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High Uric Acid-Induced Epithelial-Mesenchymal Transition of Renal Tubular Epithelial Cells via the TLR4/NF-kB Signaling Pathway

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Keywords

Uric acid · Toll-like receptor 4 · Epithelial mesenchymal transition · Inflammatory factor

Abstract

Background: Hyperuricemia is an independent risk factor for causing chronic kidney disease and contributes to kidney fibrosis. After urate crystals are deposited in the kidney, they can cause hyperuricemia nephropathy, leading to glomerular hypertrophy and renal tubular interstitial fibrosis. Recent data showed that uric acid (UA) could induce epithelial mesenchymal transition (EMT) of renal tubular cells, in which NRLP3 inflammatory pathway was involved. However, whether TLR4/NF-κB signaling pathway is also involved in EMT of renal tubular cells induced by UA is not clear. ***Methods:*** Human renal tubular epithelial cells (HK-2) were directly exposed with UA and the phenotypic transition was examined by morphological changes and the molecular markers of EMT. The activation of the TLR4/NF-κB signaling pathway induced by UA was measured by Western blot and its involvement was further confirmed by the inhibition of NF-κB activation or knockdown of toll like receptor 4 (TLR4) expression. ***Results:*** UA induced obvious morphological changes of HK-2 cell, accompanied with altered molecular markers of EMT including fibronectin, α-SMA and E-cadherin. In addition, UA significantly upregulated the gene expression of interleukin-1β and tumor necrosis factor-α in a time- and dose-dependent manner. Furthermore, UA significantly activated the TLR4/NF-κB signaling pathway in HK-2 cells, while the inhibition of the TLR4 expression by siRNA and NF-κB activation by PDTC significantly attenuated EMT induced by UA in HK-2 cells. ***Conclusions:*** UA can induce EMT in renal tubular epithelial cells by the activation of the TLR4/NF-κB signaling pathway, and the targeted intervention of the TLR4/NF-κB signaling pathway might effectively inhibit UA-induced renal interstitial fibrosis mediated by EMT.

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Introduction

Uric acid (UA) is a biochemical product of purine metabolism, which is associated with gout and kidney calculi [1]. Hyperuricemia is a very common occurrence

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in chronic kidney disease (CKD) and associated with the development and progression of CKD [2]. At the same time, hyperuricemia is an independent risk factor for causing cardiovascular disease. CKD patients with hyperuricemia have higher risk for cardiovascular and all-cause mortality [3], while lowering UA can delay the progression of CKD. When UA gets deposited in the kidney for a long time, it can cause hyperuricemia nephropathy, leading to glomerular hypertrophy and renal tubular interstitial fibrosis [4]. Thus, UA is likely an important mediator and risk marker in CKD. Recent studies suggested a role of UA in the causation and progression of kidney fibrosis, a final common pathway in CKD [5].

Nowadays, multiple mechanisms leading to hyperuricemia nephropathy have been reported, such as endothelial dysfunction, renal angiotensin system activation, oxidative stress, and tubular epithelial cell transition [6]. Among them, epithelial mesenchymal transition (EMT) plays an important role in the renal interstitial fibrosis. EMT refers to a phenotypic transition of renal tubular cells, in which renal tubular cells lose their epithelial phenotypes and acquire new characteristic features of mesenchymal phenotypes. Studies have shown that long time deposition of UA in the kidney can cause renal tubular EMT and renal interstitial fibrosis [7]. Interestingly, EMT induced by UA was found to be associated with NLRP3 inflammatory pathway in a rat model of renal fibrosis [8]. More recently, a traditional Chinese recipe was confirmed to reduce serum UA levels and ameliorate renal fibrosis by the inhibition of EMT in a rat model [9]. Thus, EMT is one of the earliest phenomena of renal fibrosis and has been considered a therapeutic target due to its reversible characteristic features [10].

Both chronic inflammation and EMT is closely associated with renal interstitial fibrosis, while inflammation can be triggered by hyperuricemia [11]. Recent studies have found that toll-like receptor 4 (TLR4) plays an essential role in the regulation of inflammation in diabetic nephropathy [12, 13], but whether it is involved in renal tubular EMT induced by hyperuricemia is not clear. The NF-κB is an important target gene of TLR4, which is responsible to produce inflammatory factors at the transcriptional level [14]. NF-κB signaling is the central cause of pathogenesis in many disease conditions, and it is one of the major targets for therapeutic intervention.

To further understand the mechanisms of UA-associated kidney disease, we investigated the effect of UA on phenotypic transition of renal tubule epithelial cells and elucidated the molecular mechanism in this work. We found that UA could induce EMT by activating the TLR4/NF-κB signaling pathway in HK-2 cells. This helps to understand the detrimental effects of UA and might provide a therapeutic target for CKD patients.

Materials and Methods

Human renal tubular epithelial cells (HK-2) were purchased from American Type Culture Collection. Antibody against α-SMA was obtained from Millipore (MA, USA), and other antibodies were purchased from BOSTER Biological Technology (Wuhan, China). Trizol RNA extraction kit, reverse transcription kits, and SYBR Green polymerase chain reaction (PCR) Master Mix were obtained from Takara (Kyoto, Japan). PDTC was obtained from Beyotime Biotechnology (Nantong, China). All the primers were obtained from Shanghai Sangon Biotechnology (Shanghai, China).

HK-2 Cell Culture and Treatment
HK-2 cells were obtained from the American Type Culture Collection (Manassas, VA, USA) and grown in Dulbecco's modified Eagle's Medium/F-12 supplemented with 10% heat-inactivated fetal bovine serum, 100 U/mL penicillin, and 100 mg/mL streptomycin (GIBCO, CA, USA) in a humidified atmosphere of 5% CO_2 at [illegible].

For [illegible] treatment, exponentially growing HK-2 cells were [illegible] 4×10^5 cells/well in 6-well culture plates. UA (Ultrapure, Sigma) was dissolved in warm media and filtered. Before UA treatment, HK-2 cells were kept in serum-free media for 12 h prior to stimulation, and then changed with complete media with increasing concentration of UA for another 2 days. As for PDTC, HK-2 cells were pretreated with PDTC (20 μM) for 12 h and then further treated with UA.

Cell Morphological Observation
HK-2 cells were treated with UA for 48 h, and morphological changes were examined under an inverted phase contrast microscope (Axiovert 200; CarlZeiss, Oberkochen, Germany), and the images were obtained by a digital camera (AxioCam HRC; Carl Zeiss).

Total RNAs Extraction
Total RNAs were isolated from HK-2 cells using Trizol reagent according to the manufacturer's instructions. The quality of the extracted RNA was monitored by formaldehyde agarose gel electrophoresis and the quantity was measured by Nanodrop spectrophotometer.

Real-Time RT-PCR
An amount of 0.5 μg RNA was reverse transcribed to cDNA according to the manufacturer's directions (Roche). Real-time PCR (RT-PCR) was performed as previously described [15]. Sequence of primers could be available upon demand. Gene expression was normalized to the corresponding β-actin level and was presented as fold change relative to that of the control.

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Western Blot Analysis

UA-treated HK-2 cells and controls were homogenized in ice-cold RIPA buffer containing complete protease inhibitors cocktail (Roche). The mixture was then centrifuged at 12,000 *g* at 4 °C for 15 min and the protein concentration in the supernatant was determined using Bradford assay. Western blotting was performed as previously described [16]. The expression of each protein was normalized by that of β-actin.

Immunofluorescence Staining

Immunofluorescence staining was performed as previously described [16]. HK-2 cells were washed and fixed in 4% phosphate-buffered paraformaldehyde (25 min at room temperature) and permeabilized with 0.2% Triton X-100 in PBS (15 min). After being washed with PBS and blocking with 5% BSA for 1 h, cells were incubated with primary antibodies against E-cadherin or α-SMA in 5% BSA overnight at 4 °C, followed by an incubation with goat anti-mouse IgG-FITC-conjugated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h at room temperature in the dark. The nucleus was counterstained with DAPI and the cells were visualized under the Axiovert 200 fluorescence microscope (Carl Ziss).

Transfection of siRNA

HK-2 cells were transfected with specific siRNA against TLR4 or control siRNA using Lipofectamine 2000 (Roche) according to the manufacturer's instructions. Then cells were recovered and further cultured in media with or without UA for an indicated period.

Statistical Analysis

SPSS 20.0 was used for statistical analysis. All data are pre ed as means ± SD. The differences between 2 groups were ete mined by *t* test. Intergroup comparisons were made us wayanalysis of variance. A *p* value less than 0.0 w co ed significant.

Results

High UA Induced Morph Changes and EMT in HK-2 Cells

HK-2 cells were tre d wi inc easing concentration of UA (0, 9, 12, and g/dL) 8 h, and the morphology of HK-2 ell we served by inverted light microscope. Com with al cultured HK-2 cells, UA-treated HK-2 re remodeled and the normal morpholog fou be with increased branches and interc ula pace instead of a spindle shape (Fig. 1a). Furtherm the changes were much apparent at higher ncen showing a dose-dependent manner. In or detect EMT induced by UA, the protein expression of ectin, α-SMA (mesenchymal cell marker) and E- dherin (epithelial marker) were detected by Western t. The results showed that the protein levels of fibronectin and α-SMA expression were significantly increased, while the epithelial marker E-cadherin expression was reduced, which was in a dose-dependent manner. This suggested that UA could induce EMT in HK-2 cells (Fig. 1b).

The Expression of Inflammatory Cytokines was Induced by High UA in HK-2 Cells

HK-2 cells were treated with increasing entra of UA (0, 9, 12, and 15 mg/dL), and total R as h vested for quantitative RT-PCR analysis. As in Figure 2, mRNA expression of interleuki (IL-1 d tumor necrosis factor-α (TNF were regulated by UA in a dose-dependent manner, mpa hat of the control group, both at 4 and after treatment. Time course response sh at t regulation of IL-1β and TNF-α could b ted at 2 h after treatment, with expression g at me points, which was clearly in a time ndent manner. These results showed that gh UA c nduce the expression of inflammato s in H cells.

NF-κB W lved in High UA-Induced EMT in HK-2 Cells

as reporte hat the phosphorylated NF-κB expres increased when NF-κB signal pathway was acti ted To detect whether NF-κB could be activated UA K-2 cells were treated with UA for 48 h and the level of pNF-κB was measured by Western blot. Compared with that of the control group, the pNF-κB xpression was significantly increased after incubated with 12 and 15 mg/dL UA (Fig. 3a). To further confirm the role of NF-κB in UA-induced EMT, PDTC, an inhibitor of NF-κB, was applied to pretreated HK-2 cells, followed by the incubation of 15 mg/dL UA. As shown in Figure 3b, the activation of NF-κB could be obviously inhibited by PDTC. Moreover, the altered protein levels of fibronectin, α-SMA, and E-cadherin induced by UA were also reversed by PDTC pretreatment. Furthermore, the expression of cytokeratin (a marker of epithelial cell) and Vimentin (an indicator of mesenchymal phenotype) was also detected by immunofluorescence. As shown in Figure 3d, e, the increased expression of Vimentin and the decreased expression of cytokeratin induced by UA were significantly reversed by PDTC pretreatment. In addition, we also measured the effect of PDTC on the expression of inflammatory cytokines. Compared with the levels of the control group, the elevated mRNA levels of IL-1β and TNF-α induced by UA were significantly attenuated by PDTC (Fig. 3f). All these data clearly showed that NF-κB was involved in UA-induced EMT in HK-2 cells.

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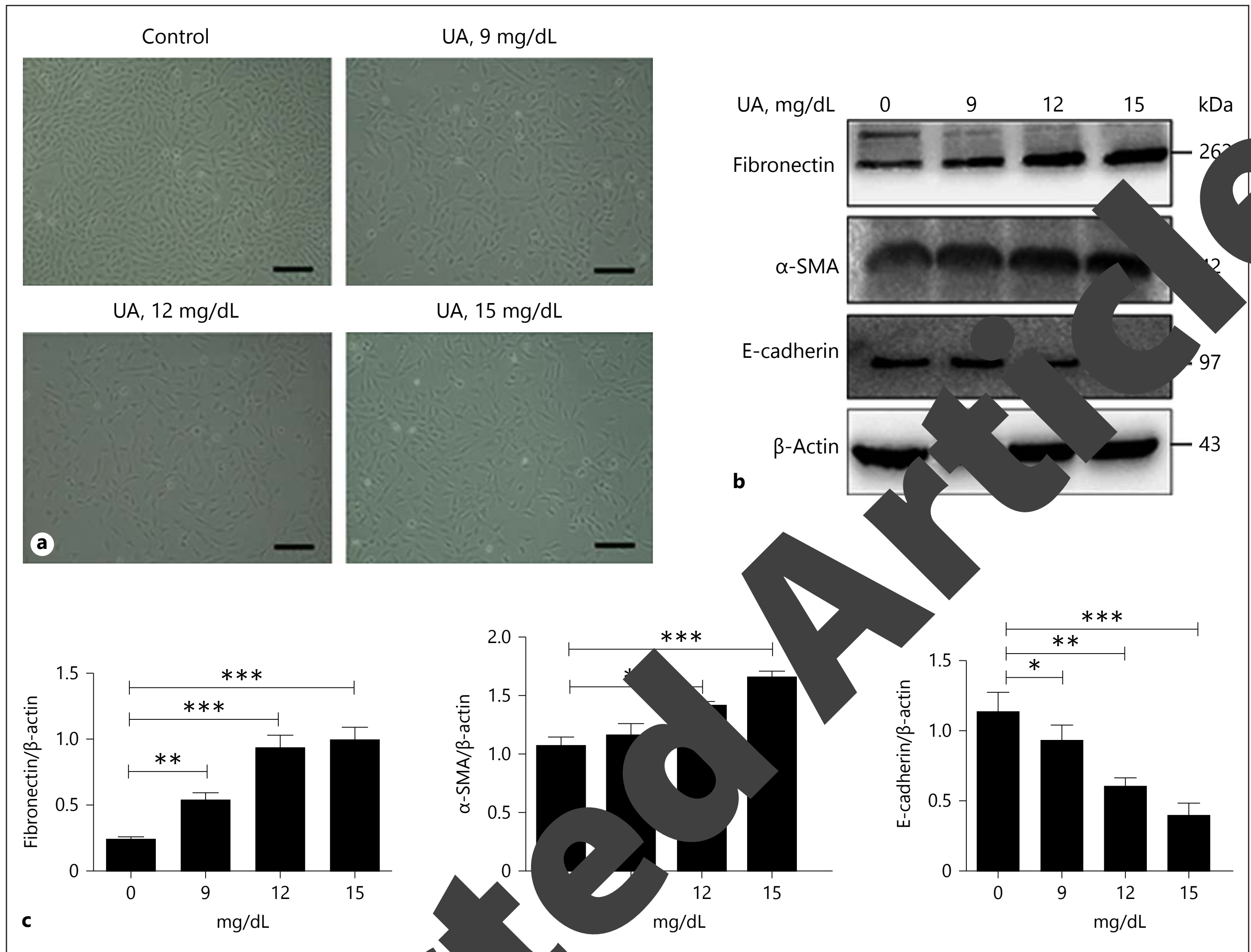

Fig. 1. High UA induced morphologica anges and MT in HK-2 cells. Human HK-2 cells were tre with n increasing concentration of UA for 48 h, and the m f HK-2 observed by inverted light micros Total proteins were isolated for Western blot to detec xpression of fibronectin, α-SMA, and E-cadherin (**b**), and the density of bands were analyzed by quantity one (**c**). The scale bar: 100 μm. β-actin protein expression was determined as a control for equal loading. Western blotting was repeated at least 3 times. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Knockdo TL uppressed High UA-Induced EMT in Cells

NF-κB ca ivated by many upstream signals. Under the dition onic inflammation, TLR4 signaling c ad ntributes to renal dysfunction [17]. Thus, e fu in igated whether TLR4 was involved in h U ced EMT in HK-2 cells. The protein ex n of TLR4 was detected by Western blot and result sho hat UA induced TLR4 expression in a dose- and me-dependent manner (Fig. 4a, b). To further confirm role of TLR4 in UA-induced EMT, we used siRNA to knockdown the expression of TLR4 in HK-2 cells prior to UA treatments. As shown in Figure 4c, the protein level of TLR4 could be efficiently downregulated by specific siRNA, and the phosphorylated NF-κB expression induced by UA was suppressed subsequently. Similarly, the protein markers of EMT induced by UA were also attenuated by TLR4 siRNA, as measured by Western blot (Fig. 4c, d). In addition, the elevated mRNA levels of IL-1β and TNF-α induced by UA were also significantly attenuated by TLR4 siRNA, while TLR4 siRNA alone had no such effects (Fig. 4e). These data suggest that the TLR4/NF-κB signal pathway plays an important role in UA-induced EMT in HK-2 cells.

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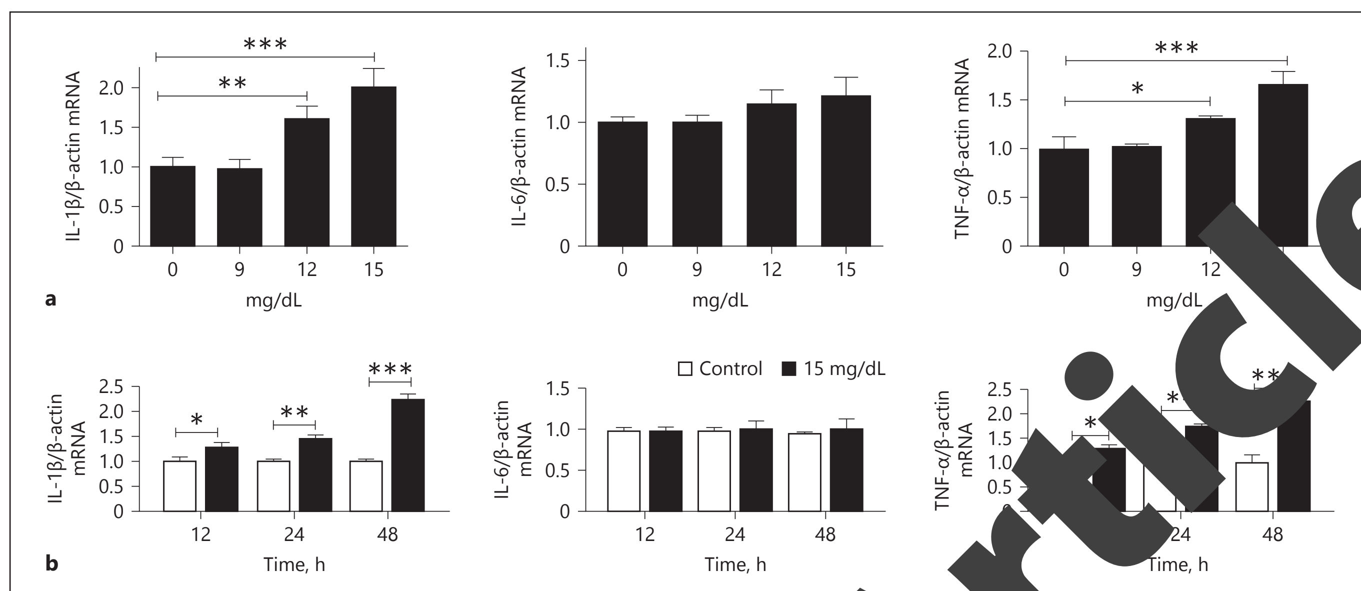
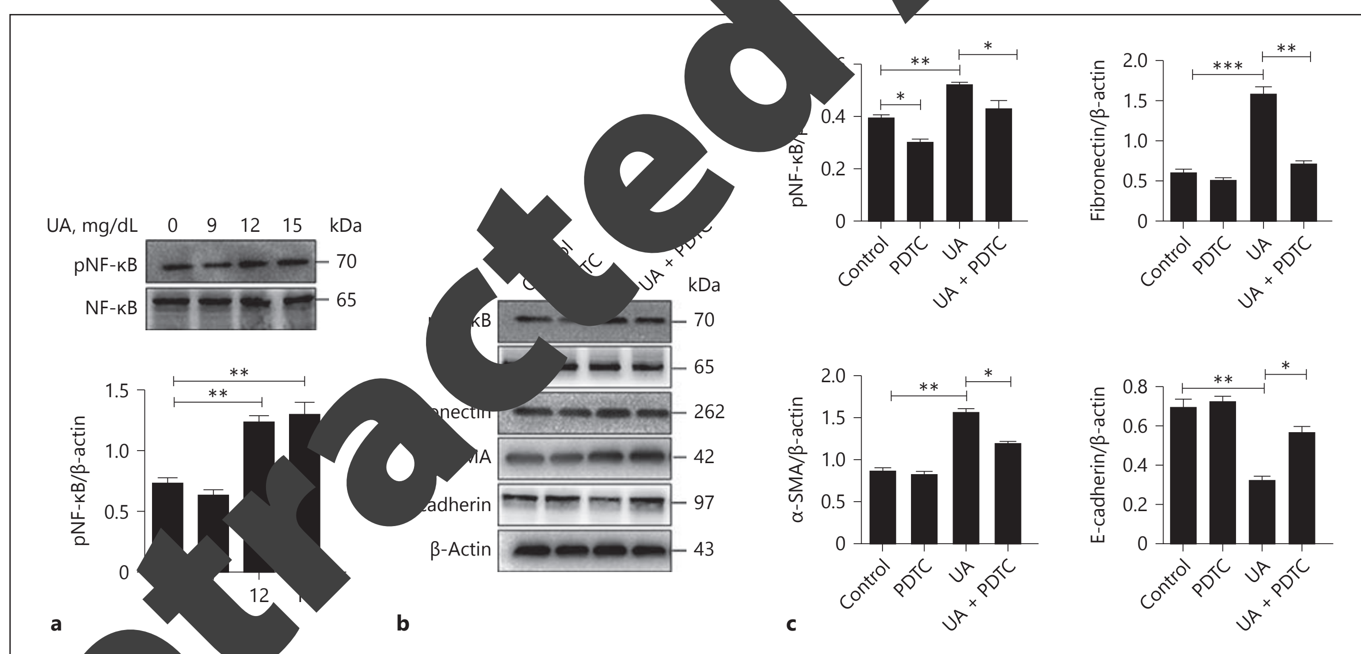


Fig. 2. Gene expression of inflammatory cytokines was induced by high UA in HK-2 cells. Human HK-2 cells were treated with increasing concentration of UA for 24 h, and the total RNAs were isolated for real-time RT-PCR to detect the gene expression of IL-1β and TN[illegible] (**a**). [illegible]lyze th[illegible]me-course response, HK-2 cells were treated [illegible] UA [illegible]/dL) for indicated periods, and then RNAs were is[illegible] fo[illegible]time RT-PCR (**b**). * $p < 0.05$, ** $p <$ [illegible]01, *** $p < 0.0$[illegible]



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(For figure d–f and legend see next page.)

[illegible]cussion

In CKD, hyperuricemia was previously thought to be a [illegible]nsequence of the declined glomerular filtration rate, rather than a risk factor for development or progression of kidney disease. However, recent epidemiological studies have shown that in healthy subjects UA itself can also promote the development of CKD [18]. When UA excretion is impaired, urate crystal deposition in kidneys, which was similar to gouty arthritis, can cause renal tubular epi-

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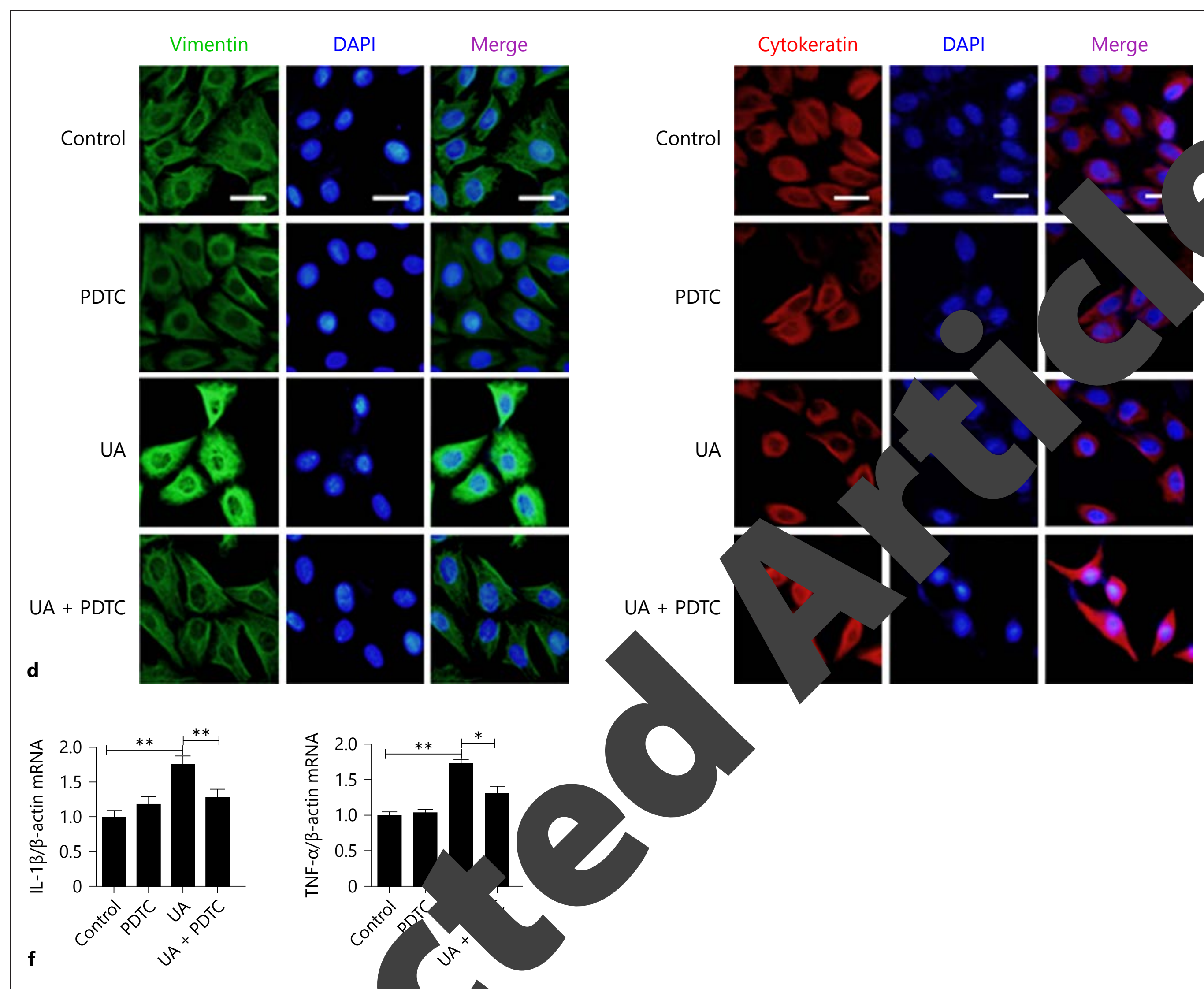

Fig. 3. NF-κB was involved in [illegible]-ind[illegible] EMT in HK-2 cells. Human HK-2 cells were [illegible]te[illegible] [illegible]n increasing concentration of UA for 24 h, and the t[illegible]l p[illegible]ns were isolated for Western blot to detect the [illegible]tein [illegible] [illegible]NF-κB (**a**). To confirm the involvement of NF[illegible] HK-2 c[illegible]ere pretreated with PDTC (20 μM) and th[illegible]n furthe[illegible]d with UA (15 mg/dL) for 24 h, and the protein le[illegible] [illegible]NF-κ[illegible]onectin, α-SMA, and E-cadherin were determi[illegible] [illegible]Western [illegible]ot (**b**), and the density of bands was analyzed by quant[illegible] Immunofluorescence staining of Vimentin and cytokeratin was carried out on similar treated HK-2 cells as described in materials and methods (**d**, **e**). For real-time RT-PCR, similar treated HK-2 cells were harvested and the gene expression of IL-1β and TNF-α was performed as described in materials and methods (**f**). The scale bar: 10 μm. Total NF-κB or β-actin protein expression was determined as a control for equal loading. Western blotting was repeated at least 3 times. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

[illegible]lial [illegible]ury, endothelial dysfunction, activation of [illegible] angiotensin system and other pathological changes such [illegible] oxidative stress. All these alterations eventually [illegible]d to renal tubular interstitial fibrosis and glomerular [illegible]erosis [19]. Chronic renal fibrosis is the common feature of CKD progression, and delaying or preventing renal fibrosis is the key intervention of CKD patients [20]. In recent years, studies of renal interstitial fibrosis are becoming more and more interesting. The EMT of renal tubule cells during the process of renal interstitial fibrosis plays a vital role [21]. However, the detailed molecular mechanisms have not been fully understood.

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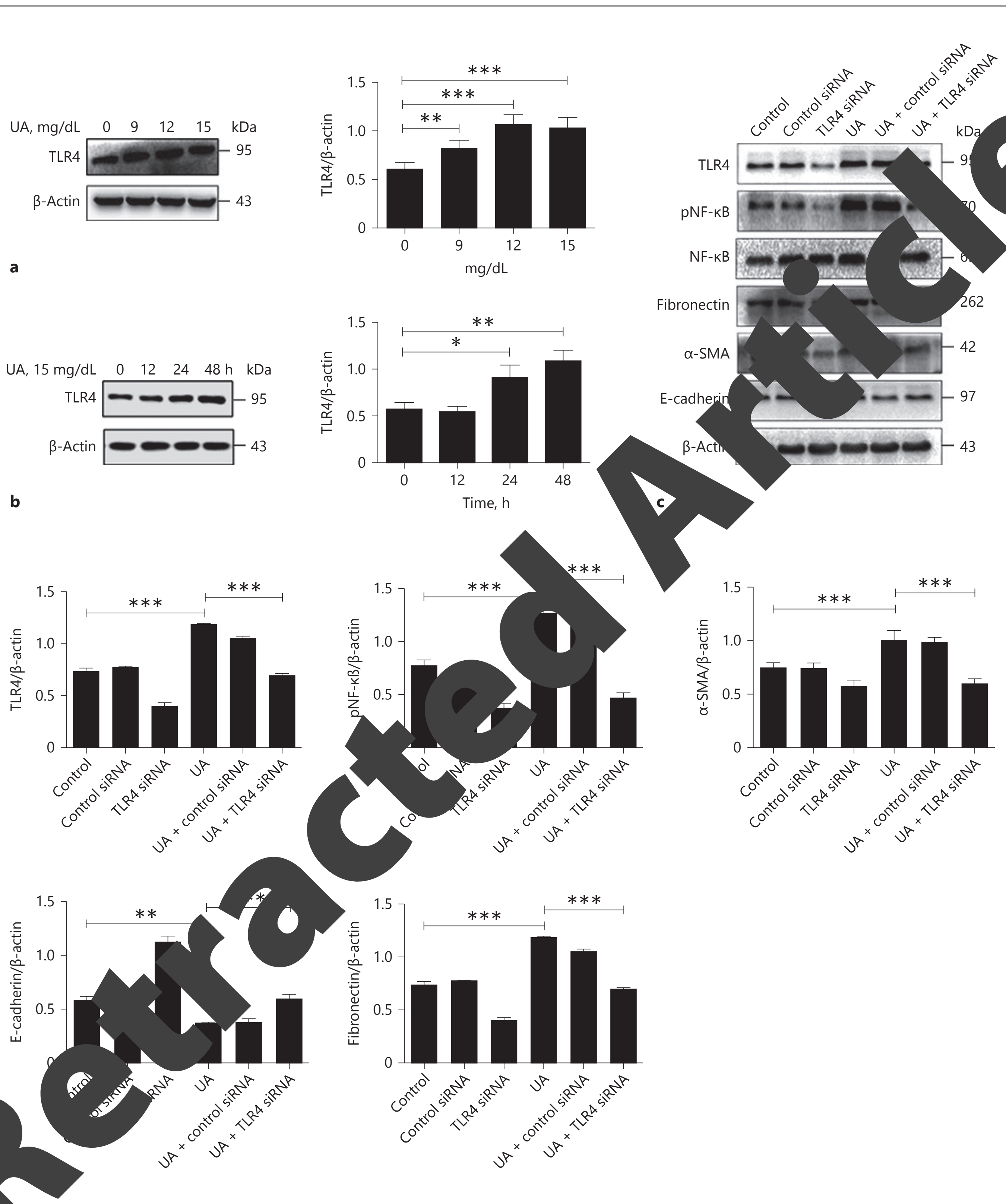

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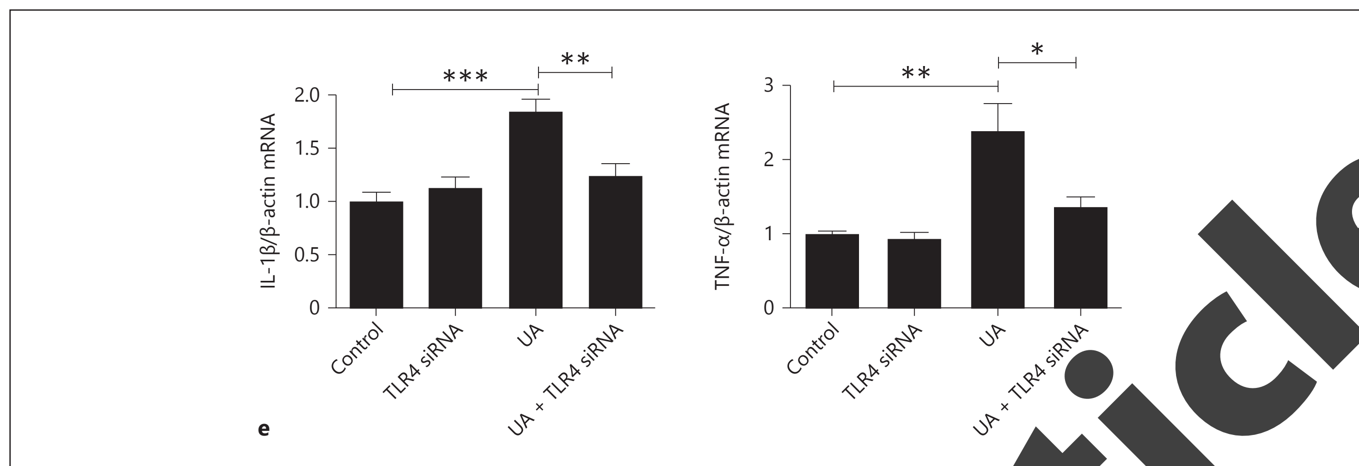

Fig. 4. Knockdown of TLR-4 suppressed high UA-induced EMT in HK-2 cells. Human HK-2 cells were treated with an increasing concentration of UA for 24 h (**a**) or with UA (15 mg/dL) for indicated periods (**b**). Total proteins were isolated for Western blot to detect the protein level of TLR4. To knockdown the expression of TLR4, HK-2 cells were transfected with TLR4 siRNA or control siRNA, and then further treated with UA for 24 h. Then the protein levels of TLR4, pNF-κB, fibro ... and E-cadherin were detected by Western blot ... the ... of bands were analyzed by quantity one ... e expression of IL-1β and TNF-α was detected ... al-time R ... (**e**). Total NF-κB or β-actin protein expres ... determin ... a control for equal loading. Western blo ... w ... ted at least 3 times. * $p < 0.05$, ** $p < 0.01$, *** $p < 0$ ...

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This study confirmed that UA could significantly induce EMT of renal tubular epithelial cells, including morphological changes as well as upregulation of mesenchymal markers. In a recent study, soluble UA was f ... to cause renal interstitial fibrosis closely associat ... wi ... the NLRP3 inflammatory pathway [22]. ... enal in ... t cells and immune-regulating cells are th ... gets o ... flammation. After injury, the renal i ... ell ... can produce a variety of cytokines and ad ... ion m ... es as well as other inflammatory mediat ... which mediated the infiltration and activation of CD ... dendritic cells, and neutrophils. The ... unological cells migrate to the kidney, and s ... se ... secreted IL-6, IL-1β, TNF-α, IL-18, IL-1 ... MC ... chemokines, leading to amplify the i ... mmato ... sponse [23]. A variety of inflamma ... cyt ... s and chemokines are associated with a ... ds of ... y diseases, including acute kidney inju ... chronic renal fibrosis. A pervious study s ... d tha ... uble UA could stimulate monocytes ... od ... IL-1β, IL-6, and TNF-α, and promote re ... al fi ... [2 ... TNF-α is a very important proinflam ... tory ... which stimulates renal tubular epithelial ... produce chemokines and recruitment of inflam ... mat ... cells [25, 26]. IL-1β and IL-6 are the vital cyto ... nes secreted by inflammatory cells in the process of re ... interstitial fibrosis, and is closely related to the development of renal fibrosis [27]. We detected the expression ... IL-6, and TNF-α mRNA by R-T PCR, and the ... sults ... ved that the expression of IL-1β and TNF-α ... was increased in a dose- and time-dependent ... except IL-6. The discrepancy of IL-6 gene expression might be related to UA concentration or the genetic background of the cell line. Except IL-6, the gene expression of other cytokines induced by UA in HK-2 cells is consistent with the finding of previous studies [28, 29].

TLR4, which is mainly expressed in glomerular mesangial cells and renal tubular epithelial cells, is a pattern recognition receptor, which is responsible for the detection and recognition of exogenous pathogen-associated molecular patterns and endogenous damage-associated molecular patterns. It is an important upstream regulatory factor of immunological and inflammatory response. As UA is thought to be a kind of damage-associated molecular pattern released from ischemic tissues and dying cells [22, 30], we speculate that UA can activate TLR4 directly. Studies have confirmed that TLR4 can promote the expression of a variety of inflammatory factors, and then activate inflammatory response by regulating the NF-κB signals. The NF-κB is an important transcription factor, involved in the immune regulation and differentiation [31]. During normal physiological conditions, the NF-κB is mainly expressed in the cell cytoplasm and inhibited by IκB. However, when cells were stimulated by cytokines,

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virus and oxidative stress, NF-κB was activated and then transferred to the nucleus to regulate gene transcription. Previous studies have showed that the NF-κB is closely related to a variety of signal transduction in cells, and also plays an important role in the process of development of renal interstitial fibrosis [32]. The NF-κB is extensively expressed in renal glomerular cells and renal tubular epithelial cells. The activation of NF-κB can mediate inflammatory response, promote renal tubular epithelial cells turn into fibroblasts cells, and upregulate the expression of a variety of inflammatory factors and chemokines including IL-6, IL-1β, and TNF-α, which results in the promotion of immune inflammatory reaction and renal tubule interstitial fibrosis [33, 34]. Our results found that UA could activate TLR4/NF-κB signaling pathways and induce EMT of renal tubular epithelial cells. At the same time, immunofluorescence also confirmed that UA can induce the upregulation of Vimentin and cytokeratin in HK-2 cells, while PDTC or TLR4 siRNA pretreatment could reverse the EMT induced by UA. The results showed that inhibition of TLR4/NF-κB signaling could reverse EMT and renal interstitial fibrosis induced by UA.

In summary, we demonstrated that UA could activate the TLR4/NF-κB signaling pathway, which in turn promoted the production of inflammatory cytokines. Subsequently, these cytokines contributed to the EMT of renal tubular epithelial cells. The inhibition of TLR4/NF-κB activation attenuated UA-induced EMT and inflam[illegible]tory cytokines expression in HK-2 cells, w[illegible] migh[illegible] a potential therapeutic target for hyperu[illegible]ia [illegible]phropathy.

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Acknowledgments

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Disclosu[illegible]

The authors [illegible]re that there are no conflicts of interest to d[illegible]e.

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