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LncRNA MALAT1 affects high glucose-induced endothelial cell proliferation, apoptosis, migration and angiogenesis by regulating the PI3K/Akt signaling pathway

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Abstract. – OBJECTIVE: To investigate the effects of long non-coding ribonucleic acid (lncRNA) metastasis-associated lung adenocarcinoma transcript 1 (MALAT1) on the high glucose-induced proliferation, apoptosis, migration and angiogenesis of endothelial cells and its potential mechanism.

MATERIALS AND METHODS: Human umbilical vein endothelial cells (HUVECs) were divided into 3 groups, including control group (medium with 5.5 mmol/L glucose), high glucose group (HG group, medium with 33.5 mmol/L glucose) and lncRNA MALAT1 knockdown group [HG + MALAT1 small interfering RNA (siRNA) group, medium with 33.5 mmol/L glucose]. Cell Counting Kit-8 (CCK-8) assay was performed to observe the proliferation of HUVECs in each group at different time points. Meanwhile, the wound-healing assay was applied to detect the migratory ability of HUVECs in each group at 0 h and 24 h. The apoptosis rate of each group of cells was measured by means of flow cytometry, and the expression of Bcl-2-associated X protein (Bax) was detected via immunofluorescence at the same time. In addition, the amount of neovascularization in each group of cells was observed through the tube formation assay. Finally, Western blotting was utilized to determine the expression level of proteins in phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathway in each group of cells.

RESULTS: Compared with that in the control group, the expression level of lncRNA MALAT1 in the HG group was elevated markedly ($p<0.05$). The proliferative capacity of HUVECs in the HG group was increased notably after knocking down lncRNA MALAT1 with siRNA ($p<0.05$). According to wound-healing assay, the knockdown of lncRNA MALAT1 could prominently reverse the declined HUVECs migratory ability induced by high glucose ($p<0.05$). Flow cytometry results manifested that the apoptosis level of HUVECs in the HG group was increased markedly, but inhibition on lncRNA MALAT1 could lower the apoptosis level evidently ($p<0.05$). The results of immunofluorescence showed that the expression of Bax in the HG + MALAT1 siRNA group was remarkably lower than that in the HG group ($p<0.05$). It was revealed in Western blotting that the knockdown of lncRNA MALAT1 could reverse the inhibition of high glucose on the PI3K/Akt signaling pathway in HUVECs ($p<0.05$).

CONCLUSIONS: Inhibiting lncRNA MALAT1 can promote endothelial cell proliferation, migration and angiogenesis and repress endothelial cell apoptosis simultaneously, whose mechanism may be related to the activation of the PI3K/Akt signaling pathway.

Key Words:
LncRNA MALAT1, PI3K/Akt, Endothelial cells, Proliferation, Apoptosis, Angiogenesis.

Introduction

Diabetes mellitus, a metabolic disease characterized by hyperglycemia and insulin resistance, is a leading cause of death around the world[1]. Currently, the global incidence rate of diabetes is rising year by year, and it is estimated that it will be increased sharply to 4.4% in 2030 compared with that in 2000 (2.8%)[2]. The constant increase in the incidence rate of the disease is caused by many factors, including rapid industrialization, urbanization and poor lifestyle[3]. The vascular complications of diabetes are the primary causes

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of death and disability of the patients, and the injuries induced thereby may affect the heart, brain, kidney, fundus and other vital organs[4]. Hyperglycemia plays a central role in the occurrence and development of vascular complications, acting as the main reason for the death of diabetic patients[5]. It can lead to vascular endothelial injury, thereby resulting in the loss of endothelial function[6]. The mechanism of diabetes-induced endothelial dysfunction is complex. Although it has been reported that high glucose-induced endothelial injury has close correlations with apoptosis, oxidative stress and abnormal glucose metabolism, the precise mechanism has not been illuminated yet[7].

Long non-coding ribonucleic acids (lncRNAs) refer to long RNA molecules with a transcript length of over 200 nucleotides[8]. LncRNAs themselves cannot encode corresponding proteins in cells, but they can regulate the expression of corresponding target genes at (post-) transcriptional level, epigenetic modification and other levels, ultimately affecting the occurrence and development of diseases[9]. Hu et al[10] have revealed that lncRNA metastasis-associated lung adenocarcinoma transcript 1 (MALAT1), as a member of lncRNAs and a competing endogenous RNA, is capable of regulating the expression of MCL-1 protein by competitive binding to micro RNA (miR)-363-3p in gallbladder cancer, finally influencing the occurrence and development of gallbladder cancer. The expression level of lncRNA MALAT1 is elevated notably in diabetic nephropathy, and the function of podocytes is improved markedly after further inhibition on the expression of lncRNA MALAT1[11]. However, the action and mechanism of lncRNA MALAT1 in high glucose-induced vascular endothelial injury have not been reported yet.

In this work, by virtue of human umbilical vein endothelial cells (HUVECs), small interfering RNA (siRNA) was utilized to construct cell lines with lncRNA MALAT1 knockdown, and the effects of lncRNA MALAT1 knockdown on HUVECs proliferation, apoptosis, migration and angiogenesis, as well as the mechanism, were further investigated.

Materials and Methods

Materials

HUVECs were purchased from the Institute of Microbiology, Chinese Academy of Sciences. Phosphate-Buffered Saline (PBS; Gibco, Grand Island, NY, USA), trypsin, fetal bovine serum (FBS; Gibco, Grand Island, NY, USA) and Roswell Park Memorial Institute-1640 medium were bought from Gibco (RPMI-1640; Grand Island, NY, USA). SiRNA was purchased from Guge Bio-Technology Co., Ltd. (Nanjing, China). The HUVECs were cultured in a cell incubator with 5% CO_2 at 37°C, and they were subjected to digestion and subculture with 0.25% trypsin-ethylenediamine tetraacetic acid (EDTA) after the culture dish was covered with the cells.

LncRNA MALAT1 Knockdown

The HUVECs in the logarithmic growth phase were immediately digested and inoculated in a cell culture dish with a 6-well plate. 12 h later (fusion: 60-80%), the complete medium was discarded, and the cells were washed with serum-free medium 2-3 times, followed by starvation in the incubator for synchronous growth. Next, the MALAT1 siRNA was dissolved in RNase-free deionized water to prepare a solution with a final concentration of 20 μmol/L. After that, the HUVECs were divided into control group (medium with 5.5 mmol/L glucose), high glucose group (HG group, medium with 33.5 mmol/L glucose) and lncRNA MALAT1 knockdown group (HG + MALAT1 siRNA group, medium with 33.5 mmol/L glucose). The prepared transfection solution was added into the well plates in sequence, mixed sufficiently and cultured for another 6 h, and then the solution was replaced with the complete medium again. The base sequences of MALAT1 siRNA are as follows: Forward: 5'-ACGATCGTAGCTAGGCGTAGTC-3', Reverse: 5'-AGCTGATCGTAGCTAGTTT-3'.

Detection of Expressions of Relevant Genes via Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

(1) The total RNA in each group of cells was extracted using TRIzol reagent method (Invitrogen, Carlsbad, CA, USA), whose concentration and purity were measured by an ultraviolet spectrophotometer, and the RNA with A_{260}/A_{280}=1.8-2.0 was eligible for use. (2) The mRNA was synthesized into complementary deoxyribonucleic acid (cDNA) through reverse transcription, and the cDNA was stored in a refrigerator at -80°C. (3) RT-PCR system: 2.5 μL 10× Buffer, 2 μL cDNA, 0.25 μL forward primer (20 μmol/L), 0.25 μL reverse primer (20 μmol/L), 0.5 μL dNTPs (10 mmol/L), 0.5 μL Taq polymerase (2×10^6 U/L) and 19 μL ddH_2O. The Reverse Transcription-Polymerase Chain Reaction (RT-PCR) amplification system was the same.

Wound Healing Assay

The cells in the logarithmic growth phase were inoculated into 96-well plates, ensuring that the number of cells in each plate was about 5×10^4. At 24 h, a wound was scratched in the middle of the well plate using the pipette tip, the shedding cells were washed away with PBS, and the medium was replaced with serum-free medium. Then, the cell migration was photographed and recorded under a high power microscope at 24 h.

Detection of Cell Apoptosis via Flow Cytometry

The HUVECs in the logarithmic growth phase were fetched, digested, prepared into suspension with 0.25% trypsin-EDTA and seeded into the medium with a 6-well plate. Loading was performed according to the operation steps in the Annexin V-fluorescein isothiocyanate (FITC) Propidium Iodide (PI) apoptosis detection kit (Beyotime, Shanghai, China), and the apoptosis rate was calculated.

Immunofluorescent Staining of Cells

First, the HUVECs were seeded into a 24-well plate containing cell slides, which, 24 h later, were harvested for immunofluorescent staining. Then, the cells were fixed with formaldehyde, and the cell membrane was destroyed using 0.2% Triton and sealed in goat serum (diluted at 1:200 with PBS). Subsequently, the anti-Bcl-2-associated X protein (Bax) antibody was diluted at 1:200 with PBS and incubated at 4°C overnight, followed by washing with PBS on a shaking table four times. Next, FITC-secondary antibody was added and incubated at 37°C for 1 h, followed by visualization of cell nuclei with 4',6-diamidino-2-phenylindole (DAPI). After color development, 6 samples were randomly selected from each group, and 5 fields of vision were randomly selected from each sample for photography under a fluorescence microscope (200×).

Vascularization Assay

The HUVECs were resuspended in the RPMI-1640 medium with high glucose and 2% FBS after digestion with trypsin, which was inoculated in the 24-well plate coated with Matrigel (BD Biosciences, Franklin Lakes, NJ, USA), with 5×10^5 cells in each well, followed by culture for another 6 h and photography under an inverted phase-contrast microscope.

Cell Counting Kit-8 (CCK-8) Proliferation Assay

The cells in the logarithmic growth phase in each group were inoculated into the 96-well plate and cultured in the incubator with 5% CO_2 at 37°C for 0, 24, 48, 72 and 96 h, followed by discarding of the medium and preparation of developing reagent in the dark using RPMI-1640 medium and Cell Counting Kit-8 (10:1) (CCK-8; Dojindo Laboratories, Kumamoto, Japan). After that, 110 μL of developing reagent was added into each well of the 96-well plate for incubation in the incubator at 37°C for 2 h, and the absorbance at 540 nm in each group was detected by the ultraviolet spectrophotometer.

Statistical Analysis

All the data were analyzed using Statistical Product and Service Solutions (SPSS) 22.0 software (IBM, Armonk, NY, USA), the measurement data were presented as mean ± standard deviation, and *t*-test was performed for comparison of data between the two groups. $p<0.05$ suggested that the difference was statistically significant.

Results

Construction of HUVECs With LncRNA MALAT1 Knockdown

First of all, the expression level of lncRNA MALAT1 in the HG group and control group was measured by means of RT-PCR. It was shown that the expression level of lncRNA MALAT1 in the HUVECs in the HG group was remarkably higher than that in the control group ($p<0.05$). After further knockdown with siRNA, the expression level of lncRNA MALAT1 in the HUVECs in HG + MALAT1 siRNA group was lowered evidently ($p<0.05$), indicating that the HUVECs with lncRNA MALAT1 knockdown were constructed successfully (Figure 1).

Identification of Proliferative Capacity of Endothelial Cells in Each Group

The proliferation of endothelial cells in each group was detected using the CCK-8 kit, and the results manifested that the proliferative capacity of the HUVECs in the HG group declined markedly at 24, 48, 72 and 96 h compared with that in the control group ($p<0.05$). However, the inhibitory effect of high glucose on the HUVECs was weakened significantly after the inhibition on lncRNA MALAT1 expression ($p<0.05$) (Figure 2).

Identification of Migratory Ability of Endothelial Cells in Each Group

In addition, the migratory ability of each group cells was determined by the wound-healing assay. It was indicated that (Figure 3) the wound healing rates

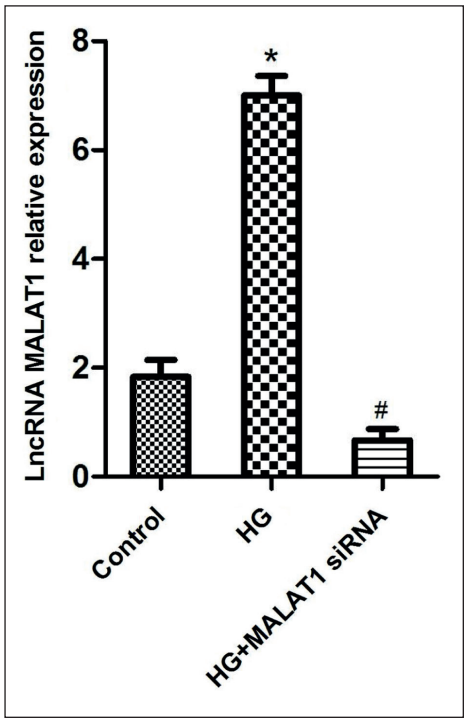

Figure 1. Construction of HUVECs with lncRNA MALAT1 knockdown. Control: control group, HG: high glucose group, HG + MALAT1 siRNA group: lncRNA MALAT1 knockdown group, *p<0.05 *vs.* Control group, #p<0.05 *vs.* HG group, with a statistical difference.

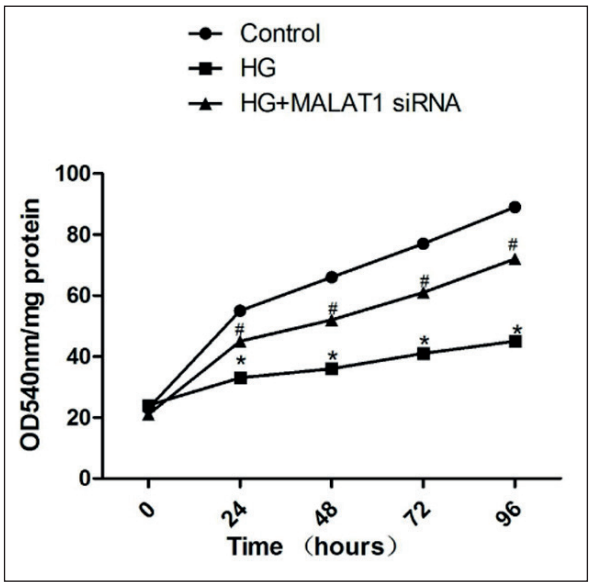

Figure 2. Effects of lncRNA MALAT1 knockdown on proliferation of HUVECs. Control: control group, HG: high glucose group, HG + MALAT1 siRNA group: lncRNA MALAT1 knockdown group, *p<0.05 *vs.* Control group, #p<0.05 *vs.* HG group, with a statistical difference.

of HUVECs at 24 h in the control group, HG group and HG + MALAT1 siRNA group were (84.82±2.98)% *vs.* (44.82±1.31)% *vs.* (67.64±3.49)%, with statistically significant differences among groups. Therefore, it is believed that knocking down lncRNA MALAT1 can enhance the migratory ability of HUVECs in high glucose conditions.

Detection of Apoptosis Level of Endothelial Cells in Each Group

Meanwhile, the apoptosis level of each group of cells was measured *via* flow cytometry. According to the results (Figure 4), in comparison

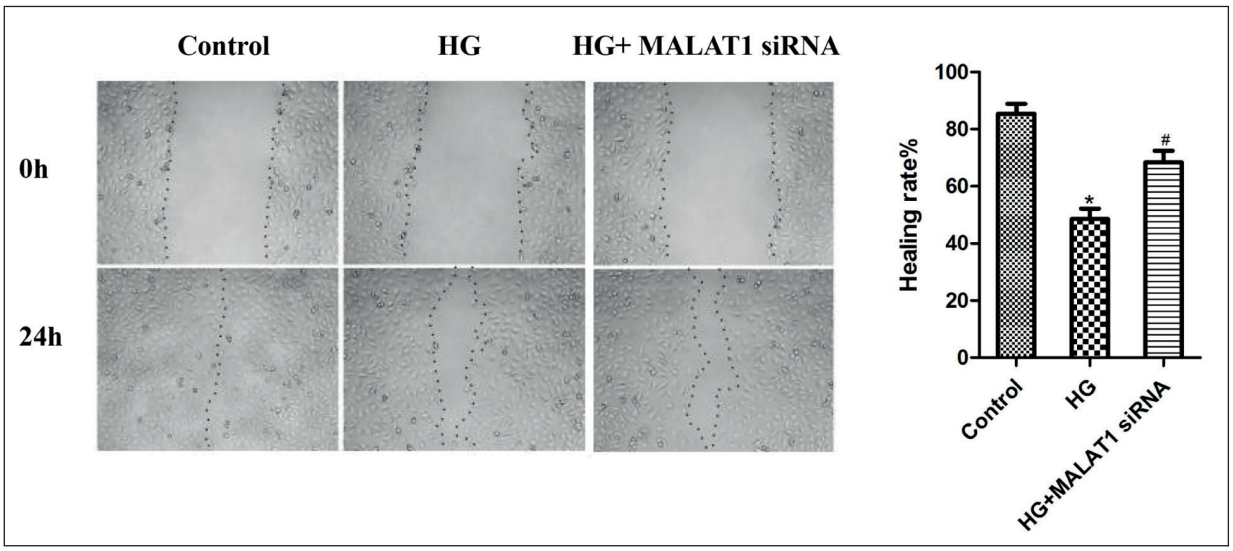

Figure 3. Effects of lncRNA MALAT1 knockdown on migration of HUVECs. Control: control group, HG: high glucose group, HG + MALAT1 siRNA group: lncRNA MALAT1 knockdown group, *p<0.05 *vs.* Control group, #p<0.05 *vs.* HG group, with a statistical difference (magnification: 10×).

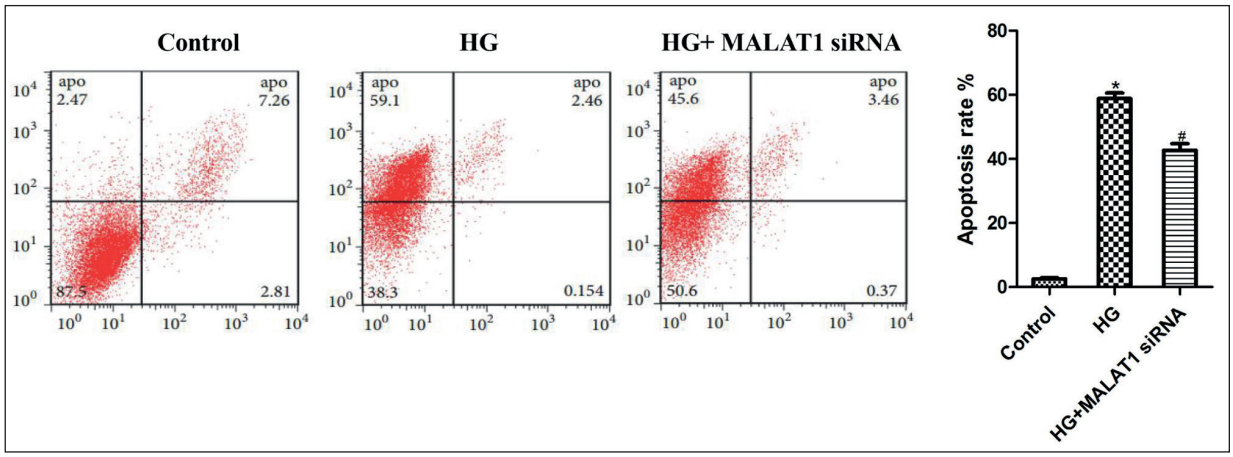

Figure 4. Effects of lncRNA MALAT1 knockdown on apoptosis of HUVECs. Control: control group, HG: high glucose group, HG + MALAT1 siRNA group: lncRNA MALAT1 knockdown group, *p<0.05 *vs.* Control group, #p<0.05 *vs.* HG group, with a statistical difference.

with that in the control group, high glucose stimulation could increase the cell apoptosis level prominently (p<0.05), but the pro-apoptotic effect of high glucose on the HUVECs was attenuated notably after repressing the expression of lncRNA MALAT1 (p<0.05).

Immunofluorescent Staining for Bax in Each Group of Endothelial Cells

To further investigate the effects of lncRNA MALAT1 on the apoptosis of HUVECs, the expression level of the pro-apoptotic protein, Bax, was detected by immunofluorescent staining. The results revealed that (Figure 5) the expression level of Bax in the HUVECs was elevated remark-

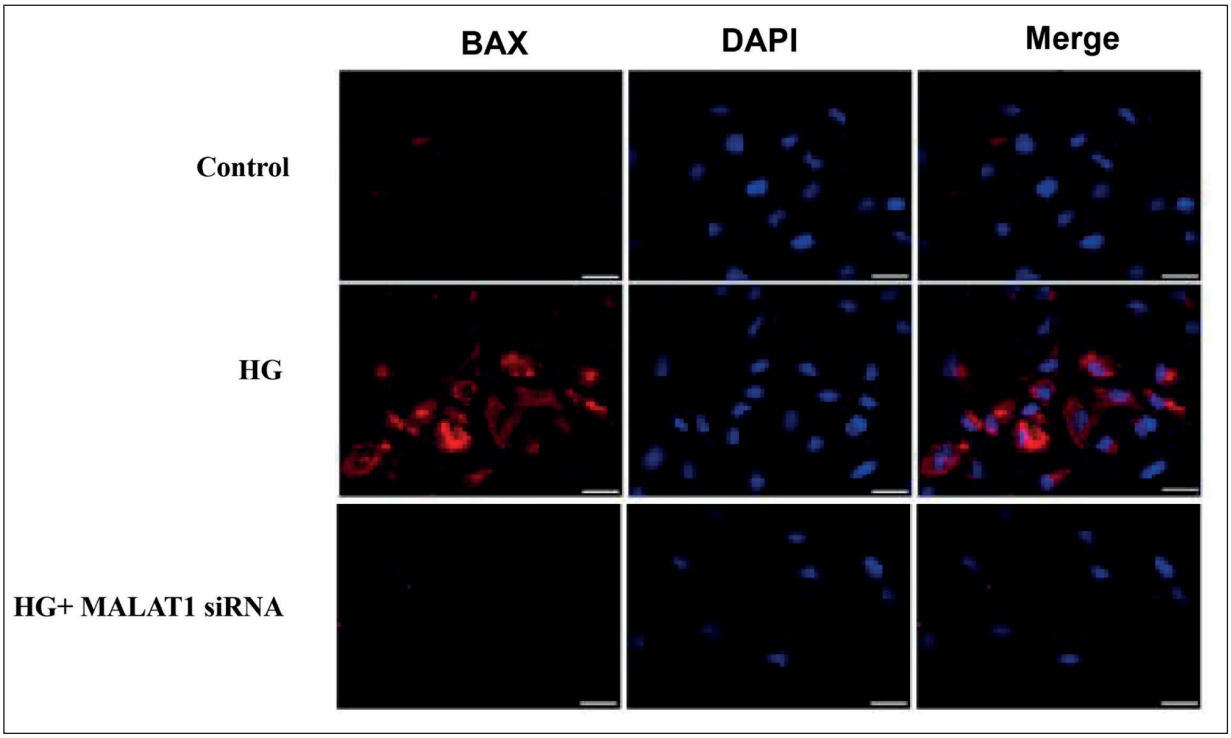

Figure 5. Effects of lncRNA MALAT1 knockdown on Bax expression in HUVECs. Control: control group, HG: high glucose group, HG + MALAT1 siRNA group: lncRNA MALAT1 knockdown group (magnification: 100×).

ably in the HG group, while it declined after lncRNA MALAT1 knockdown.

Effects of LncRNA MALAT1 Knockdown on Angiogenesis of HUVECs in High Glucose Condition

Specific Matrigel was applied to determine the angiogenesis ability of the HUVECs in each group. It was indicated that (Figure 6) the angiogenesis ability of the HUVECs was suppressed evidently in high glucose conditions ($p<0.05$), and knocking down lncRNA MALAT1 could improve the angiogenesis ability of the HUVECs prominently ($p<0.05$).

Effects of LncRNA MALAT1 Knockdown on Phosphatidylinositol 3-Kinase (PI3K)/Akt Signaling Pathway

Finally, Western blotting was adopted to detect the expression levels of PI3K, phosphorylated Akt (p-Akt) and total Akt (t-Akt) in each group of cells. According to the results (Figure 7), the protein expressions of PI3K and p-Akt in the HUVECs were inhibited markedly by the stimulation of high glucose ($p<0.05$), but the PI3K/Akt signaling pathway can be activated by repressing lncRNA MALAT1 ($p<0.05$).

Discussion

Diabetes mellitus is an important risk factor of cardiovascular disease[11]. The endothelial cells are considered to be involved in the key pathogenesis of its vascular complications. A growing amount of evidence demonstrates that hyperglycemia can trigger vascular endothelial dysfunction in the diabetic patients[12]. It has been discovered that high glucose-induced endothelial cell apoptosis plays a role in the pathogenesis accelerated by diabetes-associated atherosclerosis[13]. Besides, the medium with high glucose can also lead to endothelial cell apoptosis, and increasingly more evidence has revealed that preventing endothelial cell apoptosis can ameliorate angiogenesis and endothelial function[14]. Moreover, Fromer et al[15] have manifested that high glucose is capable of suppressing the proliferation of various endothelial cells (including HUVECs, bovine retinal endothelial cells and human dermal microvascular endothelial cells), thus decreasing local microvascular reconstruction by inhibiting the proliferation of endothelial cells and further aggravating ischemia and hypoxia in the region. Vascular reconstruction acts as a double-edged sword in the diabetic complications. On the one hand, excessive vascular reconstruction can result in excess amounts of retinal and renal vessels, thereby easily leading to rupture and bleeding of new vessels due to their fragility[16]. On the other hand, vascular reconstruction can accelerate the wound healing, improve cardiac collateral circulation and reduce and suppress postoperative rejection at the same time[17]. As a result, improving the proliferation, apoptosis, migration and angiogenesis of endothelial cells is of great significance for the vascular complications of diabetes.

PI3K is able to regulate a variety of crucial vital activities of the cells by activating downstream serine/threonine kinase (Akt)[18]. It is re-

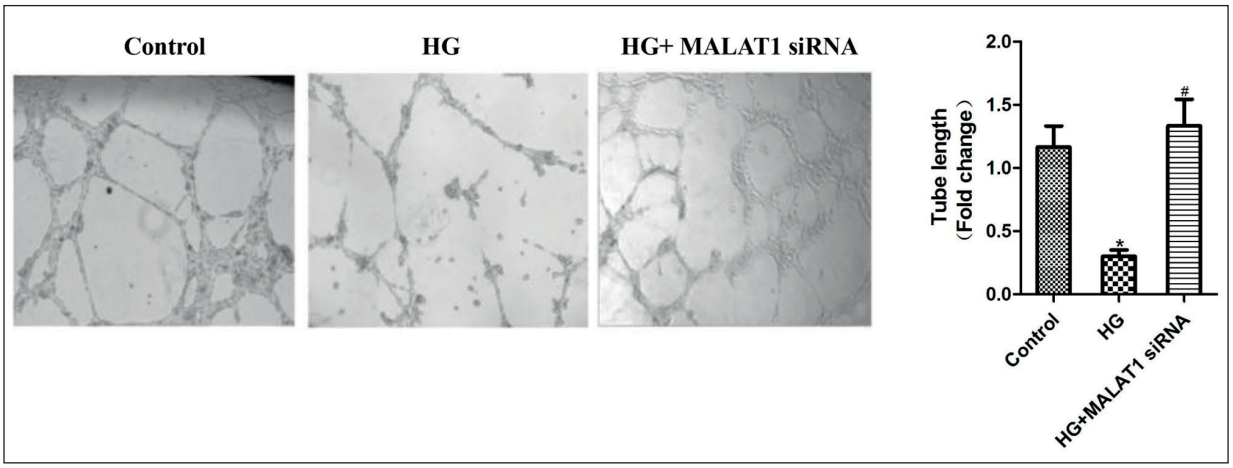

Figure 6. Effects of lncRNA MALAT1 knockdown on angiogenesis of HUVECs. Control: control group, HG: high glucose group, HG + MALAT1 siRNA group: lncRNA MALAT1 knockdown group, *$p<0.05$ *vs.* Control group, #$p<0.05$ *vs.* HG group, with a statistical difference (magnification: 200×).

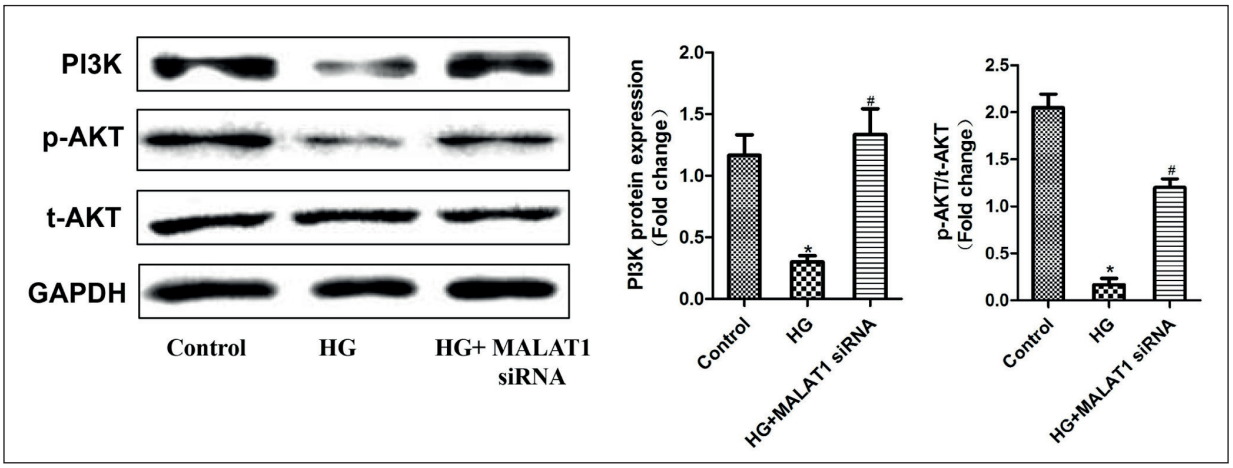

Figure 7. Effects of lncRNA MALAT1 knockdown on the PI3K/Akt signaling pathway of HUVECs. Control: control group, HG: high glucose group, HG + MALAT1 siRNA group: lncRNA MALAT1 knockdown group, *p<0.05 *vs.* Control group, #p<0.05 *vs.* HG group, with a statistical difference.

ported[19] that the Akt activation in endothelial cells can promote the survival of endothelial cells and activate endothelial nitric oxide (NO) synthase, thus generating NO. Liu et al[20] have also pointed out that the PI3K/Akt/NO pathway plays a vital role in preventing high glucose-induced endothelial injury. In this research, it was discovered for the first time that the expression level of lncRNA MALAT1 in those endothelial cells was elevated markedly when the HUVECs were stimulated by high glucose. Furthermore, the lncRNA MALAT1 in the HUVECs was knocked down using siRNA, and it was indicated that the proliferative capacity of the endothelial cells was increased significantly when the HUVECs were cultured with high glucose again. In addition, the results of wound healing assay revealed that the inhibition on lncRNA MALAT1 could strengthen the migratory ability of the HUVECs. Moreover, it was found that the apoptosis level of HUVECs with lncRNA MALAT1 knockdown was decreased compared with that of HUVECs without lncRNA MALAT1 knockdown. The protective effect of lncRNA MALAT1 siRNA on the HUVECs may be associated with the activation of the PI3K/Akt signaling pathway. Actually, previous studies have manifested that multiple lncRNAs, miRNAs or natural compounds can protect high glucose-induced HUVECs injury. For example, deoxynojirimycin is able to postpone the senescence of endothelial cells in high glucose conditions by inhibiting the production of monocyte adhesion, NF-κB signaling pathway and reactive oxygen species[21]. Ginkgolide A can reduce the high glucose-induced inflammatory responses in HUVECs by activating the STAT3 pathway[22]. Besides, Li et als[23] have discovered that the miR-221 level in HUVECs is elevated notably after high glucose induction, which can further repress the proliferation of HUVECs and damage their migratory ability simultaneously. All these findings suggest that increasing the proliferative and migratory ability of endothelial cells and inhibiting the apoptosis level have significance in improving the endothelial dysfunction. Nevertheless, there are still certain limitations in this research: 1) no animal experiments were designed for verification, 2) the Akt or PI3K was not repressed to observe the effects of lncRNA MALAT1 knockdown on the endothelial function, and 3) the direct targets on which the lncRNA MALAT1 acted were not found.

Conclusions

It is discovered in this work, for the first time, that inhibiting lncRNA MALAT1 can ameliorate the endothelial dysfunction in high glucose conditions, whose potential mechanism may be correlated with the activation of the PI3K/Akt signaling pathway.

Conflict of Interests

The authors declare that they have no conflict of interest.

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