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ORIGINAL PAPER

Lefty-1 inhibits renal epithelial–mesenchymal transition by antagonizing the TGF-β/Smad signaling pathway

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Abstract

Epithelial–mesenchymal transition (EMT) is a biological process in which tubular epithelial cells lose their phenotypes, and new mesenchymal feature are obtained. In particular, type II EMT possibly contributes to renal tissue fibrogenesis. Recent studies indicate that Lefty-1, a novel member of the TGF-β superfamily with pleiotropical and biological regulation characteristics on TGF-β and other signaling pathways, is considered to have potential fibrotic effects. However, its role in EMT, which is often a long-term consequence of renal tubulointerstitial fibrosis, remains unknown. In this study, we found that Lefty-1 alleviates EMT induction through antagonizing TGF-β/Smad pathway in vivo and in vitro. In unilateral ureteral obstruction (UUO) model mice, administration of adenovirus-mediated overexpression of Lefty-1 (Ad-Lefty-1) significantly reduced TGF-β1/Smad expression and alleviated the phenotypic transition of epithelial cells to mesenchymal cells and extracellular matrix (ECM) accumulation. In high glucose-induced rat renal tubular duct epithelial cell line (NRK-52E), EMT and ECM synthesis were alleviated with Lefty-1 treatment, which significantly inhibited TGF-β1/Smad pathway activation in UUO mice and high glucose-treated NRK-52E cells. Thus, Lefty-1 can alleviate EMT and renal interstitial fibrosis in vivo and in vitro through antagonizing the TGF-β/Smad pathway, and Lefty-1 might have a potential novel therapeutic effect on fibrotic kidney diseases.

Keywords Lefty-1 · HG · EMT · TGF-β · UUO

Abbreviations

CKD	Chronic kidney disease
HG	High glucose
UUO	Unilateral ureteral obstruction
DMEM	Dulbecco minimum essential medium
MOI	Multiplicity of infection
ANOVA	Analysis of Variance
MTT	3-(4,5-Cimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

Introduction

Renal interstitial fibrosis is a common pathway of chronic renal disease progression to end-stage renal failure (Farris and Colvin 2012; Liu 2011). The main pathological processes of renal interstitial fibrosis, includes inflammatory cell infiltration, fibroblast activation and proliferation, and loss of renal tubular epithelial cells (Duffield 2014). In renal interstitial fibrosis, EMT is increasingly considered as an important part of pathological changes (Carew et al. 2012). EMT is a complex biological process involved in embryonic development, wound healing, tumor progression, and organ fibrosis. Usually associated with organ fibrosis is type II EMT, which in renal fibrosis is associated with phenotypic transformation of renal tubular epithelial cells, loss of epithelial phenotypes, such as E-cadherin and other junctional proteins, and acquisition of new mesenchymal protein characteristics, such as alpha smooth muscle actin (α-SMA), laminin, and vimentin (Galichon et al. 2013; Kalluri and Neilson 2003; Li et al. 2015). These phenotypical changes include the loss of normal cell–cell-basement membrane contact and spindle-shaped morphology of tubular epithelial

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cells similar to that of mesenchymal/myofibroblast cells. Early studies showed that renal tubular epithelial cells could express interstitial fibroblast markers in disease states, and a growing number of evidence indicates that EMT is a major pathway that leads to the generation of myofibroblasts with ECM-producing features; approximately 30% of myofibroblasts are derived via EMT from the renal tubular epithelial cells during renal fibrosis (He et al. 2013; Kalluri and Weinberg 2009). In recent research, TGF-β1-induced EMT can be reversed via the reduction of expression and phosphorylation of Smad2/3, leading to the reduction of myofibroblast formation and ECM synthesis (Wang et al. 2017; Xu et al. 2019). Thus, EMT regulation might be a potential target for the treatment of renal interstitial fibrosis.

Increasing number of studies showed that TGF-β signaling has a key role in EMT and renal interstitial fibrosis (He et al. 2018; Park et al. 2018; Wang et al. 2019b). Multifunctional TGF-β signaling pathway is involved in diverse cellular functions, including cellular proliferation, apoptosis, differentiation, and wound healing and is a potent inducer of ECM synthesis (Gu et al. 2019; Loeffler 2019). Aberrant activation of TGF-β signaling pathway results in several renal pathological consequences, including glomerulonephritis, diabetic nephropathy, and renal tubular interstitial fibrosis (Zhou et al. 2018). Especially in renal fibrosis, TGF-β signaling pathway activation is triggered by a variety of etiology, including inflammation, immunity, and obstructive, metabolic, or other systemic diseases. TGF-β binding to its receptor II (TβRII) can activate (TβRI) kinase, resulting in Smad2 and Smad3 phosphorylation. Phosphorylated Smad2/3 with Smad4 forms a common Smad complex and transfers into the nucleus to regulate target gene transcription. As a result, activated TGF-β signaling promotes tissue regeneration, especially in EMT (Qi et al. 2018). In renal interstitial fibrosis, over-activity of TGF-β signaling promotes fibrogenesis and induces activation of other signaling pathways (Zhang et al. 2018b; Zhou et al. 2018). Thus, inhibiting the activation of TGF-β signaling pathway is a likely highly effective therapeutic target for reversing EMT and renal tubulointerstitial fibrosis.

Lefty as a novel member of the TGF-β superfamily, possesses two variants in mice, namely, Lefty-1 and Lefty-2. Lefty regulates stem cell differentiation and controls embryonic development and other functions (Tabibzadeh and Hemmati-Brivanlou 2006). Previous studies demonstrated that Lefty can inhibit decidualization of endometrium and tumor activity (Cavallari et al. 2013; Li et al. 2014; Tang et al. 2010). Furthermore, Lefty also inhibits TGF-β1 signaling via the inhibition of Smad2/3 phosphorylation and further suppresses its nuclear translocation (Ulloa and Tabibzadeh 2001). Our previous studies demonstrated that Lefty-1 has anti-inflammatory effects and regulates fibroblast transdifferentiation in renal fibrosis (Zhang et al. 2018a, 2015). Despite the beneficial properties of Lefty, it remains unknown whether it can regulate TGF-β signaling on EMT and renal tubulointerstitial fibrosis. Thus, this study aims to investigate whether Lefty-1 can alleviate EMT and attenuate renal tubulointerstitial fibrosis through the TGF-β signaling pathway.

In this study, we used a UUO model and high glucose-induced HK-2 cells to investigate the anti-fibrotic effects of Lefty-1 on kidney tissues. Cell phenotype transformed from epithelial to mesenchymal, ECM accumulation, and TGF-β signaling activity were also evaluated. Our research revealed that Lefty-1 can alleviate tubular EMT and renal tubulointerstitial fibrosis in vitro and in vivo via suppressing the TGF-β/Smad pathway, and Lefty-1 exerts therapeutic potential for fibrotic kidney diseases.

Materials and methods

Reagents and recombinant adenovirus

E-cadherin, collagen I, and α-SMA antibodies were purchased from Abcam, Inc. (Cambridge, UK). TGF-β1, Smad2/3, p-Smad2/3, p-Smad2, and p-Smad3 antibodies were purchased from Cell Signaling Technology, Inc. (Beverly, MA, USA). Except for α-SMA, which were derived from murine, all antibodies were monoclonal and derived from rabbit. Quantitative real-time polymerase chain reaction (PCR) and RevertAid First Strand cDNA Synthesis kits were obtained from TaKaRa Shuzo Co., Ltd. (TaKaRa, Otsu, Japan). Dulbecco minimum essential medium (DMEM)/high glucose medium and bovine serum albumin (BSA) were purchased from the Gibco® (Thermo Fisher Scientific, Waltham, MA, USA). Recombinant mice Lefty-1 adenovirus was packed and identified by ViGene Biosciences Co., Ltd. (Rockville, MD, USA).

Adenovirus construction

First-generation adenovirus was regulated by cytomegalovirus promoter containing mouse Lefty-1 gene (Ad-Lefty-1) using an open reading frame (ORF) Shuttling System (ViGene Biosciences Co., Ltd.). In brief, mouse Lefty-1 cDNAs were excised from the pENTR vector and inserted into a pAD-ORF transfer vector. These resulting plasmids were co-transformed into bacteria with human adenovirus type 5 (E1 and E3 deleted). The recombinant adenoviral constructs were transfected into HEK293 cells, generating recombinant adenovirus to obtain a high Ad-Lefty-1 titer (1×10^{10} plaque-forming unit (pfu)/mL). The doses were performed using a previously reports, select a higher doses used in both the mouse and cell experiments (Ghayur et al. 2012). The recombinant adenovirus

can express exogenous gene fragments of infection, as well as dividing and non-dividing cells with wide host. Using a similar approach, we obtained the vector adenovirus-mediated Lefty-1 (Ad-Lefty-1) through customized adenovirus service. The Ad-control is an empty adenovirus used as the control in vivo and in vitro.

Animals and treatments

All animal studies were performed according to the Chinese Council on Animal Care Guidelines. Approval was received from the Animal Research Ethics Board of Hubei Minzu University. On the premise of not affecting the research objectives, use anesthetics and painkillers to minimize or eliminate pain and suffering. Establish humane endpoints for all pain and suffering situations that cannot be avoided or eliminated. Death or severe pain and suffering should be avoided if possible. These include improved living conditions, diets, surgical procedures, desensitization and adaptation strategies, drug interventions or euthanasia. Monitorring of the animals at least once a day. All animals were euthanized at the end of the study with dislocation of cervical vertebra under deep anesthesia (4% to 5% isoflurane).

Six- to seven-week-old C57BL/6 mice were obtained from the Wuhan University of Laboratory Animal Research (male, weighing 20–25 g) and divided into four experimental groups ($n=7$): (1) sham, in which the mice underwent sham operation; (2) UUO (7 day); (3) UUO (7 day) + Ad-control; and (4) UUO (7 day) + Ad-Lefty-1. The mice were randomly divided into groups, and there was no difference between the groups before the experiment. Anesthesia was induced and maintained by intraperitoneal injection of pentobarbital sodium (50 mg/kg body weight) (Georgi et al. 2011). Anesthesia was induced and maintained by intraperitoneal injection of pentobarbital sodium (50 mg/kg body weight).After general anesthesia, except for the sham group, the experimental groups were subjected to UUO through a midline abdominal incision following double ligation of the upper left ureter using 4-0 silk ties. The sham-operated mice had their ureters exposed but not ligated. Their left kidneys were then wrapped with ice-cold saline-soaked gauze for 5 min after injection and then injected with 0.1 mL of cold saline containing 5.0×10^8 pfu of Ad-control or Ad-Lefty-1 through the left renal artery. The adenovirus solution with 3 U of heparin was injected using a Hamilton 800 microsyringe (Hamilton, Carnforth, UK). The mice were maintained under sterile conditions and euthanized (cervical dislocation method) on postoperative day 7. Their left and right kidneys were separately obtained for RNA and histological analyses.

Cell culture and treatment

NRK-52E cells were purchased from Cell Bank of the Chinese Academy of Sciences (Shanghai, China), cultured in DMEM medium (containing 5.5 mmol/L D-glucose, normal glucose; NG), and supplemented with 10% FBS, 1% streptomycin, and penicillin in humidified incubator with 95% air and 5% CO_2 at 37 °C. NRK-52E cells were seeded in complete medium at 70–80% confluence in six-well plates. The cells were divided into four groups as follows: the control group, high glucose (HG, 30 mmol/L) treatment group, HG with Ad-control group, and HG with Ad-Lefty-1 group. After 24 h, the complete medium was replaced with serum-free medium for 48 h prior to treatment with HG or Ad-control and Ad-Lefty-1. The efficiency and results of adenovirus transfection were verified as described previously (Zhang et al. 2018a), and these cells were washed with PBS for further analyses.

Histopathological examination

Kidney sample were processed as follows, fixed in formalin and then embedded in paraffin blocks were sectioned in 4-μm sections and stained with Hematoxylin and eosin (HE, Boster) and Masson's trichrome (Boster). Slides were examined and pictures taken using an Olympus-BX51 fluorescence upright microscope (Olympus, Tokyo, Japan) and in a double-blinded fashion. The regions of tubulointerstitial damage were examined including distal tubular cast, tubular dilatation, loss of proximal tubular brush border, endothelial damage and leukocyte infiltration. Using a well-defined renal damage morphology scoring system for evaluation of the degree of tubulointerstitial damage: 0 = normality, 1 = minimal damage involves 25% of area, 2 = mild damage involves 25–50% of area, 3 = moderate damage involves 50–75% of area and 4 = sever damage involves 75% of area.

Real-time reverse transcription PCR (RT-PCR)

Total RNA was extracted from the kidney tissues in the respective groups, reverse transcribed to cDNA, and subjected to PCR amplification. The primers are shown in the Table 1, and GAPDH was used as the reference gene. The reaction conditions were as follows: predenaturation at 95 °C for 30 s, 40 cycles of 95 °C for 5 s, and 60 °C for 40 s. Relative quantification of gene expression for both target and reference genes was performed using the $2^{-\Delta\Delta Ct}$ method based on Ct values. Real-time PCR was performed using an ABI 7500 Fast Real-Time PCR System (Applied Biosystems, Carlsbad, CA, USA) in triplicate.

Table 1 The RT-PCR primers for the mice kidney

Gene name	Gene ID	Primer sequence (5′–3′)	Amplicon size (bp)
a-SMA	NM_009610.2	F: CTGGCATCGTGCTGGACTC	291
		R: GCCCATCAGGCAACTCGTA	
E-cadherin	NM_009864.3	F: GCAGGTCTCCTCATGGCTTTGC	138
		R: CCTTCAAATCTCACTCTGCCCAGG	
Collagen I a2	NM_007743.3	F: TCCAAAGGAGAGAGCGGTAA	694
		R: GACCAGGGAGACCAAACTCA	
TGF-β1	NM_133917.3	F: ACCGCAACAACGCCATCTA	103
		R: GCCCTGTATTCCGTCTCCTT	
Smad-3	NM_016769.4	F: GTCAACAAGTGGTGGCGTGTG	150
		R: GCAGCAAAGGCTTCTGGGATAA	
GAPDH	NM_001458.4	F: TCAACGGCACAGTCAAGG	358
		R: AAGTCGCAGGAGACAACC	

α-SMA alpha-smooth muscle actin, *F* forward, *R* reverse, *TGF-β1* transforming growth factor-β1, *GAPDH* glyceraldehyde-3-phosphate dehydrogenase

Immunohistochemical analysis

All renal tissue specimens were examined for morphological changes by embedding in paraffin and serial sections (4 μm thick). Immunohistochemical examinations were performed on the sections in a blinded fashion by an examiner. The antibodies used were as follows: E-cadherin (1:300), collagen I (1:200), α-SMA (1:300), TGF-β1 (1:200) and Smad2/3 (1:200). After immunostaining with primary antibodies, the biotinylated secondary antibody binds to the first antibody, and then binds to the SABC compound to form the antigen–antibody-biotinylated secondary antibody-SABC compound. Finally, the sections were developed using 3,3′-diaminobenzidine for a positive expression area to appear in the immunohistochemical examination. The images were viewed using an Olympus-BX51 upright microscope (Olympus, Japan). An Image-Pro Plus 7.0 system was used to quantitatively analyze the positive signals.

Cellular immunofluorescence staining

NRK-52E cells were fixed on coverslips for 15 min at room temperature by using 4% paraformaldehyde. The sample was permeabilized using 1% Triton X-100 buffer for 8 min and blocked with 1% BSA in PBS buffer for 10 min at room temperature. The slides were then incubated with the primary antibodies for 1 h at 37 °C. The following polyclonal antibodies were used: anti- E-cadherin (1:500), α-SMA (1:300), collagen I (1:200), TGF-β1 (1:200) and Smad2/3 (1:200). The cells were stained with fluorescein isothiocyanate or cyanine dye (cy3)-conjugated secondary antibodies (1:100, Beyotime Biotechnology, Jiangsu, China) to visualize the primary antibodies. Finally, the nuclei were counterstained with 4,6-diamidino-2-phenylindole. The samples were quantitatively or semiquantitatively assessed by two independent investigators in a blinded manner, and each experiment was repeated at least three times. Quantitation of immunofluorescence staining was carried out on coded cell coverslips as the integrated option density (IOD) value. An Image-Pro Plus 7.0 system was used to quantitatively analyze the positive signals.

Western blot analysis

NRK-52E cells were harvested in RIPA lysis buffer containing protease inhibitor cocktail (Boster Biological Engineering, Wuhan, Hubei, China) to measure protein concentrations using the BCA assay kit (Wuhan Good Biotechnology Co., Ltd., Wuhan, Hubei, China). Protein samples (30 μg) were loaded onto each lane, resolved using 10% SDS-PAGE, and transferred onto activated polyvinylidene fluoride membranes. After blocking with 5% skim milk at room temperature for 1 h, the membranes were incubated with appropriate primary antibodies at 4 °C overnight. After incubation with fluorescence-labeled secondary antibodies (IRDye700 and IRDye800, goat anti-mouse/rabbit) for 1 h at 37 °C, immunoreactive bands were visualized using an Odyssey infrared imaging system (LI-COR Biosciences, Lincoln, NE, USA).

Statistical analysis

All statistical analyses were performed using SPSS 20.0 (IBM Corp., Armonk, NY, USA). Data are expressed as the means ± standard deviation. One-way analysis of variance, followed by the Student–Newman–Keuls test,was used for the quantitative data, whereas the Kruskal–Wallis test was used for non-normally distributed data. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Effect of Lefty-1 on tubulointerstitial damage in kidney tissues of UUO

UUO is a well-characterized experimental model of renal interstitial fibrosis (Hosohata et al. 2018). In the present study, we successfully replicated this model. As shown in (Fig. 1a), HE staining revealed progressive deterioration of tubulointerstitial damage characterized by tubular and glomerular atrophy and tubular dilation with flattened epithelium on day 7 after the kidneys were obstructed. The degree of interstitial fibrosis was assessed using Masson's trichrome staining, which revealed total collagen deposition in kidneys, which were higher at day 7 after obstruction surgery than those in sham-operated kidneys (Fig. 1b). However, Ad-Lefty-1 treatment alleviated damage to the renal interstitium, which included tubular dilation, atrophy. The impaired tubular epithelial cells significantly improved. However, damage to renal interstitium was not significantly inhibited by Ad-control. Thus, Lefty-1 ameliorated UUO-mediated renal interstitial fibrosis, and this anti-fibrotic effect is worth investigating. However, control Ad-Lefty-1 had no significant effect on renal tubular interstitial lesions. These results suggested that Lefty-1 improved UUO-mediated renal tubular interstitial damage and must be further studied for its anti-fibrosis effect.

Lefty-1 ameliorates UUO mediated ECM deposition and EMT in vivo

Emerging evidence suggests that EMT may be involved in the generation of myofibroblasts, which are the major type of interstitial cells in the tubulointerstitial fibrosis and produce large amounts of ECM components in the tubular interstitium (Grande and Lopez-Novoa 2009). We investigated the effects of Lefty-1 on the expression of E-cadherin, α-SMA, and collagen I in obstructed kidneys. Immunohistochemistry staining showed that collagenstoche-SMA protein expression in the renal interstitium and tubular epithelial cells was significantly elevated after obstructive injury, but the expression of E-cadherin decreased. However, α-SMA and collagen I downregulation and upregulation of E-cadherin were observed in the Ad-Lefty-1 group compared with that in Ad-control or sham group (Fig. 2a). Lefty-1 induced the upregulation in mRNA expression of E-cadherin and downregulation in mRNA expression of α-SMA and ColIa1 (Fig. 2b). Thus, Lefty-1 is a potent agent for alleviateing renal tubular EMT in tubulointerstitial fibrosis.

Effect of Lefty-1 on UUO-induced TGF-β signaling

In obstructive nephropathy, various damage factors, including hypoxia, microenvironmental pressure change, and inflammation lead to apoptosis and necrosis of tubular epithelial cells, accompanied by excessive proliferation of renal

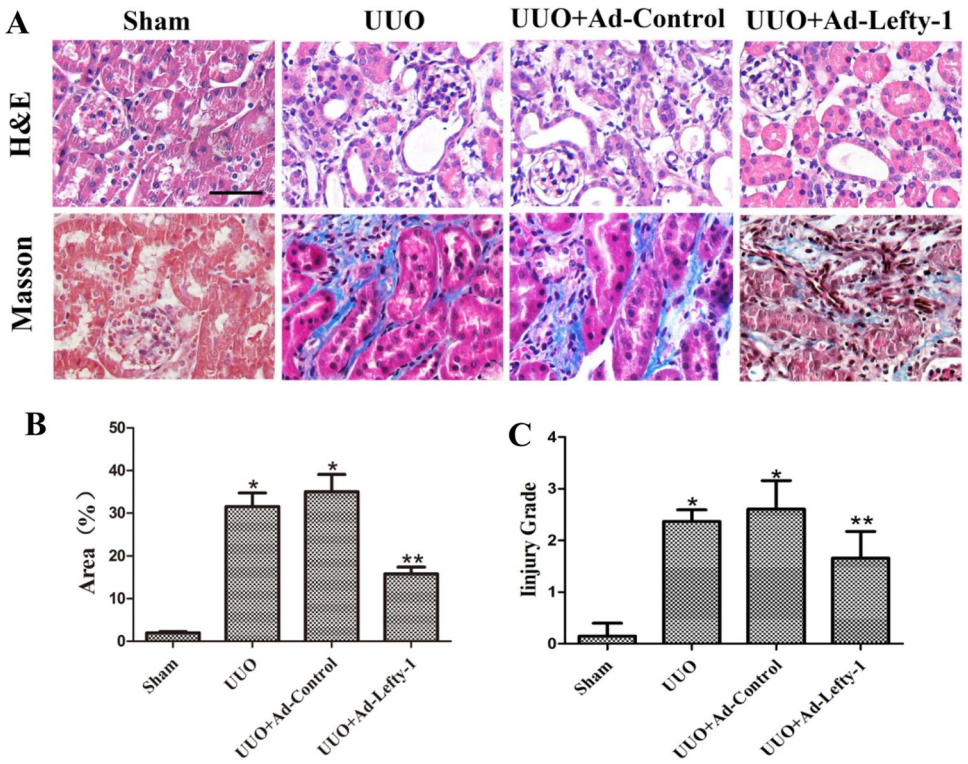

Fig. 1 Effect of Lefty-1 on UUO-induced renal deterioration of tubulointerstitial damage and fibrosis by HE and Masson's trichrome staining analyses. **a** Overexpression of Lefty-1 suppressed tubulointerstitial damage and fibrosis respectively at days 7 after UUO. **b** The analysis of Masson's trichrome staining. **c** The semi-quantitative histograms for the injury grade as shown. Experiments were performed at least three times with similar results. $n = 7$, $^{*}P < 0.05$ vs. sham, $^{**}P < 0.01$ vs. UUO or UUO + Ad-Control-treated groups

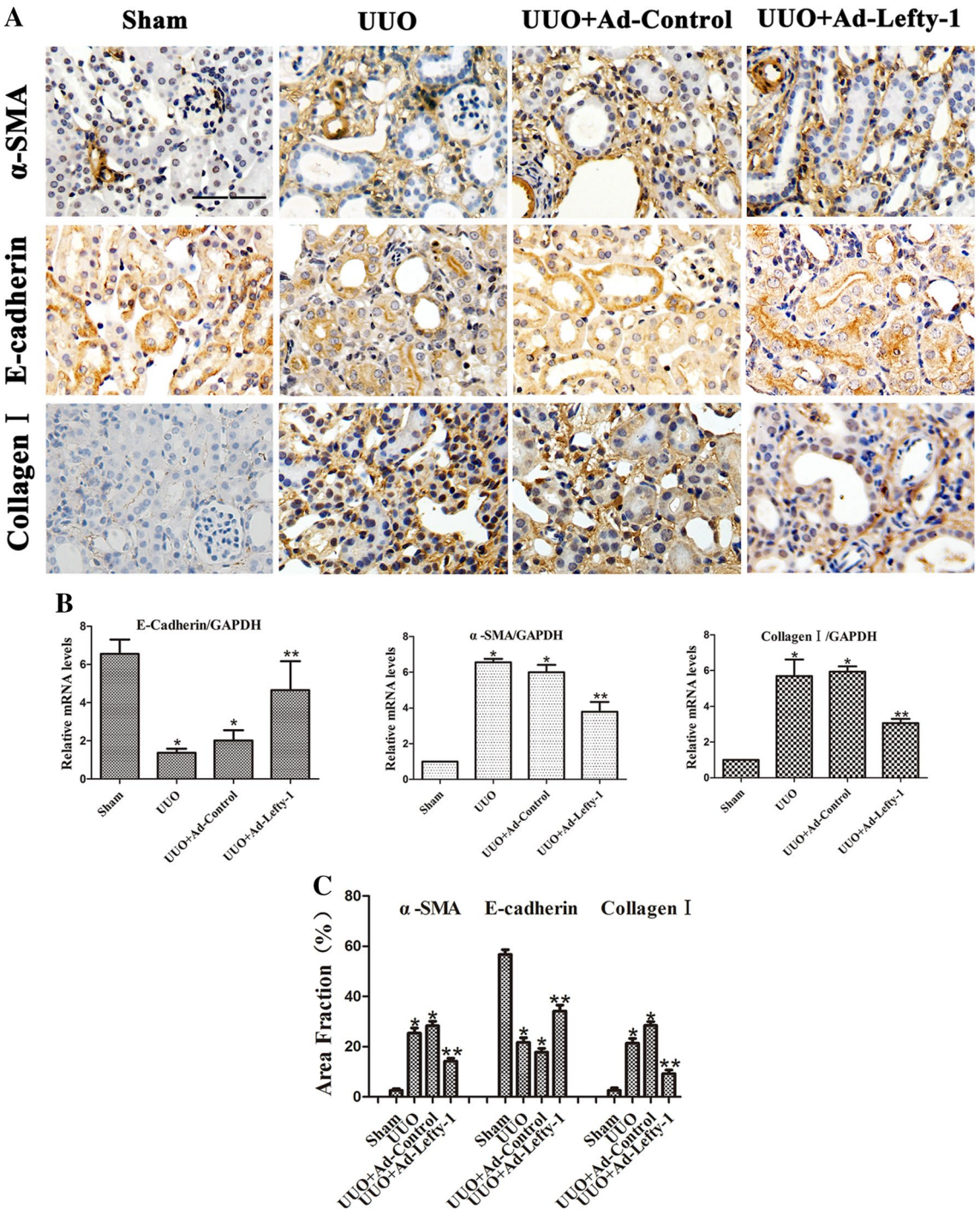

Fig. 2 Effect of Lefty-1 on UUO-induced ECM deposition and EMT in vivo by immunohistochemistry assay. **a** Overexpression of Lefty-1 reversing renal tubular EMT in tubulointerstitial fibrosis at days 7 after UUO. **b** The analysis of Real-time PCR. **c** Quantification of IHC staining-positive area by Image-Pro Plus 7.0. Results are expressed as mean ± SE for at least three independent experiments; magnification × 200; scale bar: 20 μm. $n = 7$, $^{*}P < 0.05$ vs. sham, $^{**}P < 0.01$ vs. UUO or UUO +Ad-Control-treated groups

mesenchymal cells. TGF-β-mediated signaling pathway is important renal fibrosis. Therefore, we further investigated the TGF-β signaling, which is a proliferation-relevant signaling pathway in renal tubulointerstitial fibrosis. As shown of these data, the effect of Lefty-1 on TGF-β signal was explored. In (Fig. 3a, b), TGF-β1 and the expression levels of Smad2/3 protein and mRNA were significantly increased in UUO group. The results suggested that TGF-β signaling

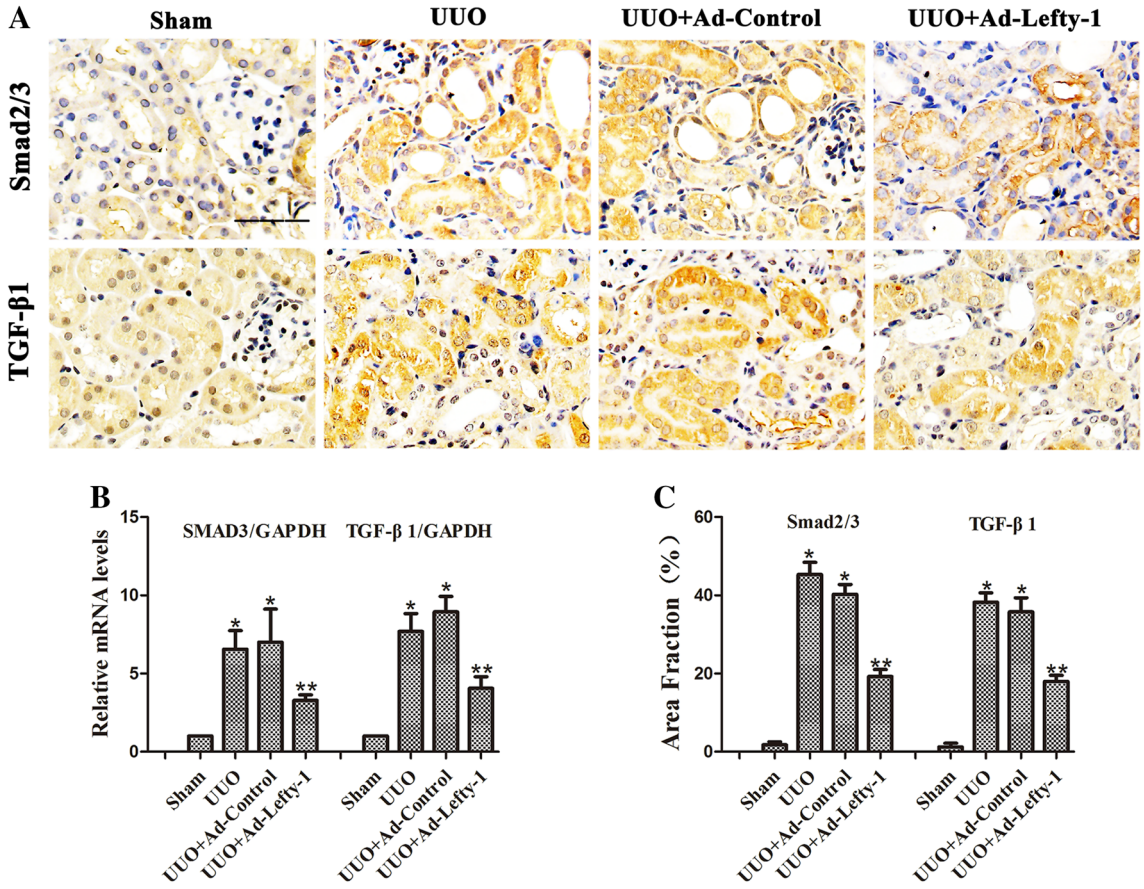

Fig. 3 Immunohistochemistry assay on effect of Lefty-1 on UUO-induced TGF-β signaling. **a** Protein expression levels of TGF-β1 and Smad2/3 were significantly increased by overexpression of Lefty-1at days 7 after UUO. **b** The analysis of Real-time PCR. **c** Quantification of IHC staining-positive area by Image-Pro Plus 7.0. Results are expressed as mean ± SE for at least three independent experiments; magnification × 200; scale bar: 20 μm. $n = 7$ $^{*}P < 0.05$ vs. sham, $^{**}P < 0.01$ vs. UUO or UUO +Ad-Control-treated groups

might be involved in EMT and tubulointerstitial fibrosis. The results showed that Ad-Lefty-1 intervention can significantly inhibit TGF-β signal; however, Ad-control interventions did not inhibit these changes. Thus, Lefty-1 might have an effect on UUO-induced TGF-β signal, but the exact mechanism needs further validation in vitro.

Effect of Lefty-1 on EMT in high glucose-induced NRK-52E cells

Previous in vivo experiments suggested that Lefty-1 exerts anti-fibrotic effects via the inhibition of aberrant activation of TGF-β signaling and tubular epithelial cell proliferation for alleviating EMT induction and amelioration of renal tubulointerstitial fibrosis, which should be further verified in vitro experiments. Thus, we investigated the effects of Lefty-1 on EMT in high glucose-induced NRK-52E cells. As shown in (Fig. 4a–c), the protein expression levels of collagen I and α-SMA were significantly elevated, and E-cadherin protein expression decreased by high glucose-challenge. Consistent with experimental results in vivo, these changes in high glucose-induced NRK-52E cells could be inhibited or even alleviated by Ad-Lefty-1 treatment. Also, Ad- control did not alleviate these changes.

Regulation of Lefty-1 on TGF-β signaling in high glucose induced NRK-52E cells

The effect of Lefty-1 on TGF-β signaling pathway was clarified by examining whether Lefty-1 can block TGF-β pathway in vitro. The protein expression of TGF-β1, Smad2/3, p-Smad2, and p-Smad3 were analyzed via immunofluorescence staining and Western blot analysis. In high glucose-treated NRK-52E cells, Ad-Lefty-1 inhibited the protein expression of TGF-β1 and Smad2/3, which are associated with TGF-β signaling pathway in immunofluorescence staining (Fig. 5a). Western blot analysis suggested that Ad-Lefty-1 could decrease the expression levels of TGF-β1, Smad2/3, p-Smad2, and p-Smad3, and the expression of EMT-related proteins were reversed (Fig. 5b, c). These

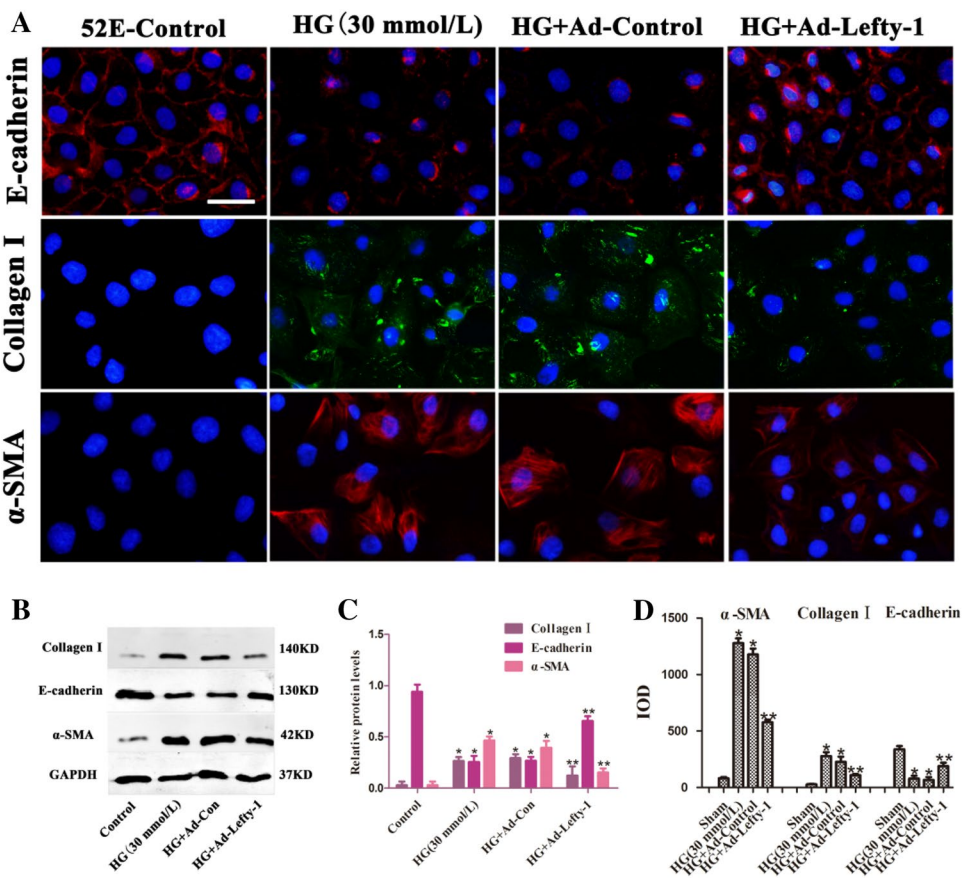

Fig. 4 The effect of Lefty-1 on EMT in high glucose-induced NRK-52E cells on immunofluorescence. **a** Overexpression of Lefty-1 reversing EMT at in vitro. **b**, **c** The analysis of Western blotting. **d** Quantification of IF staining-positive area by Image-Pro Plus 7.0 system. Immunofluorescent images of under the Olympus-BX51 fluorescence upright microscope were shown (magnification × 400). Scale bar represents 40 μm. Results are expressed as mean ± SE for at least three independent experiments. $n = 5$, $P < 0.05$ vs. control; $^{**}P < 0.01$ vs. HG (30 mmol/L) or HG +Ad-Control-treated groups

findings were similar to the previous observations in immunofluorescence staining. Thus, Smad2/3-mediated inhibition of TGF-β activation in NRK-52E cells inhibited by Lefty-1 might be the central mechanism through which Lefty-1 alleviates renal EMT.

Discussion

We investigated the effect of Lefty-1 on EMT using an experimental mice UUO model and a NRK-52E cell culture system. In vivo and in vitro experiments suggested that adenovirus-mediated Lefty-1 exerted its anti-fibrotic effects by reducing ECM component deposition and attenuating EMT to alleviate renal tubulointerstitial fibrosis. Lefty-1 could inhibit the aberrant proliferation of tubular epithelial cells, which are associated with EMT attenuating. Induction of tubular epithelial EMT is triggered by the over-activity of TGF-β signaling, which can be inhibited using Lefty-1 treatment. These findings indicated that the use of Lefty-1 as a potential treatment to alleviate renal tubulointerstitial fibrosis for new drug development.

The key pathological characteristic change of renal tubulointerstitial fibrosis is the accumulation of extensive ECM components, such as fibronectin and types I and IV collagen, in cortical interstitium and glomerular (Waasdorp et al. 2019). The activated resident fibroblasts and/or myofibroblasts lead to excessive ECM deposition, resulting in the loss of production balance and degradation of ECM components. Emerging studies have confirmed that the renal tubular epithelium through tubular EMT is a major source of myofibroblast formation (Carew et al. 2012). The role and status of EMT in renal fibrosis are unclear (Masola et al. 2019). Cell lineage tracking techniques for epithelial cells in vivo suggest that renal tubular EMT is an important event in the pathogenesis of renal tubulointerstitial fibrosis (Waasdorp et al. 2019; Xue et al. 2018; Zhu et al. 2017). EMT plays a pivotal role in physiological and pathological functions, including embryonic development, tissue regeneration, wound healing, cancer progression, and organ fibrosis. On the basis of biomarker context and functional consequences, EMTs were classified into three subtypes, especially of type II EMT as a direct contributor involved in the renal tubulointerstitial fibrosis. Type II EMT is characterized by a phenotypical change induction of epithelial cells that lose their structural polarity and cell–cell-basement membrane contacts of the epithelial cell features. The morphology gradually changed to spindle-shape similar to that of mesenchymal/myofibroblast cells and acquisition of mesenchymal

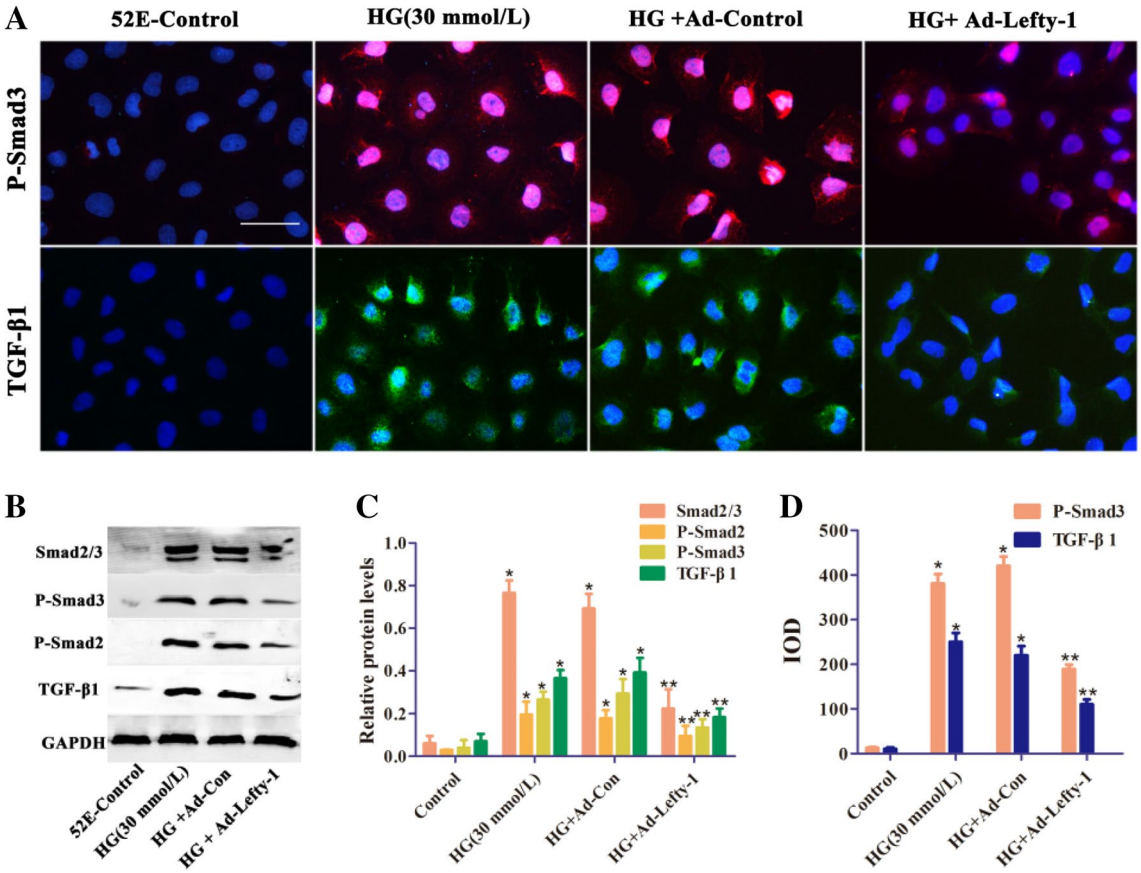

Fig. 5 Regulation of Lefty-1 on TGF-β signaling in high glucose induced NRK-52E cells. **a** Lefty-1 inhibited the protein expression of TGF-β1 and P-Smad3 in immunofluorescence staining. **b**, **c** The analysis of Western blotting. **d** Quantification of IF staining-positive area by Image-Pro Plus 7.0 system. Magnification (× 200, scale bar: 40 μm). $n = 5$, $P < 0.05$ vs. control; $^{**}P < 0.01$ vs. HG (30 mmol/L), HG + Ad-Control-treated groups

markers, such as α-SMA, undergoing various profibrotic cytokines. Thus, attenuating EMTs is a possible therapeutic intervention in preventing renal tubulointerstitial fibrosis.

At the early stages of renal injury development, tubular epithelial cells are exposed to various damage factors, especially of ischemia, hypoxia, and proteinuria. Earliest tubular epithelial cells are prone to structural and functional damage, which rapidly leads to the proliferation and generation of interstitial collagen, cell migration, and invasion capacity enhancement. A large number of chemokines and proinflammatory cytokines are produced, and fibrogenesis factors and matrix proteins are promoted, which are involved in the development of interstitial fibrosis. These impaired epithelial cells can activate a variety of pro-fibrotic cytokines, such as TGF-β, connective tissue growth factor, and Angiotensin-II. EMT, which plays an important role in the renal tubulointerstitial fibrosis, is induced. Epithelial cell proliferation might be associated with activation of proliferation-related signaling, such as TGF-β signaling. The aberrant growth and differentiation of epithelial cells might be one of the important pathological changes at early EMT (Marquez-Exposito et al. 2018). Thus, we further investigated the effects of Lefty-1 on epithelial cell proliferation and activity of TGF-β signaling.

TGF-β has been recognized a strong profibrotic cytokine in the genesis of renal tubulointerstitial fibrosis, and emerging evidence shows that TGF-β mediates several major tubular pathological events during renal tubulointerstitial fibrosis progression, including ECM component deposition, epithelial cell death, proliferation, and EMT (Jia et al. 2015). TGF-β1, as a well-known fibrogenic cytokine, initiates and completes EMT under determined conditions. The functional role of TGF-β1 in EMT and renal tubulointerstitial fibrosis is confirmed by blocking TGF-β1 with gene silencing and neutralizing TGF-β1 antibodies to ameliorate or prevent renal fibrosis (Park et al. 2018; Wang et al. 2019a). Downstream Smad proteins of TGF-β1 are closely related to stimulate fibrosis (Smad3) and inhibit fibrosis (Smad7). α-SMA-positive myofibroblasts were considered as the primary cell type responsible for EMT and is used for cellular phenotype differentiation in tubulointerstitial fibrosis. In the present study, using the mice UUO model

and NRK-52E cells induced with HG in vitro successfully reproduced EMT, which is characterized by epithelial cells of loss E-cadherin expression and increased expression of α-SMA. It also caused enhancement of TGF-β1 and Smad3 expression. This finding suggests that the activated TGF-β/Smad signaling pathway can induce transdifferentiation of NRK-52E cells into myofibroblasts. Thus, targeting multiple injurious pathways and specific antagonists for TGF-β1 or its downstream Smad proteins is effective in attenuating EMT or renal tubulointerstitial fibrosis. Therefore, blocking signal transduction and use of specific antagonists for TGF-β1 and its downstream Smad can effectively attenuate EMT and relieve the progression of renal tubular interstitial fibrosis.

Our results showed that TGF-β signaling is activated in a UUO mice model and NRK-52E cells induced by HG. Increasing number of evidence in recent years supported that the activity of TGF-β signaling is enhanced during EMT(Gong et al. 2018; Liu et al. 2018).TGF-β signaling directly promotes tubular EMT and myofibroblast formation via activating the expression of ECM components and inhibiting matrix metalloproteinases (MMPs), which are collagen-degrading enzymes(Hou et al. 2018). Our results revealed that TGF-β signaling-related molecules, such as TGF-β1, Smad2/3, and epithelial cell marker E-cadherin were co-expressed in the same areas around renal tubules. This result indicated that activated TGF-β signaling is involved in EMT induction. Further study showed that epithelial cell proliferation induced morphological and phenotypical changes. This study provides credible evidence that the over-activity of TGF-β signaling can be downregulated by Lefty-1 treatment. Lefty-1 reduced epithelial proliferation and attenuated tubular EMT. The left–right determination factor (Lefty) is an important cytokine of the TGF-β superfamily. Lefty presents two variants in mice: Lefty-1 and Lefty-2, which are orthologs of LeftyA and LeftyB in humans. These proteins control differentiation of stem cells and regulation of embryonic development (Tabibzadeh and Hemmati-Brivanlou 2006). In addition, Lefty-1 is a novel modulator of TGF-β signaling with various biological properties, such as mediation of embryonic development, stem cell differentiation, endometrium decidualization, and anticancer effects. Our previous studies revealed that Lefty-1 has anti-inflammatory effects and is capable of inhibiting fibroblast-myofibroblast transdifferentiation (Zhang et al. 2018a, 2015). Although some other subtypes of Lefty protein can attenuate EMT, the role of Lefty-1 on EMT and its underlying molecular mechanisms remain unclear. In this study, using a murine model of UUO and high glucose-induced NRK-52E cells were transfected or not with adenovirus-lefty-1.We found that Lefty-1 alleviates EMT and ECM synthesis induction through antagonizing TGF-β/Smad pathway in vivo and in vitro. Our finding suggests that Lefty-1 might attenuate EMT and ameliorate renal fibrosis via the inhibition of the TGF-β signaling pathway. Furthermore, the previous study found that LeftyA attenuates the TGF-β1-induced EMT of human renal proximal epithelial tubular cells in vitro (Li et al. 2010). We further studied the previous results and verified them in different species. This research is only an important part of our study on the anti-renal fibrosis of Lefty-1. Our research results are consistent with those of previous studies. The validity of the experiment is further confirmed. Moreover, these results showed that Lefty had anti-renal fibrosis effect on both humans and mice, and no significant difference in species was found. There is further improvement in the clinical application value of Lefty-1 in the treatment of renal fibrosis.

In conclusion, the role of Lefty-1 in attenuating EMT was confirmed in a mice model of UUO-induced renal tubulointerstitial fibrosis. Significant changes in the epithelial cell phenotype, including reduction of E-cadherin expression and enhanced a-SMA expression, were observed in NRK-52E cells induced by HG, but these changes were attenuated by Lefty-1 treatment. The underlying mechanisms of Lefty-1 on tubular EMT might be related to the regulation of TGF-β1/Smad pathway and epithelial cell proliferation. A new therapeutic target of Lefty-1 to antagonize renal fibrosis and attenuate tubular EMT was identified.

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Compliance with ethical standards

Conflict of interest The authors declare that no competing financial interests exist.

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