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Original Article

Sphingosine kinase 1 inhibition decreases the epithelial-mesenchymal transition and ameliorates renal fibrosis via modulating NF-κB signaling

Fei He[1], Maoxiao Fan[1], Yansheng Jin[1], Haiyan Wang[2], Lan Ding[1], Jianfeng Fan[1], Shuaishuai Gu[1], Wen Xu[1]

[1]*Department of Nephrology, Suzhou Wuzhong People's Hospital, Suzhou 215128, Jiangsu, China;* [2]*School of Biology and Basic Medical Sciences, Soochow University, RM702-2311, No. 199, Renai Road, Industrial Park, Suzhou 215123, Jiangsu, China*

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Abstract: Renal fibrosis is a critical process underlying the development progression of chronic kidney disease to end-stage renal disease, which has intrigued much attention. This study aimed to investigate the role of Sphingosine kinase 1 (SphK1) on epithelial-mesenchymal transition (EMT) in renal fibrosis and the potential regulatory mechanisms. In the present study, unilateral ureteral obstruction (UUO)-induced mouse renal fibrosis model was established. HE and Masson staining were employed to detect the pathological change and fibrous deposition in renal tissues respectively. Moreover, the expression of SphK1, EMT relative proteins including E-cadherin (E-cad), N-cadherin (N-cad) and vimentin as well as fibrosis marker protein α-smooth muscle actin (α-SMA) were measured by immunohistochemistry and Western blot, respectively. In vitro, SphK1 silencing was generated in TGF-β induced human renal tubular epithelial HK-2 cells. Immunofluorescence staining was applied to examine the expression of α-SMA, then the levels of EMT relative proteins and NF-κB signaling were measured using Western blot. The results revealed that notably tubulointerstitial damage and fibrous deposition were detected in the UUO mouse renal tissues. The expression level of E-cad and SphK1 were decreased coupled with an increase of N-cad, vimentin and α-SMA expression. Furthermore, after knockdown of SphK1 in TGF-β induced HK-2 cells, the E-cad expression was up-regulated while N-cad, vimentin and α-SMA expression were down-regulated remarkably. In addition, the expression levels of phospho-NF-κB p65 (p-NF-κB p65) and p-IκB-α were lowered significantly following SphK1 silencing. These findings indicated that the inhibition of SphK1 protected renal tubular epithelial cells against renal fibrosis, by contribution to decrease the EMT via blocking the NF-κB signaling. Therefore, SphK1 may serve as a therapeutic target in the future.

Keywords: Renal fibrosis, SphK1, epithelial-mesenchymal transition, NF-κB

Introduction

Renal fibrosis is a common outcome of chronic kidney disease (CKD) and main pathological basis for the progression of CKD to end-stage renal disease [1, 2]. It includes renal interstitial fibrosis and glomerular sclerosis. Renal interstitial fibrosis is characterized by aberrant activation and growth of the renal fibroblasts and the major cause of renal dysfunction [3, 4]. It is characterized by accumulation of excessive amounts of extracellular matrix proteins, which is an irreversible process [3, 5, 6]. Renal interstitial fibrosis is originated from many sources, such as tubular epithelial cells, kidney-derived fibroblasts, pericyte differentiation, and endothelial cell trans-differentiation [7, 8]. Therefore, understanding the molecular events responsible for activation of renal fibroblast may find to new approaches in the treatment of renal diseases.

Epithelial-mesenchymal transition (EMT) of renal tubular epithelial cells comprises a canonical pathological process and is of great significance for tubule-interstitial fibrosis [9, 10]. EMT is characterized by the loss of epithelial cells and their adhesion molecules such as E-cadherin (E-cad), and the increase in mesenchymal cells and their markers such as N-cadherin (N-cad), vimentin and α-smooth muscle actin (α-SMA) [11]. Mounting evidence supported that transforming growth factor-β (TGF-β) is the key regulator that controls many aspects of cel-

lular function, including differentiation, migration and fibrosis [12]. It has been reported that activation of TGF-β1/Smad pathway can promote renal fibrosis [13, 14]. In addition, in vitro experiments confirmed that overexpressed TGF-β1 in renal tubular epithelial cell line mediates Smad3 signaling pathway and increases expression of miR-21 [15].

SphK1 is evolutionary conserved enzyme that catalyzes the phosphorylation of sphingosine into endogenous sphingosine-1-phosphate (S1P) [16, 17]. It has been reported that the level of S1P was elevated in a murine model of bleomycin-induced pulmonary fibrosis, and this increase was induced by enhanced SphK1 [18]. In addition, inhibition of S1P decreases renal inflammation and fibrosis in diabetic nephropathy [19]. Mounting evidence supported that Nuclear factor kappa B (NF-κB) is a central factor in inflammation and transcriptional factor [20]. Activated NF-κB translocate from the cytoplasm into the nucleus, and then promotes the expression of its target genes [21]. Moreover, activation of NF-κB triggers a series of cellular processes, including cell proliferation, apoptosis inflammation, immunity and EMT [22, 23]. NF-κB signaling are closely linked to EMT [24, 25]. However, the role of SphK1 in renal fibrosis remains to be elucidated.

In the present study, we explored the effect of UUO in vivo. We examined the relationship between SphK1 and EMT under TGF-β1 stimulation in vitro in cultured HK-2 cells. We also explore that the effect of SphK1 on EMT-related protein in cultured HK-2 cells. We hypothesized that knockdown of SphK1 decreases the epithelial-mesenchymal transition via modulating NF-κB signaling in the fibrotic process.

Materials and methods

Animal experiments

The unilateral ureteral obstruction (UUO) model was established in male C57 black mice that weighed 20-25 g. The mice were housed on a 12-h light/12-h dark cycle. The mice were randomly divided into two experimental groups: Sham-operated control group and UUO model group with 10 mice in each group. In animals undergoing UUO, the left ureter was ligated with 8-0 nylon; in the sham mice, the ureter was left undisturbed. Mice were sacrificed by dislocation of the neck in the state of anesthesia and the kidneys were removed at day 7 for protein analysis and histological examination. This study was conducted in strict accordance with the guidelines for the Care and Use of Laboratory Animals and approved by the Ministry of Science and Technology of China. All of the study protocols were approved by the Ethics Committee on Animal Experiments of Soochow University.

Cell culture and transfection

Human renal tubular epithelial HK-2 cells were cultured in DMEM-F12 medium (3:1) supplemented with 10% fetal bovine serum (FBS, Hyclone) and maintained in a humidified 5% CO_2 atmosphere at 37°C. HK-2 cells were seeded in 6-well plates with 2×10^5/ml. SphK1 siRNA was transfected in HK-2 cells by using LipofectamineTM 2000 according to the manufacturer's instructions. After 48 h, HK-2 cells were made quiescent by culturing in serum-free medium for 24 h and then stimulated with TGF-β1 (5 ng/ml). Cell experiment were repeated at least three times.

Hematoxylin and eosin (H&E) staining

The kidney tissues were fixed with 10% buffered formalin at room temperature for 48 h, dehydrated, and embedded in paraffin. The sections were stained with hematoxylin and eosin H&E. Blinded analysis of kidney tissues was performed using a light microscope. A board-certified pathologist examined kidney tissues pathological alterations.

Masson staining

The kidney tissues were fixed with 10% buffered formalin at room temperature for 48 h, dehydrated, and embedded in paraffin. Masson trichrome staining was performed according to the protocol provided by the manufacture (Sigma, St. Louis, MO).

Immunohistochemistry

For immunohistochemistry analysis, the kidney tissue samples were fixed in formalin for 48 h. Then the tissue block was put into paraffin and next cut into slides for the desired thickness in a microtome, and was then fixed into a slide. After washing, the samples were prepared for blocking and incubating with antibody SphK1, E-cad, N-cad, vimentin and α-SMA which were

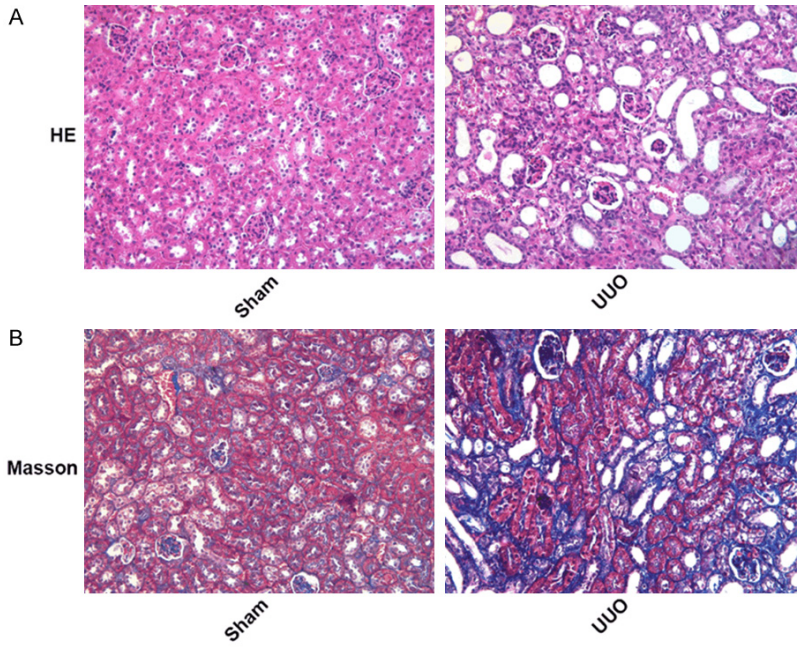

Figure 1. The pathologic changes and fibrous deposition of renal tissues after UUO injury. A. H&E staining was explored to detect the pathologic changes of renal tissues after UUO injury. B. Masson trichrome staining has been used for analysis of interstitial fibrosis (magnification, ×100).

diluted in 5% horse serum with chilled PBS at 4°C overnight. Isotype-matched IgG was used instead of primary antibody as a negative control of the staining. Sections were then incubated with diluted streptavidin-peroxidase HRP at room temperature with a staining kit, following the manufacturer's instructions. The sections were then stained with hematoxylin for 5 min and mounted and observed with a phase-contrast microscope.

Immunofluorescence assay

Renal tissue was fixed in buffered formalin, dehydrated, and embedded in paraffin. For general histology, sections were stained with PAS. For immunofluorescent staining, primary antibodies (α-SMA, 1:500) and fluorescent-conjugated secondary antibodies were applied to the sections after blocking with preimmune serum from the same species for the secondary antibody.

Western blotting assay

Cells and tissues from each group were detached with trypsin, centrifuged, and washed 2 times with pre-chilled PBS. Cell lysis buffer was subsequently added and incubated on ice for protein extraction. Protein concentration was determined using the BCA Protein Assay Kit (Beyotime Biotechnology, China). A total of 10 μg protein from each sample was loaded and separated via 12% SDS-PAGE and then transferred to a PVDF membrane (Millipore Corporation, Billerica, MA, USA). The membranes were incubated for 1 h at room temperature with 4% fat-free milk, and then incubated with an appropriate amount of primary antibody at 4°C overnight. Primary antibodies used were SphK1, α-SMA, E-cad, N-cad, vimentin, phosphor-NF-κB p65 (p-NF-κB p65), p-IκB-α and GAPDH. Detection was done by peroxidase-conjugated secondary antibodies (KPL, Gaithersburg, MD, USA) and chemiluminescence (Milipore Corporation, Temecula, CA, USA). Quantification of image density in pixel was performed using the Odyssey infrared imaging system (LI-COR Biosciences, Lincoln, NE, USA).

Statistical analysis

All the experiments were conducted at least three times. Data depicted in graphs represent the means 6 SEM for each group. Inter-group comparisons were made using one-way analysis of variance (ANOVA). Multiple means were compared using Tukey's test. The differences between two groups were determined by Student t-test. Statistical significant difference between mean values was marked in each graph. $P<0.05$ is considered significant.

Results

The pathologic changes and fibrous deposition of renal tissues in mice after UUO injury

To determine whether the renal fibrosis model was established successfully by ligation of the left ureter, H&E staining and Masson trichrome staining were used to detect the pathologic changes and fibrous deposition of kidney after UUO treatment, respectively. As shown in **Figure 1A**, there is tubular dilation in the injured

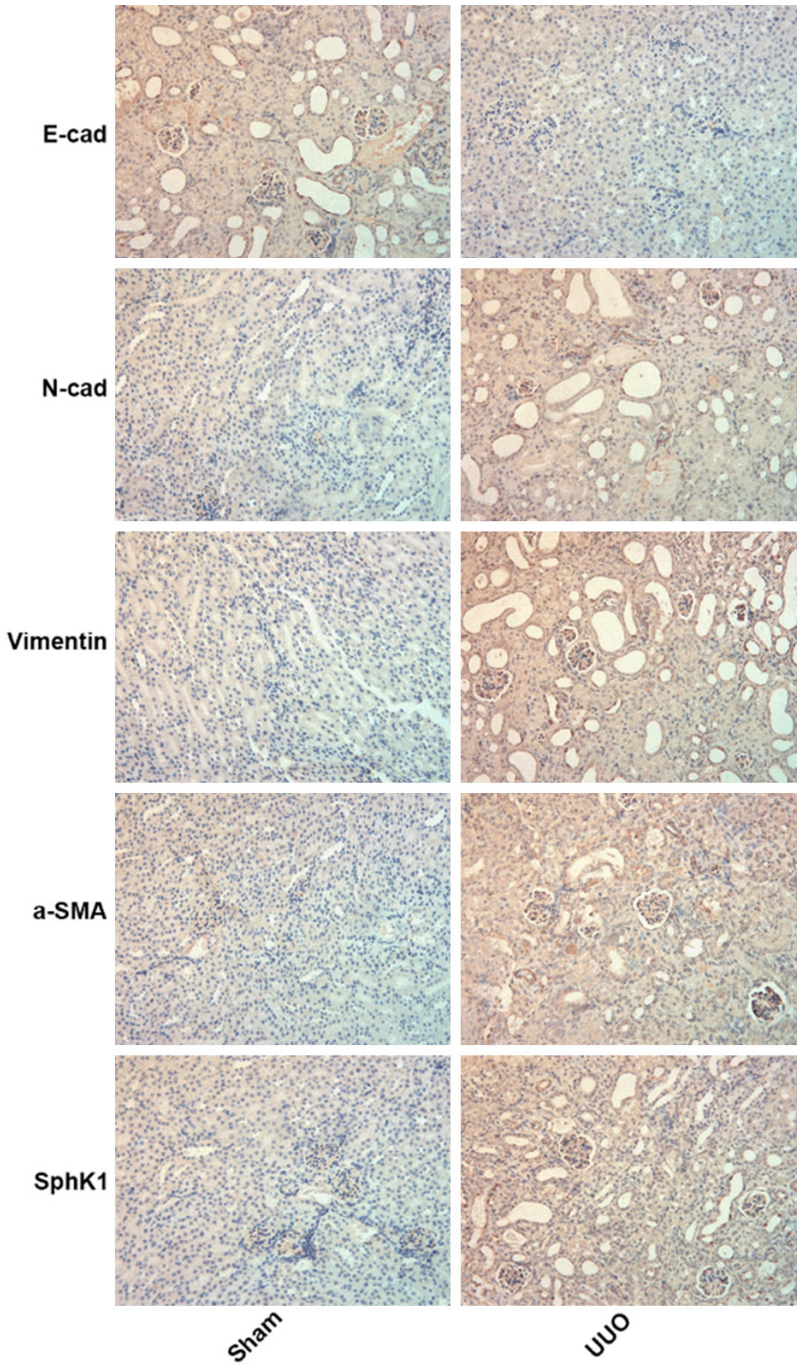

Figure 2. The expression of EMT related proteins, α-SMA and SphK1 in renal tissues after mice being treated with UUO were measured by Immunohistochemistry assay (magnification, ×200).

kidney after UUO. Masson trichrome staining has been extensively used for analysis of interstitial fibrosis. As shown in **Figure 1B**, Masson trichrome staining showed that UUO injury induced expression of interstitial collagen fibrils, and UUO resulted in a dramatic increase of collagen fibril deposition compared with sham group. These data indicated that the model of UUO was successfully established.

The expression of EMT related protein and SphK1 in renal tissues of mice after UUO treatment

The overproduction of renal tubular epithelial cells EMT is considered pivotal events in the pathogenesis of chronic renal fibrosis [26]. In our study, we further examined the effect of UUO injury on the expression of EMT related protein in kidneys. Immunohistochemistry staining showed that UUO treatment significantly reduced the expression of E-cad and increased the expression of N-cad and vimentin (**Figure 2**). Furthermore, we examined the effect of UUO injury on the expression of α-SMA, a hallmark of fibroblast activation in kidneys. We found that the expression of α-SMA was dramatically increased after UUO injury (**Figure 2**). Importantly, the expression of SphK1 was upregulated markedly in UUO group (**Figure 2**). Collectively, these data suggested that UUO injury promotes the EMT in the kidney and the expression of SphK1 was increased after UUO injury.

EMT was induced after HK-2 cells being treated with TGF-β1

To investigate whether TGF-β1 induced the EMT in HK-2 cells, HK-2 cell exposure to 5 ng/ml of TGF-β1 and the EMT related proteins and α-SMA expression levels were evaluated by western blotting assay. As shown in **Figure 3A**, TGF-β1 treatment decreased the expression of E-

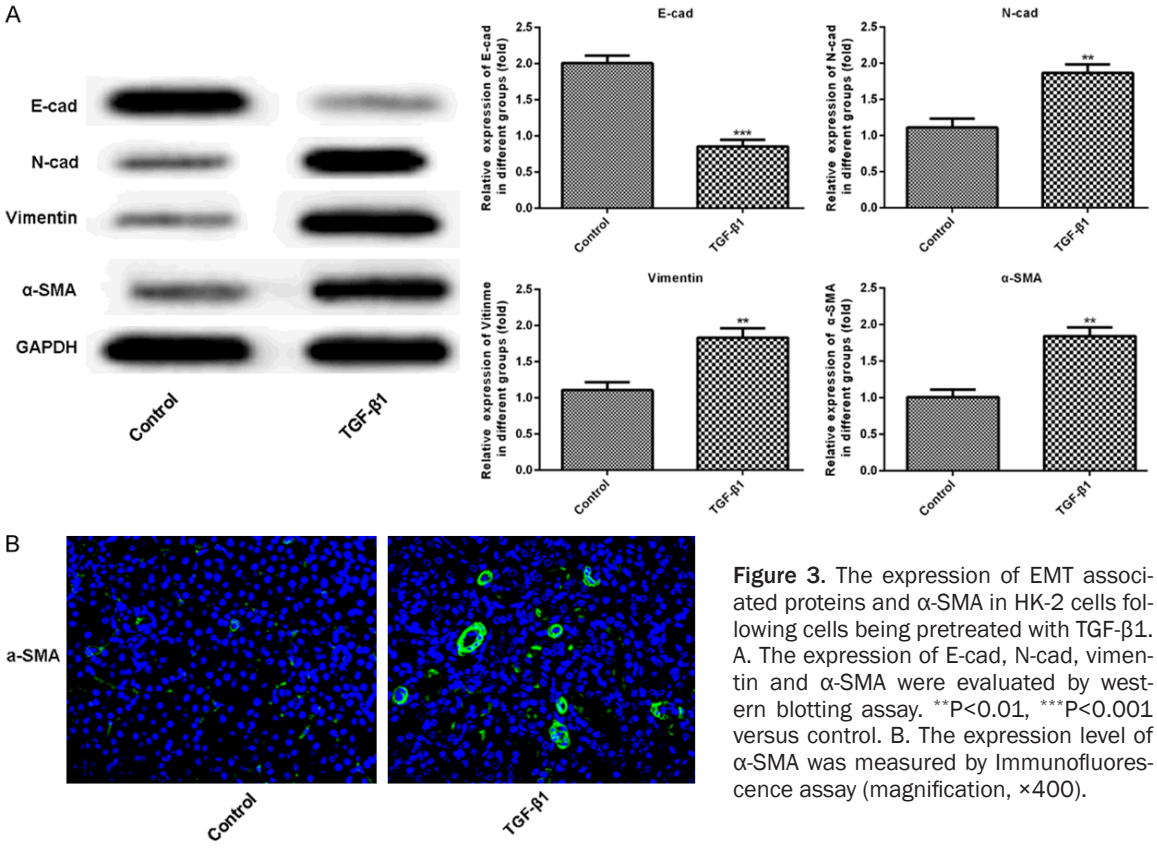

Figure 3. The expression of EMT associated proteins and α-SMA in HK-2 cells following cells being pretreated with TGF-β1. A. The expression of E-cad, N-cad, vimentin and α-SMA were evaluated by western blotting assay. **P<0.01, ***P<0.001 versus control. B. The expression level of α-SMA was measured by Immunofluorescence assay (magnification, ×400).

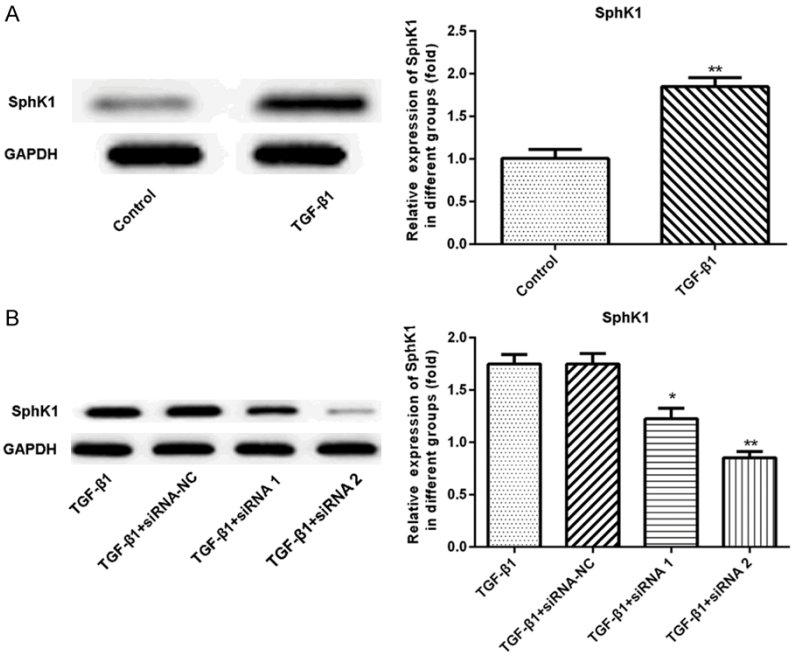

Figure 4. The expression of SphK1 in HK-2 cells following cells being pretreated with TGF-β1. A. The expression of SphK1 in HK-2 cells following cells being pretreatment with TGF-β1 was detected using Western blotting assay. **P<0.01 versus control. B. The expression of SphK1 in HK-2 cells following cells being transfected with SphK1 siRNA was measured by Western blotting assay. *P<0.05, **P<0.01 versus TGF-β1 + siRNA-NC.

cad and increased the expression of N-cad, vimentin and α-SMA in HK-2 cells. Concurrently, immunofluorescence assay showed significantly higher expression level of α-SMA in TGF-β1 induced HK-2 cells (**Figure 3B**). Above results indicated that EMT was induced after HK-2 cells being treated with TGF-β1.

SphK1 silencing suppressed EMT in HK-2 cells treated by TGF-β1

The expression of SphK1 in HK-2 cells was measured after cells being treated with TGF-β1. As presented in **Figure 4A**, the expression of SphK1 was upregulated significantly, which was in accordance with the result in vivo. To determine the effect of SphK1 in TGF-β1 in-

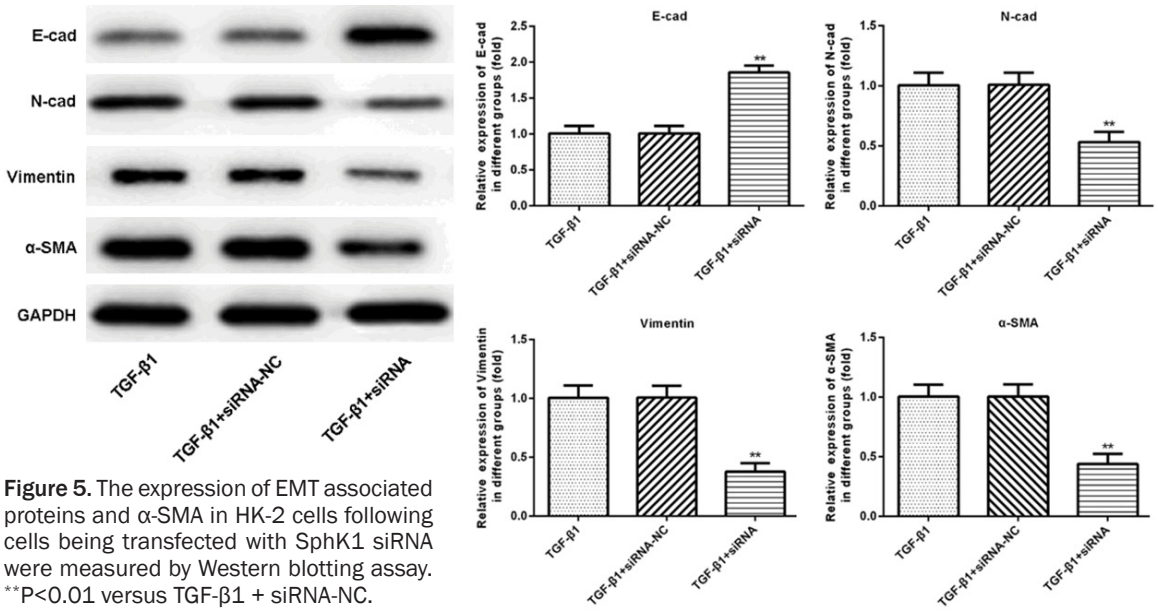

Figure 5. The expression of EMT associated proteins and α-SMA in HK-2 cells following cells being transfected with SphK1 siRNA were measured by Western blotting assay. **P<0.01 versus TGF-β1 + siRNA-NC.

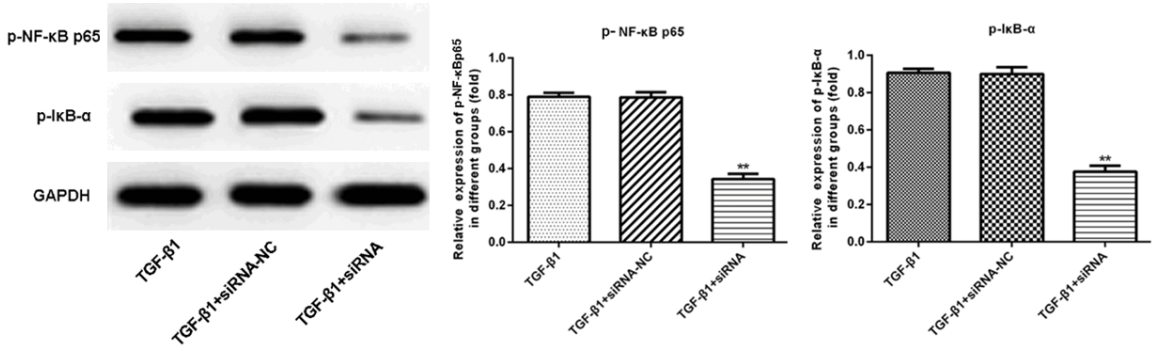

Figure 6. Knockdown of SphK1 inhibited the NF-κB signaling. The expression of p-NF-κB p65 and p-IκB-α were detected by Western blotting assay. **P<0.01 versus TGF-β1 + siRNA-NC.

duced HK-2 cells, knockdown of SphK1 was employed to perform further investigation. As shown in **Figure 4B**, silencing of SphK1 was established successfully, and we chose siRNA 2 to do the following experiment. We found that SphK1 knockdown enhanced the expression of E-cad accompanied by a reduced expression of N-cad, vimentin and α-SMA in HK-2 cells (**Figure 5**). These date manifested that knockdown of SphK1 inhibited the EMT induced by TGF-β1 in HK-2 cells.

Knockdown of SphK1 decreased the EMT and renal fibrosis via inhibiting NF-κB signaling

To the best of our knowledge, blocking of NF-κB activity and p65 nuclear transfer could inhibit the development of EMT [27]. To explore the effect of SphK1 knockdown on the NF-κB signaling following TGF-β1 treatments, western blotting assay was applied to detect the expression of p-NF-κB p65 and p-IκB-α in HK-2 cells. As shown in **Figure 6**, the expression of p-NF-κB p65 and p-IκB-α were significantly decreased after SphK1 silencing in HK-2 cells. These data suggested that knockdown of SphK1 decreased the EMT via inhibiting NF-κB signaling.

Discussion

This study provides evidence of overall effects and underlying mechanisms of SphK1 in the pathogenesis of renal fibrosis. UUO is a classic model of progressive renal fibrosis in the obstructed kidney [28]. In the present study, we showed that UUO induced significant renal

damage and renal fibrosis in mice as characterized by fibrous deposition in the mouse kidneys. Moreover, mice in the UUO group had increased the expression of EMT related protein N-cad, vimentin, and reduced the expression of E-cad, and dramatically increased the expression of α-SMA which is a hallmark of fibroblast activation in kidneys. These data suggested that UUO injury promoted the EMT of renal tubular epithelial cells and the activation of renal interstitial fibroblasts in the kidney.

SphK1 is evolutionary conserved enzyme that phosphorylates sphingosine to sphingosine 1-phosphate (S1P) and regulates endogenous levels of S1P in the sphingolipid metabolic pathway [29]. Recent studies found that SphK1 has increased the attention owing to its potential involvement in the fibrotic process of various diseases tissues including the heart, liver, lung and kidney [30-32]. In cultured podocytes, the SphK1 upregulation and fibronectin expression induced by TGF-β1 was found, and knockdown of SphK1 resulted in a reduction of fibronectin, which demonstrates that SphK1 acts as a potent pro-fibrotic cytokine in fibrotic process [33]. In this study, UUO mice and cultured HK-2 cells were used to explore the effect of SphK1 in the process of renal fibrosis. Our data found that the expression of SphK1 was significantly upregulated in UUO renal tissues and HK-2 cells treated by TGF-β1.

Renal fibrosis is a common pathological performance of CKD caused by various factor. TGF-β1 is a key member of TGF superfamily. A large number of studies have shown that TGF-β1/Smad pathway is involved in regulating renal fibrosis [34, 35]. Importantly, TGF-β1 is a critical factor in promoting fibrosis and is closely related to EMT, which supported that TGF-β1 is widely used in investigate the mechanisms of fibrotic diseases [36]. In the present study, HK-2 cell exposure to 5 ng/ml of TGF-β1.We found that TGF-β1 treatment decreased the expression of E-cad and increased the expression of N-cad, vimentin and α-SMA in the HK-2 cell, which were in accordance with the previous studies [37, 38].

EMT, a phenotypic transition of cells from the differentiated epithelial-like state to mesenchymal-like phenotype, is the underlying mechanism of cell injury in DKD [39, 40]. Renal tubular EMT is one of the major pathogenesis of renal interstitial fibrosis and of great significance for tubule-interstitial fibrosis [41]. In the present study, TGF-β1 induced the EMT and renal fibrosis by increasing SphK1 expression in HK-2 cells. And knockdown of SphK1 inhibited the EMT and renal fibrosis. In addition, emerging evidence showed that NF-κB signaling were implicated in EMT, and inhibition of this signaling contributed to protect against renal fibrosis [42, 43]. For instance, Astragaloside IV suppresses glucose-induced EMT of podocytes via the NF-κB p65 axis [24]. Interestingly, we found that SphK1 silencing significantly decreased the expression of p-NF-κB p65 and p-IκB-α. These data suggested that knockdown of SphK1 decreased the EMT via inhibiting NF-κB signaling.

In summary, the present results demonstrated that SphK1 may participate in the pathogenesis of renal fibrosis, and regulate the NF-κB signaling pathway. Inhibition of SphK1 decreases the EMT and ameliorates renal fibrosis via modulating NF-κB signaling, which demonstrated that targeting SphK1 may be a better alternative to directly suppress renal fibrosis in CKD.

Acknowledgements

All of the study protocols were approved by the Ethics Committee on Animal Experiments of Soochow University.

Disclosure of conflict of interest

None.

Address correspondence to: Fei He, Department of Nephrology, Suzhou Wuzhong People's Hospital, No. 61, Dongwu North Road, Suzhou 215128, Jiangsu, China. Tel: 18915507692; E-mail: feiyangflyfly@163.com

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