miR-212 promotes renal interstitial fibrosis by inhibiting hypoxia-inducible factor 1-α inhibitor

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Received November 4, 2019; Accepted August 18, 2020

DOI: 10.3892/mmr.2021.11828

Abstract. Renal interstitial fibrosis is one of the common causes, and a major pathological basis for the development of various types of chronic progressive renal to end-stage renal diseases. Therefore, it is important to clarify the underlying mechanisms of disease progression in order to develop effective strategies for the treatment and prevention of these pathologies. The aim of the present study was to investigate the association between microRNA (miR)-212 expression and the development of renal interstitial fibrosis, as well as analyzing the role of miR-212 in the disease. The expression of miR-212 was significantly increased in the peripheral blood of patients with renal interstitial fibrosis and in the kidney tissues of unilateral ureteral obstruction (UUO) mice. Angiotensin (Ang) II, TGF-β1 and hypoxia were found to increase the expression of miR-212 and α smooth muscle actin (α-SMA) in NRK49F cells. Ang II stimulation induced the expression of miR-212 and α-SMA in NRK49F cells, while transfection of miR-212 mimics further upregulated the expression of α-SMA. miR-212 was also revealed to target hypoxia-inducible factor 1α inhibitor (HIF1AN) and to upregulate the expression of hypoxia-inducible factor 1α, α-SMA, connective tissue growth factor, collagen α-1(I) chain and collagen α-1(III) chain, whereas HIF1AN overexpression reversed the regulatory effects of miR-212. In UUO mice, miR-212 overexpression promoted the progression of renal interstitial fibrosis, whereas inhibiting miR-212 resulted in the opposite effect. These results indicated that high expression of miR-212 was closely associated with the occurrence of renal interstitial fibrosis, and that miR-212 may promote its development by targeting HIF1AN.

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Key words: renal interstitial fibrosis, microRNA-212, inhibiting hypoxia-inducible factor 1-α inhibitor

Introduction

With an aging global population and changes in lifestyle choice, chronic kidney disease (CKD) has become a major threat to public health worldwide (1,2). Renal interstitial fibrosis is a common pathological outcome of CKD (3,4), which is associated with the destruction and disappearance of the renal tissue structure, including the glomeruli, tubules and interstitium, accompanied by excessive accumulation of extracellular matrix (ECM) (5,6). Although kidney damage caused by early-stage renal interstitial fibrosis is reversible, fibrosis during the scar-formation period is irreversible (7). Therefore, it is of great significance to explore the roles of relevant biological indicators that prevent and delay the progression of fibrosis, and to formulate therapeutic strategies and prognostic risk assessment measures for patients with renal interstitial fibrosis.

Hypoxia affects the function of kidney cells (8,9). Acute hypoxia can lead to tubular damage, causing changes in kidney function and subsequent apoptosis (10). In addition, chronic hypoxia also stimulates tubular cells, as well as the fibrosis of interstitial fibroblasts and renal microvascular endothelial cells (11). Hypoxia-inducible factor 1α (HIF-1α) is a key factor in the regulation of intracellular oxygen metabolism. Under normal conditions, HIF-1α is expressed at a low level in all tissues, whereas under hypoxic conditions, HIF-1α expression is a correlated with the increase in duration and degree of hypoxia (12,13). HIF-1α is also closely associated with tissue and organ fibrosis. A previous study revealed that surgical hepatic artery ligation in rats induces liver hypoxia and upregulates HIF-1α, causing fatty lesions and aggravating liver fibrosis (14). Also, renal hypoxia can directly induce the upregulation of HIF-1α expression and promote renal interstitial fibrosis; conversely, inhibiting HIF-1α can slow the progression of renal interstitial fibrosis (15).

In recent years, microRNAs (miRNAs/miRs) have been reported to play an important role in the field of gene expression regulation. A large number of studies have demonstrated that miRNAs are involved in a number of cellular processes, including differentiation, proliferation, apoptosis, metabolism, hematopoiesis, cardiogenesis, body morphogenesis and insulin secretion (16,17). Moreover, miRNAs also play an important role in the feedback loop of signal transduction pathways (18). Numerous studies have revealed that miRNAs may be closely

associated with kidney physiology, tumors and transplantation, as well as renal interstitial fibrosis (19,20), suggesting that these molecules serve key roles in kidney pathophysiology.

Although the relationship between various miRNAs and renal interstitial fibrosis has been reported (21,22), the role of miR-212 in renal fibrosis remains unclear. The aim of the present study was to investigate the association between miR-212 expression and renal interstitial fibrosis, as well as its effect on the expression of pro-fibrotic factors. The present study also sought to analyze the role of miR-212 in renal interstitial fibrosis by targeting hypoxia-inducible factor 1-α inhibitor (HIF1AN), so as to provide a novel research direction for the diagnosis and treatment of renal fibrosis.

Materials and methods

Peripheral blood collection. A total of 73 patients (31 males, 42 females; age range, 32-64, mean age, 47.18±11.63 years) with renal interstitial fibrosis who underwent treatment at the Second Affiliated Hospital of Fujian Medical University (Quanzhou, China) were included in the present study, along with an additional 30 healthy volunteers (17 males, 13 females, age range, 30-64, mean age 45.74±10.92 years). The recruitment criteria for renal interstitial fibrosis were as follows: i) Evidence from renal pathology in accordance with World Health Organization criteria (23). The primary diagnostic features were intimal thickening, medial hypertrophy, reduplication of the internal elastic lamina and glomerular sclerosis; ii) participants were between 18 and 60 years of age; and iii) individuals had no other glomerular diseases or systemic disorders, such as glomerulonephritis, hypertension nephropathy or diabetic nephropathy, gout or urinary stones. The healthy control samples were taken from individuals with normal blood pressure and renal function. All subjects donated ~3 ml venous peripheral blood during early morning fasting. The blood samples were placed in anticoagulation tubes containing EDTA and immediately mixed. The present study was approved by the Ethics Committee of the Second Affiliated Hospital of Fujian Medical University (Quanzhou, China) and written informed consent was provided by patients or family members of patients and the volunteers. All surgical procedures adhered to the ethical norms of clinical experiments.

Cell culture. NRK49F cells (Shanghai Cell Bank of the Chinese Academy of Sciences) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100 μg/ml penicillin and 100 μg/ml streptomycin (all Thermo Fisher Scientific, Inc.) and maintained at 37˚C (5% CO_2) in a humidified incubator.

Transfection. The NRK49F cells were seeded into a 6-well plate at a density of ~5x10^5 cells/well. Cells were sub-cultured to 60% confluence, and then transfected with 40 nM miR-negative control (NC), miR-212 mimics, NC-inhibitor or miR-212 inhibitor, HIF1AN small interfering (si)RNA, HIF1AN overexpression plasmid, or co-transfected with miR-212 mimics and HIF1AN overexpression plasmid, or the miR-212 inhibitor and HIF1AN siRNA (all from Santa Cruz Biotechnology, Inc.). Transfection was performed using Lipofectamine® 2000 (Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. After transfection, cells were cultured for 24 h in serum-free low-glucose medium (Gibco; Thermo Fisher Scientific, Inc.), and then cultured in DMEM containing Angiotensin (Ang) II (1x10^{-5} mol/l) for 72 h. The sequences used were: miR-212 mimics: 5'-ACCUUG-GCUCUAGAC UGCUUACUtt-3', miR-212 inhibitor: 5'-AGUAAGCAGUCU AGAGCCAAGGUtt-3', NC control: 5'-CUACGGCCAUUG ACUUGUC-UACUtt-3'.

Reverse transcription-quantitative (RT-q)PCR. Total RNA was extracted using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocols. cDNA was synthesized using the RevertAid™ First Strand cDNA Synthesis kit (Thermo Fisher Scientific, Inc.), qPCR was performed using the cDNA by SYBR RT-PCR kit (Takara Bio, Inc.) according to the manufacturer's protocol. and reaction conditions were 95˚C for 30 sec, 40 cycles of 95˚C for 5 sec and 60˚C for 30 sec. Relative expression was calculated using the $2^{-\Delta\Delta Cq}$ method (24). miR-212 expression was assessed relative to that of the internal reference, U6; HIF1AN, α smooth muscle actin (α-SMA), connective tissue growth factor (CTGF), collagen α-1(I) chain (COL1A1) and collagen α-1(III) chain (COL3A1) expression was quantified using GAPDH as the internal reference. The sequences of the qPCR primers were as follows: miR-212 forward, 5'-GCC TCCTGACTCCAGGTCC-3' and reverse, 5'-GCGCAAAGT GACTGGATGAA-3'; U6 forward, 5'-CTCGCTTCGGCA GCACATATACT-3' and reverse, 5'-ACGCTTCACGAATTT GCGTGTC-3'; HIF1AN forward, 5'-TTCCCGACTAGGCCC ATTC-3' and reverse, 5'-CAGGTATTCAAGGTCCCATTT CA-3'; α-SMA forward, 5'-CATCACGAACTGGGATGACAT G-3' and reverse, 5'-CATCTTCTCCCTGTTGGCTTTAG-3'; CTGF forward, 5'-TCCTTTCTGAGCAATTCACCAAG-3' and reverse, 5'-GCACACTCCGTCTTTTTCCTC-3'; COL1A1 forward, 5'-GAGGGCCAAGACGAAGACATC-3' and reverse, 5'-CAGATCACGTCATCGCACAAC-3'; COL3A1 forward, 5'-GGAGCTGGCTACTTCTCGC-3' and reverse, 5'-GGGAACATCCTCCTTCAACAG-3'; and GAPDH forward, 5'-TGTGGGCATCAATGGATTTGG-3' and reverse, 5'-ACACCATGTATTCCGGGTCAAT-3'.

Western blotting and immunohistochemistry (IHC) analysis. Total protein was extracted using RIPA lysis buffer and the protein concentration was determined using a bicinchoninic acid protein assay (both Thermo Fisher Scientific, Inc.). The proteins (50 μg/well) were separated via SDS-PAGE on 12% gels, and then subsequently transferred to polyvinylidene fluoride membranes. The membranes were blocked with 5% non-fat dry milk powder for 1 h at 37˚C, and then incubated overnight at 4˚C with the following primary antibodies (all from Abcam): Anti-HIF1AN (1:500; cat. no. ab237544), anti-HIF-1α (1:500; cat. no. ab216842), anti-α-SMA (1:500; cat. no. ab5694), anti-CTGF (1:500; cat. no. ab6992), anti-COL1A1 (1:500; cat. no. ab34710), anti-COL3A1 (1:500; cat. no. ab7778) and anti-GAPDH (1:1,000; cat. no. ab9485). A horseradish peroxidase-labeled goat anti-rabbit secondary antibody (1:1,000; cat. no. ab150077; Abcam) was then added and the membranes were incubated at room temperature for a 1 h. The protein bands were visualized with an ECL Substrate

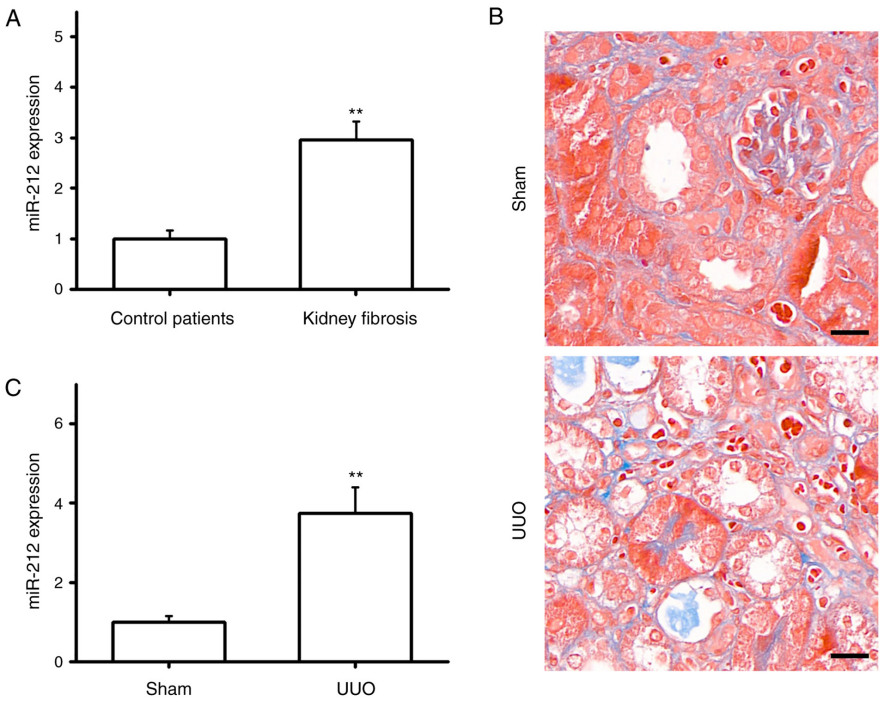

Figure 1. Association between miR-212 expression and renal interstitial fibrosis. (A) Expression of miR-212 in the peripheral blood of healthy subjects and patients with renal interstitial fibrosis was detected by RT-qPCR. (B) Masson's staining of kidneys in the Sham and UUO mice (scale bar, 20 μm). (C) Expression of miR-212 in the renal tissues of mice was detected using RT-qPCR. **P<0.01 vs. Sham. miR, microRNA; RT-qPCR, reverse transcription-quantitative PCR; UUO, unilateral ureteral obstruction.

kit (Abcam) and analyzed using ImageJ (version 1.45; National Institutes of Health).

HIF1AN detection was also assessed by IHC. In brief, the frozen sections (-20˚C) (5-μm thick) were exposed to fresh 3% hydrogen peroxide for 20 min, and then washed with PBS. The sections were incubated for 60 min at 37˚C in 5% normal blocking serum (Sigma-Aldrich; Merck KGaA), and then incubated with anti-HIF1AN (1:500; cat. no. ab237544; Abcam) overnight at 4˚C. The slides were then incubated with a secondary antibody (1:1,000; cat. no. ab150077; Abcam) for 60 min at room temperature, and with 3,3'-diaminobenzidine as a substrate. Images were captured using a light microscope (5 fields of one specimen were randomly selected; magnification, x500; Nikon Corporation).

Dual-luciferase reporter assay. TargetScan (http://www.targetscan.org) was used to predict the presence of complementary binding sites between miR-212 and the 3' untranslated region (UTR) of HIF1AN. A wild-type (WT) 3'-UTR luciferase reporter plasmid (pMIR-HIF1AN-wt) and mutant (Mut) reporter plasmid (pMIR-HIF1AN-Mut) were subsequently constructed. miR-NC, miR-212 mimics, NC-inhibitor or miR-212 inhibitor, and pMIR-HIF1AN-wt or pMIR-HIF1AN-Mut were co-transfected into NRK49F cells (6x10^4 cells/well) at 37˚C using Lipofectamine® 3000 (Invitrogen; Thermo Fisher Scientific, Inc.) for 48 h. The reporter plasmids were obtained from Promega Corporation. After transfection for 24 h, lysis buffer was added and the cells were agitated at room temperature for 15 min. The lysates were collected and transferred to an Eppendorf tube containing 100 μl LAR substrate and 20 μl passive lysis buffer. Luciferase activity was measured using a Dual-luciferase assay kit (Promega Corporation). The fluorescence intensity of each sample was calculated as the ratio of firefly to *Renilla* luciferase fluorescence.

Unilateral ureteral obstruction (UUO) mouse model. A total of 24 male C57 BL/6J mice (age, 6-8 weeks; weight, 18-22 g) were housed in a temperature-controlled environment (18-22˚C), with 50±5% humidity under a 12 h light/dark cycle and were provided with free access to food and water. The mice were randomly assigned to the Sham, UUO, UUO+miR-212 agomir and UUO+miR-212 antagomir groups (n=6 each). Mice in the UUO group were anesthetized by intraperitoneal injection of pentobarbital (50 mg/kg), and abdominal wall skin, muscle and peritoneal tissues were incised at the middle of the abdomen. Blunt forceps were used to separate the periureteral tissues and to expose the left ureter; the ureter was then ligated with 4-0 thread, and the peritoneum, muscle layer and skin were sutured layer by layer. The left ureters of the Sham group were separated without ligation, and the remaining methods were conducted in the same manner as those of the UUO group. Mice in the UUO+miR-212 agomir and UUO+miR-212 antagomir groups received an intravenous injection of 40 mg/kg miR-212 agomir or miR-212 antagomir, respectively, every 3 days after modeling; 14 days post-surgery, the mice were all sacrificed by dislocation of cervical vertebra, and the left kidneys were immediately removed.

Masson staining. The kidney tissues were fixed in 4% paraformaldehyde solution for 6-8 h at room temperature, fully automatically dehydrated, paraffin-embedded and sectioned at 5 μm. Masson lichun red acidic compound solution was then applied for 10 min, before rinsing with running water and

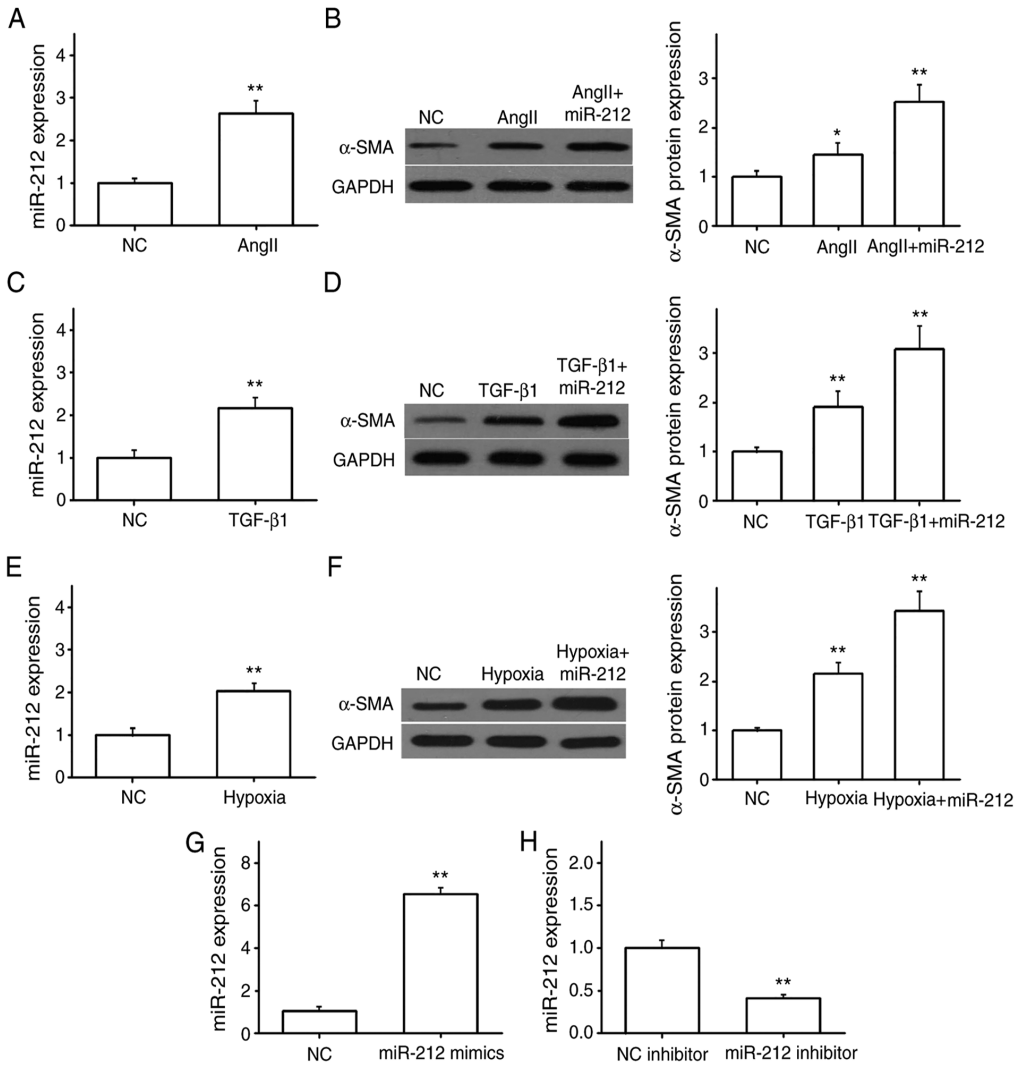

Figure 2. miR-212 induces the expression of α-SMA. (A) RT-qPCR was used to detect the expression of miR-212 in NRK49F cells after treatment with Ang II. (B) Western blotting was used to detect the expression levels of α-SMA in each group after using Ang II to stimulate NRK49F cells transfected with miR-212 mimic. (C) RT-qPCR was used to detect the expression of miR-212 in NRK49F cells after treatment with TGF-β1. (D) Western blotting was used to detect the expression levels of α-SMA in each group after using TGF-β1 to stimulate NRK49F cells transfected with miR-212 mimic. (E) RT-qPCR was used to detect the expression of miR-212 in NRK49F cells under hypoxic conditions. (F) Western blotting was used to detect expression levels of α-SMA in each group after NRK49F cells were transfected with miR-212 mimic under hypoxic conditions. (G) miR-212 mimic and (H) miR-212 inhibitor were successfully transfected into NRK49F cells. *P<0.05 and **P<0.01 vs. NC. NC, negative control; miR, microRNA; α-SMA, α smooth muscle actin; Ang II, Angiotensin II.

shaking until dry. Then, the samples were differentiated for 5 min with 1% phosphomolybdate aqueous solution. After the phosphomolybdate was removed, the samples were directly stained with aniline blue for 5 min and rinsed with water. Then, 95% alcohol, anhydrous alcohol and transparent xylene were added in sequence, and neutral gum was used to seal each specimen. All images were captured using a light microscope (magnification, x500; Nikon Corporation) and analyzed using ImageJ (version 1.45; National Institutes of Health).

Sirius red staining. The paraffin-embedded kidney sections were dewaxed with xylene, rehydrated in a descending alcohol series and stained with Sirius red staining solution for 10 min at room temperature. The sections were lightly rinsed and then stained with Mayer's hematoxylin staining solution for 8 min at room temperature, rinsed again with running water for 10 min, and then sealed with neutral gum. All images were captured using a light microscope (magnification, x500; Nikon Corporation) and analyzed using ImageJ (version 1.45; National Institutes of Health).

Statistical analysis. The data are presented as the mean ± standard deviation. The Student's t-test was used to compare the mean values between two groups. One-way analysis of variance and Bonferroni's post hoc test were used to evaluate the differences among groups. P<0.05 was considered to indicate a statistically significant difference.

Results

Association between the expression of miR-212 and renal interstitial fibrosis. The expression of miR-212 was detected in the peripheral blood of healthy subjects and patients with renal interstitial fibrosis using RT-qPCR. The results showed that compared with the healthy subjects, miR-212 expression was significantly increased in patients with renal interstitial

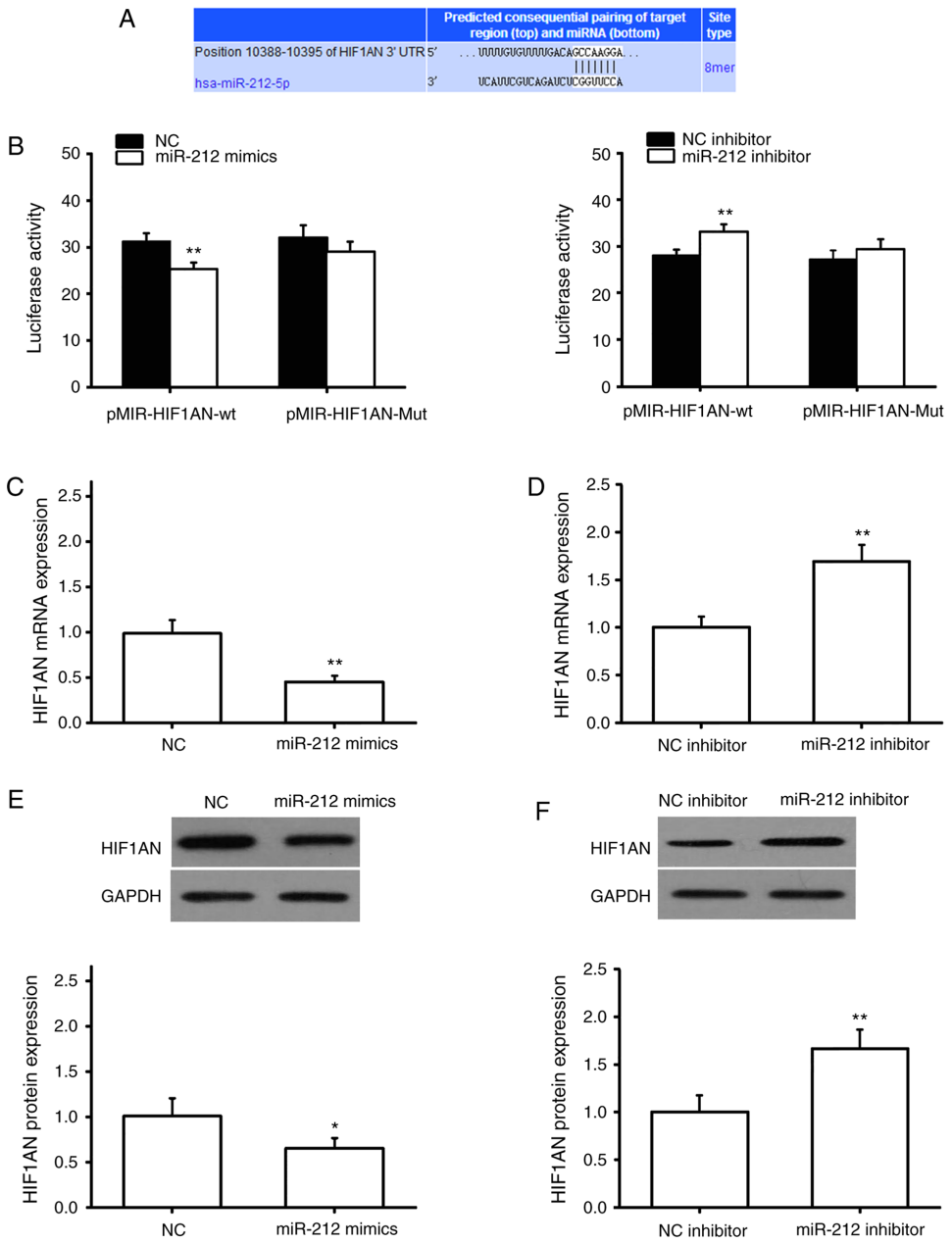

Figure 3. miR-212 negatively regulates the HIF1AN gene. (A) TargetScan was used to predict HIF1AN as a potential target gene for miR-212. (B) Luciferase activity was detected using Dual-luciferase reporter gene assays. (C and D) Expression of HIF1AN mRNA was detected by reverse transcription-quantitative PCR. (E and F) Protein expression of HIF1AN was detected by western blotting. *P<0.05 and **P<0.01 vs. NC. NC, negative control; miR, microRNA; HIF1AN, hypoxia-inducible factor 1-α inhibitor; wt, wild-type; Mut, mutant.

fibrosis (Fig. 1A). Following construction of a UUO mouse model, Masson's staining was performed on the resulting kidney tissue sections. The results revealed that the glomeruli and renal tubules were intact in the Sham group, and the renal tubular epithelial cells were regularly arranged. However, the UUO mice exhibited obvious atrophic tubular dilatation, disordered tubule arrangement and a large amount of densely interwoven blue collagen fibers deposited in the renal interstitium (Fig. 1B), indicating that the UUO mouse model had been successfully constructed. The RT-qPCR results indicated that the expression of miR-212 in the kidneys of the UUO mice was significantly higher than in those of the Sham group (Fig. 1C). These results indicated a potential association between aberrant miR-212 expression and renal interstitial fibrosis.

miR-212 induces the expression of α-SMA. RT-qPCR was used to determine the expression levels of miR-212 in NRK49F cells following Ang II stimulation for 72 h. The results showed that the expression of miR-212 was significantly increased in Ang II-treated NRK49F cells compared with untreated cells (Fig. 2A). miR-212 mimics or inhibitor were transfected into NRK49F cells to up- or downregulate miR-212, and compared with that of the control group, miR-212 expression was significantly increased in cells transfected with miR-212 mimics (Fig. 2G), but significantly decreased following transfection with miR-212 inhibitors (Fig. 2H). These findings confirmed that transfection was successful. Cells transfected with miR-NC or miR-212 mimics were then stimulated with Ang II. Compared with the untreated controls, the western blotting

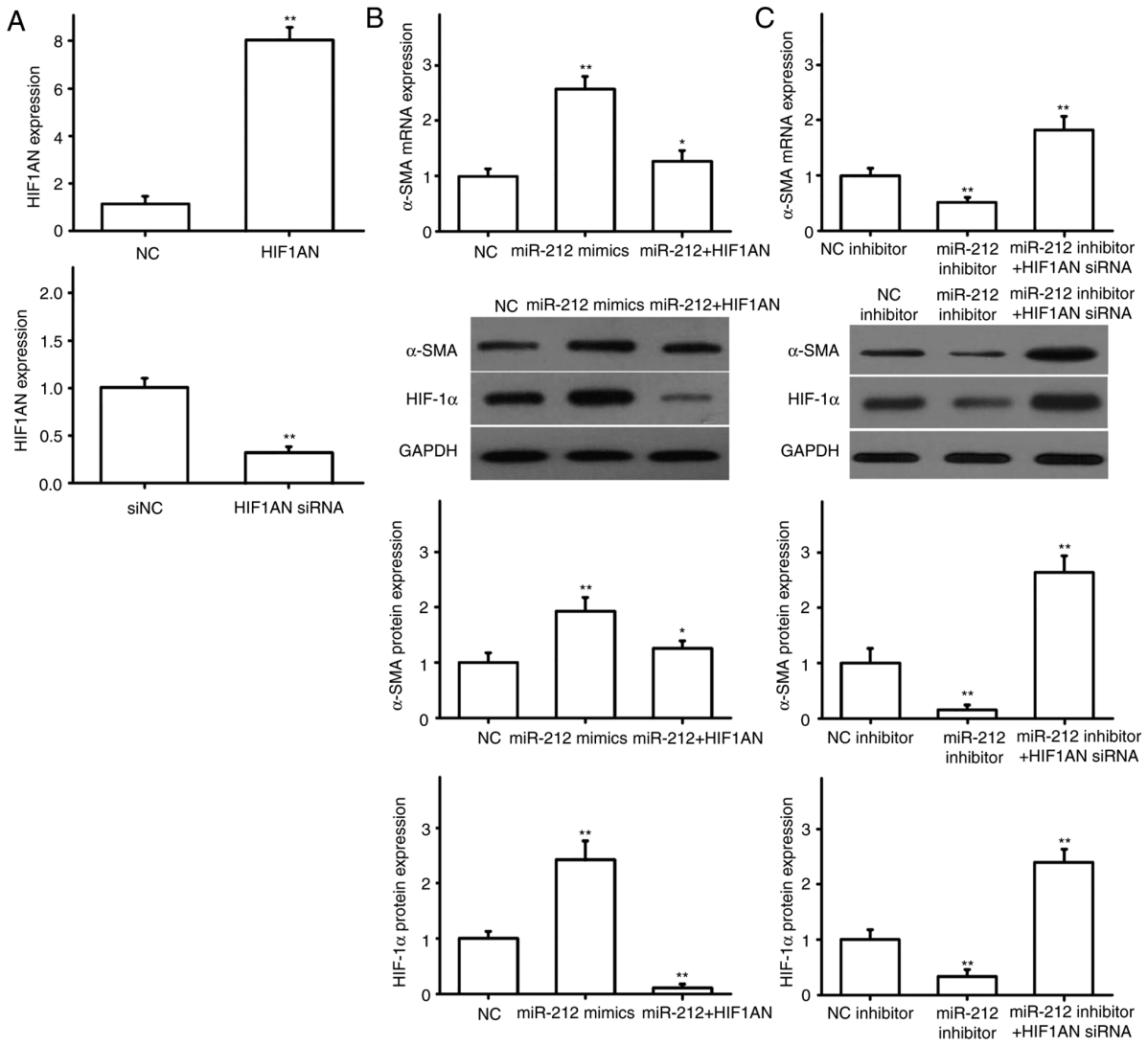

Figure 4. Regulation of pro-fibrotic factors by miR-212. (A) HIF1AN siRNA and HIF1AN overexpression plasmid were successfully transfected into cells. (B and C) Expression of α-SMA and HIF-1α. *P<0.05 and **P<0.01 vs. NC. NC, negative control; miR, microRNA; α-SMA, α smooth muscle actin; HIF-1α, hypoxia-inducible factor 1-α; siRNA, small interfering RNA; HIF1AN, hypoxia-inducible factor 1-α inhibitor.

results revealed that Ang II induced the expression of α-SMA, which was further upregulated by miR-212 overexpression (Fig. 2B). On the other hand, NRK49F cells stimulated with TGF-β1 for 72 h, or cultured under hypoxic conditions, also expressed significantly increased levels of miR-212 and α-SMA, and overexpression of miR-212 further upregulated α-SMA protein expression (Fig. 2C-F). These results suggested that following NRK49F cell activation, miR-212 expression is significantly increased, which subsequently promotes the expression of α-SMA.

miR-212 targets and negatively regulates HIF1AN. TargetScan was used to predict potential target genes of miR-212, and the results showed that the 3'-UTR region of HIF1AN contains a complementary binding site for miR-212 (Fig. 3A). NRK49F cells were subsequently co-transfected with pMIR-HIF1AN-wt or pMIR-HIF1AN-Mut, together with miR-NC, miR-212 mimics, an NC-inhibitor or miR-212 inhibitor. The results of the Dual-luciferase reporter assay showed that miR-212 mimics significantly inhibited the luciferase activity of the pMIR-HIF1AN-wt plasmid, whereas transfection with an miR-212 inhibitor exerted the opposite result; however, neither had a significant effect on the luciferase activity of the pMIR-HIF1AN-Mut plasmid (Fig. 3B). The effects of miR-212 on the expression of HIF1AN were detected by RT-qPCR and western blotting. The results suggested that the expression of HIF1AN mRNA and protein was significantly decreased in NRK49F cells following miR-212 upregulation (Fig. 3C and E). By contrast, HIF1AN mRNA and protein expression were significantly increased following miR-212 inhibition (Fig. 3D and F). These results indicated that miR-212 may negatively regulate the expression of the target gene, HIF1AN.

miR-212 regulates the expression of pro-fibrotic factors. NRK49F cells were transfected with miR-NC or miR-212 mimics, HIF1AN siRNA, HIF1AN overexpression plasmid, and co-transfected with miR-212 mimics and HIF1AN. The transfection efficiency for HIF1AN siRNA and the overexpression plasmid were detected by RT-qPCR. The results showed

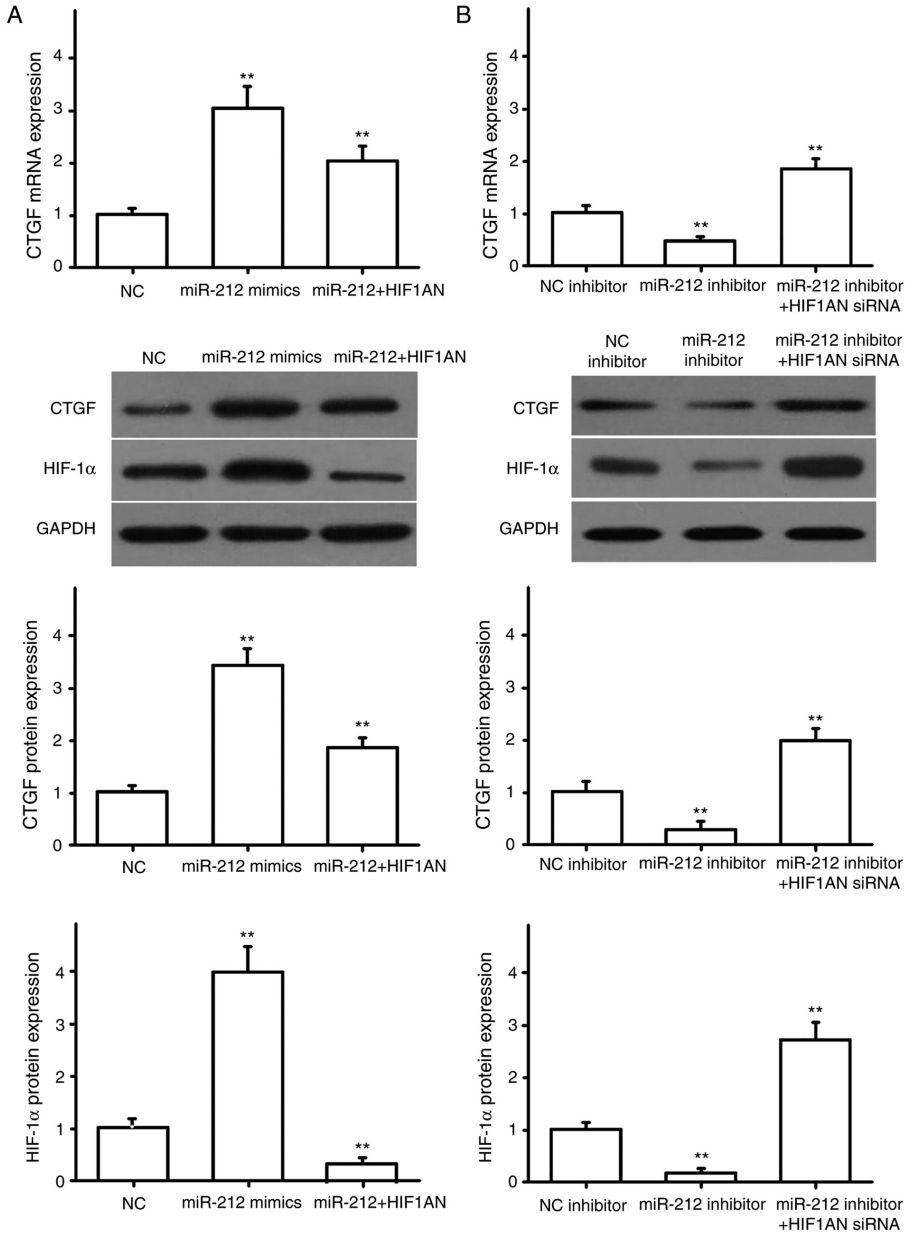

Figure 5. Regulation of pro-fibrotic factors by miR-212. (A and B) Expression of CTGF and HIF-1α. **P<0.01 vs. NC. NC, negative control; miR, microRNA; CTGF, connective tissue growth factor; HIF-1α, hypoxia-inducible factor 1-α; siRNA, small interfering RNA; HIF1AN, hypoxia-inducible factor 1-α inhibitor.

the HIF1AN siRNA and HIF1AN overexpression plasmid were successfully transfected (Fig. 4A). The expression of α-SMA, CTGF, COL1A1 and COL3A1 were then detected by RT-qPCR and western blotting. The results indicated that miR-212 overexpression promoted the expression of α-SMA (Fig. 4B), CTGF (Fig. 5A), COL1A1 (Fig. 6A) and COL3A1 (Fig. 7A), and that HIF1AN upregulation reversed these effects (Figs. 4-7). After downregulating the expression of miR-212 in NRK49F cells, the expression levels of pro-fibrotic factors, including α-SMA (Fig. 4C), CTGF (Fig. 5B), COL1A1 (Fig. 6B) and COL3A1 (Fig. 7B), were also significantly reduced, and inhibiting HIF1AN expression reversed this effect (Figs. 4-7). Concurrently, HIF-1α protein expression was also altered with the expression of miR-212, and HIF-1α was found to be negatively regulated by HIF1AN (Figs. 4-7). These results suggested that miR-212 may be involved in the regulation of renal interstitial fibrosis by inhibiting HIF1AN gene expression.

Role of miR-212 in renal interstitial fibrosis in UUO mice. The role of miR-212 in renal interstitial fibrosis was further studied using an *in vivo* mouse UUO model. Masson's staining revealed that compared with the UUO mice, those in the UUO+miR-212 agomir group exhibited increased tubular dilatation and fibrosis. On the contrary, mice in the UUO+miR-212 antagomir group possessed slightly dilated renal tubules and the pathological changes to the interstitium were alleviated. Sirius red staining also indicated that upregulating miR-212 expression promoted renal interstitial fibrosis in UUO mice, whereas downregulating miR-212 produced an inhibitory effect. Furthermore, the IHC results showed that the expression of HIF1AN in the kidney tissue of UUO mice was notably reduced, and that the overexpression of miR-212 could further inhibit HIF1AN expression. By contrast, inhibiting miR-212 expression upregulated HIF1AN expression (Fig. 8A). In addition, miR-212 agomirs induced the expression of COL1A1

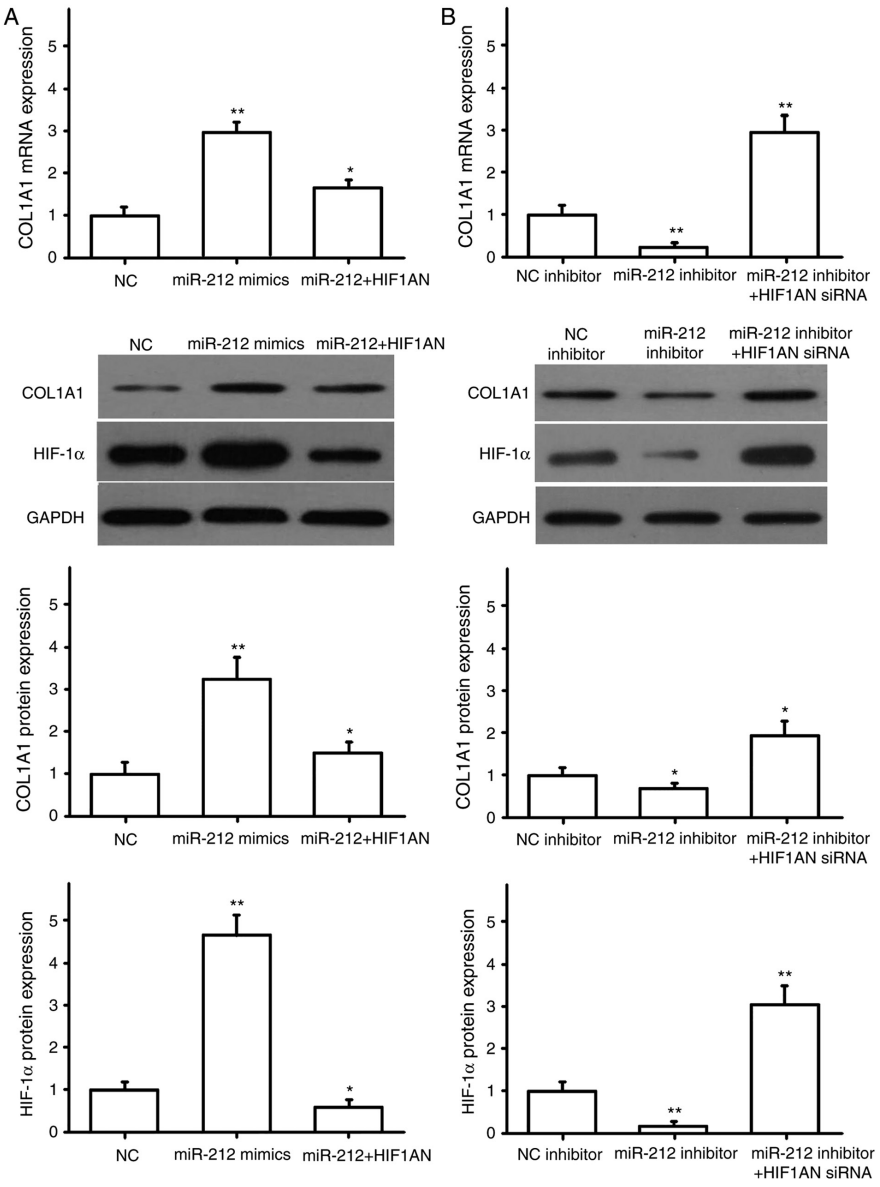

Figure 6. Regulation of pro-fibrotic factors by miR-212. (A and B) Expression of COL1A1 and HIF-1α. *P<0.05 and **P<0.01 vs. NC. NC, negative control; miR, microRNA; COL1A1, collagen α-1(I) chain; HIF-1α, hypoxia-inducible factor 1-α; siRNA, small interfering RNA; HIF1AN, hypoxia-inducible factor 1-α inhibitor.

and COL3A1 in the kidney tissues of the UUO mice, whereas miR-212 antagomirs had the opposite effect (Fig. 8B and C). RT-qPCR showed that the miR-212 agomirs and miR-212 antagomirs were successfully transfected in mice (Fig. 8D). Collectively, the results of the present study indicated that miR-212 serves a catalytic role in the progression of renal interstitial fibrosis (Fig. 9).

Discussion

The pathological progression of renal interstitial fibrosis involves multiple factors, and the primary clinical manifestations include glomerular sclerosis, tubular atrophy, abnormal composition and excessive deposition of ECM and renal interstitial fibrosis (25,26). As a result of progressively in-depth research, increasing numbers of miRNAs have been found to be associated with renal fibrosis (27-29). miRNAs are a highly conserved class of non-coding single-stranded RNAs, 19-25 nucleotides in length, that inhibit gene expression at the post-transcriptional level by associating with the 3'-UTRs of their target genes. miR-200a has been reported to inhibit the development of renal interstitial fibrosis by targeting TGF-β2 (30), and miR-29b can inhibit fibrosis by regulating the expression of collagen genes COL1A1, COL3A1 and COL4A1 in mice kidney medullary epithelial cells (31). Other studies have indicated that miR-21 is highly expressed in the renal tissues of patients with renal fibrosis (29). Although the association between specific miRNAs and renal interstitial fibrosis has already been reported (32,33), the role and influence of miR-212 in renal interstitial fibrosis are yet to be elucidated. In the present study, miR-212 was found to be significantly elevated in the peripheral blood of patients with renal interstitial fibrosis, compared with that of the healthy controls. Moreover, the expression of miR-212 in the kidney tissues of UUO mice was also significantly higher compared with in those of the sham group, indicating a potential association

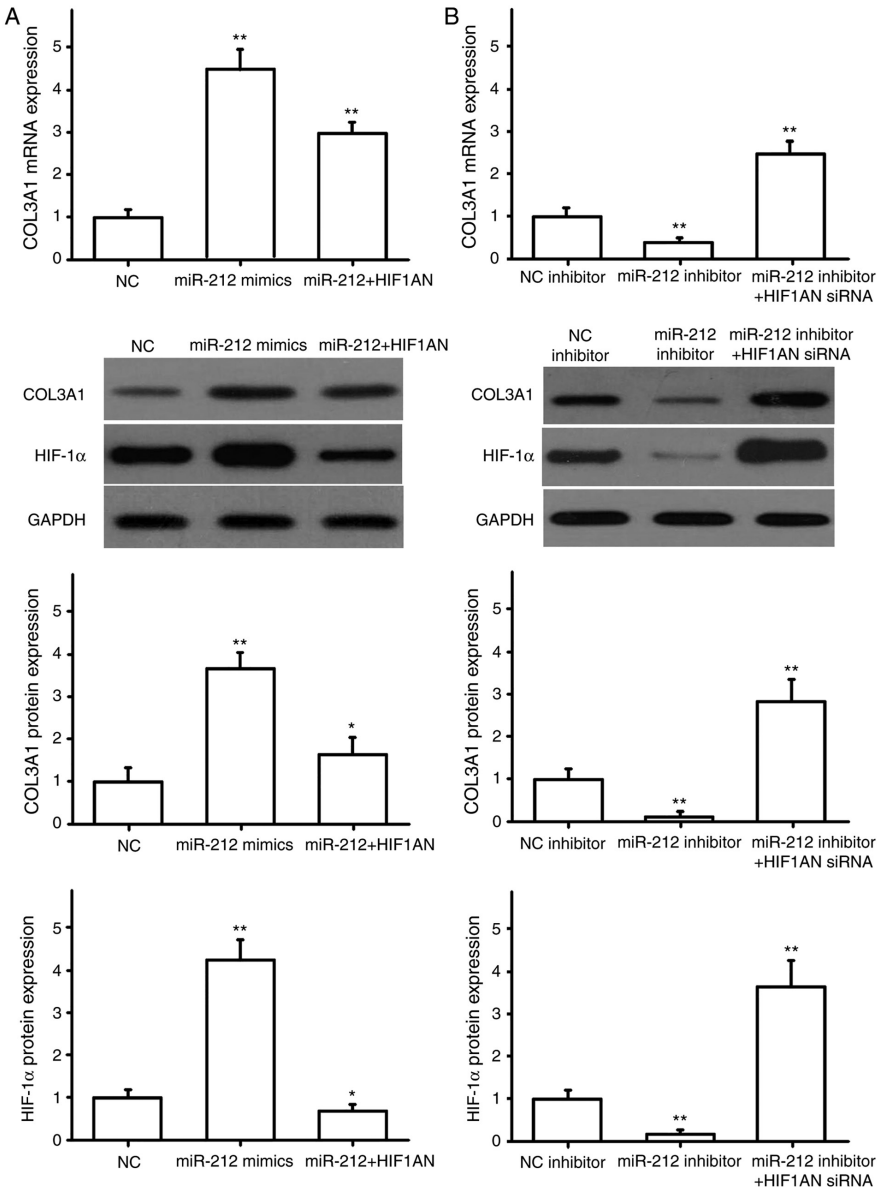

Figure 7. Regulation of pro-fibrotic factors by miR-212. (A and B) Expression of COL3A1 and HIF-1α. *P<0.05 and **P<0.01 vs. NC. NC, negative control; miR, microRNA; COL3A1, collagen α-1(III) chain; HIF-1α, hypoxia-inducible factor 1-α; siRNA, small interfering RNA; HIF1AN, hypoxia-inducible factor 1-α inhibitor.

between alterations in miR-212 expression and renal interstitial fibrosis.

Ang II promotes the proliferation of renal interstitial fibroblasts, induces myofibroblast (MFB) activation, increases ECM secretion and promotes renal interstitial fibrosis (26,34). As such, continuous intravenous infusion of Ang II can induce renal tubular atrophy, renal interstitial expression of α-SMA and ECM accumulation (35,36). MFBs are the primary synthesizers of the ECM and TGF-β in the renal interstitium, and their large numbers are closely associated with the degree of renal interstitial fibrosis (37). In the present study, the expression of miR-212 and α-SMA in NRK49F cells was found to increase significantly following stimulation with Ang II, TGF-β1 or hypoxia, and transfection with miR-212 mimics further upregulated the expression of α-SMA protein. These results suggested that the overexpression of miR-212 may promote the activation of NRK49F cells.

Hypoxia plays an important role in the occurrence and development of renal interstitial fibrosis (38-40), and increased expression of HIF-1α is a direct indicator of hypoxia (41). Numerous studies have reported that the excessive activation of HIF-1α is a risk factor for the development of kidney disease (42-44). The degree of pathological changes to the renal tissue is also directly proportional to the number of HIF-1α-positive cells, with more positive cells indicating a higher degree of damage (45). Matoba *et al* (46) found that HIF-1α is an important regulator of renal hardening in diabetic nephropathy. A previous study has also demonstrated that HIF-1α is highly expressed during kidney damage caused by poisoning, which promotes renal fibrosis (47). Higgins *et al* (48) reported that HIF-1α promoted the epithelial-to-mesenchymal transition of renal tubular epithelial cells, as well as renal fibrosis. The cause of chronic Ang II-induced kidney injury may be the excessive activation of HIF-1α, of

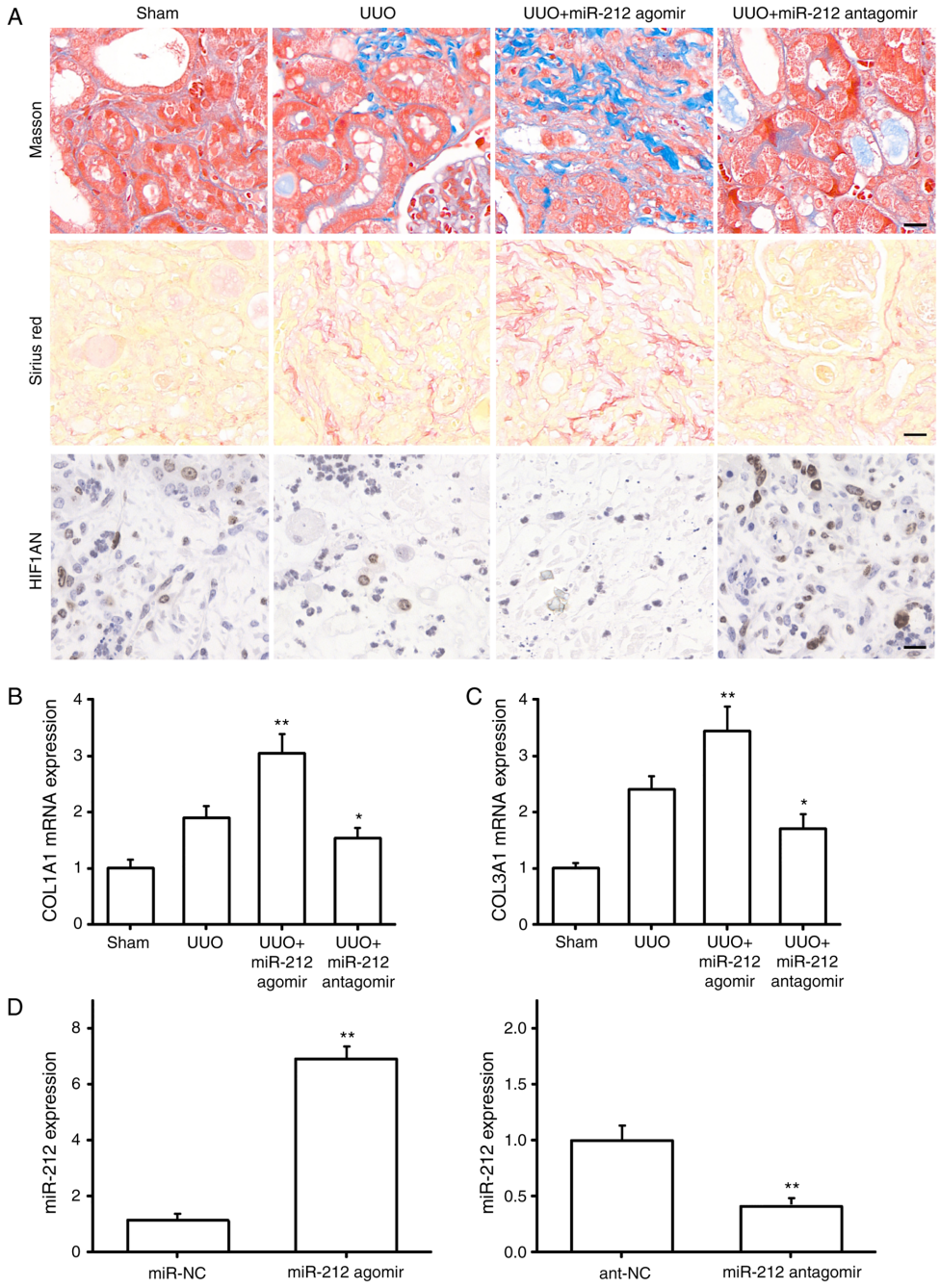

Figure 8. Role of miR-212 in renal interstitial fibrosis in UUO mice. (A) Masson's staining, Sirius red staining and immunohistochemical analysis (scale bar, 20 μm). mRNA expression of (B) COL1A1 and (C) COL3A1 in the kidney tissues of mice were detected by reverse transcription-quantitative PCR. (D) miR-212 agomirs and miR-212 antagomirs were transfected into mice. *P<0.05 and **P<0.01 vs. Sham or NC group. NC, negative control; miR, microRNA; COL1A1, collagen α-1(I) chain; COL3A1, collagen α-1(III) chain; UUO, unilateral ureteral obstruction; HIF1AN, hypoxia-inducible factor 1-α inhibitor.

which HIF1AN is an important inhibitor. In the present study, the HIF1AN gene was predicted to be a potential target of miR-212. In addition, Dual-luciferase reporter, RT-qPCR and western blotting confirmed that miR-212 directly targets and negatively regulates the 3'-UTR of HIF1AN.

Connective tissue growth factor is associated with the progression of fibrosis in the kidney, skin, lung and liver, and is expressed at the highest degree in the kidney (49-51). CTGF is one of the downstream factors of the TGF-β pro-fibrotic signaling pathway, which stimulates cellular proliferation and ECM formation and promotes renal interstitial fibrosis (52). The primary components of the ECM include collagen I and III, which are heavily involved in renal interstitial fibrosis (53,54). In the present study, the overexpression of miR-212 was found to regulate the expression of HIF-1α and pro-fibrotic factors, such as α-SMA, CTGF, COL1A1 and COL3A1, whereas the upregulation of HIF1AN reversed the promotive effects of miR-212. Furthermore, downregulating the expression of miR-212 significantly reduced that of HIF-1α and the aforementioned pro-fibrotic factors, which was reversed by inhibiting HIF1AN expression. The present study further found that miR-212 is involved in the regulation of renal interstitial fibrosis by inhibiting its target gene, HIF1AN. Therefore, the role of miR-212 in renal interstitial fibrosis was

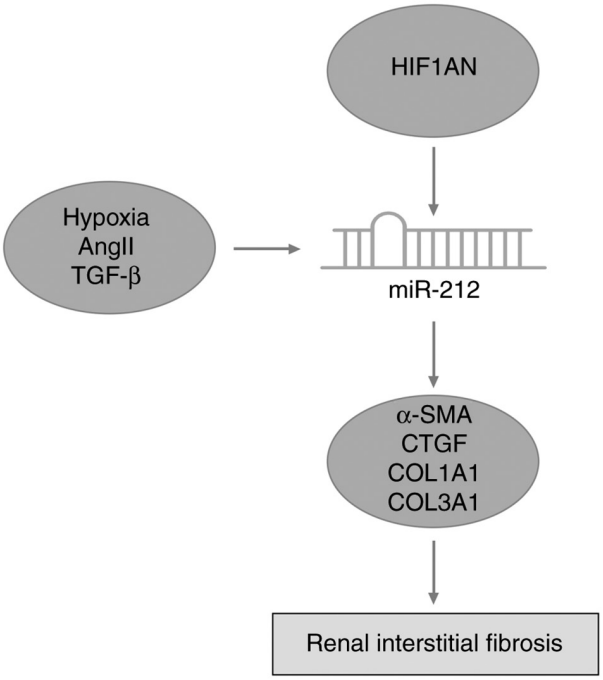

Figure 9. Schematic diagram of the proposed molecular mechanisms of miR-212-mediated renal damage. miR, microRNA; HIF1AN, hypoxia-inducible factor 1-α inhibitor; Ang II, Angiotensin II; COL1A1, collagen α-1(I) chain; COL3A1, collagen α-1(III) chain; α-SMA, α smooth muscle actin; CTGF, connective tissue growth factor.

further investigated by constructing an *in vivo* UUO model. The results showed that the degree of tubular dilatation and fibrosis were increased in UUO mice overexpressing miR-212, in which the expression of HIF1AN was notably reduced and the levels of COL1A1 and COL3A1 were significantly increased. On the contrary, low expression levels of miR-212 inhibited fibrosis and decreased the expression levels of COL1A1 and COL3A1, but upregulated the expression of HIF1AN in UUO mice. These findings were consistent with the *in vitro* findings, indicating that miR-212 plays a catalytic role in the progression of renal interstitial fibrosis.

There are some limitations to the present study. miR-212 expression was not detected in the renal biopsy tissues; investigating miR-212 in human renal tissues would be more beneficial and directly confirm the conclusions drawn herein. Also, miR-212 has other target genes, including recombinant mothers against decapentaplegic homolog 4 (55) and frizzled family receptor 5 (56), and so it was hypothesized that HIF1AN is not the only downstream target of miR-212 in the regulation of renal fibrosis. Systemic analysis of the downstream genes of miR-212 will be key to further investigations.

In conclusion, high expression levels of miR-212 were closely associated with the occurrence of renal interstitial fibrosis, and miR-212 induced the expression of fibroblast growth-promoting factors and promoted fibrosis in UUO mice. Furthermore, miR-212 was revealed to play an important role in renal interstitial fibrosis by targeting HIF1AN.

Acknowledgements

Not applicable.

Funding

The present study was supported by the Quanzhou Science and Technology Bureau (grant no. 2018Z125).

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

YZ and YTL designed the study and wrote the manuscript. YZ, GXZ and LSC performed the experiments. YTL and SHS analyzed the data and YZ drafted the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of the Second Affiliated Hospital of Fujian Medical University (approval no. 201939) and written informed consent was provided by patients or family members of patients and the volunteers. All surgical procedures adhered to the ethical norms of clinical experiments.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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