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MEG3 damages fetal endothelial function induced by gestational diabetes mellitus via AKT pathway

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Abstract. – OBJECTIVE: To explore the role of maternally-expressed gene 3 (MEG3) in fetal endothelial dysfunction induced by gestational diabetes mellitus (GDM) and its underlying mechanism.

PATIENTS AND METHODS: Human umbilical vein endothelial cells (HUVECs) were extracted from GDM pregnancies and normal pregnancies. Cell proliferation, apoptosis, migration and angiogenesis of HUVECs were detected by cell counting kit-8 (CCK-8), enzyme-linked immunosorbent assay (ELISA), wound healing and tube formation assay, respectively. MEG3 expressions in HUVECs extracted from 16 GDM pregnancies and 18 normal pregnancies were detected by quantitative real-time polymerase chain reaction (qRT-PCR). Besides, angiogenesis and MEG3 expression in HUVECs treated with glucose were detected, respectively. Proliferation, apoptosis, migration and angiogenesis were also detected after HUVECs were transfected with MEG3 lentivirus. Target genes of MEG3 were predicted by bioinformatics method and further verified by luciferase reporter gene assay. The protein expression of possible signaling pathway was detected by Western blot.

RESULTS: HUVEC cells extracted from GDM pregnancies presented increased apoptosis and decreased proliferation, migration and angiogenesis compared with those from healthy pregnancies. Meanwhile, MEG3 was overexpressed in HUVECs extracted from GDM pregnancies compared with that of healthy pregnancies. High dose of glucose treatment led to reduced angiogenesis and elevated MEG3 expression in HUVECs. MEG3 overexpression further promoted apoptosis, but inhibited proliferation, migration and angiogenesis of HUVECs. By bioinformatics and luciferase reporter gene assay, microRNA-370-3p was found to be the target gene of MEG3 and directly targeted on AFF1. Moreover, MEG3 overexpression led to downregulated microRNA-370-3p and upregulated AFF1 mainly through inhibiting PI3K/AKT pathway.

CONCLUSIONS: MEG3 is overexpressed in HUVECs extracted from GDM pregnancies. MEG3 damages fetal endothelial function through targeting microRNA-370-3p and AFF1 via PI3K/AKT pathway.

Key Words:
Gestational diabetes mellitus, Human umbilical vein endothelial cells, MEG3, MicroRNA-370-3p, PI3K/AKT.

Introduction

Gestational diabetes mellitus (GDM) is a condition in which a woman without diabetes develops high blood sugar levels during pregnancy[1]. GDM is one of the most common complications during pregnancy. Many studies have shown that both genetic and environmental factors may contribute to GDM. GDM severely threatens physical health of mothers and babies, and could lead to fetal hyperglycemia and increased risks of future cardiovascular diseases. Among them, destruction of endothelial function is an important factor of GDM pathogenesis[2-4].

Under normal circumstances, endothelium can inhibit contraction of vascular smooth muscle, the proliferation of smooth muscle cells, platelet aggregation, leukocyte adhesion and thrombosis. Endothelial dysfunction may lead to decreased secretion of vasodilator substances, increased release of vasoconstrictor substances, adhesion molecules and growth factors, thereafter promoting the occurrence and development of atherosclerosis. Among them, NO, endothelin-1 and E-selectin are specific cytokines secreted by endothelial cells. Functionally, they exert an important role in maintaining the normal function of endothelial cells. Under the high glucose en-

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vironment, imbalanced secretion of these cytokines participates in endothelial cell damage[5,6]. Non-coding RNAs are derived from the transcription of the genome, including microRNAs, snoRNAs, siRNAs, tRNAs, rRNAs, and lncRNAs (long non-coding RNAs)[7,8]. With the discovery of lncRNAs with specific functions, such as H19 and Xist, researches on non-coding RNA have been well recognized[9]. LncRNAs are involved in the occurrence and development of many diseases. Previous studies have focused on the roles of lncRNAs in cancer, cardiovascular disease, dyspnea and neurodegenerative diseases. However, few studies have been carried out to explore the effect of lncRNA on GDM.

H19 is the first lncRNA reported to be associated with GDM, which affects insulin secretion by altering the cellular function of islet cells[10]. MEG3 is downregulated in many tumors such as gastric cancer[11], liver cancer[12] and bladder cancer[13]. MEG3 is closely related to the incidence and progression of various tumors. However, the role of MEG3 in GDM has not been fully elucidated. The primary purpose of this study was to investigate the effect of MEG3 on fetal endothelial dysfunction induced by GDM.

Patients and Methods

Sample Collection

A total of 16 GDM pregnancies admitted in Obstetrics Department, Woman's Hospital, School of Medicine, Zhejiang University and People's Hospital of Rizhao from July 2014 to July 2017 were selected as GDM group. Meanwhile, 18 healthy pregnancies without GDM diagnosed with oral glucose tolerance test (OGTT) in the same period were selected as control group. Exclusion criteria were applied in pregnancies younger than 18 years, combined with other medical conditions, exposed to harmful substances during pregnancy, abnormal placenta or umbilical cord, blood group incompatibility or multifetation. Pregnancies in both groups underwent delivery in our hospital. Basic characteristics of enrolled pregnancies were listed in Table I. No significant differences in age and body weight between the two groups were found. This study was approved by the Ethics Committee of Woman's Hospital; School of Medicine; Zhejiang University and People's Hospital of Rizhao. Signed written informed consents were obtained from all participants before the study.

Cell Culture of HUVECs

15-20 cm of umbilical cord was collected within 12 h after delivery of healthy and GDM pregnancies. Under sterile conditions, the umbilical cord was washed with phosphate-buffered saline (PBS) and the residual blood in the lumen of the umbilical cord vein was extruded. One end of the umbilical cord was clamped using hemostatic forceps, whereas the other end was injected with 0.25% trypsin. After completely filling with trypsin, the umbilical cord was placed in a 37°C incubator for 10 min. The digestive fluid was collected, centrifuged, and maintained in Dulbecco's Modified Eagle Medium (DMEM, HyClone, South Logan, UT, USA) containing 20% fetal bovine serum (FBS, Gibco, Rockville, MD, USA). Cells were then resuspended in DMEM and placed in a 5% CO_2 incubator at 37°C. Culture medium was replaced 24 h later.

Cell Apoptosis

Cell apoptosis was detected using enzyme-linked immunosorbent assay (ELISA). HUVECs were resuspended in 200 μL of loading buffer and centrifuged at 1000 r/min for 10 min. 20 μL of cell suspension was then seeded in the pre-coated plates, followed by incubation with 80 μL of

Table I. Demographic clinical features of study subjects.

Variable	Control (n=18)	GDM (n=16)
Age (years, mean, SE)	31.91 (6.65)	35.42 (6.67)
Weight (kg, mean, SE)	75.53 (15.15)	73.32 (14.13)
Smokers (n)	1	1
Obese (n)	2	3
Hypertensive (n)	1	1
Epidural delivery (n)	3	5
Pregnancy at risk (n)	0	1
Baby birth weight (kg, mean, SE)	3.16(0.43)	3.44(0.6)

No statistically significant differences were found between the two groups for any of the parameters reported above.

an immunoreactive solution containing anti-DNA-POD, anti-histone-biotin and incubation solution. After incubation for 2 h at room temperature, 100 μL of substrate buffer was added, followed by cell apoptosis detection using a microplate reader (Bio-Rad, Hercules, CA, USA).

Cell Counting Kit-8 (CCK-8) Assay

Transfected cells were collected and seeded into the 96-well plates at a dose of 5.0×10^3/well, with 5 replicates in each group. After cell culture for 6 h, the activity of the adherent cells was measured. Briefly, 20 μL of CCK-8 solution (Dojindo Laboratories, Kumamoto, Japan) was added into each well. Cells were incubated at 37°C for 2-3 h in the dark. Absorbance (OD) values at the wavelength of 450 nm were detected by the microplate reader (Bio-Rad, Hercules, CA, USA).

Wound Healing Assay

Cells were seeded into the 6-well plate at a density of 5.0×10^5/well. 5 wounds were made in the monolayer with an interval of 0.5-1.0 cm using a pipette tip. Subsequently, non-adherent cells were expelled after washing with phosphate-buffered saline (PBS) three times. The wound area was captured 24 h later.

Tube Formation Assay

200 μL of diluted Matrigel was added into the pre-cooled 24-well plate and placed in the incubator for 30 min. HUVECs were seeded on the Matrigel at a density of 1.0×10^5/mL. Tube formation was observed and captured using an inverted microscope every 3 h.

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

The total RNA was extracted from cells by TRIzol method (Invitrogen, Carlsbad, CA, USA) and then transcribed into complementary Deoxyribose Nucleic Acid (cDNA). The reverse transcription reaction was carried out in strict accordance with the instructions of SYBR Green Real-Time PCR Master Mix (Invitrogen, Carlsbad, CA, USA), with a total reaction volume of 10 μL. Primers used in this experiment were as the follows: MEG3, F: 5'-CTGCCCATCTACACCTCACG-3', R: 5'-CTCTCCGCCGTCTGCGCTAGGGGCT-3'.

Cell Transfection

Cell transfection was performed when the cell confluence was up to 60-80% according to the instructions of Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA, USA). Briefly, transfection solution and Lipofectamine™ 2000 were diluted in serum-free DMEM, respectively. 20 min later, cells were added with the mixture for the following cell transfection. A single-strand oligo of MEG3 was synthesized and the splicing products were cloned into T vectors for constructing LV-MEG3.

Luciferase Reporter Gene Assay

Wild-type MEG3, mutant-type MEG3, wild-type AFF1 and mutant-type AFF1 were constructed, respectively. Cells were seeded in the 96-well plate, followed by co-transfection of 50 pmol/L microRNA-370-3p mimics or negative control, 80 ng wild-type MEG3 or mutant-type MEG3 and wild-type AFF1 or mutant-type AFF1, respectively. Luciferase activity was determined using single photon detector (Bio-Rad, Hercules, CA, USA).

Western Blot

The total protein was extracted by the radioimmunoprecipitation assay (RIPA) lysate (Yeasen, Shanghai, China). The concentration of each protein sample was determined by a bicinchoninic acid kit (BCA) (Abcam, Cambridge, MA, USA). Briefly, total protein was separated by a sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) under denaturing conditions and then transferred to polyvinylidene difluoride (PVDF) membranes (Merck Millipore, Billerica, MA, USA). Membranes were blocked with 5% skimmed milk for 1h, followed by the incubation of specific primary antibodies (Cell Signaling Technology, Danvers, MA, USA) overnight. After washing with Tris-Buffered Saline-Tween (TBS-T, Yeasen, Shanghai, China) 3 times, membranes were then incubated with the secondary antibody (Cell Signaling Technology, Danvers, MA, USA) at room temperature for 1 h. Immunoreactive bands were exposed by enhanced chemiluminescence (ECL) method.

Statistical Analysis

SPSS (Statistical Product and Service Solutions) 13.0 software (IBM, Armonk, NY, USA) was introduced for statistical analysis. The quantitative data were represented as mean ± standard deviation ($\bar{x}\pm s$). The *t* test was used for comparing differences between the two groups. $p<0.05$ was considered statistically significant.

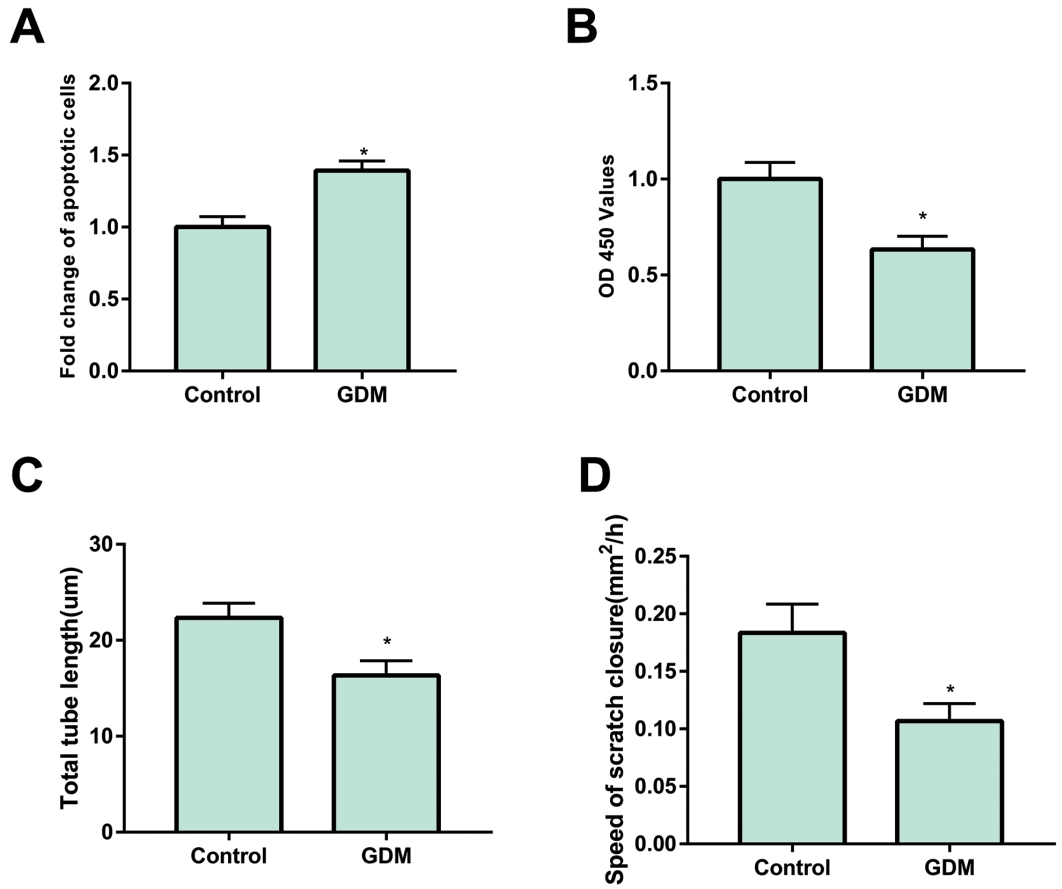

Figure 1. GDM damages fetal endothelial function. ***A,*** Cell apoptosis of HUVECs extracted from GDM pregnancies were higher than those extracted from normal pregnancies. ***B,*** Cell proliferation of HUVECs extracted from GDM pregnancies were lower than those extracted from normal pregnancies. ***C,*** Angiogenesis of HUVECs extracted from GDM pregnancies were lower than those extracted from normal pregnancies. ***D,*** Cell migration of HUVECs extracted from GDM pregnancies were lower than those extracted from normal pregnancies.

Results

GDM Damaged Fetal Endothelial Function

Third to fifth-passage HUVECs were selected for the following experiments. Increased apoptosis (Figure 1A) and decreased proliferation (Figure 1B) were found in HUVECs extracted from GDM pregnancies compared with those from normal pregnancies. Besides, decreased tube formation (Figure 1C) and migration (Figure 1D) were observed in HUVECs extracted from GDM pregnancies than those of controls, indicating that GDM severely damages fetal endothelial function.

MEG3 Was Overexpressed in HUVECs Extracted from GDM Pregnancies

MEG3 was overexpressed in HUVECs extracted from GDM pregnancies compared with that of healthy pregnancies (Figure 2A). Tube formation assay was performed in HUVECs after treatment with 5 mM, 12.5 mM and 25 mM glucose, respectively. We found that tube formation ability was remarkably increased after treatment of 12.5 mM glucose, whereas decreased after treatment of 25 mM glucose (Figure 2B). Besides, remarkable elevation of MEG3 expression was found when HUVECs were treated with 25 mM glucose (Figure 2C). Subsequently, LV-MEG3 plasmid was constructed and transfection efficacy was verified by qRT-PCR (Figure 2D). Overexpression of MEG3 led to increased apoptosis (Figure 2E), decreased viability (Figure 2F), tube formation (Figure 2G) and migration (Figure 2H) of HUVECs. The above data all indicated that overexpressed MEG3 in HUVECs extracted from GDM pregnancies damages epithelial function.

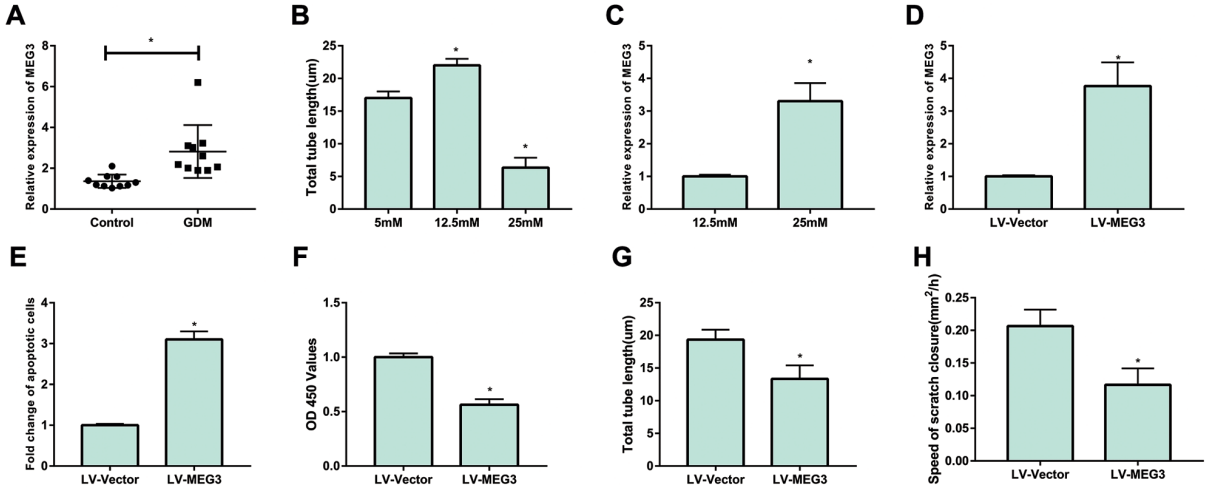

Figure 2. MEG3 was overexpressed in HUVECs extracted from GDM pregnancies. ***A,*** MEG3 was overexpressed in HUVECs extracted from GDM pregnancies than that of healthy pregnancies. ***B,*** Tube formation ability was remarkably increased after treatment of 12.5 mM glucose, whereas decreased after treatment of 25 mM glucose (Figure 2B). ***C,*** MEG3 expression was significantly increased when HUVECs were treated with 25 mM glucose. ***D,*** LV-MEG3 plasmid was constructed and transfection efficacy was verified by qRT-PCR. ***E,*** Overexpression of MEG3 led to increased apoptosis of HUVECs. ***F,*** Overexpression of MEG3 led to decreased viability of HUVECs. ***G,*** Overexpression of MEG3 led to decreased tube formation of HUVECs. ***H,*** Overexpression of MEG3 led to decreased migration of HUVECs.

MEG3 Regulated AFF1 Expression Through Endogenous Competition

We predicted target genes of MEG3 by Starbase and microRNA-370-3p was screened out. To further verify the binding condition of microRNA-370-3p with MEG3, wild-type MEG3 and mutant-type MEG3 were constructed (Figure 3A, left). Luciferase reporter gene assay showed that the luciferase activity of wild-type MEG3 was remarkably lower in HUVECs transfected with microRNA-370-3p mimics than that of mutant-type MEG3 (Figure 3A, right). AFF1 was predicted as the target gene of microRNA-370-3p by bioinformatics method. Subsequently, wild-type AFF1 and mutant-type AFF1 were constructed (Figure 3B, left). Lower luciferase activity of wild-type AFF1 was found in HUVECs transfected with microRNA-370-3p than that of mutant-type AFF1 (Figure 3B, right).

Overexpression lentivirus of MEG3 was transfected into HUVECs and the transfection efficacy was verified (Figure 3C). We observed a significant reduction of AFF1 expression after transfection of microRNA-370-3p mimics (Figure 3D). On the contrary, protein expression of AFF1 was remarkably increased after MEG3 overexpression (Figure 4A), indicating that MEG3 regulates AFF1 expression through endogenous competition.

MEG3 Inhibited PI3K/AKT Pathway

It is reported that PIK3/AKT pathway is involved in cell proliferation and apoptosis, which is of great significance in the survival of epithelial cells. Hence, we speculated whether MEG3 could regulate PI3K/AKT pathway. In the present study, we detected expression levels of p-PI3K, PI3K, p-AKT and AKT in HUVECs after MEG3 overexpression. The results of Western blot showed that overexpressed MEG3 remarkably inhibited PI3K/AKT pathway-related genes (Figure 4B).

Discussion

GDM is the disease condition of glucose intolerance that occurs during pregnancy, which is the major cause of the increased prevalence and mortality of pregnant women and perinatal infants[14]. With the economic development and lifestyle change, the incidence of GDM has increased annually. GDM is harmful to mothers and babies. However, the pathogenesis of adverse effects of GDM on fetal epithelium damage has not been fully elucidated.

LncRNA regulates gene expressions at the epigenetic, transcriptional, and post-transcriptional levels. Functionally, lncRNAs participate in various physiological processes, including tumor development[15]. Recent studies have found that

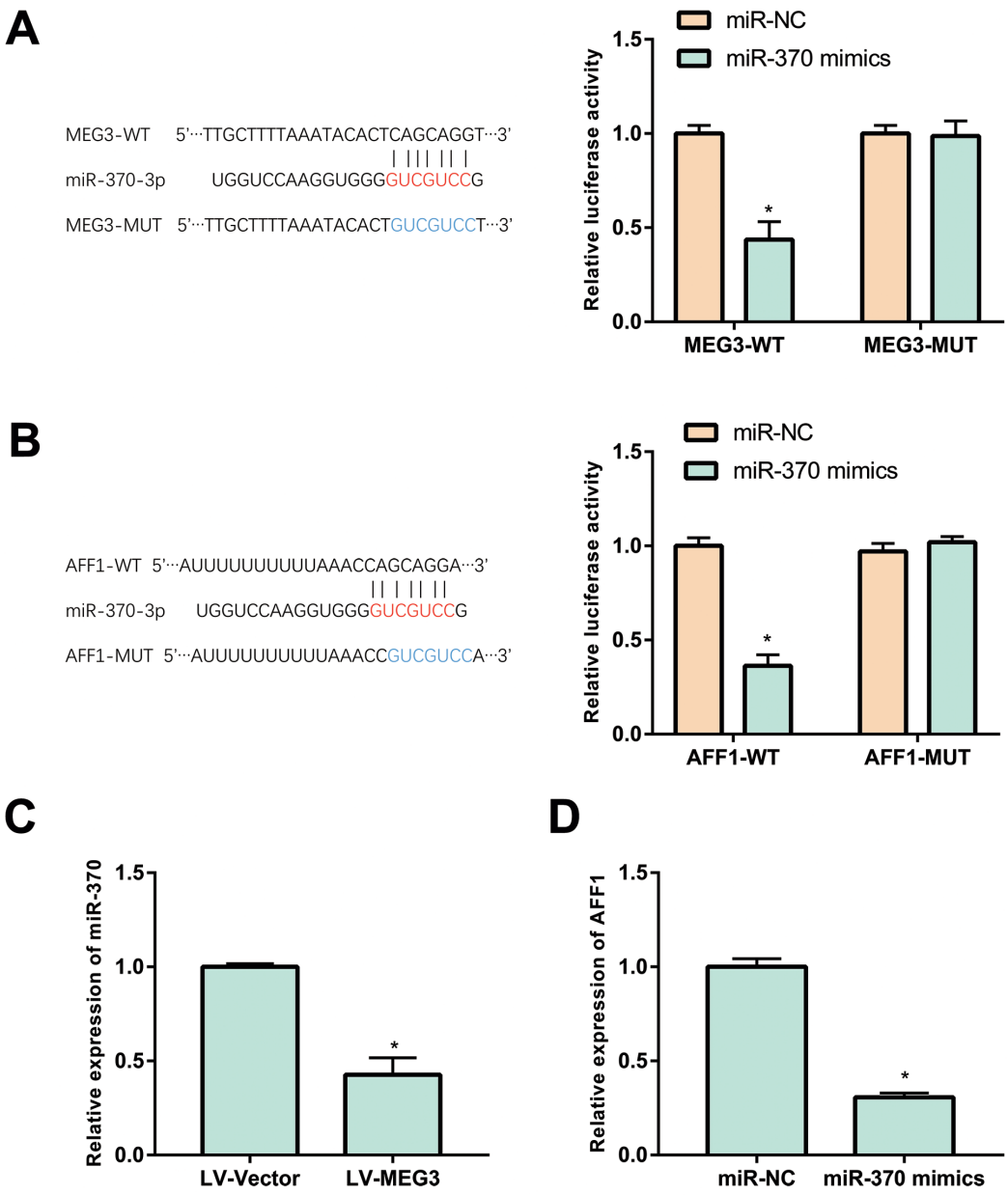

Figure 3. MEG3 regulated AFF1 expression through endogenous competition. ***A,*** MEG3 was directly bound to microRNA-370-3p. ***B,*** AFF1 was directly bound to microNRA-370-3p. ***C,*** Overexpression of MEG3 decreased microRNA-370-3p expression. ***D,*** Overexpression of microRNA-370-3p decreased mRNA level of AFF1.

some lncRNAs exert an essential role in GDM, such as MALAT1 and maternally-expressed gene 3 (MEG3)[16]. MEG3 is an imprinting gene located at the long arm of human chromosome 14. 32[17,18]. MEG3 is widely expressed in pituitary gland, brain tissue, meninges[19], lungs[20], and liver[21]. On the contrary, MEG3 is rarely expressed in tumor tissues. Overexpression of MEG3 could inhibit proliferation and growth of tumors[22]. Dysfunction of vascular endothelial cells induced by diabetes leads to changes in barrier function, cell adhesion and apoptosis. In addition, diabetes is likely to change the overall performance of vascular endothelial cells[23]. Accumulating evidence has demonstrated that endothelial dysfunction in type 1 and type 2 diabetes is a key factor in diabetic retinopathy, nephropathy, and atherosclerosis[24]. In our study, vascular endothelial cell function was impaired by GDM. Overexpressed MEG3 in HUVECs further damaged

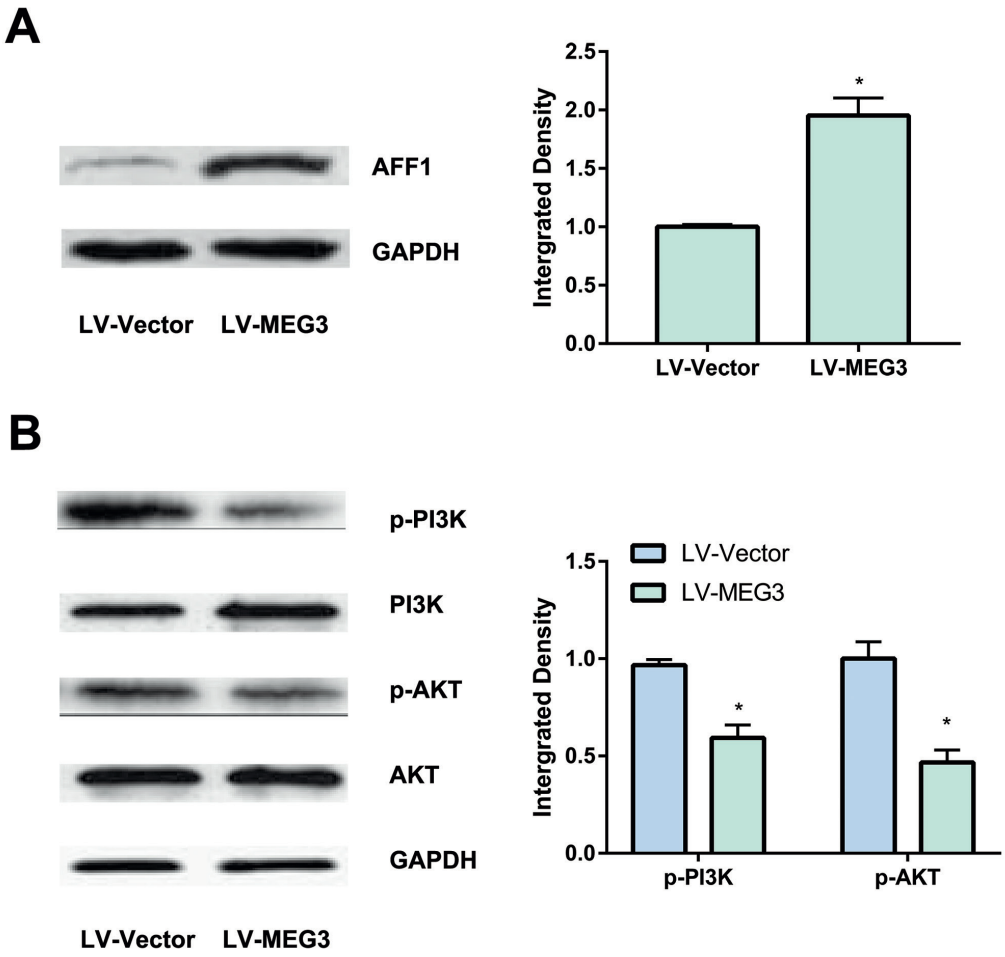

Figure 4. MEG3 inhibitedPI3K/AKT pathway ***A,*** Overexpressed MEG3 promoted AFF1 expression. ***B,*** Overexpressed MEG3 inhibited PI3K/AKT pathway.

proliferative and migrate abilities of vascular endothelial cells, thereafter progressing into neonatal type 2 diabetes and cardiovascular diseases. MicroRNAs are single-stranded non-coding RNAs with 18-23 nucleotides in length. They are highly conserved during evolution and exert a regulatory role at the epigenetic level. Mature miRNAs inhibit translation of the target gene or directly degrade the target mRNA by binding to an RNA-induced silencing complex (RISC)[25,26]. Some certain microRNAs have been found to be differentially expressed in GDM patients, including microRNA-21, microRNA-29a, microRNA-132, microRNA-222 and microRNA-375[27]. Our results found that microRNA-370-3p bound to MEG3 and AFF1 to participate in regulating GDM development. Relative studies have demonstrated that hyperglycemia can induce endothelial cell damage, including endothelial cells in human or animal kidneys, retina, myocardium and human umbilical vein[28]. It is currently believed that oxidative stress, increased intracellular Ca^{2+}, mitochondrial failure, intracellular fatty acid metabolism, and inhibited PI3K/AKT pathway are involved in endothelial cell damage resulted from hyperglycemia[29]. Here, our data confirmed that exogenous overexpression of MEG3 inhibited PI3K/AKT pathway.

Conclusions

We showed that MEG3 is overexpressed in HUVECs extracted from GDM pregnancies. MEG3 damages fetal endothelial function through targeting microRNA-370-3p and AFF1 *via* PI3K/AKT pathway.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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