Original Paper

Identification of Tisp40 as an Essential Regulator of Renal Tubulointerstitial Fibrosis via TGF- β /Smads Pathway

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Key Words

Tisp40 • Ischemia-reperfusion • Renal tubulointerstitial fibrosis • TGF- β • Smad

Abstract

Background: Tisp40, a transcription factor of the CREB/CREM family, is involved in cell proliferation, differentiation and other biological functions, but its role in renal tubulointerstitial fibrosis is unknown. **Methods:** In our study, we investigated the effects of Tisp40 on extracellular matrix (ECM) accumulation, epithelial-mesenchymal transition (EMT) and the underlying molecular mechanisms in transforming growth factor- β (TGF- β)-stimulated TCMK-1 cells by quantitative real-time polymerase chain reaction (qPCR), Western blot analysis and immunofluorescence *in vitro*, and further explored the role of Tisp40 on renal fibrosis induced by ischemia-reperfusion (I/R) by qPCR, Western blot analysis, hydroxyproline analysis, Masson trichrome staining and immunohistochemistry staining *in vivo*. **Results:** The data showed that Tisp40 was upregulated in a model of renal fibrosis induced by I/R injury (IRI). Upon IRI, Tisp40-deficient mice showed attenuated renal fibrosis compared with wild-type mice. Furthermore, the expression of α -smooth muscle actin, E-cadherin, fibronectin, and collagen I was suppressed. Tisp40 overexpression aggravated ECM accumulation and EMT in the TGF- β -stimulated TCMK-1 cell line, whereas the opposite occurred in cells treated with small interfering RNA (siRNA) targeting Tisp40. Importantly, it is changes in the Smad pathway that attenuate renal fibrosis. **Conclusion:** These findings suggest that Tisp40 plays a critical role in the TGF- β /Smads pathway involved in this process. Hence, Tisp40 could be a useful therapeutic target in the fight against renal tubulointerstitial fibrosis.

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Introduction

Statistics suggest that the global incidence rate of chronic kidney disease (CKD) due to various causes is 8-16%, and renal tubulointerstitial fibrosis is considered a common

outcome of CKD [1, 2]. Approximately 60% of patients who survive acute kidney injury (AKI) develop CKD [3]. Research shows that AKI accelerates the development of CKD, while patients with CKD have a higher probability of suffering from AKI [4, 5]. Ischemia-reperfusion (I/R) injury (IRI), whose pathological changes are acute renal tubular apoptosis and necrosis, is a main cause of AKI. Epithelial-mesenchymal transition (EMT) is a determinant of renal tubulointerstitial fibrosis [6, 7], which represents a loss of epithelial markers, including E-cadherin, and an increase in mesenchymal markers, such as alpha smooth muscle actin (α -SMA) [8-10]. In the process of renal tubulointerstitial fibrosis, tubular epithelial cells that encounter various injury stimuli undergo a de-differentiation [11-13]. The short-term injured de-differentiated tubular epithelial cells can proliferate and re-differentiate to restore the structural and functional stability of nephrons [14]. However, under severe and repetitive stimulation, the de-differentiated tubular epithelial cells release profibrotic mediators that cause a transformation from tubulointerstitial cells, including fibroblasts and pericytes, to scar-producing cells, leading to the accumulation and deposition of extracellular matrix (ECM) [10-13, 15]. Renal function can recover after ischemic injury, but the accumulation and deposition of ECM combined with the proliferation of fibroblasts leads to renal tubulointerstitial fibrosis, which progresses to CKD [4, 16]. Evidence indicates that TGF- β plays a key role and activates downstream Smad signaling in mediating the process of renal tubulointerstitial fibrosis [17-19].

Recently, Tisp40 and other endoplasmic reticulum (ER) membrane-bound bZIP domain-containing transcription factors, which have a transmembrane (TM), a transcription-activation, and a bZIP domain and share a region of high sequence homology with activating transcription factor 6 (ATF6), have been identified [20]. Mouse Tisp40 encodes Tisp α /Atce1 and Tisp40 β , which differ in starting point, downstream or upstream of the first exon. In humans, Tisp40 is located in 1q21.3, encoding 395 amino acids [21]. As a transcription factor, Tisp40 can bind to a variety of response elements, such as CRE response element, NF-Kb response element and unfolded protein response element (UPRE), to initiate the transcription of multiple genes [20]. Tisp40 (also named CREB3L4, AlbZIP, or ATCE1) disruption activates caspase 12 via the endoplasmic reticulum stress protein Grp78/BiP and the membrane protein ribosome-associated 4 (RAMP4), leading to the apoptosis of meiotic/postmeiotic germ cells and the aggregation and acetylation of sperm in epididymis [22]. In humans, Tisp40 is highly expressed in the prostate. Its expression level in prostate cancer tissue is higher than in normal tissue and epithelial tumor tissue, and androgens can induce the expression of Tisp40 in a dose-dependent manner in a prostate cancer cell line [21, 23]. Compared with normal tissues, HCC tissues express more Tisp40 protein [24]. However, its role in the differentiation of renal tubular cells and renal tubulointerstitial fibrosis is unknown.

In this study, we demonstrate a novel role of Tisp40 in renal tubulointerstitial fibrosis. We find that the loss of Tisp40 in the kidney prevents I/R-induced tubulointerstitial fibrosis by inhibiting ECM accumulation and EMT, and we further demonstrate that this inhibitory action is mediated by a reduction in Smad2/3 phosphorylation. These results show that in all probability, Tisp40 is a potential target gene for the treatment of renal tubulointerstitial fibrosis.

Materials and Methods

Animals and Surgical Protocols

All surgical procedures were conducted according to the Guide for the Care and Use of Laboratory Animals (1996) and approved by the Institutional Animal Care and Use Committee of Wuhan University. Male C57BL/6 mice (20-25g) were obtained from the Center of Experimental Animals in Wuhan University Medicine College (Hubei, China). Tisp40-knockout mice crossed into a C57BL/6 background were purchased from RIKEN BioResource Center (Stock NO. RBRC01942). The kidney

morphology and function is no appreciable defect in phenotypically normal Tisp40^{-/-} mice. All mice caged in a standard temperature-controlled room with alternating 12-h light/dark cycles, free access to water and standard laboratory diet. The mice were randomly divided into 4 groups: (1) wild-type sham-operated; (2) wild-type renal I/R; (3) Tisp40-knockout sham-operated; (4) Tisp40-knockout renal I/R (n = 6 per group).

At 10 weeks of age, Tisp40-knockout and wild-type C57BL/6 mice were abdominal anaesthetized with Phenobarbital Sodium (60mg/Kg). After induction of anesthesia, the mice were placed abdomen on a heated table to keep a rectal temperature of 37–38°C. The occurrence of loss of righting reflex proved to desired depth of surgical anesthesia. A left flank subcostal incision was made approximately 1–1.5 cm, followed by careful dissection to remove the perihilar adipose tissue for the exposed blood vessels. Afterwards we use a microvascular clamp the blood vessels as little perihilar fat as possible for 30min. Then, a 0.5cm lowered incision was made in the right, and the right kidney underwent an identical surgical procedure. If the induction of ischemia is successful, the appearance of kidney will change from red to dark purple within 1–2 minutes gradually. Layer sutured and incision closed with a suture, the postoperative animals were carefully transferred into an incubator which was set at a fixed temperature. Sham-operated mice (wild-type sham-operated+Tisp40-knockout sham-operated) underwent the same procedure without clamping of the renal blood vessels. At 10 weeks post operatively, the animals were sacrificed by pentobarbital sodium overdose. After removed under fully maintained anesthesia, the left kidneys were snap-frozen in liquid nitrogen and stored at –80°C or fixed in 4% paraformaldehyde for biochemical analysis and pathological evaluation.

Hydroxyproline Analysis

Samples of kidneys of wild-type sham-operated, wild-type renal I/R, Tisp40-knockout sham-operated and Tisp40-knockout renal I/R mice were hydrolyzed by 6mol/L HCl at 110°C for 18 h, and then centrifuged at 18,000 g for 2 min, followed by frozen-drying. The content of hydroxyproline in each group was analyzed with a hydroxyproline analysis kit (Sigma-Aldrich).

Masson Trichrome Staining

Samples from paraffin-embedded tissues were prepared at 4 µm thickness, stained with Masson's trichrome. Masson trichrome staining, a standard for visualizing fibrosis in tissue, provides tissue morphology, allowing evaluation of localization and severity of deposition of extracellular matrix.

Immunohistochemistry Staining

Kidney sections (4-µm) were deparaffinized and then hydrated in graded ethanol followed by blocked with 3% hydrogen peroxide for 15 min at room temperature. The sections were then treated with 10% normal goat serum in Tris-buffered saline for 30 min at 37°C. Subsequently, they were incubated overnight at 4°C with the following primary antibodies against Tisp40 (1:100; sc-54800; Santa), alpha smooth muscle actin (1:100; ab5694; Abcam), E-cadherin (1:50; 610181; BD Biosciences), collagen I (1:150; ab34710; Abcam), fibronectin (1:200; ab2413; Abcam), phosphor(p) Smad2/3 (D27F4; 1:100; Cell signaling technology) and Snail (1:300; ab180714; Abcam). Afterwards the sections were washed three times in PBS and incubated with horseradish peroxidase (HRP)-conjugated anti-rabbit/mouse IgG secondary antibodies (Dako; Glostrup, Denmark.) for 10 min at 37°C. A dark brown color reaction was developed with 3,3'-diaminobenzidine (Beyotime Institute of Biotechnology, Shanghai, China) in the presence of 3% hydrogen peroxide, and terminated by washing twice in distilled water. The percentage of area stained represents the ratio of the summed absolute areas of staining versus the total tissue.

Cell culture and Treatment

Renal tubular epithelial cell line TCMK-1/wildtype (TCMK-1/wt) (ATCC number CCL-139) were cultured in DMEM (GDM12800, Gino bio-medical technology Co., Ltd.) supplemented with 10% fetal bovine serum (FBS; Gibco, Gaithersburg, MD, USA) and 1% penicillin- streptomycin at 37°C under a 95% air and 5% CO₂ condition. A total of 1.5×10⁶ cells/ml cells were transferred to 6-well plates for group comparison study. After the cells which reached 70% confluence were provided a 12h starvation, TGF-β (Millipore, London, UK) was added into the medium at a final concentration of 10ng/ml for 2hrs or 24hrs.

Total RNA isolation and quantitative real-time PCR

Total RNA of renal samples and TCMK-1 cells was extracted with TRIzol® Reagent (Invitrogen, 15596026), according to the manufacturer's protocol. RNA underwent purification with DNase (Fermentas, EN0521), the purity of which was assessed by determining the absorbance ratio at A260/A280. Total RNA was reverse transcribed into cDNA using RevertAid Reverse Transcriptase (Fermentas, EP0442), dNTP (Fermentas, R0191) and RiboLock RNase Inhibitor (Fermentas, E00381). Quantitative real-time PCR (qPCR) was performed using KAPA SYBR FAST qPCR Kit Master Mix(2X) ABI Prism™ (KAPA, KK4605) and Real-Time PCR Systems instrumentation and software (ABI stepone plus). Primer sequences for the renal samples were as follows: Tisp40 forward 5'-TACCTGAAGCCCCAACTACAAA-3', and reverse, 5'-GTGCCCTGCCACATGATAAA-3'; α-SMA forward 5'-CGAAACCACCTATAACAGCATCA-3', and reverse, 5'-GCGTTCTGGAGGGGCAAT-3'; fibronectin forward, 5'-AGGCTGGATGATGGTGGACT-3', and reverse, 5'-CGGCTGAAGCACTTTGTAGAG-3'; collagen I forward, 5'-CCCCCGCGCAAGAGT-3', and reverse, 5'-ATTGTGGCAGATACAGATCAAGC-3'; E-cadherin forward, 5'-TTCAAAGTGGCGACAGACGG-3', and reverse, 5'-GGGTACACGCTGGGAAACAT-3'; Snail forward, 5'-TCTGCACGACCTGTGGAAAG-3', and reverse, 5'-GCAGTGGGAGCAGGAGAATG-3'; TGF-β forward, 5'-TAATGGTGGACCGCAACAAC-3', and reverse, 5'-CACTCAGGCGTATCAGTGGG-3'; (Invitrogen). Expression of all target genes was normalized to that of β-actin, which had the following primer sequences: Forward 5'-CTGAGAGGGAAATCGTGGCT-3' and reverse, 5'-CCACAGGATTCCATACCCAAGA-3' (Invitrogen). The relative mRNA expression levels were determined using the 2-ΔΔCq method [25].

Western blotting

Proteins from renal tissues or cultured TCMK-1 cells were extracted with RIPA Lysis Buffer (P0013B, Beyotime Institute of Biotechnology). The protein concentration was determined using a BCA protein assay kit (K763-KIT, Amresco). The protein (50 μg) loaded in each lane was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by electroblotting onto polyvinylidene difluoride membranes (EMD Millipore, Billerica, MA, USA), and then blocked with 5% non-fat milk and incubated overnight at 4°C with the following primary antibodies against Tisp40 (1:100; sc-54800; Santa), α-SMA (1:100; ab5694; Abcam), E-cadherin (1:100; 610181; BD Biosciences), collagen I (1:100; ab34710; Abcam), fibronectin (1:100; ab2413; Abcam), TGF-β (1:100; 18978-1-AP; PTG), phosphor(p)Smad2/3 (D27F4; 1:100; Cell signaling technology), Smad2 (1:100; 12570-1-AP; PTG), Smad3 (1:100; 25494-1-AP; ATG) and Snail (1:100; ab180714; Abcam). Washed three times with Tris-buffered saline containing Tween-20 (TBST), the membranes were incubated with HRP-conjugated goat anti-rabbit/mouse polyclonal secondary antibody (1:100; LK2001/ LK2003; Sungene Biotech.) for 1 h at room temperature. Target proteins were visualized and detected using Odyssey Infrared Imaging system Model 9120 (LI-COR Biotechnology, Lincoln, NE, USA) with Quantity-One software (Bio-Rad Laboratories, Inc.).

Establishment of a stable cell line

Fragments were retrieved by 1% agarose gel electrophoresis using an Agarose Gel Extraction Kit (Omega, D2500), after double-enzyme digestion of pUC57-Tisp40 and pLVX-mCMV-ZsGreen-IRES-Puro (Wuhan Viraltherapy Technologies Co. Ltd) by EcoRI and SpeI (NEB), followed by connection by T4 DNA Ligase (BM121, Transgen BioTech, Beijing). The ligation product was transformed into JM109 (Wuhan Viraltherapy Technologies Co. Ltd). The plasmid was extracted using a Small Plasmid Extraction Kit (EM101, Transgen BioTech, Beijing) after the inoculation and amplification of single colonies, and the bacterial fluid was verified by sequencing. The sequencing primer was CMV-F, whose primer sequence is 5'-CGCAAATGGGCGGTAGCGGTG-3'. Then, 293T cells were co-transfected with pLVX-Tisp40-ZsGreen-Puro (recombinant plasmid) or pLVX-ShRNA2-Puro (control plasmid) to obtain high-titer lentiviral (rLV-Tisp40 and rLV-ShRNA2) containing the target gene using a Lentiviral Packaging Kit (R003, Wuhan Viraltherapy Technologies Co. Ltd). When the TCMK-1 cells reached 80-90% confluence, a total of 5×10⁵ cells/ml cells were transferred to cell plates, which were transfected with rLV-Tisp40 and rLV-ShRNA2 on the basis of MOI=20 the next day. Two days after transfection, lentivirus-infected TCMK-1 cells were selected using complete medium (DMEM supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin) containing 10 μg/ml puromycin. TCMK-1/Tisp40 (Tisp40-overexpression group) and TCMK-1/vector cells (empty vector group) were obtained.

Small-interfering RNA

RNA oligonucleotides for scramble (forward, 5'-CCUACGCCACCAAUUCGdTdT-3') (Bioneer); mouse siRNA1-Tisp40 (forward, 5'-CCCAUAUGAUACACUGCUCAGAAA-3'), mouse siRNA2-Tisp40 (forward, 5'-GGCCAACCUUCGGACUCAUCUCUAU-3'), mouse siRNA3-Tisp40 (forward, 5'-AGUAGAAGUUGGCCACUCCAUGGG-3') [26] (Invitrogen) were synthesized. Each siRNA (100nM) was transfected into TCMK-1/wt cells using Lipofectamine RNA iMAX (Invitrogen) for at least 48h; cells were then harvested for RNA and protein preparation. The siRNA which had the strongest inhibitory effect was applied to this study.

Immunofluorescence staining

Cells were fixed in 4% paraformaldehyde for 30 min at room temperature and subsequently permeabilized with 0.1% Triton X-100 (BIOSHARP) for 10 min at room temperature. Following blocking in 10% donkey serum (ANT051; antGene), the cells were incubated with primary antibodies targeting fibronectin (1:200; ab2413; Abcam) overnight at 4°C. The cells were then cultured with Alexa Fluor®594 donkey anti-rabbit IgG (1:400; A20207) and Alexa Fluor®594 donkey anti-mouse IgG (1:400; A21203; both Invitrogen) for 30min at 37°C. The cell nuclei were stained with 4'6'-diamino-2-phenylindole dihydrochloride (216276, Roche).

Statistical analysis

All data are presented as the mean ± standard deviation (s.d). Analysis of variance was used to evaluate statistical significance. P-values < 0.05 were regarded significant. All experiments were performed at least 3 times. Statistical analysis was conducted using SPSS 19.0.

Results

Expression of Tisp40 is elevated in the kidney of I/R-induced mice in vivo, and TGF-β increased the expression of Tisp40 in TCMK-1 cells dose-dependently and time-dependently

First, we investigated whether the expression of Tisp40 in the kidney is changed by I/R. We found that the expression of Tisp40 was upregulated at both the mRNA level and the protein level in the kidneys of I/R-induced mice (Fig. 1A, 1B and 1C). Furthermore, IHC staining showed that Tisp40 expression was significantly higher in I/R-induced renal tubular epithelial cells (Fig. 1D and 1E). Consistently, TCMK-1/wt cells treated with TGF-β also showed elevated expression of Tisp40, while unconditioned TCMK-1/wt cells showed only limited expression of Tisp40 (Fig. 1F, 1G and 1H). Exogenous TGF-β increased the expression of Tisp40 in TCMK-1 cells. In addition, we confirmed that TGF-β increased the expression of Tisp40 dose-dependently (0.1, 1, or 10 ng/ml) and time-dependently (from 0 to 48 h) by Western blot (Fig. 1I, 1J and 1K). These results indicated that Tisp40 might be critical in renal fibrosis.

To determine the effects of Tisp40 on TGF-β-stimulated renal cellular fibrosis, we established a stable cell line, TCMK-1/Tisp40 (Fig. 1L), and knocked down endogenous Tisp40 by transfecting TCMK-1/wt cells with siRNA-Tisp40. We constructed three kinds of siRNA-Tisp40, and siRNA3-Tisp40 proved to have the strongest inhibitory effect based on Western blot (Fig. 1M and 1N).

Tisp40 enhanced ECM accumulation in cultured TGF-β-stimulated TCMK-1 cells

Next, we investigated the effects of Tisp40 on ECM accumulation in cultured TGF-β-stimulated TCMK-1 cells. Fibronectin and collagen I were examined by Western blotting and qPCR, and fibronectin was also examined by immunofluorescence staining. Following treatment with TGF-β, the expression levels of fibronectin and collagen I increased significantly in cultured TCMK-1/Tisp40 cells compared to the control and the empty vector group (Fig. 2A, 2B, 2C, 2D, 2I and 2K) but decreased significantly in a cultured Tisp40-deficient TCMK-1 cell line (Fig. 2E, 2F, 2G, 2H, 2J and 2L). These results indicated that Tisp40 enhanced ECM accumulation in the cultured TGF-β-stimulated

Fig. 1. Tisp40 is upregulated in I/R kidneys and TGF- β -induced TCMK-1 cell line, and TGF- β increased expression of Tisp40 in TCMK-1 cells dose-dependently and time-dependently. (A-C) Tisp40 measured by representative real-time reverse transcriptase polymerase chain reaction and Western blot analysis in the kidney of wild-type mice after ischemia-reperfusion (I/R); D, Tisp40 staining; Brown staining indicates immunopositivity ($\times 400$); E, quantitative analysis of Tisp40 (brown area %) from the sections; (F-H) Tisp40 measured by representative real-time reverse transcriptase polymerase chain reaction and Western blot analysis in TCMK-1/wt, TCMK-1/Tisp40, TCMK-1/vector cells after treatment of TGF- β ; (I-K) Tisp40 measured by western blot analysis in TCMK-1/wt cells treated with different dose (0.1, 1, or 10 ng/ml) and period (4, 8, 12, 24, 48h) of TGF- β ; L, establishment of TCMK-1/Tisp40 (Tisp40-overexpression group) and TCMK-1/vector cells (empty vector group); (M, N) Tisp40 measured by western blot analysis in TCMK-1/wt cells transfected by control siRNA, siRNA1-Tisp40, siRNA2-Tisp40 and siRNA3-Tisp40 (all 100 nM), then treated with TGF- β . Protein level of β -actin was used as internal loading control. The data are shown as the mean $\pm$ s.d. * $P < 0.05$, ** $P < 0.01$ versus sham-operation (Sham) or control (Con) (0 ng/ml TGF- β); # $p < 0.05$, ## $p < 0.01$ versus TGF- β -induced TCMK-1/wt cells.

TCMK-1 cell line, which supports the hypothesis that Tisp40 could be critical in renal fibrosis.

Tisp40 aggravated EMT in TGF- β -stimulated TCMK-1 cells

To confirm the effect of Tisp40 on EMT in TCMK-1 cells, the expression levels of α -SMA (mesenchymal marker) and E-cadherin (epithelial marker) were examined by Western blotting and qPCR. Cellular exposure to TGF- β resulted in a significant EMT,

Fig. 2. Tisp40 enhanced ECM accumulation in TGF- β -induced TCMK-1 cell line. (A-D, I) fibronectin and collagen I measured by representative real-time reverse transcriptase polymerase chain reaction and Western blot analysis in TCMK-1/wt, TCMK-1/Tisp40, TCMK-1/vector cells treated with or without TGF- β ; (E-G, J) fibronectin and collagen I measured by representative real-time reverse transcriptase polymerase chain reaction and Western blot analysis in TCMK-1/wt cells transfected by control siRNA or siRNA-Tisp40 (both 100nM), then treated with or without TGF- β ; K, fibronectin detected by immunofluorescence in TCMK-1/wt and TCMK-1/Tisp40 cells treated with or without TGF- β ($\times 400$), fibronectin (red) and DAPI (blue); L, fibronectin detected by immunofluorescence in TCMK-1/wt cells transfected by control siRNA or siRNA-Tisp40 (both 100nM), then treated with or without TGF- β ($\times 400$), fibronectin (red) and DAPI (blue). Protein level of β -actin was used as internal loading control. The data are shown as the mean $\pm$ s.d. * $P < 0.05$, ** $P < 0.01$ versus corresponding control (Con) (0 ng/ml TGF- β); # $p < 0.05$, ## $p < 0.01$ versus TGF- β -induced TCMK-1/wt cells or scramble.

characterized by a decrease in E-cadherin and an increase in α -SMA, which aggravated by Tisp40 overexpression compared with the control and empty vector group (Fig. 3A, 3B, 3C, 3D and 3I) and relieved by Tisp40 silencing compared to the scramble group (Fig. 3E, 3F, 3G, 3H and 3J). These results suggest that Tisp40 aggravates the loss of the epithelial marker E-cadherin and increases the expression of the myofibroblast marker α -SMA in TGF- β -stimulated renal epithelial cells, which reinforces the possibility Tisp40 could be critical in renal fibrosis.

Tisp40 knockout protects against I/R-induced renal fibrosis

After determining the effects of Tisp40 on TGF- β -stimulated renal cellular fibrosis *in vitro*, we investigated the protective effect of Tisp40 deficiency against renal fibrosis *in vivo* using Tisp40-knockout (Tisp40 $^{-/-}$) and wild-type (Tisp40 $^{+/+}$). We compared renal pathologic changes in renal fibrosis mice of both genotypes induced by I/R. We confirmed that Tisp40 was not expressed in the kidneys of Tisp40 $^{-/-}$ mice by Western blot and qPCR (Fig. 4A, 4B and 4C). Masson trichrome staining and hydroxyproline analysis suggested that the kidneys of wild-type mice exhibited significantly elevated atrophy and necrosis of tubular and interstitial fibrosis after induction by I/R. By contrast, the renal fibrosis in Tisp40 $^{-/-}$ mice induced by I/R was markedly attenuated (Fig. 4D, 4E and 4F). Furthermore, IHC staining revealed that, relative to Tisp40 $^{+/+}$

Fig. 3. Tisp40 aggravated EMT in TGF-β-induced TCMK-1 cell line. (A-D, I) α-SMA and E-cadherin measured by representative real-time reverse transcriptase polymerase chain reaction and Western blot analysis in TCMK-1/wt, TCMK-1/Tisp40, TCMK-1/vector cells treated with or without TGF-β; (E-G, J) α-SMA and E-cadherin measured by representative real-time reverse transcriptase polymerase chain reaction and Western blot analysis in TCMK-1/wt cells transfected by control siRNA or siRNA-Tisp40 (both 100nM), then treated with or without TGF-β. Protein level of β-actin was used as internal loading control. The data are shown as the mean ± s.d. *P<0.05, *P<0.01 versus corresponding control (Con) (0 ng/ml TGF-β); #p<0.05, ##p<0.01 versus TGF-β-induced TCMK-1/wt cells or scramble.

Fig. 4. Tisp40-knockout mice are protected against I/R-induced renal fibrosis. (A-C) Tisp40 measured by representative real-time reverse transcriptase polymerase chain reaction and Western blot analysis in kidneys of wild-type mice and Tisp40-knockout mice induced by ischemia-reperfusion (I/R) or not; D, Masson staining (×400); E, quantitative analysis of the extent of collagen fibrils (blue area %) from the sections; F, hydroxyproline analysis. Protein level of β-actin was used as internal loading control. The data are shown as the mean ± s.d. *P<0.05, *P<0.01 versus corresponding sham-operation (Sham); #p<0.05, ##p<0.01 versus I/R-induced wild-type.

Fig. 5. Tisp40-knockout mice are protected against I/R-induced ECM accumulation. A, fibronectin staining; B, collagen I staining; Brown staining indicates immunopositivity ($\times 400$). C, quantitative analysis of fibronectin (brown area %) from the sections; D, quantitative analysis of collagen I (brown area %) from the sections; (E-H) fibronectin and collagen I measured by representative real-time reverse transcriptase polymerase chain reaction and Western blot analysis in kidneys of wild-type mice and Tisp40-knockout mice induced by ischemia-reperfusion (I/R) or not. Protein level of β -actin was used as internal loading control. The data are shown as the mean $\pm$ s.d. * $P < 0.05$, ** $P < 0.01$ versus corresponding sham-operation (Sham); # $p < 0.05$, ## $p < 0.01$ versus I/R-induced wild-type.

mice, Tisp40 $^{-/-}$ mice exhibited significantly lower levels of ECM accumulation, collagen I and fibronectin (Fig. 5A, 5B, 5C and 5D). The mRNA and protein expression was further examined by qPCR and Western blot (Fig. 5E, 5F, 5G, 5H and 5I). In addition, we explored the difference of EMT in mice of both genotypes induced by I/R, and compared with Tisp40 $^{-/-}$ mice, Tisp40 $^{+/+}$ mice showed a more serious EMT, characterized by a decrease in E-cadherin and an increase in α -SMA (Fig. 6).

The inhibitory effect of Tisp40 deficiency on renal fibrosis requires modulation of Smad2 and Smad3 phosphorylation

Considering the well-characterized role of Smad2 and Smad3 as key downstream mediators in the TGF- β signaling pathway of renal fibrosis [27], we investigated whether the inhibitory signal-mediated process of Tisp40 deficiency in renal fibrosis requires Smad2/3. To study signal transduction pathways, we first confirmed that the levels of both total and phosphor-Smad2/3 in kidneys are higher in I/R-induced mice than in control mice (Fig. 7A, 7B, 7C and 7D). We next found that there was no difference in TGF- β , Smad2 and Smad3 in I/R-induced kidneys between Tisp40 $^{-/-}$ mice and wild-type mice (Fig. 7G, 7H, 7I, 7J and 7L), but both pSmad2 and pSmad3 were significantly inhibited in the kidneys of Tisp40 $^{-/-}$ mice induced by I/R (Fig. 7E, 7F, 7K and 7L). These results suggested that Tisp40 deficiency provides a protective effect against renal

Fig. 6. Tisp40-knockout mice are protected against I/R-induced ECM accumulation. A, α -SMA staining; B, E-cadherin staining; Brown staining indicates immunopositivity ($\times 400$); C, quantitative analysis of α -SMA (brown area %) from the sections; D, quantitative analysis of E-cadherin (brown area %) from the sections; (E-H) α -SMA and E-cadherin measured by representative real-time reverse transcriptase polymerase chain reaction and Western blot analysis in kidneys of wild-type mice and Tisp40-knockout mice induced by ischemia-reperfusion (I/R) or not. Protein level of β -actin was used as internal loading control. The data are shown as the mean $\pm$ s.d. * $P < 0.05$, ** $P < 0.01$ versus corresponding sham-operation (Sham); # $p < 0.05$, ## $p < 0.01$ versus I/R-induced wild-type.

fibrosis induced by IRI without modulating the renal expression of TGF- β , Smad2 and Smad3 rather than inhibiting the phosphorylation of Smad2 and Smad3 in I/R-induced kidneys. In our *in vitro* study, Tisp40 overexpression upregulated the expression of pSmad2/3 without modulation of Smad2 and Smad3 (Fig. 8A, 8B, 8C and 8G). In contrast, when Tisp40 was knocked down by transfection with siRNA-Tisp40, the TGF- β -induced pSmad2/3 level was reduced (Fig. 8D, 8E, 8F and 8H). These results provided further evidence that renal fibrosis suppressed by Tisp40 deficiency is mediated by the downregulation of Smad2/3 phosphorylation.

Inhibition of Tisp40 decreases Snail signaling

Snail has been shown to be a key EMT-regulatory gene in tubular epithelial cells [28]. In the process of EMT, the expression of E-cadherin and α -SMA was proven years ago to be concomitant with Snail protein [29, 30]. Considering the relationship between Snail and EMT, we then investigated the expression of Snail *in vitro* and *in vivo* to examine the underlying mechanism of Tisp40 deficiency-inhibited EMT. To investigate whether Tisp40 affects Snail signaling, we first determined that TGF- β promoted the expression of Snail. The expression of TGF- β -stimulated Snail in TCMK-1/Tisp40 cells was markedly higher than that in the control and empty vector group (Fig. 9F, 9G and 9J). In contrast, when Tisp40 was knocked down by transfection with siRNA-Tisp40, the TGF- β -induced Snail level was reduced (Fig. 9H, 9I and 9K). These results *in vitro* tentatively confirmed our assumption that the inhibition of Tisp40 decreases Snail signaling. Snail was significantly inhibited in the I/R-induced kidneys of Tisp40-knockout mice compared with wild-type mice (Fig. 9A, 9B, 9C, 9D and 9E), which confirms that Tisp40 deficiency is mediated by Snail downregulation to inhibit EMT.

Fig. 7. IRI activates TGF- β /Smad signaling, and Tisp40-deficiency inhibits I/R-induced renal fibrosis by modulating Smad2 and Smad3 phosphorylation without modulating renal expression of TGF- β directly. (A-D) Smad2, Smad3 and pSmad2/3 measured by western blot analysis in the kidney of wild-type mice after ischemia-reperfusion (I/R); E, pSmad2/3 staining; Brown staining indicates immunopositivity ($\times 400$); F, quantitative analysis of pSmad2/3 (brown area %) from the sections; (G, H, L) TGF- β measured by

representative real-time reverse transcriptase polymerase chain reaction and Western blot analysis in kidneys of wild-type mice and Tisp40-knockout mice induced by ischemia-reperfusion (I/R) or not; (I-L) Smad2, Smad3 and pSmad2/3 measured by western blot analysis in kidneys of wild-type mice and Tisp40-knockout mice induced by ischemia-reperfusion (I/R) or not. Protein level of β -actin was used as internal loading control. The data are shown as the mean $\pm$ s.d. * P < 0.05, ** P < 0.01 versus corresponding sham-operation (Sham); # p < 0.05, ## p < 0.01 and ^ p > 0.05 versus I/R-induced wild-type.

Discussion

Renal IRI develops easily into chronic renal failure through inflammatory interstitial fibrosis, whose mechanism has not been fully clarified [2-4]. Thus, studying the biological networks, especially transcription factors, is an indispensable process in research on developing therapies to inhibit and delay kidney fibrosis against kidney disease. Our study showed for the first time that Tisp40 (41 kDa) is involved in renal tubulointerstitial fibrosis. Tisp40 deficiency inhibited TGF- β -stimulated EMT and ECM accumulation in renal tubular epithelial cells and prevented pathological changes of renal fibrosis by I/R. Furthermore, the inhibitory effect of Tisp40 deficiency on renal fibrosis was related to the downregulation of Smad2/3 phosphorylation.

ER-resident transcription factors are involved in the regulation of various biological processes, including cell maturation, differentiation and maintenance of cellular homeostasis [31]. Tisp40, as a member, has been proven to play an important role in the differentiation of intestinal goblet and Paneth cells [32] and in spermatogenesis [22, 33]. In this study, we explored the mechanism of Tisp40's involvement in renal fibrosis. We showed that Tisp40 expression was elevated following I/R; in other words, the expression of Tisp40 was increased by ischemic injury. The tubular epithelial

Fig. 8. Tisp40-deficiency inhibits TGF- β -induced cellular fibrosis by modulating Smad2 and Smad3 phosphorylation. (A-C, G) Smad2, Smad3 and pSmad2/3 measured by western blot analysis in TCMK-1/wt, TCMK-1/Tisp40, TCMK-1/vector cells treated with or without TGF- β ; (D-F, H) Smad2, Smad3 and pSmad2/3 measured by western blot analysis in TCMK-1 cells transfected by control siRNA or siRNA-Tisp40 (both 100nM), then treated with or without TGF- β . Protein level of β -actin was used as internal loading control. The data are shown as the mean $\pm$ s.d. * P <0.05, ** P <0.01 versus corresponding control (Con) (0 ng/ml TGF- β); # p <0.05, ## p <0.01 and ^ p >0.05 versus TGF- β -induced TCMK-1/wt cells or scramble.

Fig. 9. Tisp40-deficiency decreases Snail signaling. A, Snail staining; Brown staining indicates immunopositivity ($\times 400$); B, quantitative analysis of Snail (brown area %) from the sections; (C-E) Snail measured by representative real-time reverse transcriptase polymerase chain reaction and Western blot analysis in kidneys of wild-type mice and Tisp40-knockout mice induced by ischemia-reperfusion (I/R) or not; (F, G, J) Snail measured by representative real-time reverse transcriptase polymerase chain reaction and Western blot analysis in TCMK-1/wt, TCMK-1/Tisp40, TCMK-1/vector cells treated with or without TGF- β ; (H, I, K) Snail measured by representative real-time reverse transcriptase polymerase chain reaction and Western blot analysis in TCMK-1 cells transfected by control siRNA or siRNA-Tisp40 (both 100nM), then treated with or without TGF- β . Protein level of β -actin was used as internal loading control. The data are shown as the mean $\pm$ s.d. * P <0.05, ** P <0.01 versus sham-operation (Sham) or control (Con) (0 ng/ml TGF- β); # p <0.05, ## p <0.01 versus I/R-induced wild-type, TGF- β -induced TCMK-1/wt cells or scramble.

cells exposed to injury stimulation undergo de-differentiation and release profibrotic mediators [10-13, 15]. Excessive exposure to profibrotic cytokines leads to the acquisition of mesenchymal markers and the loss of epithelial cells [34]. As a central component of the cell adhesion junction, E-cadherin is required for epithelial formation to maintain epithelial homeostasis. The loss of E-cadherin and excessive α -SMA are the most important aspects of EMT [35, 36].

To explore whether Tisp40 plays a beneficial role in renal fibrosis, we conducted an experiment *in vitro*. We found that the expression levels of α -SMA, fibronectin, and collagen I was increased and the expression of E-cadherin attenuated in cultured TCMK-1/Tisp40 cells, which demonstrates aggravated EMT and ECM accumulation. To further confirm that Tisp40 is critical in renal fibrosis, we measured the TGF- β -stimulated expression of genes related to fibrosis in cultured renal tubular epithelial cells transfected with siRNA-Tisp40. As expected, the knockdown of Tisp40 blocked the expression of fibrosis-related genes, suggesting a strong possibility that Tisp40 is involved in I/R-induced renal fibrosis and that Tisp40 deficiency has a protective effect against I/R-induced tubulointerstitial fibrosis. This conclusion was further confirmed *in vivo*. The IR-induced atrophy and necrosis of tubular and tubulointerstitial fibrosis was markedly attenuated in Tisp40 knockout mice compared with wild-type mice.

TGF- β , a typical fibrogenic cytokine associated with EMT and ECM protein accumulation in renal diseases, is elevated not only in human patients but also in animal models of renal fibrosis [17, 18]. Given the crucial role of TGF- β in renal fibrosis, we asked how TGF- β correlated with Tisp40. We found that TGF- β time- and dose-dependently increased the expression levels of Tisp40 in cultured renal cells. In a variety of renal diseases, highly expressed TGF- β can phosphorylate downstream factors Smad2/3 to induce the development of tubulointerstitial fibrosis, causing EMT and increasing ECM accumulation [37-39]. TGF- β exerts multiple biological effects by initiating canonical and non-canonical pathways, among which Smads signaling is recognized as crucial in renal tubulointerstitial fibrosis. During fibrogenesis, Smad2/3 are highly activated and are associated with Smad7 inhibition via an ubiquitin E3-ligase-dependent degradation mechanism [40]. Multiple previous studies have demonstrated renal fibrosis can be effectively attenuated both *in vivo* and *in vitro* by blocking TGF- β /Smad signaling in a variety of ways (neutralizing antibodies, antisense oligonucleotides and inhibitors) [41-43]. In this study, we confirmed that Smad2/3 were activated in I/R-induced kidneys and cultured TGF- β -stimulated renal tubular epithelial cells. In addition, we asked whether the protective effect of Tisp40 deficiency is mediated by modulating the renal expression of TGF- β directly. However, no change in the expression of TGF- β was detected between Tisp40-deficient mice and wild-type mice. This result suggests that the antifibrotic effect of Tisp40 deficiency does not suppress the renal expression of TGF- β directly. Subsequently, our research results confirmed that Smad2 and Smad3 expression showed no difference between Tisp40-deficient mice and wild-type mice as well as different groups *in vitro*. In other words, in both *in vivo* and *in vitro* experiments, Smad2/3 show no relationship to Tisp40. Interestingly, in Tisp40-deficient I/R mice, pSmad2/3 were expressed at lower levels. Consistently, Smad2/3 phosphorylation was reduced in TGF- β -stimulated TCMK-1/wt cells treated with siRNA-Tisp40 but increased in TCMK-1/Tisp40 cells. These results showed that the downregulation of Tisp40 facilitated the phosphorylation of Smad2/3 but had no direct effect on Smad2 and Smad3.

The zinc finger transcription factor Snail is a key molecule that triggers the process of tubular EMT. Snail can bind to a specific DNA sequence (CANNTG, where N is any nucleotide), called E-boxes, in the promoter of the E-cadherin gene to repress E-cadherin transcription [44, 45]. Given the role of Snail in EMT, we investigated the correlation between Snail and Tisp40. We confirmed that Snail was activated in I/R-induced kidneys and cultured TGF- β -stimulated renal tubular epithelial cells. Moreover, we found that Tisp40 was closely related to Snail, and Tisp40 downregulation decreased the

expression of Snail. These results suggest that Tisp40 deficiency decreases Snail protein expression *in vitro* and *in vivo*. The mechanism by which Tisp40 deficiency inhibits renal EMT and ECM accumulation remains unknown. Most likely, multiple pathways are involved in the inhibitory effect of Tisp40 deficiency, and Smad2/3 activation and Snail expression may be the major events during the process. Tisp40 downregulation relieves renal tubulointerstitial fibrosis by suppressing the TGF- β /Smad2/3 pathway and subsequently inhibiting Snail expression. It is also possible that Snail might also be a potential target of Tisp40. The exact target of Tisp40 and the mechanism by which Tisp40 affects renal tubulointerstitial fibrosis need to be further elucidated.

Previous studies have shown that MAPKs (p38, ERK, JNK) can regulate the phosphorylation of Smad2/3 driven by TGF- β [46-48]. In considering the role of MAPKs in phosphorylating Smad2/3, we cannot exclude the possibility that Tisp40 deficiency inhibits renal fibrosis via MAPKs or that one or several MAPK signaling processes are involved in the antifibrotic effect of Tisp40-deficiency. These possibilities should be investigated in our future studies.

In conclusion, our results show that Tisp40 is involved in the process of renal fibrosis. The knockdown of Tisp40 inhibits EMT and ECM accumulation. This reduction is associated with the downregulation of Smad2/3 phosphorylation. In addition, Snail, an important downstream mediator of the TGF- β /Smads signaling pathway, is important in the process. Given the above, Tisp40 could be a useful therapeutic target in the fight against renal interstitial fibrosis.

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Disclosure Statement

All author declare no conflicts of interest.

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