

RESEARCH ARTICLE

Effect of pro-inflammatory interleukin-17A on epithelial cell phenotype inversion in HK-2 cells *in vitro*

Li Liu^{1,6}, Fu-gang Li^{1,6,a}, Man Yang³, Li Wang^{1,2}, Yue Chen¹, Li Wang⁴, Wen Ji², Jun-ming Fan^{1,2,5,6}

¹ Department of Nephrology, Affiliated Hospital of Luzhou Medical College, Luzhou City 646000, Sichuan Province, China

² Department of Nephrology, West China Hospital of Sichuan University, Chengdu City 610041, Sichuan Province, China

³ Department of Nephrology, Shanghai general hospital, Shanghai Jiao Tong University, Shanghai City 200080, China

⁴ Department of Central Service, West China Hospital of Sichuan University, Chengdu City 610041, Sichuan Province, China

⁵ State Key Laboratory of Biotherapy of Human Disease, West China Hospital, Sichuan University, Chengdu City 610041, Sichuan Province, China.

⁶ People's Hospital of Jianyang, Ziyang City 641400, Sichuan Province, China.

Correspondence

<junmingfan@163.com>

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ABSTRACT. Background: Renal interstitial fibrosis (RIF) is a pathological change common to a variety of chronic renal diseases, ultimately progressing to end-stage renal failure. It is believed that epithelial cell phenotype inversion plays an important role in RIF, which is characterized by expression of the mesenchymal marker α -SMA, loss of the epithelial marker E-cadherin, and enhanced secretion of extracellular matrix. IL-17, a newly discovered pro-inflammatory cytokine, has recently been reported to play an important role in tissue fibrosis, involving pulmonary, liver, intestine and skin tissues. This study aimed to investigate whether IL-17A, a member of the IL-17 family, can induce epithelial cell phenotype inversion, and to explore the molecular mechanism of this phenotype inversion, *in vitro*. **Methods:** HK-2 cells were cultured and incubated with IL-17A. Cell proliferation was measured by CCK-8 assay, and the secretion of types I and III collagen was detected by ELISA in dose-dependent and time-dependent experiments. To find out whether IL-17A can induce epithelial cell phenotype inversion, HK-2 cells were stimulated with 80 ng/mL of IL-17A and 10 ng/mL of TGF- β 1 as a positive control, for 72 h. To explore the potential signaling pathway, anti-TGF- β 1 antibody was added before IL-17A treatment. At the same time, anti-TGF- β 1 antibody alone was added to the medium as the negative control group. The expression of types I and III collagen, α -SMA and E-cadherin proteins, and mRNA was measured by real-time PCR, western blotting and immuno-histochemistry. **Results:** IL-17A promoted the proliferation of HK-2 cells and secretion of types I and III collagen in a dose-dependent and time-dependent manner. Compared with the normal control, IL-17A could stimulate the expression of α -SMA, types I and III collagen, and suppressed the expression of E-cadherin in HK-2 cells. Incubation of IL-17A with TGF- β 1 antibody decreased significantly the expression of α -SMA, but increased the expression of E-cadherin in HK-2 cells. **Conclusion:** Our results suggest that IL-17A might promote the proliferation of HK-2 cells and secretion of extracellular matrix, and induce epithelial cell phenotype inversion via a TGF- β 1-dependent pathway. Blocking the pro-inflammatory cytokine IL-17A might be a potential target for the treatment of fibrotic kidney disease.

Keywords: Interleukin-17A, renal interstitial fibrosis, EMT, TGF- β 1

Renal interstitial fibrosis (RIF) is the pathological basis and the common pathway of progressive renal disease. It results in functional deterioration and eventual loss of renal function, regardless of the diverse initial causes, and eventually develops into end-stage renal failure [1-4]. RIF is characterized by an increase in inflammatory cells and extracellular matrix (ECM), tubular atrophy etc. [5]. In addition, the increasing accumulation of ECM plays an important role in RIF, as it fills the interstitium leading to nephron loss and declining kidney function [6].

ECM is mainly composed of collagen, glycoprotein and proteoglycan [7]. It is not only a cell scaffold, but also directly affects cellular adhesion, metabolism, nutrition, proliferation and differentiation. Generally, the synthesis and degradation of ECM are in a dynamic equilibrium state. When this balance becomes disrupted, the matrix components accumulate, leading eventually to progressive fibrosis. The ECM-producing cells in kidney include fibroblasts, mesangial cells and renal tubular epithelial cells, but the excessive accumulation of ECM is derived mainly from myofibroblasts [8, 9], which are a special kind of cell with an ultrastructure somewhere between fibroblasts and smooth muscle cells.

^aThe authors contributed equally to this article.

Myofibroblasts have the ability to proliferate and secrete collagen, which plays an important role in RIF. During the process of RIF, myofibroblasts can secrete types I, III and IV collagen, proteoglycan, and glycoproteins such as fibronectin and laminin. Moreover, myofibroblasts are also a major source of ECM-degrading proteases such as matrix metalloproteinases (MMP) considered to be the most important medium in the degradation of ECM [10]. Therefore, myofibroblasts not only synthesize large amounts of ECM, they also inhibit its degradation, eventually leading to excessive accumulation of ECM in renal interstitium.

The origin of the myofibroblast is not yet fully understood: some scholars believe that the renal tubular epithelium is an important source. Tubular epithelial cells could transform into myofibroblasts via an epithelial cell phenotype inversion pathway, which is an important pathogenesis of RIF [11-14]. Transdifferentiation refers to the process whereby cells lose their characteristic phenotype and obtain a new one. This is characterized by a change in cell morphology, phenotype and function. The main manifestations of epithelial cell phenotype inversion in kidney include: tubular epithelial cells losing markers, such as E-cadherin [15], and acquiring interstitial cell markers, such as alpha-SMA [15], and cells showing greater proliferation and secretion abilities.

Interleukin-17 (IL-17) is a newly discovered cytokine, secreted by newly defined CD4⁺T cells called Th17 cells, which are different from Th1 and Th2 cells [16, 17]. The IL-17 family includes IL-17A, IL-17B, IL-17C, IL-17D, IL-17E and IL-17F [18]. IL-17A usually refers to IL-17. IL-17, a multifunctional cytokine, that might promote expression of inflammatory factors such as IL-6, chemotactic factors such as MCP-1 and MMP, leading to infiltration of inflammatory cells and tissue destruction [19, 20]. IL-17 is also involved in proliferation, maturation and migration of neutrophils [20], and it stimulates the production of IL-6, IL-8 and prostaglandin E2 by epithelial cells, mesothelial cells and fibroblast cells. It has been reported that IL-17 might play a catalytic role in pulmonary fibrosis, liver fibrosis and myocardial fibrosis, but the effect of IL-17 on renal fibrosis is not clear. This study aims to investigate the influence of IL-17 on epithelial cell phenotype inversion, proliferation and secretion of types I and III collagen in HK-2 cells *in vitro*, and to explore the possible molecular mechanism.

MATERIALS AND METHODS

Cell culture

The human proximal tubular epithelial (HK-2) cell line was a gift from Professor Xu Yong (Endocrinology, Affiliated Hospital of Luzhou Medical College, China). The HK-2 cells were cultured in DMEM/F12 (Hyclone, USA) containing 10% heat-inactivated fetal calf serum (FBS; Hyclone, USA), 100 U/mL penicillin and 100 mg/mL streptomycin, at 37°C in a 5% CO₂ atmosphere in six-well plastic plates, 96-well plates with plastic coverslips coated with type I collagen. The media were changed every two days, and the cells were passaged when they grew to 80% confluency.

Experimental protocol

The recombinant human IL-17A and anti-TGF-β1 antibody (TGF-β1 antibody) were purchased from R&D Systems (Minneapolis, MN, USA). Recombinant human TGF-β1 (TGF-β1) was purchased from PeproTech (USA). These were dissolved according to the manufacturer's protocol, and were then diluted with cell culture medium before use.

For the dose-dependent experiment, HK-2 cells were cultured with 0, 20, 40, 80, 160, 320 ng/mL of IL-17A for 72h. For time-dependent experiments, the cells were cultured with the optimal concentration of IL-17A for 24, 48, and 72h. Cell proliferation was then measured using a Cell Counting Kit-8 assay (Sigma-Aldrich, St. Louis, MO, USA). The secretion of types I and III collagen were measured using a sandwich enzyme-linked immunosorbent assay (ELISA).

To find out whether IL-17A could induce cell phenotype inversion, the cells were stimulated with 80 ng/mL of IL-17A or 10 ng/mL of TGF-β1 as a positive control (according to our previous study; [22]) for 72 h. To study the signaling pathway, anti-TGF-β1 antibodies, as inhibitor, were added separately before IL-17A incubation. Simultaneously, anti-TGF-β1 antibody was added to the medium as the negative control. The expression of α-SMA and E-cadherin protein, and mRNA was assessed by real-time PCR, western blotting and immunohistochemistry.

Cell Counting Kit-8 (CCK-8) assay

The HK-2 cells were seeded in 96-well plates at a density of 1×10^4 cells per well and left for 12 h, after which cells were starved in serum-free medium for 24h and then incubated with different concentrations of IL-17A for 48h. Cell proliferation was assessed using the Cell Counting Kit-8 (CCK-8) assay (Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer's instructions. Briefly, after treatment, 10 ul of CCK-8 were added to each well and incubated at 37°C for 2 h. The optical density was read at a wavelength of 450 nm with a microplate reader (Biotech, USA).

Immunocytochemistry

HK-2 cells (1×10^5) cultured under plastic cover slips for 72 hours were rinsed with PBS, fixed in 2% paraformaldehyde for 30 minutes, and permeabilized in 0.1% (v/v) Triton X-100 for 10 minutes (when detecting α-SMA, but not E-cadherin), incubated with 0.3% (v/v) H₂O₂ for 30 minutes to inactivate endogenous peroxidases, and then with 5% BSA for 30 minutes at 37°C to block nonspecific binding. After that, cells were incubated with anti-α-SMA monoclonal antibody (1:200; Abcam, USA) and anti-E-cadherin polyclonal antibody (1:100; Abcam, USA) overnight at 4°C, then with horseradish peroxidase (HRP)-conjugated goat anti-mouse-immunoglobulin (IgG) (1:200, Santa Cruz, USA) for 30 min at 37°C. The cells were then stained with diaminobenzidine (DAB; Boster, China). In this way, the primary antibodies were stained by sequential incubations to produce a brown color. All procedures were performed

at room temperature. The number of HK-2 cells stained with the anti- α -SMA or anti-E-cadherin antibodies was determined by counting the number of positively-stained cells in a total of at least 100 cells under high power ($\times 400$) in each cell spot. Data from five experiments are expressed as the mean \pm standard deviation (SD). For all analyses, the observer was blinded to the coded slides.

Real-time polymerase chain reaction (PCR)

HK-2 cells (1×10^5) were cultured under the different conditions as described above. To measure the mRNA levels for α -SMA, E-cadherin, and TGF- β 1, total RNA was extracted from cells using the Trizol method (Invitrogen, Carlsbad, CA, USA). cDNA was synthesized from total RNA using the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific Fermentas, Vilnius, Lithuania). Real-time PCR was performed using the SYBR Green real-time PCR Master Mix (Applied Biosystems, Foster City, CA) routinely. GAPDH was employed as an internal reference. The following gene-specific primers were used: β -actin, forward 5'-TCACCCA-CACTGTGCCCATCTACGA-3', reverse 5'-CAGCGGAACCGCTCATTGCCAATGG-3'; α -SMA, forward 5'-TCCCTTGAGAAGAGTTACGA-3', reverse 5'-CCCCTGATAGGACATT-3'; E-cadherin, forward 5'-TGGACCGAGAGAGTTTCC-3', reverse 5'-AATATGGTGTATACAGC-CTC-3'; The amplified fragments of β -actin, α -SMA, E-cadherin were 295bp, 202bp, 250bp in length, respectively. The real-time PCR conditions were as follows: samples were incubated at 95°C for 5 minutes, followed by 40 cycles with 30 seconds of denaturation at 94°C, 30 seconds annealing at 60°C and 30 seconds extension at 72°C, then 5 minutes extension at 72°C. Afterwards, the amplified products were separated by gel electrophoresis on 2% agarose gel and visualized using ethidium bromide on an ultraviolet transilluminator. The intensity values were normalized to the value of GAPDH, and analyzed using the Quantity One software (Bio-Rad, USA).

Enzyme-linked immunosorbent assay (ELISA)

The concentrations of types I and III collagen were determined by ELISA. Briefly, HK-2 cells were plated overnight in culture medium at a density of 1×10^5 cells/well on 6-well dishes. Plated cells were synchronized overnight in serum-free medium and then treated as mentioned above. After isolation, types I and III collagen were measured using the ELISA kits, according to the manufacturer's instructions. The expression of types I and III collagen were normalized for the amount of cellular protein in each well.

Western blotting

The samples were normalized for total protein content with a Pierce BCA protein assay kit (Thermo Fisher Scientific, Rockford, IL, USA): 30 μ g aliquots were suspended in an equal volume of 2 \times Laemmli buffer containing 2-mercaptoethanol and boiled for 5 min before SDS-PAGE. Proteins were transferred to Immobilon-P membranes (Millipore, Billerica, MA, USA), blocked with skimmed milk (5%), and incubated with various pri-

mary antibodies recognizing E-cadherin (Abcam, USA), α -SMA (Abcam, USA), β -actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Antibody detection was accomplished via horseradish peroxidase-conjugated protein A or goat anti-rabbit immunoglobulin G (Santa Cruz Biotechnology, Santa Cruz, CA, USA), a chemiluminescent substrate (Millipore, Billerica, MA, USA), and exposure to X-ray film (Eastman Kodak Co., Rochester, NY, USA).

Statistical analysis

All the experiments were performed at least three times. All data were presented as mean \pm standard deviation (SD). Statistical differences between groups were evaluated by independent Student's *t*-tests using SPSS software (version 16.0; SPSS, Chicago, IL, USA). A *P*-value of <0.05 was considered significant.

RESULTS

Cell proliferation in HK-2 cells treated with IL-17A

To investigate the effect of IL-17A on cell proliferation in HK-2 cells, dose-dependent and time-dependent experiments were performed. Compared with the normal control, IL-17A, within the range of 0~80 ng/mL, could stimulate the proliferation of HK-2 cells in a dose-dependent manner, but the proliferation began to decline with 160 ng/mL of IL-17A, which was assessed by CCK-8 assay (*figure 1A*). Therefore, we chose the 80 ng/mL concentration of IL-17A as the optimum for further time-dependent study. We observed that 80 ng/mL of IL-17A stimulated cell proliferation in a time-dependent manner (*figure 1B*). Ultimately, 80 ng/mL of IL-17A with 72 h treatment was chosen as the best condition for future studies.

The secretion of types I and III collagen in HK-2 cells treated with IL-17A

To evaluate the effect of IL-17A on the secretion of collagen I and III in HK-2 cells, we stimulated HK-2 cells with IL-17A, and tested the concentration of collagen I and III in the supernatant using an ELISA. The results showed that within the range of 0~320 ng/mL, IL-17A could stimulate the production of collagen I and III, although the effect became weakened with concentrations over 160 ng/mL (*table 1*). In time-dependent experiments, IL-17A stimulated the production of types I and III collagen at 24, 48 and 72 h (*table 1*).

Effect of IL-17A, TGF- β 1 and TGF- β 1 antibody on protein expression of α -SMA, Col I, Col III and E-cadherin in HK-2 cells

Western blotting and immunocytochemistry were used to measure the protein expression of α -SMA and E-cadherin in HK-2 cells.

In comparison with the normal control, IL-17A and TGF- β 1 significantly stimulated the expression of α -SMA (mesenchymal marker) protein (*figure 2C*), induced the secretion of collagen I and III (*figures 2D, E*), but decreased the expression of E-cadherin (the epithelial marker) protein

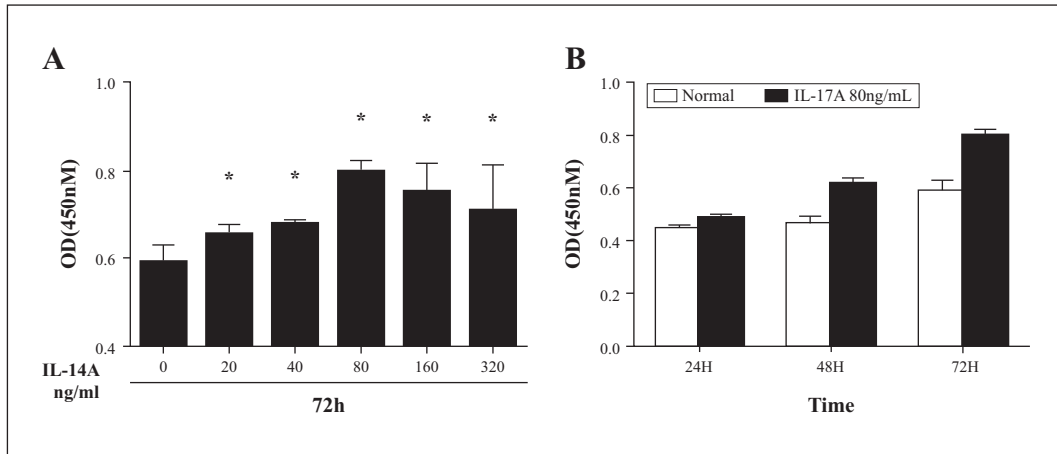


Figure 1

Effect of IL-17A on cell proliferation in HK-2 cells. (A) In dose-dependent experiments, Doses from 20 to 80 ng/mL of IL-17A promoted proliferation of HK-2 cells in a dose-dependent manner, and cell proliferation began to decline when the concentration of IL-17A was higher than 80 ng/mL as determined using a CCK-8 assay. (B) IL-17(80 ng/mL) promoted proliferation of HK-2 cells in a time-dependent manner as determined using a CCK-8 assay. * $p < 0.05$, compared with control.

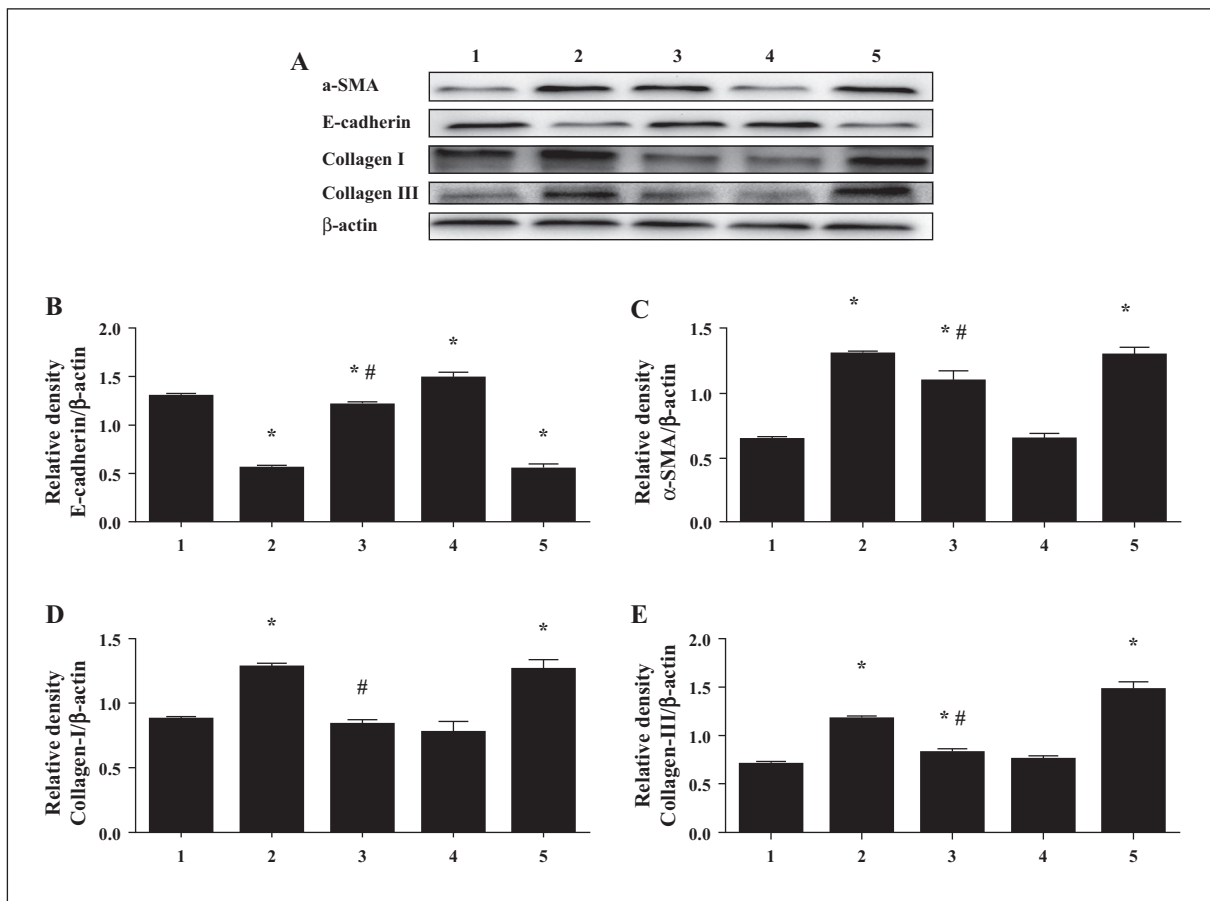


Figure 2

Effect of IL-17A, TGF-β1 and TGF-β1 antibody on protein expression of α-SMA, collagen I and III and E-cadherin in HK-2 cells as shown by western blotting. HK-2 cells were incubated with or without IL-17A (80 ng/mL) for 72 h. TGF-β1 antibody (2 μg/mL) was added 2 h before the IL-17A treatment. TGF-β1(10 ng/mL) was the positive control. The expression of E-cadherin, collagen I and III, and α-SMA was analyzed by Western blotting. (A) The results of western blotting. (B) IL-17A and TGF-β1 decreased expression of E-cadherin protein; blocking TGF-β1 with TGF-β1 antibody, expression of E-cadherin protein recovered in HK-2 cells treated with IL-17A; TGF-β1 antibody alone promoted expression of E-cadherin protein. (C) IL-17A and TGF-β1 stimulated expression of α-SMA protein; blocking TGF-β1 with TGF-β1 antibody weakened expression of α-SMA protein. (D, E) IL-17A and TGF-β1 induced production of collagen I and III in HK-2 cells, but with the addition of TGF-β1 antibody at the same time, production of collagen I and III both decreased. * $p < 0.05$, compared with control. # $p < 0.05$, compared with IL-17A group. Notes: 1 normal control group. 2. IL-17A group. 3. IL-17A+TGF-β1 antibody group. 4. TGF-β1 antibody group. 5. TGF-β1 group.

(figure 2B) as shown by western blotting. With TGF-β1 antibody blocking TGF-β1, the expression of E-cadherin recovered partially, and expression of α-SMA, collagen I and III.

Immunohistochemistry showed a similar effect. IL-17A and TGF-β1 progressed the generation of α-SMA protein (figure 3B), although they suppressed the expression of E-cadherin protein (figure 3A). If TGF-β1 antibody was

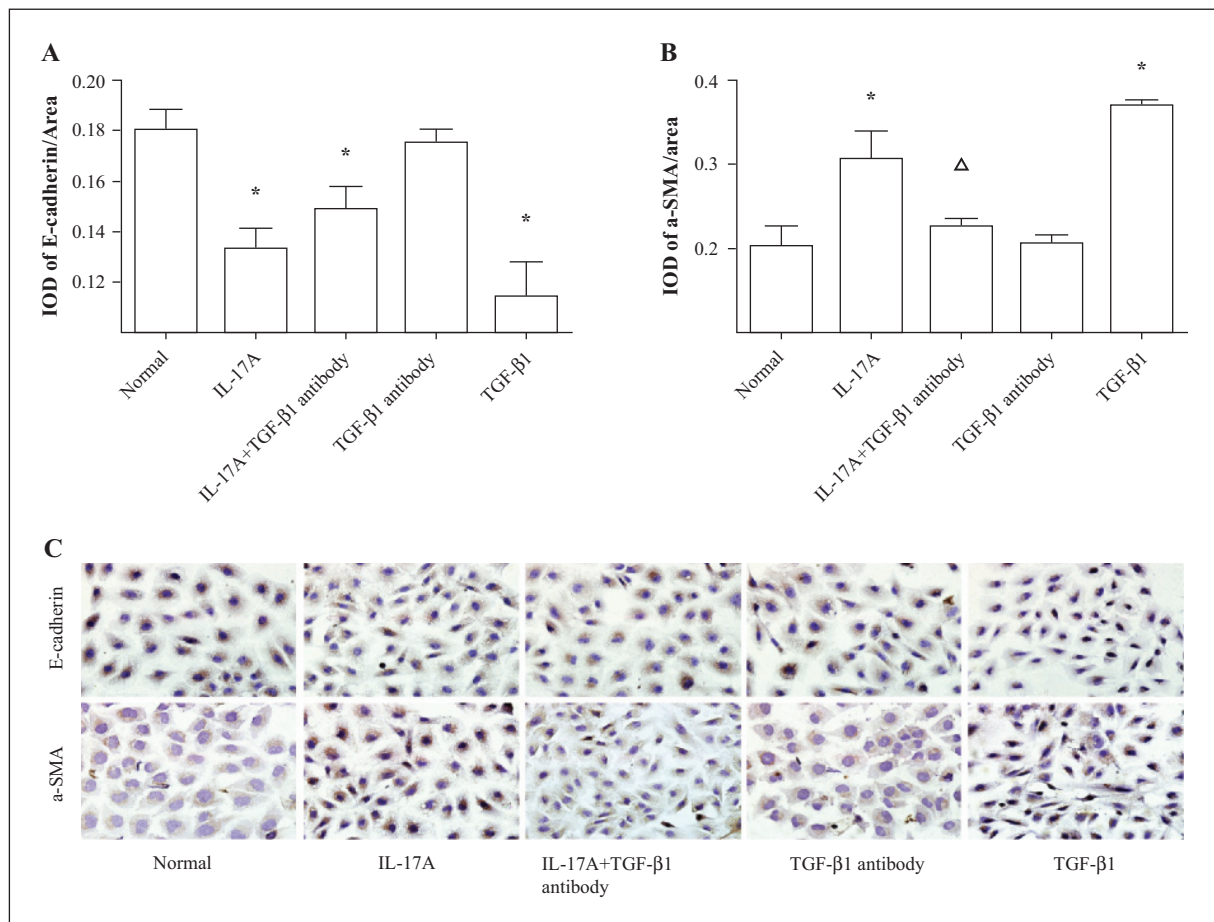


Figure 3

Effect of IL-17A, TGF- β 1 and TGF- β 1 antibody on protein expression of α -SMA and E-cadherin in HK-2 cells by immunocytochemistry. HK-2 cells were incubated with or without IL-17A (80 ng/mL) for 72 h. TGF- β 1 antibody (2 μ g/mL) was added 2 h before the IL-17A treatment. TGF- β 1 (10 ng/mL) was the positive control. **A**) IL-17A and TGF- β 1 attenuated expression of E-cadherin protein; Blocking TGF- β 1 with TGF- β 1 antibody, caused expression of E-cadherin protein recover. **B**) IL-17 and TGF- β 1 promoted expression of α -SMA; blocking TGF- β 1 with TGF- β 1 antibody decreased α -SMA expression. * p <0.05, compared with control. Δ p <0.05, compared with IL-17A group.

added before IL-17A incubation, the cells could restore E-cadherin expression, and attenuate the expression of α -SMA.

Effect of IL-17A, TGF- β 1 and TGF- β 1 antibody on mRNA expression of α -SMA and E-cadherin

RT-PCR was used to measure the mRNA expression of α -SMA and E-cadherin in HK-2 cells. In comparison with the normal control, IL-17A and TGF- β 1 significantly downregulated the mRNA expression of E-cadherin (the epithelial maker) (figure 4A) and increased the mRNA expression of α -SMA (mesenchymal maker) (figure 4B). Compared with the IL-17A group, the mRNA expression of E-cadherin was upregulated, and expression of α -SMA decreased with TGF- β 1 antibody incubation in HK-2 cells.

DISCUSSION

Inflammation is a major factor in RIF, resulting in tubular damage and accumulation of extracellular matrix. Tubular epithelial cells are the main constituent of renal interstitium, and are the main cells that suffer damage in early disease. With the progression of kidney disease, tubular epithelial cells are activated and their morphology changes.

Previous studies have found that renal tubular epithelial cells can transform into myofibroblasts, which participate in the development of RIF [21, 22]. This transformation process, epithelial cell phenotype inversion, is characterized by renal tubular epithelial cells gradually losing their markers, such as E-cadherin, and acquiring ectomesenchymal markers, such as α -SMA. α -SMA is considered a marker protein of myofibroblasts, and it is not only a symbol of epithelial cell phenotype inversion, it is closely related to the degree of fibrosis and progress of kidney disease. Myofibroblasts are rare in normal kidney tissues, but in the process of renal fibrosis, a large number of myofibroblasts infiltrate the renal tissues. Myofibroblasts may secrete types I, III, IV collagen, in particular types I and III, as well as other matrix components. In addition, the myofibroblasts may also synthesize and secrete metal protease inhibitors to inhibit the degradation of extracellular matrix, resulting in development of RIF.

A variety of pro-inflammatory cytokines can induce cells to produce abundant collagen components, leading to RIF. IL-17 is a newly discovered, pro-inflammatory cytokine. It participates widely in the development of a variety of inflammatory diseases and autoimmune diseases. Some studies have shown that IL-17 might promote the secretion of collagen in some cells. The relationship between IL-17 and tissue fibrosis has gradually become a hot topic.

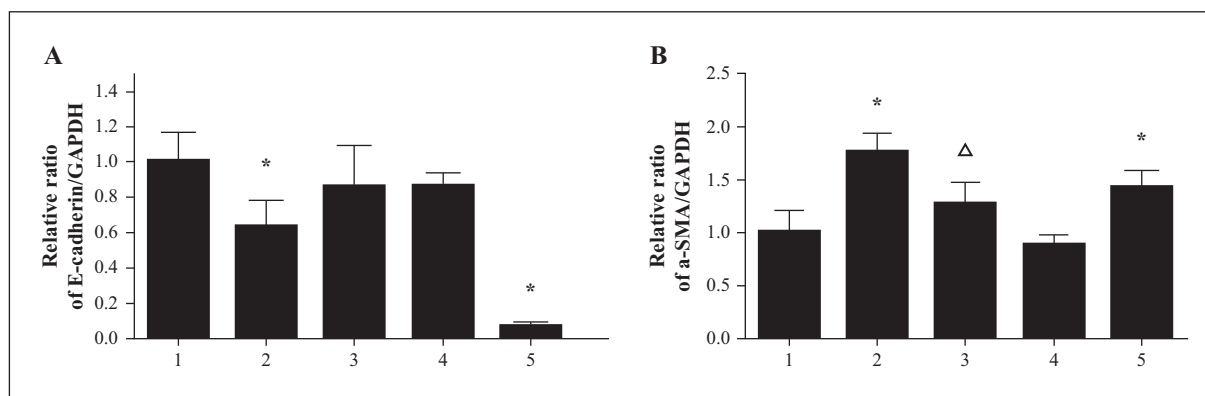


Figure 4

Effect of IL-17A, TGF-β1 and TGF-β1 antibody on mRNA expression of α-SMA and E-cadherin in HK-2 cells by RT-PCR. HK-2 cells were incubated with or without IL-17A (80 ng/mL) for 72 h. TGF-β1 antibody (2 μg/mL) was added 2 h before IL-17A treatment. TGF-β1 (10 ng/mL) was the positive control. **A**) IL-17A and TGF-β1 downregulated mRNA expression of E-cadherin. Blocking TGF-β1 with TGF-β1 antibody, the mRNA expression of E-cadherin recovered in HK-2 cells treated with IL-17A. **B**) In IL-17A and TGF-β1 groups, the mRNA expression of α-SMA increased. On blocking TGF-β1 with TGF-β1 antibody, the mRNA expression of α-SMA decreased. * $p < 0.05$, compared with control. Δ $p < 0.05$, compared with IL-17A group.

Notes: 1. normal control group. 2. IL-17A group. 3. IL-17A+TGF-β1 antibody group. 4. TGF-β1 antibody group. 5. TGF-β1 group.

IL-17 might promote the proliferation of lung fibroblasts, synthesis of collagen I and III, and increase the expression of mRNA and protein of α-SMA in bleomycin-induced lung fibrosis [23]. An investigation concerning expression of IL-17 in patients with chronic hepatitis B, cirrhosis, hepatocellular carcinoma, chronic liver failure and normal human, showed that IL-17 expression was mainly located in the portal area, and was correlated positively with inflammation grade and the stage of fibrosis [24]. A report about the role of IL-17 signaling in the pathogenesis of liver fibrosis showed that IL-17 could facilitate production of IL-6, IL-1β, and TNF-α by inflammatory cells, increase the expression of TGF-β1, and directly induce collagen type I production in hepatic stellate cells [25]. In Crohn's disease-associated intestinal fibrosis, IL-17A was significantly overexpressed in strictured compared with non-strictured Crohn's disease tissues. It significantly inhibited myofibroblast migration, significantly upregulated MMP-3, MMP-12, tissue inhibitor of metalloproteinase-1, and collagen production by myofibroblasts from strictured Crohn's disease tissues [26]. In the study of skin fibrosis, the loss of IL-17A significantly attenuated bleomycin-induced skin fibrosis: daily bleomycin injections induced the expression of IL-17A in the skin and potent IL-17A producers in splenic CD4+ T cells from wild-type mice; Furthermore, a skin fibroblast cell line expressed increased TGF-β, CTGF, and collagen after the addition of recombinant IL-17A [27]. To sum up these results, we found that IL-17A is closely involved in liver, lung, intestine, skin tissue fibrosis, but until now the role of IL-17 in renal fibrosis has remained unknown. In the present study, we found that IL-17A can promote cell proliferation and production of collagen I and III in HK-2 cells in a time- and dose-dependent manner. In addition, IL-17A can promote expression of α-SMA at the protein and gene levels, decrease the expression of E-cadherin, and mediate epithelial cell phenotype inversion in HK-2 cells. In conclusion, these results suggested that IL-17A could induce tubular cell phenotype inversion and take part in RIF.

Most scholars believe that TGF-β is a key mediator in the pathogenesis of renal fibrosis. The TGF-β superfamily is composed of more than 35 members, including TGF-β,

BMPs, growth differentiation factor, etc. They are involved in cell differentiation, migration and adhesion, maintaining homeostasis in tissues [28, 29]. There are three different forms of TGF-β (β1, β2 and β3) in mammals, they all show similar biological activities, but for tissue fibrosis, it is TGF-β1 that plays a major role [30]. TGF-β1 mediates progressive renal fibrosis mainly via the TGF-β1/Smads signaling pathway, in which Smad3 plays a central role for renal epithelial cell phenotype inversion [31-34]. A number of cytokines such as IL-1, IL-6, IL-4 might also induce epithelial cell phenotype inversion via the TGF-β signaling pathway. In pulmonary fibrosis, IL-17A increased the synthesis and secretion of collagen and promoted the epithelial-mesenchymal transition in a TGF-β1-dependent manner. It also attenuated the starvation-induced autophagy, with autophagy modulators regulating collagen degradation [35]. In the present study, in HK-2 cells incubated with IL-17A and TGF-β1 neutralizing antibodies, the expression of collagen I and III and α-SMA was significantly lower than in the IL-17A only group, however, the expression of E-cadherin significantly recovered. These results suggest that IL-17A might mediate epithelial cell phenotype inversion in kidney by a TGF-β1 signaling pathway. This, of course, needs further research.

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