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ANGPTL4 participates in gestational diabetes mellitus via regulating Akt pathway

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Abstract. – **OBJECTIVE:** To explore ANGPTL4 expressions in patients with gestational diabetes mellitus (GDM) and its underlying mechanism.

PATIENTS AND METHODS: We first detected serum expressions of ANGPTL4 in GDM patients and healthy pregnancies. Subsequently, effects of ANGPTL4 knockdown on apoptosis, proliferation, and cell cycle in 3T3-L1 cells were determined, respectively. Effects of ANGPTL4 on glucose uptake and adipocyte differentiation were also evaluated, respectively. The cytokine secretion in adipocytes transfected with sh-ANGPTL4 was detected by quantitative Reverse Transcriptase-Polymerase Chain Reaction (qRT-PCR) and enzyme-linked immunosorbent assay (ELISA). Furthermore, effects of ANGPTL4 knockdown on NF-kB and Akt pathway were detected by Western blot.

RESULTS: ANGPTL4 was down-regulated in serum of GDM patients. *In vitro* experiments suggested that down-regulated ANGPTL4 inhibited apoptosis and promoted proliferation of 3T3-L1 cells. Meanwhile, down-regulated ANGPTL4 significantly inhibited glucose uptake and Akt pathway. However, ANGPTL4 expression did not affect cell cycle and adipocyte differentiation. Detection of inflammatory cytokines suggested that down-regulated ANGPTL4 resulted in increased expressions of inflammatory cytokines and activation of NF-kB pathway.

CONCLUSIONS: ANGPTL4 is down-regulated in GDM and may participate in the GDM development by promoting insulin resistance and secretion of inflammatory cytokines.

Key Words

ANGPTL4, Inflammatory factors, Insulin resistance, Gestational diabetes mellitus.

Introduction

Gestational diabetes mellitus (GDM) is a condition in which a woman develops high blood sugar levels during pregnancy. The incidence of GDM reported in different countries is 2%-12%, which has been risen annually[1]. More than 80% of pregnant women with diabetes mellitus are diagnosed as GDM. GDM significantly increases morbidity and mortality of pregnancies and fetuses, which seriously leads to a long-term impact on GDM mothers and their offspring[2,3]. The incidence of type 2 diabetes mellitus in GDM mothers within 20 years after pregnancy is up to 40%. More importantly, the risks of obesity and diabetes are greatly increased in their offspring[4]. Therefore, it is of great importance to explore the underlying mechanism of GDM.

Recently, great progress has been made in the investigation of the pathogenesis of GDM. So far, adipocyte dysfunction, changes in placenta and hormones, inflammation and oxidative stress are considered to be the possible pathogenesis of GDM[5]. Adipokines, including leptin, adiponectin, resistin, visfatin, etc., are closely related to insulin resistance (IR)[6]. Recent studies have found that many pregnancies experience abnormal secretion of adipokines, which exerts a crucial role in the occurrence, development, and prognosis of IR and GDM. Previous studies[7,8] have specifically described changes and effects of leptin and adiponectin on GDM. Effects of other adipokines such as resistin, visfatin, RBP4, and vaspin on pregnancy complications remain controversial[9,10]. Further studies are urgently needed to explore the possible interactions between adipokines and inflammatory factors.

The human ANGPTL4 gene is located on 19p131 with 7 exons and 6 introns. The full-length complementary Deoxyribose Nucleic Acid (cDNA) of human ANGPTL4 is 1943 bp. Additionally, the open reading frame of ANGPTL4

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contains 1218 bp and encodes 406 amino acids. ANGPTL4 protein is mainly expressed in adipose tissue and embryo with organ specificity[11]. ANGPTL4 is also found to be expressed in lung, kidney, liver, pituitary, skeletal muscle, and heart[12,13]. Currently, there is a controversy about the effect of ANGPTL4 on diabetes. Some scholars believed that ANGPTL4 can improve IR by increasing insulin sensitivity[14]. However, Mandard et al[15] found that overexpression of ANGPTL4 decreased glucose tolerance. Therefore, the relationship between ANGPTL4 and diabetes still requires to be further confirmed. In the present study, we explored ANGPTL4 expressions in GDM patients and its underlying mechanism.

Patients and Methods

Patients

Patients diagnosed as GDM in Obstetrics Department, Jining No. 1 People's Hospital from June 2015 to May 2017 were selected as GDM group. Healthy pregnancies in the same period were selected as control group. The study was approved by the Ethics Committee of Jining No. 1 People's Hospital and all subjects signed the informed consent. Fasting venous samples before natural delivery or caesarean surgery were collected and centrifuged, followed by preservation in liquid nitrogen. For diagnosing GDM, all pregnancies at 24-30 weeks of gestation were screened for glucose test. If plasma glucose was over 7.8 mmol/L 1 h after breakfast, a 75 g oral glucose tolerance test (OGTT) was required after one-week normal diet[1].

Cell Culture

Mouse 3T3-L1 cells were purchased from Cell Bank of Chinese Academy of Sciences (Shanghai, China). 3T3-L1 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) medium supplemented with 10% fetal bovine serum (FBS, Gibco, Rockville, MD, USA), 100 U/mL penicillin and 100 μg/mL streptomycin, and maintained in a 5% CO_2 incubator at 37°C. Fresh medium was replaced every 2 to 3 days. The adipocyte differentiation procedure was as follows: cells were cultured in DMEM medium containing 10% FBS, 1 μM dexamethasone, 0.5 mM IBMX, and 10 μg/ml insulin for 4 days. Normal medium containing 10 μg/mL insulin was then changed for 3-day incubation. Cytokine stimulation procedure was as follows: at 10 days differentiation, cells were starved in serum-free medium for 16 h. Cells were then cultured for 24 h after adding 10 ng/mL IL-1β in the medium. Expressions of inflammatory cytokines were finally detected.

Cell Transfection

Cells in LV-Vector group and LV-shANGPTL4 group were transfected with empty vector and lentivirus containing ANGPTL4 shRNA, respectively. Cell transfection was performed by Lipofectamine 2000 (Thermo Fisher Scientific, Inc. Waltham, MA, USA) based on the manufacturer's recommendations. Briefly, cells were seeded into a 6-well plate and then transfected with the above-mentioned plasmids when cell confluence was up to 60%. Lentivirus used in the study was purchased from GenePharm (Shanghai, China).

Cell Counting Kit-8 (CCK-8) Assay

Transfected cells were collected and seeded into a 96-well plate at a dose of 5×10^3/mL. After 24 h-inoculation, 10 μL of CCK-8 (Dojindo, Kumamoto, Japan) solution was added into each well at 0 h, 24 h, 48 h, and 72 h, respectively. The absorbance (OD) values at the wavelength of 450 nm were accessed with a microplate reader.

Cell Apoptosis

Transfected cells were digested with EDTA-free trypsin and washed with phosphate-buffered saline (PBS). 100 μL of 1×binding buffer was added. Subsequently, 5 μL of Annexin V-FITC was added and maintained at room temperature without light for 10 min. 400 μL of 1×binding buffer was added for gentle mixture. The apoptosis rate was analyzed by flow cytometry.

Cell Cycle

Transfected cells were harvested and prepared for cell suspension. Cells were fixed with ice-cold 70% ethanol overnight. For cell cycle assay, cells were centrifuged, washed twice with PBS and incubated with 150 μL of propidium iodide (PI) in the dark for 30 min. Finally, the specific distribution of cell cycle was determined by analyzing 10000 events by flow cytometry.

Oil Red O Staining

Cells were seeded into a 6-well plate with preplaced slices, followed by cell differentiation for 6 days. For oil red O staining, cells were fixed with 4% formaldehyde and washed twice with 60% isopropanol. Fixed cells were incubated with oil red O solution at room temperature for 30 min. Images of cells were captured with an inverted microscope.

Enzyme-Linked Immunosorbent Assay (ELISA)

Samples were added in the coated well for 30-min incubation. Thereafter, enzyme-labeled antibody was added for culturing at 37°C. Cells were washed and stained for 15 min. Subsequently, 0.05 mL of sulfuric acid was added to terminate the reaction. OD value at the wavelength of 450 nm was measured on the ELISA detector.

Glucose Uptake

At 10 days differentiation, cells were washed with PBS and maintained at 37°C for 20 min after 0.5 mM 2-deoxy-D-[2,6–^{3}H] glucose (1.5 mCi/well, Moravek Biochemicals, CA, USA) was added into each well. Pre-cooled PBS was used to wash cells for terminating the reaction. Thereafter, cells were lysed in 200 µL of lysis buffer for scintillation counting with a liquid scintillation counter.

RNA Extraction and Quantitative Reverse Transcriptase-Polymerase Chain Reaction (qRT-PCR)

The mRNAs of cells were extracted by TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and then reversely transcribed to cDNAs. The reaction conditions were as follows: denaturation at 95°C for 1 min, followed by annealing at 95°C for 30 s, and extension at 60°C for 40 s, for a total of 40 cycles. Each sample was repeatedly performed for 3 times. Primers used in this study were as follows: ANGPTL4: F: GTCCACCGACCTCCCGTTA; R: CCTCATGGTCTAGGTGCTTGT; IRS1: F: ACAAACGCTTCTTCGTACTGC; R: AGTCAGCCCGCTTGTTGATG; IRS2: F: CGGTGAGTTCTACGGGTACAT; R: TCAGGGTGTATTCATCCAGCG; Akt1: F: AGCGACGTGGCTATTGTGAAG; R: TCAGGGTGTATTCATCCAGCG; FoxO1: F: CCAAGAGGTGAGTGCTTCCC;R:CTGTTGTTCAGACTCTCTCCCT; IL-6: F: ACTCACCTCTTCAGAACGAATTG; R: CCATCTTTGGAAGGTTCAGGTTG; MCP1: F: CTGCAAGAGACTTCCATCCAG; R: AGTGGTATAGACAGGTCTGTTGG.

Western Blot

The total protein of the transfected cells was extracted. The concentration of each protein sample was determined by a bicinchoninic acid (BCA) kit (Pierce, Rockford, IL, USA). Briefly, 50 µg of total protein was separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) under denaturing conditions and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). Membranes were blocked with 5% skimmed milk, followed by the incubation of specific primary antibodies overnight. Membranes were then incubated with the secondary antibody at room temperature for 1 h. Immunoreactive bands were exposed by enhanced chemiluminescence (ECL) method.

Statistical Analysis

ImageJ and Statistical Product and Service Solutions (SPSS19.0, IBM, Armonk, NY, USA) statistical software were used for data analysis. Measurement data were expressed as mean ± standard deviation ($\bar{x}\pm s$). Comparison of measurement data was conducted using *t*-test. $p<0.05$ was considered statistically significant.

Results

ANGPTL4 Expressions Were Decreased in Serum of GDM Patients

30 GDM patients and 30 healthy pregnancies were selected in this study. No significant difference in age was found (29.12 ± 3.67 years in GDM group *vs.* 28.92 ± 2.65 years in control group, $p>0.05$). However, BMI (kg/m^2) of GDM patients was significantly higher than that of control group (27.1 ± 1.4 in GDM group *vs.* 23.9 ± 2.0 in control group, $p<0.05$). Lower serum levels of ANGPTL4 were observed in GDM patients than those of healthy pregnancies (Figure 1A). For *in vitro* experiments, ANGPTL4 expression was remarkably decreased after cells were transfected with si-ANGPTL4 (Figure 1B). Knockdown of ANGPTL4 inhibited apoptosis in 3T3-L1 cells (Figure 1C). CCK-8 assay showed that the cell proliferation was increased in a time-dependent manner, which achieved the peak at 72 h after transfection (Figure 1D). ANGPTL4 knockdown, however, did not affect the cell cycle of adipocytes (Figure 1E). The above results suggested that abnormally expressed ANGPTL4 may be involved in the occurrence and development of GDM.

Down-Regulated ANGPTL4 Promoted IR

We next investigated effects of ANGPTL4 on glucose metabolism in adipocytes. ANGPTL4 knockdown significantly reduced glucose uptake capacity in GDM group compared to that of control group (Figure 2A), suggesting the IR occurrence. Moreover, ANGPTL4 knockdown remarkably increased FoxO1 expression, but did not affect expressions of IRS1, IRS2, and Akt1 (Figure 2B).

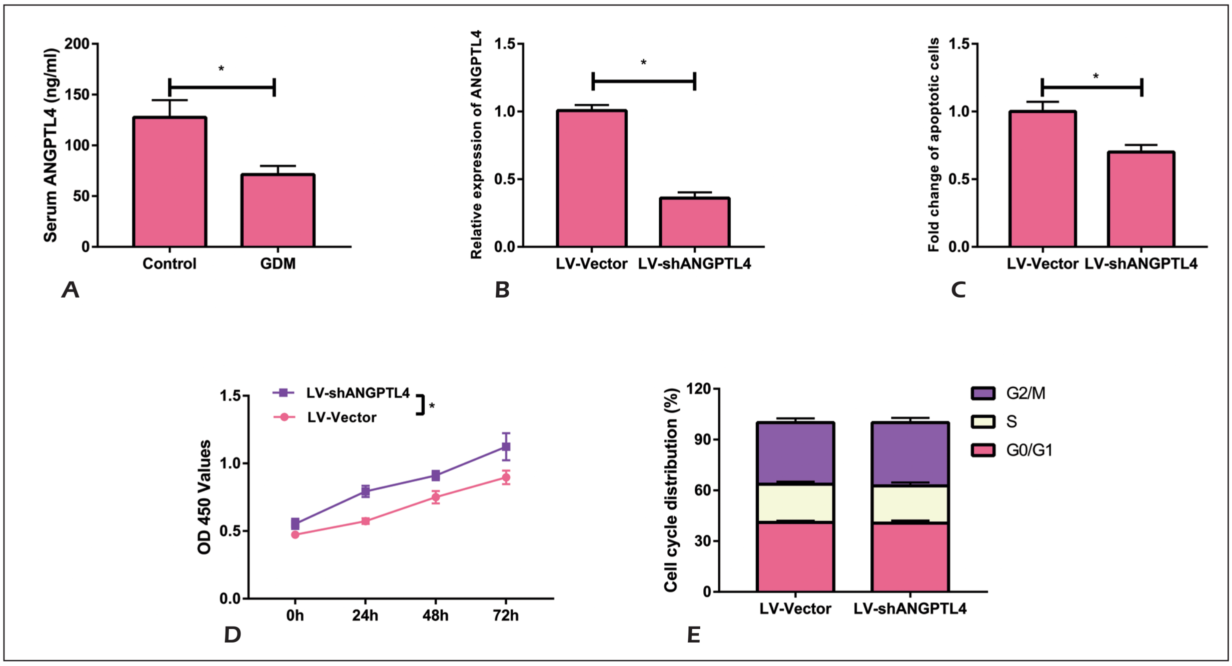

Figure 1. ANGPTL4 expressions were decreased in serum of GDM patients. **A**, Serum expressions of ANGPTL4 in GDM patients and healthy pregnancies; **B**, ANGPTL4 expression after lentivirus transfection; **C**, Cell apoptosis after ANGPTL4 knockdown; **D**, Cell proliferation after ANGPTL4 knockdown; **E**, Cell cycle after ANGPTL4 knockdown.

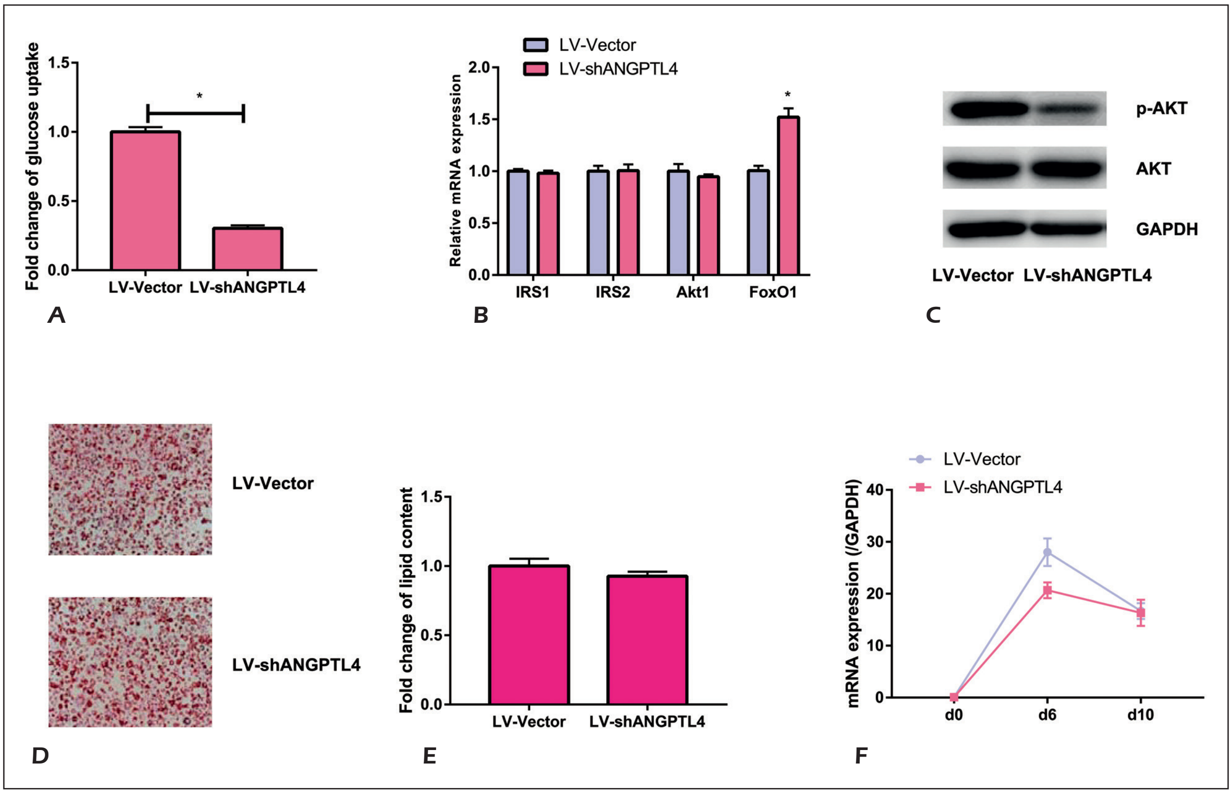

Figure 2. Down-regulated ANGPTL4 decreased the glucose uptake. **A**, Glucose uptake detection after ANGPTL4 knockdown; **B**, The mRNA levels of key genes in insulin signaling pathway after ANGPTL4 knockdown; **C**, Phosphorylation level of Akt detected by Western blot; **D-E**, Cell differentiation and mRNA level of Fabp4 after ANGPTL4 knockdown.

Previous authors have shown that FoxO1 is an important substrate for Akt. Akt phosphorylation is capable of regulating cell proliferation, metabolism, and other essential functions[16]. Therefore, we examined the phosphorylation level of Akt and found that ANGPTL4 knockdown inhibited Akt expression (Figure 2C). Furthermore, we speculated whether ANGPTL4 can affect adipocyte differentiation. Our results indicated that ANGPTL4 could not affect the adipocyte differentiation (Figure 2D). Although Fabp4 expression, a key gene during cell differentiation, was remarkably increased on the 6th day of adipocyte differentiation, no significant difference in Fabp4 expression was found between GDM group and control group (Figure 2E). These data indicated that ANGPTL4 may participate in GDM development *via* regulating insulin sensitivity of adipocytes by Akt pathway.

Down-regulated ANGPTL4 increased expressions of inflammatory cytokines

Scholars have suggested that IL-1β is upregulated in GDM patients[17]. Therefore, we investigated whether ANGPTL4 regulates expressions of inflammatory cytokines after IL-1β stimulation. Our data demonstrated that not only mRNA levels of IL-6 and MCP-1 (Figure 3A), but also IL-6 and TNF-α levels in the cell supernatant (Figure 3B) were remarkably increased after ANGPTL4 knockdown. Western blot results revealed that ANGPTL4 knockdown activated NF-kB pathway (Figure 3C). The above results indicated that ANGPTL4 knockdown participates in GDM development by promoting the secretion of inflammatory cytokines in adipocytes.

Discussion

Gestational diabetes mellitus (GDM) is a common and serious complication during pregnancy, which poses a great risk to mother and child. With changes of lifestyle and dietary structure, the incidence of GDM is rapidly increasing. Currently, insulin resistance (IR) and chronic low-grade inflammation are considered to be the major factors of GDM[3]. Adipose tissue is well recognized as its involvement in obesity, carbohydrate and lipid metabolism, and inflammation[18,19]. Dysfunctional adipocytes would lead to IR[20]. 3T3-L1 cell line derived from Swiss-3T3 mouse embryonic cells, has a single differentiation potential, which could spontaneously differentiate into mature adipocytes[21]. 3T3-L1 cell line can effectively simulate the function of living adipose tissues and adipocyte differentiation, which is a very ideal cell line for *in vitro* experiments[22]. So far, 3T3-L1 cells have been widely applied in experimental researches on glycometabolism and lipid metabolism.

Insulin signaling dysfunction is the main pathophysiological mechanism of IR. Abnormal signaling transduction after binding of insulin with its receptor results in decreased glucose uptake and hepatic glucose synthesis[23]. Akt is a member of the serine/threonine protein kinase family, the downstream of PI3K. Binding of insulin to the corresponding receptor activates the phosphorylation of Akt, which in turn transfers into the cytoplasm. Phosphorylated Akt further activates its downstream substrate cascade reaction, thus participating in glucose transport, gluconeogenesis inhibition, glycogen synthesis, glycolysis, protein

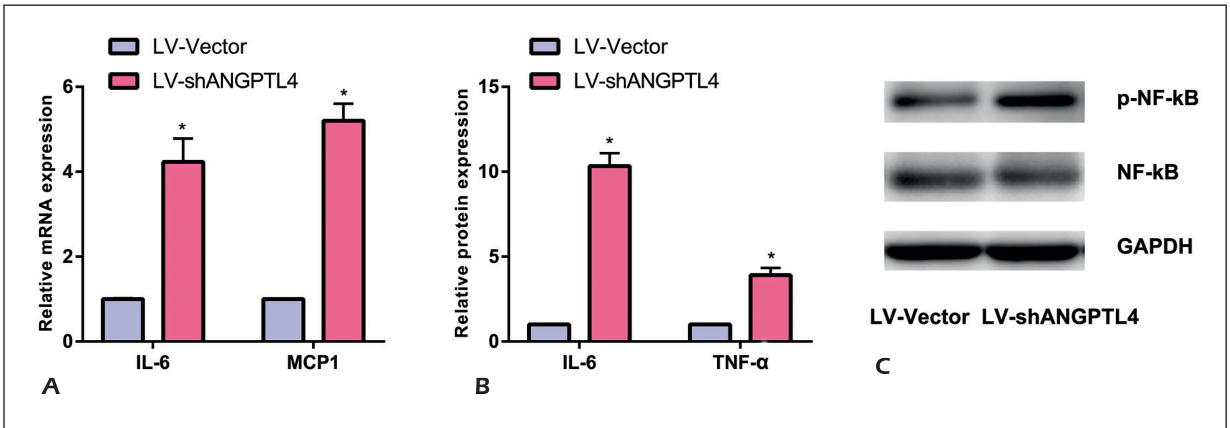

Figure 3. ANGPTL4 knockdown promoted expressions of inflammatory cytokines. **A**, The mRNA levels of inflammatory cytokines in adipocytes after ANGPTL4 knockdown; **B**, The protein levels of inflammatory cytokines in adipocytes after ANGPTL4 knockdown; **C**, Phosphorylation level of NF-kB after ANGPTL4 knockdown.

synthesis, and anti-apoptotic process[24]. FoxO1 is an important substrate of PI3K/Akt pathway, the activity of which is regulated by phosphorylation, ubiquitination, acetylation, and other post-translational modifications. Transcriptional activity of phosphorylated FoxO1 is inhibited in the nucleus, which negatively regulates insulin signaling pathway[16]. In this study, we found that ANGPTL4 knockdown significantly inhibits glucose uptake in adipocytes, which may be explained by the IR promotion *via* decreased Akt phosphorylation and increased FoxO1 expression.

NF-kB is a widespread transcription factor in almost all types of tissues and cells, which is involved in regulating inflammatory responses. Inflammatory cytokines are produced in adipocytes by oxidative stress, thereby activating the inflammatory signaling pathway. Previous researches suggested that activated NF-κB increases expressions of TNF-α and IL-6 in 3T3-L1 cells, thus exacerbating IR and chronic inflammation[25,26]. Our study also found that ANGPTL4 knockdown can stimulate the secretion of inflammatory cytokines *via* NF-kB pathway, including IL-6 and TNF-α.

Conclusions

We found that ANGPTL4 is down-regulated in GDM and may participate in the GDM development by promoting insulin resistance and secretion of inflammatory cytokines.

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Conflict of Interests

The authors declared no conflict of interest.

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