

Astragaloside IV/lncRNA-TUG1/TRAF5 signaling pathway participates in podocyte apoptosis of diabetic nephropathy rats

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Objective: This study aims to figure out the mechanism of astragaloside IV (AS-IV) in the protection of podocyte apoptosis in diabetic nephropathy (DN) rats.

Materials and methods: Streptozotocin (STZ) was used to induce diabetes in rats, and the diabetic rats were treated with 5 mg/kg/d of AS-IV for 12 weeks. Albuminuria level, relative TUG1 and TRAF5 levels, and TRAF5 and cleaved-caspase-3 protein levels were examined by ELISA, quantitative reverse transcription (qRT)-PCR, and Western blot analyses, respectively. The interaction between TUG1 and TRAF5 was confirmed by RNA pull-down and RNA precipitation. TUNEL assay was used to detect podocyte apoptosis.

Results: Compared with control rats, DN rats had higher albuminuria and TRAF5 levels and lower TUG1 level. AS-IV treatment attenuated albuminuria and TRAF5 levels and improved TUG1 level in DN rats. TUG1 was downregulated and TRAF5 was upregulated in high-glucose-treated MPC5 cells, and AS-IV ameliorated the TUG1 level. In addition, TUG1 interacted with TRAF5, and TUG1 overexpression promoted degradation of TRAF5 protein. Besides, AS-IV modulated TRAF5 expression through regulating TUG1. AS-IV decreased podocyte apoptosis via the TUG1/TRAF5 pathway. Finally, in vivo experiment proved that si-TUG1 abrogated the protective effect of AS-IV on DN.

Conclusion: AS-IV attenuated podocyte apoptosis and protected diabetic rats from DN via the lncRNA-TUG1/TRAF5 pathway.

Keywords: astragaloside IV, diabetic nephropathy, TUG1, TRAF5, albuminuria

Introduction

Diabetic nephropathy (DN) is a common and serious complication of diabetes, which leads to the end-stage renal disease (ESRD).¹ Studies have shown that podocyte apoptosis played a key role in the pathogenesis of glomerulosclerosis and albuminuria in DN.²⁻⁴ It has been reported that TRAF5-mediated nuclear factor- κ B (NF- κ B) activation was involved in the pathogenesis of chronic kidney disease, and overexpression of TRAF5 remarkably suppressed cell viability and induced mice podocytes apoptosis.⁵ Therefore, in this study, we focused on TRAF5 to find effective treatments of DN.

Studies have shown that DN was regulated by abnormal lncRNAs, such as lncRNA Erbb4-IR, lncRNA PVT1, and lncRNA MALAT1.⁶⁻⁸ lncRNA TUG1 is a widely expressed lncRNA that participates in cancers, metabolic disorders, cardiovascular disorders, etc.⁹⁻¹¹ Recently, Duan et al¹⁰ reported that lncRNA TUG1 was downregulated in the DN rat model, which revealed extracellular matrix accumulation in DN via the microRNA-377/PPAR γ pathway. In addition, researchers found that lncRNA TUG1 could regulate mitochondrial function in podocytes in the diabetic milieu.¹²

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What's more, lncRNA TUG1 could directly target CUGBP elav-like family member 1 (CELF1), and TUG1 knockdown could remarkably accelerate CELF1 expression,¹³ which indicated that lncRNA TUG1 could target proteins. Therefore, we speculated that lncRNA TUG1 might regulate podocyte apoptosis in DN by targeting TRAF5.

Radix astragali is the root of *Astragalus membranaceus* Bunge that plays important roles in protecting liver, such as eliminating toxins, preventing cancers, and protecting cells from oxidative stress.¹⁴ It was one of the most commonly used natural traditional Chinese medicine in China and other Asian areas, and mainly used as a tonic, which was first recorded in *Ben Cao Gang Mu*.¹⁵ Astragaloside IV (AS-IV) is the main active constituent of Radix astragali that has been used to cure DN of diabetes.¹⁶ Recently, researchers have found that AS-IV could improve DN through regulating mitochondrial quality control network.³ AS-IV could reduce the endoplasmic reticulum stress-induced apoptosis of podocytes through upregulating sarco/endoplasmic reticulum Ca²⁺-ATPase (SERCA) expression in DN.¹⁷ However, the underlying mechanism of AS-IV in the treatment of DN is not fully revealed.

This study aims to figure out the mechanism of AS-IV in the protection of podocyte apoptosis in DN rats. Based on the important role of AS-IV in the reduction of podocyte apoptosis and our preliminary experiment result of the regulation of TUG1 on TRAF5 expression, we speculated that AS-IV might suppress TUG1 expression to increase TRAF5 and thus reduce podocyte apoptosis in DN.

Materials and methods

Establishment of DN rats

Male Sprague Dawley rats (~6-week old, 200 g) were purchased from Experimental Animal Center of Sichuan University and housed in a 12 hours light/dark cycle at 23°C±1°C with free access to water. Streptozotocin (STZ) (65 mg/kg) was intraperitoneally injected into rats to induce DN in rats. An equal volume of citrate buffer (0.1 M) was injected into control rats. After 48 hours, blood samples were collected to detect the blood glucose level. If the blood glucose level was >300 mg/dL, the rat could be confirmed as a diabetic rat. Two weeks after STZ injection, the rats were divided into control rats (n=6), STZ-induced DN rats (DN group) (n=6), and DN rats treated with AS-IV (AS-IV group) (n=6). AS-IV was purchased from Sinopharm Chemical Reagent (CAS number: 84687-43-4, HPLC ≥98%) (Shanghai, China). Two weeks after STZ injection, rats in the AS-IV group received AS-IV treatment at 5 mg/kg/d via oral gavage for 12 weeks. Rats in control and DN groups received

an equal volume of citrate buffer at the same time. Urine was collected at the end of 12 weeks and centrifuged at 800×g for 10 minutes for the following experiments. Glomerular hypertrophy, increased mesangial matrix, thickened capillary basement, and glomerulosclerosis were obvious in the DN group.

si-TUG1 (3'-AUCGAGUGCCGAGAAAAGU-5'), pcDNA-TUG1 (3'-GTTCTAACCCCTTGACGAT-5'), and their negative controls (NCs) were synthesized by Shanghai Genechem (Shanghai, China). One week after STZ injection, rats received an injection of 100 μL of lentiviral vector carrying si-control or si-TUG1 at a lentivirus titer of 5×10⁷ TU/mL. The animal experiment was approved by the ethics committee of North Sichuan medical college and was performed according to the guidelines by the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Cell culture and transfection

Conditionally immortalized mouse podocytes (MPC5) were purchased from the American Type Culture Collection (Manassas, VA, USA) and cultured in RPMI 1640 medium (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Thermo Fisher Scientific) and antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin) (Sigma-Aldrich Co., St Louis, MO, USA) at 37°C with 5% CO₂ atmosphere for 24 hours. Then, the cells were divided into normal glucose (NG) group, high glucose (HG) group, and HG+AS-IV group. In the NG group, cells were incubated in RPMI 1640 medium supplemented with 5 mM glucose. In the HG group, cells were incubated in RPMI 1640 medium supplemented with 30 mM glucose. In the HG+AS-IV group, cells incubated in HG medium were treated with 100 μg/mL of AS-IV for 24 hours. si-TUG1 and si-control were transfected into MPC5 cells in the HG+AS-IV group using lipofectamine 2000 (Thermo Fisher Scientific).

ELISA

Albuminuria concentration in urine was detected by rat albuminuria ELISA kit (Nanjing Jinyibo Biotechnology Co. Ltd, Nanjing, Jiangsu, China) according to the manufacturer's instructions.

Quantitative reverse transcription PCR (qRT-PCR)

Total RNAs were isolated from renal tissues or MPC5 cells using Trizol Reagent (Thermo Fisher Scientific) and

inversely transcribed into cDNA using SuperScript™ IV First-Strand Synthesis System (Thermo Fisher Scientific). Power SYBR™ Green PCR Master Mix (Thermo Fisher Scientific) was used to measure TUG1 and TRAF5 expression, which were expressed as a function of threshold cycle (Ct) and analyzed by the $2^{-\Delta\Delta Ct}$ method.

Western blot

RIPA buffer (Thermo Fisher Scientific) was used to lyse renal tissues or MPC5 cells on ice. Protein samples were isolated by 12% SDS-PAGE and then transferred to polyvinylidene fluoride (PVDF) membrane (Thermo Fisher Scientific). The membrane was blocked with 5% skim milk for 2 hours and incubated with primary antibody against TRAF5 (Thermo Fisher Scientific), cleaved-caspase 3 (Thermo Fisher Scientific), and β -actin (Abcam, Cambridge, MA, USA) overnight. The secondary horseradish peroxidase-conjugated antibody (Abcam) was added and incubated for 2 hours. β -Actin was used as an internal control. The blots were visualized by ChemiDoc MP imaging system (Bio-Rad Laboratories Inc., Hercules, CA, USA).

RNA pull-down

The biotin-labeled lncRNA-TUG1 was transcribed in vitro with Biotin RNA Labeling Mix (Hoffman-La Roche Ltd., Basel, Switzerland) and T7 RNA polymerase (Hoffman-La Roche Ltd.). Cell lysate was prepared by 1.5×10^7 cells in RIP buffer and then mixed with biotin-labeled lncRNA-TUG1 RNAs incubated at 4°C for 1 hour. The streptavidin agarose beads (Thermo Fisher Scientific) were added to each binding reaction and incubated at room temperature for 1 hour, and Western blot was used to determine the retrieved proteins.

RNA precipitation (RIP)

The RIP assay was used to determine the interaction between lncRNA-TUG1 and TRAF5 by the Magna RIP RNA-Binding Protein Immunoprecipitation Kit (EMD Millipore, Billerica, MA, USA) according to the manufacturer's instructions. qRT-PCR was used to detect TUG1 and TRAF5 in the precipitates, with the NC group as control.

TUNEL assay

MPC5 cell apoptosis was detected by ApopTag Plus In Situ Apoptosis Fluorescein Detection Kit (EMD Millipore). Cells were fixed with 1% polytetrafluoroethylene in PBS for 10 minutes at room temperature. Cooled ethanol was used to permeabilize fixation cells. Then, the cells were immersed in equilibration buffer. The working strength TdT enzyme

was added and incubated at 37°C for 1 hour. The stop buffer was added to stop the reaction. Cells were washed with PBS and incubated with anti-digoxigenin conjugate at room temperature for 30 minutes in the dark.

Ubiquitination assay

In denaturing conditions, TRAF5 was immunoprecipitated using the TRAF5 antibody (Thermo Fisher Scientific). Then, TRAF5 protein was purified and immunoblotted with anti-ubiquitin antibody (Cell Signaling Technology, Danvers, MA, USA).

Statistical analysis

The data are analyzed by SPSS software (version 18.0; SPSS Inc., Chicago, IL, USA) and presented as mean \pm standard error. Statistical analysis was performed with Student's *t*-test or one-way analysis of variance (ANOVA), with *P*-value < 0.05 considered statistically significant.

Results

AS-IV protected diabetic rats from DN

As shown in Figure 1A, STZ-induced DN rats had higher albuminuria concentration (expressed as mg/d) compared with control rats, and AS-IV treatment significantly decreased albuminuria concentration in DN rats. TUG1 level in kidney tissue was significantly decreased in DN rats compared with control rats. After AS-IV treatment, the TUG1 level in kidney tissue was significantly increased in DN rats (Figure 1B). Moreover, the TRAF5 mRNA and protein level in kidney tissue was significantly upregulated in DN rats compared with control rats. After AS-IV treatment, the TRAF5 mRNA and protein level in kidney tissue was significantly decreased in DN rats (Figure 1C).

AS-IV improved lncRNA-TUG1 expression in HG-induced MPC5 cells

Compared with the NG group, HG significantly decreased the TUG1 level, and AS-IV treatment increased the TUG1 level in HG-induced MPC5 cells (Figure 2A). The TRAF5 mRNA and protein level was significantly upregulated in the HG group than the NG group. After AS-IV treatment, the TRAF5 mRNA and protein level was significantly decreased (Figure 2B).

lncRNA-TUG1 interacted with TRAF5

As shown in Figure 3A and B, TRAF5 was observed in TUG1 complexes, and lncRNA-TUG1 accumulation was detected in TRAF5 precipitation samples. In addition, lncRNA-TUG1 knockdown increased the TRAF5 protein level,

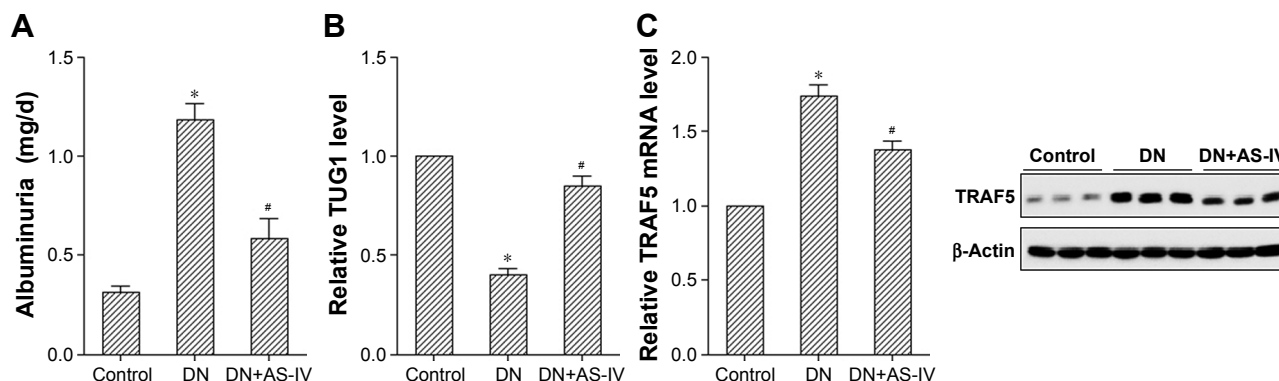


Figure 1 AS-IV protected diabetic rats from DN. **Notes:** After 2 weeks of STZ injection, AS-IV treatment was started and lasted for 12 weeks. **(A)** ELISA was performed to measure albuminuria concentration. Albuminuria concentration was increased in the DN group compared with that in the control group. After AS-IV treatment, albuminuria concentration was significantly decreased. **(B)** qRT-PCR showed that the TUG1 level was decreased in the DN group compared with that in the control group. After AS-IV treatment, the TUG1 level was significantly increased. **(C)** qRT-PCR and Western blot showed that the TRAF5 mRNA and protein level was upregulated in the DN group compared with that in the control group. After AS-IV treatment, the TRAF5 mRNA and protein level was significantly decreased. * $P < 0.05$ vs control and # $P < 0.05$ vs DN. **Abbreviations:** AS-IV, astragaloside IV; DN, diabetic nephropathy; ELISA, enzyme-linked immunosorbent assay; qRT-PCR, quantitative reverse transcription PCR; STZ, streptozotocin.

and lncRNA-TUG1 overexpression decreased the TRAF5 protein level in 293 T cells (Figure 3C). There was no significant difference in TRAF5 mRNA between lncRNA-TUG1 knockdown and overexpression (Figure 3D). With the treatment of cycloheximide (125 μg/mL), lncRNA-TUG1 overexpression promoted the degradation of TRAF5 protein (Figure 3E). This degradation effect was time dependent. The effect of TUG1 overexpression on other TRAFs (TRAF2, TRAF3, and TRAF6) showed no significant changes in TRAF2, TRAF3, and TRAF6 expressions (Figure S1), which suggested that TUG1 specifically regulate TRAF5.

AS-IV regulated TRAF5 expression via TUG1

Figure 4A shows that the TUG1 level was significantly decreased in HG-treated MPC5 cells. After AS-IV treatment, the lncRNA-TUG1 level was significantly increased, and si-TUG1 reversed this promotion effect. TRAF5 mRNA and protein level was increased in HG-treated MPC5 cells. After AS-IV treatment, the TRAF5 mRNA and protein level was significantly downregulated, and si-TUG1 rescued this inhibition effect (Figure 4B). The effect of lncRNA-TUG1 on the ubiquitination of TRAF5 showed that AS-IV upregulated

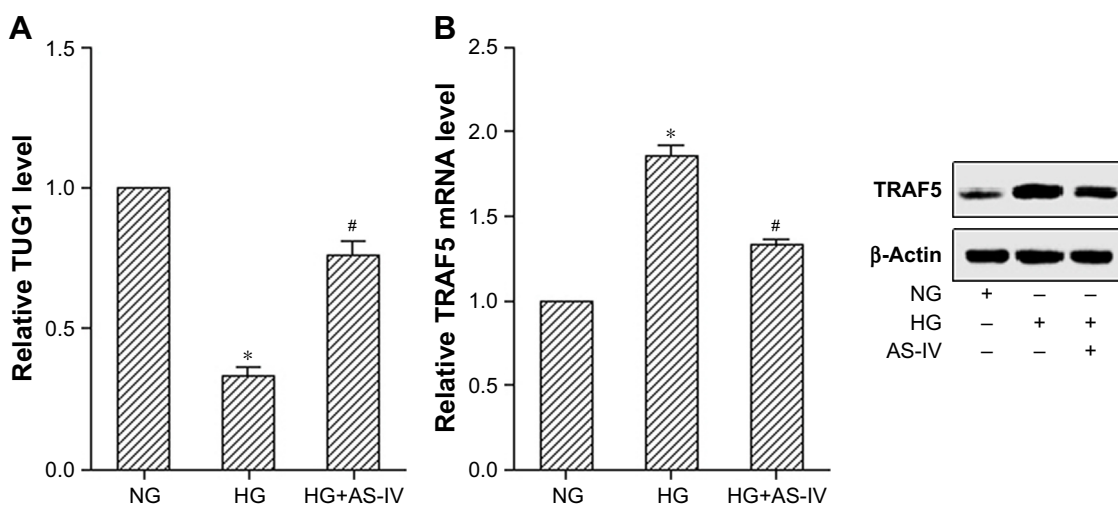


Figure 2 AS-IV improved lncRNA-TUG1 expression in HG-induced MPC5 cells. **Notes:** MPC5 cells were divided into NG group, HG group, and HG+AS-IV group. **(A)** lncRNA-TUG1 expression was significantly decreased in the HG group than the NG group. After AS-IV treatment, lncRNA-TUG1 expression was significantly increased. **(B)** TRAF5 mRNA and protein level was upregulated in the HG group than the NG group. After AS-IV treatment, the TRAF5 mRNA and protein level was significantly decreased. * $P < 0.05$ vs NG and # $P < 0.05$ vs HG. **Abbreviations:** AS-IV, astragaloside IV; HG, high glucose; NG, normal glucose.

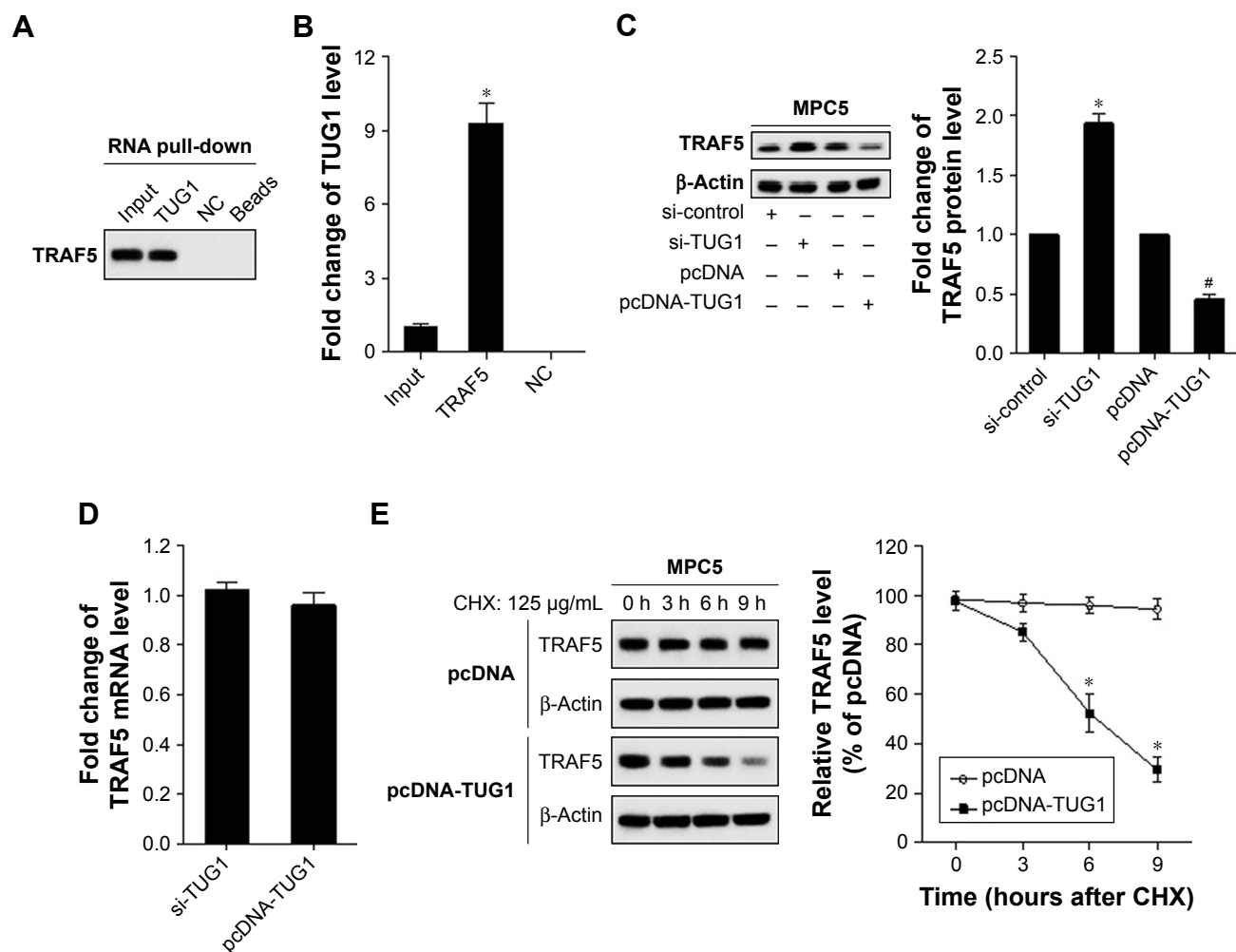


Figure 3 IncRNA-TUG1 interacts with TRAF5.

Notes: (A) RNA pull-down assay was used to detect TRAF5 in TUG1 complexes. (B) qRT-PCR showed IncRNA-TUG1 accumulation in TRAF5 precipitation samples. * $P < 0.05$ vs NC or IgG. (C) IncRNA-TUG1 knockdown increased TRAF5 protein level, and IncRNA-TUG1 overexpression decreased TRAF5 protein level in 293 T cells; * $P < 0.05$ vs si-control, # $P < 0.05$ vs pcDNA. (D) IncRNA-TUG1 knockdown or overexpression did not change TRAF5 mRNA level. (E) With the treatment of CHX (125 $\mu\text{g}/\text{mL}$), IncRNA-TUG1 overexpression promoted degradation of TRAF5 protein. * $P < 0.05$ vs control.

Abbreviations: h, hour; NC, negative control; qRT-PCR, quantitative reverse transcription PCR.

TUG1 expression and increased ubiquitination of TRAF5, thus decreased TRAF5 expression (Figure S2). These results suggested that AS-IV decreased the TRAF5 level through regulating TUG1.

AS-IV regulated podocyte apoptosis via TUG1/TRAF5

As shown in Figure 5A, podocyte apoptosis in the HG group was significantly increased than the NG group. After AS-IV treatment, podocyte apoptosis was significantly decreased, and si-TUG1 rescued the inhibition effect. At the same time, protein level of cleaved-caspase-3 in the HG group was significantly upregulated than the NG group. After AS-IV treatment, protein level of cleaved-caspase-3 was significantly decreased, and si-TUG1 rescued the inhibition effect (Figure 5B).

si-TUG1 abrogated the protective effect of AS-IV on DN rats

DN rats had higher albuminuria concentration compared with control rats, AS-IV treatment significantly decreased albuminuria concentration in DN rats, and si-TUG1 rescued this inhibition effect, indicating that si-TUG1 abrogated the protective effect of AS-IV on DN (Figure 6A). In renal tissue, we found that the TUG1 level was significantly decreased in the DN group than the control group, whereas TRAF5 protein level was increased. After AS-IV treatment, the TUG1 level was significantly increased in the DN group than the control group, whereas TRAF5 protein level was decreased. si-TUG1 reversed the promotion effect of AS-IV on TUG1 and the inhibition effect of AS-IV on TRAF5 (Figure 6B).

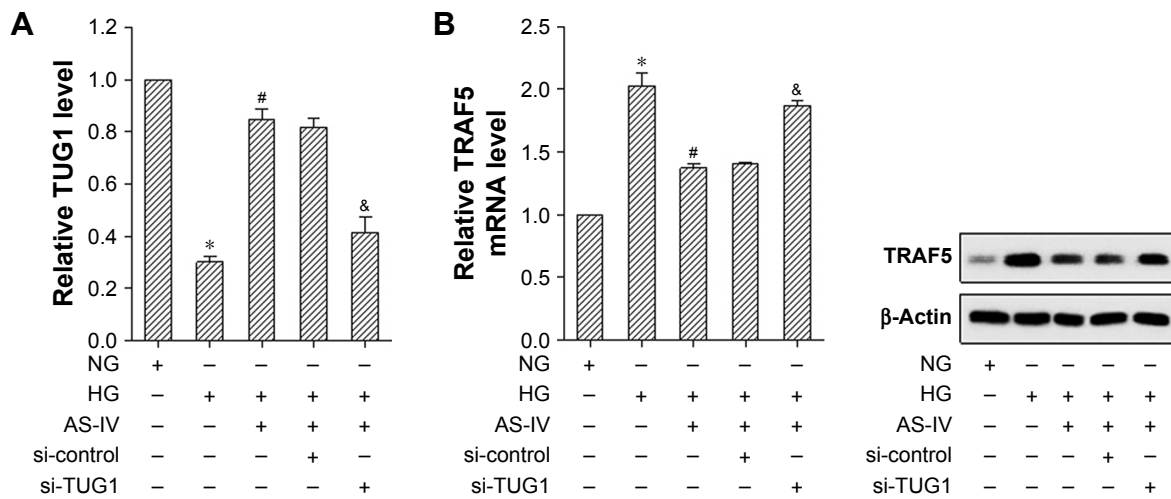


Figure 4 AS-IV regulated TRAF5 expression via lncRNA-TUG1. **Notes:** MPC5 cells were divided into NG, HG, HG+AS-IV, HG+AS-IV+si-control, and HG+AS-IV+si-TUG1 groups. **(A)** lncRNA-TUG1 level was significantly decreased in HG-treated MPC5 cells. After AS-IV treatment, lncRNA-TUG1 level was significantly increased, and si-TUG1 reversed this promotion effect. **(B)** TRAF5 mRNA and protein level was increased in HG-treated MPC5 cells. After AS-IV treatment, the TRAF5 mRNA and protein level was significantly downregulated, and si-TUG1 rescued this inhibition effect. * $P < 0.05$ vs NG, # $P < 0.05$ vs HG, and & $P < 0.05$ vs si-control. **Abbreviations:** AS-IV, astragaloside IV; HG, high glucose; NG, normal glucose.

Discussion

Apoptosis of podocytes is determinant in DN, and the identification of potential molecular targets that regulate podocyte apoptosis is important for the prevention and treatment of DN. Podocytes belong to epithelial cells and consist the renal glomerular filtration barrier, which have limited ability to regenerate and repair.¹⁸ Numerous studies have shown that podocyte injury occurred in DN through different signaling pathways, such as lncRNA LINC01619/miR-27a/FOXO1,

TGFβ1-PI3K/AKT, TNF-α-ROS-p38MAPK pathways, etc.¹⁹⁻²¹ When podocyte injury occurs, the renal glomerular filtration barrier cannot be maintained, which leads to proteinuria.²² The present study explored a new pathway from AS-IV to TRAF5 in reducing podocytes apoptosis for the treatment of DN.

Traditional Chinese Medicine can prevent and treat various diseases that have a history of thousands of years in China.²³ Radix astragali is an important Chinese herb that

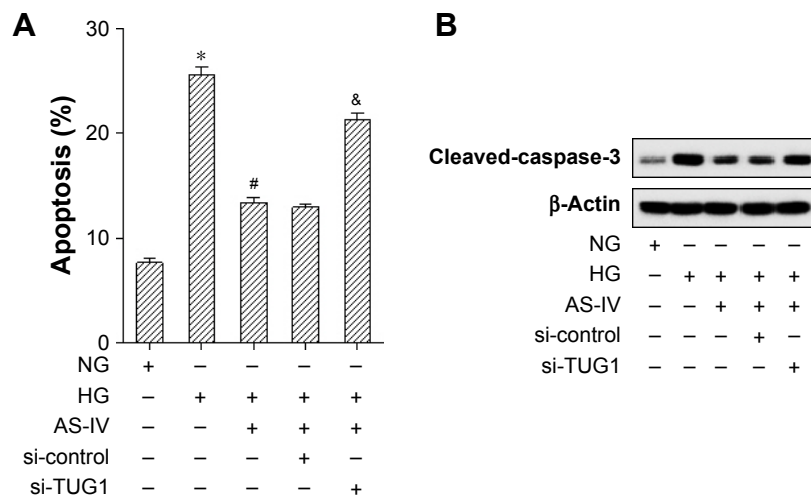


Figure 5 AS-IV regulated podocyte apoptosis via TUG1/TRAF5. **Notes:** MPC5 cells were divided into NG, HG, HG+AS-IV, HG+AS-IV+si-control, and HG+AS-IV+si-TUG1 groups. **(A)** Podocyte apoptosis in the HG group was significantly higher than the NG group. After AS-IV treatment, podocyte apoptosis was significantly decreased, and si-TUG1 rescued the inhibition effect. **(B)** Protein level of cleaved-caspase-3 in the HG group was significantly upregulated than the NG group. After AS-IV treatment, protein level of cleaved-caspase-3 was significantly decreased, and si-TUG1 rescued the inhibition effect. * $P < 0.05$ vs NG, # $P < 0.05$ vs HG, and & $P < 0.05$ vs si-control. **Abbreviations:** AS-IV, astragaloside IV; HG, high glucose; NG, normal glucose.

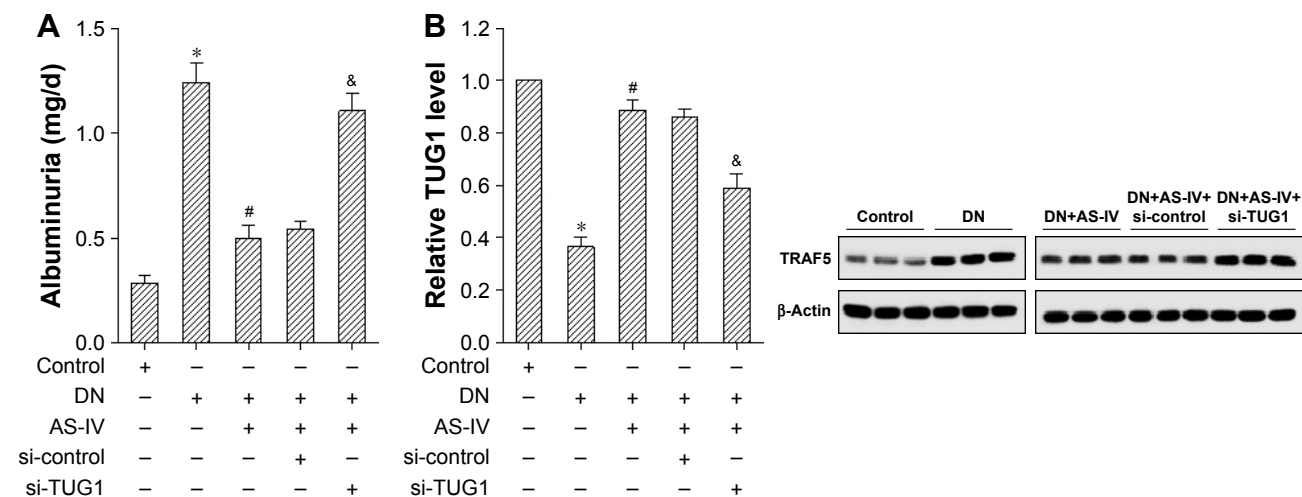


Figure 6 si-TUG1 abrogated the protective effect of AS-IV on DN rats.

Notes: After 2 weeks of STZ injection, AS-IV treatment was started and lasted for 12 weeks. Rats were divided into control, DN, DN+AS-IV, DN+AS-IV+si-control, and DN+AS-IV+si-TUG1 groups. si-control or si-TUG1 (100 μ L) was infused into caudal vein at the lentivirus titer of 5×10^7 TU/mL. **(A)** ELISA assay showed that albuminuria concentration in the DN group was increased than that of the control group. After AS-IV treatment, albuminuria concentration was significantly decreased, and si-TUG1 rescued this inhibition effect, indicating that si-TUG1 abrogated the protective effect of AS-IV on DN. **(B)** In renal tissue, the lncRNA-TUG1 level was decreased in the DN group than the control group, whereas the TRAF5 protein level was increased. After AS-IV treatment, the lncRNA-TUG1 level was increased in the DN group than the control group, whereas the TRAF5 protein level was decreased. si-TUG1 reversed the promotion effect of AS-IV on TUG1 and the inhibition effect of AS-IV on TRAF5. * $P < 0.05$ vs control, # $P < 0.05$ vs DN, and $P < 0.05$ vs DN+AS-IV+si-control.

Abbreviations: AS-IV, astragaloside IV; DN, diabetic nephropathy; STZ, streptozotocin.

has many functions, such as eliminating toxins, preventing cancers, and protecting cells from oxidative stress.^{24,25} AS-IV is a main active constituent of Radix astragali, and many in vitro and in vivo experiments showed that it has anti-diabetic, anti-inflammatory, and anti-hypertensive effects.^{26,27} Besides, many reports have proved that AS-IV can protect podocytes from HG-induced DN through modulating different pathways, such as reducing SERCA2-dependent ER stress,²⁸ downregulating TRPC6,²⁹ and attenuating Toll-like receptor four expression.³⁰ However, the underlying mechanism of AS-IV in the protection of podocytes is still not fully revealed.

lncRNA is found to be vital regulators that regulate autophagy, apoptosis, and differentiation. Studies have proved that lncRNAs can regulate podocytes injury in DN. For example, lncRNA MALAT1 was overexpressed in STZ-induced DN mice and could interplay with β -catenin to HG-induced podocyte injury.³¹ lncRNA TUG1 was decreased in podocytes from diabetic mice and regulated mitochondrial function in podocytes, and overexpression of TUG1 in podocytes reduced diabetes-induced ROS formation and albuminuria.^{12,32} Therefore, we assumed that AS-IV could increase the expression of TUG1 in podocytes and reduce albuminuria concentration. Our results showed that AS-IV reduced albuminuria concentration and increased TUG1 level, which further decreased podocyte apoptosis.

In addition, we proved that TUG1 could target TRAF5, a molecule that could induce mice podocytes apoptosis, and AS-IV could suppress TRAF5 via regulating TUG1.

Conclusion

AS-IV/lncRNA-TUG1/TRAF5 signaling pathway participates in modulating podocyte apoptosis and thus alleviates podocyte apoptosis of DN rats. This study first discovered the interaction between TUG1 and TRAF5 and revealed the treatment of AS-IV through TUG1/TRAF5, which provided potential targets for preventing DN.

Disclosure

The authors report no conflicts of interest in this work.

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Supplementary materials

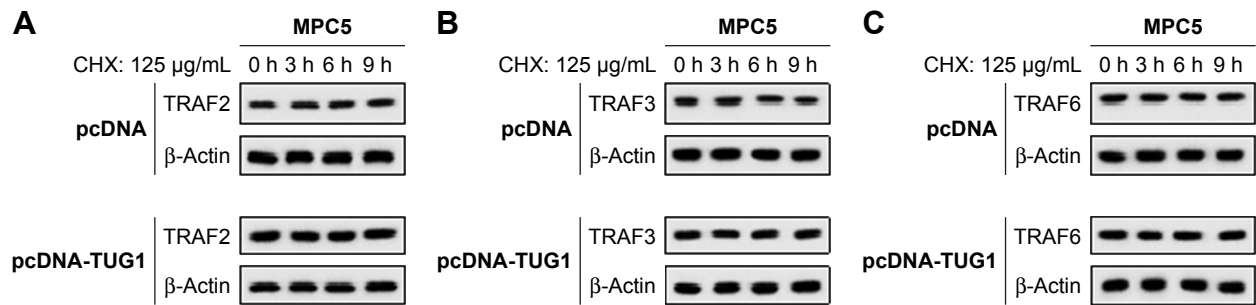


Figure S1 The effect of TUG1 overexpression on other TRAFs (TRAF2, TRAF3, and TRAF6) expressions.

Notes: (A) The effect of TUG1 overexpression on TRAF2 expression. (B) The effect of TUG1 overexpression on TRAF3 expression. (C) The effect of TUG1 overexpression on TRAF6 expression.

Abbreviation: h, hours.

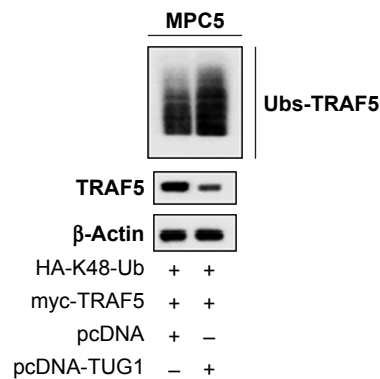


Figure S2 The effect of lncRNA-TUG1 on the ubiquitination of TRAF5.

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