Smad3-Mediated Upregulation of miR-21 Promotes Renal Fibrosis

Xiang Zhong,*† Arthur C.K. Chung,* Hai-Yong Chen,‡ Xiao-Ming Meng,‡ and Hui Y. Lan*‡

*Li Ka Shing Institute of Health Sciences and Departments of †Chemical Pathology and ‡Medicine and Therapeutics, the Chinese University of Hong Kong, Hong Kong, People’s Republic of China

ABSTRACT

TGF-β/Smad signaling plays a role in fibrogenesis, but therapies targeting TGF-β are ineffective in treating renal fibrosis. Here, we explored the therapeutic potential of targeting TGF-β-induced microRNA in the progression of renal fibrosis. Microarray analysis and real-time PCR revealed upregulation of miR-21 in tubular epithelial cells (TECs) in response to TGF-β. Lack of Smad3, but not lack of Smad2, prevented cells from upregulating miR-21 in response to TGF-β. In addition, Smad3-deficient mice were protected from upregulation of miR-21 and fibrosis in the unilateral ureteral obstruction model. In contrast, conditional knockout of Smad2 enhanced miR-21 expression and renal fibrosis. Furthermore, ultrasound-microbubble-mediated gene transfer of a miR-21-knockdown plasmid halted the progression of renal fibrosis in established obstructive nephropathy. In conclusion, these data demonstrate that Smad3, but not Smad2, signaling increases expression of miR-21, which promotes renal fibrosis. Inhibition of miR-21 may be a therapeutic approach to suppress renal fibrosis.


Renal fibrosis is the common end stage of many kidney diseases and is characterized by interstitial extracellular matrix (ECM) and myofibroblast accumulation accompanied with destruction of the renal tubules. In the progression of fibrosis, TGF-β, a central mediator in the development of fibrosis, upregulates many fibrogenic genes (e.g., those of ECM proteins) positively by Smad2 or Smad3, but negatively by Smad7. However, therapies that target TGF-β are noneffective and have very limited effects on renal fibrosis.

Recent advances in the study of TGF-β biology have shown that TGF-β regulates several microRNAs (miRNAs) to control epithelial-to-mesenchymal transition (EMT) and fibrosis. miRNAs are noncoding, short (approximately 22 nucleotides long) RNAs that regulate gene expression by the translational repression or the induction of mRNA degradation. With regard to the kidney, recent reports describe miR-192 as being upregulated in diabetic and obstructive nephropathy, as well as in kidney cells, in response to TGF-β. In mesangial cells, miR-192 controls TGF-β-induced collagen expression by downregulating Smad interacting protein 1 (SIP1) expression. These findings suggest that miRNAs are candidates for mediators of TGF-β-driven fibrosis and EMT.

miR-21 is a prominent miRNA implicated in the genesis and progression of human cancers. TGF-β is characterized to upregulate miR-21 expression, and in mice, miR-21 is detected in liver, heart, kidneys, and lungs, thereby suggesting that miR-21 may be involved in TGF-β-induced fibrotic events in these organs. The first report of the functional importance of miR-21 in...
fibrosis was in heart failure. The upregulation of miR-21 is observed in the cardiac fibroblasts of failing hearts, and miR-21 antagonort treatment in a mouse model of cardiac hypertrophy prevents interstitial fibrosis and improves cardiac function.

miR-21 expression is elevated in patients with idiopathic pulmonary fibrosis and in mice with bleomycin-induced lung fibrosis. Treatment with miR-21 antisense oligonucleotides attenuates the severity of experimental lung fibrosis in mice. However, the functional role of miR-21 in renal fibrosis has not been fully investigated.

In the study presented here, we hypothesized that miR-21 regulates the progression of renal fibrosis in response to TGF-β. We used in vitro and in vivo studies to demonstrate that miR-21 was upregulated in the tubular epithelial cells (TECs) in response to TGF-β1 and the fibrotic kidneys. This upregulation depends on activation of TGF-β/Smad3 signaling. We also used ultrasound-microbubble-mediated gene transfer to deliver miR-21 knockdown plasmids into the living kidney to ameliorate renal fibrosis in a mouse model of obstructive nephropathy. More importantly, knockdown of miR-21 expression halted the progression of established renal fibrosis in the mice.

RESULTS

Activation of TGF-β/Smad Signaling Is Essential for the Upregulation of miR-21 during Renal Fibrosis In Vitro

To search for TGF-β-related miRNAs in renal injury, we utilized microarray-based analysis to determine miRNA expression profiles in a rat TEC line (NRK52E) after TGF-β1 treatment. Similar to our and others’ studies, expression levels of miR-192 were upregulated and miRNAs of miR-200 and miR-29 families were downregulated (Figure 1A). A novel finding was that the miR-21 expression in TECs was upregulated after TGF-β1 treatment. This result provides the first in vitro evidence that miR-21 expression may play a role in renal fibrosis.

To confirm this relationship, we determined whether TGF-β1 upregulated the miR-21 expression in NRK52E cells. Real-time PCR demonstrated that TGF-β1, but not the control, induced miR-21 expression in a time- and dosage-dependent manner, peaking at 24 hours with an optimal dose at 2 ng/ml (Figure 1B and C). This upregulation was blocked by a neutralizing anti-TGF-β antibody, implying that TGF-β signaling was necessary for miR-21 expression. Pretreatment with cycloheximide, a protein synthesis inhibitor, suppressed TGF-β1-induced miR-21 expression (Figure 1D). All of these results strongly suggested that TGF-β signaling played a vital role in miR-21 expression.

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The microarray results from normal and Smad3 knockdown (KD) TECs revealed that the suppression of Smad3 prevented TGF-β-induced miR-21 expression (Figure 2A). To further elucidate the specific role for Smad signaling in miR-21 expression, we examined miR-21 expression in vitro in TECs, in which Smad2 or Smad3 expression were specifically knocked down. As shown in Figure 2B, TGF-β1-induced miR-21 expression was significantly suppressed in Smad3, but it was enhanced in Smad2 KD TECs. This observation was further
Figure 2. Smad3 induces, but Smad2 inhibits, miR-21 expression during renal fibrosis. Differential mediation of Smad2 and Smad3 on miR-21 expression during renal fibrosis. (A) miRNA expression profile in NRK52E cells treated with TGF-β1 for 24 hours compared with control determined by Agilent miRNA array. Data are expressed as the fold increase against normal kidneys. (B) Real-time PCR analysis demonstrates that KD of Smad3 in TECs blocks TGF-β1-induced miR-21 expression whereas Smad2 KD promotes miR-21 expression.
confirmed in Smad2 or Smad3 knockout (KO) mouse embryonic fibroblasts (MEFs). TGF-β1-induced miR-21 mRNA expression was abolished in MEFs lacking Smad3, but it was aggravated in Smad2 KO MEF cells (Figure 2C).

Three potential Smad binding sites (SBSs) were located in the miR21 promoter (Figure 2D). Chromatin immunoprecipitation (ChIP) assays demonstrated that Smad3 physically interacted with two sites—SBS1 and SBS2—in the absence and presence of TGF-β treatment (Figure 2E). However, phospho-Smad3 appeared to physically interact with SBS1 and SBS2 after TGF-β treatment (Figure 2F and Supplemental Figure 1). In contrast, phospho-Smad2 did not interact with SBS1 or SBS2. In addition, we also examined whether Smad4 interacted with the miR-21 promoter. As expected, Smad4 also physically interacted with SBS1 and SBS2 after TGF-β treatment (Figure 2F, Supplemental Figure 1), suggesting that phospho-Smad3 binding may be the critical step in the regulation of miR-21 expression.

Because Smad proteins have been demonstrated to promote the biogenesis of miR-21, we therefore examined the accumulation of primary miR-21 (pri-miR-21) and precursor miR-21 (pre-miR-21) after TGF-β treatment. At 3 hours after TGF-β treatment, an induction of pri- and pre-miR-21 was observed (Figure 2, G and H). Expression levels of pri- and pre-miR-21 were reduced at 12 hours and rose again at 24 hours after TGF-β treatment. Interestingly, Smad2 and Smad3 differentially regulated the post-transcriptional modification of miR-21. Induction of pri- and pre-miR-21 was enhanced in Smad2 KD TECs, but it was inhibited in Smad3 KD cells (Figure 2, G and H).

The role of TGF-β/Smad in regulating miR-21 expression was further determined in vivo by an examination of the mouse kidneys of a unilateral ureteral obstruction (UUO) induced in Smad3 WT and KO mice or conditional Smad2 KO mice. As described in several previous reports, including our own studies, 11,25,26 mice null for Smad3 were protected against renal tubulointerstitial fibrosis, but the deletion of Smad2 enhanced tubulointerstitial fibrosis after UUO. For example, the expression of TGF-β1, the key mediator of fibrosis, was significantly reduced in the fibrotic kidneys of Smad3 KO mice, but it was promoted in conditional Smad2 KO mice (Figure 2, I and J). Consistent with the results from the in vitro studies, miR-21 expression was also downregulated in the fibrotic kidneys of Smad3 KO mice, but it was further enhanced in conditional Smad2 KO mice (Figure 2, I and J), demonstrating that miR-21 is positively regulated by Smad3 but negatively regulated by Smad2.

miR-21 is an Important Mediator of TGF-β-Induced Renal Fibrosis In Vitro

We next determined the role of miR-21 in renal fibrosis in vitro by the transient transfection of an miR-21 mimic or anti-miR-21 into TECs. As shown in Figure 3A, mRNA expression levels of collagen I, fibronectin, and α-smooth muscle actin (α-SMA) induced by TGF-β1 were significantly enhanced by miR-21 mimic treatment but were inhibited by anti-miR-21 treatment. However, we detected adverse effects on TECs, including cell death (data not shown), when miR-21 was knocked down by anti-miR or the stable expression of miR-21 small hairpin RNA (shRNA). This phenomenon has been reported for cancer cells in which downregulation of miR-21 expression prevents proliferation and promotes apoptosis.27-29 To bypass this undesirable side effect, we instead used NRK52E cell lines stably expressing inducible vectors for miR-21 overexpression or miR-21 shRNA in which transgene expression was regulated in the presence of doxycycline (Dox). We empirically determined 0.5 μg/ml as the optimal dose of Dox for miR-21 overexpression and KD (Figure 3, Bi and Ci). Dox induction caused a 2-fold overexpression of miR-21 whereas the KD efficiency of miR-21 expression almost reached 50% (Figure 3, Bi and Ci).

miR-21 overexpression further aggravated the TGF-β1-induced expression of collagen I, fibronectin, and α-SMA in NRK52E cells (Figure 3D), but miR-21 KD significantly attenuated the TGF-β1 effects (Figure 3E). These results provide strong evidence that miR-21 plays a role in TGF-β1-mediated renal fibrosis and it is a compelling rationale for a therapeutic approach targeting miR-21 to block the progression of renal fibrosis by delivering a KD construct into fibrotic kidneys.

Ultrasound-Microbubble-Mediated Gene Transfer to the Kidney

Before investigating the functional role of miR-21 during renal fibrosis in a mouse model, we delivered miR-21 KD and Tet
Targeting miR-21 Has Therapeutic Effects on Renal Fibrosis in Mice

We then investigated whether KD of miR-21 prevented renal fibrosis in a UUO model by delivering miR-21 KD and Tet-repressor-expressing plasmids into kidneys, followed by ultrasound transducer treatment directly onto the left kidney and the ligation of the left ureter (Figure 5A). Renal expression of TGF-β1, the key mediator of renal fibrosis, was induced at day 7 after UUO (Figure 5B). However, the delivery of miR-21 KD plasmids into the ligated kidneys was able to downregulate renal TGF-β1 expression, suggesting that renal fibrosis in ligated kidneys may be suppressed (Figure 5B). At day 7 after...
UUO, miR-21 expression was significantly increased (Figure 5, C and D). Results from in situ hybridization demonstrated that miR-21 expression was mainly located in the tubulointerstitial area where fibrosis occurred (Figure 5D). This induction was attenuated by approximately 20% in the presence of miR-21 shRNA treatment (Figure 5, C and D). No effect was observed when control plasmids were used or no ultrasound treatment was applied (Figure 5, B and C). Compared with normal mice, the ligated kidney demonstrated typical histologic features of hydropnephrosis, fibrosis, and inflammatory cell infiltration, which were reduced in mice treated with miR-21 shRNA (Figure 5E). We then examined the expression of fibrotic markers in the ligated kidney. There was a minimal accumulation of collagen I, fibronectin protein, and α-SMA-positive cells in the normal kidney (Figure 5, E and F). At day 7 after UUO, an abundant accumulation of collagen I and fibronectin and α-SMA-positive cells was observed in the ligated kidneys, and this accumulation was significantly reduced after treatment with miR-21 shRNA (Figure 5, E and F).

Real-time PCR and Western blot analyses showed a significant increase in the expression levels of collagen I, fibronectin, and α-SMA in mice at day 7 after UUO compared with normal kidneys (Figure 6A through 6C). At the same time, treatment with miR-21 shRNA resulted in a reduction of the expression levels of collagen I, fibronectin, and α-SMA compared with the untreated diseased mice (Figure 6A through 6C). These findings, which were consistent with our immunohistochemical findings, suggest that the reduction of miR-21 levels is effective to attenuate renal fibrosis by decreasing ECM synthesis and deposition.

We next examined the therapeutic effect of miR-21 shRNA on established renal fibrosis by delivering miR-21 shRNA plasmids directly into mice at day 4 after UUO (Figure 7A). Similar to the prevention study, upregulation of TGF-β1 expression was already observed at day 4 after UUO (Figure 7B). Treatment with miR-21 shRNA plasmids resulted in a reduction of TGF-β1 expression in the ligated kidneys, suggesting that knocking down miR-21 expression might ameliorate renal fibrosis during kidney diseases (Figure 7B). miR-21 expression was further increased at day 10 after UUO. However, treatment of miR-21 shRNA significantly reduced the expression levels of miR-21 at day 10 to levels similar to those found at day 4 after UUO (Figure 7B). No effect was observed when control plasmids were used or when no ultrasound treatment was applied (Figure 7, B and C).

Compared with mice at day 4 after UUO, the ligated kidney at day 10 after UUO demonstrated more severe features of hydropnephrosis, fibrosis, and inflammatory cell infiltration (Figure 7D). However, these pathologies were ameliorated in the mice treated with miR-21 shRNA. At day 10 after UUO, the mice also developed severe tubulointerstitial fibrosis, as was demonstrated by an abundant accumulation of collagen I and fibronectin with numerous α-SMA-positive cells, when compared with mice at day 4 after UUO (Figure 7, D and E). In contrast, miR-21 shRNA treatment reduced this accumulation.

Consistent with the results from the immunohistochemical findings, real-time PCR and Western blot analyses also demonstrated that compared with the ligated kidney at day 4 after UUO, there was a 10% to 20% increase in mRNA and protein expression levels of collagen I, fibronectin, and α-SMA (Figure 7E).
expression of fibrotic markers at day 10 after UUO (Figure 8A through 8C), whereas treatment with miR-21 shRNA reduced the mRNA and protein levels of these markers to the levels obtained at day 4 after UUO (Figure 8A through 8C). These findings suggest that the reduction of miR-21 levels halted the progression of renal fibrosis, as evidenced by a reduction of ECM synthesis and the deposition and number of α-SMA-positive cells.

DISCUSSION

TGF-β upregulates many of the genes involved in fibrosis and it also regulates EMT; thus, a blockade of TGF-β signaling in the experimental models prevents the progression of kidney diseases.6–8,34–36 Because TGF-β1 is an essential gene for many processes (e.g., the TGF-β1 KO mice die of massive inflammation),37 the identification and targeting the TGF-β/Smad-specific miRNAs related to fibrosis may be a better approach for combating kidney diseases. A novel and potentially clinically important finding from this study is that the miR-21 expression was significantly induced in TECs by TGF-β1. This upregulation of miR-21 was mediated by Smad3 but prevented by Smad2 via altering the biogenesis of miR-21. More importantly, our study demonstrated for the first time that miR-21 mediated Smad3-dependent renal fibrosis, and that suppression of miR-21 by shRNA blocked the progression of renal fibrosis. Targeting miR-21 may be a better alternative to directly suppress TGF-β-mediated fibrosis in kidney diseases.

Our recent findings that Smad3 mediates, but Smad2 inhibits, renal and cardiovascular fibrosis in vivo and in vitro under diabetic and hypertensive conditions21,23,26 suggest that Smad2 and Smad3, downstream effectors of TGF-β signaling, may play reciprocal roles in regulating TGF-β target genes. It has been reported that TGF-β1 is able to induce miR-21 expression.9,13,14 However, it is still unknown how Smad2 and Smad3 participate in this regulation. We have recently demonstrated that Smad3, but not Smad2, regulates miR-192 expression through interacting with the promoter of miR-192.11 However, the regulation of miR-21 expression by TGF-β/Smad3 signaling appears to use another mechanism; this is because Smad3 constitutively interacts with the miR-21 promoter (Figure 2). Treatment of TGF-β1 induced the interaction between phospho-Smad3 and the miR-21 promoter, suggesting that the TGF-β/Smad3 signaling is essential for miR-21 transcription. Indeed, a new protein synthesis is required for TGF-β1 to induce tubular miR-21 expression (Figure 1). The accumulation of pre-miR-21 shortly after TGF-β treatment is consistent with a report by Davis et al. that Smad proteins positively control the conversion of miR-21 from pre-miR-21.13,14 Most impor-

Figure 5. Delivery of miR-21 shRNA plasmids attenuates renal fibrosis in mice. (A) Schematic diagram of the experimental approach. (B) Real-time PCR results show that TGF-β1 expression in ligated kidneys increases at day 7 after UUO but reduces after gene transfer of miR-21 shRNA plasmids. (C) Real-time PCR results show that miR-21 expression in ligated kidneys increases at day 7 after UUO but reduces after gene transfer of miR-21 shRNA plasmids. (D) In situ hybridization shows that miR-21 is upregulated in the UUO kidneys and its expression is remarkably reduced in the UUO kidneys with ultrasound-mediated miR-21 gene therapy. (E) Histology and immunohistochemistry. (F) Quantitative analysis of immunohistochemical staining. Results from histology and immunohistochemistry demonstrate that renal fibrosis is ameliorated in mice treated with shRNA plasmids (KD plasmid) as evidenced by a reduction of the expression of collagen I, fibronectin, and α-SMA in day-7 UUO kidneys. Each bar represents the mean ± SEM for at least eight mice. ***P < 0.001 compared with normal mice; ##P < 0.01 as indicated. Ctl plasmid, control plasmid; G, glomerulus. Magnification: ×200.
tantly, this is the first demonstration that Smad2 and Smad3 play differential roles in regulating the biogenesis of miRNAs; this is because Smad3 induces, but Smad2 prevents, the expression of pre-miR-21 after TGF-β treatment (Figure 2). However, our results differ from those of Davis et al., who did not detect significant change in pri-miR-21 expression. This difference may be attributable to different cell types (TECs versus vascular smooth muscle cells) because TGF-β stimulation can lead to an increase of pri-miR-21 levels in isolated TECs. Taken together, the differential regulation of miR-21 biogenesis by Smad2 and Smad3 may account for our recent findings that Smad3 plays a pathologic role whereas Smad2 plays a protective role in fibrosis during renal and cardiovascular diseases.

Figure 6. Delivery of miR-21 shRNA plasmids reduces the expression of fibrotic markers in ligated kidneys. (A) Real-time PCR, (B) representative Western blots, and (C) quantitative analysis of Western blots. Expression of collagen I, fibronectin, and α-SMA in ligated kidneys increases at day 7 after UUO but reduces after gene transfer of miR-21 shRNA plasmids (KD plasmid). Each bar represents the mean ± SEM for at least eight mice. **P < 0.01, ***P < 0.001 compared with normal mice; ###P < 0.001 as indicated. Ctl plasmid, control plasmid.
In the study presented here, we demonstrate that miR-21 participates in renal fibrosis because miR-21 positively regulates the expression of ECM and α-SMA in TECs and fibrotic kidneys. However, the action of miR-21 on fibrosis relies on the activation of TGF-β signaling, as evidenced that without TGF-β1 treatment, the alternation of miR-21 expression has no effects on the expression of fibrotic markers (Figure 3). In this respect, our findings differ from those relating to human pulmonary fibroblasts. In pulmonary fibroblasts, the addition of an miR-21 mimic induces fibronectin and α-SMA expression levels when the fibroblasts are not treated with TGF-β1. This discrepancy is probably attributable to the difference in cell types. Overexpression of miR-21 may be able to initiate fibrogenesis in fibroblasts whereas TGF-β-induced EMT may be required for TECs to respond to miR-21 action in fibrogenesis. These findings imply that miR-21 alone is not sufficient to initiate fibrotic events in the TECs and the function of miR-21 in fibrosis may be to promote the fibrotic activity of TGF-β1.

It remains unclear how miR-21 affects fibrosis. Studies within hearts and lungs demonstrate that miR-21 is expressed more in cardiac fibroblasts than in cardiomyocytes during cardiac fibrosis or in myofibroblasts during lung fibrosis. In this study, miR-21 was shown to be mainly expressed by TECs and fibroblasts/myofibroblasts where fibrosis occurred. Thus, in addition to miR-21 expression by fibroblasts or myofibroblasts as seen in cardiac and lung fibrosis, TECs were also a major cell type expressing miR-21 during renal fibrosis.

Studies in cardiac fibrosis have demonstrated that Sprouty (SPRY) and phosphatase and tensin homolog (PTEN) are targets of miR-21. SPRY is a potent inhibitor of Ras/MEK/extracellular regulated kinase. The suppression of miR-21 reduces interstitial fibrosis by reducing extracellular-regulated kinase-mitogen activated protein kinases because SPRY is no longer suppressed by miR-21. On the other hand, miR-21 also suppresses PTEN expression during cardiac remodeling, and inhibition of PTEN activity attenuates TGF-β-induced fibrosis in lung fibroblasts. During renal fibrosis, miR-21 may possibly activate the expression of fibrogenic genes by targeting SPRY or PTEN. Results of this study add new information that miR-21 expression is required for TGF-β-induced fibrosis during renal diseases.

Similarly, miR-192 also mediates TGF-β/Smad3-driven renal fibrosis. However, its role in kidney diseases is still controversial. Different studies show that treatment of TGF-β1 in mesangial cells and TECs can increase or reduce miR-192 expression levels. Opposite expression patterns of miR-192 are also found in mouse models of diabetic nephropathy. Further studies should be done to clarify its role in renal fibrosis.
renal diseases. However, the role of miR-21 in fibrosis is clearer. Combining the results from this study, miR-21 plays a pathologic role in cardiac, lung, and renal fibrosis. The suppression of miR-21 is to successfully reduce fibrosis in rodent models of heart, lung, and kidney diseases, suggesting that targeting miR-21 should possess a therapeutic potential to ameliorate the fibrosis.

Another important finding in this study was that suppression of renal miR-21 expression into fibrotic kidneys successfully blocked the progression of renal fibrosis. In both of the prevention and intervention studies, treatment with miR-21 shRNA was able to attenuate the enhancement of renal miR-21 expression and the expression of fibrotic markers in diseased kidneys. Our results supported the previous findings that miR-21 plays an important role in fibrosis in the heart and the lungs. Targeting miR-21 should provide significant therapeutic effects on fibrosis in various diseases. However, the major obstacle to the use of miR-21 as a therapeutic agent for fibrotic diseases is that the downregulation of miR-21 expression induces cell death. A recent study in renal ischemia reperfusion injury and our study (data not shown) have demonstrated similar results. Thus, the critical issue for targeting at miR-21 in renal diseases is to successfully deliver miR-21 KD plasmid into the kidney without generating any adverse effects. To retain most of the therapeutic effects without triggering unacceptable apoptotic effects, we used a Dox-regulated miR-21 shRNA plasmid with a weak H1 promoter to minimize the extent of cell death. Although the KD efficiency of miR-21 was weaker (20% to 30%) in the in vitro and in vivo studies, treatment with miR-21 shRNA still had a significant therapeutic effect on renal fibrosis, thereby reducing the expression levels of the fibrotic markers in diseased kidneys and treating the TECs significantly. Most importantly, the administration of these Dox-regulated plasmids had only minor adverse effects on the TECs. For gene delivery, the intraperitoneal injection of miR-21 antisense oligonucleotides to suppress renal fibrosis is discouraged because there is no control on the effects of miR-21 KD throughout the whole body. At the same time, intratracheal installation or delivery through a jugular vein catheter can be used only to treat fibrotic diseases in the lungs and heart, respectively. We therefore used an ultrasound-microbubble-mediated gene transfer to deliver miR-21 shRNA plasmids to ligated kidneys because it has been shown to be a safe, effective, and controllable gene therapy in renal fibrosis in animal models. In the study presented here, we were able to reduce renal miR-21 expression and fibrosis in diseased kidneys by delivering miR-21 KD plasmids by using this ultrasound-based miRNA therapy system, thus demonstrating that the specific targeting of the renal miR-21 expression is an effective therapeutic tool for combating renal fibrosis.

In conclusion, TGF-β1 positively regulates miR-21 expression by Smad3 and negatively regulates it by Smad2 via miRNA biogenesis. The findings of this study support the notion that miR-21 acts as a downstream mediator of TGF-β/Smad3-driven renal fibrosis. Delivery of the Dox-inducible miR-21 shRNA plasmid by using the ultrasound-based miRNA therapy system may be a useful alternative thera-

![Figure 8. Delivery of miR-21 shRNA plasmids reduces the expression of fibrotic markers in ligated kidneys. (A) Real-time PCR, (B) representative Western blots, and (C) quantitative analysis of Western blots. Expression of collagen I, fibronectin and α-SMA in ligated kidneys increases at day 10 after UUO but reduces after gene transfer of miR-21 shRNA plasmid (KD plasmid). Each bar represents the mean ± SEM for at least eight mice. *P < 0.05, **P < 0.01, ***P < 0.001 compared with normal mice; #P < 0.05, ##P < 0.01, ###P < 0.001 as indicated; †P < 0.05, ††P < 0.01, †††P < 0.001 as indicated. Ctl plasmid, control plasmid.](image-url)
peutic treatment to suppress the fibrotic events in different renal diseases.

CONCISE METHODS

Cell Culture
The normal rat TEC line, NRK52E, (ATCC), characterized MEFs from Smad3 wild-type (WT) or KO embryos, and Smad2 WT or KO embryos were maintained in DMEM/LG containing 0.5% fetal bovine serum with human TGF-β1 (R&D Systems, Minneapolis, MN) at concentrations of 0, 0.25, 0.5, 1.0, 2.0, and 4.0 ng/ml in the presence or absence of a neutralizing TGF-β antibody (10 μg/ml; R&D Systems) for periods of 0, 3, 6, 12, and 24 hours for miR-21 detection. NRK52E cell lines with stable expression of Smad2 and Smad3 shRNA were generated and characterized as described previously. Pretreatment with 20 μM cycloheximide (Sigma, St Louis, MO) was applied 30 minutes before TGF-β1 stimulation.

Transient Transfection with miRNAs
NRK52E cells were transfected with 30 nM of miR-21 mimic, anti-miR-21 (Ambion, Austin, TX), or negative control 1 precursor miRNAs (Ambion) in six-well plates using siPort Neo-FX (Ambion) according to the manufacturer’s instruction. The cells were then stimulated with human TGF-β1 (R&D Systems) at 2 ng/ml for 6, 12, and 24 hours in serum-free medium following our protocols.

Construction of Inducible Cell Lines of miR-21 Overexpression and KD
The Dox-regulated miR-21-expressing plasmid was generated using Tet-on system plasmids. A rat miR-21 cDNA was PCR amplified by the Tet-on system plasmids. A rat miR-21 cDNA was PCR amplified by forward primer 5'-GAC GAT CCA ACC AGA GAC GTG TTT GCT CTT-3' and reverse primer 5'-CTT AGT CGA GAC GAC TAC CCC AACTTC C-3' (underlined indicates BamHI and Sall sites, respectively). The PCR-amplified product was then cloned into a plTRE2-hygro vector (Clontech) to obtain pTRE2-miR21. G. Vario (Cerylid, Melbourne, Australia) generously provided a Tet-on plasmid, pEFpuro-Tet-on. For KD plasmids, gene-specific insert sequences for miR-21 (sense, CTG ATG TTG ACT CTG GAA TCT; antisense, AGA TTA CGA AGT CAA CAT CAG) were synthesized and subcloned into pSuperior-puro vector (OligoEngine, Seattle, WA) to generate a pSuperior-miR-21 shRNA construct in accordance with the manufacturer’s instructions. The vector synthesizes shRNA transcripts that target the stem loop region of pri-miRNA. A small interfering RNA without a predicted target site in the rodent genome was inserted into pSuperior-puro as a negative control. All plasmids were generated by using the EndoFree Plasmid kit (Qiagen, Valencia, CA) in accordance with the manufacturer’s instruction. pSuperior-miR-21 and pcDNA6/TR (Invitrogen, Carlsbad, VA) or pTRE2-miR-21 and pEFpuro-Tet-on were cotransfected into NRK52E cells using Lipofectamine 2000 (Invitrogen). Dox was added at an optimal concentration (0.5 μg/ml) for 48 hours, and the cells were then stimulated with human TGF-β1 (R&D Systems) at 2 ng/ml for 6, 12, and 24 hours in serum-free medium in accordance with our own published procedures.

RNA Extraction, RT-PCR, and Quantitative RT-PCR Analysis
Trizol reagent (Invitrogen) was used to isolate total RNA from the cultured cells and kidney tissues in accordance with the manufacturer’s instructions. Template cDNA was prepared using reverse transcriptase, and miR-21 expression was quantified by real-time PCR by the Taqman miRNA assay (Applied Biosystems, Foster City, CA) with small nuclear RNA U6 as an endogenous control for normalization in accordance with manufacturer’s instructions (see http://jcb.rupress.org/cgi/content/full/139/2/541/F1). Real-time RT-PCR analysis of the fibrotic markers was performed as described previously. Primers for primary-miR-21 are 5'-CCC TGT TCA TTT TGT TTT GCT-3' and 5'-TGG TAC AGC CAT GCA GAT GT-3', and primers for pre-miR-21 are 5'-TGT CGG GTA GCT TAT CAG AC-3' and 5'-TGT CAG ACA GCC CAT CGA CT-3'. Ratios for mRNA/glycer-aldehyde 3-phosphate dehydrogenase (GAPDH) miRNA were calculated by using the ΔΔCt method for each sample and expressed as the mean ± SEM. For detecting the expression of the puromycin-resistant gene, we used RT-PCR by using primers (TCA CCG AGC TGC AAG AAC TCT and CCC ACA CCT TGC CGA TGT). The PCR was performed in a total volume of 20 μl containing 20 mmol/L Tris-HCl, 50 mmol/L KCl, 1.25 mmol/L MgCl2, 0.2 mmol/L dNTP, 0.5 mmol/L of each primer, 1 U of TaqDNA polymerase, and 0.5 μl cDNA. The mRNA expression of GAPDH, a housekeeping gene, was considered to be an internal reference. Cycle parameters were as follows: 95°C for 3 minutes, 25 cycles (98°C for 30 seconds, 60°C for 40 seconds, and 72°C for 60 seconds), and 72°C for 5 minutes. The ordinary PCR products were separated on a 2% agarose gel.

miRNA Expression Analyses
Total RNA was extracted using the miReasy miRNA isolation kit (Qiagen). The quality of RNA samples was first assessed by Bioanalyzer (Agilent Technologies, Palo Alto, CA) and then labeled for microarray. A rat miRNA array (G4473A, Agilent Technologies) was used to identify the upregulated miRNAs in TECs in response to TGF-β1 stimulation. The microarray data were analyzed using GeneSpring GX 11 (Agilent Technologies). A differential miRNA expression was considered statistically significant when values in the treated cells were more than double those of the controls in two replicate experiments. ANOVA was used to compare the average values of the miRNA probes in all samples and it yielded significant P values (<0.05) in all cases.

ChIP Analysis
ChIP was performed as described previously11 with primary antibodies against phospho-Smad2, phospho-Smad3 (Cell Signaling, Danvers, MA), and Smad4 (Santa Cruz Biotechnology, Santa Cruz, CA). Precipitated DNAs were detected by PCR using specific primers: SBS1: 5'-TGA GAA GAA GTC CCA CAT TTA TCA CC-3' and 5'-AGG GAG GCC AGT TTT TTT TT-3', SBS2: 5'-CTT CCA TCT TCT TCT TGA CGT TCC-3' and 5'-CCC ATC CCC CTG AGA AGA C-3', SBS3: 5'-CGT CTT CTC AGG GGG ATG-3' and 5'-GCC TCC CCA ATG TGC TAA T-3'.
Obstructive Kidney Disease Model

A UUO kidney disease model was induced in eight Smad3 WT/KO mice, Smad2 floxed mice, and conditional Smad2 KO mice by left ureteral ligation in accordance with a previously described procedure. The experimental procedures were approved by the Chinese University of Hong Kong’s Animal Experimental Ethics Committee.

Ultrasound-Mediated Gene Transfer of Inducible miR-21 shRNA Plasmids into the Ligated Kidneys

The mixture of miR-21 KD plasmid and pDNA6/TR was combined with Sonovue (Bracco, Milan, Italy) at a ratio of 1:1 (vol/vol) as described previously. The mixed solution (400 µl) was then injected via the tail vein of eight C57Bl/L6 mice (20 to 22 g body weight, 10 weeks old). Immediately after injection, the ultrasound transducer (Therasonic, Electro-Medical Supplies, Wantage, United Kingdom) was directly applied onto the left kidney with the ultrasound media with a continuous wave output of 1 MHz at 1 W power output for a total of 5 minutes. The same procedure was also applied to the eight control animals that received the mixed solution containing the same amount of empty control plasmids (pSuperior/pCDNA6). To induce transgene expression, a dose of Dox (200 µg/ml in 200 µl volume) was injected into the peritoneal cavity immediately after the ultrasound-mediated gene transfer and followed by additional Dox in drinking water (200 µg/ml) until sacrifice. A group of eight age-matched normal rats was used as a normal control. For the prevention study, a UUO model was induced immediately after the KD plasmid transfer in ten mice. The mice were sacrificed on day 7 after UUO, and their kidney tissues were collected for analysis. For the intervention study, groups of eight UUO mice on day 4 received ultrasound-microbubble-mediated miR-21 KD plasmid transfer. All mice were euthanized on day 10 after UUO for analysis and their kidney tissues were collected for analysis. The Chinese University of Hong Kong’s Animal Experimental Ethics Committee approved the experimental procedures.

Western Blot Analysis

Western blot analysis was performed as described previously with primary antibodies against collagen I (Southern Tech, Birmingham, AL), fibronectin (Dako, Carpinteria, CA), α-SMA (Sigma, St. Louis, MO), and GAPDH (Chemicon, Temecula, CA).

Histology and Immunohistochemistry

Changes in renal morphology were examined in methyl Carnoy-fixed, paraffin-embedded tissue sections (4 µm) stained with hematoxylin and eosin or periodic acid–Schiff. Immunostaining was performed in paraffin sections using a microwave-based antigen retrieval technique. The antibodies used in this study included collagen I (Southern Tech), fibronectin (Dako), α-SMA (Sigma), and KIM-1 (Abcam, Cambridge, MA). An isotype-matched rabbit IgG (Sigma) was used as negative controls throughout the study. All slides were counterstained with hematoxylin for the nuclei.

In Situ Hybridization

Specific 5’ digoxigenin (DIG)-labeled antisense-locked nucleic acid oligonucleotides for Mus musculus-miR-21 (5’-TCA ACA TCA GTC TGA TAA GCT A-3’) and a scramble probe (5’-GTG TAA CAC GTC TAT ACG CCC A-3’) as a negative control were purchased from Exiqon (Vedbaek, Denmark). The detailed procedure for in situ hybridization was done in accordance with the manufacturer’s instruction. In brief, 5-µm slides were prepared from formalin-fixed, paraffin-embedded kidney tissues. After deparaffinization and deproteinization (10 µg/ml) for 8 minutes, the slides were prehybridized with 1X hybridization buffer without probe. The hybridization was performed overnight in a 1X hybridization buffer (30 to 70 µl) with DIG-antisense miR-21 probe at 45°C. After washing, the slides were blocked and incubated with alkaline phosphatase-conjugated anti-DIG Fab fragments (1:1500; Roche) and visualized for color detection.

Statistical Analysis

Each experiment was repeated at least 3 times throughout the study. Data were expressed as the mean ± SEM and analyzed using one-way ANOVA with the Newman–Keuls comparison program from GraphPad Prism 5.0 (GraphPad Software, San Diego, CA).

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DISCLOSURES

None.

REFERENCES


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