehyde-3-phosphate dehydrogenase. **P < 0.05** compared with the contralateral (CON) group, n = 4 mice per group.

**Figure S2.** AMPKα1 is induced in the interstitial cells of the obstructed kidneys. Representative immunophotographs of kidney sections from wild-type mice after 7 days of unilateral ureteral obstruction (UUO) stained for AMPKα1 (brown) and counterstained with hematoxylin (blue). Original magnification ×400. **P < 0.05** compared with the contralateral (CON) group, n = 4 mice per group.

**Figure S3.** Phosphorylated AMPKα1 localization to α-smooth muscle actin (α-SMA)-positive myofibroblasts in the obstructed kidneys. Representative immunophotographs of kidney sections from wild-type mice after 10 days of unilateral ureteral obstruction (UUO) stained for p-AMPKα1 (red), α-SMA (green), and 4′,6-diamidino-2-phenylindole (blue). Bar = 20 μm.

**Figure S4.** Generation of mice with tamoxifen-inducible global (CAG-Cre) or fibroblast-specific (Col1-Cre) deletion of AMPKα1. Schematic representation of the genetic crosses to (A) globally delete or (B) fibroblast-specifically delete AMPKα1. (C) Primers for genotyping.

**Figure S5.** AMPKα1 is deleted in cultured CAG-Cre+AMPKα1lox/lox fibroblasts after treatment with 4-hydroxytamoxifen. Primary mouse kidney fibroblasts from CAG-Cre+AMPKα1lox/lox mice were treated with 1 μM 4-hydroxytamoxifen for 1 to 3 days. Western blotting showed AMPKα1 levels in kidney fibroblasts after 4-hydroxytamoxifen treatment.

**Figure S6.** Coflin is phosphorylated in the interstitial cells of the kidneys with unilateral ureteral obstruction (UUO). Representative photomicrographs of kidney sections from wild-type mice after 5 days of UUO stained for phosphorylated coflin (brown) and counterstained with hematoxylin (blue). Original magnification ×400.

**Figure S7.** Global deletion of AMPKα1 abolishes coflin phosphorylation in the kidneys with unilateral ureteral obstruction (UUO). Representative photomicrographs of kidney sections from CAG-CreAMPKα1lox/lox (CTRL) and CAG-Cre+AMPKα1lox/lox (GAKO) mice after 10 days of UUO stained for phosphorylated coflin (brown) and counterstained with hematoxylin (blue). Original magnification ×400.

**Figure S8.** Global deletion of AMPKα1 abolishes coflin phosphorylation in the kidneys with IRL. Representative photomicrographs of kidney sections from CAG-CreAMPKα1lox/lox (CTRL) and CAG-Cre+AMPKα1lox/lox (GAKO) mice after 14 days of ischemia-reperfusion injury (IRI) stained for phosphorylated coflin (red) and counterstained with 4′,6-diamidino-2-phenylindole (blue). Original magnification ×400.

**Figure S9.** Coflin is phosphorylated in α-smooth muscle actin (α-SMA)-positive cells in the kidney with unilateral ureteral obstruction (UUO). Representative photomicrographs of paraffin kidney sections from wild-type mice after 10 days of UUO stained for p-coflin (red), α-SMA (green), and 4′,6-diamidino-2-phenylindole (blue). Original magnification ×400.

**Figure S10.** Coflin is phosphorylated in α-smooth muscle actin (α-SMA)-positive cells in the kidney with unilateral ureteral obstruction (UUO). Representative photomicrographs of paraffin kidney sections from wild-type mice after 5 days of UUO stained for AMPKα1 (red), phosphorylated coflin (green), and 4′,6-diamidino-2-phenylindole (blue). Original magnification ×400.

**REFERENCES**


