Interference of KLF9 relieved the development of gestational diabetes mellitus by upregulating DDAH2

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ABSTRACT

Gestational diabetes mellitus (GDM) is a situation where glucose intolerance is found in pregnant women without a previous diagnosis of diabetes. The role of Kruppel-like factor 9 (KLF9) has not been investigated in GDM, which constituted the aim of our study. HTR8/SVneo cells were induced by high glucose (HG) and pregnant mice were treated with streptozocin (STZ) to establish GDM model in vitro and in vivo, respectively. The expression level of KLF9 was detected by real-time PCR, immunohistochemical staining, and Western blot. Cell viability, apoptosis, inflammation, and oxidative stress were investigated by cell counting kit-8 (CCK-8), TUNEL, enzyme-linked immunosorbent assay (ELISA) and oxidative stress detection kits, respectively. The interaction of KLF9 with dimethylarginine dimethylaminohydrolase 2 (DDAH2) was predicted by bioinformatic tools and confirmed by luciferase reporter assay and chromatin immunoprecipitation (ChIP). The expression of KLF9 was increased in the placental tissues of GDM patients and HG-induced HTR8/SVneo cells. Silencing of KLF9 increased cell viability, reduced cell apoptosis, and suppressed inflammation and oxidative stress in HG-induced HTR8/SVneo cells. KLF9 could bind to DDAH2 promoter and negatively regulate DDAH2 expression. Inhibition of DDAH2 partly weakened the effects of KLF9 silencing on cell apoptosis, inflammation, and oxidative stress. The suppressive effects of KLF9 silencing on blood glucose and insulin concentration in vivo were also abolished by DDAH2 knockdown. In conclusion, we provided evidence that interference of KLF9 could hinder the development of GDM by alleviating cell apoptosis, inflammation, and oxidative stress through upregulating DDAH2, which might instruct the targeting therapies against GDM.

Abbreviations: KLF9: Kruppel-like factor 9; DDAH2: dimethylarginine dimethylaminohydrolase 2; GDM: gestational diabetes mellitus; ELISA: enzyme-linked immunosorbent assay; CCK-8: cell counting kit-8; ChIP: chromatin immunoprecipitation; sh: short hairpin; HG: high glucose; PBS: phosphate-buffered saline; DAPI: 4, 6-diamidino-2-phenylindole; IL-6: Interleukin-6; TNF-α: tumor necrosis factor-α; ROS: reactive oxygen species; MDA: malondialdehyde; SOD: superoxide dismutase; wt: wild-type; mut: mutant

Introduction

Gestational diabetes mellitus (GDM) is a situation where glucose intolerance is found in pregnant women without a previous diagnosis of diabetes and is linked to a probable resolution after the end of the pregnancy [1]. Recent statistics has noted that this disease threatens the health of roughly 1 in 6 live births [2]. At present, the incidence of GDM is still increasing since the criteria for screening and diagnosis of GDM has not gained universal acceptance [3]. The mechanism of the occurrence and development of GDM has not been fully clarified, which renders the study of GDM pathogenesis of theoretical significance for clinical treatment.

Kruppel-like factors (KLFs) are the transcription factors that are able to regulate a wide range of biological processes, including development, differentiation, and cell apoptosis [4,5]. KLF9, first recognized as a transcriptional repressor of the rat Cyp1a1, can modulate diverse biological processes to affect the progression of various diseases [6]. Evidence has emerged to suggest that knockdown of KLF9 can reduce hyperglycemia [7]. Studies have shown that upregulation of...
KLF9 can aggravate high glucose-induced podocyte injury, and can aggravate myocardial ischemic injury by increasing oxidative stress [8,9]. However, there is currently no research on the regulatory role of KLF9 in GDM.

Through the analysis of JASPAR database, it was found that KLF9 could bind to the promoter sequence of dimethylarginine dimethylaminohydrolase 2 (DDAH2). DDAH2 is a major enzyme that can degrade dimethylarginine, which is an endogenous NOS inhibitor and inhibits the generation of NO. Thus, DDAH2 has the potential ability to regulate oxidative stress. Coincidentally, GDM often causes inflammation and oxidative stress in trophoblastic cells [10,11]. In addition, dysregulation of DDAH2 was also involved in diabetes and its complications [12,13]. Therefore, it is curious that whether DDAH2 plays an important role in GDM.

In this study, we speculated that KLF9 might transcriptionally regulate DDAH2 and influence the development of GDM. Here, it was firstly found that KLF9 was greatly upregulated in the placenta tissue of GDM. Inhibition of KLF9 potently alleviated inflammatory response and oxidative stress in GDM model in vitro and in vivo. Our data suggest that KLF9 is useful as a potential target for the management of GDM progression.

Materials and methods

Patients and placenta tissue preparation

Human placenta tissues were obtained from 36–42-week pregnant women from Huai’an Maternal and Child Health Hospital, from 2018.01 to 2019.12. Placenta tissues were obtained from 5 pregnant women with normal pregnancy (Control group) and another five patients with GDM (GDM group). All subjects with a history of other complications were excluded from this study. The study was approved by our ethics committee, and all patients had signed informed consent.

Immunohistochemical staining

The sections of placental tissues were fixed overnight in 4% formaldehyde, embedded in paraffin, sectioned and deparaffinized with xylene. Then, the EnVisionTM Detection Kit (Dako Diagnostics, Switzerland) was used to stain the sections, following which was the incubation with anti-KLF9 (1:300, HPA029308, Atlas) overnight at 4°C. Images were obtained with a light microscope (Olympus, Japan).

Cell culture and treatment

The human trophoblast HTR8/SVneo cell line, which was obtained from BeNa Culture Collection (Beijing, China), was used for in vitro experiments. Cells were cultured in DMEM/F-12 supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin in a humidified incubator containing 5% CO2 at 37°C. High glucose (HG) at the concentration of 25 mmol/L was used to incubate the cells for 24 h to simulate the GDM environment in cells, and the normal glucose level (5 mmol/L) was used to treat cells as the control [14].

The specific short hairpin RNA (shRNAs) targeting KLF9 (sh-KLF9-1 and sh-KLF9-2) and DDAH2 (sh-DDAH2#1 and sh-DDAH2#2) and their respective negative control (sh-NC) were designed and synthesized by GenePharma (Shanghai, China). For transfection, the HTR8/SVneo cells were inoculated in 6-well culture plates and transfections were conducted using the Lipofectamine™ 2000 Transfection reagent (Invitrogen, USA) according to the manufacturer’s instructions.

GDM mice model establishment

The 4-week-old C57BL/6 J mice were purchased from Vitalriver (Beijing, China). This study was approved by the Ethics Committee of Huai’an Maternal and Child Health Hospital. Mice were randomly divided into two groups: mice in the Control group were given the normal diet and mice in the GDM group were received high-sugar and high-saturated fat combined diet. After 8 weeks of feeding, female and male mice were caged in a 2:1 number ratio, and the successful mating was further confirmed by the evidence of vaginal suppository the next morning, which was counted as the Day 0 of pregnancy.
On Day 5, 0.25% streptozotocin (STZ) at the concentration of 35 mg/kg was used to treat GDM group [14,15], and the same volume of phosphate-buffered saline (PBS) was intraperitoneally injected into the Control group. The lentiviral vector silencing KLF9 were injected to the mice of the GDM group via tail veins to explore the regulatory mechanism of KLF9 on the progression of GDM. On Day 8, the blood glucose level of higher than 13.5 mmol/L was observed, indicating that the GDM mice models were established successfully.

Pathological Tests on Mouse Models
Body weights of mice were monitored and recorded in the morning after 12 hour fasting at 0, 5, 8, 12 and 18 days of pregnancy. A certain amount of 20% glucose solution (2 g glucose/kg) was injected intraperitoneally, and the blood glucose levels and insulin content were then measured at indicated days after glucose injection.

Bioinformatics analysis
The shared binding sites between KLF9 and DDAH2 were analyzed using JASPAR (http://jaspar.genereg.net) and their correlation was predicted by GEPIA (http://gepia.cancer-pku.cn) database.

Real-Time PCR analysis
Total RNA was extracted from HTR8/SVneo cells using Trizol reagent (Invitrogen, USA) and then was converted to cDNA by the cDNA Reverse Transcription Kit (Invitrogen, USA). The SYBR Green PCR Master Mix (TaKaRa, Japan) was used for the implementation of qPCR on StepOne Plus real-time PCR system (Thermo Fisher, USA). All qPCRs were carried out in triplicate for each sample and GAPDH was used as endogenous control. Primers are as follows: KLF9: forward 5’-TACATGGACTTCGTGGCTGC-3’, reverse 5’-AGGGCCGTTCACCTGTATGC-3’. DDAH2: forward 5’-GCAACGACTAGGTCTGCAGCTTC-3’, reverse 5’-GGTACCGTAGAGACAGCGAAGTC-3’. GAPDH: forward 5’-GTCTTCACATTACGGAGAAG-3’, reverse 5’-TCATGGATGACCTTGCCAG-3’.

Cell counting kit-8
HTR8/SVneo cells were inoculated in 96-well plate (2 × 10⁵ cells/well) for attachment. Thereafter, 10 μL cell counting kit-8 reagent (CCK-8, Beyotime, China) was added to each well at 37°C for a further incubation of 4 h, and the optical density at 450 nm was read through a microplate reader (Bio-Rad).

TUNEL
TUNEL assay was performed using the In Situ Cell Death Detection Kit (Roche, Switzerland) as per the manufacturer’s protocol. Cell nuclei was counterstained with DAPI (4, 6-diamidino-2-phenylindole). The number of TUNEL-positive cells was counted under a fluorescent inverted microscope.

Enzyme-linked immunosorbent assay (ELISA)
Interleukin-6 (IL-6), IL-1β and tumor necrosis factor-α (TNF-α) were measured by ELISA kits in line with the manufacturer’s instructions (R&D Systems, Minneapolis, MN, USA).
Detection of NOS and NO

The detection of NOS and NO was conducted, respectively, by NOS activity assay kit and NO activity assay kit (Nanjing Jiancheng Biotechnology, China).

Determination of oxidative stress

The reactive oxygen species (ROS, Beyotime, China), malondialdehyde (MDA, Beyotime, China) content and activities of superoxide dismutase (SOD, Beyotime, China) in the serum of GDM mice model were conducted by corresponding commercial kits following the manufacturer’s instructions.

Dual luciferase reporter assay

With the help of JASPAR and GEPIA prediction tools, KLF9 is shown to be associated with DDAH2. The wild-type (wt) and mutant (mut) of DDAH2 were inserted into the vector (Promega, USA). After that, HTR8/SVneo cells were seeded in 6-well plate cultured for 24 h and then co-transfected with sh-KLF9 or sh-NC and DDAH2-WT or DDAH2-MUT using Lipofectamine 3000 reagent (Invitrogen, USA). The experiment was performed with Dual Luciferase Reporter Gene Assay Kit (Abnova, USA), followed by detection of Renilla luciferase as well as Firefly luciferase intensity.

Chromatin immunoprecipitation (ChIP)

SimpleChIP kit (Cell Signaling Technology, USA) was used to conduct ChIP assay. The HTR8/SVneo cells were fixed with 1% formaldehyde to cross-link histones to DNA and sonicated to yield chromatin fragments of 200–500 base pairs. Specific immunoprecipitation reactions were performed using anti-KLF9, with Normal Rabbit IgG (CST, USA) as a negative control. After decrosslinking, the target DNA was then purified. One percent Pre-enriched chromatin (input) served as the percentage input for the quantification of samples. The quantification of target DNA obtained in ChIP was performed by Real-Time PCR.

Statistical analysis

Data were analyzed by Graphpad prism 6.0 (GraphPad Software, La Jolla, CA, USA) and expressed as mean ± SD. One-way analysis of variance followed by Tukey’s test was used to analyze the differences. p < 0.05 was considered as statistically significant.

Results

KLF9 was high expressed in GDM cells and tissues

To explore the function of KLF9 in GDM, we first detected the expression of KLF9 in GDM cells and tissues. Notably, the expression of KLF9 in the placental tissues of GDM patients was elevated, compared with control (Figure 1a). Meanwhile, immunohistochemical staining presented the consistent result (Figure 1b). The expression of KLF9 was then measured in HTR8/SVneo cells, and an upregulated expression of KLF9 was observed in HG-induced HTR8/SVneo cells, compared to the control (Figure 1c-d).

Inhibition of KLF9 increased the cell viability and reduced the inflammatory response and oxidative stress in HG-induced HTR8/SVneo cells

To examine the mechanism by which KLF9 exerts effects on the cellular behaviors, we silenced KLF9 and chose the sh-KLF9-2 with lower KLF9 expression for the following experiments (Figure 2a-b). It was obviously found in Figure 2c-e that in HG-induced HTR8/SVneo cells, inhibition of KLF9 increased the cell viability and suppressed the apoptosis of HTR8/SVneo cells, accompanied with the upregulated Bcl-2 and the downregulated cleaved caspase-3/caspase-3 and cleaved caspase-9/caspase-9, compared with HG group. Meanwhile, the inflammation of HG-induced HTR8/SVneo cells was suppressed by KLF9 knockdown, as illustrated by reduced TNF-μ, IL-1β, IL-6, p-p65 and COX-2 expression upon KLF9 inhibition (Figure 3a-b). Furthermore, sh-KLF9 inhibited the ROS level and increased the NOS and NO levels as compared with the HG group (Figure 3c-e). Thus, these data suggested that inhibition of KLF9 increased the cell viability and...
reduced the inflammatory response and oxidative stress in HG-induced HTR8/SVneo cells.

**KLF9 transcription inhibits the expression of DDAH2**

The underlying mechanism by which KLF9 exerts effects on the HG-induced HTR8/SVneo cells was further investigated. As exhibited in Figure 4a, the binding sites between the transcription factor KLF9 and DDAH2 promoter were predicted. After that, dual-luciferase reporter assay and CHIP demonstrated the association of KLF9 with DDAH2 promoter (Figure 4b-c). Subsequently, the expression of DDAH2 was found to be downregulated in HTR8/SVneo cells upon HG induction (Figure 4d-e). In addition, HTR8/SVneo cells that were transfected with sh-KLF9 presented a higher expression of DDAH2 than that in sh-NC group (Figure 4f-g), demonstrating that KLF9 could bind to DDAH2 promoter and negatively regulate DDAH2 expression.

**DDAH2 is involved in the inhibition of sh-KLF9 on the oxidative stress and inflammation of HG-induced HTR8/SVneo cells**

To confirm whether DDAH2 is the casual link between KLF9 expression and the abnormal changes in the HG-induced HTR8/SVneo cellular behaviors, DDAH2 was silenced and sh-DDAH2#2 was chosen for the following experiments due to its higher transfection efficacy (Figure 5a-b). As observed in Figure 5c-e, sh-KLF9 increased the cell viability while reduced the apoptosis of HG-induced HTR8/SVneo cells, which was partially abrogated by sh-DDAH2 (Figure 5c-e). The detection of oxidative stress by measuring related markers implied that the oxidative stress inhibited by sh-KLF9 was reversed by sh-DDAH2 (Figure 6a-c). ELISA and Western blot analysis indicated the expression of proinflammatory factors and inflammation-related markers reduced by sh-KLF9 was increased by sh-DDAH2, implying the involvement of DDAH2 in the inhibitory role of sh-KLF9 in HG-induced HTR8/SVneo cells (Figure 6d-e). Thus, DDAH2 is involved in the inhibition of sh-KLF9 on the oxidative stress and inflammation of HG-induced HTR8/SVneo cells.

**The impact of KLF9/DDAH2 axis on the in vivo GDM mice model**

An *in vivo* study was conducted to observe the effects of KLF9/DDAH2 axis on GDM. In comparison to the control group, an evident increase in the weights of GDM mice was observed, whereas injection with sh-KLF9 greatly reduced this
elevation, which was partly weakened by the coinjection with sh-DDAH2 in the tail vein of GDM mice model (Figure 7a). The blood glucose was increased while insulin level was decreased in GDM mice, but interference with KLF9 rescued these changes (Figure 7b-c). Nevertheless, KLF9 inhibition-rescued blood glucose and insulin level of GDM mice were attenuated by DDAH2 inhibition. The detection of oxidative stress-related markers by commercial kits and release of inflammatory factors by ELISA suggested that sh-KLF9 suppressed the oxidative stress and inflammation in GDM mice, which was abated by sh-DDAH2 (Figure 7d-g). Consistent with the results in in vitro GDM model, the above findings implied the participation of KLF9/DDAH2 axis in GDM progression.

**Discussion**

GDM is a heterogeneous disorder that occurs during the pregnancy of women [16]. This disease has grown to be a burdensome health issue as it presents long-term consequences for offspring and consumes large expenditures for its treatment [17,18]. A better understanding of underlying
mechanism pertaining to GDM may contribute to the identification of molecular targets for management of this disease. This study aimed to investigate the role of transcription factor KLF9 in GDM by constructing both the in vitro and the in vivo GDM model.

KLF9 has been recognized as an important transcriptional factor that is involved in the progression of diabetes-related diseases. For example, microarray analysis indicated KLF9 as a potential biomarker for diabetic kidney disease as its upregulated level was observed in this disease [19]. KLF9 regulated by miR-30d was also involved in the diabetic cardiomyopathy [20]. This study suggested for the first time that KLF9 mRNA was increased both in the placental tissues of GDM patients and in HG-induced HTR8/SVneo cells, suggesting its involvement in the development of GDM.

To further ensure the functional effects of KLF9 in GDM, a series of in vitro and in vivo experiments were conducted. One of the main findings was that KLF9 interference increased the cell viability and decreased the apoptosis of HG-induced HTR8/SVneo cells.

**Figure 3.** Inhibition of KLF9 reduced the inflammatory response and oxidative stress in HG-induced HTR8/SVneo cells. (a) The expression of inflammatory cytokines in HG-induced HTR8/SVneo cells transfected with sh-KLF9 was detected by ELISA. (b) The expression of p-p65 and COX-2 in HG-induced HTR8/SVneo cells transfected with sh-KLF9 was detected by Western blot. The detection of (c) ROS, (d) NOS and (e) NO by corresponding kits. *, **, ***p < 0.05, 0.01, 0.001 vs HG; ##, ###p < 0.01, 0.001 vs HG+sh-NC.
SVneo cells. Inflammation during pregnancies shows close association with the initiation of GDM [14], which prompts our further analysis on the expression of inflammation-associated markers in HG-induced HTR8/SVneo cells. Reduced levels of proinflammatory cytokines, including TNF-α, IL-1β, and IL-6, and inflammation-associated markers p-p65 and COX-2 suggested that KLF9 inhibition suppressed the inflammation in GDM. In addition, GDM placenta shows lack of capability to cope with oxidative stress [21]. Correspondingly, elevated ROS and decreased NOS and NO by HG induction was in part restored upon sh-KLF9 transfection in HG-induced HTR8/SVneo cells. Therefore, interference of KLF9 could protect HTR8/SVneo cells against HG-induced cell apoptosis, inflammatory response and oxidative stress, which was also verified in in vivo experiments.

Interestingly, a binding relationship between transcriptional factor KLF9 and DDAH2 promoter was demonstrated in this study. DDAH is positively associated with NOS and NO through an inverse association with ADM, suggesting the enhancement of DDAH in promoting oxidative stress [22]. The expression of DDAH2 was found to be remarkably decreased in the adipose tissue of diabetic rats [23]. DDAH2 enhances pancreatic insulin release by transcriptional regulation of secretagogin in mice [24]. There was also a report indicating the participation of DDAH activity in endothelial dysfunction in diabetic rats or cell damage upon HG incubation [25,26]. Concurrently, downregulated level of DDAH2 was observed in HG-induced HTR8/SVneo cells, and KLF9 could negatively regulate DDAH2 expression. In addition, the protective

Figure 4. KLF9 transcription inhibits the expression of DDAH2. (a) The binding sites between KLF9 and DDAH2 promoter was predicted by JASPAR. (b) The luciferase activity of the DDAH2 detected by dual luciferase reporter assay. ***p < 0.001 vs sh-NC. (c) The combination between KLF9 and DDAH2 promoter detected by ChIP. ***p < 0.001 vs IgG. The (d) protein and (e) mRNA levels of DDAH2 in HG-induced HTR8/SVneo cells. **, ***p < 0.01, 0.001 vs control. The (f) protein and (g) mRNA levels of DDAH2 in HG-induced HTR8/SVneo cells transfected with sh-KLF9. ***p < 0.001 vs sh-NC.
effects of KLF9 silencing on HG-induced HTR8/SVneo cells and STZ-induced GDM mice were partly weakened by inhibition of DDAH2, suggesting the possibility that KLF9 might exert protective effects on GDM by regulating the DDAH level. This finding was in coincidence with the previous one which implied that the upregulation of DDAH2 alleviated endothelial dysfunction and that targeting DDAH2 might also be deemed as a positive factor for diabetes treatment [27]. However, some limitations still exist in the present study. Firstly, a larger sample size is beneficial to better verify these findings. Secondly, the downstream pathway of DDAH2 relating to inflammation, oxidative stress, and apoptosis in GDM is still unclear, which is deserved to be explored in our future work.

**Conclusion**

In conclusion, by the construction of *in vitro* and *in vivo* GDM models and a series of loss-of-function assays, we provided pieces of evidence that interference of KLF9 could hinder the development of GDM by alleviating cell apoptosis, inflammation and oxidative stress through upregulating DDAH2, which may instruct the targeting therapies against GDM.
Figure 6. DDAH2 is involved in the inhibition of sh-KLF9 on the oxidative stress and inflammation of HG-induced HTR8/SVneo cells. The detection of (a) ROS, (b) NOS and (c) NO in HG-induced HTR8/SVneo cells co-transfected with sh-KLF9 and sh-DDAH2 detected by corresponding kits. The (d) inflammatory factors and (e) expression of inflammation-related markers detected respectively by ELISA and Western blot. ***p < 0.001 vs HG; ###p < 0.001 vs HG+sh-NC; +, ++, +++p < 0.05, 0.01, 0.001 vs HG+sh-KLF9+ sh-NC.

Figure 7. The impact of KLF9/DDAH2 axis on the in vivo GDM mice model. (a) The body weights, (b) blood glucose and (c) insulin level were measured in GDM mice injected into sh-KLF9 and sh-DDAH2. (d) The oxidative stress indexes and (e) inflammatory cytokines detected in GDM mice using their corresponding commercial kits. ***p < 0.001 vs Control; ###p < 0.001 vs GDM+sh-NC; +, ++p < 0.05, 0.01 vs GDM+sh-KLF9+ sh-NC.
Availability of data and material
All the generated/analyzed data have been included in this article.

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Ethics approval
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Consent to participate
All patients had signed informed consent.

Consent for publication
All authors have read the final version of manuscript and approved for publication.

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