Original Paper

Ets-1 Targeted by MicroRNA-221 Regulates Angiotensin II-Induced Renal Fibroblast Activation and Fibrosis

Jia Di^{a,b} Lei Jiang^a Yang Zhou^a Hongdi Cao^a Li Fang^a Ping Wen^a Xiurong Li^b
Chunshun Dai^a Junwei Yang^a

^aCenter for Kidney Disease, 2nd Affiliated Hospital, Nanjing Medical University, Nanjing, ^bDepartments of Nephrology, 3rd Affiliated Hospital, Soochow University, Changzhou, China

Key Words

Angiotensin II • Fibroblast • Renal fibrosis • microRNA

Abstract

Background: Fibroblast activation is one of the most important mechanisms for Angiotensin II (Ang II) in promoting renal fibrosis. Transcription factor Ets-1 is recognized to play a key role in kidney diseases. However, the role and mechanisms of Ets-1 in Ang-II induced fibroblast activation and kidney fibrosis are not fully understood. **Methods:** Mice were treated with Ang II via osmotic mini-pumps or Ang II expression plasmid (pAng II). Cultured normal rat kidney interstitial fibroblast (NRK-49F) cells were incubated with Ang II. Role of Ets-1 in renal fibrosis and fibroblast activation were assessed by Western blot, Immunohistochemical staining, MTT, Boyden chamber and Immunofluorescence staining. Effects of miR-221 on Ets-1 and fibroblast activation were investigated by MTT, Boyden chamber, Western blot and Q-PCR. **Results:** We found that Ets-1 was up-regulated in fibrotic kidneys. Similarly, Ang II could activate NRK-49F cells as demonstrated by up-regulated α -SMA and fibronectin(FN) expression and enhanced cell proliferation and migration. Ang II also induced Ets-1 expression in NRK-49F cells in a dose and time dependent manner. Knock-down of Ets-1 by RNA interference attenuated Ang II-induced activation of NRK-49F cells. Ets-1 was previously reported as a target of microRNA-221 (miR-221). In Ang II-induced fibrotic kidney, miR-221 was down-regulated. Similar results were observed in Ang II treated NRK-49F cells. Ectopic expression of miR-221 mimic attenuated the up-regulation of Ets-1 by Ang II in NRK-49F cells, which further prevented the activation of NRK-49F cells. However, the inhibitor of miR-221 aggravated Ang II induced Ets-1 expression and NRK-49F cells activation. **Conclusions:** Our study suggests that miR-221/Ets-1 axis takes an important role in mediating AngII induced interstitial fibroblast activation and renal fibrosis.

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Junwei Yang

Center for Kidney Disease, 2nd Affiliated Hospital, Nanjing Medical University,
262 Zhongshan North Road, Nanjing 210003, Jiangsu Province (China)
Tel.+86-25-83345110, Cell 86-13655178822, E-Mail jwyang@njmu.edu.cn

Introduction

The prevalence of CKD is increasing worldwide [1, 2]. Renal interstitial fibrosis is an inevitable result of virtually all progressive kidney diseases. Solid evidence has pointed out that the key mediator of fibrotic tissue remodeling is the activation of ECM-producing myofibroblasts [3], which originated from local interstitial fibroblasts, pericytes, bone marrow-derived circulating fibrocytes, local mesenchymal stem cells, endothelium, and injured epithelium [4]. Among them, activation of local interstitial fibroblasts have been recognized as the majority contributor [5]. Under physiological conditions, fibroblasts stay in the kidney interstitium and maintain the homeostasis of interstitial matrix and adjacent tissue. Once activated, they acquire the myofibroblast phenotype characterized by α -SMA expression and other markers [5-7]. These activated fibroblasts may proliferate and invade the periglomerular and peritubular spaces, contributing to matrix deposition in the tubulointerstitial area. Mechanical stress, cytokines and various other factors have been identified to induce fibroblasts activation [8, 9]. However, the intracellular molecular mechanisms regulating fibroblast activation remain largely unknown.

Ang II emerges as a major profibrogenic cytokine of tubulointerstitial fibrosis. Emerging evidences have shown that AngII could upregulate many profibrotic factors such as TGF- β 1, CTGF [10], induce epithelial mesenchymal transition, activate inflammatory cells and produce extracellular matrix [11]. One of the most important mechanisms is stimulating fibroblast activation. Ets-1 is an important member of the Ets transcription factor family that participates in the regulation of a wide variety of biological processes [12-14]. Previous investigations demonstrated that Ets-1 participated in Ang II-induced ROS generation and inflammation [15, 16]. However, the role and mechanisms of Ets-1 in Ang-II induced fibroblast activation and kidney fibrosis are still not understood.

MicroRNAs (miRNAs) are a family of endogenous noncoding RNA with 20–22 nucleotides in length. They have been proved to induce degradation or repress translation of target mRNAs. It is reported that up to 30% of mammalian protein-encoding genes are the targets of miRNAs [17]. Recent investigations demonstrated that Ets-1 was a target of miR-221 in Ang II-induced endothelial inflammation and migration [18]. Large amounts of data demonstrated that miR-221 regulated various kinds of cancer [19-22]. However, the role of miR-221 in renal fibrosis remains unclear. It has been reported that miR-221 was downregulated in fibrotic kidney after unilateral ureteral occlusion (UUO) [23]. In this study, we detected miR-221 expression in Ang II-induced fibrotic kidney and Ang II treated NRK-49F cells. Consistent with miR-221 was downregulated after UUO, we found that miR-221 was decreased while Ets-1 was upregulated after AngII infusion. In cultured NRK-49F cells, AngII could also downregulate miR-221 and increase Ets-1 expression. We further demonstrated that miR-221 regulated Ets-1 expression and involved in AngII induced fibroblast activation and renal fibrosis.

Materials and Methods

Ethics statement

All of the following details of the study were approved by the responsible ethics committee of Nanjing Medical University (Permit Number: KY2012020). The written informed consent was supplied by the patients before the study.

The animal ethics approval was obtained from the Animal Care and Use Committee of Nanjing Medical University. All experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals adopted by the Institutional Animal Care and Use Committee (IACUC) to ensure the proper care and welfare of animals involved in research. All the mice were housed in single cages and the housing room was maintained at a 12 h light/dark cycle (lights on at 8.00 h). Food and water were provided ad libitum. Mice were sacrificed under anesthesia. All the animal experiments were made to minimize suffering and reduce the number of animals used.

Animal models

Male CD1 or female Balb/c mice that weighed 20–22 g were acquired from the Specific Pathogen-Free Laboratory Animal Center of Nanjing Medical University. They were housed in the animal facilities of the Kidney Disease Center of the 2nd Affiliated Hospital of Nanjing Medical University. Animals were treated humanely in accordance with NMAC guidelines and approved protocols of the Nanjing Medical University Institutional Animal Use and Care Committee. Ang II (sigma) was infused at the rate of 1 µg/kg/min by a miniosmotic pump (Alzet) for 4 weeks in male CD1 mice. The Ang II expression plasmid (pAng II) was injected intravenously at 1mg/kg of body weight using a hydrodynamics-based *in vivo* gene transfer approach in female Balb/c mice. To euthanize the mice, pentobarbital sodium (150–200 mg/kg, intraperitoneally) was used at 4- weeks after the treatment; serum and urine were collected and the kidneys were harvested.

Cell culture and treatment

The rat renal interstitial fibroblast (NRK-49F) cells were obtained from American Type Culture Collection (Manassas, VA, USA). Cells were cultured in DMEM/F12 medium supplemented with 10% fetal bovine serum (Invitrogen, San Diego, CA). For Ang II treatment, NRK-49F cells were seeded at 70% confluence in complete medium containing 10% fetal bovine serum. Twenty-four hours later, the cells were changed to serum-free medium and incubated for 16h. Thereafter, Ang II was added at different concentrations for various periods of time as indicated. The cells were then collected at different time points for further characterization.

Human renal biopsy specimens

Renal biopsy specimens were obtained from patients undergoing diagnostic evaluation at the Division of Nephrology, The Second Affiliated Hospital of Nanjing Medical University. A total of 6 subjects were selected from our database. All biopsy specimens were evaluated by a single pathologist who was unaware of the results of molecular studies. The patients (n = 3) with mild tubulointerstitial fibrosis whose serum creatinine were about 77 ± 12.4 µmol/l were chosen as a control group; The patients (n = 3) with obvious tubulointerstitial fibrosis whose serum creatinine were about 160 ± 28.5 µmol/l were chosen as a renal fibrosis group.

MTT assay

Cells were transfected with the miR-221 mimics or inhibitors and Ets-1 siRNA or negative control for 24 hours before the treatment of Ang II at different concentrations and then seeded at 1 × 10³ cells per well in 96-well plates. After a further culture of 24–72h, MTT solution (dissolved in phosphate buffer saline) was added to the wells containing cells (final concentration 0.5 mg/ml). The cells were incubated for 4 h and the medium was removed from the cells and then an aliquot was added to formazan dissolved in 0.1% dimethylsulfoxide (DMSO) 150 µl. The absorbance was measured at 490 nm using a microplate reader and the arithmetic mean OD of 10 wells for each group was calculated. The experiment was repeated at least 3 times.

Boyden chamber for motility assay

Cells were transfected with the miR-221 mimics or inhibitors and Ets-1 siRNA or negative control for 24 hours and then added onto the upper chamber of the transwell insert (8 µm, Corning-Costar Corp, Corning, New York, USA). After 24h of incubation with or without Ang II in the lower chamber at 37°C, filters were fixed with 3% paraformaldehyde in PBS and stained with 0.1% Coomassie blue in 10% methanol and 10% acetic acid, and the upper surface of the filters was carefully wiped with a cotton-tipped applicator. Cells that migrated across the Transwell filter pores were counted in 5 nonoverlapping × 10 fields and photographed with a Nikon microscope. The experiments were performed in triplicate.

Western Blot

The preparation of whole-cell lysates and kidney tissue homogenates and western blot analysis of protein expression were carried out using previous routine procedures [24]. The primary antibodies used were as follows: anti-α-smooth muscle actin (A5691, Sigma-Aldrich), anti-fibronectin (F3648, Sigma-Aldrich), anti-Ets-1 (sc-350, Santa Cruz), and anti-actin (A5385; Sigma Aldrich). Quantification was performed by measurement of the intensity of the signals with aid of National Institutes of Health Image software package.

RNA transfection

MiR-221 mimics or inhibitors and their negative control RNA (purchased from Qiagen), as well as Ets-1 small interfering (si) RNA or negative control (Integrated Biotech Solutions, Shanghai, China), were transfected into NRK-49F cells using Lipofectamine 2000 reagent (Invitrogen) following the protocols provided by the manufacturer. After transfection, cells were incubated at 37°C in a CO₂ incubator for 24h until they were ready for further treatment or assay.

Quantitative polymerase chain reaction

Total RNA was prepared using a TRIzol RNA isolation system according to the instructions by the manufacturer (Invitrogen). The first strand of cDNA was synthesized using 2 µg of RNA in 20 µl of reaction buffer using MLV-RT (Promega, Madison, WI). Quantitative polymerase chain reaction (Q-PCR) was performed using an Applied Biosystems 7300 Sequence Detection system. The expression levels of target miRNAs were normalized to U6. The CT data were determined using default threshold settings and the mean CT was determined from the duplicate PCRs. The ratio to sham-control group was calculated by using the equation $2^{-\Delta CT}$, in which $\Delta CT = CT_{\text{Sample}} - CT_{\text{control}}$. The sequences of primer pairs were as follows: Ets-1 (forward) 5'-GGGTGGGAGGAAAACAGTTT-3' and (reverse) 5'-CCTTCCATTAAAGTC-CATCTTTGTT-3'; b-Actin (forward) 5'-CAG CTG AGA GGG AAA TCG TG-3' and (reverse) 5'-CGT TGC CAA TAG TGA TGA CC-3'. The PCR products were size fractionated on a 1% agarose gel and detected by NA-green (D0133, Beyotime) staining. All the primers were acquired from QIAGEN.

Immunohistochemical and Immunofluorescence Staining

Immunohistochemical staining of kidney sections was performed according to an established protocol [24]. In brief, paraffin-embedded sections were stained with anti-Ets-1 (sc-350, Santa Cruz) using the Vector M.O.M. immunodetection kit, according to the protocol by the manufacturer (Vector Laboratories, Burlingame, CA). Indirect immunofluorescence staining was carried out according to the procedures described previously [24]. Briefly, cells were incubated with the specific primary antibodies described above, followed by staining with FITC or TRITC-conjugated secondary antibody. Cells were double stained with DAPI to visualize the nuclei. Slides were viewed with a Nikon Eclipse E80i microscope equipped with a digital camera (DS-Ri1, Nikon). In each experimental setting, immunofluorescence images were captured with identical light exposure parameters.

Morphological Studies

Tissue sections from the mice were prepared at 3 µm thickness by routine procedure. Sections were stained with hematoxylin/eosin or periodic acid Schiff for general histology. Slides were viewed with a Nikon Eclipse E80i microscope equipped with a digital camera (DS-Ri1, Nikon).

Statistical analysis

Western blotting, RT-PCR, and immunofluorescence staining were all repeated at least three times independently. For Western blot analysis, quantitation was performed by scanning and analyzing the intensity of the hybridization signals using NIH I ImageJ software. Data collected were expressed as mean ± SEM. Statistical analyses of data were performed using Sigma Stat software (Jandel Scientific Software). Comparison between groups was made using one-way ANOVA, followed by the t test. P < 0.05 was considered significant.

Results

Ets-1 Expression is induced in the fibrotic kidney induced by Ang II

To confirm the role of Ets-1 in Ang II induced renal interstitial fibrosis, we tested the Ets-1 expression in Ang II plasmid injected mice. Compared with pcDNA3 injection, Ets-1 protein level was increased in kidney tissues of the Ang II plasmid injection group (Fig. 1A and B). Immunohistochemical staining revealed that Ang II plasmid injection induced Ets-1 protein expression both in glomerular and renal interstitium (Fig. 1C though E). Consistent with the western blot and immunohistochemical staining results, Ets-1 mRNA was increased after Ang II plasmid injection compared with those injected with empty vector (Fig. 1F). We

Fig. 1. Ang II increases Ets-1 Expression *in vivo*. (A) Female Balb/c mice were injected with Ang II expression plasmid through the tail vein. Western blot analysis shows the results of Ets-1 proteins expression in the kidneys of each group. Representative pictures show the results of four animals per group. (B) Graphic presentation of relative Ets-1 abundance normalized to actin. * $P < 0.05$ versus control mice injected with pcDNA3. (C) Representative micrographs show kidney morphology by H/E staining. (D) Representative photomicrographs

of sections stained with periodic acid Schiff. (E) Immunohistochemical staining of the localization of Ets-1 in kidneys. (F) Quantitative real-time PCR analysis for Ets-1 mRNA. * $P < 0.05$ versus control mice injected with pcDNA3. (G and H) Immunohistochemical staining of the localization of Ets-1 in Renal biopsy specimens.

also detected Ets-1 expression in Renal biopsy specimens. Immunohistochemical staining of Ets-1 in the renal biopsies demonstrated the upregulation of Ets-1 in the obvious renal fibrosis group (Fig. 1H) compared with mild tubulointerstitial fibrosis group (Fig. 1G).

Ang II induces the activation of NRK-49F cells

Activation of fibroblasts is the central event in AngII induced renal fibrosis. These activated fibroblasts had biological functional changes, and demonstrated by α -SMA expression, extracellular matrix (ECM) production, enhanced cell proliferation and migration. To determine the effects of Ang II on proliferation and migration of NRK-49F cells, cells were exposed to Ang II at different concentrations as indicated, and cell survival was assessed by the MTT assay. As shown in Fig. 2A, Ang II induced cell proliferation in a dose-dependent manner. Few pores in Transwell filters were filled with cell extensions after 2 days (Fig. 2B) whereas cells displayed a much more migratory property after incubating of Ang II (Fig. 2C). Next we investigated the effects of Ang II on FN and α -SMA expressions. As shown in Fig. 2 (E and F), Ang II markedly induced FN and α -SMA expressions in NRK-49F cells both in time and dose dependent manner. Similar results were also obtained in immunofluorescent staining assay Fig. 2 (G and H).

Fig. 2. Ang II induces the activation of NRK-49F cells. (A) MTT assay showing proliferation of NRK-49F cells after different concentration of Ang II treatment. (B and C) Boyden chamber for motility assay showing enhanced migration of NRK-49F cells after Ang II treatment. B: incubation without Ang II. C: incubation with Ang II. (D) Graphic presentation of the numbers of cells or cell extensions migrated through the pores of the filters after incubation with or without Ang II for 2 days. The data are presented as mean $\pm$ S.E. of three experiments. * $P < 0.05$ versus control. (E) Western blot showing FN and α -SMA expression in the NRK-49F cells after different time of Ang II treatment. (F) Western blot showing FN and α -SMA expression in the NRK-49F cells after different concentration of Ang II treatment. (G) Representative micrographs of immunofluorescent staining of FN in NRK-49F cells. (H) Representative micrographs of immunofluorescent staining of α -SMA in NRK-49F cells.

(G) Representative micrographs of immunofluorescent staining of FN in NRK-49F cells. (H) Representative micrographs of immunofluorescent staining of α -SMA in NRK-49F cells.

Ang II induces Ets-1 expression in NRK-49F cells

In vivo experiment, correlation was found between Ets-1 and renal fibrosis. It would be interesting to investigate whether Ets-1 is induced in cultured NRK-49F cells after Ang II treatment. Our results suggested that Ets-1 mRNA expression was obviously increased after Ang II treatment in both time-dependent and dose-dependent manner except at the dose of 10^{-5} M Fig. 3 (A and B). Consistent with the results of quantitative real-time PCR, western blot analysis showed that Ets-1 protein was also increased at 3 h after Ang II treatment and maintained a high level 24 h after treatment (Fig. 3C). Ang II induced Ets-1 protein expression in a dose-dependent manner in a range of 0– 10^{-6} M (Fig. 3D). Similar results were also obtained in immunofluorescent staining assay. Resting NRK-49F cells expressed little Ets-1 whereas Ang II stimulated NRK-49F cells displayed abundant Ets-1 in the cytoplasm and nucleus (Fig. 3G). Therefore, Ets-1 expression was induced after Ang II treatment in NRK-49F cells.

Knockdown of Ets-1 attenuates Ang II-induced activation of NRK-49F cells

To verify the role of Ets-1 in Ang II induced fibroblast activation. We examined whether knockdown of Ets-1 acted to repress Ang II-induced cell proliferation, migration and FN, α -SMA expression. As shown in Fig. 4A and 4B, immunoblotting and immunofluorescent

Fig. 3. Ang II induces Ets-1 expression in NRK-49F cells. (A and B) Quantitative real-time PCR analysis was used for Ets-1 mRNA analysis. A: after different time of Ang II treatment. B: after different concentration of Ang II treatment. (C and D) Western blot analysis was used for showing Ets-1 expression. C: after different time of Ang II treatment. D: after different concentration of Ang II treatment. (E and F) Graphic presentation of relative Ets-1 abundance normalized to actin. E: after different time of Ang II treatment. F: after different concentration of Ang II treatment. (G) Representative immunofluorescent staining images for Ets-1 in NRK-49F cells. The data are presented as mean $\pm$ S.E. of three experiments. * $P < 0.05$ versus control.

staining showed that the protein level of Ets-1 was significantly down-regulated in cells transfected with Ets-1 siRNA. Fig. 4C shows the result of Ets-1 mRNA levels in NRK-49F cells by Q-PCR analysis, consistent with the western blot results. In addition, Ets-1 knockdown significantly reduced cell proliferation induced by Ang II (Fig. 4D). The migration of NRK-49F cells induced by Ang II was also inhibited by knockdown of Ets-1 (Fig. 4E-F). Furthermore, inhibition of Ets-1 abolished FN and α -SMA expressions induced by Ang II (Fig. 4G-H), suggesting Ets-1 is required for NRK-49F cells activation. Similar results were also obtained in immunofluorescent staining assay (Fig. 6I). In a word, these results suggested that Ets-1 was involved in Ang II-induced activation of NRK-49F cells.

Ang II induces down-regulation of miR-221 in vivo and in vitro

Our results indicated that Ets-1 has a critical role in mediating Ang II induced renal fibrosis, but how it is regulated remains obscure. Since TargetScan predicted the existence of evolutionarily conserved binding sites for miR-221 in the 3' untranslated region (UTR) of Ets-1 (Fig. 5A) and it was confirmed by luciferasereport in obesity [25]. So we examined the miR-221 expression in fibrotic kidney induced by Ang II. Quantitative real-time PCR results indicated that miR-221 expression was down regulated both in mice treated with AngII infusion via osmotic minipump and mice injected with Ang II plasmid (Fig. 5B-C). Next we

Fig. 4. Knockdown of Ets-1 attenuates Ang II-induced activation of NRK-49F cells. Western blot analysis (A) and immunofluorescent staining (B) showing Ets-1 protein abundance after Ets-1 small interfering RNA (siRNA) transfection. (C) Q-PCR showing Ets-1 mRNA abundance after Ets-1 siRNA transfection. (D) MTT assay showing proliferation of NRK-49F cells. (E) Graphic presentation of the numbers of cells or cell extensions migrated through the pores of the filters. * $P < 0.05$ versus control. (F) Boyden chamber for motility assay showing reduced migration of NRK-49F cells after Ets-1 small interfering RNA (siRNA) transfection. (G) Western blot analysis showing FN and α -SMA expression. (H) Graphic presentation of relative FN and α -SMA abundance normalized to actin. * $P < 0.05$ versus control. (I) Representative immunofluorescent staining images for FN and α -SMA in NRK-49F cells.

investigated miR-221 expression in NRK-49F cells after Ang II treatment. As shown in Fig. 5(D and E), miR-221 was significantly decreased in time and dose-dependent manner. We further investigated the role of miR-221 on Ets-1 expression in NRK-49F cells. As shown in Fig. 5 (F and G), transfection of miR-221 mimic significantly decreased Ang II-induced Ets-1 expression, whereas transfection of miR-221 inhibitor induced Ets-1 expression.

MiR-221/Ets-1 axis is involved into Ang II induced NRK-49F cells activation

To further investigate the role of miR-221/Ets-1 axis in kidney fibrosis, we observed the role of miR-221 in Ang II induced NRK-49F cells activation. NRK-49F cells were transfected with miR-221 mimic or inhibitor before Ang II treatment. As shown in Fig. 6 (A and B), miR-221mimic transfection resisted Ang II induced migration in NRK-49F cells, whereas miR-221 inhibitor promoted this effect. Fig. 6 (C and E) shows that miR-221mimic transfection resisted to Ang II-induced activation of NRK-49F cells, whereas miR-221 inhibitor promoted phenotype changes viewed as induction of α -SMA and FN expression Fig. 6 (D and F). From the discussion above, it was indicated miR-221/Ets-1axis is involved in Ang II induced NRK-49F cells activation.

Fig. 5. Ang II induces down-regulation of miR-221 *in vivo* and *in vitro* (A) Predicted con-

sequential pairing of Ets-1 and miR-221. (B) Male CD1 mice

were subcutaneously embed-

ded with a mini pump infused

with purified Ang II protein.

Quantitative real-time PCR

showing miR-221 expression

in kidneys. * $P < 0.05$ versus

control. (C) Female Balb/c

mice were injected with Ang

II expression plasmid through

the tail vein. Quantitative

real-time PCR showing miR-

221 expression in kidneys. * $P < 0.05$ versus control mice

injected with pcDNA3. (D)

Quantitative real-time PCR

showing miR-221 expression

after different time of Ang II

treatment in NRK-49F cells.

* $P < 0.05$ versus control. (E)

Quantitative real-time PCR

showing miR-221 and Ets-1

after different dose of Ang II

treatment in NRK-49F cells.

* $P < 0.05$ versus control. (F)

Western blot analysis showing

Ets-1 expression after miR-

221 miminc or inhibitor treat-

ment in NRK-49F cells with or

without Ang II treatment. (G)

Graphic presentation of relative

Discussion

Renal fibrosis is an inevitable outcome of almost all progressive chronic kidney disease. Maladaptive activation of the Renin-angiotensin-aldosterone system (RAAS) is a key regulator of renal fibrosis [26], and inhibition of RAAS system could attenuate glomerulosclerosis and interstitial fibrosis. As the main effector of RAAS, Ang II has been considered as a renal growth factor with many effects on the kidney as modulating cell growth and extracellular matrix (ECM) synthesis. Accumulated evidences have shown that Ang II is a profibrotic cytokine and activation of fibroblast to myofibroblast plays a crucial role in promoting renal fibrosis induced by Ang II [27, 28]. However, the cellular and molecular mechanisms of this process are still under investigation. In this study, we presented evidence suggesting that Ets-1, targeted by miR-221, acts as an important regulator implicated in the activation of renal fibroblast induced by Ang II.

Many transcription factors are involved in the pathogenesis of CKD and mediate the complex pathways in response to Ang II. Ets-1 is a member of the Ets family of transcription factors that share a highly conserved DNA-binding domain. Although it is reported to be

Fig. 6. MiR-221/Ets-1 axis is involved into Ang II induced NRK-49F cells. (A) Graphic presentation of the numbers of cells or cell extensions migrated through the pores of the filters after miR-221-mimic or miR-221-inhibitor transfection along with Ang II treatment (B) Boyden chamber for motility assay showing migration of NRK-49F cells after miR-221-mimic or miR-221-inhibitor transfection along with Ang II treatment. (C) Western blot analysis showing miR-221-mimic transfection reduced FN and α -SMA expression. (D) Western blot analysis showing miR-221-inhibitor transfection increased FN and α -SMA expression. (E) Graphic presentation of relative FN and α -SMA abundance normalized to actin incubated with or without Ang II after miR-221-mimic transfection. (F) Graphic presentation of relative FN and α -SMA abundance normalized to actin incubated with or without Ang II after miR-221-inhibitor transfection. * $P < 0.05$ versus control. # $P < 0.05$ versus Ang II treatment.

required for the normal development of the mammalian kidney, many recent studies have described that Ets-1 is up-regulated in a wide range of renal diseases and regulate numerous genes such as MMP-1, -3, -9, TIMP-1, urokinase-type plasminogen activator(u-PA), MCP-1 and PAI-1 [29-33]. However, most studies mainly focus on glomerular and mesangial cells. The role of Ets-1 in renal interstitial fibroblast activation and tubulointerstitial fibrosis remains largely unknown. Our results displayed that Ets-1 was induced by Ang II in rat renal interstitial fibroblast cells accompanied by fibroblast activation. Knockdown of Ets-1 significantly inhibited this effect. To our knowledge, this is the first report describing the critical role of Ets-1 in Ang II induced interstitial fibroblast activation. More over, we also first described that Ang II plasmid injection could increase Ets-1 expression, which is consistent with the results of Ang II infusion mice model reported by previous study [34]. To further confirm the role of Ets-1 in tubulointerstitial fibrosis, we detected the Ets-1 expression in renal biopsies of patients with mild or obvious tubulointerstitial fibrosis. Our data suggested

Ets-1 was upregulated in fibrotic renal biopsy specimens. Taken together, our findings would suggest that Ets-1 is an important mediator of Ang II in tubulointerstitial fibrosis.

In previous studies, we have demonstrated that miR-30e and miR-200 family were involved in renal fibrosis [35, 36]. MiR-221 was found under-expressed in Unilateral Ureteral Obstruction(UUO) mouse model using miRNA array [23]. Our further investigation found that miR-221 was downregulated *in vivo*. Similar results were also obtained in NRK-49F cells treated with Ang II. Therefore it is suggested that miR-221 downregulation might be an important facilitator in renal fibrosis. Target screening and previous luciferase assays have confirmed Ets-1 is the target of miR-221 [25]. In this study, the expression of miR-221 was inversely related to the Ets-1 expression treated with Ang II *in vivo* and *in vitro*. Inhibition of miR-221 increased Ets-1 levels and was accompanied by the activation of NRK-49F similar to Ang II treatment. In contrast, overexpression of miR-221 hindered this phenotypic change by downregulation of Ets-1 expression. Taken together, our results indicate that miR-221 has an important role in mediating Ang II-induced interstitial myofibroblast activation and kidney fibrosis by target Ets-1. These findings may provide new therapeutic targets in fibrotic kidney diseases.

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