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TUG1 promotes diabetic atherosclerosis by regulating proliferation of endothelial cells via Wnt pathway

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Abstract. – OBJECTIVE: To explore the specific role of TUG1 in regulating the occurrence and progression of diabetic atherosclerosis and its underlying mechanism.

PATIENTS AND METHODS: TUG1 expressions in coronary artery disease (CAD) tissues, normal arterial tissues, endothelial cells induced by high-dose glucose and tumor necrosis factor-α (TNF-α) were detected by quantitative Real-time polymerase chain reaction (qRT-PCR). The effects of TUG1 on proliferation, migration and cell cycle of human umbilical vein endothelial cells (HUVECs) were detected by cell counting kit-8 (CCK-8), transwell assay and flow cytometry, respectively. Subsequently, protein expressions of proliferation-related genes, cell cycle-related genes and Wnt pathway-related genes were detected by Western blot after altering TUG1 expression in HUVECs. Further rescue experiments were carried out to explore whether TUG1 could regulate diabetic atherosclerosis via Wnt pathway.

RESULTS: Overexpressed TUG1 was found in CAD tissues and endothelial cells induced by high-dose glucose and TNF-α compared with those of controls. TUG1 overexpression remarkably promoted proliferation, migration and cell cycle of HUVECs. Protein expressions of β-catenin and c-Myc were upregulated by overexpression of TUG1. Rescue experiments indicated that XAV-939, the inhibitor of Wnt pathway, could partially reverse the increased proliferative and migratory changes in HUVECs induced by TUG1 overexpression.

CONCLUSIONS: We found that overexpressed TUG1 stimulates proliferation and migration of endothelial cells via Wnt pathway, thereby promoting the occurrence and progression of diabetic atherosclerosis.

Key Words:
TUG1, Diabetic atherosclerosis, Proliferation, Migration.

Introduction

Diabetes mellitus is a disease where high blood sugar levels occur from decreased insulin signaling, either by a lack of insulin secretion in Type 1 diabetes or insulin resistance in Type 2 diabetes[1]. Atherosclerosis is the cause of a majority of cardiovascular events and is accelerated by diabetes and the metabolic syndrome. Epidemiological studies have provided convincing evidence that the risk of cardiovascular disease (CVD) is increased in the presence of diabetes, which is related to the extent of glycemic control. Many risk factors are associated with the metabolic syndrome and CVD. Diabetic atherosclerosis is marked by the appearance of plaques consisting of leukocytes, lipids, smooth muscle cells and low-density lipoprotein (LDL) cholesterol in the arterial walls. Deteriorative disease condition leads to ischemia-causing cerebrovascular accidents and myocardial infarctions in diabetic patients. A large number of studies have confirmed that various pathogenic factors are involved in the occurrence of vascular endothelial dysfunction and pro-inflammatory reactions. These pathogenic factors include persistent hyperglycemia, hyperlipidemia, obesity, etc. Atherosclerosis is a complex chronic polygenic disease[2,3], in which fiber

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components and lipids are deposited in medium-sized arteries and arterial walls[4-6]. So far, the specific pathogenesis of atherosclerosis remains unclear. Relative studies have suggested that endothelial cell damage exerts an essential role in the formation and pathological progression of atherosclerosis. Endothelial cell damage destroys the endothelial integrity and thereafter leads to atherosclerosis through lipid deposition[7-9]. However, the molecular mechanism of endothelial cell damage is rarely reported.

LncRNAs (long non-coding RNAs) are non-coding, small RNAs with 200 nt in length[10]. Recent studies have found that lncRNA regulates gene expressions at transcriptional and post-transcriptional level. LncRNA participates in the occurrence and development of multiple diseases[11-13]. In recent years, the specific function of lncRNA in cardiovascular disease has been well recognized. Accumulating evidence has demonstrated that some certain lncRNAs could regulate the development of cardiovascular diseases[14]. Previous studies have found that lncRNA TUG1 can regulate the proliferation and apoptosis of tumor cells, which is served as a potential tumor biomarker and therapeutic target[15,16]. Although TUG1 function in cardiovascular disease has not been reported, TUG1 is highly expressed in vascular endothelial cells, suggesting its potential role in the regulation of endothelial cells[17]. Meanwhile, disordered vascular endothelium induced by chronic inflammation ultimately leads to the formation of atheromatous plaque, which is the key factor in the pathogenesis of coronary artery disease (CAD)[18].

Our study aims at investigating the effect of TUG1 on proliferation and migration of endothelial cells to provide a theoretical basis for improving clinical outcomes of diabetic atherosclerosis.

Patients and Methods

Subjects and Sample Collection

A total of 15 CAD patients diagnosed in The Affiliated Hospital of Medical School, Ningbo University from June 2012 to June 2017 were enrolled. CAD tissues and corresponding normal arterial tissues were surgically resected and immediately preserved in liquid nitrogen. This study was approved by the Affiliated Hospital of Medical School, Ningbo University Ethics Committee and all subjects were informed consent.

Cell Culture and Transfection

Primary human umbilical vein endothelial cells (HUVECs) were isolated from umbilical vein and cultured in ECM (endothelial cell medium) containing 10% FBS (fetal bovine serum), 100 U/mL penicillin and 100 μg/mL streptomycin (HyClone, South Logan, UT, USA). The umbilical tissues were collected from healthy pregnancies in Obstetrics and Gynecology Department, the Affiliated Hospital of Medical School, Ningbo University. HUVECs were cultured in a 5% CO_2 incubator at 37°C. Culture medium was replaced every two days.

Cells in logarithmic growth phase were seeded in the 6-well plates. TUG1 lentiviruses (LV-TUG1 and LV-Vector) were transfected in HUVECs according to the instructions of lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). LV-TUG1 and LV-Vector were constructed by Gene Pharma (Shanghai, China).

Cell Treatment

HUVECs were treated with low-dose glucose (5 mM) or high-dose glucose (25 mM) medium when the cell confluence was up to 70-90%. After culturing for 48 h, cells were harvested on ice for the following experiments.

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

Total RNA in treated cells was extracted using TRIzol method (Invitrogen, Carlsbad, CA, USA) for reverse transcription according to the instructions of PrimeScript RT reagent Kit (TaKaRa, Otsu, Shiga, Japan). QRT-PCR was then performed based on the instructions of SYBR Premix Ex Taq TM (TaKaRa, Otsu, Shiga, Japan), with 3 replicates in each group. The specific qRT-PCR reaction parameters were: 94°C for 30 s, 55°C for 30 s and 72°C for 90 s, for a total of 40 cycles. Primers used in the study were as follows: TUG1, F: 5'-TAGCAGTTCCCCAATCCTTG-3', R: 5'-CACAAATTCCCATCATTCC-3'; PCNA, F: 5'-CCTGCTGGGATATTAGCTCCA-3', R: 5'-CAGCGGTAGGTGTCGAAGC-3'; cyclin D1, F: 5'-GCTGCGAAGTGGAAACCATC-3', R: 5'-CCTCCTTCTGCACACATTTGAA-3'; GAPDH (glyceraldehyde 3-phosphate dehydrogenase), F: 5'-AGCCACATCGCTCAGACAC-3', R: 5'-GCCCAATACGACCAAATCC-3'.

Cell Counting Kit-8 (CCK-8) Assay

Transfected HUVECs were seeded into 96-well plates at a density of 1×10^4/μL. 10 μL of CCK-8 solution (cell counting kit-8, Dojindo, Kumamoto, Japan) was added in each well after cell culture for 0, 24, 48 and 72 h, respectively. The absorbance at 450 nm of each sample was measured by a microplate reader (Bio-Rad, Hercules, CA, USA). Each group had 5 replicates.

Western Blot

Total protein was extracted from treated cells by RIPA (radioimmunoprecipitation assay) solution (Beyotime, Shanghai, China). The protein sample was separated by electrophoresis on 10% SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis) and then transferred to polyvinylidene difluoride membrane (PVDF) (Millipore, Billerica, MA, USA). After membranes were blocked with skimmed milk, they were incubated with primary antibodies (Cell Signaling Technology, Danvers, MA, USA) overnight at 4°C. The membranes were then washed with TBST (Tris-buffered Saline and Tween 20) 3 times and followed by the incubation of secondary antibody at room temperature for 1 h. The protein blot on the membrane was exposed by enhanced chemiluminescence (ECL) (Thermo Fisher Scientific, Waltham, MA, USA).

Cell Cycle Detection

HUVECs were collected and adjusted to a density of 1×10^5/mL. Subsequently, HUVECs were fixed with pre-cooled ethanol overnight, washed with PBS (phosphate buffer saline) twice and incubated with 100 μL of RNase A at 37°C in the dark. 30 min later, cells were stained with 400 μL of propidium iodide (PI). Cell cycle was detected using flow cytometry (Partec AG, Arlesheim, Switzerland) at the wavelength of 488 nm. Each experiment was performed in triplicate.

Transwell Assay

Transfected HUVECs were centrifuged, washed with PBS (phosphate-buffered saline) three times and resuspended in serum-free medium. Cell density was adjusted to 8×10^3 /mL. For transwell assay, 200 μL of cell suspension and 600 μL of culture medium were added in the upper and lower chamber, respectively. After cell cultured for 48 h, cells were washed with PBS, fixed with ethanol and stained with crystal violet. 6 randomly selected fields were observed and captured using an inverted microscope (Nikon, Tokyo, Japan).

Statistical Analysis

SPSS (Statistical Product and Service Solutions) 20.0 statistical software (IBM, Armonk, NY, USA) was used for data analysis. GraphPad Prism 7 (La Jolla, CA, USA) was introduced for figure editing. Measurement data were expressed as mean ± standard deviation ($\bar{x}\pm s$) and compared using the *t*-test. $p<0.05$ considered the difference was statistically significant.

Results

High-Dose Glucose Treatment Upregulated TUG1 Expression

TUG1 expression in 15 CAD tissues and normal arterial tissues were detected by qRT-PCR. We found that TUG1 was overexpressed in CAD tissues compared with that of normal arterial tissues (Figure 1A). Subsequently, HUVECs were treated with low-dose glucose (5 mM) or high-dose glucose (25 mM) for 0, 24, 48 and 72 h, respectively. CCK-8 results indicated that high-dose glucose treatment remarkably promoted HUVECs proliferation than that of low-dose glucose treatment (Figure 1B). QRT-PCR results showed that TUG1 expression was remarkably upregulated in HUVECs treated with high-dose glucose in comparison with those low-dose glucose treatment (Figure 1C). To further explore the effect of TUG1 on regulating HUVECs proliferation, we detected the proliferative ability in HUVECs induced with 50 ng/mL TNF-α (Tumor Necrosis Factor-α) at different time points. The data demonstrated that TNF-α significantly increased cell proliferation in a time-dependent manner (Figure 1D). Meanwhile, TNF-α also upregulated TUG1 expression in HUVECs (Figure 1E).

Overexpressed TUG1 Promoted Proliferation and Migration of HUVECs

To explore the specific role of TUG1 in regulating HUVECs functions, TUG1 lentivirus and negative control were first constructed. Transfection efficacies of corresponding lentiviruses were verified by qRT-PCR (Figure 2A). CCK-8 results indicated that overexpressed TUG1 remarkably promoted cell proliferation of HUVECs (Figure 2B). In addition, both mRNA and protein levels of PCNA were ele-

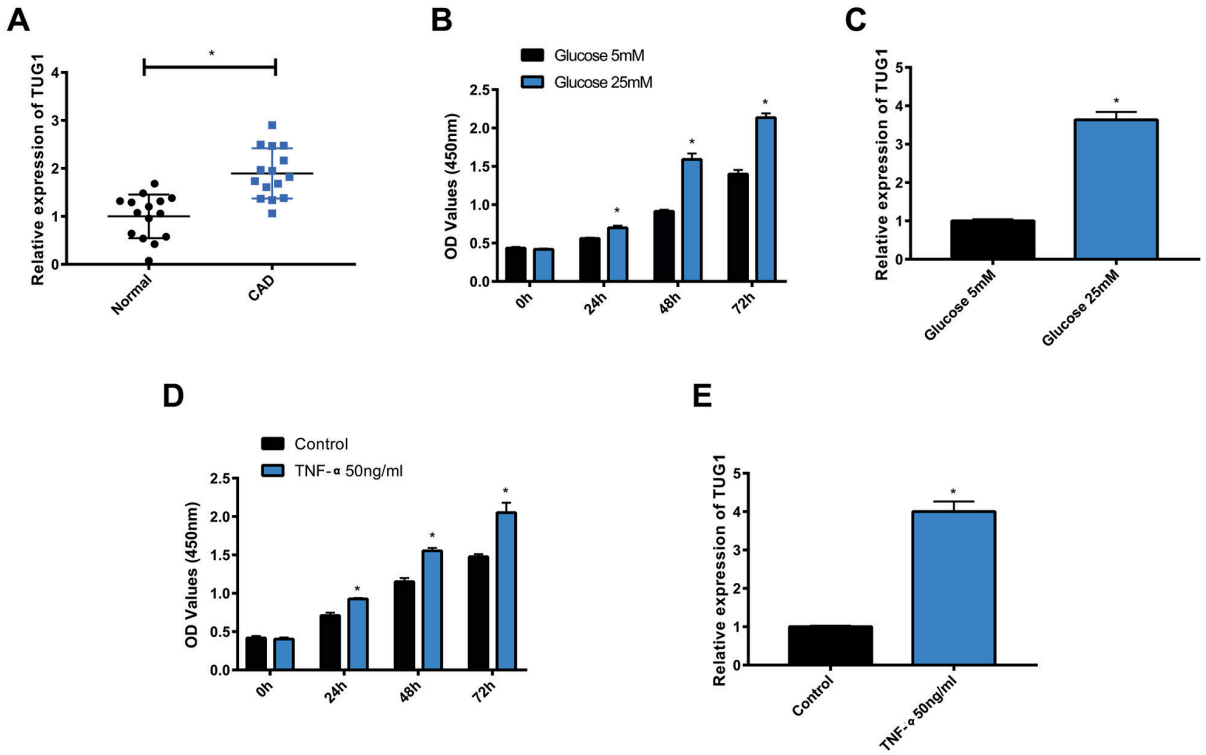

Figure 1. High-dose glucose treatment upregulated TUG1 expression. ***A,*** TUG1 was overexpressed in CAD tissues than that of normal arterial tissues. ***B,*** CCK-8 results indicated that high-dose glucose treatment remarkably promoted HUVECs proliferation than that of low-dose glucose treatment. ***C,*** TUG1 expression was remarkably upregulated in HUVECs treated with high-dose glucose. ***D,*** TNF-α significantly increased cell proliferation in a time-dependent manner. ***E,*** TNF-α treatment upregulated TUG1 expression in HUVECs.

vated after LV-TUG1 transfection in HUVECs (Figure 2C and 2D). Subsequently, we detected cell cycle of HUVECs after transfection of LV-TUG1 or LV-Vector, respectively. Flow cytometric results elucidated that TUG1 overexpression promoted cell cycle of HUVECs (Figure 2E). Both mRNA and protein levels of cyclin D1 were also elevated by TUG1 overexpression (Figure 2F and 2G). Transwell results suggested that cell invasion of HUVECs was increased by TUG1 overexpression (Figure 2H).

Overexpressed TUG1 Activated Wnt Pathway

Further experiments revealed that TUG1 overexpression upregulated the Wnt pathway-related genes, including β-catenin and c-Myc (Figure 3A). On the contrary, expressions of β-catenin and c-Myc in HUVECs were remarkably inhibited by Wnt pathway inhibitor XAV-939 (Figure 3B), suggesting that TUG1 might regulate diabetic atherosclerosis *via* Wnt pathway.

TUG1-Induced Cell Proliferation Was Inhibited Via Wnt Pathway

XAV-939 partially reversed TUG1-induced cell proliferation (Figure 4A). Similarly, upregulated PCNA and cyclin D1 were reversed by XAV-939 treatment (Figure 4B and 4C). Increased migratory ability induced by TUG1 overexpression was rescued by XAV-939 treatment (Figure 4D), indicating that TUG1 promotes HUVECs proliferation and migration *via* Wnt pathway.

Discussion

The response to the retention hypothesis outlines the initial stages of atherosclerotic lesion formation. The hypothesis proposes that proteoglycan-mediated lipoprotein retention plays a critical step in the initiation of atherosclerosis development. The etiology of atherosclerosis initiation and progression has been reviewed extensively. It is questioned, however, whether the pathoge-

nic mechanisms for atherosclerotic lesion development differ for diabetes *vs.* other risk factors. Comorbidities induced by type 2 diabetes lead to the metabolic syndrome. The incidence of the atherosclerotic disease is also increased in type 1 diabetes that is less frequently associated with other risk factors of CVD. Atherosclerosis is the primary reason for death and disability in diabetic patients. Dysregulation of metabolism in diabetes adversely affects every cellular element within the vascular wall.

In the present study, we first detected TUG1 expression in CAD tissues and normal arterial tissues. Overexpressed TUG1 was observed in CAD tissues than that of normal arterial tissues. Furthermore, TUG1 expression was also upregulated in proliferative HUVECs induced by high-dose glucose and TNF-α. Overexpressed TUG1 remarkably promoted protein expressions of proliferation-related and cell cycle-related genes in HUVECs. The Wnt pathway-related genes, β-catenin and c-Myc

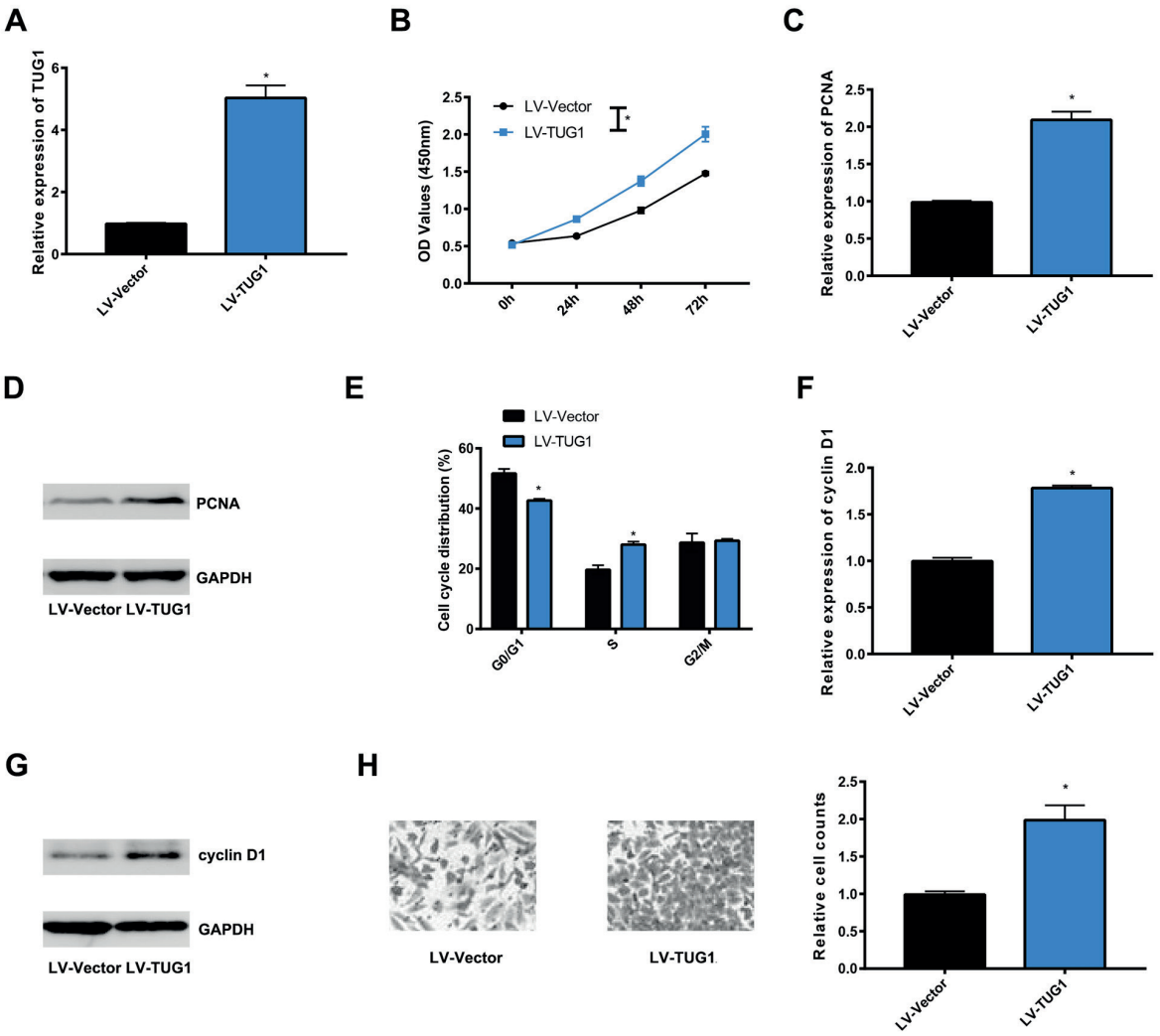

Figure 2. Overexpressed TUG1 promoted proliferation and migration of HUVECs. ***A,*** Transfection efficacies of LV-TUG1 and LV-Vector were verified by qRT-PCR. ***B,*** CCK-8 results indicated that overexpressed TUG1 remarkably promoted cell proliferation. ***C, D,*** The mRNA (***C***) and protein (***D***) levels of PCNA were elevated after LV-TUG1 transfection. ***E,*** Flow cytometric results elucidated that TUG1 overexpression promoted cell cycle. ***F, G,*** The mRNA (F) and protein (G) levels of cyclin D1 were elevated by TUG1 overexpression. H. Transwell results suggested that cell invasion of HUVECs was increased by TUG1 overexpression (20×).

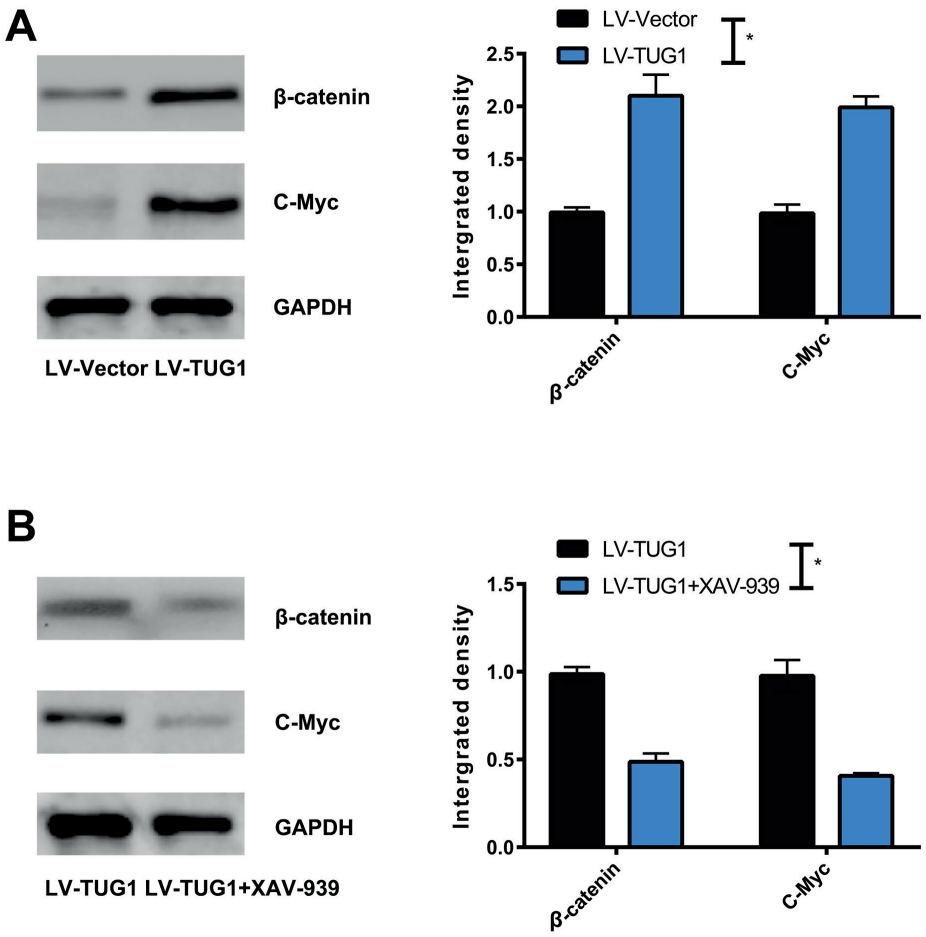

Figure 3. Overexpressed TUG1 activated Wnt pathway. ***A,*** TUG1 overexpression upregulated Wnt pathway-related genes, β-catenin and c-Myc. ***B,*** Expressions of β-catenin and c-Myc in HUVECs were remarkably inhibited by Wnt pathway.

were upregulated after TUG1 overexpression. Finally, rescue experiments demonstrated that TUG1 promoted proliferation and migration of endothelial cells *via* Wnt pathway, which may be the possible mechanism of TUG1 in regulating diabetic atherosclerosis.

A significant amount of evidence has elucidated that lncRNA may exert a regulatory role in the pathophysiology of vascular diseases. LncRNA has been served as the biomarker in target therapies. Previous studies have reported that lncRNA TUG1 is involved in mouse retinal development[19]. Upstream of lncRNA TUG1 in tumor cells is directly regulated by p53[20]. In recent years, TUG1 has been found to be differentially expressed in various tumors, affecting the proliferation and apoptosis of tumor cells. TUG1 is also correlated with the prognosis of tumor patients[21-23]. In this study, we found that TUG1 expression in CAD tissues and endothelial cells induced by high-dose glucose was remarkably increased than that of normal controls. Overexpression of TUG1 in HUVECs can promote proliferation, migration and cell cycle, thereafter stimulating diabetic atherosclerosis development.

Wnt pathway is a highly conserved signal pathway involving a series of biological processes, such as cell proliferation, differentiation and anti-apoptosis. β-catenin is a key factor in Wnt pathway and maintains a phosphorylation state in inactivated Wnt pathway. However, phosphorylation of β-catenin is blocked and accumulated after activation of Wnt pathway. Sub-

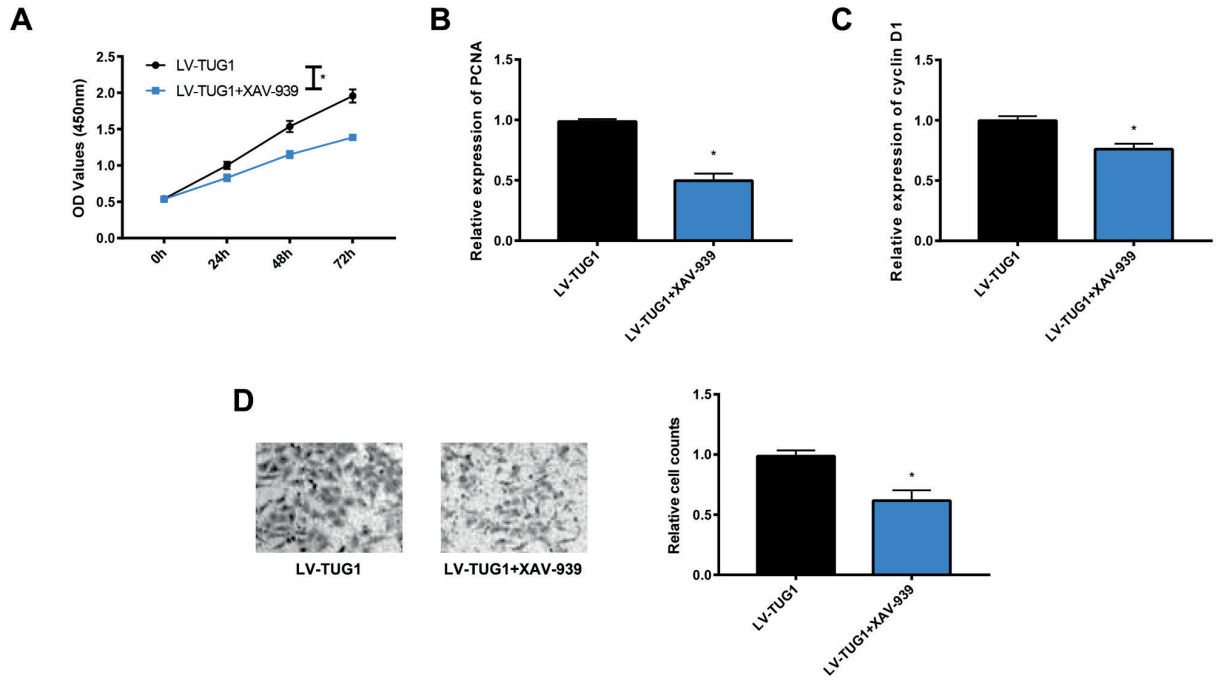

Figure 4. TUG1-induced cell proliferation was inhibited *via* Wnt pathway. ***A,*** XAV-939 partially reversed TUG1-induced cell proliferation. ***B, C,*** Upregulated PCNA (***B***) and cyclin D1 (***C***) were reversed by XAV-939 treatment. ***D,*** Increased migratory ability induced by TUG1 overexpression was rescued by XAV-939 treatment (20×).

sequently, accumulated nuclear β-catenin induces target genes in Wnt pathway, thus inhibiting apoptosis and stimulating proliferation. Activated Wnt pathway transmits signals to the downstream cells *via* binding to the corresponding receptors. In our study, overexpressed TUG1 upregulated protein expressions of β-catenin and c-Myc in HUVECs, suggesting the activation of Wnt pathway.

Conclusions

We showed that overexpressed TUG1 stimulates proliferation and migration of endothelial cells *via* Wnt pathway, thereby promoting the occurrence and progression of diabetic atherosclerosis.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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