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ORIGINAL ARTICLE

LncRNA SNHG16 regulates RAS and NF-κB pathway-mediated NLRP3 inflammasome activation to aggravate diabetes nephropathy through stabilizing TLR4

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Abstract

Aims LncRNA SNHG16 and Toll-like receptor-4 (TLR4) participate in diabetes nephropathy. This study investigated whether SNHG16 regulates diabetic renal injury (DRI) via TLR4 and its related mechanism.

Methods Diabetic mice and high glucose (HG)-induced HRMCs were used to examine the expressions of SNHG16 and TLR4. The SNHG16 expression, cytokines, reactive oxygen species, MDA, SOD, GSH, and fibrosis-related proteins were evaluated in HG-induced HRMCs transfected with sh-NC or sh-SHNG16. RNA immunoprecipitation and RNA pull-down determined the interaction between SNHG16 and EIF4A3 or TLR4 and EIF4A3. We used HG-treated HRMCs or diabetic mice to investigate the roles of TLR4 or SNHG16 in renal injuries.

Results Both SNHG16 and TLR4 were upregulated in diabetic conditions. HG increased serum Scr and BUN, led to significant fibrosis, increased inflammation- and renal fibrosis-related proteins in mice, and increased ROS, MDA, and decreased SOD and GSH in HRMCs. SNHG16 silencing diminished HG-upregulated SNHG16, decreased HG-increased cytokines secretion, ROS, MDA, and fibrosis but increased SOD and GSH. RIP and RNA pull-down confirmed that SNHG16 recruits EIF4A3 to stabilize TLR4 mRNA. TLR4 knockdown alleviated HG-induced renal injuries by suppressing RAS and NF-κB-mediated activation of NLRP3 inflammasomes. SNHG16 knockdown alleviated HG-induced renal injuries in HG-induced HRMCs or diabetic mice. Interestingly, TLR4 overexpression reversed the effects of SNHG16 knockdown. Mechanistically, SNHG16 knockdown alleviated HG-induced renal injuries by suppressing TLR4.

Conclusion SNHG16 accelerated HG-induced renal injuries via recruiting EIF4A3 to enhance the stabilization of TLR4 mRNA. The SNGHG16/ELF4A3/TLR4 axis might be a novel target for treating DRI.

Keywords LncRNA SNHG16 · Diabetic nephropathy · High glucose · RAS · TLR4 · NRLP3 inflammasome

Abbreviations

LncRNA	Long non-coding RNAs
SNHG16	Small nucleolar RNA host gene 16
RAS	Renin-angiotensin system
HG	High glucose
TLR4	Toll-like receptor 4
NRLP3	NLR family pyrin domain containing 3
EIF4A3	Eukaryotic translation initiation factor 4A3
DRI	Diabetic renal injury
DN	Diabetes nephropathy
RIP	RNA immunoprecipitation
HRMCs	Human renal mesangial cells
ROS	Reactive oxygen species
MDA	Malondialdehyde
SOD	Superoxide dismutase
GSH	Glutathione
MAPK	Mitogen-activated protein kinase
ACE	Angiotensin-converting enzyme
AT	Angiotensin
DCFH-DA	Dichlorodihydrofluorescin diacetate
FN	Fibronectin

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Introduction

Diabetic nephropathy (DN) is a severe complication of diabetes which can lead to end-stage renal deficits [22]. Due to the complex diabetic conditions, the rising frequency of DN is one of the most reasons for diabetes-related kidney failures [23]. Although the exact reason for DN remains unknown, diabetes-related high blood glucose and pressure are among the factors contributing to DN, characterized by hyperfiltration, increased albuminuria, and subsequent cell apoptosis and functional defects in podocytes [26]. Recent studies also showed that high glucose could induce high levels of cytokines, such as IL-8, in patients with T2D, which might induce podocyte damage by activating IL-8/CXCR1/2 [17]. Although many effective interventions are applied in controlling DN progressions, such as blockers for the renin–angiotensin–aldosterone system (RAAS), blood pressure and glucose management, and avoiding alcohol drinking and smoking [26]. Developing a new therapeutic method for DN remains challenging to reduce the high frequency of DN.

Several long non-coding RNA (lncRNA) is involved in the pathophysiological progression of DN. For instance, lncRNA MALAT1 induced podocyte apoptosis [35]. LncRNA lnc-ISG20 enhanced renal fibrosis via miR-486-5p/NFAT5 during the development of diabetic nephropathy [4]. LncRNAs such as PVT1 [30] and NEAT1 ([29] regulate oxidative stress, inflammation, and accumulation of extracellular matrix in DN. Similarly, the previous studies showed that LncRNA SNHG16 contributed to HG-induced podocyte injuries and fibrogenesis [10, 12]. A recent experiment showed that overexpression of SNHG16 in retinal endothelial cells facilitated the endothelial cell proliferation, migration, and angiogenesis via the activation of NF-κB and PI3K/AKT pathways [2]. Additional studies pointed out that SNHG16 aggravates DN through damaging cellular proliferation [10] and enhanced fibrogenesis [12]. However, there are fewer reports related to inflammation. Therefore, more investigations are required to uncover the functions of SNHG16 in HG-induced inflammatory response in DN.

Toll-like receptors (TLRs) are widely expressed in human tissues, including the kidney. A previous study found that TLR1/2/3/4 and TLR6 are mainly expressed in human kidneys' tubular epithelial cells and mesangial cells [1]. During renal injuries and overexpression of TLR4, a significant member of the TLR family was found in the population of penetrating inflammatory cells [1]. In the renal parenchymal cells, the activated TLR4 could activate the p38 MAPK pathway to synthesize inflammatory cytokines, including TNF-α and interleukin (IL)-1β and -6 [33]. The activation of TLR4/NF-κB signaling plays a critical role in LPS-induced inflammatory response in cardiomyocytes via increasing the NLRP3 inflammasome [25]. Besides inflammatory response, NLRP3 also regulates Th cell fate. Its dysregulation is closely related with the poor outcomes in cardiac-transplanted patients [3]. Therefore, the investigation of NRLP3 signaling is a novel target to delineate how inflammation influences the progression of diabetic kidney diseases. Although the molecular mechanisms of TLR4 in DN remain elusive, the previous studies showed that TLR4 activation significantly contributed to the progression of diabetic inflammation and renal fibrosis in DN [7, 15]. The renin-angiotensin system (RAS) controls blood pressure and body fluid homeostasis [9]. A recent study indicated that RAS blocking improved the outcome of DN-related renal injuries [16]. Increasing evidence showed that RAS upregulated TLR4 to activate the p38 MAPK signaling pathway to produce proinflammatory cytokines, chemokines, and profibrotic elements in DN. As feedback, the TLR4/MAPK pathway also induces the expression of RAS molecules, including angiotensin-converting enzyme (ACE) and angiotensin (AT). However, the definite mechanism related to the interaction of RAS and TLR4 is still unknown.

EIF4A3 is an RNA helicase of the DEAD-box family, which can control mRNA splicing, supervise mRNA quality and regulate RNA metabolism [32]. Evidence showed that EIF4A3 contributed to the pathological progression of diseases like HPV-related cancer [19] and circRNA circPPMIF-modulated M1 macrophage activation in type 1 diabetes mellitus [34]. The online bioinformatics analysis found that TLR4 interacted with eukaryotic initiation factor 4A-III (EIF4A3), which lncRNAs can recruit to stabilize proteins and participate in RNA splicing and influencing the downstream cascades [32]. Therefore, we hypothesized that the interaction between TLR4 and EIF4A3 possibly participated in the pathological progression of DN.

This study aimed to disclose whether SNHG16 contributed to HG-induced renal injuries by EIF4A3-mediated TLR4 expression and the involved potential mechanism.

Materials and methods

Animals

We purchased C57BL6 background db/db male mice and their littermates (db/m) from the Animal Center at the Dongguan Tung wah Hospital, Guangdong, China. The db/db mice comprised five groups: db/db group ($n=8$), db/db + sh-NC($n=8$, db/db mice receiving sh-NC lentivirus), db/db + sh-SNHG16 ($n=8$, db/db mice receiving sh-SNHG16 lentivirus), db/db + sh-SNHG16 + oe-NC ($n=8$, db/db mice receiving sh-SNHG16 lentivirus and pcDNA3.1 empty

vector), and db/db + sh-SNHG16 + oe-TLR4 ($n = 8$, db/db mice receiving sh-SNHG16 lentivirus and pcDNA3.1-TLR4 vector), The control group db/m mice received the injection of sterile 1 × phosphate-buffered saline (PBS). All mice were housed in environment-controlled conditions and had free access to water and food. All experiments were supervised by the Animal Welfare and Ethics Committee of Dongguan Tung wah Hospital. (1) Mice were sacrificed at 12-week to evaluate the expressions of SNHG16 and TLR4 under diabetic conditions. (2) The mice subjected to vehicle control, sh-NC, sh-SNHG16, sh-SNHG16 + pcDNA3.1 vector, or sh-SNHG16 + pcDNA3.1-TLR4 at the age of 12 weeks were killed at the end of 20 weeks. After sacrifice, the kidneys and blood were sampled for further analysis.

Measurement of blood glucose

Blood was sampled from the caudal vein puncture of mice on days 6, 10, 14, 18, and 22. The blood glucose was monitored using veterinary blood glucose meters (DSI, MN, USA).

Biochemical analysis

To determine the characteristics of the diabetic animals, we used an automatic IDEXX VetTest biochemical analyzer (IDEXX Laboratories, Maine, USA) to measure urine proteins and blood serine creatinine (Scr) and urea nitrogen (BUN).

Hematoxylin–eosin (H&E) and periodic acid-Schiff (PAS) staining

After harvesting from each mouse, the kidneys were fixed in 4% paraformaldehyde (PFA). After embedding in paraffin, the kidneys were sectioned into 5 μm thickness. H and E were performed using a commercial kit (H-3502, Vector Laboratories, Inc, CA, USA). Periodic acid-Schiff staining was conducted according to the detailed protocol of the PAS stain kit (C0142M, Beyotime Biotechnology, Shanghai, China). After counterstaining, acidic ethanol differentiation, permeabilization, and the slides were imaged using an OMAX Binocular Laboratory Compound Microscope (M82EZ-C50S, AmScope, CA, USA).

Human renal mesangial cells

Human renal mesangial cells (HRMCs) were purchased from creative bioarray (CSC-7719W, NY, USA). HRMCs were maintained in RMPI-1640 medium plus 10% fetal bovine serum (FBS-02-0050, Rockland Immunochemicals, Inc, NJ, USA) and 1 × penicillin-streptomycin solution (15140122, Thermo Fisher Scientific, CA, USA) at 37 °C 5% CO_2 incubator. Cells were used between the 3rd and 7th passages in low serum (2% FBS) after serum starvation for 24 h.

Cell transfection

We purchased commercial constructs including pLKO.1 empty vector (sh-NC), pLKO.1-SNHG16 (sh-SNHG16 (#1, #2, and #3), pcDNA3.1 empty vector, pcDNA3.1-SNHG16, pcDNA3.1-TLR4, pSuper.puro empty vector (si-NC), pSuper.puro-NC vector (si-NC), pSuper.puro-EIF4A3 vector (si-EIF4A3) from Sangon Biotech Co., Ltd. (Shanghai, China). For lentivirus production, LV-MAX™ Lentiviral Production System (A35684) was purchased from Thermo Fisher Scientific, CA, USA. pcDNA3.1 vector was transfected into HRMCs using Lipofectamine 3000 reagent.

qRT-PCR

Total RNAs were isolated from mice kidney tissues or HRMCs using TRIzol reagent (15596026, Thermo Fisher Scientific, CA, USA). To study the effect of SNHG16 on TLR4 mRNA stability, we treated RNA extraction with actinomycin D (10 μg/mL), then collected samples at 0, 3, 6, 9, 12 h. After RNA extraction, we detected the levels of TLR4 mRNA. 500 ng of total RNAs were reversely transcripted into cDNA. The used primers listed in Table 1 were synthesized by Generay Biotech Co Ltd (Guangzhou, China). β-actin and U6 snRNA were the internal controls of the TLR4 gene and the SNHG16, respectively. The levels of genes were quantitatively analyzed by the $2^{-\Delta\Delta CT}$ method.

Measurement of ROS, MDA, SOD, and GSH

We purchased OxiSelect Intracellular ROS Assay Kit from Amyjet Scientific Inc (Green Fluorescence, KA4074, Wuhan, Hubei, China), and Lipid Peroxidation (MDA) Assay Kit (S0131S), Total Superoxide Dismutase Assay Kit (S0088), Total Glutathione Assay Kit (S0052) from Beyotime Biotechnology (Shanghai, China). To detect ROS, we incubated cells with 2′, 7′-Dichlorodihydrofluorescin diacetate (DCFH-DA) at a final concentration of 100 μM DCFH-DA for 60 min at 37 °C. After removing the solution, cells were washed with 1 × DPBS 3 times. Subsequently, cells were imaged by an inverted OM900-T microscope (AmScope, CA, USA). For the measurement of MDA, SOD, and GSH, the serums from the whole blood of mice were separated, or the cells were harvested with cell lysate. Then, the concentrations of MDA, SOD, and GSH were detected according to the detailed protocols from the commercial kits.

Table 1 Primers for qRT-PCR

Primer for qPCR		
Human lncSNHG16	Forward	5′-TGCCTCAGGAAGTCTCTTGC -3′
	Reverse	5′-ACAGCACTTAACCAAGCCCT-3′
Human TLR4	Forward	5′-AGTTGATCTACCAAGCCTTGAGT -3′
	Reverse	5′-GCTGGTTGTCCCAAAATCACTTT-3′
Human β-actin	Forward	5′-CATGTACGTTGCTATCCAGGC-3′
	Reverse	5′-CTCCTTAATGTCACGCACGAT -3′
Human U6 snRNA	RT-primer	5′-CGCTTC ACGAATTTGCGTGTCAT-3′
Human U6 snRNA	Forward	5′-GCTTCGGCAGCACATATACTAAAAT-3′
	Reverse	5′-CGCTTCACGAATTTGCGTGTCAT-3
Mouse lncSNHG16	Forward	5′-TGCTAAGTGGCAGATTCCCG-3′
	Reverse	5′-ACCAGATAGAGGCATCCCGA-3′
Mouse TLR4	Forward	5′-AGGCACATGCTCTAGCACTAA-3′
	Reverse	5′-AGGCTCCCCAGTTTAACTCTG-3′
Mouse β-actin	Forward	5′-GGCTGTATTCCCCTCCATCG -3′
	Reverse	5′-CCAGTTGGTAACAATGCCATGT -3′
Mouse U6 snRNA	RT-primer	5′-AACGCTTCACGAATTTGCGT-3′
Mouse U6 snRNA	Forward	5′-CTCGCTTCGGCAGCACA-3′
	Reverse	5′-AACGCTTCACGAATTTGCGT-3′

ELISA assay

We detect tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), interleukin-1β (IL-1β), and interleukin-8 (IL-8) in the serum from mice or cell culture media to using the commercial enzyme-linked immunosorbent assay (ELISA) kit purchased from Beyotime Biotechnology (Guangzhou, Guangdong, China).

RNA immunoprecipitation (RIP) assays

To determine whether lncRNA SNHG16 or TLR4 could interact with EIF4A3, we performed the RNA immunoprecipitation using an EZ-Magna RNA-binding protein immunoprecipitation kit (17–700, Sigma-Aldrich, MO, USA) according to the manufacture's instruction. The antibodies for RIP assays were rabbit anti-EIF4A3 antibody (ab32485) and rabbit anti-human IgG (ab6715) from Abcam (MA, USA).

RNA pull-down

We performed the RNA pull-down assay using a magnetic RNA-protein pull-down kit (20164, Thermo Scientific™, CA, USA). Briefly, HRMCs were transfected with biotinylated-SNHG16, -TLR4, or -control. After 48 h, the extracted total RNA was incubated with 100 nmol streptavidin magnetic beads. The bound RNAs were extracted using TRIzol reagent. The levels of EIF4A3 were detected by qRT-PCR.

Western blot

Total proteins were extracted after the different groups of treatments by RIPA buffer (Beyotime, Nantong, China) supplemented with 100 μM phenylmethylsulfonyl fluoride (PMSF) (P0013C, Beyotime Biotechnology, Nantong, China). The protein concentrations were measured using a bicinchoninic acid (BCA) assay kit (Beyotime Biotechnology, Nantong, China). Twenty micrograms of total proteins (20 μg) were dissolved on 12% SDS-PAGE gel and transferred to nitrocellulose membrane (FFN03, Beyotime, Nantong, China). After blocking, the individual primary antibody was applied. The used primary antibodies were mouse anti-TLR4 (ab22048, CA, USA), rabbit anti-collagen I (ab260043, CA, USA), rabbit anti- CTGF (ab6992, CA, USA), rabbit anti-fibronectin (ab2413, CA, USA), rabbit anti-TGF beta 1 (ab215715, CA, USA), rabbit anti-ACE2 (ab108252, CA, USA), rabbit anti-angiotensin II (Ang II, ab239995, CA, USA), rabbit anti-angiotensin II Type 1 (ab239995, CA, USA),rabbit anti-NF-kB p65 (phosphor-S536) (ab76302, CA, USA), rabbit anti- NF-kB p65 [E379] (ab32536, CA, USA), rabbit anti- NLRP3 [EPR23094-1] (ab263899, CA, USA), rabbit anti-cleaved-caspase1 (89332, Cell Signaling Technology, Inc, MA, USA), and rabbit anti-GAPDH [EPR16891] (ab181602, CA, USA). After three washes by 1 × TBST, the membranes were incubated with goat anti-rabbit IgG H&L (HRP) (ab7090, Abcam, MA, USA) and goat anti-mouse IgG H and L (DyLight® 488) (ab96879, Abcam, MA, USA). After three washes by 1 × TBST, the

membranes were visualized using an ECL Substrate Kit (ab133409, Abcam, MA, USA). The protein bands were analyzed with ImageJ 1.58 (NIH, USA).

Statistical analysis

All data were expressed as means ± standard deviation (Mean ± SD). The unpaired student t test was used to analyze the difference between the two groups using SPSS 20.0 (SPSS, Chicago, IL, USA). One-way ANOVA analyzed the differences among multiple groups. $P < 0.05$ was considered statistically significant.

Results

SNHG16 and TLR4 expressions are upregulated in the diabetic kidney

To test the roles of SNHG16 and TLR4 in the development of diabetic kidneys, we developed the db/m and db/db mice. The db/db mice exhibited significantly higher blood glucose (Fig. 1A), urinary protein, (Fig. 1B), and blood Scr and BUN (Fig. 1C) compared with those of db/m mice. H and E and PAS staining showed db/db mice exhibited increased tubular and interstitial fibrosis, hyperplasia of the mesangial matrix, and thickness of basement membrane relative to those of db/m mice (Fig. 1D). Meanwhile, db/db mice exhibited

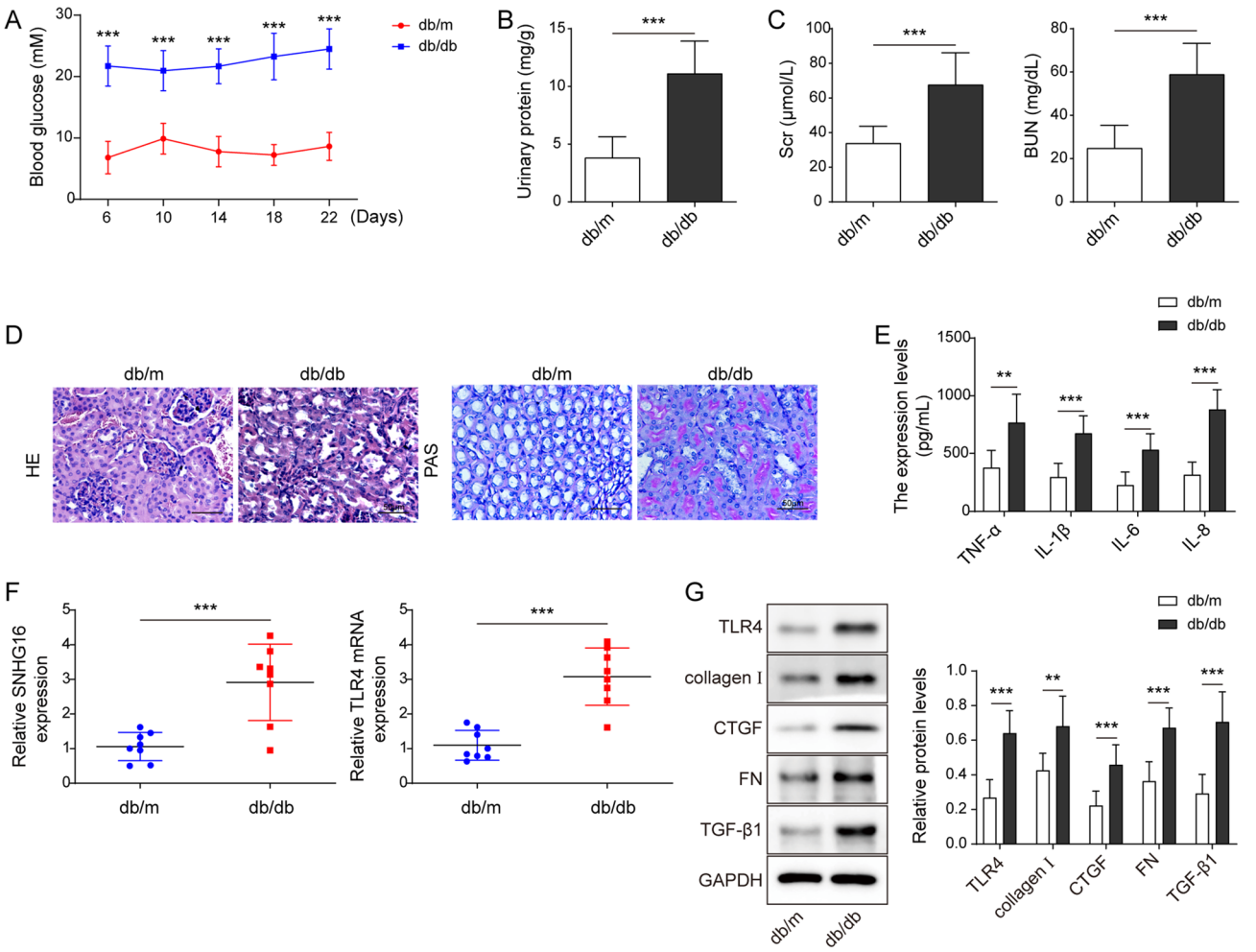


Fig. 1 SNHG16 and TLR4 expressions are upregulated in the diabetic kidney. Blood samples were collected on day 6, 10, 14, 18, and 22 from the caudal vein puncture of db/m or db/db mice. Then, an automatic biochemical analyzer measured blood glucose levels (**A**), protein content in mice urine (**B**), serine creatinine, and BUN (**C**). Renal tissues were examined by HE and PAS staining (**D**). The levels of cytokines in sera were detected by ELISA©. The gene expressions of SNHG16 and TLR4 in the rental tissues were measured by qRT-PCR (**F**). The protein levels of TLR4, collagen 1, CTGF, FN, and TGF-β1 in renal tissues were detected by western blot (**G**). Data are representative of three independent experiments. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ versus the corresponding control

enhanced inflammatory cytokines (Fig. 1E), upregulated levels of SNHG16 and TLR4 mRNA (Fig. 1F), as well as protein levels of TLR4, collagen 1, CTGF, FN, and TGF-β1 (Fig. 1G). All these data suggested that the expression of SNHG16 and TLR4 might participate in the progression of diabetic nephropathy.

High glucose upregulates the expressions of SNHG16 and TLR4 in human renal mesangial cells (HRMCs)

Subsequently, we developed the in vitro human diabetic nephropathy models using HRMCs after exposure to HG. Our results revealed that HRMCs after exposure to HG exhibited the increased secretions of cytokines (Fig. 2A), remarkably increased production of reactive oxygen species (ROS) (Fig. 2B), and lipid peroxidation MDA level (Fig. 2C), decreased levels of SOD and GSH (Fig. 2C). Additionally, we found that HG exposure induced higher protein levels of collagen 1, CTGF, FN, TGF-β1 (Fig. 2D), and TLR4 (Fig. 2F), as well as the mRNA levels of SNHG16 and TLR4 (Fig. 2E) relative to control. HG exposure led to the increased expressions of SNHG16 and TLR4.

Knockdown of SNHG16 alleviates HG-induced injuries in HRMCs

Subsequently, we transfected an SNHG16 knockdown plasmid (pLKO.1-SNHG16) or its parallel pLKO.1 empty vector into HRMCs with or without HG-treatment and investigated the effect of SNHG16 on the pathological conditions of HRMCs. Our data showed that SNHG16 was significantly decreased after transfection of pLKO.1-SNHG16 compared with that of the empty vector group (Fig. 3A). Further

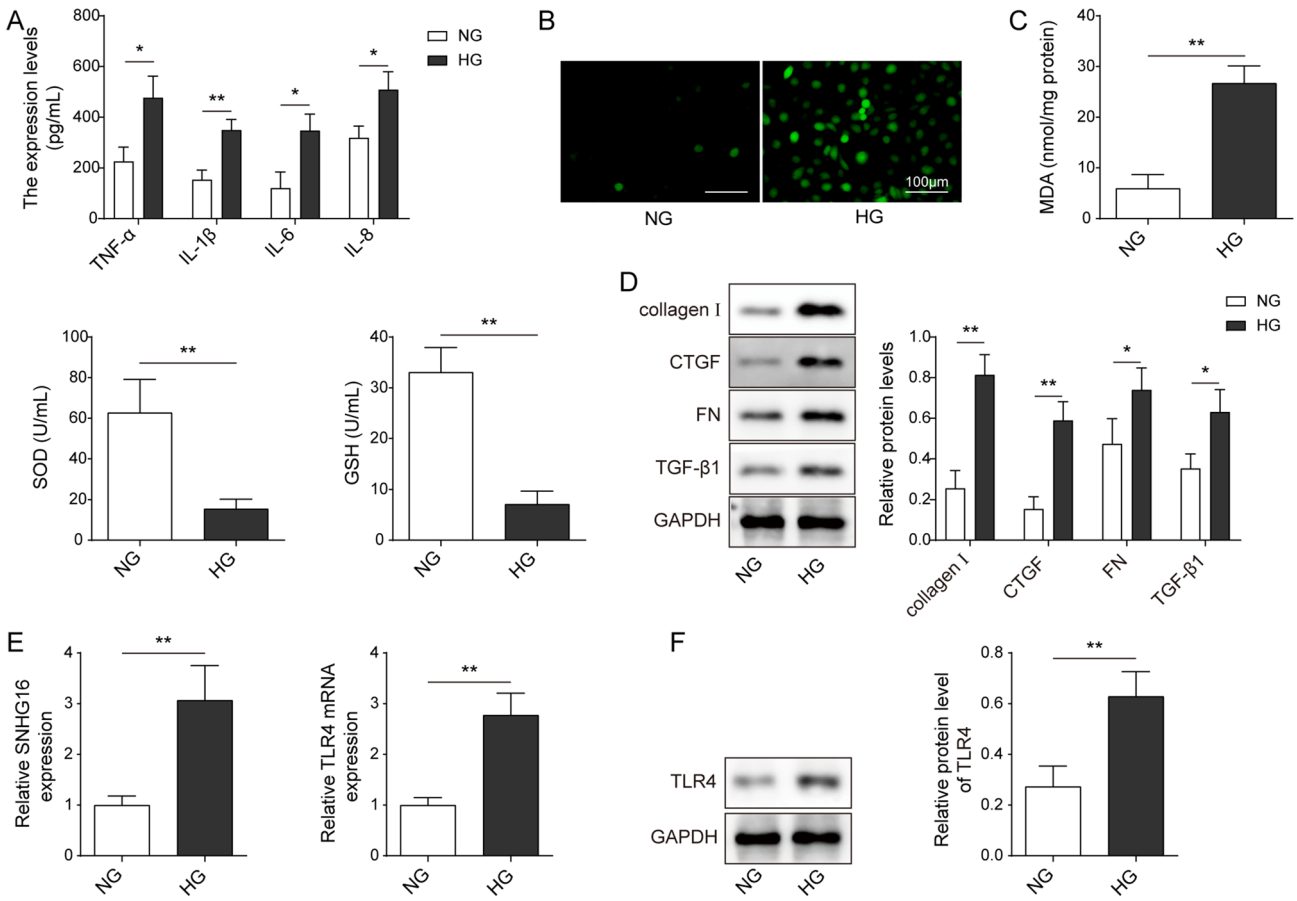


Fig. 2 High glucose upregulates the expressions of SNHG16 and TLR4 in human renal mesangial cells (HRMCs). Human Renal Mesangial Cells (HRMCs) were treated with normal or high glucose for 24 h. The levels of cytokines were measured by ELISA assay (**A**). ROS production was detected in the normal or high glucose-treated HRMCs (**B**). The synthesis of malondialdehyde, superoxide dismutase, and glutathione were detected using commercial kits (**C**). The protein levels of collagen 1, connective tissue growth factor (CTGF), fibronectin (FN), and transforming growth factor-beta 1 (TGF-β1) were detected by western blot (**D**). SNHG16 and TLR4 mRNA levels were detected by qRT-PCR (**E**). TLR4 protein level was detected by western blot (**F**). $*P<0.05$, $**P<0.01$, $***P<0.001$ versus the corresponding control. Data are representative of three independent experiments

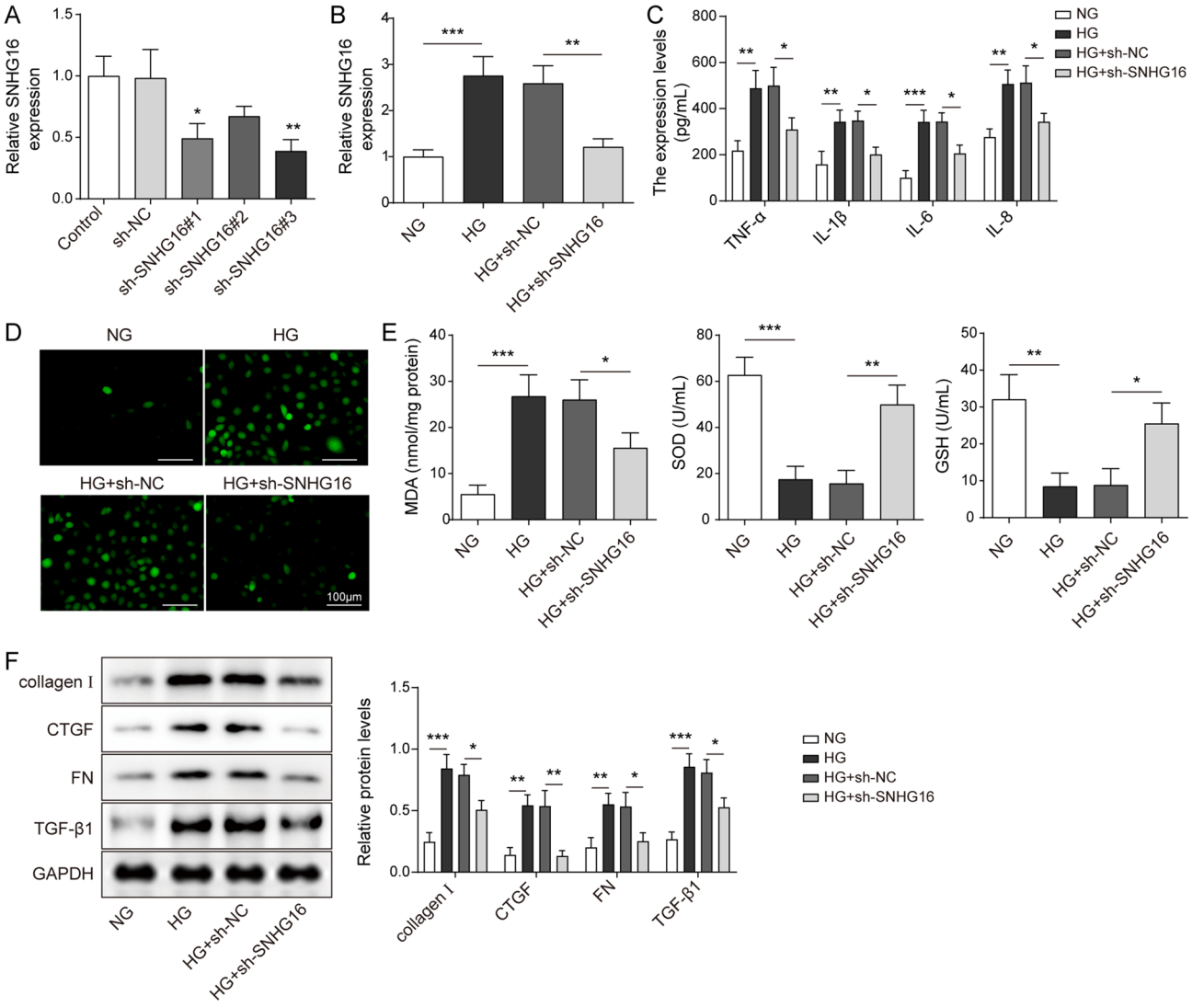


Fig. 3 Knockdown of SNHG16 alleviates high glucose-induced injuries in HRMCs. HRMCs were transfected with shNC or shSNHG16 #1/#2/#3 for 48 h. Then, cells were treated with normal or high glucose for 24 h. The transfection efficiency was measured by qRT-PCR (**A**). SNHG16 mRNA levels were measured in each group by qRT-PCR (**B**). The cytokines were measured by ELISA (**C**). ROS levels were evaluated by DCFDA staining using a ROS detection assay kit (**D**). The levels of malondialdehyde (MDA), superoxide dismutase (SOD), and glutathione (GSH) were measured using the commercial kits (**E**). The protein levels of collagen 1, CTGF, FN, and TGF-β1 were detected by western blot (**F**). $*P<0.05$, $**P<0.01$, $***P<0.001$ versus the corresponding control. Data are representative of three independent experiments

experiments showed that the expression of SNHG16 was significantly upregulated after HG exposure, while knockdown of SNHG16 remarkably downregulated SNHG16 in HG-treated HRMCs (Fig. 3B). Subsequent assays indicated that HG exposure induced high levels of inflammatory cytokines (Fig. 3C), leading to the increased accumulation of reactive oxygen species (ROS) (Fig. 3D) and MDA (Fig. 3E) and decreased expressions of SOD and GSH (Fig. 3E). In contrast, knockdown of SNHG16 reversed the effects of HG in HRMCs (Fig. 3C–E). Further western blot assay revealed that HG caused a high level of renal fibrosis-related proteins (collagen 1, CTGF, FN, and TGF-β1), SNHG16 knockdown reversed the effects of HG (Fig. 3F). All those results suggested that knockdown of SNHG16 alleviated HG-induced injuries in HRMCs.

SNHG16 stabilizes TLR4 mRNA by recruiting EIF4A3

We performed the RIP and RNA pull-down assays to find out the potential downstream molecules of SNGH16. The RIP assay showed that both SNHG16 and TLR4 bound to EIF4A3 proteins (Fig. 4A). Meanwhile, the RNA pull-down assay indicated that the ELF4A3 remarkably was enriched by both bio-SNHG16 and bio-TLR4 relative to the

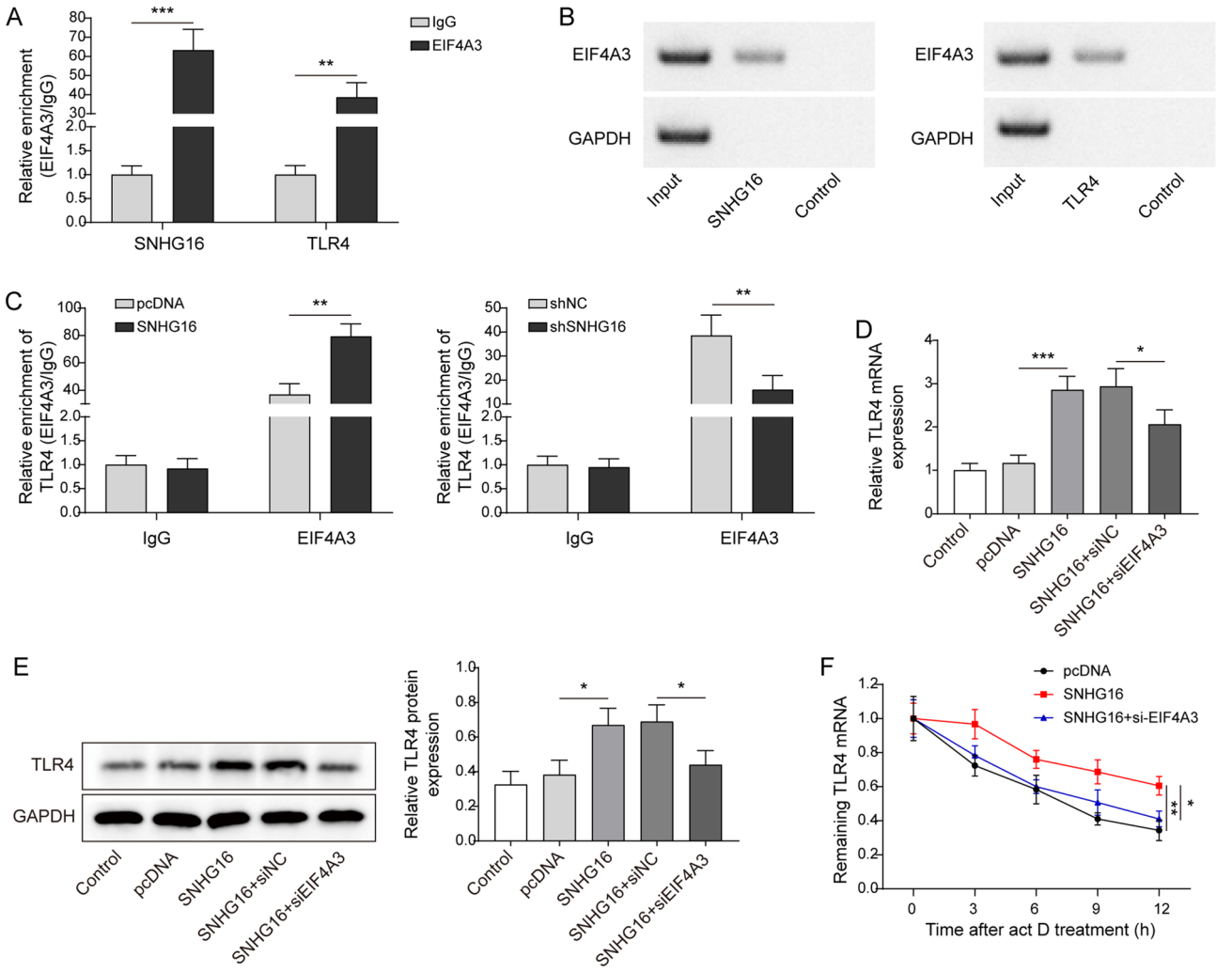


Fig. 4 SNHG16 stabilizes TLR4 mRNA by recruiting EIF4A3. HRMCs were transfected with pcDNA3.0, pcDNA3.0-SNHG16, pcDNA3.0-TLR4, shNC or shSNHG16, siNC or siEIF4A3 for 48 h. The interactions between SNHG16 and EIF4A3 or between TLR4 and EIF4A3 were determined by RNA immunoprecipitation (**A**). The bindings of SNHG16 or TLR4 to EIF4A3 were determined by RNA pull-down assay (**B**). TLR4 mRNA levels were evaluated by RIP assay using anti-IgG and anti-EIF4A3 antibodies (**C**). TLR4 mRNA or protein levels were detected by qRT-PCR or western blot (**D**, **E**). The stabilities of TLR4 mRNA were detected in actinomycin-treated HRMCs after transfection of pcDNA3.1, pcDNA3.1-SNHG16 single or together with si-EIF4A3 (**F**). $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ versus the corresponding control. Data are representative of three independent experiments

bio-control group (Fig. 4B), meaning that both SNHG16 and TLR4 interacted with EIF4A3. After overexpression of SNHG16, we found that the enrichment of TLR4 mRNAs by EIF4A3 was markedly increased, while the enrichment of TLR4 mRNA by EIF4A3 was significantly decreased when SNHG16 was knockout, indicating that SNHG16 increased the TLR4 levels by EIF4A3 protein (Fig. 4C). The additional experiment showed that overexpression of SNHG16 promoted TLR4 expression, which was significantly attenuated by EIF4A3 knockdown (Fig. 4D–E). The mRNA stability assay using transcription inhibitor actinomycin D showed that overexpression of SNHG16 stabilized the TLR4 mRNA, while knockdown of EIF4A3 promoted the degradation of TLR4 mRNA (Fig. 4F), indicating that SNHG16 stabilized TLR4 mRNA by recruiting EIF4A3 protein.

Knockdown of TLR4 alleviates the HG-induced injuries in HRMCs by suppressing RAS and NF-κB to attenuate the activation of the NLRP3 inflammasome

Then, we further characterized the role of TLR4 in HG-induced renal injuries. qRT-PCR confirmed that the transfection of TLR4 siRNA significantly decreased the TLR4 expression (Fig. 5A). HG significantly upregulated TLR4 expression at both mRNA and protein levels, but TLR4

silencing antagonized the upregulation of TLR4 at both mRNA and protein levels (Fig. 5B, C). The ELISA assays showed that HG led to significant secretion of cytokines, but knockdown of TLR4 diminished the levels of inflammatory cytokines (Fig. 5D). Further experiments exhibited that HG led to significantly increased productions of ROS and MDA but decreased expressions of SOD and GSH in HRMCs (Fig. 5E, F). However, when TLR4 was knockdown, the effects of HG on ROS, MDA, SOD, and GSH were reversed (Fig. 5E, F). Similarly, HG-induced high levels of renal fibrosis-related proteins (collagen 1, CTGF, FN, and TGF-β1), but TLR4 knockdown diminished HG-induced renal fibrosis (Fig. 5G). To further discover the underlying mechanism related to TLR4 in HG-induced renal injuries, we detected HG-induced pathological changes in the renin-angiotensin system (RAS) and the inflammasome. Our western blot assays exhibited that HG caused high activations of ACE, Ang II, AT1R, p-p65, and NLRP3, as well as cleaved-caspase1. TLR4 knockdown reversed the trend (Fig. 5H, I), indicating that knockdown of TLR4 protected HG-induced injuries by disrupting the blood system and increasing inflammation.

SNHG16 promotes high glucose-induced cell injuries in HRMCs by regulating TLR4-mediated RAS and NF-κB to activate the NLRP3 inflammasome

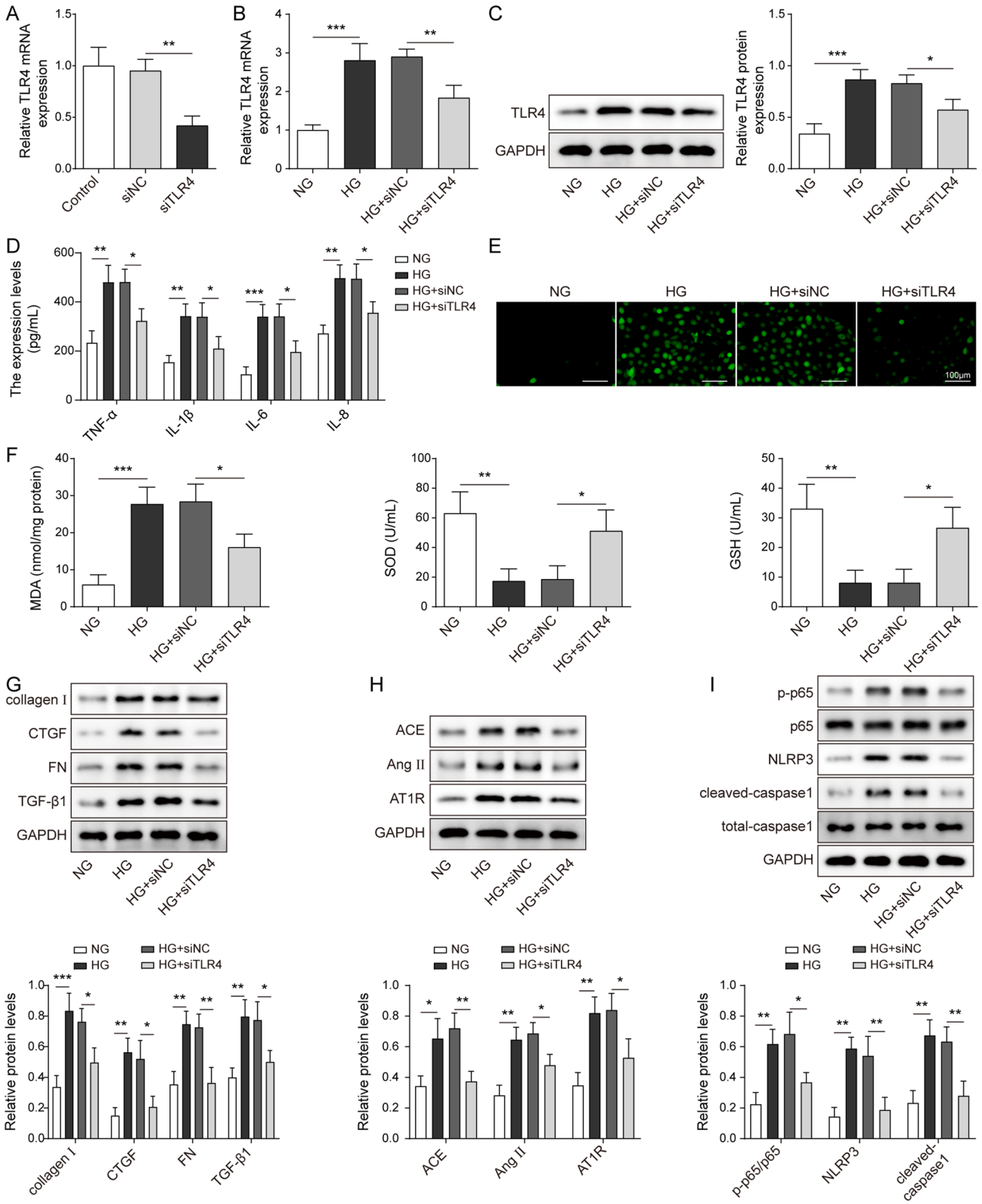
To disclose the mechanism of SHNG16 in HG-induced renal injuries, we performed siRNA-based SNHG16 knockdown in HG-induced HRMCs and found that SNHG16 knockdown largely diminished HG-increased SNHG16 (Fig. 6A). Meanwhile, overexpression of TLR4 could not rescue SNHG16 silencing-induced low levels of SNHG16 (Fig. 6A). Interestingly, SNHG16 knockdown significantly decreased HG-upregulated TLR4, which was complemented by TLR4 overexpression (Fig. 6B, C). Additional experiments showed that knockdown of SNHG16 markedly reduced inflammatory cytokines, ROS productions, and MDA and increased the synthesis of SOD and GSH proteins (Fig. 6D–F). However, overexpression of TLR4 attenuated the protective roles of SNHG16 in HG-treated HRMCs by the increased ROS, cytokines, and MDA and the decreased SOD and GSH (Fig. 6D–F). Furthermore, we found that SNHG16 knockdown distinctly diminished HG-induced renal fibrosis and RAS-related proteins, but those protective roles were reversed when TLR4 was overexpressed (Fig. 6G, H). Importantly, we identified that knockdown of SNHG16 abolished HG-induced the increased p-p65, NLRP3, and cleaved caspase1. However, overexpression of TLR4 reversed the inhibitory effects of SNHG16 knockdown (Fig. 6I). Therefore, those results suggested that SNHG16 promotes renal injuries in HRMCs under HG conditions, possibly through directly regulating TLR4-mediated RAS and NF-κB- drove NLRP3-inflammasome.

Knockdown of SNHG16 alleviates kidney injuries in db/db mice by suppressing TLR4

Subsequently, we generated the diabetic mice models and investigated the effects of SNHG16 in vivo. Our data showed that SNHG16 knockdown decreased the blood glucose levels in mice, but TLR4 overexpression compensated for SNHG16 knockdown-induced the decreased glucose level (Fig. 7A). The biochemical assays found that diabetic mice exhibited higher levels of urinary protein, Scr and BUN relative to those of control (Fig. 7B, C). Knockdown of SNHG16 decreased the levels of urinary protein, Scr and BUN in diabetic mice, but overexpression of TLR4 observably increased the urinary protein, Scr and BUN when SNHG16 was silenced (Fig. 7B, C). H and E and PAS staining showed that HG led to noticeable tubular and interstitial fibrosis, hyperplasia of the mesangial matrix, and increased thickness of basal membrane of mice renal tissue. Meanwhile, the knockdown of SNHG16 alleviated HG-induced damages. However, TLR4 overexpression reversed the protective role of SNHG16 knockdown (Fig. 7D). Our ELISA assay further found that knockdown of SNHG16 distinctly attenuated the inflammatory cytokine levels in diabetic mice compared with the control group. Relative to the control group, overexpression of TLR4 markedly enhanced the cytokine secretion in diabetic mice subjected to SNHG16 knockdown (Fig. 7E). We also measured the expressions of SNHG16 and TLR4. Our qRT-PCR results validated that SNHG16 and TLR4 were significantly increased in diabetic conditions, but knockdown of SNHG16 decreased both SNHG16 and TLR4. However, overexpression of TLR4 could not upregulate SNHG16 but significantly upregulated TLR4 (Fig. 7F). Our western blot further revealed that diabetes mice exhibited higher levels of TLR4, collagen 1, CTGF, FN, TGF-β1, RAS proteins ACE, Ang II, AT1R, p-p65, NLRP3, and cleaved caspase-1 than those of normal mice (Fig. 7G–I). SNHG16 silencing dramatically attenuated HG-induced effects. Significantly, TLR4 overexpression observably diminished the protective roles of SNHG16 knockdown (Fig. 7G–I). All those results suggested that knockdown of SNHG16 alleviated renal injuries in diabetic mice probably via suppressing TLR4.

Discussions

DN is one of the chronic inflammation and microvascular deficits. The previous studies evidenced that inflammation is a significant cause of DN development [27]. Our data revealed that lncRNA SNHG16 contributed to renal injury


A
Relative TLR4 mRNA expression
Control
siNC
siTLR4
B
Relative TLR4 mRNA expression
NG
HG
HG+siNC
HG+siTLR4
C
TLR4
GAPDH
Relative TLR4 protein expression
D
The expression levels (pg/mL)
TNF-α
IL-1β
IL-6
IL-8
E
100μm
F
MDA (nmol/mg protein)
SOD (U/mL)
GSH (U/mL)
G
collagen I
CTGF
FN
TGF-β1
Relative protein levels
H
ACE
Ang II
AT1R
I
p-p65
p65
NLRP3
cleaved-caspase1
total-caspase1
p-p65/p65
cleaved-caspase1

◂**Fig. 5** Knockdown of TLR4 alleviates the high glucose-induced injuries in HRMCs by suppressing RAS and NF-κB to attenuate the activation of the NLRP3 inflammasome. HRMCs were transfected with siNC or siTLR4 for 48 h, then administrated by normal or high glucose for a further 24 h. The TLR4 mRNA levels were measured (**A**). TLR4 mRNA or protein levels were measured by qRT-PCR or western blot (**B**, **C**). The protein levels of cytokines were analyzed by ELISA (**D**). DCFDA staining evaluated the ROS levels (**E**). MDA, SOD, and GSH levels were assessed using commercial kits (**F**). Western blot detected the protein levels of collagen 1, CTGF, FN and TGF-β1 (**G**), ACE, Ang II and AT1R (**H**), p-p65, p65, NLRP3, cleaved-caspase1, and total-caspase 1 (**I**). $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ versus the corresponding control. Data are representative of three independent experiments

via regulating TLR4, thus activating the RAS system and NF-κβ-mediated NLRP3 inflammasome. Our findings further identified that SNHG16 upregulated TLR4 expression by recruiting EIF4A3 to stabilize TLR4 mRNA and decreased the degradation of TLR4 mRNA. Knockdown of SNHG16 or TLR4 alleviated HG-induced renal injuries, providing a new therapeutic target for ND treatment.

LncRNAs are non-coding RNAs that act as primary regulators to regulate gene expressions at transcriptional, post-transcriptional, translational, or post-translational levels [36]. Recently, accumulating evidence found that lncRNAs participate in many pathological progressions of DN, such as podocyte apoptosis [13], renal fibrosis [8], oxidative stress [21], and inflammation [30]. In the current study, our experiments revealed that SNHG16 was dramatically increased in both diabetic renal tissues and HG-induced HRMCs. Further investigations found that knockdown of SNHG16 decreased the release of cytokines, ROS, MDA, and fibrosis-related proteins and increase SOD and GSH, indicating that SNHG16 might be a potential target for diabetic renal injuries.

LncRNAs exhibit functional roles, possibly through direct binding its downstream mRNA sequences to silence gene expression or directly interacting with proteins to regulate the downstream genes (X. [36]. Recently, it was found that lncRNA NEAT1 promoted the TLR4 expression by directly binding and inhibiting miR-302b-3p [6]. This study found that both SNHG16 and TLR4 were upregulated in diabetic conditions. Hence, we wondered whether SNHG16 regulated TLR4. Renin-angiotensin system (RAS) supervises blood pressure and fluid homeostasis. During diabetic conditions, HG can significantly upregulate RAS-related angiotensin II, contributing to the progression of diabetic angiopathy [24]. A previous study verified that HG-induced high levels of angiotensin II increased the expression of TLR4 [20]. Later experiments also showed that high glucose upregulated TLR4 expression, subsequently regulating the Ang II expression [5]. Increasing evidence identified that inflammatory response might be the critical regulator linking the mutual regulation between TLR4 and Ang II activations [5]. Our study found that HG upregulated both TLR4 and RAS-related enzymes ACE, Ang II, and AT1R, and knockdown of TLR4 decreased the ACE, Ang II, and AT1R. All those results indicated that HG-induced activation of RAS and TLR4 exhibited mutual regulation and promotion. However, their regulatory relationship still needs to be further explored.

Several studies confirmed that inflammation-related signaling pathways such as IL-8-CXCR1/2 axis [17] and TLR4 signaling were potential therapeutic target for diabetic kidney disease. A recent study showed that some drugs like panobinostat might attenuate inflammation-related glomerulonephritis through regulating the differentiation of human renal progenitor cells into podocytes to restore the glomerular filtration barrier [18]. Accumulating studies indicate that the NLRP3 inflammasome is a promising therapeutic target for treating ND [31]. Suppression of NLRP3 inflammasome alleviated podocyte damage [28] and autophagy [11]. A previous study had validated that high glucose mediates the activation of NLRP3 inflammasome via the TLR4/NF-κB signaling pathway [14]. Our experiments showed that HG-induced NLRP3 inflammasome correlated with the level of TLR4. After TLR4 was silenced in HG-induced HRMCs, we found HG-induced NLRP3 was remarkably downregulated. In contrast, when TLR4 was overexpressed, NLRP3 expression was reversed in HG-induced HRMCs and diabetic renal tissues. All those results revealed that the activation of TLR4 contributed to the development of NLRP3 inflammasome under diabetic conditions.

In conclusion, our study validated that lncRNA SNHG16 aggravated DN via the activations of RAS and TLR4, which subsequently promote NF-κB-dependent activation of the NLRP3 inflammasome. Mechanistically, SNHG16 upregulated TLR4 mRNA by recruiting EIF4A3 and increasing the stabilization of TLR4 mRNA. Our findings suggest that SNHG16/TLR4/NRLP3 axis might be a novel therapeutic target for treating DN.

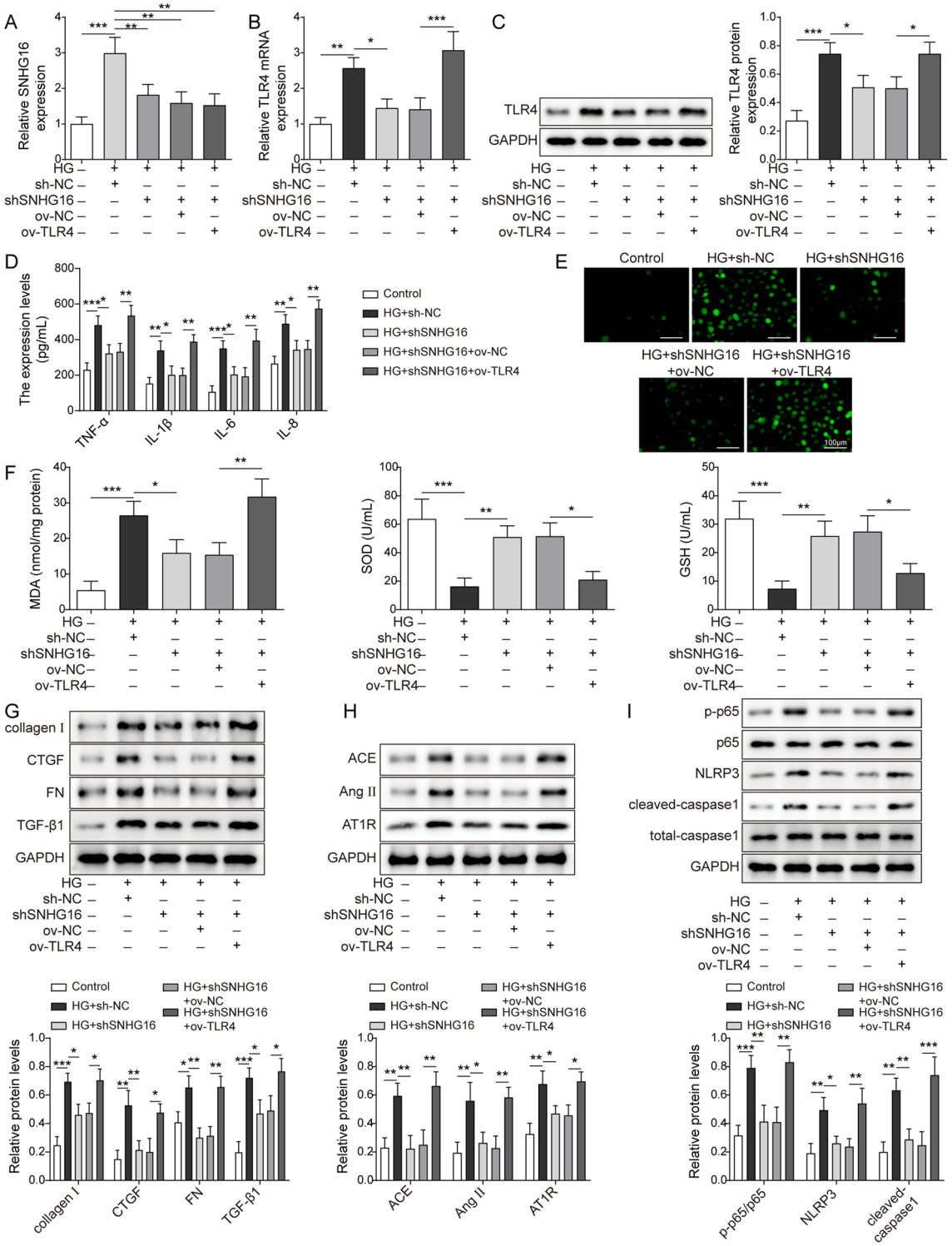


Fig. 6 SNHG16 promotes high glucose-induced cell injuries in HRMCs by regulating TLR4-mediated RAS and NF-κB to activate the NLRP3 inflammasome. HRMCs were transfected with shNC, shSNHG16, shSNHG16 + ov-NC or shSNHG16 + ov-TLR4 for 48 h, then were treated with normal or high glucose for a further 24 h. The SNHG16 mRNA levels were detected by qRT-PCR (**A**). TLR4 mRNA and protein levels were detected by qRT-PCR or western blot (**B**, **C**). The protein levels of cytokines TNF-α, IL-6, IL-1β, and IL-8 were detected by ELISA assay (**D**). ROS, MDA, SOD, and GSH were analyzed using a corresponding commercial kit (**E**, **F**). Western blot detected the protein levels of collagen 1, CTGF, FN, and TGF-β1 (**G**), ACE, Ang II, and AT1R (**H**), p-p65, p65, NLRP3, cleaved- and total-caspase 1 (**I**). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus the corresponding control. Data are representative of three independent experiments

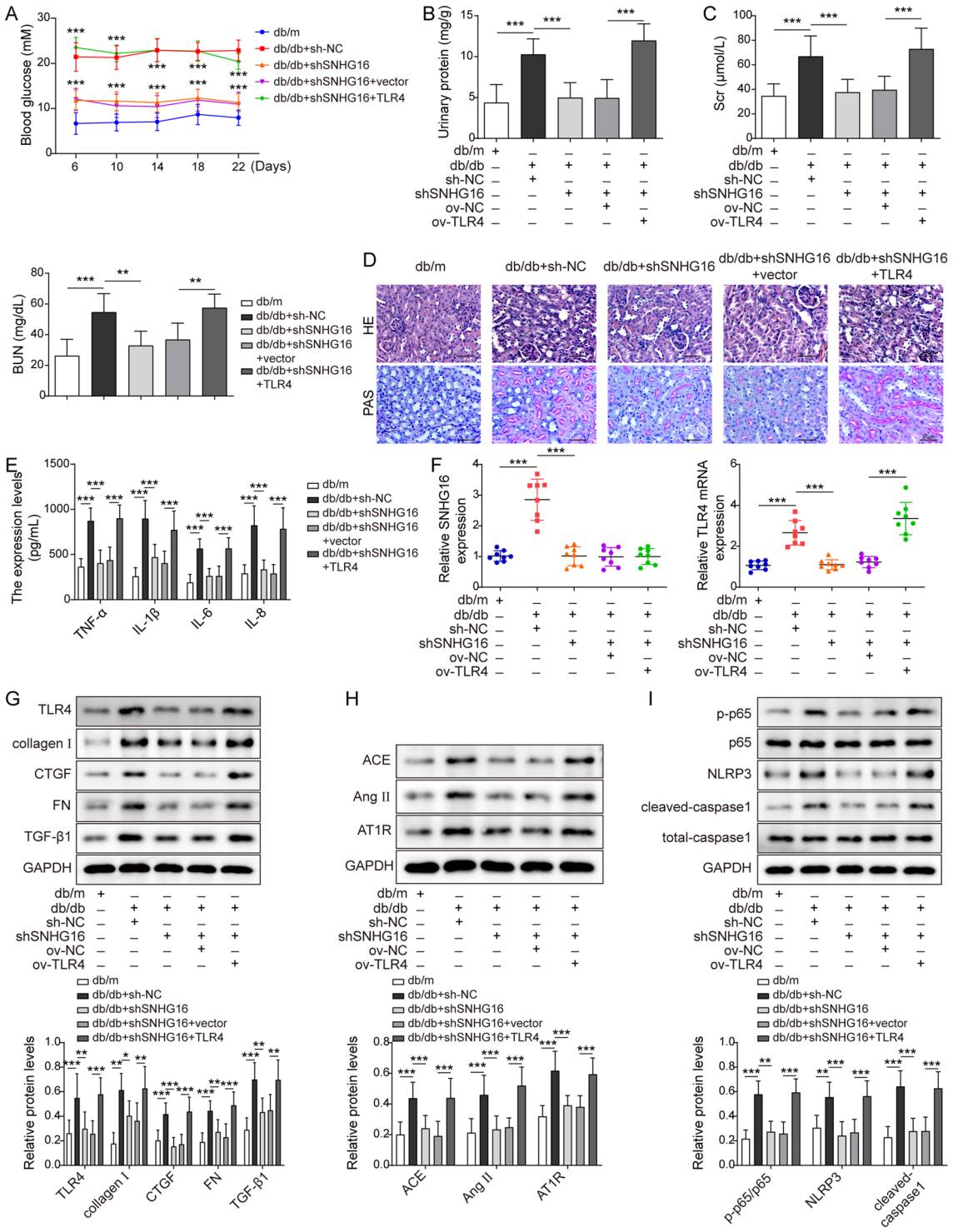


Fig. 7 Knockdown of lncRNA SNHG16 alleviates kidney injuries in db/db mice by suppressing TLR4. The db/db mice were injected with sh-NC, sh-SNHG16, sh-SNHG16 + VE, or sh-SNHG16 + TLR4 lentivirus. A glucometer detected the blood glucose levels (**A**). An automatic biochemical analyzer analyzed the urine protein contents of mice (**B**). An automatic biochemical analyzer analyzed serum creatinine (SCR) or blood urea nitrogen (BUN) (**C**). Hematoxylin and eosin staining (HE) and periodic acid-Schiff (PAS) assay analyzed the changes in histological structures and glycogen accumulation (**D**). The cytokines in the sera of mice were determined by ELISA assay (**E**). The SNHG16 and TLR4 mRNA levels were detected by qRT-PCR (**F**). Western blot detected the protein levels of TLR4, collagen 1, CTGF, FN, and TGF-β1 (**G**), ACE, Ang II, and AT1R (**H**), p-p65, p65, NLRP3, cleaved-caspase1, and total-caspase1 (**I**).*$P<0.05$, **$P<0.01$, ***$P<0.001$ versus the corresponding control. Data are representative of three independent experiments

Author contributions YL contributed to conceptualization; methodology; supervision; MZ contributed to validation; formal analysis; HZ contributed to investigation; resources; NX contributed to data curation; YW contributed to writing—original draft; SD contributed to visualization; XS contributed to writing—review and editing; project administration; funding acquisition.

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Data availability All data collected and analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

Ethics approval and consent to participate All experiments were supervised by the Animal Welfare and Ethics Committee of Dongguan Tung wah Hospital.

Consent for publication All the authors approved the publication.

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