

Expression and function of lncRNA MALAT1 in gestational diabetes mellitus

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D – writing the article; E – critical revision of the article; F – final approval of the article

Advances in Clinical and Experimental Medicine, ISSN 1899–5276 (print), ISSN 2451–2680 (online)

Adv Clin Exp Med. 2020;29(8):903–910

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Funding sources

None declared

Conflict of interest

None declared

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Received on October 22, 2018

Reviewed on January 24, 2019

Accepted on April 30, 2020

Published online on August 12, 2020

Abstract

Background. Gestational diabetes mellitus (GDM) severely threatens maternal and fetal health. Long non-coding RNA (lncRNA) participates in the regulation of various cellular processes.

Objectives. Previous studies have identified the role of lncRNA MALAT1 in diabetic retinopathy-related inflammation. However, the role of lncRNA MALAT1 in GDM has not been reported yet.

Material and methods. Real-time polymerase chain reaction (RT-PCR) was used to measure the lncRNA MALAT1 expression level in placental tissues from GDM patients and from a normal pregnant group. Placental trophoblastic-derived cell line HTR8 cells were divided into a control group, an siRNA negative control group and a MALAT1 siRNA group. The cells underwent RT-PCR analysis of lncRNA MALAT1 expression, an MTT assay of cell proliferation, and a transwell assay of cell invasion and migration. In addition, enzyme-linked immunosorbent assay (ELISA) was used to analyze the level of tumor necrosis factor α (TNF- α) and interleukin 6 (IL-6). Western blotting was used to measure the changes of the tumor growth factor β (TGF- β)/nuclear factor-kappa B (NF- κ B) signaling pathway.

Results. Gestational diabetes mellitus placental tissues showed higher lncRNA MALAT1 expression compared to a normal control group ($p < 0.05$). After siRNA intervention, lncRNA MALAT1 showed decreased expression in the trophoblastic layer; inhibited trophoblastic cell proliferation, migration, or invasion; decreased the secretion of inflammatory factors TNF- α and IL-6; and suppressed the expression of TGF- β and NF- κ B compared to that of the control and siRNA-NC groups ($p < 0.05$).

Conclusions. Gestational diabetes mellitus appears to upregulate lncRNA MALAT1. Downregulation of lncRNA MALAT1 inhibits inflammation and suppresses the proliferation, invasion and migration of GDM placental trophoblastic cells, possibly by modulating the TGF- β /NF- κ B signaling pathway.

Key words: gestational diabetes mellitus, lncRNA MALAT1, TGF- β /NF- κ B signal pathway, cell proliferation, cell migration

Cite as

Zhang Y, Qu L, Ni H, et al. Expression and function of lncRNA MALAT1 in gestational diabetes mellitus. *Adv Clin Exp Med*. 2020;29(8):903–910. doi:10.17219/acem/121524

DOI

10.17219/acem/121524

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Introduction

Gestational diabetes mellitus (GDM) is frequently caused by abnormal glucose resistance or impairment during the gestational period, and mainly presents as elevated fasting blood glucose and insulin resistance, accompanied with inflammation, increased secretion of inflammatory factors and abnormal body immunity.^{1,2} Gestational diabetes mellitus is a unique subtype of diabetes which occurs in pregnant women worldwide.³ With differential incidence, European countries usually have a GDM prevalence of about 4% and China now has a GDM incidence of about 3%. With lifestyle change and more focus on gestational nutrition, GDM incidence has been continuously increasing over the years.^{4,5} Gestational diabetes mellitus patients have elevated glucose levels and glucose catabolic disorder, caused by insulin resistance and leading to cellular glucose metabolic disorder. On the other hand, GDM can cause increased production of reactive oxygen species (ROS) and oxygen free radicals, further leading to oxidative stress and maternal/fetal injury. Elevated blood glucose during pregnancy can cause vascular disease, impair vascular endothelial proliferation and inhibit placental angiogenesis, leading to abnormal placenta blood circulation and placental abnormality caused by ischemia/hypoxia as the result of pathological changes in the placental cells.^{6–8} In short, GDM can cause placental abruption, gestational hypertension, premature delivery, or congenital abnormality, leading to severe and long-lasting threats to maternal/fetal health.^{9,10} Recent studies have found that long noncoding RNA (lncRNA) participates in the regulation of various cellular processes. For example, lncRNA MALAT1 has been shown to play roles in diabetic retinopathy and related inflammation.¹¹ However, whether lncRNA MALAT1 plays a role in the development and pathogenesis of GDM remains poorly understood. In the present study, we aimed to uncover the role of lncRNA MALAT1 in GDM and to provide a theory for a novel therapeutic target in the treatment of GDM.

Material and methods

Research subject recruitment

A total of 78 women with a confirmed diagnosis of GDM in Yantai Yuhuangding Hospital (China) between June 2016 and December 2017 were selected for the study. All GDM patients were diagnosed with an oral glucose tolerance test (OGTT) from gestation weeks 24 to 28. All patients matched the diagnosis criteria of GDM as stipulated by the World Health Organization (WHO) in 2013.⁹

The exclusive criteria included¹⁰ multiple pregnancy; incompatibility of maternal and fetal blood types; history of surgical operations (including laparoscopic surgery, open surgery and keyhole surgery); complications such as inflammation, malignant tumor, severe diabetes, organ failure, systemic immune disorder, or malignant tumor complications. Furthermore, women who were receiving chemotherapy or radiotherapy or had other gestational complications – such as hypertension, exposure to toxic substances during pregnancy, or an abnormal placental or umbilical cord – were also excluded from the study. A cohort of 38 normal pregnant women was recruited as the normal control group. Informed consent was obtained from all participants prior to the study. This study has been approved by the ethical committee of Yantai Yuhuangding Hospital (China). All placental samples were collected from the maternal side of the placenta after delivery. Small tissue cubes were collected and kept in cryopreserved tubes in liquid nitrogen.

Major equipment and reagents

Placental trophoblastic derived cell line HTR8 was purchased from ATCC (CRL-3271; Manassas, USA). An RNA extraction kit and reverse transcription kit were purchased from R&D Systems, Inc. (Minneapolis, USA). Other common reagents were purchased from Sangon Biotech (Shanghai, China). Real-time polymerase chain reaction (RT-PCR) reagent was purchased from Thermo Fisher (Waltham, USA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS) and penicillin-streptomycin were purchased from Hyclone (Chicago, USA). Dimethyl sulfoxide (DMSO) and MTT powder were purchased from Gibco (Waltham, USA). Trypsin-EDTA digestion buffer was purchased from Hyclone. Western blot reagents were purchased from Beyotime (Haimen, China). Enhanced chemiluminescence (ECL) reagent was purchased from Amersham Biosciences (Little Chalfont, UK). Rabbit anti-human nuclear factor-kappa B (NF- κ B) monoclonal antibody, rabbit anti-human tumor growth factor β (TGF- β) monoclonal antibody, and mouse anti-rabbit horseradish peroxidase (HRP)-conjugated IgG secondary antibody were purchased from Cell Signaling Technology (Leiden, the Netherlands). Enzyme-linked immunosorbent assay (ELISA) kits for measuring the level of tumor necrosis factor α (TNF- α) and interleukin 6 (IL-6) were purchased from Cell Signaling Technology. siRNA was synthesized by Gimma Gene (Shanghai, China). A transwell chamber was purchased from Corning (New York, USA). A RT-PCR cyclor was purchased from Applied Biosystems (Waltham, USA). A Gene Amp PCR System 2400 DNA amplification cyclor was purchased from Perkin Elmer (Waltham, USA). A 371 CO₂ cell incubator was purchased from Thermo Fisher.

HTR8 cell culture and grouping

An HTR8 cell line kept in liquid nitrogen was resuscitated and cultured for passage. Cells in log-growth phase at generations 3–8 were used for assays. The HTR8 cells were kept in 90% high-glucose (25 mmol/L) complete medium plus 10% FBS for attached growth. The cells were then randomly assigned into 3 groups: a control group, which was cultured under normal conditions; an siRNA-NC group that was transfected with lncRNA MALAT1 negative control (NC) plasmid; and an siRNA group which received lncRNA MALAT1 siRNA transfection.

Liposome transfection of lncRNA MALAT1 siRNA into HTR8 cells

HTR8 cells were transfected with lncRNA MALAT1 siRNA or siRNA-NC. The sequence for lncRNA MALAT1 siRNA was 5'-GAGGA GUGCA GUUCU UCA-3', and the sequence for siRNA-NC was 5'-GACAG UGUGA UUCUA-3'. The cells were seeded into 6-well plates and cultured until 70–80% confluence was reached. Plasmid for lncRNA MALAT1 siRNA or NC control was added into 200 µL of serum-free DMEM for 15 min at room temperature for incubation, which was added into a dilution buffer along with Lipo2000 reagent and incubated for 30 min at room temperature. Serum was removed from the culture medium, and the cells were rinsed gently in PBS. Another 1.6 mL of serum-free DMEM was added into the system, and the cells were incubated in a chamber at 37°C with 5% CO₂ for 6 h. Serum-containing DMEM medium was then used for 48-hour continuous incubation for the assays.

RT-PCR for lncRNA MALAT1 in GDM placental tissues and HTR8 cells

Trizol reagent was used to extract total RNA from the placental tissues of GDM patients or normal placental tissues, or from HTR8 cells in all groups. The RNA was then reverse transcribed into cDNA according to the instructions of the test kit. The primers were designed using PrimerPremier v. 6.0 (Premier Biosoft, San Francisco, USA) based on the target gene sequence, and they were synthesized by Invitrogen (Carlsbad, USA), as shown in Table 1. Real-time PCR was performed on the target genes under the following conditions: 55°C for 1 min, followed by 35 cycles consisting of 92°C for 30 s, 58–60°C for 45 s, and 72°C for 45 s. Data was collected to calculate the threshold

cycle (Ct) values of all samples and standard samples using fluorescent quantification. Based on the CT values of the standard samples, the standard curve was plotted and semi-quantitative analysis was performed using the 2^{-ΔCt} method.

MTT assay for cell proliferation of all groups

HTR8 cells in the log-growth phase were inoculated into 96-well plates using DMEM medium with 10% FBS at a density of 5 × 10³/well. After 24 h of incubation, the supernatant was discarded and all wells were filled with 20 µL of sterile MTT. Three replicated wells were performed at each time point. After 4 h of continuous incubation, the supernatant was discarded and 150 µL of DMSO was added into each well for 10 min of vortex mixing. After complete resolution of violet crystals, absorbance (A) values were measured at a wavelength of 570 nm under a microplate reader in order to calculate the proliferation rate of all groups.

Transwell chamber assay for measuring invasion from all groups of cells

A transwell invasion assay was performed according to the instruction manual. Serum-free medium was used. After 24 h, the transwell chamber was pre-coated with 1:5 50 mg/L Matrigel dilution buffer on the chamber bottom and the upper phase of the membrane, and the chamber was air-dried at 4°C. The interior of the chamber was filled with 500 µL of DMEM containing 10% FBS, and the exterior was filled with 100 µL of serum-free medium with the prepared cell suspensions. Three replicated wells were used for each group. Transwell chambers were placed into a 24-well plate. A Matrigel-free chamber was employed for incubation of the control group. After 48 h, the transwell chamber was rinsed in PBS, and the cells on the membrane were removed. The chamber was fixed with iced ethanol and stained with crystal violet. Cells at the lower phase of the membrane were enumerated and the experiment was repeated 3 times.

Transwell chamber for cell migration

The cell migration of all groups of cells was measured in serum-free medium using the transwell chamber according to the instructions of the test kit.

Table 1. Primer sequence

Gene	Forward primer 5'-3'	Reverse primer 5'-3'
GAPDH	AGTAGTCACCTGTTGCTGG	TAATACGGAGACCTGTCTGGT
lncRNA MALAT1	A CTTACATGTCGCC TTGG	TCAAAGCTGGTACAGCCA

Western blot for TGF- β /NF- κ B signal pathway activity

Total proteins were extracted from all groups of HTR8 cells. In brief, lysis buffer was added and proteins were quantified using the Bradford method at -20°C storage for western blot. The proteins were separated using 10% SDS-PAGE and transferred to the NC membrane at 100 mA for 1.5 h. Primary antibody (TGF- β diluted to 1:2000 and NF- κ B monoclonal antibody diluted to 1:2000) was added for overnight incubation at 4°C . The following day, the membrane was rinsed in phosphate-buffered saline with Tween (PBST) and was incubated in goat anti-rabbit secondary antibody diluted to 1:2000 for 30 min at room temperature in the dark. After PBST rinsing, ECL substrate was added for development for 1 min, followed by X-ray exposure. Protein imaging software and Quantity One software (Bio-Rad, Hercules, USA) were used to scan the X-ray film and measure the band density. All experiments were repeated 4 times ($n = 4$) for statistical analysis.

ELISA for inflammatory cytokine expression in cell culture supernatant

All samples were measured by the expression of inflammatory cytokines TNF- α and IL-6 in the supernatant from the cell cultures using ELISA kits and following the test kit manuals. In brief, a 96-well plate was filled with 50- μL serially diluted standard samples to prepare the standard curve. Fifty-microliter test samples were added to the wells in triplicate. The plate was then washed and dried. Each well was filled with dilution buffer for washing in a vortex for 30 s. Each well then had 50 μL of enzyme-labelled reagent added to it, except for the blank control well. After gentle mixing and incubation at 37°C for 30 min, the plate was rinsed 5 times. Chromogenic substrate A and B (50 μL each) were subsequently added into each well for gentle mixing and 37°C incubation for 10 min. Fifty microliters of quenching buffer was added into each well in order to stop the reaction. The optical density (OD) values of each well were measured at a wavelength of 450 nm using a microplate reader.

Statistical processing

SPSS v. 16.0 (SPSS Inc., Chicago, USA) software was used for statistical analysis. The measurements are presented as means \pm standard deviation (SD). One-way analysis of variance (ANOVA) was used for comparisons of the means among multiple groups. Statistically significant values were recognized when $p < 0.05$.

Results

Characteristics of patients and controls

All patients were aged between 22 and 41 years (average age: 27 ± 5.2 years) with a body mass index (BMI) of 24.5 ± 3.2 . The controls – 38 normal pregnant women – were aged between 21 and 42 years (average age: 26 ± 5.1 years) and had a BMI of 25.1 ± 4.6 . No significant differences were observed regarding age or BMI between the control and GDM groups.

Expression of lncRNA MALAT1 in GDM

Real-time PCR was used to measure the expression of lncRNA MALAT1 in GDM, which showed that it was significantly higher in placental tissues than in normal pregnant placentas ($p < 0.05$; Fig. 1).

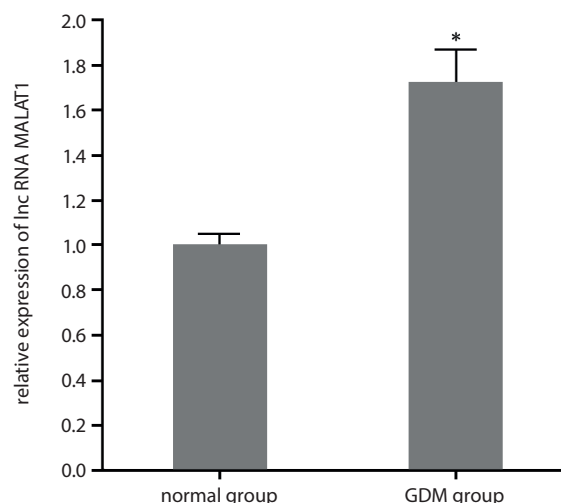


Fig. 1. Expression of lncRNA MALAT1 in GDM

* $p < 0.05$ compared to the control group.

Effects of siRNA transfection on the expression of lncRNA MALAT1 in trophoblastic cell HTR8

Compared to the control group and the siRNA-NC group, the HTR8 cells had significantly decreased expression of lncRNA MALAT1 thanks to transfection of lncRNA MALAT1 siRNA ($p < 0.05$; Fig. 2).

Effects of lncRNA MALAT1 knockdown on HTR8 cell proliferation

To investigate the effect of lncRNA MALAT1 on the proliferation of trophoblastic HTR8 cells, we performed an MTT assay and found that the transfection of lncRNA MALAT1 siRNA for 48 h significantly suppressed the proliferation of HTR8 cells in comparison to the control group ($p < 0.05$; Fig. 3), indicating that downregulation of lncRNA MALAT1 could inhibit HTR8 cell proliferation.

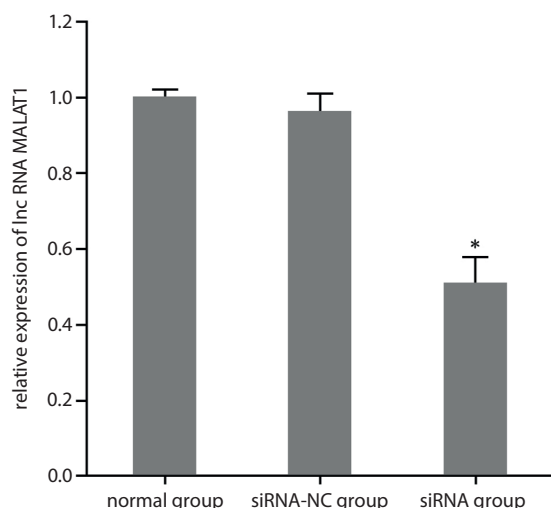


Fig. 2. Expression of lncRNA MALAT1 in trophoblastic cell line HTR8

* $p < 0.05$ compared to the control group.

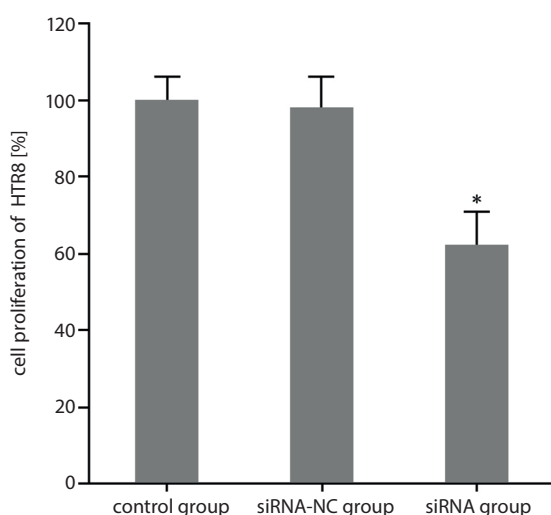


Fig. 3. Effects of lncRNA MALAT1 knockdown on the proliferation of trophoblast cell line HTR8

* $p < 0.05$ compared to the control group.

Effect of lncRNA MALAT1 knockdown on migration of trophoblast HTR8 cells

We next investigated the effect of lncRNA MALAT1 on the migration of trophoblastic HTR8 cell using a transwell assay and found that transfection of lncRNA MALAT1 siRNA for 48 h significantly inhibited the migration of HTR8 cells when compared to the control group ($p < 0.05$; Fig. 4).

Effects of lncRNA MALAT1 knockdown on invasion of trophoblast HTR8 cell

We also used the transwell approach to analyze the effect of lncRNA MALAT1 knockdown on the invasion of trophoblastic cell line HTR8 and found that the transfection of lncRNA MALAT1 siRNA significantly inhibited invasion of HTR8 cells after 48 h compared to the control group ($p < 0.05$; Fig. 5).

Effects of lncRNA MALAT1 knockdown on inflammatory cytokine expression in the supernatant of trophoblastic cell line HTR8

The ELISA approach was used to measure the expression of inflammatory cytokines in the supernatant of trophoblastic cell line HTR8 and demonstrated that transfection of lncRNA MALAT1 siRNA for 48 h significantly inhibited TNF- α and IL-6 secretion from HTR8 cells in comparison with the control group ($p < 0.05$; Fig. 6).

Effects of lncRNA MALAT1 knockdown on the TGF- β /NF- κ B signaling pathway

Western blot was performed to observe the effect of lncRNA MALAT1 knockdown on the TGF- β /NF- κ B signaling pathway in trophoblastic cell line HTR8. It revealed that the transfection of lncRNA MALAT1 siRNA significantly reduced the expression of TGF- β and NF- κ B when compared to the control group ($p < 0.05$; Fig. 7).

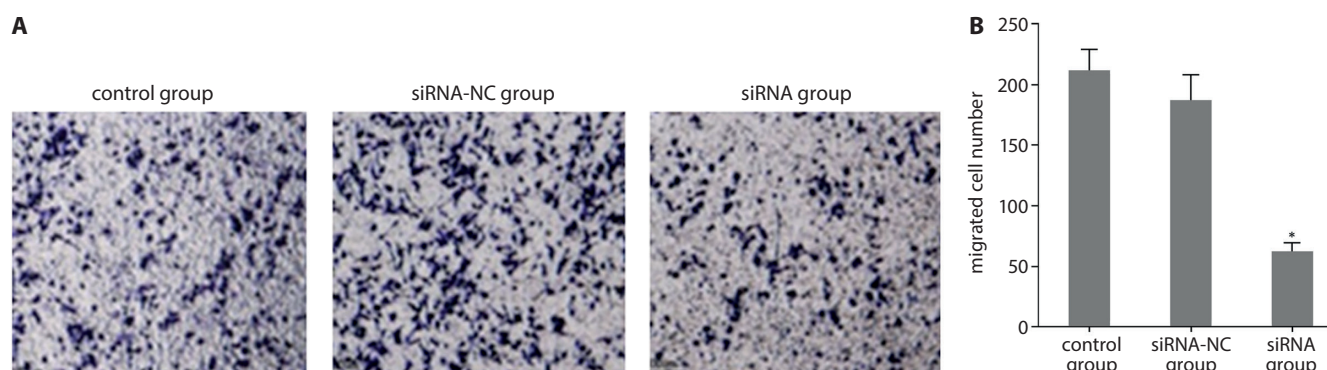


Fig. 4. Effects of lncRNA MALAT1 knockdown on trophoblastic cell HTR8 migration. A. Transwell assay for analyzing the effect of lncRNA MALAT1 knockdown on HTR8 cell migration. B. Analysis for HTR8 cell migration

* $p < 0.05$ compared to the control group.

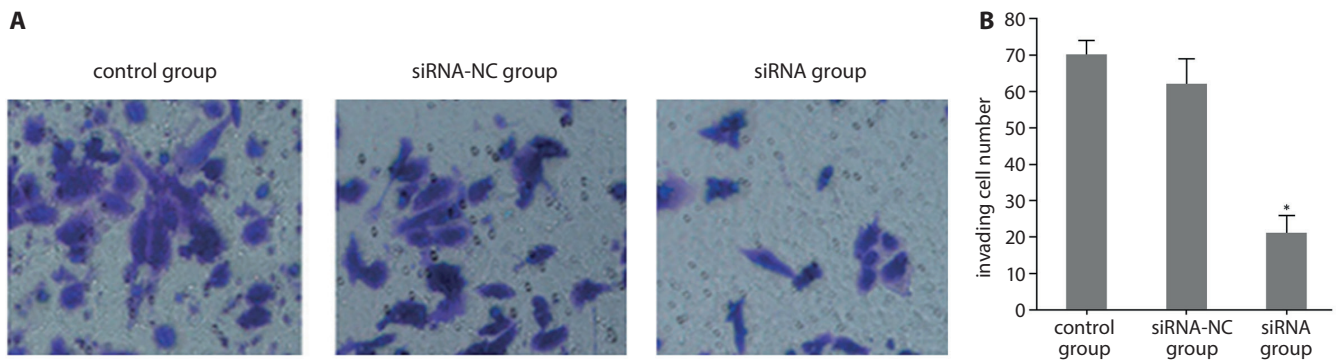


Fig. 5. Effects of lncRNA MALAT1 knockdown on invasion of trophoblast cell HTR8. **A.** Transwell chamber assay for analyzing the effect of lncRNA MALAT1 knockdown on HTR8 cell invasion. **B.** Statistical analysis for HTR8 cell invasion

* $p < 0.05$ compared to the control group.

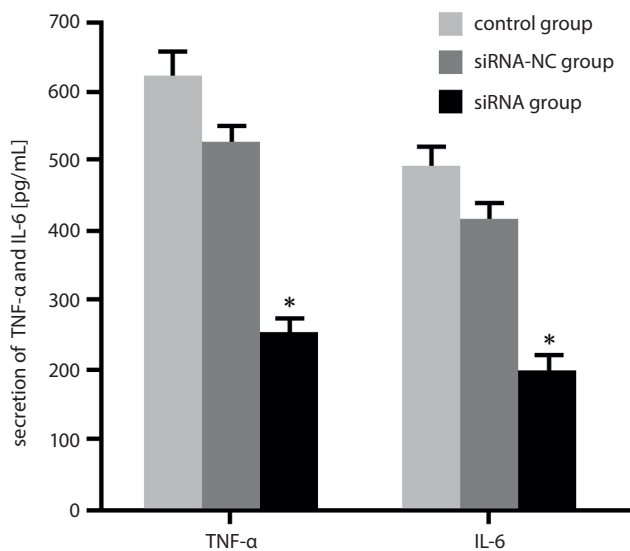


Fig. 6. Effect of lncRNA MALAT1 knockdown on inflammatory cytokine factor of trophoblastic cell line HTR8

* $p < 0.05$ compared to the control group.

Discussion

Long non-coding RNA was discovered several years ago.¹² Large amounts of non-coding RNA transcripts have been found in eukaryotes and they occupy the majority of the human genome.^{13,14} Long non-coding RNA refers to those transcripts longer than 200 nt. Due to the irrelevance to protein coding, lncRNA was initially recognized as “background noise” of gene transcription without any biological function. However, through deeper studies, lncRNA has been found to regulate and participate in gene expression regulation at the epigenetic, transcriptional and post-transcriptional levels.¹⁵ Long non-coding RNA can regulate various physiological events at the genetic level, including chromatin modification, genomic imprinting, nuclear trafficking, chromosomal gene silencing, and transcriptional activation.¹⁶ In addition, lncRNA can regulate both physiological and pathological conditions of cells, including growth, proliferation, cell cycle, and cell apoptosis, making it a critical regulatory factor

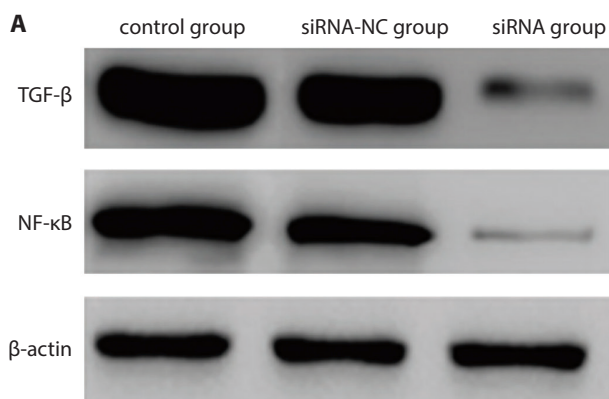
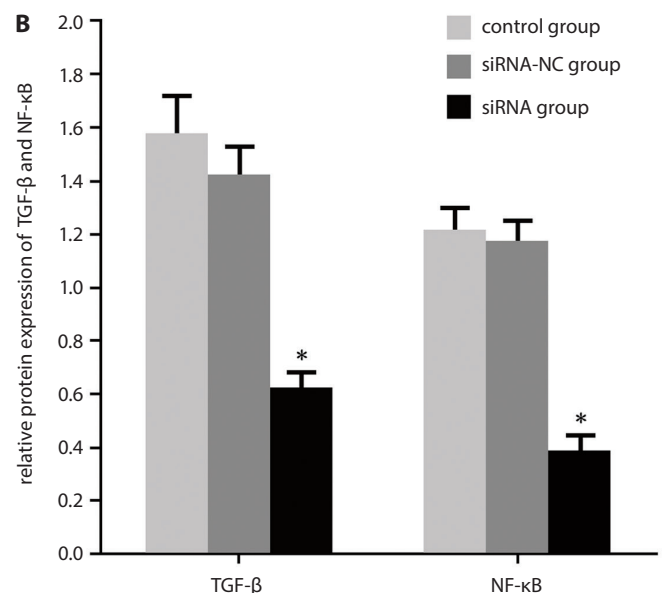


Fig. 7. Effect of lncRNA MALAT1 knockdown on the TGF-β/NF-κB signaling pathway in trophoblastic HTR8 cells. **A.** Western blot for the effect of lncRNA MALAT1 knockdown on the TGF-β/NF-κB signaling pathway in trophoblastic cell line HTR. **B.** Statistical analysis for the TGF-β/NF-κB signaling pathway

* $p < 0.05$ compared to the control group.



for human disease onset and progression.¹⁷ However, few studies have been performed regarding its role in GDM. In diabetes, lncRNA MALAT1 has been found to be abnormally expressed,¹¹ though the exact role and function of lncRNA MALAT1 in GDM remains poorly understood. This study aimed to investigate the expression of lncRNA MALAT1 in GDM placental tissues. Its expression was found to be upregulated in GDM, indicating that it might be involved in GDM pathogenesis and may affect maternal/fetal health.

We further analyzed the role of lncRNA MALAT1 in GDM and its functional mechanism. As the only maternal–fetal interface and the site for exchange of substances, the placenta is critical for maintaining and protecting normal fetal development.¹⁸ The placenta can provide a route for the transportation of nutrients between maternal and fetal bodies, and it is an important organ for gas exchange, blood circulation and endocrine function. The placenta is also important for investigating glucose metabolism and endocrine disease.^{19,20} This study thus selected placental trophoblastic cell line HTR8, which was cultured under a high-glucose environment to mimic the conditions of the GDM placental trophoblastic layer. To investigate the role and function of lncRNA MALAT1 in GDM, we transfected lncRNA MALAT1 siRNA into placental trophoblastic cell HTR8 under high-glucose conditions and found that lncRNA MALAT1 downregulation in placental trophoblastic HTR8 cells under high-glucose conditions inhibited the proliferation, migration and invasion potency of HTR8 cells. These results indicate that lncRNA MALAT1 might affect placental functions in GDM by regulating the biological behaviors of placental trophoblastic cells. On the other hand, pro-inflammation in GDM frequently leads to disease progression and body immune disorder.²¹ This study also demonstrated that lncRNA MALAT1 downregulation can inhibit inflammation, possibly by suppressing the secretion of inflammatory factors. The TGF- β /NF- κ B signaling pathway has been demonstrated to play a role in transcriptional regulation, it is closely related with cell ontogenesis, invasion and metastasis, it can regulate the cytokine network, and it is closely correlated with GDM pathogenesis.²² The findings of this study are consistent with this, showing that the downregulation of lncRNA MALAT1 inhibited the TGF- β /NF- κ B signaling pathway, suggesting that lncRNA MALAT1 affects the biological behaviors of HTR8 cells, possibly through regulating the TGF- β /NF- κ B signaling pathway. However, the exact mechanism by which lncRNA MALAT1 regulates the TGF- β /NF- κ B signaling pathway remains unclear. In addition, the exact molecular mechanism by which lncRNA MALAT1 is involved in the pathogenesis of GDM remains poorly understood. Future studies are required to investigate these issues in order to provide more evidence for GDM pathogenesis and clinical treatment.

Conclusions


The GDM patients present upregulation of lncRNA MALAT1. Downregulation of lncRNA MALAT1 inhibits the proliferation, invasion and migration of GDM placental trophoblastic cells, possibly by modulating the TGF- β /NF- κ B signaling pathway.


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