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ORIGINAL ARTICLE

hsa_circ_0003218 Mitigates Trophoblast Dysfunction in Gestational Diabetes by Regulating TLR4/MyD88/NF-κB and NLRP3 Inflammasome

Xiao-Feng Zhang [1, *], Luo-Hong Wei [1, *], Yu-Dan Wu [1], Xiao-Hui Li [1], Zhi-Fen Wang [1], Ya-Ru Ju [2]

[*] *These authors contributed equally to this work*
[1] *Department of Obstetrics, The Fourth Hospital of Shijiazhuang, Shijiazhuang City, Hebei Province, China*
[2] *Department of Perinatal Center, The Fourth Hospital of Shijiazhuang, Shijiazhuang City, Hebei Province, China*

SUMMARY

Background: **This investigation sought to determine hsa_circ_0003218's role and mechanism in trophoblast dysfunction during gestational diabetes mellitus (GDM).**
Methods: **The study involved forty pregnant women, comprising twenty with GDM and twenty with normal pregnancies. hsa_circ_0003218 expression levels in serum and placental tissues were detected by RT-qPCR. hTR8/SVneo cells were exposed to high glucose (HG) *in vitro* and assayed for proliferation, apoptosis, migration, and invasion by CCK-8, flow cytometry, and Transwell tests, respectively. Inflammatory factors were detected by ELISA. TLR4/MyD88/NF-κB cascade and NLRP3 inflammasomes-associated proteins were detected by Western blot.**
Results: **hsa_circ_0003218 was lowly expressed in placental tissues from GDM patients and HG-treated trophoblasts. hsa_circ_0003218 overexpression lessened HG-induced inhibition of trophoblast proliferation, migration, and invasion, and stimulation of apoptosis and inflammatory factor production. Furthermore, hsa_circ_0003218 prevented the activation of both the TLR4/MyD88/NF-κB cascade and the NLRP3 inflammasome.**
Conclusions: **hsa_circ_0003218 improves trophoblast function in GDM by blocking the TLR4/MyD88/NF-κB cascade and preventing NLRP3 inflammasome activation.**
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Correspondence:
Ya-Ru Ju
Department of Perinatal Center
The Fourth Hospital of Shijiazhuang
No. 91, Xueyuan Road, Shijiazhuang City
Hebei Province, 050000
China
Email: laoju1965@outlook.com

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KEYWORDS

hsa_circ_0003218, TLR4/MyD88/NF-κB cascade, NLRP3 inflammasomes, gestational diabetes mellitus, trophoblasts

INTRODUCTION

Gestational diabetes mellitus (GDM), which is characterized by glucose intolerance, is the most frequent medical issue encountered during pregnancy [1]. The International Diabetes Federation reported that, globally, GDM affected about 14% of pregnancies in 2017, with a higher rate of 21% in Asia [2]. In China, the GDM incidence was 11.91%, which is significantly higher compared to Japan, Korea, and Thailand, where the prevalence was below 8% [3]. Apart from the immediate perinatal dangers, GDM, marked by hyperglyce-

mia and carbohydrate metabolism disorders, increases the risk of metabolic diseases, in addition to immediate perinatal risks [4,5]. During pregnancy, the placenta facilitates the exchange of nutrients, gases, and blood between the mother and the fetus [6]. Abnormal placental function contributes to adverse pregnancy outcomes associated with GDM [7,8]. Furthermore, trophoblasts with normal biological functions are essential for the development of the placenta [9,10]. Thus, targeting trophoblast dysfunction might provide a valuable strategy for understanding GDM pathogenesis.

circRNAs are non-coding RNAs characterized by covalently closed loops, which lack 3′ and 5′ ends, making them resistant to RNase R [11]. In mammalian cells, circRNAs demonstrate high stability along with species-, tissue-, cell-, or disease-specific expression patterns [12] and have significant functions in a range of biological processes [13-15]. These features render circRNAs as ideal biomarkers for promising therapeutic interventions [16]. Recent findings indicate that aberrantly expressed circRNAs are related to disease conditions, including GDM [17].

Identified as a novel functional circRNA, hsa_circ_0003218 is derived from the BMPR2 gene and located at chr2:203329531-203332412, showing differential expression in patients with GDM [18]. However, the specific regulatory mechanism and function of hsa_circ_0003218 in GDM have yet to be fully clarified.

Previous research established and identified the HTR8/SVneo cell line, which is an immortalized line from human chorionic trophoblasts [19]. In this investigation, HTR8/SVneo cells exposed to high glucose (HG) were used to replicate the T2DM condition *in vitro*, aiming to explore hsa_circ_0003218's role and molecular mechanisms in trophoblast dysfunction.

MATERIALS AND METHODS

This study recruited pregnant women diagnosed at the Fourth Hospital of Shijiazhuang. Forty pregnant women who had regular prenatal check-ups were included, with 20 diagnosed with GDM and the remaining 20 being normal. All participants were free from pregnancy complications and received a 75-g oral glucose tolerance test during the 24 - 28 weeks of pregnancy. GDM is diagnosed according to the guidelines from the International Association of Diabetes and Pregnancy Research Group. This study was approved by the Fourth Hospital of Shijiazhuang´s Ethics Committee. Written informed consent was obtained from all patients.

Cell culture and treatment

The HTR8/SVneo human placental trophoblasts (Jining Cell Culture Center, Shanghai, China) were conserved in RPMI 1640 medium (Thermo Fisher Scientific, CA, USA), enriched with 10% fetal bovine serum (FBS; Solarbio, Shanghai, China) and 1% streptomycin-penicillin (Solarbio), maintained at 37°C in an atmosphere containing 5% CO_2.

The full cDNA sequence of hsa_circ_0003218 was amplified and cloned into the pCD5-ciR overexpression vector (Geneseed, Guangzhou, China) to create oe-hsa_circ_0003218, using a vector without the hsa_circ_0003218 sequence as a control. Geneseed synthesized specific siRNA (si-hsa_circ_0003218) targeting hsa_circ_0003218 and negative control siRNA (si-NC). HTR8/SVneo cells (2×10^5 cells/well) at 70 - 80% confluence were transfected using Lipofectamine 2000 (Invitrogen, CA, USA).

Glucose exposure (Solarbio) was performed on HTR8/SVneo cells to mimic T2DM conditions. The cells were incubated for 48 hours in a medium containing either 5 mmol/L glucose (normal glucose) or 25 mmol/L glucose (HG) [20].

CCK-8 assay

HTR8/SVneo cells (3×10^3 cells/well), transfected and/or treated, were plated in 96-well plates and detected with CCK-8 solution (10 μL/well, Beyotime, Shanghai, China). After 2 hours, the absorbance at 450 nm was detected on a microplate reader.

Flow cytometry

After HTR8/SVneo cells were transfected and/or treated as indicated, they underwent two washes with 1 × PBS, followed by resuspension in 1 × buffer, achieving 1 × 106 cells/mL. Subsequently, 100 μL of the cell mixture was combined with 5 μL of Annexin V-fluorescein isothiocyanate and propidium iodide each (BD Bioscience, NJ, USA), maintained at 37°C to shield it from light. Apoptosis was assessed by FACSCantoII flow cytometer (BD Bioscience).

Transwell assay

Matrigel (500 ng/μL, BD Biosciences) was applied or omitted on the transwell chamber (Costar, MA, USA) to analyze cell invasion and migration. The upper chamber received an introduction of HTR8/SVneo cell suspension (1×10^5 cells/mL or 5×10^5 cells/mL, in 150 μL of serum-free medium for migration or invasion), accompanied by the addition of 600 μL of RPMI 1640 medium with 10% FBS in the lower chamber. After 24 hours, cells traversing the membrane were stabilized using paraformaldehyde and colored with 0.1% crystal violet (Beyotime). Subsequently, the dyed cells were recorded in photographs and counted [21].

ELISA

The cells were planted in 6-well plates (Corning), followed by collecting the medium. After centrifuging the gathered medium at 1,000 × g for 20 minutes, TNF-α, IL-1β, and IL-6 were measured using ELISA kits (Ml-bio, Shanghai, China). A microplate reader (MD Spectra Max M3; Molecular Devices, CA, USA) was employed to measure the optical density at 450 nm.

Table 1. Primers for RT-qPCR.

Gene	Sequence (5' - 3')
hsa_circ_0003218	F: TGGAACATACCGTTTCTGCTGT
	R: AACGCACATAGCCGTTCTTG
GAPDH	F: AGGTCGGAGTCAACGGATTT
	R: TGACGGTGCCATGGAATTTG

GAPDH glyceraldehyde 3-phosphate dehydrogenase.

Table 2. Clinical characteristics of normal and GDM groups.

Characteristic	Normal (n = 20)	GDM (n = 20)	p-value
Maternal age (years)	30.05 ± 2.86	31.58 ± 2.79	0.0949
Pre-pregnancy BMI (kg/m^2)	20.54 ± 1.50	21.84 ± 1.67	0.0135
Gestational age (weeks)	38.41 ± 0.95	38.22 ± 0.98	0.5373
FPG-OGTT (mmol/L)	4.56 ± 0.39	5.17 ± 0.52	0.0002
1Hr-OGTT (mmol/L)	7.62 ± 0.77	10.60 ± 1.21	< 0.0001
2Hr-OGTT (mmol/L)	6.40 ± 0.63	8.85 ± 1.04	< 0.0001
Birth weight of newborn (g)	3,325 ± 336.8	3,380 ± 304.6	0.5912

GDM gestational diabetes mellitus, BMI body mass index, FPG fasting plasma glucose, OGTT oral glucose tolerance test, Hr hour.

RT-qPCR

The extraction of total RNA adhered to the guidelines of the Trizol kit (Invitrogen), utilizing the NanoDrop 8000 (Thermo Fisher Scientific) for assessing RNA concentration and purity. Subsequently, 3 μg of RNA underwent reverse transcription into cDNA using either the PrimeScript RT reagent kit or the PrimeScript miRNA cDNA Synthesis Kit (Takara, Japan), succeeded by RT-qPCR employing the SYBR Green PCR Master Mix (Thermo Fisher Scientific). The PCR procedure proceeded under these thermal cycling conditions: starting with denaturation at 95°C for 5 minutes, followed by 45 sequences of 94°C for 10 seconds, primer pair annealing at 55°C for 20 seconds, and 72°C for 30 seconds, culminating in a final elongation at 72°C for 10 minutes. The comparative fold changes were evaluated through the $2^{-\Delta\Delta Ct}$ technique. Sangon Biotech, based in Shanghai, China, manufactured the primers (Table 1).

Western blot

Using radio-immunoprecipitation lysis buffer and phenylmethylsulfonyl fluoride, total protein was extracted, and the concentration was assessed with BCA protein concentration assay kit (Solarbio). Each sample had equal protein amounts (10 - 20 μg) separated via SDS-PAGE using 8%, 12%, and 15% gels, followed by transfer to polyvinylidene fluoride membranes. Following an hour-long blocking using 5% skimmed milk, the membranes underwent exposure to primary antibodies at 4°C throughout the night, then to horseradish peroxidase-linked secondary antibodies (1:3,000, Solarbio) at 37°C for an hour. The membranes were then observed using enhanced chemiluminescence (Solarbio) to generate luminescence. The primary antibodies included NLRP3 (1:1,000, ABclonal, Wuhan, China), MyD88 (1:1,000, ABclonal), TLR4 (1:1,000, ABclonal), ASC (1:1,000, ABclonal), cleaved caspase-1 (1:500, ABclonal), p-NF-κB p65 (1:1,000, Cell Signaling Technology), NF-κB p65 (1:1,000, Cell Signaling Technology, MA, USA), and GAPDH (1:1,000, Abcam, MA, USA).

RNase R digestion

Around 3 μg of total RNA underwent a 20-minute treatment with or without 3 U/μg RNase R (Epicenter, WI, USA) and was then purified using the RNeasy MinElute Cleanup Kit (Qiagen, Tokyo, Japan). The abundance of BMPR2 mRNA and hsa_circ_0003218 was detected by RT-qPCR.

Statistical analysis

By using SPSS software (version 18.0; SPSS, Inc.), statistical analysis was conducted, and data are shown as mean ± standard deviation. To examine differences between multiple groups, one-way ANOVA and Tukey's post-test were applied. The statistical significance between the two groups was assessed using the unpaired

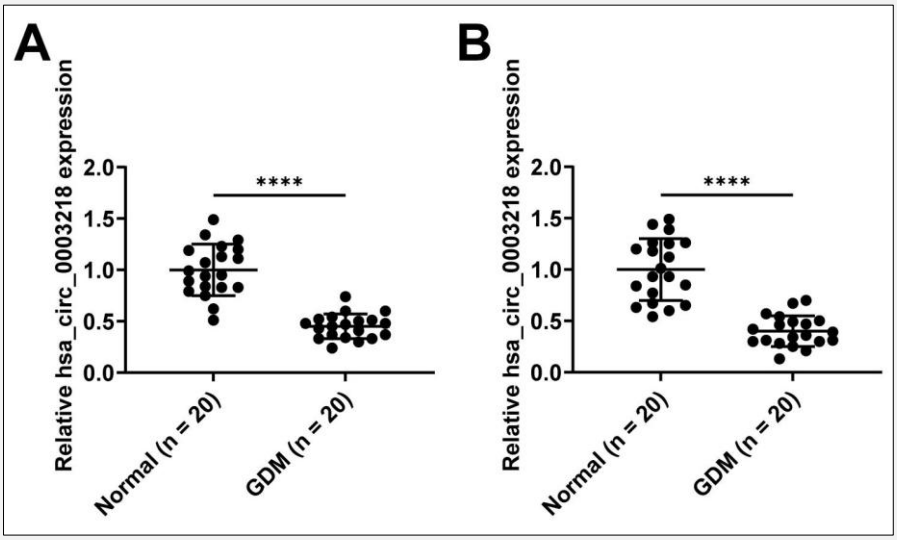

Figure 1. hsa_circ_0003218 expression is reduced in serum and placental tissues of GDM patients.

A/B) RT-qPCR for hsa_circ_0003218 expression.
*** indicates p < 0.05, ** indicates p < 0.01, *** indicates p < 0.001, **** indicates p < 0.0001.**

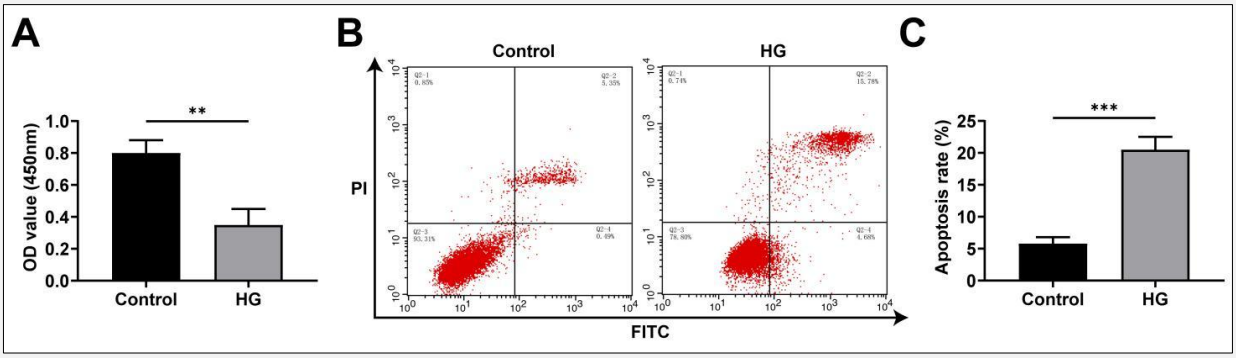

Figure 2. HG inhibits trophoblast proliferation and promotes apoptosis.

A) CCK-8 to detect cell proliferation, B/C) Flow cytometry to detect apoptosis.
*** indicates p < 0.05, ** indicates p < 0.01, *** indicates p < 0.001, **** indicates p < 0.0001.**

Student´s *t*-test. Statistical significance was indicated by $p < 0.05$.

RESULTS

hsa_circ_0003218 expression is reduced in serum and placental tissues of GDM patients

In the GDM group, both blood glucose levels and pre-pregnancy body mass index were greater than those in

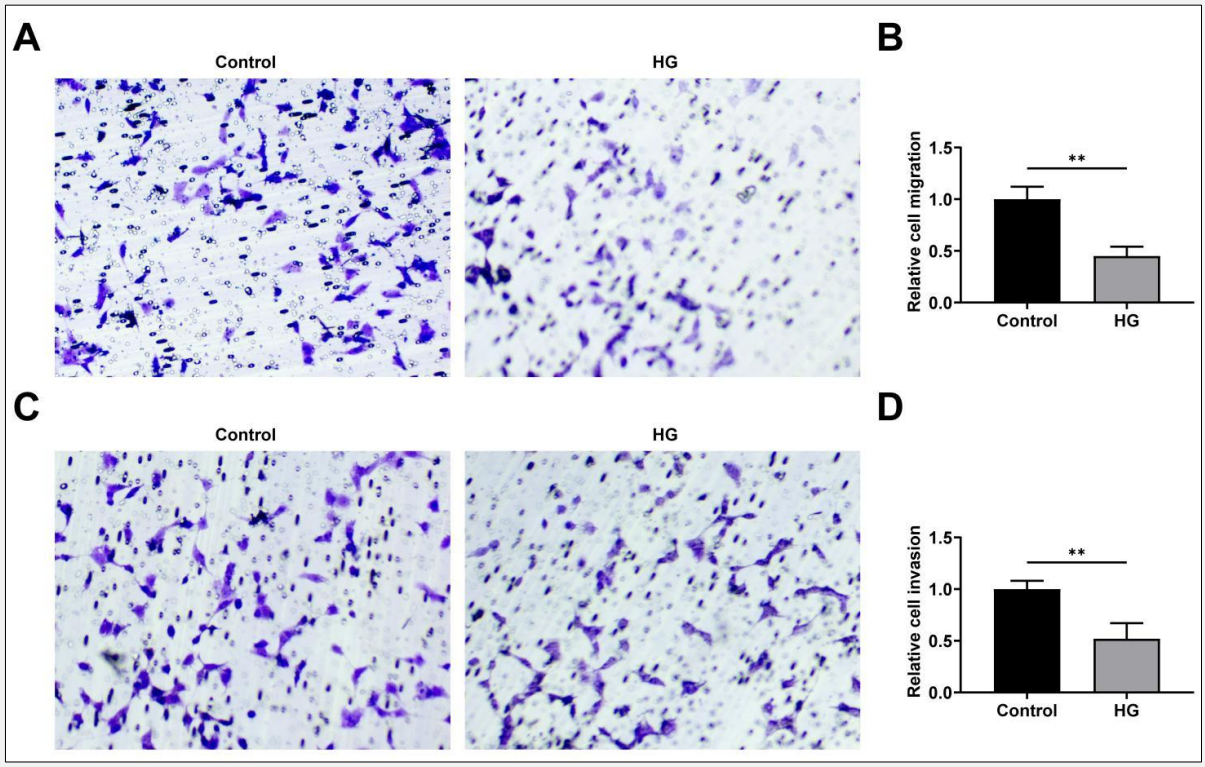

Figure 3. HG inhibits trophoblast migration and invasion.

A - D) Transwell to detect cell migration and invasion.
*** indicates p < 0.05, ** indicates p < 0.01, *** indicates p < 0.001, **** indicates p < 0.0001.**

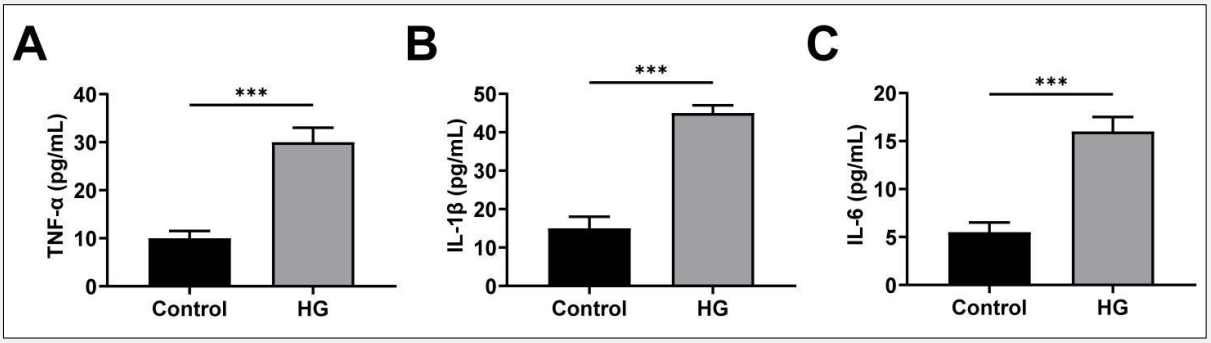

Figure 4. HG promotes trophoblast inflammation.

A - C) ELISA to detect inflammatory factors (TNF-α, IL-1β, and IL-6).
*** indicates p < 0.05, ** indicates p < 0.01, *** indicates p < 0.001, **** indicates p < 0.0001.**

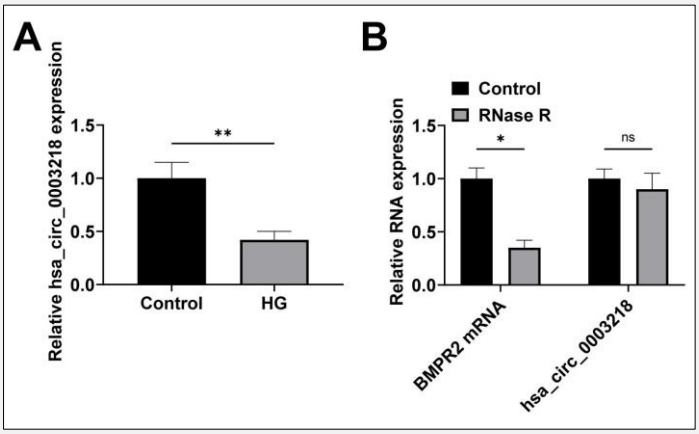

Figure 5. hsa_circ_0003218 expression is downregulated in HG-treated trophoblasts.

A) RT-qPCR for hsa_circ_0003218 expression, B) RNase R digestion experiment.
*** indicates p < 0.05, ** indicates p < 0.01, *** indicates p < 0.001, **** indicates p < 0.0001.**

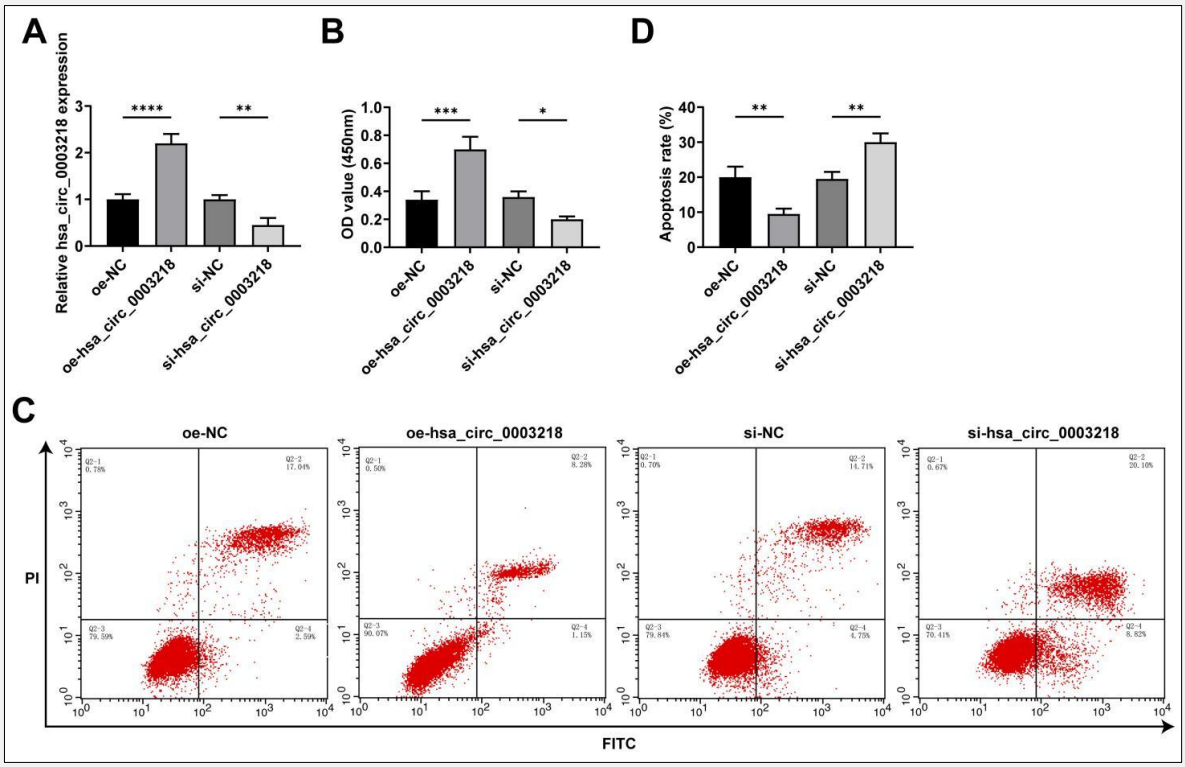

Figure 6. Upregulation of hsa_circ_0003218 ameliorates HG-induced trophoblast dysfunction.

A) RT-qPCR for hsa_circ_0003218 expression, B) CCK-8 to detect cell proliferation, C/D) Flow cytometry to detect apoptosis.
*** indicates p < 0.05, ** indicates p < 0.01, *** indicates p < 0.001, **** indicates p < 0.0001.**

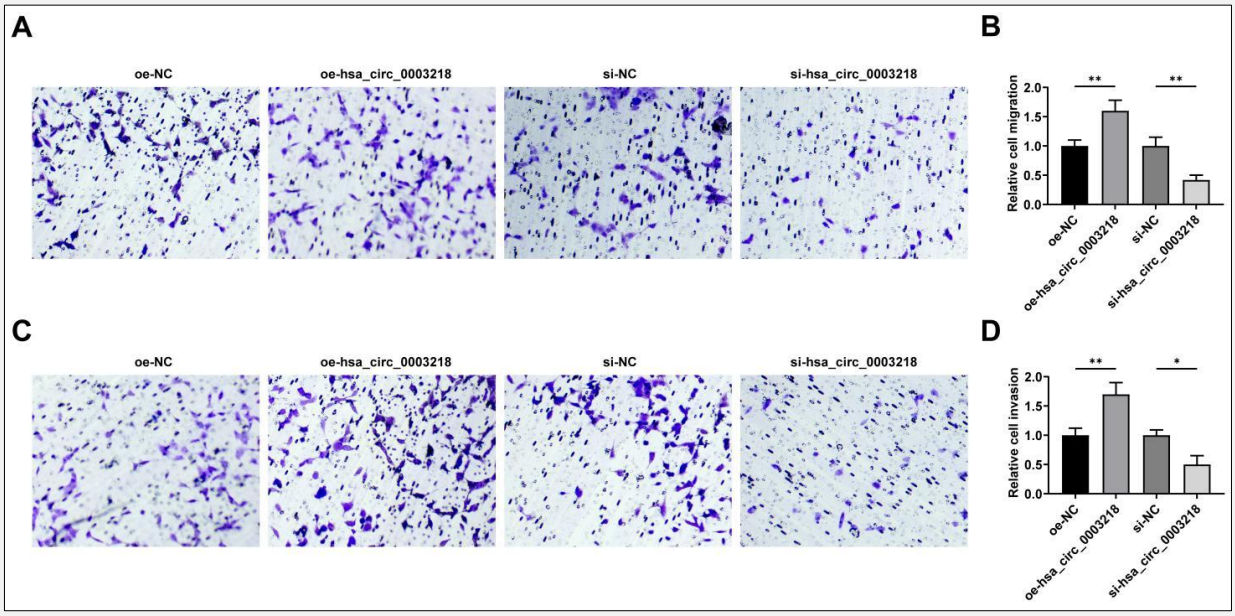

Figure 7. Upregulation of hsa_circ_0003218 ameliorates HG-induced trophoblast dysfunction.

A - D) Transwell to detect cell migration and invasion.
*** indicates p < 0.05, ** indicates p < 0.01, *** indicates p < 0.001, **** indicates p < 0.0001.**

the normal group (Table 2). RT-qPCR results exhibited that hsa_circ_0003218 expression was reduced in serum and placental tissues of GDM patients (Figure 1A, B).

HG inhibits trophoblast proliferation, migration, and invasion and promotes apoptosis and inflammation

HTR8/SVneo cells underwent glucose exposure to replicate T2DM conditions. HG was shown to inhibit the proliferation of HTR8/SVneo cells as detected by CCK-8 (Figure 2A). Flow cytometry detected cell apoptosis, revealing that HG increased apoptosis in HTR8/SVneo cells (Figure 2B, C). Moreover, Transwell analysis demonstrated that HG impeded HTR8/SVneo cell migration and invasion (Figure 3A - D). HG also resulted in higher levels of TNF-α, IL-1β, and IL-6 in the supernatants from HTR8/SVneo cell cultures (Figure 4A - C).

hsa_circ_0003218 expression is downregulated in HG-treated trophoblasts

RT-qPCR results exhibited that hsa_circ_0003218 expression was downregulated in HG-treated HTR8/SVneo cells (Figure 5A). In addition, the circular property of hsa_circ_0003218 was studied through RNase R digestion, and the findings indicated that hsa_circ_0003218 resisted RNase R digestion, in contrast to the significant reduction of linear BMPR2 (Figure 5B).

Upregulation of hsa_circ_0003218 ameliorates HG-induced trophoblast cell dysfunction

Given that hsa_circ_0003218 is lowly expressed in HG-treated trophoblasts, it is hypothesized that hsa_circ_0003218 regulates trophoblast function during GDM. To test this hypothesis, oe-hsa_circ_0003218, oe-NC, si-hsa_circ_0003218, or si-NC were transfected into HTR8/SVneo cells, and then the transfected HTR8/SVneo cells were subjected to HG treatment. Transfecting with oe-hsa_circ_0003218 diminished the inhibitory effect of HG on hsa_circ_0003218 expression, while transfecting with si-hsa_circ_0003218 further inhibited hsa_circ_0003218 expression (Figure 6A). According to CCK-8 and flow cytometry results, overexpressing hsa_circ_0003218 counteracted the reduction in proliferation and rise in apoptosis of HTR8/SVneo cells caused by HG, whereas knocking down hsa_circ_0003218 had the opposite effect (Figure 6B - D). Meanwhile, transwell experiments confirmed that upregulation of hsa_circ_0003218 lessened the HG-induced inhibition of HTR8/SVneo cell migration and invasion, while downregulation of hsa_circ_0003218 intensified this inhibition (Figure 7A - D). Furthermore, the inflammatory factors induced by HG were reduced following the upregulation of hsa_circ_0003218 but increased when hsa_circ_0003218 was downregulated (Supplementary Figure 1A - C).

hsa_circ_0003218 overexpression inhibits HG-induced TLR4/MyD88/NF-κB cascade activation in trophoblasts

Further exploration was carried out on the regulatory mechanism of hsa_circ_0003218. Western blot results exhibited that TLR4, MyD88, and p-NF-κB p65 proteins were elevated in HG-treated HTR8/SVneo cells (Supplementary Figure 2A - D). Elevating hsa_circ_0003218 decreased TLR4, MyD88, and p-NF-κB p65 proteins induced by HG, whereas reducing hsa_circ_0003218 increased these levels (Supplementary Figure 3A - D).

hsa_circ_0003218 inhibits HG-induced NLRP3 inflammasome activation in trophoblast cells

The role of hsa_circ_0003218 in NLRP3 inflammasomes was also examined. According to Western blot results, HG-treated HTR8/SVneo cells exhibited elevated NLPR3, ASC, and cleaved caspase 1 protein expression (Supplementary Figure 4A, B). hsa_circ_0003218 overexpression decreased the HG-induced NLPR3, ASC, and cleaved caspase 1 protein expression levels, while downregulating hsa_circ_0003218 further increased their expression (Supplementary Figure 5A, B).

DISCUSSION

The placenta serves as the connection between mother and fetus, supporting intrauterine life and offering nutritional, hormonal, and immune functions to aid fetal growth [22]. The placenta is now more recognized as a target organ of GDM, with maternal hyperglycemia in GDM impacting its structure and potentially causing functional changes that could lead to fetal malformation and miscarriage [23,24]. Proper functioning of trophoblasts is crucial for placenta development, and issues with cell migration, invasion, and growth inhibition might contribute to placental maldevelopment [20]. circRNAs have been recognized as being involved in trophoblast dysfunction [21,25].

In this study, hsa_circ_0003218 was lowly expressed in placental tissues from GDM patients and HG-treated trophoblast cells. HG treatment hindered trophoblast proliferation, migration, and invasion and facilitated apoptosis and the generation of inflammatory factors. Importantly, hsa_circ_0003218 overexpression significantly reversed HG-induced trophoblast dysfunction. Therefore, it was concluded that hsa_circ_0003218 ameliorated HG-induced trophoblast dysfunction, proposing that hsa_circ_0003218 may have a protective role in GDM progression.

Evidence points to the possibility that inflammatory cytokines activated downstream of TLR4 are associated with GDM pathogenesis [26-28]. As a member of the white matter family, TLR can be utilized to prevent diseases and epidemics, serving as a bridge between innate and acquired immune responses [29]. TLR4 is the most significant receptor for mediating the response to endotoxins/lipopolysaccharides and the release of inflammatory cytokines [30]. Downstream target factors, such as NF-κB, are activated by TLR4 through the MyD88-mediated cascade, and NF-κB is transported to the nucleus where it induces the expression of specific genes, resulting in the production of cytokines like IL-1β, IL-6, and TNF-α, which regulate cell proliferation and differentiation [31].The TLR4/MyD88/NF-κB axis is essential for controlling GDM, as demonstrated [32,33]. The innate immune system heavily depends on the NLRP3 inflammasome, composed of NLRP3, ASC, and procaspase-1 [34]. NLRP3 inflammasome activation by a range of endogenous and exogenous pattern receptors results in the release of mature IL-1β and IL-18, which then regulate inflammatory diseases [35,36]. Earlier research has shown a significant rise in the NLRP3 inflammasome in the placentas of individuals with GDM, indicating a strong link between the activated NLRP3 inflammasomes and GDM development [37-39]. In this study, TLR4/MyD88/NF-κB cascade- and NLRP3 inflammasome-associated proteins were increased in HG-treated trophoblasts, whereas upregulating hsa_circ_0003218 inhibited HG-induced activation of TLR4/MyD88/NF-κB cascade and NLRP3 inflammasomes in trophoblasts.

However, there are limitations to this study. The functions of the TLR4/MyD88/NF-κB cascade and NLRP3 inflammasomes in trophoblasts exposed to HG were not examined at first. Our conclusions were not validated through animal experiments, and further research is needed to explore the potential role of hsa_circ_0003218 in regulating other cascades.

CONCLUSION

In summary, this study proposes for the first time that hsa_circ_0003218 alleviates HG-induced trophoblast dysfunction through the regulation of the TLR4/MyD88/NF-κB cascade and NLRP3 inflammasomes. This research offers fresh perspectives on targeted treatment approaches for patients with GDM.

Availability of Data and Materials:

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Ethical Approval Statement:

The present study was approved by the Ethics Committee of the Fourth Hospital of Shijiazhuang (No. 20230047), and written informed consent was provided by all patients prior to the study start. All procedures were performed in accordance with the ethical standards of the Institutional Review Board and the Declaration of Helsinki and its later amendments or comparable ethical standards.

Declaration of Interest:
The authors have no conflicts of interest to declare.

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