The feedback loop between miR-21, PDCD4 and AP-1 functions as a driving force for renal fibrogenesis

Qi Sun1, Jiao Miao1, Jing Luo1, Qi Yuan1, Hongdi Cao1, Weifang Su1, Yang Zhou1, Lei Jiang1, Li Fang1, Chunsun Dai1,* Ke Zen2,* and Junwei Yang1,*

ABSTRACT
Renal fibrosis is a final common pathway of chronic kidney disease. Sustained activation of fibroblasts is considered to play a key role in perpetuating renal fibrosis but the driving force in the perpetuation stage is only partially understood. To date, some investigations have specifically identified overexpression of microRNA 21 (miR-21) in the progression of kidney fibrosis. Nevertheless, the precise role of miR-21 in fibroblast activation remains largely unknown. In this study, we found that miR-21 was significantly upregulated in activated fibroblasts and that it maintained itself at constant high levels by employing an auto-regulatory loop between miR-21, PDCD4 and AP-1. Persistently upregulated miR-21 suppressed protein expression of Smad7 and, eventually, enhanced the TGF-β1/Smad pathway to promote fibroblast activation. More importantly, we found miR-21 sequestration with miR-21 antagonist or AP-1 inhibitors attenuated unilateral ureteral obstruction (UUO)-induced renal fibrosis. miR-21-knockout mice also suffered far less interstitial fibrosis in response to kidney injury. Altogether, these data suggest that miR-21 is a main driving force of fibroblast activation and keeps its high expression level by employing a double negative autoregulatory loop. Targeting this aberrantly activated feedback loop may provide new therapeutic strategy in treating fibrotic kidneys.

KEY WORDS: Fibroblasts, MicroRNA, miR-21, Renal fibrosis, miR-21/PDCD4/AP-1

INTRODUCTION
Organ fibrosis is an inappropriate wound-healing response, when normal tissue remodeling fails to terminate (Hernandez-Gea and Friedman, 2011). It is frequently associated with fibroblast activation to synthesize and secrete components of extracellular matrix (ECM) (Mack and Yanagita, 2015; Honda et al., 2013). Following kidney injury, the resident fibroblasts are stimulated and undergo phenotypic transition into scar-forming myofibroblasts, and produce a large amount of ECM components (Inoue et al., 2010; Asada et al., 2011; Humphreys et al., 2010). This continuous excessive deposition of ECM proteins results in fibrous scars and distorts the architecture of kidney tissue, ultimately leading to the loss of kidney function (Feist et al., 2014; Wight and Potter-Perigo, 2011; Genovese et al., 2014). Despite fundamental advances in understanding the pathophysiology of renal fibrosis, the driving force for the persistent fibrogenic activities is only little understood, and definitive therapies to effectively prevent and/or treat this disorder remain limited.

MicroRNAs are a set of ~22 nucleotide noncoding RNAs that regulate gene expression predominantly by repressing translation or facilitating degradation of target mRNAs (Godwin et al., 2010; Kato and Natarajan, 2012; Thum et al., 2008). Aberrant expression of miRNAs is associated with initiation and progression of numerous pathologic processes including renal cancer, diabetic nephropathy and renal injury, suggesting that modulation of dysregulated miRNAs in vivo may attenuate the manifestation of these diseases (Jung et al., 2009; Chau et al., 2012; Wei et al., 2013). To date, some investigations have identified that specific patterns of miRNA expression, particularly miR-21 overexpression, plays a critical role in the progression of kidney fibrosis (Gomez et al., 2015; Lai et al., 2015; Srivastava et al., 2013; Li et al., 2013; Zhou et al., 2013). Nevertheless, how miR-21 is dysregulated in fibrotic kidney and, moreover, what the precise role of miR-21 is in fibroblast activation remains largely unknown.

In the present study, we explored the role of miR-21 in the pathogenesis and progression of kidney fibrosis, and reported the effect of antagonim-21 as a novel therapeutic treatment to attenuate renal fibrosis. We have previously found that miR-21 is consistently upregulated in the development of kidney fibrosis (Zhou et al., 2013). Here, we identified that miR-21 was increased robustly on the first day in mice with unilateral ureteral obstruction (UUO), and was maintained at constant high levels by employing an auto-regulatory loop between microRNA-21 (miR-21), programmed cell death protein 4 (PDCD4) and activation protein-1 (AP-1), to which we hereafter refer to as miR-21/PDCD4/AP-1. The persistently upregulated miR-21 reduced expression of Smad7 and, eventually, enhanced signaling through transforming growth factor-β1 (TGF-β1)/Smad pathway to promote fibrosis progression. More importantly, we found that deletion of the gene encoding miR-21 or acute sequestration of miR-21 with miR-21 antagonist or AP-1 inhibitors in mouse kidneys attenuated the pathological response to UUO injury. Altogether, these data suggest that miR-21 is a central mediator in the pathogenesis of renal fibrosis and a potential target in the treatment of fibrotic kidneys.

RESULTS
miR-21 is upregulated in kidneys with UUO-induced fibrosis
To delineate the role of miRNAs in kidney fibrosis, we used the murine renal fibrosis model induced by UUO, which is characterized by a slow initial injury that results in progressive interstitial fibrosis. In this study, we performed Solexa sequencing analysis to identify
miRNA expression in control and obstructive kidneys. The expression abundance of almost 1000 miRNAs in kidney samples was compared. The Solexa results showed that expression levels of 639 miRNAs was altered in obstructed kidneys as early as at the first day after UUO injury (Fig. 1A). Out of 36 upregulated miRNAs, miR-21 demonstrated the greatest increase in expression (Fig. 1B). Elevated expression of miR-21 had been noted previously; however, the expression profile in different cell types remains elusive. miRNA in situ hybridization (ISH) was then performed on kidney sections to locate upregulated miR-21 (Fig. 1C). We detected only weak miR-21 signals in normal kidney, whereas the hybridization signal was greatly enhanced in obstructive kidney. The miR-21 signal was widespread throughout the UUO kidney, particularly in proximal tubule epithelium and tubulointerstitial fibroblasts, which all contained detectable amounts of miR-21. The scrambled miR-21 probe showed no positive signal on the kidney sections during the experiments (data not shown). Dysregulation of miR-21 was then confirmed by reverse transcription–quantitative polymerase chain reaction (RT-qPCR). As shown in Fig. 1D, expression levels of miR-21 increased robustly as early as day 1 (3.00±1.24) and were maintained at high levels until day 7 (7.67±1.98) after UUO injury. Upregulation of miR-21 in renal cortex correlated with progression...
of fibrosis, highlighting the involvement of miR-21 in fibrotic activation possible.

**Anti-miR-21 oligonucleotide ameliorates kidney fibrosis**

To further confirm that miR-21 participated in and promoted the progression of kidney fibrosis, chemically modified anti-miR-21 oligonucleotid (antagomir-21) was administered through tail-vein injection into mice the day before UUO injury to knockdown miR-21 expression in vivo. Kidneys were harvested at day 7 after the UUO injury. UUO7d mice have shown significant tubulointerstitial pathology, characterized by interstitial fibrosis, tubular injury and peritubular capillaries destruction (Fig. 2A). H&E-stained images of whole kidney showed interstitial fibrosis, and PAS-stained images showed tubule injury. Staining for collagen I (COL1A1), fibronectin (FN) and α-SMA showed deposition in the extracellular matrix (ECM). Compared with control antagomir, administration of antagonim-21 led to less interstitial fibrosis in the UUO model, and total matrix deposition was much reduced (Fig. 2B). Consistent with the reduction of histological staining of fibrosis in kidneys, the protein levels in the ECM of UUO mice were also significantly attenuated after antagonim-21 treatment (Fig. 2C,D).

To determine the knockout efficiency of miR-21, we performed RT-qPCR in control- and antagomir-21-treated mice. As shown in Fig. 2E, miR-21 was strikingly sequestered by antagonim-21 in obstructed kidneys. In line with miR-21, mRNA levels of COL1A1, FN and α-SMA (Fig. 2F) were also downregulated after miR-21 sequestration. Accordingly, these findings from animal models demonstrated that miR-21 stimulates ECM expansion and is involved in the pathogenesis of kidney fibrosis.

**miR-21 directly enhances activation of NRK-49F cells**

Given the attenuation of kidney fibrosis and α-SMA (a marker of myofibroblast trans-differentiation) gene expression in antagonim-21-treated mice, we sought to determine whether miR-21 facilitates fibrogenesis through promoting fibroblast activation. To understand the role of miR-21 in these renal fibroblast precursors when they differentiate into myofibroblasts, we incubated rat kidney fibroblasts (NRK-49F cells) with TGF-β1, a cytokine known to stimulate fibroblast differentiation in vitro. After exposure to TGF-β1, normal NRK-49F cells showed notable increase in miR-21 expression (Fig. 3A) and underwent trans-differentiation into myofibroblasts, as demonstrated by the transcriptional and translational upregulation of myofibroblast markers COL1A1, FN and α-SMA (Fig. 3B-D). As the miR-21 level increased with the upregulation of extracellular matrix expression, it demonstrated that the miR-21 levels correlated with the activation status of fibroblasts.

To determine whether miR-21 directly regulates fibroblast activity, we transfected miR-21 mimics or inhibitors into NRK-49F cells to modulate miR-21 content during fibroblast differentiation. As shown in Fig. 3A, TGF-β1-dependent induction of miR-21 was attenuated by miR-21 inhibitors, and augmented by miR-21 mimics. Meanwhile, mRNA expression of the myofibroblast phenotypic markers COL1A1, FN and α-SMA was downregulated after miR-21 attenuation, and upregulated with miR-21 mimics transfection (Fig. 3B). In line with the mRNA change, knockdown of miR-21 reduced COL1A1, FN and α-SMA protein levels, while an increase of the miR-21 content inversely upregulated them (Fig. 3C,D). This result was further confirmed by immunofluorescence staining (Fig. 3E,F). These data suggest that miR-21 participates in fibroblast activation by regulating the expression of phenotypic and functional genes of myofibroblasts.

**miR-21 enhances kidney fibrosis by regulating the TGF-β1/Smad signaling pathway**

To investigate the mechanism by which miR-21 promotes fibroblast activation in the injured kidney, we examined the expression profiles of TGF-β1 signaling mediators in NRK-49F cells after the transfection of miR-21 mimics or inhibitors. As shown in Fig. 4A,B, increasing miR-21 levels enhanced Smad3 phosphorylation in response to stimulation with TGF-β1, whereas knockdown of miR-21 attenuated the levels of phosphorylated Smad (p-Smad). It has been reported that Smad7 exhibits antagonistic effects on Smad3 phosphorylation (Dooley et al., 2003), and is a direct target of miR-21 (Zhang et al., 2017; McClelland et al., 2015). To further investigate whether miR-21 directly bound to 3′-UTR of Smad7, a fragment of human Smad7 3′-UTR containing the putative miR-21 binding sequence was fused into a luciferase reporter plasmid (Fig. 4C), and co-transfected with miR-21 mimics into NRF-49F cells by using Lipofectamine 2000. The luciferase assay showed that miR-21 mimics only reduced luciferase activity in cells containing wild-type 3′-UTR, but not in cells containing mutant 3′-UTR. This result indicates the direct binding of miR-21 to the 3′-UTR region of Smad7. The effects of miR-21 on Smad7 expression levels were further analyzed by western blot. miR-21 inhibitors increased Smad7 level, whereas miR-21 mimics decreased it. We also observed that miR-21 inhibitors reversed TGF-β1-induced Smad7 downregulation in NRK-49F cells (Fig. 4D,E). Together, these data suggest a role of miR-21 in the regulation of Smad3 phosphorylation by modulating Smad7 expression.

**Inhibition of AP-1 activity attenuates miR-21-induced NRK-49F cell activation**

Transcription factor activating protein-1 (AP-1) is a heterodimeric complex that primarily comprises nuclear oncoproteins c-Jun and Fra-1 (FOSL1). AP-1 can be activated and phosphorylated at the N-terminal region of c-Jun by JNK, a member of MAPK family (Rui et al., 2012). After activation, AP-1 binds to the TPA-responsive element (TRE) and induces transcription of a variety of genes involved in multiple cellular processes, including proliferation, differentiation, transformation and apoptosis (Shaullian, 2010; Vesely et al., 2009). It has been noted that AP-1 is one of the transcription factors that activate the transcription of miR-21 (Zhou et al., 2011). We therefore determined the role of AP-1 in miR-21 activation in activated fibroblasts. NRK-49F cells were cultured with two different AP-1 inhibitors: SP600125, which interferes with c-Jun phosphorylation (Bennett et al., 2001), and SR11302, an intrinsic AP-1 inhibitor (Huang et al., 1997). The expression level of miR-21 was significantly downregulated in SP600125- and SR11302-treated cells compared with the DMSO-treated group (Fig. 5A). To exclude the off-target effects of chemical inhibitors, we transfected small interfering RNA (siRNA) against c-Jun (si-c-Jun) into NRK-49F cells. As expected, miR-21 levels were downregulated (Fig. 5B) in response to reduced c-Jun mRNA levels. Therefore, inhibition of AP-1 activity can suppress miR-21 expression in renal fibroblasts.

Next, to test the role of AP-1 in fibroblast activation, we inhibited AP-1 activation in NRK-49F cells with SP600125. We confirmed that levels of phosphorylated c-Jun (p-c-Jun) were reduced in activated NRK-49F cells upon inhibition of AP-1 (Fig. 5C,D). A similar reduction of FN and α-SMA protein levels was showed in Fig. 5E,F. Consistent with western blot results, immunofluorescence staining also showed that the presence of SP600125 significantly alleviated the expression of the
Fig. 2. Antagomir-21 prevents UUO kidney fibrosis. (A) Representative images of renal cortices from day 7 UUO-injured mice (UUO7d) with and without antagomir-21 treatment (antagomir-21+UUO7d and antago-ctrl+UUO7d, respectively) showing H&E staining, and staining for PAS, COL1A1; FN; α-SMA/laminin. Scale bars: 50 μm. n=5 per group. (B) Semi-quantitative analyses of fibrosis areas stained for COL1A1, FN and α-SMA shown in A. (C) Western blot analysis for FN and α-SMA. Actin is shown as internal control. (D) Densitometric quantification of FN and α-SMA protein levels shown in C. (E,F) Quantitative RT-qPCR analysis for miR-21 (E), COL1A1, FN and α-SMA (F) in whole kidneys. n=5 per group. *P<0.05, **P<0.01. Error bars represent ±s.d.
myofibroblast phenotypic markers FN and α-SMA when compared with DMSO control treatment (Fig. 5G,H). Therefore, these data indicated that inhibition of AP-1 activity suppressed fibroblast activation by repressing miR-21 expression.

**Feedback regulation of miR-21 by miR-21/PDCD4/AP-1**

Computer analysis and previous reports showed that programmed cell death 4 (PDCD4) is a potential target gene of miR-21 (Frankel et al., 2008). To test this hypothesis, we incubated NRK-49F cells with TGF-β1 or vehicle for 48 h, and the PDCD4 protein level was determined by western blot. As expected, treatment with TGF-β1 decreased PDCD4 protein levels (Fig. 6A). To further confirm that PDCD4 is a target gene of miR-21 during TGF-β1-induced myofibroblast transdifferentiation, we performed the miR-21 loss-of- and gain-of-function experiments in NRK-49F cells. As shown in Fig. 6B, miR-21 inhibitors reversed the downregulation of PDCD4 protein synthesis mediated by TGF-β1, whereas miR-21 mimics promoted PDCD4 degradation. It has been reported that PDCD4 inhibits AP-1-dependent transcription by inhibiting the phosphorylation of c-Jun (Zhang et al., 2013). Thus, we determined the modulating effects of miR-21 on c-Jun phosphorylation in activated NRK-49F cells. miR-21 inhibitors downregulated levels of p-c-Jun, whereas miR-21 mimics increased them (Fig. 6A,B).

We then determined the function of PDCD4 in fibroblasts by knocking down PDCD4 expression using specific PDCD4 siRNA. When the protein synthesis of PDCD4 was downregulated, we observed increased levels of p-c-Jun (Fig. 6C,D) and myofibroblastic phenotypic marker FN and α-SMA (Fig. 6E,F). Immunofluorescence staining confirmed the western blot results (Fig. 6G,H). Together, these results showed that, miR-21-mediated PDCD4 degradation maintained maximal p-c-Jun levels in NRK-49F cells, thereby further promoting miR-21 expression.

**Attenuation of AP-1 activity ameliorated renal fibrosis**

We next determined the role of AP-1 in renal fibrosis in vivo. Results shown in Fig. 7A,B first confirmed the consistently elevated levels of p-c-Jun and c-Jun in fibrotic kidney samples from UUO mice. Next, to test the role of AP-1 in renal fibrogenesis, we inhibited AP-1 activation in UUO mice by using SP600125 (Fig. 7C,D). Compared with the DMSO-treated control group, we found a less significant induction of miR-21 in SP600125-treated mice 7 days after the injury (Fig. 7E). A similar reduction of COL1A1, FN and...
α-SMA protein levels was shown in Fig. 7F,G. Consistent with western blot results, histological staining also showed that SP600125 treatment significantly alleviated extracellular matrix expression compared to treatment with DMSO control (Fig. 7H). Therefore, these data indicate that inhibition of AP-1 activity suppressed renal fibrosis by repressing miR-21 expression and preventing fibroblast activation.

**miR-21 deficiency reduced UUO-induced renal fibrosis**

To gain further insights into whether miR-21 is a potential target in renal fibrosis, we purchased miR-21 knockout mice (B6;129S6-Mir21atm1Yoli/J), and crossed them with C57BL/6J background mice twice. Mice with heterozygous or homozygous miR-21 deletion in C57BL/6J background were fertile and appeared phenotypically normal, similar to previous reports (Hatley et al., 2010). We subjected 20 miR-21-null mice along with 22 of their wild-type littermates to UUO surgery. Kidneys were analyzed at days 1, 3 and 7 after the surgery to assess renal pathology. As Fig. 8A shows, there was far less interstitial fibrosis in miR-21−/− mice compared to their wild-type littermates. Both WT and miR-21−/− mice had increased accumulation of ECM at day 3, but miR-21−/− mice showed far lower levels of COL1A1, FN and α-SMA protein than the WT group (Fig. 8B-D). These observations were supported further by immunohistochemistry IHC and immunofluorescence (Fig. 8E).

This result confirmed a substantial increased expression of ECM proteins in WT mice, but far less in miR-21−/− mice at 3 and 7 days after treatment. Taken together, these results suggest that miR-21 deficiency protects mice against renal fibrosis.

**DISCUSSION**

Renal fibrosis is a common pathway of chronic kidney injury. During the injury, the resident fibroblasts are stimulated and trans-differentiated into myofibroblasts that produce high amounts of ECM components and, ultimately, lead to the loss of kidney function. Sustained activation of fibroblasts is considered to play a key role in perpetuating renal fibrosis, but the driving force of fibroblast activation is only partially understood. Aberrant expression of miRNAs is associated with numerous pathologic processes. Previous studies, including those from our laboratory, showed that miR-21 regulates lung, liver, kidney and heart fibrosis by enhancing the fibrogenic activity or by promoting the proliferation of interstitial fibroblasts (Hernandez-Gea and Friedman, 2011; Thum et al., 2008; Zhou et al., 2013). Nevertheless, the precise role of miR-21 in fibroblast activation remains largely unknown. In this study, by using a murine renal fibrosis model induced by UUO, we demonstrated that upregulated miR-21 was a main driving force of fibroblast activation by functioning in an autoregulatory loop together with PDCD4/AP-1 and by being a promising candidate for anti-fibrotic therapy.
We first performed Solexa sequencing to identify miRNA expression in murine interstitial fibrotic kidneys. Solexa results showed 639 differently expressed miRNAs in obstructive kidneys. Among them, miR-21 demonstrated the greatest increase of expression out of 36 upregulated miRNAs. To validate these sequencing results, we carried out ISH and RT-qPCR assays to confirm the rapid and abundant induction of miR-21 in kidney sections. The greatest intensity of miR-21 in kidney was in proximal tubule epithelium but miR-21 was widespread throughout the whole kidney. Because activation of fibroblasts is a key event during fibrosis development, and because fibroblasts also contained detectable amounts of miR-21, we assumed that miR-21 participates in renal fibrosis by regulating myofibroblast trans-differentiation. It must be noted that, although the mechanisms of fibroblast activation are overwhelming (Jiang et al., 2013a,b; Ponnusamy et al., 2014; Miller et al., 2011; Vasquez et al., 2010; Flack et al., 2006; Jarvis et al., 2006), there are still controversial questions that remain to be answered, particularly with the driving force of constant activation of fibroblasts in renal fibrosis. In addition, there is currently no study that has addressed how miR-21 expression is elevated during fibroblast activation and, moreover, how the miR-21 expression is maintained at a high level.

Extensive studies have suggested an important role of miRNA networks in the promotion of disease progression. This miRNA-mediated gene expression might have the capacity to promote the irreversible activation of some signaling pathways, to facilitate a cellular phenotype switch under pathological conditions and, ultimately, contributing to disease progression. Our previous study reported such an miRNA network, the miR-30e/UCP2 axis,

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**Fig. 5. Inhibition of AP-1 activity attenuates miR-21-induced activation of NRK-49F cells.**

(A) Quantitative RT-qPCR analysis of miR-21 levels in NRK-49F cells treated with AP-1 inhibitors SR11302 or SP600125, with or without treatment with TGF-β1 (to upregulate expression of miR-21). (B) Quantitative RT-qPCR analysis of miR-21 levels in NRK-49F cells transfected with si-c-Jun or negative control (ctrl) with or without treatment with TGF-β1. (C) Western blot analysis of phosphorylated c-Jun (p-c-Jun) in NRK-49F cells treated with or without the AP-1 inhibitor SP600125. (D) Densitometric quantification of p-c-Jun protein results in C. (E) Western blot analysis of FN and α-SMA in NRK-49F cells treated with or without SP600125. (F) Densitometric quantification of the FN and α-SMA results shown in E. (G,H) Representative images of NRK-49F cells immunostained for FN (G) or α-SMA (H) in the presence of SP600125, DMSO+TGF-β1 or SP600125+TGF-β1. FN, green; α-SMA, red; DAPI, blue. Scale bars: 10 μm. n=5 per group. *P<0.05, **P<0.01. Error bars represent ±s.d.
which has an important role in mediating TGF-β1-induced epithelial-mesenchymal transition and kidney fibrosis (Jiang et al., 2013a,b). This study here sheds light on another axis, the miR-21/PDCD4/AP-1 autoregulatory loop, which plays a role in myofibroblast trans-differentiation. For the first time, we report that miR-21 was upregulated in activated fibroblasts. But, how miR-21 expression increases during fibroblast activation is still elusive. As a transcriptional factor, AP-1 was considered to be involved in hepatic and pulmonary fibrogenesis, and to predominantly occur in myofibroblasts (Zhang et al., 2013; Dube et al., 2009). miR-21 was suggested to be a transcriptional target of AP-1 (Fujita et al., 2008; Frezzetti et al., 2011). Moreover, PDCD4, a direct target of miR-21, can inhibit AP-1 activity by preventing c-Jun phosphorylation. Thus, miR-21-mediated PDCD4 inhibition contributes to the increase of AP-1 activity, which in turn promotes miR-21 transcription. Given that AP-1 can drive miR-21 transcription and miR-21-mediated PDCD4 downregulation is necessary for maximal AP-1 activity, we speculate that miR-21 maintained its high level by employing this miR-21/PDCD4/AP-1 self-promoted loop during fibroblast activation and renal fibrosis.

Recently, Smad7 mRNA was found to be a sequence-dependent target of miR-21. Smad2/3 is the primary signal mediator of the TGF-β1 signaling pathway and plays a pivotal role in pro-fibrogenic gene expression. Smad7 is an inhibitor of Smad2/3 phosphorylation and inhibits fibrosis. Our studies have shown that Smad7 was
Fig. 7. See next page for legend.
downregulated during fibrogenesis, because of the high level of miR-21 in fibroblasts. Thus, miR-21 generated through the miR-21/PDCD4/AP-1 feedback loop can directly decrease the anti-fibrotic Smad7 expression, connecting this self-promoting feedback loop with the fibrogenic mechanism (Fig. 8F). Therefore, the miR-21/PDCD4/AP-1 feedback loop might be the major impetus to maintain the consistent activation of fibroblasts, resulting in the continuous production of fibrogenic ECM.

In addition, it has been implicated that miR-21 promotes fibrosis through regulation of multiple signaling pathways (Zhong et al., 2011; Zhang et al., 2013; McClelland et al., 2015). Several studies pointed out that, the transcription factor peroxisome proliferator-activated receptor alpha (PPARα; also known as PPARα), and its downstream FA oxidation pathways are key targets for miR-21 function in tubule epithelium (Chau et al., 2012). In the normal proximal tubule, FA oxidation is required to maintain the high metabolic demands of a bunch of transporters involved in reabsorption from, or secretion into, the tubular lumen. Suppression of this important pathway appears to be another approach, by which miR-21 exerts its negative influence in CKDs. In addition to regulating lipid metabolism, PPARα has been confirmed to interfere negatively with the binding activity of AP-1 to DNA (Delerive et al., 1999). Thus, the miR-21-facilitated AP-1 activity may act, at least in part, by inhibiting PPARα expression. miR-21 also silences mitochondrial UCP enzymes, which regulate uncoupling of respiration and therefore prevent ROS generation (Gomez et al., 2015). Therefore, preventing miR-21 activation either genetically or by use of silencing oligonucleotides also limits ROS-mediated damage and injury amplification in the kidney.

In summary, our data here suggest that the self-promoted miR-21/PDCD4/AP-1 feedback loop is a common driving force behind different progressive diseases. In activated fibroblasts, miR-21, PDCD4 and AP-1 form a doubly negative feedback loop during renal fibrosis. Once activated, the circuit maintains the stable activation of fibroblasts from the quiescent stage into the large ECM-producing state, and this finding provides additional understanding of fully activated myofibroblasts. In this regard, targeting of this aberrantly activated feedback loop might provide a new therapeutic strategy in treating renal fibrogenesis.

MATERIALS AND METHODS

Animal models
Animal studies were conducted in strict accordance with the principles and procedures approved by the Committee on the Ethics of Animal Experiments of Nanjing Medical University (China). Mice were housed under a 12-h light/12-h dark cycle with free access to food and water. miR-21−/− mice on C57BL/6J genetic background were obtained from heterozygous breeding after miR-21-null mice on the B6129SF1/J background were crossed to C57BL/6J mice for two generations. To mitigate minor genetic variations, animals from the same litter were utilized. At 8 weeks, male miR-21−/− (B6;129S6-Mir21atm1Yoli/J; The Jackson Laboratory) and CD-1 mice, were randomly assigned into different groups (five mice per group) with or without unilateral ureteral obstruction (UUO) for 7 days. UUO was performed using an established procedure (Yang and Liu, 2001). Briefly, under general anaesthetics, complete left ureteral obstruction was performed by doubly ligating of the left ureter using 4-0 silk after creating a midline abdominal incision. Mice of the sham group had their ureter exposed and manipulated but not ligated. Mice received antagonist-21 or control (antago-ctrl) at 50 mg/kg in PBS by tail vein injection at different times. Mice were killed at day 7 after the injury, and the obstructed kidneys were removed for further investigation. In some experiments, SP600125 (55567, Sigma) or control vehicle DMSO was injected through the tail vein.

Cell culture and treatment
The rat renal fibroblast cell line NRK-49F was purchased from American Type Culture Collection (ATCC, CRL-1570/1571™). NRK-49F cells were maintained in DMEM-F12 medium supplemented with 10% fetal bovine serum (Gibco) at 37°C in an atmosphere containing 5% CO2. For cell treatment, 50 nM miR-21 mimic (GenePharm), miR-21 inhibitor (GenePharm) or siRNAs (si-c-Jun, si-PDCD4; Invitrogen) were transfected into NRK-49F cells cultured on 6-well plates with Lipofectamine 2000 (Invitrogen), according to the manufacturer’s instruction. After 6 hours of transfection, cells were stimulated with 5 ng/ml TGF-β1 for 48 h and collected for RT-qPCR or western blot analysis. siRNAs were designed and synthesized by Invitrogen and are listed in Table S2.

Western blot
Protein samples were quantified using a BCA kit (Thermo Scientific, Rockford, IL). 20 μg of each sample was separated by using 10% SDS-PAGE and transferred onto polyvinylidene difluoride (PVDF) western blotting membranes (Roche Diagnostics). The membranes were incubated with primary antibodies overnight at 4°C. The primary antibodies were used as follows: anti-c-Jun (#9165, Cell Signaling; dilution 1:1000), anti-p-c-Jun (#3270, Cell Signaling; dilution 1:1000), anti-PDCD4 (#9535, Cell Signaling; dilution 1:1000), anti- phospho-Smad3 (#9520, Cell Signaling; dilution 1:1000), anti-Smad3 (#9523, Cell Signaling; dilution 1:1000), anti-Smad7 (sc-11392, Santa Cruz; dilution 1:1000) and anti-GAPDH (sc-23323, Santa Cruz; dilution 1:1000). Horseradish peroxidase anti-mouse, anti-goat and anti-rabbit (Sigma; dilutions 1:5000) were used as secondary antibodies. Quantification was performed by measuring signal intensity by using ImageJ (NIH, Bethesda, MD).

miRNA in situ hybridization
miRNA in situ hybridization (ISH) was performed by using the miRCURY LNA™ microRNA ISH Optimization Kit (Exiqon, Vedbaek, Denmark) for formalin-fixed, paraffin-embedded kidney samples, according to the protocol by the manufacturer. Briefly, sections (10 mm thick) were prepared, followed by deparaffinization in xylene and ethanol. The slides were incubated with 15 ng/ml of proteinase-K (Exiqon) for 20 min at 37°C. After washing and dehydrating, slides were hybridized with double digoxigenin-labeled, LNA™ miR-21 probe, LNA™-scrambled miRNA probe, LNA™ U6 snRNA probe or LNA miR-126 probe (positive control) (Exiqon) for 1 h at 55°C. The slides were washed with standard saline citrate buffer, and then incubated with the kit blocking solution for 15 min, followed by incubation with anti-digoxigenin reagent for 60 min, alkaline phosphatase substrate for 2 h at 30°C, and the kit KTB2 buffer twice for 5 min. The slides were mounted with mounting medium, and the results analyzed by light microscopy (Nikon Eclipse 80i).
Fig. 8. miR-21 deficiency reduces UUO-induced renal fibrosis. (A) Western blot analysis of COL1A1, FN and α-SMA protein level in WT of miR-21-null UUO kidneys. (B-D) Densitometric quantification of COL1A1 (B), FN (C) and α-SMA (D) protein results in A. (E) Representative images showing histological staining of renal cortices from kidneys of WT mice or of miR-21-null UUO7d mice. Scale bars: 50 μm. n=5 per group. *P<0.05, **P<0.01. Staining was H&E, and for PAS, COL1A1, FN and α-SMA as indicated. Error bars represent ±s.d. (F) Scheme of the miR-21/PDCD4/AP-1 autoregulatory loop during fibroblast activation. miR-21-induced PDCD4 degradation contributes to AP-1 activity which - in turn - then promotes miR-21 transcription, revealing a miR-21 autoregulatory self-promoted mechanism during myofibroblast activation.
transcribed to produce cDNA using miScript RT II buffer (Qiagen). The mix was incubated at 37°C for 60 min, followed by 95°C for 5 min. RT-PCR was performed using miScript HiSpec buffer (Qiagen) and the 7300 Sequence Detection System (Applied Biosystems). The mixtures were incubated at 95°C for 15 min, followed by 40 cycles of 94°C for 15 s, 55°C for 30 s and 70°C for 34 s. All reactions were run in triplicate. mRNA levels were normalized to glyceraldehyde-3-phosphatedehydrenase (GAPDH) and microRNA level was normalized to U6 snRNA. All miR-21 primers were purchased from Qiagen. Other primer sets purchased from GenePharma are listed in Table S1.

**Immunofluorescence staining**

Indirect immunofluorescence staining was performed as previously described (Yang and Liu, 2001). Briefly, kidney sections or cells cultured on coverslips were washed twice with cold PBS and fixed with cold methanol-acetone (1-1) for 10 min at −20°C. Following three extensive washes with PBS, slides were blocked with 0.1% Triton X-100 and 2% normal donkey serum (Gibco) in PBS buffer for 40 min at room temperature and then incubated with the specific primary antibodies as previously described (Zhou et al., 2013), followed by staining with AlexaFluor 488-conjugated secondary antibody (Sigma) for 1 h. Slides were double-stained with DAPI to visualize the nuclei. At the end of the process, slides were viewed with a Nikon Eclipse 80i microscope equipped with a digital camera (DS-R1i, Nikon). In each experimental setting, immunofluorescence images were captured with identical exposure settings.

**Luciferase reporter assay**

A luciferase reporter assay was performed, as previously described (Sun et al., 2013). To test the binding of miR-21 to its target gene Smad7, a fragment of human SMAD7 3′-untranslated region (UTR), containing a presumed miR-21 complementary site (seed sequence, 5′-AUAAGCUA-3′), was amplified by PCR using human genomic DNA as a template. The PCR products were inserted into the pMIR-REPORT plasmid (Applied Biosystems), and efficient insertion was confirmed by sequencing. To test the binding specificity, we mutated the complementary site from 5′-AUAAGCUA-3′ to 5′-UUAUCGAU-3′. For the luciferase reporter assays, 1 μg firefly luciferase reporter plasmid or β-gal vector (Applied Biosystems) were transfected into NRK-49F cells cultured in six-well plates using Lipofectamine 2000 (Invitrogen). The β-gal vector was used as a transfection control. 24 h after transfection, the cells were assayed using a luciferase assay kit (Promega, Madison, WI).

**Morphological assessment**

Kidney tissues were immersed in 4% neutral-buffered formaldehyde at 4°C for 48 h. The tissues were paraffin-embedded, processed for light microscopy, and divided into sections (3 mm thick) that were then stained with H&E for general histological analysis and periodic acid–Schiff (PAS)/Masson’s trichrome for extracellular matrix deposition. Pictures were taken with a Nikon Eclipse 80i microscope equipped with a digital camera.

**Statistical analysis**

Animals were randomly assigned to control and treatment groups. All images of western blots were representatives of at least three independent experiments. RT-qPCR assays were performed in triplicate. Data shown are the mean±s.d. of three or more independent experiments. Differences were considered statistically significant at *P<0.05 or **P<0.01 and assessed by using Student’s t-test.

**Competing interests**

The authors declare no competing or financial interests.

**Author contributions**

Conceived and designed the experiments: Q.S., K.Z., J.Y. Performed the experiments: Q.S., J.M., J.L., Q.Y., H.C., W.S., Y.Z., L.J., L.F. Software: Q.S.; Validation: Q.S., J.M., J.L., Q.Y., H.C., W.S., Y.Z., L.J., L.F. Formal analysis: Q.S.; Investigation: Q.S.; Resources: Q.S.; Data curation: Q.S., J.M., J.L., Q.Y., H.C., W.S., Y.Z., L.J., L.F. Writing - original draft: Q.S.; Writing - review & editing: Q.S.; Visualization: Q.S.; Supervision: Q.S., C.D., K.Z., J.Y.; Project administration: Q.S., K.Z., J.Y.; Funding acquisition: Q.S., J.Y.

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**Supplementary information**

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