

Original Article

MiR-23b inhibited the effects of IL-17 on renal tubule epithelial cell via targeting IKK α

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Abstract: The inflammatory cytokine interleukin 17 (IL-17) is an important contributor of glomerulonephritis (GN) chronicity. miR-23b regulates cell metabolism and cancer development, but the effects of miR-23b on GN are still need to be illustrated. Here we reported that miR-23b was downregulated and inversely correlated with IKK α expression in GN tissues. Additionally, miR-23b suppressed IL-17-induced NF- κ B activation and the expression of proinflammatory and profibrotic mediators expression in HK-2. Furthermore, we identified that miR-23b inhibited the effects of IL-17 on HK-2 via targeting the IKK α . These results prompt us to further understand the molecular regulation of miRNAs in glomerulonephritis.

Keywords: Renal tubule epithelial cell, glomerulonephritis, miR-23b, interleukin 17, IKK α

Introduction

Glomerulonephritis (GN) encompasses a group of diseases that are characterized by inflammatory glomerular processes and clinically usually present with a nephritic or a nephrotic syndrome, as well as a deterioration of the renal function [1]. The most forms of GN begin with activation of the innate immune response and lead to autoimmunity [2]. Several lines of evidence indicate that interleukin-17 (IL-17)-producing T cells (Th17 cells) is involved in the renal inflammatory cascade associated with glomerulonephritis [3]. Th17 immune response play a key role in renal inflammatory disease [4].

MicroRNAs (miRNAs) are small non-coding RNA molecules that regulate gene expression. It has been reported that miRNAs participate in the progression of various forms of chronic kidney disease (CKD) [5]. miR-26a regulates podocyte differentiation and cytoskeletal integrity in autoimmune glomerulonephritis [6]. miR-155 is a new therapeutic target in crescentic GN [7]. However, the function of miRNAs in renal tubule epithelial cell during glomerulonephritis pathogenesis is still need to be illustrated.

Here, we identified that miR-23b was downregulated and inversely correlated with IKK α expression in GN tissues. Moreover, miR-23b suppressed IL-17-induced NF- κ B activation via targeting the IKK α in renal tubule epithelial cell. These results prompt us to further understand the molecular regulation of miRNAs in glomerulonephritis.

Materials and methods

Renal biopsy

Glomerulonephritis (GN) (n = 7) patients and healthy controls (n = 5) received a percutaneous renal biopsy guided by B ultrasound. The renopuncture tissues were stored in the fridge (-80°C). All experiments were approved by the research ethics committee of The First Affiliated Hospital of Huzhou University. Written informed consent was obtained from all patients.

Cell culture

The human proximal tubular epithelial (HK-2) cell line was purchased from Shanghai Institute of Cell Biology, Chinese Academy of Sciences (Shanghai, China). The HK-2 cells were cultured in Keratinocyte Serum Free Medium (K-SFM)

Table 1. Primer sequences

Genes	Forward primer (5'-3')	Reverse primer (5'-3')
CTGF	CAGCATGGACGTTCTGCTG	AACCACGGTTTGGTCCTTGG
CD44	CTGCCGCTTTGCAGGTGTA	CATTGTGGGCAAGGTGCTATT
IL-6	ACTCACCTCTTCAGAACGAATTG	CCATCTTTGGAAGGTTTCAGGTTG
IL-8	TTTGTCCAAGGAGTGCTAAAGA	AACCCTCTGCACCCAGTTTTC
COL I	GAGGGCCAAGACGAAGACATC	CAGATCACGTCATCGCACAAAC
COL III	GGAGCTGGCTACTTCTCGC	GGGAACATCCTCCTTCAACAG
IKK α	ATGAAGAAGTTGAACCATGCCA	CCTCCAGAACAGTATTCCATTGC
GAPDH	GGAGCGAGATCCCTCCAAAT	GGCTGTTGTCACTTCTCATGG

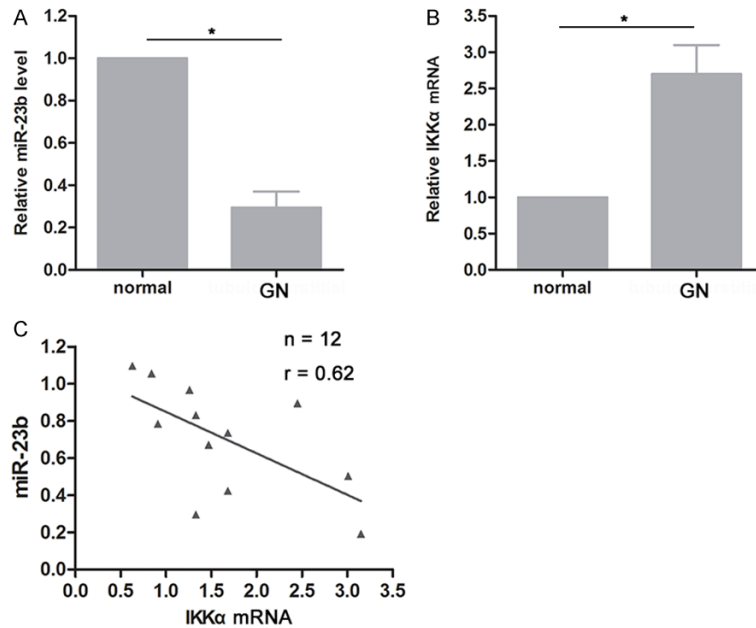


Figure 1. MiR-23b was decreased and inversely correlated with IKK α mRNA expression in glomerulonephritis patients. A. Real-time PCR was performed to examine the expression of miR-23b in glomerulonephritis (GN) ($n = 7$) patients and healthy controls ($n = 5$). Data are showed as means \pm SEM. $*P < 0.05$, Student's t test. B. The level of IKK α mRNA was analyzed by real-time PCR. Data are showed as means \pm SEM. $*P < 0.05$, Student's t test. C. The correlation between miR-23b expression and the IKK α mRNA level was evaluated using Spearman's correlation analysis. $n = 12$; $*P < 0.05$.

(Invitrogen) containing 10% heat-inactivated fetal calf serum (FBS; Gibco), Gentamicin/Amphotericin Solution (Gibco), 100 IU/ml penicillin and 100 mg/ml streptomycin maintained at 37°C in humidified air containing 5% CO₂. Cells were stimulated with 80 ng/ml of IL-17 (peprotech) for 48 h.

Real-time PCR

Total RNA was extracted from tissue and cell samples using Trizol (Invitrogen). The mRNA levels for CTGF, CD44, IL-6, IL-8, collagen I, colla-

gen III, and IKK α were measured by real-time PCR (Step One, Applied Biosystems). The PCR primer sequences were listed in **Table 1**. The relative mRNA expression level of each gene was normalized against GAPDH. TaqMan miRNA probes (Applied Biosystems) were used to perform human miR-23b and U6 real-time PCR assays according to the manufacturer's instructions. Real-time PCR was performed using the TaqMan PCR kit on the ABI Step One Real-Time PCR System (Applied Biosystems). The level of miR-23b was normalized to U6.

Western blotting

Cells were lysed in RIPA lysis buffer (Beyotime). Total protein was quantified using a BCA Protein Assay Kit (Beyotime), and an equal amount of protein was resolved by SDS-PAGE and transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore). The blots were blocked within 5% BSA. The following primary antibodies were applied used according to the manufacturer's instructions. Antibodies against IKK α , GAPDH, p-p65 and p65 were purchased from Santa Cruz Biotechnology (Santa Cruz). The ap-

propriate secondary antibodies (Santa Cruz) were used for 1 h at room temperature, and the signals were revealed using ECL kit (Thermo Scientific).

Plasmids and cell transfection

Human IKK α cDNA were generated by PCR and cloned into BamHI/XhoI site of pcDNA3 (Invitrogen). miR-23b mimic or inhibitor and their controls were purchased from GenePharma Biotechnology (Shanghai). Cells were transiently transfected with the Lipofectamine 2000 reagent

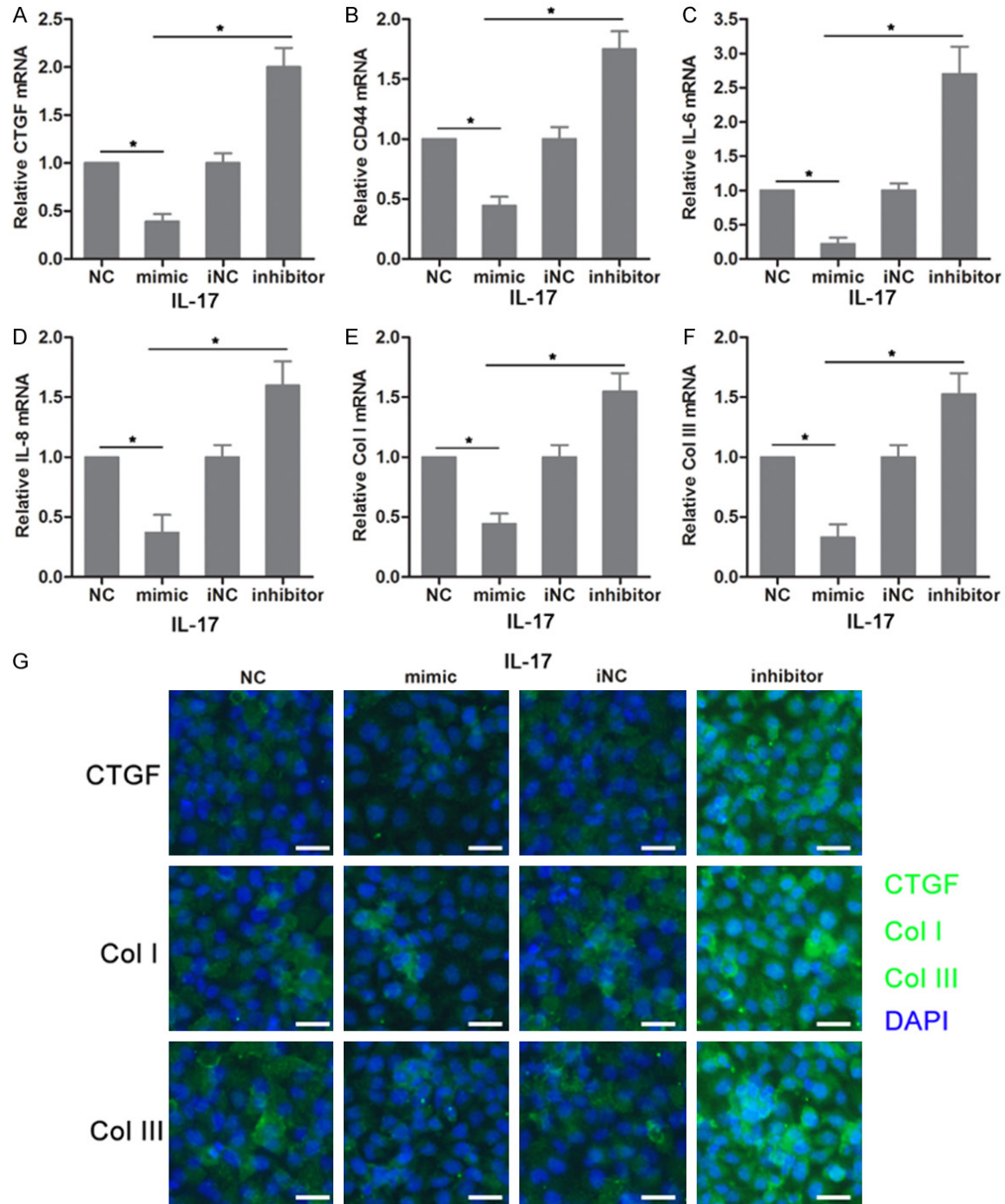


Figure 2. MiR-23b suppressed the effects of IL-17 on renal tubule epithelial cell. The expression of CTGF (A), CD44 (B), IL-6 (C), IL-8 (D), collagen I (E), and collagen III (F) mRNA determined by real-time PCR in renal tubule epithelial cell HK-2, which were transfected with the miR-23b mimic, miR-23b inhibitor or the relative controls (NC and iNC) and then incubated with IL-17 inflammatory cytokine. Data are representative of three independent experiments (means \pm SEM). * $P < 0.05$, ANOVA. (G) Immunofluorescence assay was performed on HK-2 cells stained with antibody directed against CTGF, collagen I, and collagen III (green) and DAPI (blue). The white bar indicates 10 μ m.

(Invitrogen) according to the manufacturer's instructions. Transfected cells were harvested

after 48 h for RNA isolation and western blotting.

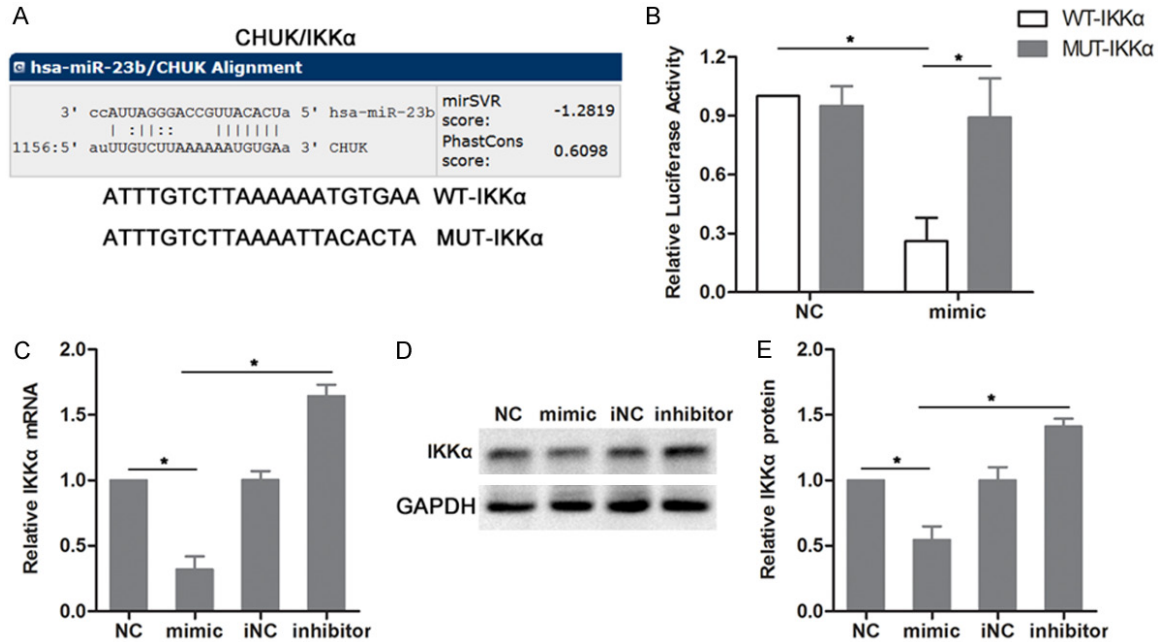


Figure 3. IKK α was the direct target of miR-23b. **A.** Schematic representation of the predicted interaction of miR-23b with wild type IKK α 3'UTR site and the point-mutated IKK α 3'UTR site. **B.** Luciferase activity determined in HK-2 cells that were first transfected with the indicated wild-type or point-mutated 3'UTR reporter constructs (WT-IKK α or MUT-IKK α) and then transduced with miR-23b or control mimic. Data are representative of three independent experiments (means \pm SEM). * P < 0.05, ANOVA. **C.** Real-time PCR of IKK- α mRNA expression in HK-2 cell transfected with the miR-23b mimic, miR-23b inhibitor or the relative controls. Data are representative of three independent experiments (means \pm SEM). * P < 0.05, ANOVA. **D.** Western blotting of IKK- α protein expression in HK-2 cell transfected with the miR-23b mimic, miR-23b inhibitor or the relative controls. **E.** Relative intensity measurements provide a quantitative means of examining protein bands on the western blotting. Data are representative of three independent experiments (means \pm SEM). * P < 0.05, ANOVA.

Luciferase assay

The wild type and mutation of IKK α -3'UTR were cloned into the pmirGLO (Promega), named pmirGLO-WT-IKK α or pmirGLO-MUT-IKK α . Twenty-four hours after transfection, the medium was removed and the cells were lysed and assayed with a Dual Luciferase Reporter Assay Kit (Promega) according to the manufacturer's instructions. Firefly luciferase activity was measured and normalized to Renilla luciferase.

Immunofluorescence assay

Cells on slides were fixed in 4% paraformaldehyde and washed three times with PBS, then incubated with PBS containing 0.1% Triton X-100 and 1% normal serum for 30 min at room temperature. The primary and secondary antibodies was diluted as follows: rabbit anti-CTGF (1:500), anti-collagen I (1:200), anti-collagen III (1:300), anti-p-p65 (1:250) (Abcam, USA), Alexa Fluor 594-conjugated goat anti-rabbit IgG (1:400), and Alexa Fluor 488-conju-

gated goat anti-rabbit IgG (1:400) (Thermo Fisher Scientific, USA). Nuclear DNA was dyed with DAPI (Thermo Fisher Scientific, USA). For all immunochemistry experiments, negative staining controls were carried out by omitting the primary antibody.

Statistical analysis

Values are showed as the means \pm SEM. The one-way analysis of variance (ANOVA) test and student's t test were used for statistics. The correlation between miR-23b and IKK α expression levels was determined by Spearman's correlation. Values of P < 0.05 were considered statistically significant.

Results

MiR-23b expression was decreased and inversely correlated with IKK α in glomerulonephritis patients

To explore the effects of miR-23b on renal inflammation, we examined the expression of

MiR-23b targeted IKK α in renal epithelial cell

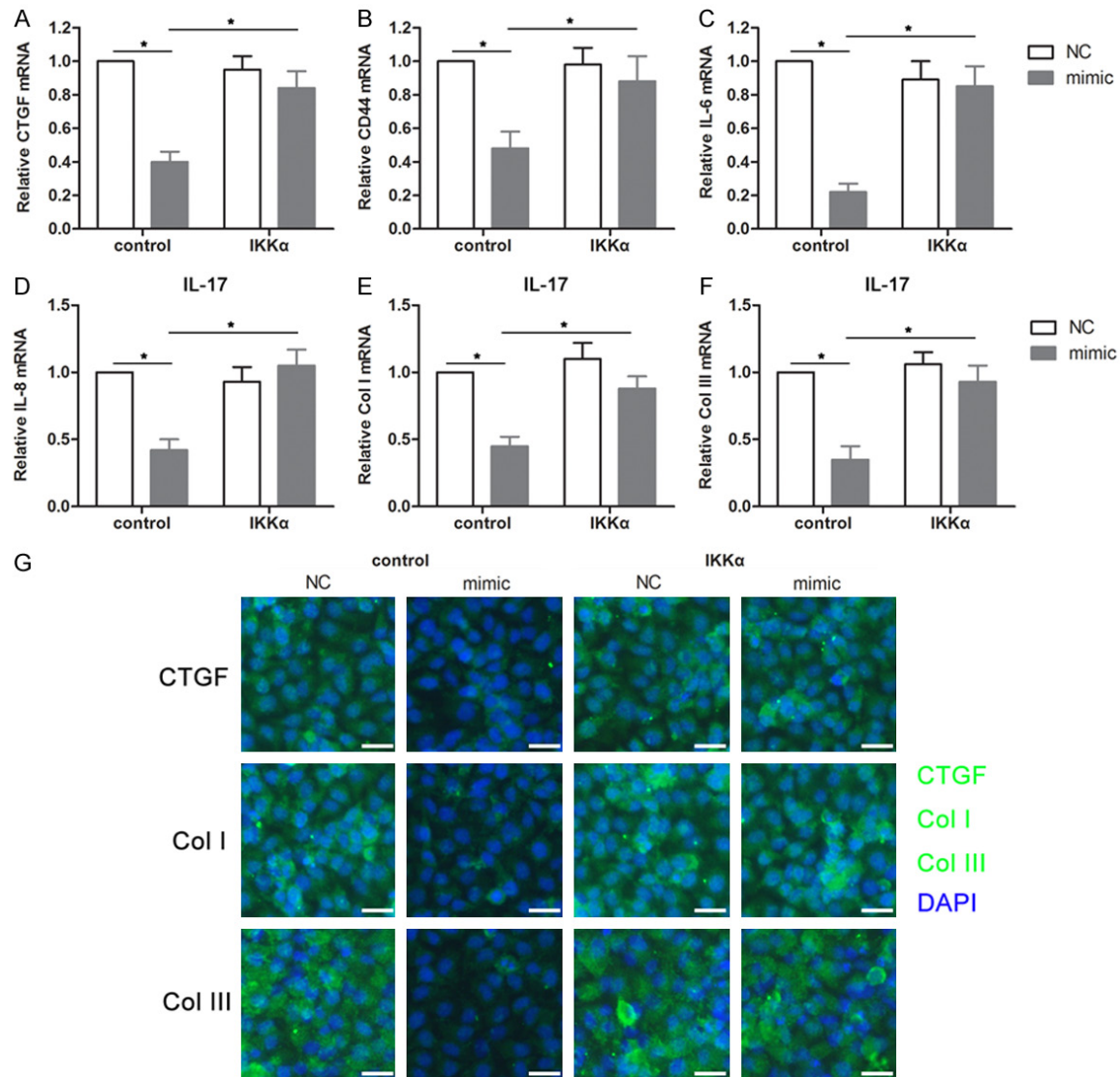


Figure 4. IKK α was functional targets of miR-23b. (A) Expression of CTGF (A), CD44 (B), IL-6 (C), IL-8 (D), collagen I (E), and collagen III (F) mRNA were determined by real-time PCR in HK-2 cell transfected with pCDNA-IKK α or control plasmid and then transfected with the miR-23b mimic, or NC and then incubated with IL-17 inflammatory cytokine. Data are representative of three independent experiments (means \pm SEM). * P < 0.05, ANOVA. (G) Immunofluorescence assay was performed on HK-2 cells stained with antibody directed against CTGF, collagen I, and collagen III (green) and DAPI (blue). The white bar indicates 10 μ m.

miR-23b in renopuncture tissues from glomerulonephritis patients. Findings showed that the expression of miR-23b was significantly decreased in GN tissues compared with control tissues (Figure 1A). Then, we hypothesized that miR-23b was also involved in the regulation of IKK α in glomerulonephritis. Therefore, real-time PCR was performed to determine the expression of IKK α in GN tissues and control tissues. The results showed that IKK α mRNA was significantly increased in GN tissues (Figure 1B). Moreover, IKK α expression was inver-

sely correlated with miR-23b expression in renopuncture tissues (Figure 1C).

MiR-23b suppressed the effects of IL-17 on renal tubule epithelial cell

IL-17 is an early initiator of inflammation. It induces the release of several renal injury mediators in renal tubule epithelial cell, such as CTGF, CD44, IL-6, IL-8, collagen I, and collagen III. Therefore, we wondered whether miR-23b suppressed the induction of CTGF, CD44, IL-6, IL-8,

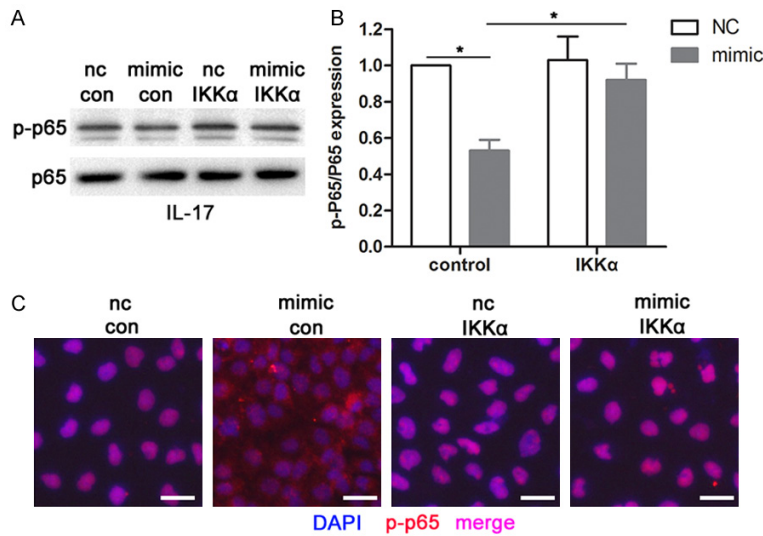


Figure 5. MiR-23b suppressed IL-17-induced NF- κ B activation. A. The western blotting of NF- κ B activity in HK-2 cell transfected with the miR-23b mimic or NC and then incubated with IL-17 for the 30 min. Data represent three independent experiments. B. Relative intensity measurements provide a quantitative means of examining protein bands on the western blotting. Data are representative of three independent experiments (means \pm SEM). * P < 0.05, ANOVA. C. Immunofluorescence assay was performed on HK-2 cells stained with antibody directed against p-p65 (red) and DAPI (blue). The white bar indicates 10 μ m.

collagen I, and collagen III expression by IL-17 in renal tubule epithelial cell HK-2. As shown in **Figure 2**, miR-23b mimic inhibited IL-17-induced upregulated expression of CTGF, CD44, IL-6, IL-8, collagen I, and collagen III in HK-2 cells. However, miR-23b inhibitor enhanced IL-17-induced upregulated expression of CTGF, CD44, IL-6, IL-8, collagen I, and collagen III in HK-2 cells. Collectively, these data suggested that miR-23b suppressed the effects of IL-17 on renal tubule epithelial cell. Furthermore, the immunofluorescence assay showed that miR-23b mimic inhibited IL-17-induced upregulated expression of CTGF, collagen I, and collagen III; however, miR-23b inhibitor enhanced IL-17-induced upregulated expression of CTGF, collagen I, and collagen III in HK-2 cells (**Figure 2G**).

IKK α is the direct target of miR-23b

We used TargetScan and miRanda online software to verify potential targets of miR-23b. MiR-23b was predicted to have seed regions able to bind to the 3'UTR of IKK α (CHUK). To confirm whether miR-23b directly targets IKK α 3'UTR, we cloned the wild-type (WT) IKK α 3'UTR or a mutant variant into a luciferase reporter vector pmirGLO (**Figure 3A**). The results of luciferase activity assay showed that miR-23b suppress-

ed the luciferase activity of the WT IKK α 3'UTR, while mutation of the miR-23b binding sites blocked this suppressive effect (**Figure 3B**). Real-time PCR and western blotting assays demonstrated that transfection of miR-23b mimic in HK-2 inhibited IKK α mRNA and protein expression, while the miR-23b inhibitor elevated the expression of IKK α mRNA and protein levels (**Figure 3C** and **3D**). Collectively, these results proposed that the IKK α could be the target of miR-23b.

MiR-23b inhibited the effects of IL-17 on HK-2 via targeting the IKK α

To further explore the influences of IKK α and miR-23b on glomerulonephritis pathogenesis, we co-transfected HK-2 cells with pCDNA-IKK α

plasmid and miR-23b mimic. The overexpression of IKK α reversed the miR-23b-imposed inhibitory effects on IL-17-induced the upregulated of CTGF, CD44, IL-6, IL-8, collagen I, and collagen III mRNA levels in renal tubule epithelial cell HK-2 (**Figure 4**). Furthermore, the immunofluorescence assay showed that overexpression of IKK α reversed the miR-23b-imposed inhibitory effects on IL-17-induced the upregulated of CTGF, collagen I, and collagen III levels in HK-2 cells (**Figure 4G**). The results suggested that IKK α was the target of miR-23b and contributed to the suppressive effects of miR-23b. IL-17 activates NF- κ B/p65 pathway and NF- κ B/p65 are required for IL-17-induced gene expression. Following, we further examined the phosphorylation of p65 by western blotting analysis. The data showed that transfection of miR-23b mimic in HK-2 cells incubated with IL-17 decreased the phosphorylation of p65, while overexpression of IKK α blocked this suppressive effect (**Figure 5A** and **5B**). Furthermore, the immunofluorescence assay showed that transfection of miR-23b mimic in HK-2 cells incubated with IL-17 decreased the nuclear translocation of p-p65, while overexpression of IKK α blocked this suppressive effect (**Figure 5C**). These results indicated that miR-23b inhibited

IL-17-mediated gene expression via targeting the IKK α in renal tubule epithelial cell HK-2.

Discussion

Although miR-23b regulates cell metabolism and cancer development [8, 9], a role for miR-23b in GN was previously unknown. Here we showed that miR-23b was decreased and inversely correlated with IKK α in GN patients' tissues.

IL-17 induces the release of several proinflammatory and profibrotic mediators in renal tubule epithelial cell [10, 11]. CTGF is a profibrotic mediator and promotes renal fibrogenesis [12]. CD44 is a cell surface glycoprotein involved in the pathologic progression of fibrosis and cancer [13]. pro-inflammatory cytokines IL-6 and IL-8 can induce the activation of the NF- κ B pathway [14]. The types I and III collagen contribute to myofibroblasts infiltrate the renal tissues in the process of renal fibrosis [15]. Our results suggested that decreased miR-23b expression might contribute to the progress of GN.

Findings reported that miR-23b suppresses IL-17-associated autoimmune inflammation by targeting TAB2, TAB3 and IKK α [16]. IKK α , a kinase of the NF- κ B signaling [17], has been reported to have an important role in GN [18, 19]. Additionally, NF- κ B activation has been implicated in the pathogenesis of GN and blockade of the IKK signal complex has attracted considerable attention [19-22]. Our results discovered that the IKK α could be the target of miR-23b in renal tubule epithelial cell. Furthermore, the IKK α overexpression reversed the miR-23b-imposed inhibitory effect on IL-17-mediated proinflammatory and profibrotic mediators expression and NF- κ B activation.

Taken together, our study demonstrated that miR-23b regulated IL-17-mediated proinflammatory and profibrotic mediators expression via targeting IKK α in glomerulonephritis. Our findings may be useful for future studies exploring therapeutic approaches for glomerulonephritis.

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Disclosure of conflict of interest

None.

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