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TAK1 may promote the development of diabetic nephropathy by reducing the stability of SnoN protein

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ABSTRACT

Aims: This study aimed to investigate the role of transforming growth factor-β-activated protein kinase 1(TAK1) in the development of diabetic nephropathy (DN) by regulating the protein stability of Ski-related novel protein N(SnoN).

Main methods: A combination of in vivo and in vitro model systems was used to investigate how TAK1 regulated the expression of SnoN protein in DN. The study determined the effects of modulating the expression or activity of TAK1 on the SnoN protein level and its influence on the epithelial–mesenchymal transition (EMT) and extracellular matrix (ECM) deposition.

Key findings: Under the high-glucose condition, the activation of TGF-β1/TAK1-induced phosphorylation and ubiquitination of SnoN protein resulted in reduced SnoN protein level as a consequence of enhanced SnoN degradation, which promoted EMT and ECM deposition in renal tubular epithelial cells. The study showed that TAK1 impaired SnoN protein level by decreasing the protein stability of SnoN.

Significance: TAK1 mediated the phosphorylation of SnoN, resulting in SnoN ubiquitination and eventual degradation, which enhanced EMT and ECM deposition to promote renal fibrosis during DN.

1. Introduction

Diabetic nephropathy (DN) is one of the main causes of end-stage renal disease [1]. The pathogenesis of DN is complex and not fully understood. Transforming growth factor-β1(TGF-β1) is a cytokine, and TGF-β1/Smad signal is recognized as a key pathway contributing to the pathogenesis of renal fibrosis in DN. Renal tubular epithelial cells can be induced by TGF-β1/Smad signal to undergo epithelial–mesenchymal transition (EMT) and increase the deposition of extracellular matrix (ECM), thereby promoting kidney fibrosis [2–4].

Ski-related novel protein N (SnoN), as an important negative regulator of TGF-β1/Smad signal, inhibits the expression of TGF-β1 target gene by interacting with Smads protein [5,6]. Our previous studies showed that SnoN protein was downregulated in renal tubular cells under high-glucose (HG) condition and in kidney tissues of rats with diabetes mellitus (DM), resulting in hyperactive TGF-β1/Smad signal, increased EMT of renal tubular epithelial cells, and increased deposition of ECM. These studies suggested that the downregulation of SnoN protein played an important role in the pathogenesis of DN [7,8].

Previous studies [9–12] showed that TGF-β1 treatment resulted in a rapid and significant reduction in SnoN protein, which was mainly driven by E3 ubiquitin ligases, such as Smad ubiquitination regulatory factor 2(Smurf2). The activation of TGF-β1/Smad signal could promote SnoN protein ubiquitination and subsequent degradation by the proteasome, resulting in the reduction in SnoN protein level. In view of the fact that SnoN protein has an important biological function similar to the “switch” of TGF-β1/Smad signal pathway, it was presumed that the level of SnoN protein was strictly regulated in terms of transcriptional activation and protein stability.

Transforming growth factor-β-activated protein kinase 1(TAK1) is a

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member of the Mitogen-activated protein kinase (MAP3K) family and functions as a serine/threonine kinase. It is a downstream factor of TGF-β1 signaling pathway and also known as mitogen-activated protein kinase kinase kinase 7(Map3k7) [13,14]. On stimulation by TGF-β1, TAK1 is released from the cell membrane to form an active complex with TAK1-binding (TAB1) protein. As expected, the overexpression of TAB1 further activated TAK1 [15,16].TAK1 has been shown to be vital in regulating the stability of SnoN protein [17]. This study speculated that TAK1 could phosphorylate SnoN protein with TGF-β1 stimulation, and phosphorylated SnoN protein might be required for SnoN degradation via ubiquitination mediated by TGF-β1. Therefore, it was hypothesized that high glucose might mediate the phosphorylation of SnoN protein by activating TAK1, impairing the stability of SnoN to facilitate its degradation via ubiquitination, resulting in the decrease in SnoN protein level in renal tubular epithelial cells, and ultimately promoting TGF-β1/Smad pathway–mediated renal tubulointerstitial fibrosis in DN.

This study demonstrated that the activation of TAK1 induced phosphorylation and ubiquitination of SnoN protein, resulting in the downregulation of E-cadherin and induction of mesenchymal markers and Collagen I (Col-I), Collagen III (Col-III), Collagen IV (Col-IV) and promoting the invasion and migration of renal tubular epithelial cells under DM condition. These results indicated that TAK1 participated in DN.

2. Materials and methods

2.1. Antibodies and kit

The antibodies used in this study and their sources were as follows:TAK1(CST, USA), *p-TAK1*(*Thr184/187*)(CST, USA), TAB1(CST, USA), SnoN(Santa Cruz, USA), Desmin(Santa Cruz, USA), TGF-β1(Santa Cruz, USA), *p-Smad2*(*Ser465/467*) (CST, USA), *p-Smad3*(*Ser423/425*) (CST, USA), E-cadherin(Santa Cruz, USA), Smurf2(Santa Cruz, USA), CollagenI(Sigma, USA), CollagenIII(Sigma, USA), CollagenIV(Sigma, USA), anti-ubiquitylation (Abcam, USA), anti-phosphoserine (Bioss, China), and 5Z-7-oxozeaenol (Tocris, England). The Dynabeads™ Protein G Immunoprecipitation Kitw (Thermo Fishe, rUSA), streptavidin–peroxidase immunohistochemical staining kit(Solarbio, China), urine protein concentration kit Meidikang (Shaoxing, China), blood glucose determination kit(Johnson & Johnson, USA), triglyceride determination kit(Sigma, USA), cholesterol determination kit (Sigma, USA), horseradish peroxidase–conjugated goat anti-rabbit immunoglobulin G (IgG), horseradish peroxidase–conjugated goat anti-mouse IgG(Pumei, China), total RNA extraction kit(Tiangen, China), Revert Aid First Strand cDNA Synthesis Kit (Fermentas, Lithuania), SYBR Prime Scriptreal-time polymerase chain reaction (RT-PCR) kit (Tiangen, China), bicinchoninic acid (BCA) protein assay kit (Tiangen, China), and ultrasensitive enhanced chemiluminescence kit(Smart, China), Dimethyl sulfoxide(DMSO)(Solarbio, China).

2.2. Animal model

A total of 16 healthy and specific-pathogen-free male Sprague–Dawley rats, weighing180 ± 20 g, were provided by Beijing HuaFuKang Bioscience Co., Ltd. (Beijing, China) and housed in the animal center of Guizhou Medical University (Guizhou, China). The study was conducted in accordance with the guidelines of the National Health and Medical Research Council of China's Code and the Animal Experimental Ethics Committee of Guizhou Medical University (No. 1702230) for the care and use of animals for scientific purpose. All rats were randomly divided into the diabetic group (DM group, $n = 8$) and normal control group (NC group, $n = 8$). Diabetic rats were produced by injecting 0.01 mol/L streptozotocin (STZ, prepared with sterile citric acid–sodium citrate buffer, pH 4.5; Sigma) into the tail vein at a dose of 55 mg/kg. The fasting blood glucose level of all rats was detected after 72 h. The rats with blood glucose level ≥16.7 mmol/L and glucose in their urine were considered to be diabetic, indicating that the diabetic rat model was established successfully. The rats in the NC group were age-matched and injected with an equal volume of solvent. The rats in each group were given a normal diet and unlimited drinking water. After 8 weeks, 24-h urine of each rat was collected in a metabolic cage, and the total volume of urine was recorded before the rats were sacrificed. The rats were fasted for 6–8 h before being anesthetized using diethyl ether, and their femoral arteries were punctured to collect blood samples, which were centrifuged at 4 °C to separate serum. Urine and serum were stored at −20 °C for measuring urine protein and biochemical indices. The kidneys of each rat were harvested; one was fixed with 4% paraformaldehyde for paraffin sections, and the other was snap-frozen in liquid nitrogen and stored at −80 °C for use in molecular studies. The KW/BW ratio was assessed as: kidneys weight(mg)/body weight(g) each rat.

2.3. Biochemical analysis

The oxidase method was used to measure serum glucose, and the Coomassie Brilliant Blue method was used to measure urine protein. Total cholesterol (TC) and triglycerides were determined using enzymatic assay kits. All tests were analyzed using Bayer 1650 automatic biochemical analyzer (Beckman, USA), according to the manufacturer's protocol. Urine protein excretion (mg/24 h) was assessed as follows: urine protein (mg/mL) × urine volume (mL)/24 h.

2.4. Histological analysis

Paraformaldehyde-fixed renal tissues were embedded in paraffin, and the sections were prepared using standard methods. Hematoxylin–eosin (H&E) staining was used for morphological and structural analyses. Masson's trichrome staining was used to detect renal tissue fibrosis [16]. The images were taken and then the second block was adjusted according to the original image so that the blue part appeared clearly. After all the images were analyzed, the average value of Area% was calculated. Data were statistically analyzed using Image-Pro Plus 6.0 software.

2.5. Immunohistochemistry

The sections were deparaffinized in xylene, rehydrated in decreasing percentage of ethanol solutions, and incubated in 3% $H_2O_2$for 20 min at 37 °C. They were then washed three times with phosphate-buffered saline for 5 min. Antigen retrieval was performed in a microwave oven, and the sections were then blocked in 5%bovine serum albumin. The sections were incubated overnight with an anti-Col-I primary antibody(1:150 dilution) at 4 °C. Following washing, the sections were incubated with affinity-purified biotinylated goat anti-rabbit antibody. The secondary antibody used was affinity-purified biotinylated goat anti-rabbit or anti-mouse IgG. The protein was visualized using diaminobenzidinetetrahydrochloride as a chromogen. Tissue sections were counterstained with Mayer's hematoxylin. These stained slides were observed under a microscope and photographed. The distribution and expression of positive protein in rat kidney tissues were detected by measuring the mean value of positive staining area. Data were statistically analyzed using Image-Pro Plus 6.0 software.

2.6. Cell culture

The rat renal proximal tubule epithelial cells(NRK-52E cells) were purchased from Jennio Biotech and cultured in Dulbecco's modified Eagle's medium (DMEM, Hyclone, UT, USA) containing 10% fetal bovine serum (Gibco, Invitrogen, CA, USA) and 5.5 mmol/L normal glucose unless otherwise indicated. The cultures were maintained at 37 °Cin the presence of 5% CO_2.For normal-glucose (NG) condition, the

cells were cultured in DMEM containing 2% FBS and 5.5 mmol/L of glucose. For HG condition, the cells were cultured in DMEM containing 2% FBS and 25 mmol/L of glucose. 5Z-7-oxozeaenol[(5Z)-7o] was dissolved in dimethyl sulfoxide (DMSO) to prepare 500 mM stock solutions. Equal volumes of DMSO were used for controls.NRK-52E cells were typically pretreated with (5Z)-7o(2 mM) for 0.5 h, followed by incubation with HG condition for 48 h.

2.7. Transfection

Plasmid extraction was performed according to the manufacturer's protocol. Transfection began when50%–60% of cells fused. A DMEM culture medium (no serum, no bio-antibiotics), polyethyleneimine, and plasmid (*Map3k7 shRNA* plasmid*: Lv-Map3k7shRNA*, *Map3k7/TAB1* plasmid*: p2CMV-Map3k7-Tab1*, Supplementary Material Fig. 1) were added, mixed, and placed in a sterile environment for 20 min. The culture medium was discarded. The liquid was put in an Eppendorf tube and blended, with which the cells were incubated at 37 °C in the presence of 5% CO_2 for 6 h. The solution was discarded. The DMEM culture medium (no serum, no bio-antibiotics) was added, and the cells were incubated at 37 °C in the presence of 5% CO_2 overnight. Then, the culture medium was discarded and the cells were washed with PBS. The culture media of different treatments were added as needed, and the cells were incubated at 37 °C in the presence of 5%CO_2 for 48 h.

2.8. Wound healing assay

The cells were cultured in a 6-well plate and overnight to form uniform monolayers. Scratch wounds were generated using a pipette tip (NEST, USA). The cultures were maintained at 37 °C in the presence of 5% CO_2 and photographed at 0 h and 24 h time points. The area of the scratch changed over time and the scratch area of each field of view and the percentage of wound healing were calculated. Data were statistically analyzed using Image-Pro Plus 6.0 software.

2.9. Transwell invasion assay

The cells were placed in 24-well Transwell plate. Then, 200 μL of serum-free media was added to the upper chamber, and 400 μL of media containing 10% FBS to the lower chamber. The plates were incubated at 37 °C in the presence of 5% CO_2 for 12 h. The cells were photographed after fixation with paraformaldehyde and stained with hematoxylin. Direct cell count was used. Five visual fields were randomly selected for each culture hole, and the cells were photographed and counted.

2.10. Real time-quantitative PCR (RT-qPCR)

RNA was purified from cells or tissues using trichloromethane, isopropanol, and ethyl alcohol according to the manufacturer's protocol (Tiangen, China). First-strand cDNA was synthesized from 5-μg total RNA in renal tissuesor3-μg total RNA in NRK-52E cells using the Revert Aid First Strand cDNA Synthesis Kit (Fermentas, Lithuania) and stored at −20 °C. An SYBR Prime Script RT-PCR Kit (Tiangen, China) was used to measure TAK1 and β-actin transcription levels with the following primers:

TAK1 forward (5′-TCTGGATGTCCCTGAGATCGT-3′).

TAK1 reverse (5′-GCTCACCTGACCAGGTTCTG-3′).

β-actin forward (5′-ACCACCATGTACCCAGGCAT-3′).

β-actin-reverse (5′-CCGGACTCATCGTACTCCTG-3′).

Relative quantification of gene expression was performed by the $2^{-\Delta\Delta Ct}$ method using β-actin as internal control.

2.11. Western blot analysis

Total protein lysates from cells and tissues were prepared in Radio Immunoprecipitation Assay(RIPA) (Solarbio, China) and quantified using the Bicinchoninic acid(BCA) kit (Beyotime, China). Equal amounts of proteins were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis(SDS-PAGE) and transferred onto a Poly vinylidene fluoride(PVDF) membrane. The membranes were incubated at room temperature for 1 h in blocking buffer (5% fat-free milk in Tris-buffered saline containing Tween-20) and subsequently overnight at 4 °C with primary antibodies diluted in blocking buffer as follows: SnoN (1:500), E-cadherin(1:500), α-Smooth muscle actin(α-SMA)(1:800), β-actin(1:4000), TAK1(1:800), *p-TAK1*(*Thr184/187*)(1:800), *p-Smad2(Ser465/467)(1:1000)*, *p-Smad3*(*Ser423/425*)(*1:1000*), and TGF-β1(1:500). Signals were detected by chemiluminescence after incubation with appropriate secondary antibodies. Images were acquired using the Bio-Rad gel imaging system(Bio-Rad, CA, USA), and the band intensity was quantified using the Quantity One 4.6 software (Bio-Rad).

2.12. Co-immunoprecipitation

Total protein lysates were prepared in 50 mM Tris-HCl, pH 7.4, 0.2 M NaCl, 2 mM Ethylene Diamine Tetraacetic Acid(EDTA), 0.5%NP-40, 50mMNaF, 0.5 mM Na_3VO_4, 20 mM sodium pyrophosphate, 1 mM Phenylmethanesulfonyl fluoride(PMSF), 10 μg/mL aprotinin, 10 μg/mL leupeptin, 1 mM Dithiothreitol(DTT), and 150 mM NaCl. Co-immunoprecipitation was performed by adding Dynabeads-Ab complexes to equal amounts of protein lysates and incubating the mixtures overnight at 4 °C. Following washing of the co-immunoprecipitate complexes, the samples were subjected to Western blot analysis.

2.13. Statistical analysis

All data were expressed as mean ± standard deviation ($\bar{x} \pm s$). The SPSS19.0 software(IBM Corp, Armonk, NY, USA) was used for statistical analysis. Statistical analysis between the groups was performed using an unpaired Student *t*-test, and comparison among multiple groups was performed using one-way analysis of variance followed by Student–Newman–Keulsq-test. $P < 0.05$ was considered statistically significant.

3. Results

3.1. High glucose activated TGF-β1/Smad and TGF-β1/TAK1 pathways, and decreased SnoN protein levels, accompanied by renal tubular interstitial fibrosis in vitro and in vivo

Western blot analysis demonstrated that the expression of TGF-β1, *p-Smad3* (*Ser423/425*), and *p-Smad2* (*Ser465/467*) protein increased remarkably in NRK-52E cells under HG condition for 24 or 48 h ($P < 0.05$; Fig. 1a–b). Meanwhile, no difference was found in the expression of Smad2 and Smad3 between groups. The expression of TAK1 in NRK-52E cells cultured under NG and HG conditions was determined by RT-qPCR and Western blot analysis. Increased levels of TAK1 mRNA transcripts were observed in cells cultured in HG compared with those cultured in NG after 24 and 48 h ($P < 0.05$; Fig. 1c). The levels of TAK1 and *p-TAK1*(*Thr184/187*) proteins were confirmed by Western blot analysis to be higher under HG condition ($P < 0.05$; Fig. 1d–e). The protein levels of Col-III and Desmin were also upregulated in NRK-52E cells cultured under HG compared with NG condition ($P < 0.05$; Fig. 1f–g). In contrast, the protein levels of SnoN and E-cadherin were lower under HG compared with NG condition ($P < 0.05$; Fig. 1d–g). Except for SnoN, these changes occurred in a time-dependent manner, with the differences being more prominent after culture in HG for 48 h.

To confirm that DN was successfully replicated in the DM rat model, the biochemical analysis of blood sera and urine from these animals, as well as the histological evaluation of their kidneys, was performed. As shown in Supplementary Material Table 1, KW/BW, blood glucose, 24-h urine protein, total cholesterol, and triglyceride levels were

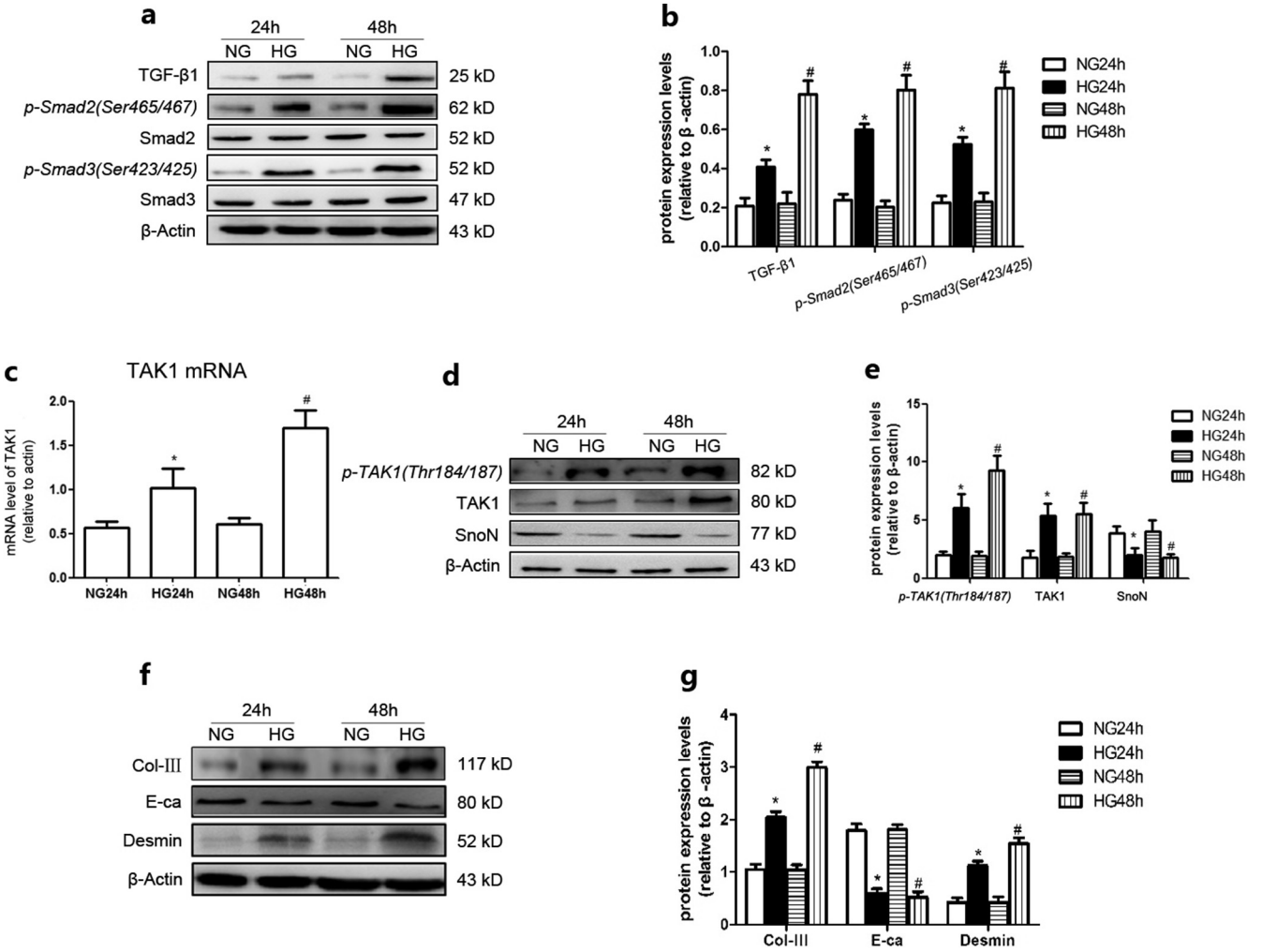

Fig. 1. High glucose activated TGF-β1/Smads and TGF-β1/TAK1 pathways, and decreased SnoN protein levels, accompanied by renal tubular cells fibrosis. High glucose activated TGF-β1/Smads pathway in NRK-52E cells. Representive (a) Western blot and (b) quantitative data are revealed the expression of TGF-β_1, *p-Smad2*(*Ser465/467*), Smad2, *p-Smad3*(*Ser423/425*), Smad3. High glucose increase TAK1 mRNA expression and activity in NRK-52E cells, while reduced SnoN protein level in NRK-52E cells. (c) Graphical presentations show the relative abundance of TAK1mRNA after normalization with β-actin mRNA analysed by RT-qPCR. Representive (d) Western blot and (e) quantitative data are revealed the expression of *p-TAK1*(*Thr184/187*), TAK1, and SnoN. High glucose enhanced fibrosis in NRK-52E cells. Representive (f) Western blot and (g) quantitative data are revealed the expression of Col-III, *E*-ca, and Desmin. NG: Normalglucose; HG: Highglucose; Col-III: CollagenIII; E-cadherin: E-ca. *, #means data of differed significantly in comparison with that of the NG group and HG24h group;$^{*}P < 0.05$;$^{\#}P < 0.05$.$\bar{x} \pm s$, $n = 3$.

significantly higher in the DM group compared with the NC group ($P < 0.05$). H&E(Fig. 2a) and Masson($P < 0.05$; Fig. 2b and d) staining showed an increase in mesangial matrix and thickness of the glomerular basement membrane in 8-week diabetic rats. Moreover, renal tubular interstitial fibrosis was observed, with part of renal tubular epithelial cells atrophied or lost and inflammatory cells increased and infiltrated in widened tubulointerstitium. The kidneys of animals in the DM group showed increased immunohistochemical positivity for Col-I compared with those from animals in the NC group (Fig. 2c and e). Consistent with the immunohistochemical studies, Western blot analysis revealed increased expression of Col-IV in the kidneys of rats in the DM group compared with those in the NC group(Fig. 2f–g). It was concluded that DM rats displayed biochemical and histological features of DN.

TGF-β1 signal was robustly activated in the DM group, as evident by the increase in the levels of *p-Smad2*(*Ser465/467*) and *p-Smad3*(*Ser423/425*)($P < 0.05$; Fig. 2i–j). The levels of TAK1mRNA were significantly higher in the DM group compared with the NC group ($P < 0.05$; Fig. 2h), similar to the findings on NRK-52E cells cultured under HG compared with NG condition. The protein levels of TAK1 and *p-TAK1*(*Thr184/187*) were also higher in the DM group. In contrast, the protein levels of SnoN were lower in the DM group compared with the NC group ($P < 0.05$;Fig. 2k–l). These results suggested the existence of activation of TGF-β1/Smad and TGF-β1/TAK1 axis and a remarkable decrease in SnoN protein level, accompanied by renal fibrosis in rats with DM.

3.2. TAK1 downregulated the expression of SnoN and promoted EMT and ECM deposition

To investigate the relationship between the expression of TAK1 and SnoN in the development of DN, the expression of TAK1 in NRK-52E cells was knocked down by transfecting *Map3k7shRNA* plasmid and especially inhibiting TAK1 activity by pre-treatment with 5Z-7-oxozeaenol((5z)-7o)(2 mM). It was confirmed by RT-qPCR and Western blot analysis that *Map3k7shRNA* plasmid mediated the knockdown of the expression of TAK1, resulting in a decrease in TAK1 mRNA and protein levels and also in *p-TAK1*(*Thr184/187*) protein levels ($P < 0.05$;Fig. 3a–c), which is similar to the expression of *p-TAK1*(*Thr184/187*) in NRK-52E cells pre-treated with(5Z)-7o(2 mM)

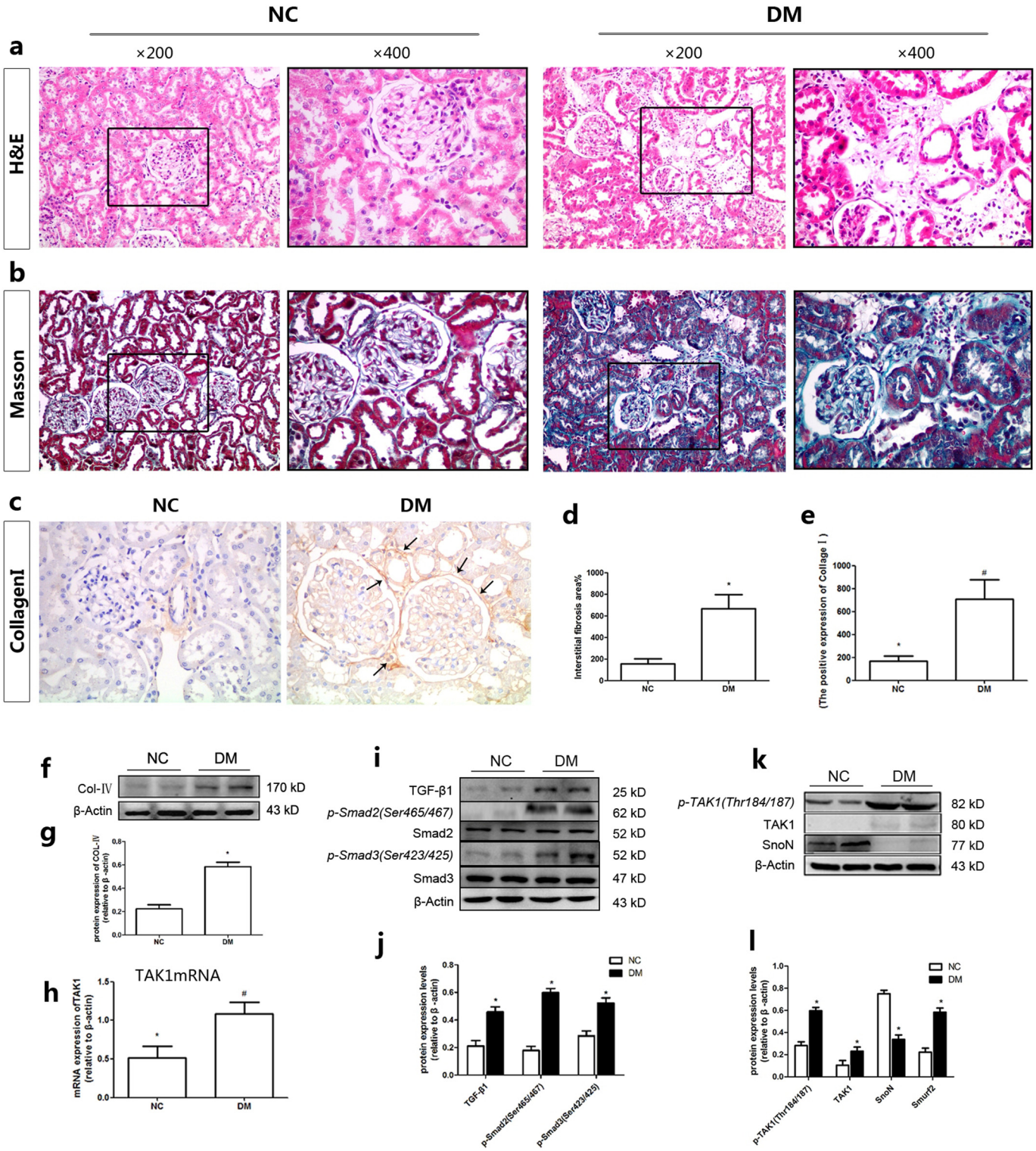

Fig. 2. DM activated TGF-β1/Smads and TGF-β1/TAK1 pathways, decreased SnoN protein levels, accompanied by renal tubular interstitial fibrosis in DM rats.DM enhanced renal fibrosis in diabetic rats. (a and b) Histological changes of kidneys in the NC group and DM group (hematoxylin–eosin staining, and Masson stains, magnification ×200), black box indicates the enlarged area of details for fibrosis (magnification ×400). (d) Graphical presentations show the interstitial fibrosis area. Representive (c) Immunohistochemical staining (magnification ×200) and (e) quantitative data are revealed the expression of Col-I in the kidney tissues in different groups. Arrows (→) indicate positive expression. Representive (f) Western blot and (g) quantitative data are revealed the expression of Col-IV in the kidney tissues in different groups. (h) Graphical presentations show the relative abundance of TAK1mRNA after normalization with β-actin mRNA analysed by RT-qPCR. Representive (i) Westernblot and (j) quantitative data are revealed the expression of TGF-β_1, *p-Smad2*(*Ser465/467*), Smad2, *p-Smad3*(*Ser423/425*), Smad3 in the kidney tissues in different groups. Representive (k) Western blot and (l) quantitative data are revealed the expression of *p-TAK1*(*Thr184/187*), TAK1, and SnoN in the kidney tissues in different groups. NC: normal control; DM: diabetes Mellitus; H&E: Hematoxylin-eosin staining; Col-I: CollagenI; Col-IV: CollagenIV, *means data of differed significantly in comparison with that of the NC group;*$P < 0.05$.$\bar{x} \pm s$, $n = 8$.

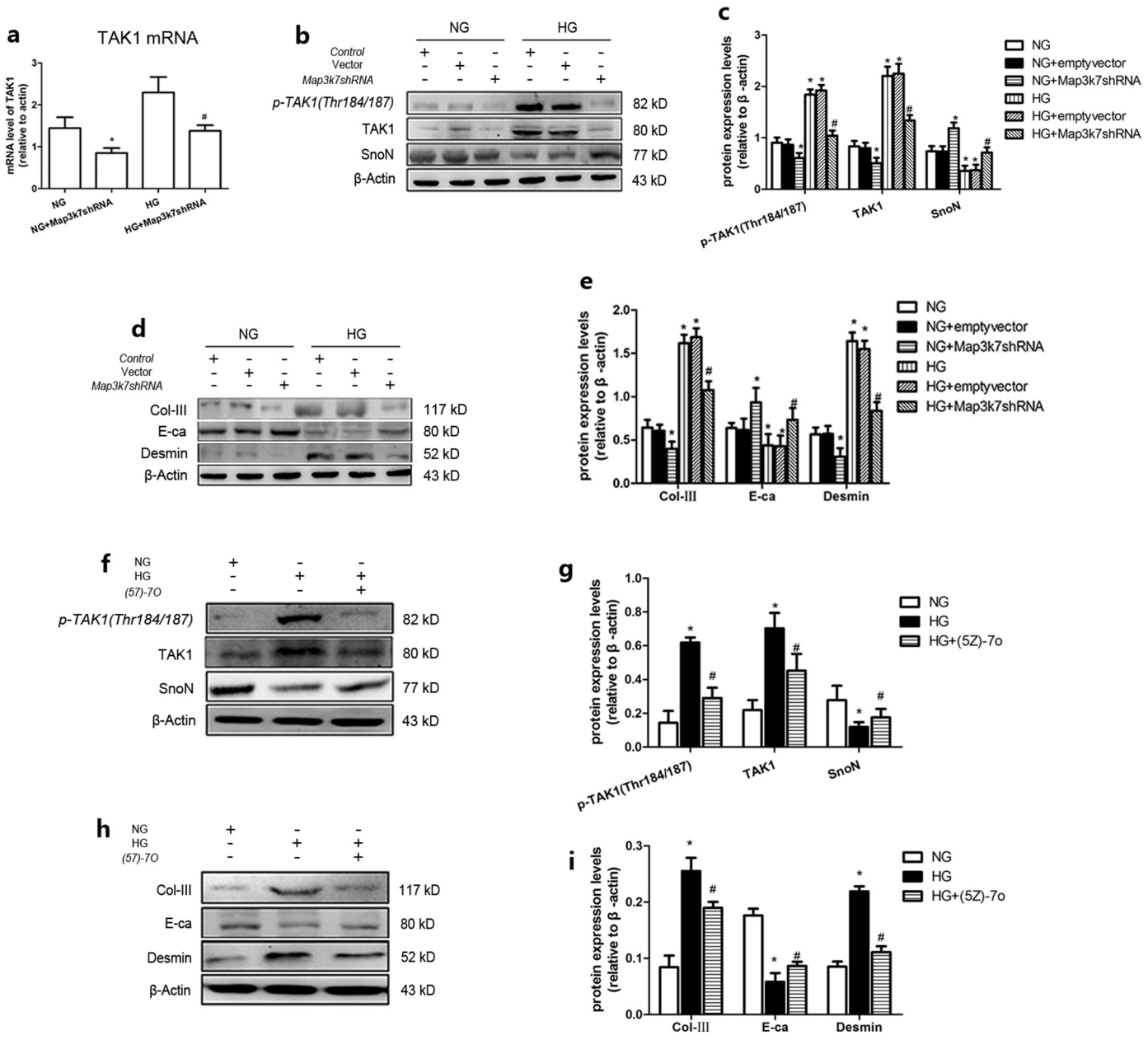

Fig. 3. Inhibition of TAK1 expression and activity increased SnoN protein levels and ameliorated renal tubular interstitial fibrosis. Knock down TAK1 by transfection of *Map3k7shRNA*plamid. (a) Graphical presentations show the relative abundance of TAK1mRNA after normalization with β-actin mRNA analysed by RT-qPCR. Representive (b) Western blot and (c) quantitative data are revealed the expression of *p-TAK1*(*Thr184/187*), TAK1, and SnoN in NRK-52E cells under different conditions. Representive (d) Western blot and (e) quantitative data are revealed the expression of Col-III, *E*-ca, and Desmin in NRK-52E cells under different conditions. Activity of TAK1 suppressed by (5Z)-7o(2 mM) pre-treatment. Representive (f) Western blot and (g) quantitative data are revealed the expression of *p-TAK1*(*Thr184/187*), TAK1, and SnoN. Representive (h) Western blot and (i) quantitative data are revealed the expression of Col-III, E-ca, and Desmin in NRK-52E cells under different conditions.(5Z)-7o:5Z-7-oxozeaeno. *, #means data of differed significantly in comparison with that of the NG group and HG group;*$P < 0.05$; #$P < 0.05$.$\bar{x} \pm s$, $n = 3$.

($P < 0.05$;Fig. 3f–g). After the protein levels or activity of TAK1 were impaired, decreased levels of Col-III and Desmin ($P < 0.05$;Fig. 3d–e, h–i) were observed in NRK-52E cells. However, the levels of SnoN and E-cadherin were significantly higher than the those in cells cultured in HG medium ($P < 0.05$;Fig. 3b–i).

On the contrary, transfecting with overexpression *Map3k7/TAB1* plasmid resulted in an increase in the expression of TAK1 compared with respective controls (under NG and HG conditions) ($P < 0.05$;Fig. 4a–c). In the TAK1/TAB1-overexpressed group, the expression levels of *p-TAK1*(*Thr184/187*), TAK1, TAB1, Col-III, and Desmin increased, while those of SnoN and E-cadherin were decreased ($P < 0.05$;Fig. 4b–e).

The role of TAK1 in NRK-52E cell migration and invasion was characterized by performing scratch wound healing and Transwell invasion assays. HG promoted wound healing as indicated by a more rapid scratch wound closure compared with NG. The inhibition of TAK1 by knockdown or (5Z)-7o decreased the rate of wound closure. On the contrary, wound healing was accelerated in the TAK1/TAB1-overexpressed group, with the wound almost completely filled at the 24-h time point, while a significant gap still existed in the control group ($P < 0.05$;Fig. 5a and c). These results were further confirmed in Transwell migration assay. In this assay, HG-induced cell migration was suppressed by TAK1 knockdown or inhibition with(5Z)-7o.Similar to the scratch wound healing assay, the TAK1/TAB1-overexpressed group

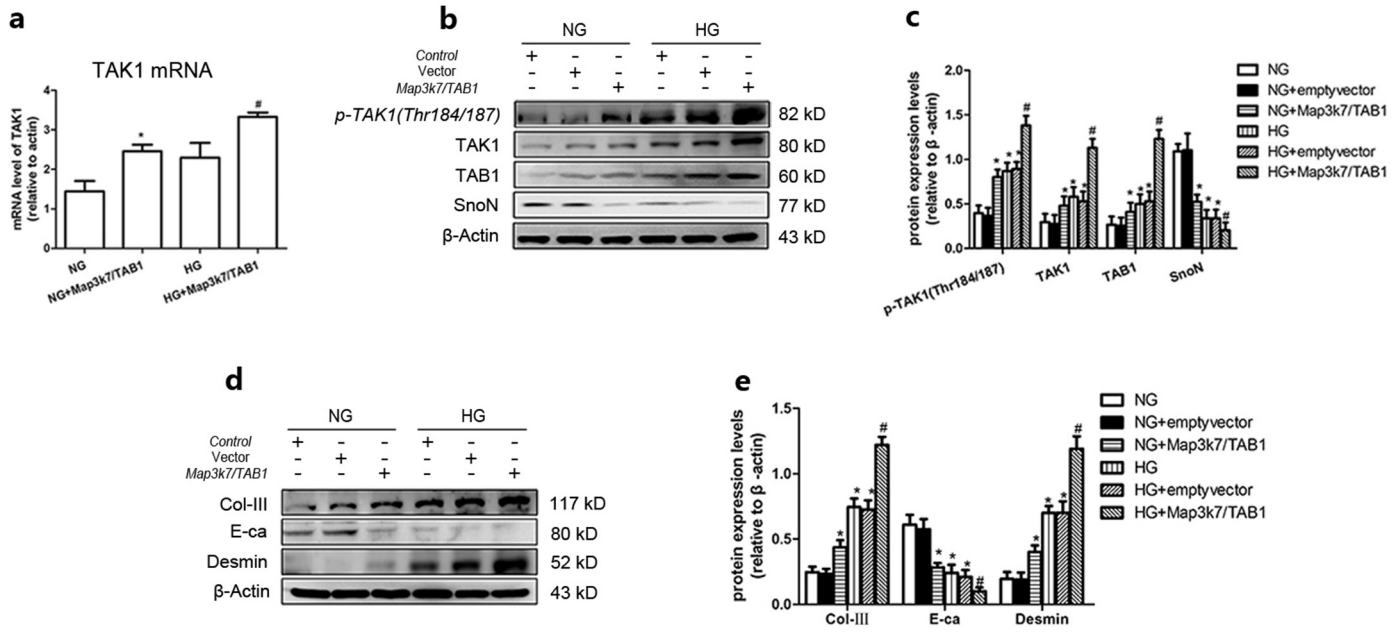

Fig. 4. Augment of TAK1 expression and activity reduced SnoN protein levels and promote renal tubular interstitial fibrosis. Augment of TAK1 expression and activity by transfection of *Map3k7/Tab1*plamid. (a) Graphical presentations show the relative abundance of TAK1mRNA after normalization with β-actin mRNA analysed by RT-qPCR. Representive (b) Western blot and (c) quantitative data are revealed the expression of *p-TAK1*(*Thr184/187*), TAK1, and SnoN in NRK-52E cells under different conditions. Representive (d) Western blot and (e) quantitative data are revealed the expression of Col-III, E-ca, and Desminin NRK-52E cells under different conditions.*, #means data of differed significantly in comparison with that of the NG group and HG group;*$P < 0.05$;#$P < 0.05$.$\bar{x} \pm s$, $n = 3$.

showed further increase in cell migration compared with HG treatment alone ($P < 0.05$; Fig. 5b and d). These results indicated that TAK1 could promote NRK-52E cell migration and invasion.

3.3. TAK1 modulated the phosphorylation and ubiquitination of SnoN

To investigate SnoN regulation in relation to TAK1, SnoN protein was immunoprecipitated from NRK-52E cells under different treatment conditions and its phosphorylation and ubiquitination levels were measured. High glucose induced the phosphorylation and ubiquitination of SnoN. The levels of SnoN phosphorylation and ubiquitination were further induced in the TAK1/TAB1-overexpressed group. As expected, TAK1 knockdown or inhibition with (5Z)-7o resulted in reduced phosphorylation and ubiquitination of SnoN protein (Fig. 6a). This could potentially be related to the time-dependent increase in the levels of Smurf2, an E3 ligase that can mediate the ubiquitination of target protein ($P < 0.05$;Fig. 6b–c). Also, in vivo, the expression levels of SnoN protein decreased and the phosphorylation and ubiquitination of SnoN significantly increased in the DM group (Fig. 6d). However, in the kidneys of diabetic rats, increased protein expression of Smurf2 was noted at the protein level ($P < 0.05$;Fig. 6e–f). These results suggested that TAK1 played an important role in regulating the protein stability of SnoN by modulating SnoN phosphorylation, which propelled Smurf2-mediated ubiquitin degradation of SnoN protein.

4. Discussion

DN is a complex disease driven by many factors still not fully understood. TGF-β1 has been recognized as a principal fibrogenic factor, and the TGF-β1/Smad pathway plays a key role in renal fibrosis. TGF-β1/Smad signal promotes the synthesis of ECM and induces phenotypic changes in renal tubular cells [18]. SnoN is a nuclear transcriptional co-repressor that negatively regulates TGF-β1/Smad signal [5,19]. Our previous studies showed that HG could reduce the expression level of SnoN protein in renal tubular cells, thereby limiting the negative feedback effect of SnoN on TGF-β1/Smad signal [20]. These data suggested that SnoN could inhibit the occurrence of EMT of renal tubular epithelial cells under HG condition, thus having a protective role against the development of renal fibrosis and DN. This study validated that TGF-β1/Smad signal was activated and the expression level of SnoN reduced in NRK-52E cells cultured in HG. The expression of markers of EMT and ECM also increased under HG condition, consistent with a condition that favored renal fibrosis and the development of DN. These results supported the model in which aberrantly activated TGF-β1/Smad signal under HG condition as a result of reduced expression of SnoN led to enhanced EMT and ECM deposition.

Our previous study also found that the increase in the mRNA expression of SnoN was not consistent with the protein expression in DN [21]. The protein expression level was not coupled with the expression of SnoN mRNA, as TGF-β1 led to a reduction in SnoN rapidly and significantly [21–23]. This decrease was mainly related to Smurf2, E3 ubiquitin enzyme, which specifically recognized SnoN and was involved in activating Smad-mediated ubiquitination and degradation of SnoN [9]. Protein has the ability to resist the influence of various factors and maintain their biological activity, referred to as the stability of protein, which mainly depends on the spatial structure of protein [24]. Most proteins are in the dynamic process of synthesis and degradation in biological cells, which is affected by the intracellular environment, and this dynamic characteristic is the prerequisite for realizing its physiological function. However, the mechanisms underlying the reduction in the stability of SnoN protein in DN are unknown.

TAK1 has been shown to have an important role in regulating the stability of SnoN protein. Kajino et al. [15] showed that TGF-β1 increased the activity of TAK1 and decreased the expression of SnoN protein. Further, the present study also found that TAK1 was a phosphorylated SnoN protein kinase. Further, the ubiquitination of SnoN protein increased and the protein levels decreased significantly. This study speculated that in response to treatment with TGF-β1, TAK1 interacted and phosphorylated SnoN at multiple sites, leading to increased degradation via ubiquitination. Under DM conditions, reduced SnoN protein levels were observed, accompanied by elevated levels of TAK1 mRNA and protein. The reduction in SnoN levels under HG condition in DM mice coincided with an increase in its phosphorylation and ubiquitination. The polyubiquitin modification of proteins

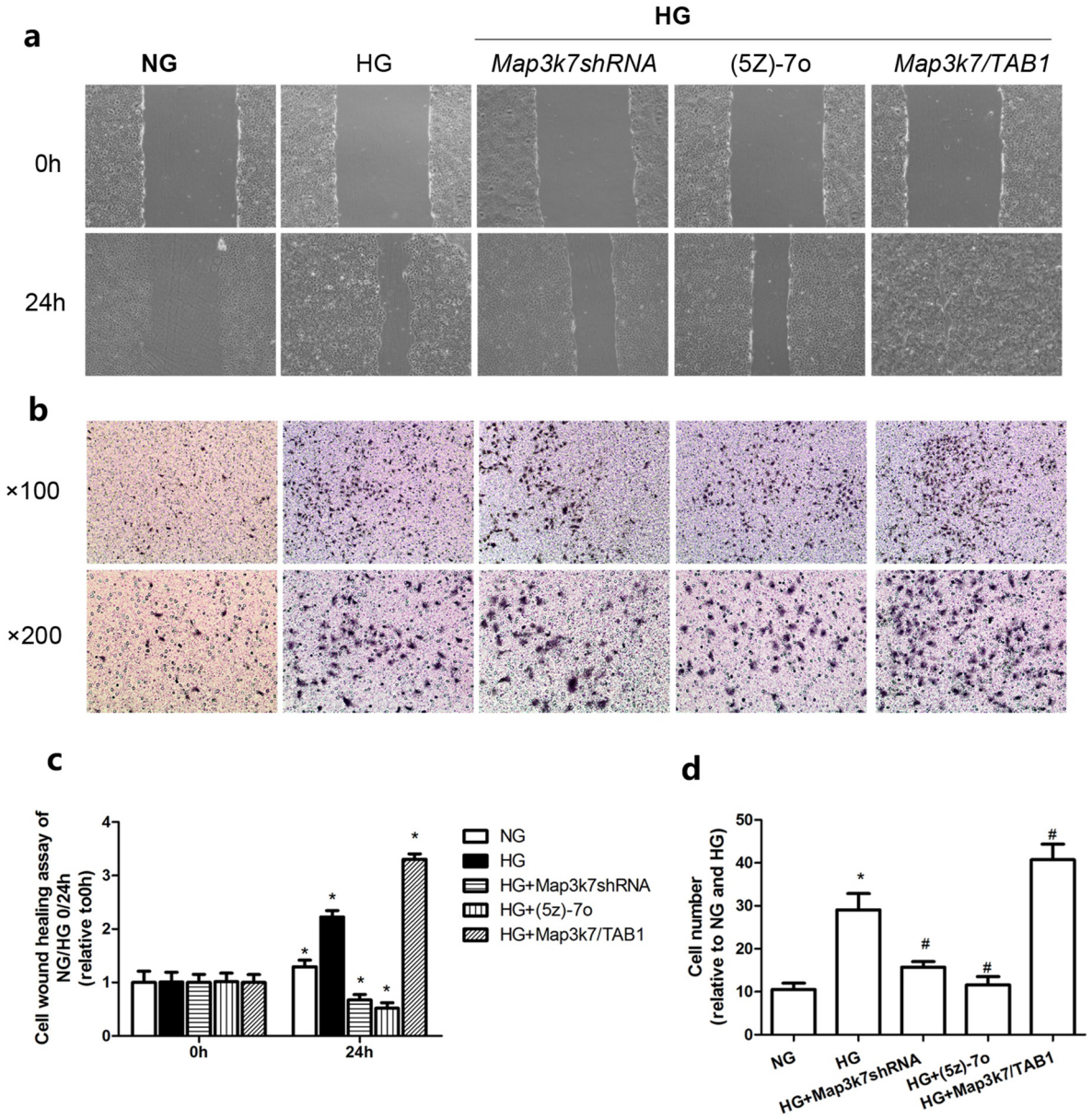

Fig. 5. TAK1 promotes migration and invasion of renal tubular cells *under* high glucose condition. (a and c) Wound Healing Assay(magnification ×200) and (b and d) Transwell Invasion Assay(magnification×100, ×200) indicated TAK1 promotes migration and invasion of NRK-52E cells under different conditions.(5Z)-7o:5Z-7-oxozeaeno.*, #means data of differed significantly in comparison with that of the NG group and HG group;*$P < 0.05$;#$P < 0.05$.$\bar{x} \pm s$, $n = 3$.

mediates the proteasome degradation of proteins, and the increase in the levels of ubiquitinated SnoN protein are consistent with the increase in degradation, resulting in a decrease in SnoN protein levels. The data suggested that the stability of SnoN protein level might be reduced by TAK1-mediated phosphorylation, which marked the ubiquitination and degradation of SnoN by the proteasome.

The study next investigated the relationship between TAK1 and the stability of SnoN protein in NRK-52E cells cultured under HG or NG condition. Under HG condition, the knockdown of TAK1 by specific shRNA or inhibition of TAK1 activity by (5Z)-7o restored the expression of SnoN, leading to a reduction in EMT and ECM deposition. In contrast, the activation of TAK1 further reduced the expression of SnoN and promoted EMT and ECM deposition. It was speculated that during the development of DN, the aberrant activation of TGF-β1 induced the expression of TAK1, which in turn phosphorylated SnoN, leading to its ubiquitination and subsequent degradation.

5. Conclusions

The results suggested that HG could activate the TGF-β1/TAK1 pathway, which induced the phosphorylation of SnoN and promoted its ubiquitination and degradation by the proteasome, leading to reduced SnoN protein levels in renal tubular epithelial cells. This ultimately led to the promotion of renal tubular interstitial fibrosis, a common pathological feature of DN.

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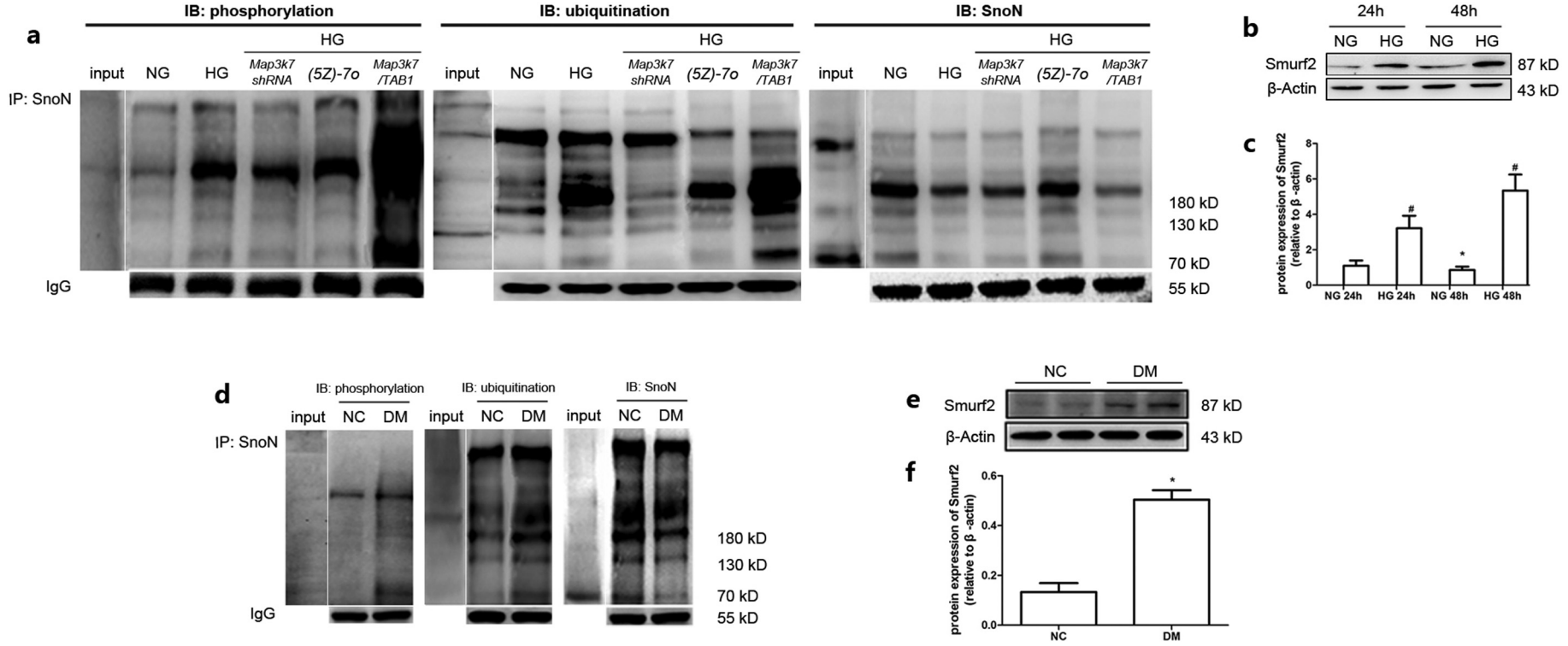

Fig. 6. TAK1 modulates phosphorylation and ubiquitination of SnoN in renal tubular cells during DN. (a) Immunoprecipitation shows the phosphorylation and ubiquitination of SnoN in NRK-52E cells under different conditions. Representive (b) Western blot and (c) quantitative data are revealed the expression of Smurf2 in NRK-52E cells under different conditions for 24 and 48 h. $\bar{x} \pm s$, $n = 3$. (d) Immunoprecipitation shows the phosphorylation and ubiquitination of SnoN in the kidney tissues in different groups. Representive (e) Western blot and (f) quantitative data are revealed the expression of Smurf2 in the kidney tissues in different groups. $\bar{x} \pm s$, $n = 4$. (5Z)-7o:5Z-7-oxozeaeno.*, #means data of differed significantly in comparison with that of the NG group and HG group;$^{*}P < 0.05$;$^{\#}P < 0.05$.

Disclosure statement

The authors declare no conflicts of interest.

Author contributions

Yuanyuan Wang and Yanwen Mao contributed equally to this study.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lfs.2019.04.058.

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