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LncRNA TUG1 inhibits the proliferation and fibrosis of mesangial cells in diabetic nephropathy *via* inhibiting the PI3K/AKT pathway

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Abstract. – OBJECTIVE: To elucidate the potential function of long non-coding RNA (lncRNA) TUG1 in the progression of diabetic nephropathy (DN) and the underlying mechanism.

MATERIALS AND METHODS: Rat diabetes mellitus (DM) model was established by streptozocin (STZ) administration. *In vivo* levels of TUG1 and relative genes in the phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT) pathway in DM rats and control rats were determined by the quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). Moreover, levels of kidney weight, 24 h-urine protein, blood urea nitrogen and serum creatinine in DM rats and controls were detected. Mesangial cells were subjected to induction of high-level glucose. Relative levels of TUG1 and relative genes in the PI3K/AKT pathway in mesangial cells were determined as well. Through Cell Counting Kit-8 (CCK-8) and 5-Ethynyl-2'-deoxyuridine (EdU) assay, the regulatory effect of TUG1 on the proliferative ability of mesangial cells induced with high-level glucose was evaluated. Finally, expression changes in the PI3K/AKT pathway and extracellular matrix (ECM)-related genes in mesangial cells were determined.

RESULTS: TUG1 was downregulated in DM rats and mesangial cells induced with high-level glucose. Compared with controls, DM rats presented higher levels of kidney weight, 24 h-urine protein, blood urea nitrogen and serum creatinine, which were markedly reduced after TUG1 overexpression *in vivo*. Moreover, overexpression of TUG1 downregulated TGF-β1, FN, and COL-IV, and inhibited the activation of the PI3K/AKT pathway.

CONCLUSIONS: TUG1 is downregulated in DN. The overexpression of TUG1 could suppress the proliferation and ECM accumulation of mesangial cells via inhibiting the PI3K/AKT pathway.

Key Words:
TUG1, PI3K/AKT pathway, Diabetic nephropathy, Mesangial cells, Proliferation, Fibrosis.

Introduction

In recent years, diabetic nephropathy (DN) has become the leading cause of end-stage renal disease (ESRD), which is also the major cause of death in DM patients worldwide[1,2]. Glomerular hypertrophy is the most prominent manifestation of early-stage DN, accompanied by an increased mesangial matrix, widened mesangial area and abnormal accumulation of extracellular matrix (ECM). These pathological lesions result in glomerular sclerosis and tubulointerstitial fibrosis, which are the key features in the progression of DN[3,4]. Nevertheless, the pathogenesis of DN remains unclear, and current therapeutic strategies are ineffective. It is of clinical significance to clarify the pathogenesis of DN and to search for novel therapeutic targets.

Non-coding RNAs are categorized into long non-coding RNA (lncRNA) (>200 nt) and short noncoding RNAs (≤200 nt) based on the length, both of which are involved in diverse physical or pathological progressions. Non-coding RNAs have been extensively studied in disease progression. Several non-coding RNAs have been applied as therapeutic targets for individualized treatment. As a common transcript in the genome, lncRNA barely has a protein-encoding function. However, lncRNA is widely involved in the pathological processes[5,6]. Relevant studies[7-9] have identified the role of lncRNAs in tumors and other diseases, including DN.

LncRNA TUG1 is located in chromosome 22q12, which was initially reported to participate in the development of the retina and the formation of photoceptors[10]. Later, TUG1 was found to be able to mediate tumor progression. The knockdown of TUG1 influences the proliferation and apoptosis phenotype, suggesting the potential function of TUG1 in the occurrence and progres-

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sion of tumors[11,12]. It is reported that p53 directly acts on TUG1. The knockdown of TUG1 mediates the proliferative ability of tumor cells by upregulating HOXB7 level and activating protein kinase B (AKT) and mitogen-activated protein kinase (MAPK) pathway[13]. Besides, TUG1 affects physical function *via* interacting with miRNAs. For instance, upregulated TUG1 in vascular endothelial cells of gliomas affects the permeability of the blood-brain barrier through interacting with miR-144, thereby affecting the therapeutic efficacies of chemotherapy drugs[14]. In addition to the tumor field, TUG1 is upregulated in patients with multiple sclerosis (MS), exerting a potential value in the disease progression[15]. However, the specific function of TUG1 in DN has not been reported yet. This study mainly explored the regulatory role of TUG1 in the progression of DN, which provides novel directions in the clinical treatment.

Materials and Methods

Establishment of DM Model in Rats

Sprague Dawley (SD) rats were provided by the Shanghai Laboratory Animal Center (Shanghai, China) and habituated for one week. After fasting overnight, rats were administrated with a single dose of 1% streptozocin (STZ, 55 mg/kg) (Sigma-Aldrich, St. Louis, MO, USA) through the tail vein. Rats in control group were administrated with the same volume of normal saline. After 72 h of administration, the blood sample was collected from the tail vein and rats with over 16.7 mmol/L blood glucose were considered to be qualified. Qualified DM rats were randomly assigned into three groups, with the administration of 30 mg/kg/d LV-TUG1, LV-NC or no special treatment, respectively. Body weight, levels of 24 h-urine protein, blood urea nitrogen and serum creatinine of rat were recorded every two weeks. 8 weeks later, rats were sacrificed to harvest kidneys. Kidney weight was recorded. KW/BW=kidney weight (mg)/body weight (g). This investigation was approved by the Animal Ethics Committee of the Second Military Medical University Animal Center.

Cell Culture

Mouse-derived mesangial cells were provided by Cell Bank, Chinese Academy of Science (Shanghai, China). Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Rockville, MD, USA) containing 25 mmol/L, 5 mmol/L and 0 mmol/L of glucose, respectively and were maintained in a 5% CO_2 incubator at 37°C. Cell passage was performed up to 80% of confluence.

RNA Extraction and Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

Total RNA was extracted from cells and tissues using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). RNA was subjected to determination of purity and concentration using a spectrophotometer. The complementary Deoxyribose Nucleic Acid (cDNA) was obtained by reverse transcription of RNA and amplified by PCR at 95°C for 2 min, followed by 40 cycles at 95°C for 15 s and 60°C for 30 s. Relative levels were calculated using the $2^{-\Delta\Delta Ct}$ method. The primer sequences were as follows: TUG1: F: TAGCAGTTCCCCAATCCTTG; R: CACAAATTCCCATCATTCCC; TGF-β1: F: CAGGCTGCTCTAACGATGAA; R: CAGGAATCCCAGAAACAACC; FN: F: CAGCCCCTGATTGGAGTC; R: TGGGTGACACCTGAGTGAAC; COL-IV: F: CATGTTCAGCTTTGTGGACCT; R: GCAGCTGACTTCAGGGATGT.

Western Blotting

Proteins were extracted from kidney tissues and loaded for electrophoresis. After transferring on a polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA), it was blocked in 5% of skim milk for 2 h, incubated with primary antibodies at 4°C overnight and secondary antibodies for 2 h. Bands were exposed by enhanced chemiluminescence (ECL) and analyzed by Image J Software (NIH, Bethesda, MD, USA).

5-Ethynyl-2'-Deoxyuridine (EdU) Assay

Cells seeded in the 96-well plate were labeled with 10 μmol/L of EdU (Beyotime, Shanghai, China) for 2 h. Subsequently, cells were fixed in 4% of paraformaldehyde for 15 min and incubated in phosphate-buffered saline (PBS) containing 0.5% of Triton- X100 for 20 min. After washing with PBS containing 3% of bovine serum albumin (BSA; Sigma-Aldrich, St. Louis, MO, USA), 100 μL of dying solution was applied per well for 30-min incubation in the dark and nuclei staining with 4',6-diamidino-2-phenylindole (DAPI; Beyotime, Shanghai, China) for 5 min. Apollo-positive cells and DAPI-positive cells were captured using a confocal laser scanning microscope. The ratio of EdU-positive cells was finally calculated.

Cell Transfection

One day prior to transfection, cells were seeded in the 6-well plate with 5.5×10^5 cells per well. Cells were transfected with LV-TUG1 or LV-Vector with MOI=15. Fresh DMEM was replaced 8 h later.

Cell Counting Kit-8 (CCK-8)

Cells were seeded in a 96-well plate at a dose of 1.0×10^5/mL. At the appointed time points, 10 μL of CCK-8 solution (Dojindo, Kumamoto, Japan) was applied per well. After incubation for 2 h, the recorded absorbance at 450 nm using a microplate reader was used for plotting the growth curve.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 17.0 (SPSS Inc., Chicago, IL, USA) was used for all statistical analysis. Data were represented as mean ± standard deviation. The *t*-test was used to analyze the intergroup differences. $p<0.05$ indicated a significant difference.

Results

TUG1 Was Downregulated in DM Rats

The expression pattern of TUG1 was first examined in DM rats and controls. As the qRT-PCR data revealed, TUG1 was lowly expressed in DM rats relative to controls (Figure 1A). Similarly, TUG1 was downregulated in mesangial cells induced with high-level glucose than that of controls (Figure 1B). Previous researches reported the crucial role of phosphatidylinositol 3-kinase (PI3K)/AKT pathway in DN. Here, the protein levels of p-PI3K and p-AKT were upregulated in DM rats and mesangial cells induced with high-level glucose, indicating the activated PI3K/AKT pathway (Figure 1C and 1D).

Overexpression of TUG1 Alleviated Kidney Lesions in DM Rats

To elucidate the biological function of TUG1 in DN, DM rats were administrated with LV-Vector or LV-TUG1, respectively. DM rats presented higher levels of kidney weight, 24 h-urine protein, blood urea nitrogen and serum creatinine than controls (Figure 2A-2D). In particular, DM rats administrated with LV-TUG1 had lower levels of the above-mentioned indicators than those administrated with the LV-Vector (Figure 2A-2D). These results demonstrated that TUG1 overexpression could alleviate kidney lesions in DM rats.

Overexpression of TUG1 Suppressed Proliferation of Mesangial Cells

The *in vitro* effect of TUG1 on the proliferative ability was further explored. Transfection efficacy of LV-TUG1 was firstly verified, which markedly increased TUG1 level in mesangial cells. Transfection of LV-TUG1 markedly suppressed proliferative rate in mesangial cells induced with high-level glucose than that of controls (Figure 3A). With the prolongation of cell culture, the viability was gradually elevated (Figure 3B). Identically, EdU assay also suggested that TUG1 overexpression remarkably inhibited the proliferative rate (Figure 3C).

Overexpression of TUG1 Suppressed ECM Secretion of Mesangial Cells

QRT-PCR data illustrated that mRNA levels of TGF-β1, FN, and COL-IV were upregulated after high-level glucose induction in mesangial cells, while they were downregulated after TUG1 overexpression (Figure 4A-4C). Western blot analysis obtained the same trends influenced by TUG1 at their protein levels (Figure 4D). It is reported that the PI3K/AKT pathway exerts a crucial role in the proliferation and ECM accumulation of mesangial cells. Here, overexpression of TUG1 remarkably downregulated protein levels of p-PI3K and p-AKT in mesangial cells induced with high-level glucose, suggesting the inhibited PI3K/AKT pathway (Figure 4E).

Discussion

DN is a serious diabetic microvascular complication. Recent studies have shown the involvement of lncRNAs in glomerular mesangial fibrosis and hyperplasia of DN. LncRNA-ENSMUST00000147869 is upregulated in diabetic kidney tissues, and the silence of its expression enhances the proliferation and fibrosis of mesangial cells[16]. LncRNA-CYP4B1-PS1-001 is downregulated in early-stage DN, and its upregulation suppresses the proliferation and fibrosis of mesangial cells[17]. LncRNA PVT1 is upregulated in mesangial cells induced with high-level glucose, which increases the relative levels of Fn, Col. IV, and TGF-β1. Knockdown of PVT1 remarkably downregulates ECM-related genes[18,19]. It is believed that abnormally expressed lncRNAs greatly affect the mesangial proliferation and fibrosis of DN. In this study, TUG1 was lowly expressed in DM rats and mesangial cells induced

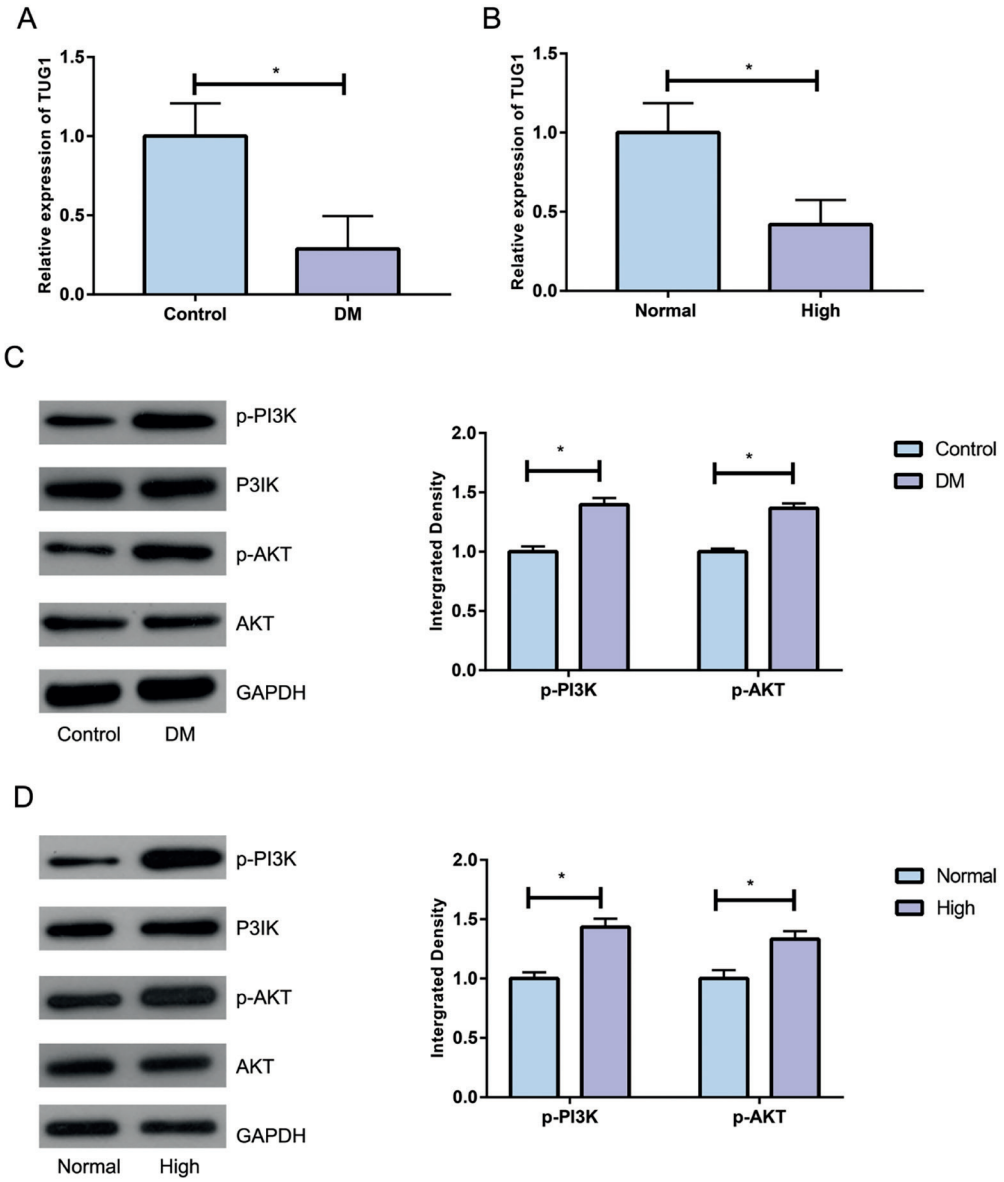

Figure 1. TUG1 was downregulated in DM rats. ***A,*** TUG1 level was downregulated in DM rats compared to controls determined by qRT-PCR. ***B,*** TUG1 level was downregulated in mesangial cells induced with high-level glucose determined by qRT-PCR. ***C,*** Western blot analyses of p-PI3K, PI3K, p-AKT, and AKT in DM rats and controls. ***D,*** Western blot analyses of p-PI3K, PI3K, p-AKT, and AKT in mesangial cells induced with high-level glucose and controls.

with high-level glucose. Moreover, TUG1 overexpression inhibited the proliferative rate of mesangial cells and downregulated ECM-related gene expressions, suggesting the protective role of TUG1 in DN. To further investigate the molecular mechanism of TUG1 in the progression of DN, the relative genes in the PI3K/AKT pathway were determined.

Studies have shown that high-level glucose induces ECM deposition in the glomerulus by activating the PI3K/AKT pathway[20]. As an intracellular phosphatidylinositol kinase, PI3K is a key signaling molecule in biological activities. AKT is a direct downstream of PI3K and belongs to the serine/threonine protein kinase. Phosphorylated AKT is the activation form that further influences the downstream genes to participate in cellular behavior regulations[21,22]. Relative studies have shown that the PI3K/AKT pathway is activated after the occurrence of DN. Downstream

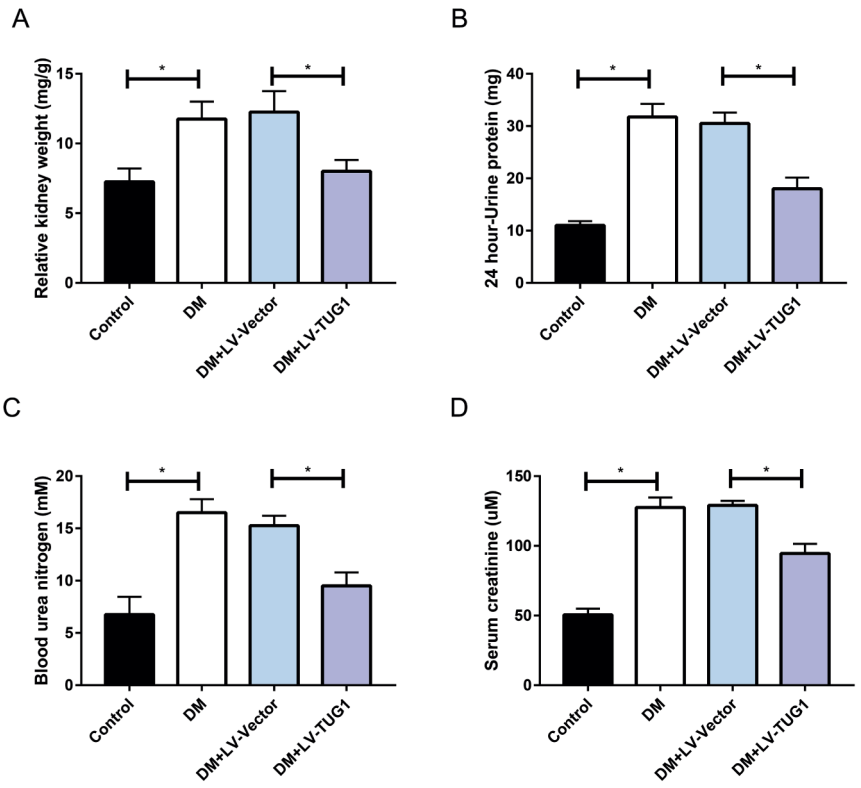

Figure 2. Overexpression of TUG1 alleviated kidney lesions in DM rats. Overexpression of TUG1 reduced levels of kidney weight (***A***), 24 h-urine protein (***B***), blood urea nitrogen (***C***) and serum creatinine (***D***) in DM rats.

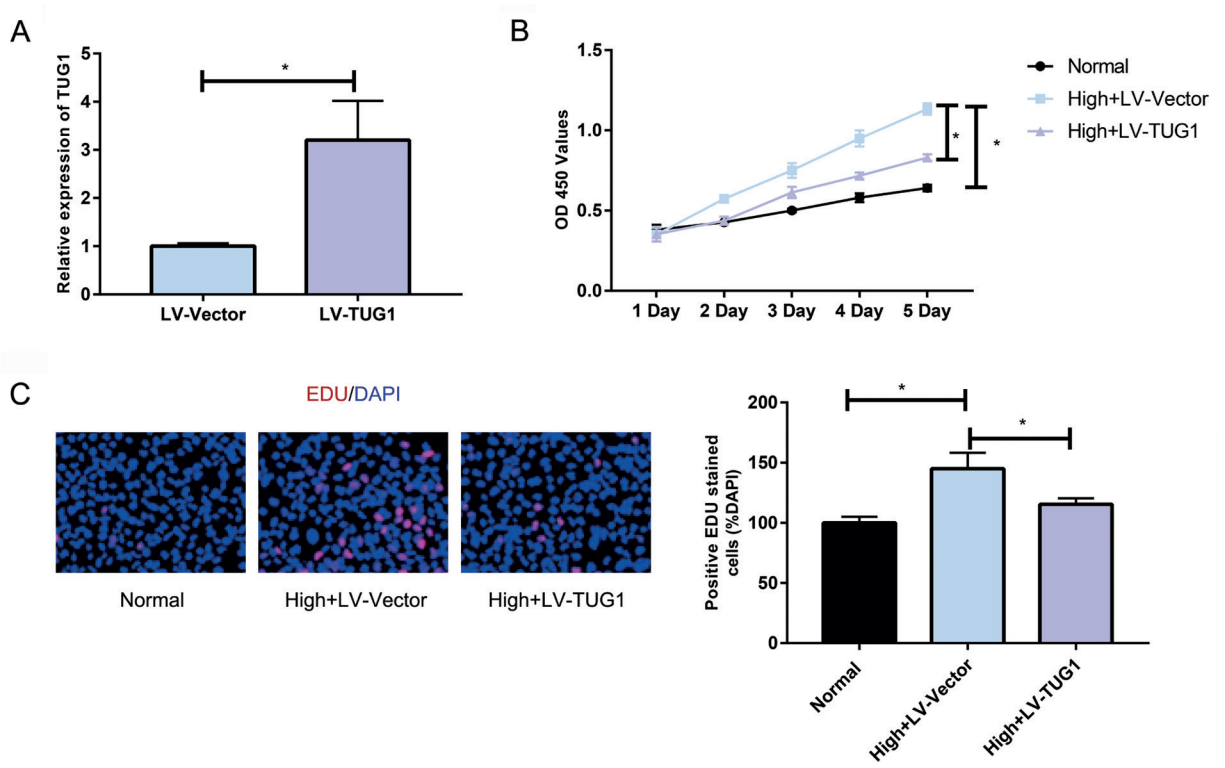

Figure 3. Overexpression of TUG1 suppressed proliferation of mesangial cells. ***A,*** Transfection efficacy of LV-TUG1 in mesangial cells determined by qRT-PCR. ***B,*** CCK-8 assay determined the proliferative rate in normal cells, high-glucose treated mesangial cells transfected with LV-Vector or LV-TUG1. ***C,*** EdU assay determined the proliferative rate in normal cells, high-glucose treated mesangial cells transfected with LV-Vector or LV-TUG1.

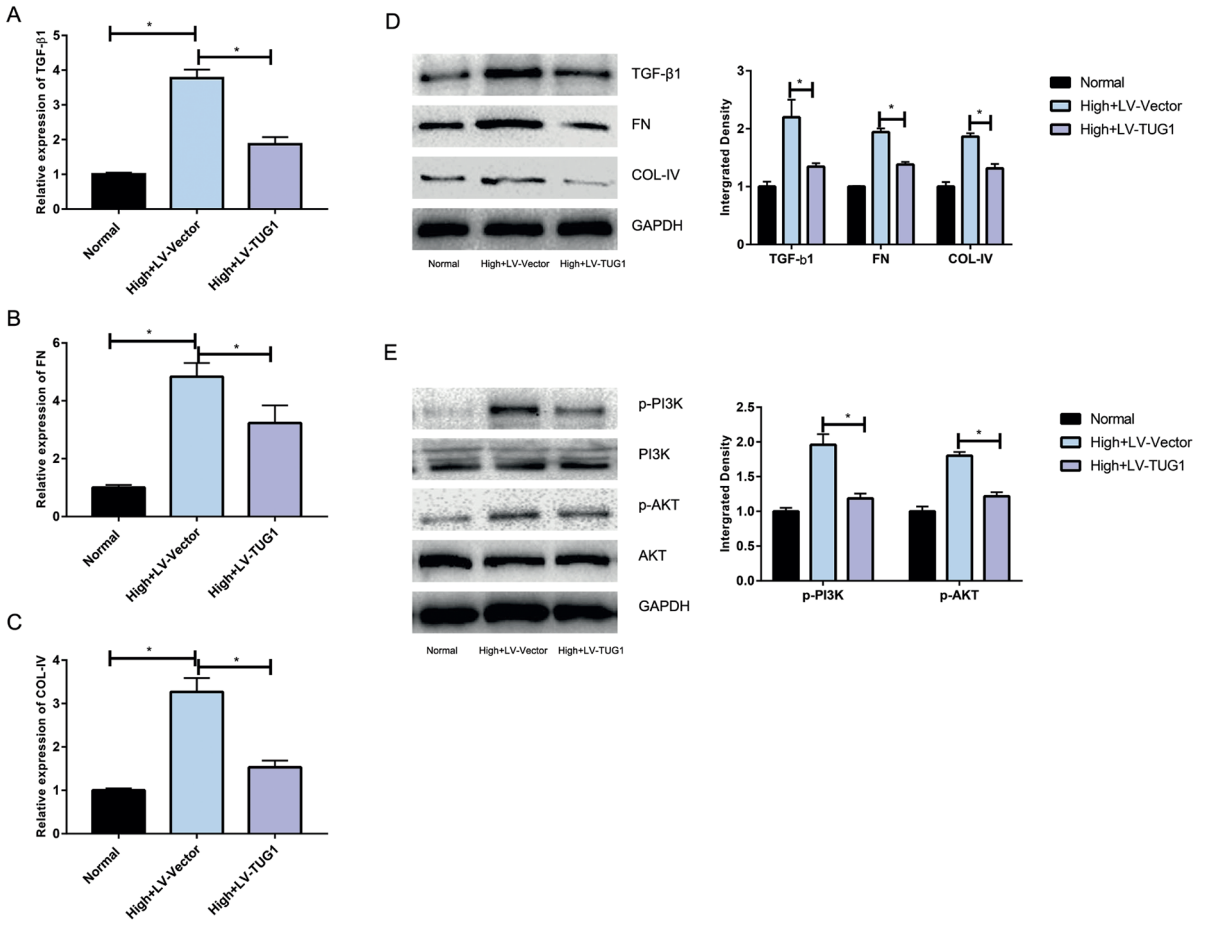

Figure 4. Overexpression of TUG1 suppressed ECM secretion of mesangial cells PCR. The mRNA levels of TGF-β1 (***A***), FN (***B***) and COL-IV (***C***) in normal cells, high-glucose treated mesangial cells transfected with LV-Vector or LV-TUG1 determined by qRT-PCR. ***D,*** Western blot analyses of TGF-β1, FN, and COL-IV in normal cells, high-glucose treated mesangial cells transfected with LV-Vector or LV-TUG1. ***E,*** Western blot analyses of p-PI3K, PI3K, p-AKT, and AKT in normal cells, high-glucose treated mesangial cells transfected with LV-Vector or LV-TUG1.

phosphorylated factors subsequently enhance ribosome biosynthesis, proliferative ability and inhibit apoptosis. These genetic changes lead to podocyte apoptosis, the proliferation of glomerular endothelial cells and mesangial cells, as well as the ECM accumulation, eventually aggravating fibrosis and sclerosis degree of the kidney[23]. This study revealed that the PI3K/AKT pathway was activated by high-level glucose induction, which was further inhibited by TUG1 overexpression.

Some limitations of this study should be noteworthy. The rat DM model was established by the STZ administration. Verification of TUG1 role in db/db mice with spontaneous type 2 DM would enhance the credibility of our conclusions.

Conclusions

TUG1 is downregulated in DN. The overexpression of TUG1 could suppress the proliferation and ECM accumulation of mesangial cells *via* inhibiting the PI3K/AKT pathway.

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Conflict of Interests

The Authors declare that they have no conflict of interests.

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