Mechanism associated with aberrant lncRNA MEG3 expression in gestational diabetes mellitus

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Abstract. Gestational diabetes mellitus (GDM) is a common metabolic condition during pregnancy. Long non-coding RNAs (lncRNAs) have been found to serve critical roles in GDM development; however, the role of lncRNA maternally expressed gene 3 (MEG3) in GDM remains unclear. Therefore, the aim of the present study was to investigate the expression and role of MEG3 in GDM, and to further explore the underlying mechanism. The levels of lncRNA MEG3 in the blood and placental villous tissues of pregnant women with GDM was measured using reverse transcription-quantitative PCR. Bioinformatics analysis and dual luciferase reporter assays were performed to investigate the association between lncRNA MEG3 and microRNA (miR)-345-3p. Transfection was subsequently performed on HTR-8/SVneo cells, a human chorionic trophoblast cell line, to assess the role of lncRNA MEG3 in GDM. In particular, cell viability, cellular migratory/invasive ability and cell apoptosis were analyzed using MTT assay, Transwell assay and flow cytometry, respectively. Compared with pregnant women without GDM, lncRNA MEG3 levels were significantly elevated in the blood and placental villous tissues of GDM pregnant women. miR‑345‑3p was identified to be a direct target of lncRNA MEG3 using dual luciferase reporter assay, which was found to be reduced in pregnant women with GDM. Further analysis demonstrated that lncRNA MEG3 overexpression significantly inhibited HTR‑8/SVneo cell viability, and prevented cell migration and invasion in addition to inducing cell apoptosis. In contrast, lncRNA MEG3 knockdown significantly increased HTR-8/SVneo cell viability, promoted cell migration/invasion and reduced cell apoptosis. Inhibiting miR-345-3p expression negated all the observed physiological effects of lncRNA MEG3 knockdown on HTR-8/SVneo cells. In conclusion, lncRNA MEG3 levels were abnormally upregulated in GDM, which participated in the development and progression of GDM by regulating human chorionic trophoblast cell physiology. Therefore, lncRNA MEG3 may be a potential diagnostic and therapeutic target for GDM.

Introduction

Gestational diabetes mellitus (GDM) is a common metabolic condition during pregnancy that is characterized by glucose intolerance, leading to varying degrees of hyperglycemia (1,2). GDM accounts for >80% of pregnant women with diabetes, whilst pregnancies complicated by non-gestational diabetes are <20% (3). The incidence of GDM varies around the world due to the use of varying diagnostic criteria and environmental factors (4,5). However, incidences of GDM in China had been increasing annually with economic development and changes in lifestyle (6,7). In 2017, 21.3 million or 16.2% of live births had some form of hyperglycaemia in pregnancy with ~85.1% due to GDM. (8). The adverse effects of GDM on pregnant women and infants should not be underestimated as hyperglycemia during pregnancy increases the risk of adverse outcomes in the mother and fetus (9). In particular, infants born from pregnant women with GDM are more susceptible to obesity, metabolic and cardiovascular complications during childhood and adulthood (10,11). In addition, ~50% of women with a history of GDM go on to develop type 2 diabetes within five to ten years post-delivery (8). Therefore, GDM research has become a subject of interest in recent years globally.

Currently, oral glucose tolerance test is a commonly used clinical diagnostic tool for GDM. However, this test is usually performed between 24 and 28 or 32 weeks of gestation (12). This timeframe restricts pregnant women from altering diet and exercise regimens, or to access medical interventions; hence increasing the risk of GDM in late pregnancy. Therefore, there is an urgent demand for developing novel diagnostic approaches for the accurate and rapid prediction of GDM.

Long non-coding RNA (lncRNAs) is a type of functional RNA molecule consisting of >200 nucleotides, which lacks the ability to encode proteins (13). LncRNAs can regulate protein-coding genes in a number of ways, including forming complexes with target genes, as well as promoting the degradation and inhibiting the translation and modification of target microRNAs (miRNA). LncRNAs can regulate the expression of genes at epigenetic, transcriptional and post-transcriptional levels, and are widely involved in most physiological and pathological processes in the body (14). Studies have suggested
that the aberrant expression of lncRNAs is closely associated with the development of complex diseases, including cancer, cardiovascular diseases, nervous system diseases and diabetes (15-18).

LncRNA maternally expressed gene 3 (MEG3) is a known imprinted gene that is ~1.6 kbp in length and located on human chromosome 14q32.2 (19). Imprinting of this gene is controlled by upstream intergenic differentially methylated region (20). In recent years, a number of studies have demonstrated that lncRNA MEG3 is involved in the development and progression of diseases, including retinopathy, osteoarthritis and cardiovascular disease (21-23). It was previously found that lncRNA MEG3 may serve a role in microvascular dysfunction that is associated with diabetes (24). In addition, the upregulation of lncRNA MEG3 promotes insulin resistance in the liver by increasing forkhead box O1 expression (25). In mouse pancreatic β-cells, lncRNA MEG3 promotes the expression of v-maf musculoaponeurotic fibrosarcoma oncogene family protein A and affects insulin production by inhibiting the expression of RAD21 cohesin complex component, structural maintenance of chromosomes 3 or transcriptional regulator SIN3A (26). These studies indicate that lncRNA MEG3 is closely associated with insulin resistance. However, the role of lncRNA MEG3 in the development of GDM and its potential molecular mechanism remain unclear. Therefore, the present study aimed to investigate the expression and role of lncRNA MEG3 in GDM, and to explore any potential underlying mechanism.

Materials and methods

Clinical samples. A total of 20 paired blood samples and matched placental villous tissues were collected from pregnant women between 23 and 37 years old with or without GDM at Weifang People's Hospital (Weifang, China) between May 2015 and May 2017. Fasting peripheral blood was collected from all pregnant women following 28 weeks of gestation, whilst the placental tissues were obtained after delivery. Pregnant women with the following conditions were excluded from the present study: i) Abnormal blood lipid (triglyceride, low-density lipoprotein cholesterol and high-density lipoprotein cholesterol) levels, hypertension, and chronic liver and kidney diseases; ii) endocrine diseases, including, thyroid disease, adrenal cortical disease, obesity, osteoporosis, diabetes and hyperthyroidism prior to pregnancy; iii) pregnant women currently undergoing long-term drug treatments (such as sodium levethoxypine) that affect the metabolism of carbohydrates; and iv) other pregnancy complications (pregnancy-induced hypertension, pre-eclampsia, pregnancy with chronic nephritis). GDM was diagnosed if the fasting plasma glucose (FPG) level was ≥5.1 mmol/l. GDM was excluded if the FPG level was ≤4.4 mmol/l. Women with an FPG level ≥4.4 mmol/l but ≤5.1 mmol/l underwent a 75 g oral glucose tolerance test (OGTT). In such cases, a diagnosis of GDM was made when at least one glucose value was elevated (FPG ≥5.1 mmol/l, 1-h OGTT ≥10.0 mmol/l or 2-h OGTT ≥8.5 mmol/l). Inclusion criteria: Women with DGM who did not fulfill any of the exclusion criteria were involved in the present study. Written informed consent was obtained from each patient and the present study was approved by the Ethics Committee of Weifang People's Hospital.

Cell culture. HTR-8/SVneo cells, a human chorionic trophoblast cell line, were purchased from Shanghai Huzhen Industrial Co., Ltd. (cat. no. HZ-CC337655). Cells were cultured in RPMI 1640 medium (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.) and 1% streptomycin-penicillin solution (Beyotime Institute of Biotechnology), and incubated in a humidified atmosphere under 5% CO₂ at 37°C.

Cell transfection. The MEG3 sequence was synthesized based on the MEG3 sequence and then sub-cloned into the pcDNA3.1 vector (pcDNA-MEG3; Shanghai GeneChem Co., Ltd.). The empty pcDNA3.1 vector was used as a control (pcDNA-control). For MEG3 knockdown, the MEG3-shRNA plasmid (sh-MEG3 sequence, 5'-GAGAGGTTTTT CACTGGTATCTATGGCA-3'; pGFP-C-shLenti Vector; cat. no. TL 320132C) and the scrambled shRNA plasmid (sh-control; pGFP-C-shLenti Vector; cat. no. TR30021) were purchased from OriGene. HTR-8/SVneo cells were seeded into 6-well plates (1x10⁴ cells/well) and cultured at 37°C for 24 h. The cells were subsequently transfected with 100 ng pcDNA-control, 100 ng pcDNA-MEG3, 100 ng sh-control, 100 ng sh-MEG3, 100 nM miR-345-3p inhibitor (cat. no. HmiR-AN0437-AM01; GeneCopoeia, Inc.), 100 nM inhibitor control (cat. no. CmiR-AN0001-SN; GeneCopoeia, Inc.) or 100 ng sh-MEG3 + 100 nM miR-345-3p inhibitor using Lipofectamine® 2000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocols. Transfection efficiency was assessed using reverse transcription-quantitative PCR (RT-qPCR) 48 h after transfection.

Bioinformatics analyses. Bioinformatics analysis (http://starbase.sysu.edu.cn/index.php) (27) was performed to predict potential binding sites between Inc-MEG3 and miR-345-3p using the ‘miRNA-lncRNA’ search function.

Luciferase reporter assay. The putative binding sequences of MEG3-wild type (WT; 5'-CCAGAGCTGTTCA GGG-3') and MEG3-mutant (MUT; 5'-AACCGAGGCG GACAAA-3') were respectively cloned into a pmirGLO vector (Promega Corporation). HTR-8/SVneo cells were co-transfected with 50 nM either miR-345-3p mimics (HmiR0210-MR03; GeneCopoeia, Inc.) or mimic control (cat. no. CmiR0001-MR03; GeneCopoeia, Inc.) and 100 ng either MEG3-WT or MEG3-MUT using Lipofectamine® 2000. Following 48 h transfection, Dual-Luciferase Reporter Assay System (Promega Corporation) was used to detect luciferase activity, according to the manufacturer's protocols. All firefly luciferase activities were normalized to Renilla luciferase activity.

Cell viability assay. HTR-8/SVneo cell viability (1x10⁴ cells/well) was measured using MTT assay. Following 48 h transfection, 20 µl MTT solution (0.5 mg/ml) was added into each well. After 4 h incubation at 37°C, 150 µl dimethyl sulfoxide (DMSO) (Sigma-Aldrich; Merck KGaA) was used to dissolve the formazan crystals. Finally, cell viability was detected by determining absorbance values at 570 nm using a FLUOstar® Omega Microplate Reader (BMG Labtech GmbH).
**Apoptosis analysis.** Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) apoptosis detection kit [cat. no. 70-AP101-100; Multisciences (Lianke) Biotech Co., Ltd.] was used to assess cell apoptosis. Briefly, 48 h after cell transfection, HTR-8/SVneo cells (1x10^6) were collected though centrifugation (1,000 x g) at 4˚C for 5 min, then the cells were stained using 5 µl Annexin V-FITC and 5 µl PI for 30 min at room temperature in the dark. Lastly, apoptotic cells were analyzed using a flow cytometer (BD Biosciences) with WinMDI soft-ware (version 2.5; Purdue University Cytometry Laboratories; www.cyto.purdue.edu/flowcyt/soft-ware/Catalog.htm).

**Transwell assay.** The invasive and migratory abilities of HTR-8/SVneo cells were measured using Transwell inserts (Corning Inc.) with or without Matrigel (BD Biosciences), respectively. For cell invasion assay, Transwell inserts were pre-coated with Matrigel and incubated at 37˚C for 5 h. Following 48 h transfection, HTR-8/SVneo cells (2x10^5) suspended in 100 µl serum-free medium were seeded into the upper chamber, whilst 500 µl medium supplemented with 20% FBS was added into the lower chamber. The cells were then incubated for 48 h at 37˚C. The migratory or invasive cells were subsequently fixed with 100% methanol at room temperature for 20 min, stained with 0.1% crystal violet at 37˚C for 20 min and counted under an inverted light microscope (magnification, x100) in five randomly-selected fields.

**RNA extraction and RT-qPCR.** Total RNA was extracted from blood, tissues and cells using the TRIzol reagent (Thermo Fisher Scientific, Inc.) according to manufacturer's protocols and the cDNA of miR-345-3p was synthesized using the TaqMan™ miRNA reverse transcription kit (Thermo Fisher Scientific, Inc.) according to manufacturer's protocols. The temperature protocol for the reverse transcription reaction consisted of primer annealing at 25˚C for 5 min, cDNA synthesis at 42˚C for 60 min and termination at 80˚C for 2 min. miR-345-3p levels were quantified using TaqMan™ human MiRNA assay kit (Thermo Fisher Scientific, Inc.) according to manufacturer's protocols. cDNA synthesis and subsequent qPCR of lncRNA MEG3 were performed using SYBR® Premix Ex Taq™ (Tli RNaseH Plus; Takara Biotechnology Co., Ltd.) according to manufacturer's protocols. The following thermocycling conditions were used for PCR: Initial denaturation at 95˚C for 45 sec, followed by 40 cycles of 95˚C for 10 sec and 52˚C for 35 sec. U6 and GAPDH were used as the endogenous controls for miR-345-3p and lncRNA MEG3, respectively. The primer sequences used for qPCR were as follows: lncRNA MEG3 forward, 5'-CTG CCCCCTACACCTCAGC-3' and reverse, 5'-CTCTCAGCC GTCTGCCCTAGGGGCT-3'; GAPDH forward, 5'-CTTTTGG TATCCTGGAGAAGCTC-3' and reverse, 5'-GTAGAGGCA GGGATGATTTCT-3'; U6 forward, 5'-GCTTCGGCACGA CATATACTAAAAT-3' and reverse, 5'-CGGCTTCAACAATTTTGTCTCAT-3'; miR-345-3p forward, 5'-GGTGTGGG ATTTGTTGTTAGAGT-3' and reverse, 5'-AACAAACAC AATCCCATACACACTAC-3'. Relative gene expression was calculated using the 2^[-ΔΔCt] method (28).

**Western blotting.** Total protein was extracted from cells using the radioimmunoprecipitation assay buffer kit (Thermo Fisher Scientific, Inc.). Protein concentration was quantified using a bicinchoninic acid assay kit (Thermo Fisher Scientific, Inc.) prior to separation via SDS-PAGE on a 10% gel at 30 µg protein/ lane. The separated proteins were then transferred onto polyvinylidene fluoride membranes and blocked with 5% non-fat milk at room temperature for 1 h. Following blocking, the membranes were then incubated with primary antibodies against Bcl-2 (cat no. 4223), Bax (cat no. 5023) and β-actin (cat no. 4970; all dilution: 1:1,000; Cell Signaling Technology, Inc.) at 4˚C overnight. The membranes were washed with phosphate-buffer saline (PBS)-0.05% Tween 20 5 times and then incubated with a horseradish peroxidase-conjugated anti-rabbit immunoglobulin G secondary antibody (cat no. 7074; dilution: 1:2,000; Cell Signaling Technology, Inc.) at room temperature for 2 h. The protein bands were visualized using an enhanced chemiluminescence kit (Applygen Technologies, Inc.) according to manufacturer's protocols. Densitometry was performed using the ImageJ software (version 1.38X; National Institutes of Health).

**Statistical analysis.** Data were presented as the mean ± SD. Statistical analysis was performed using the SPSS software (version 18.0; SPSS, Inc.). Differences between multiple groups were analyzed using one-way ANOVA followed by Tukey's post-hoc test, and differences between two groups were analyzed using Student's t-test. P<0.05 was considered to indicate a statistically significant difference.

**Results**

**LncRNA MEG3 is highly expressed in blood and placental villous tissues of pregnant women with GMD.** The lncRNA MEG3 levels in the blood and placental villous tissues of pregnant women were measured using RT-qPCR. LncRNA MEG3 levels in the blood and placental villous tissues from pregnant women with GDM were significantly upregulated compared with those without GDM (Fig. 1A and B). This suggest that lncRNA MEG3 may serve a role during GDM.

miR-345-3p is a target of lncRNA MEG3. Bioinformatics analysis (http://starbase.sysu.edu.cn/index.php) showed miR-345-3p may be a target of lncRNA MEG3 (Fig. 2A). Luciferase assay was used to confirm whether lncRNA MEG3 could regulate miR-345-3p expression by acting as molecular sponge. Luciferase activity was significantly reduced in cells co-transfected with the miR-345-3p mimic and MEG3-WT compared with cells co-transfected with mimic control (miR-control) and MEG3-WT. However, no significant differences were observed between the luciferase activity of cells co-transfected with mimic control (miR-control) and MEG3-MUT and that in cells co-transfected with the miR-345-3p mimic and MEG3-MUT (Fig. 2B). This finding suggests that miR-345-3p is a direct target of lncRNA MEG3.

miR-345-3p is downregulated in the blood and placental villous tissues of pregnant women with GMD. The levels of miR-345-3p in the blood and placental villous tissues of pregnant women with or without GMD were next measured using RT-qPCR. miR-345-3p levels in the blood (Fig. 3A) and placental villous tissues (Fig. 3B) of women with GMD was...
significantly reduced compared with pregnant women without GDM.

**LncRNA MEG3 overexpression inhibits viability, migration and invasion of placental trophoblast cells, and induces apoptosis.** The effects of LncRNA MEG3 overexpression on human choriocarcinoma trophoblast cells (HTR-8/SVneo cells) was next investigated by transfecting HTR-8/SVneo cells with pcDNA-control or pcDNA-MEG3. Following 48-h transfection, efficiency was evaluated using RT-qPCR. pcDNA-MEG3 transfection significantly increased the levels of LncRNA MEG3 in HTR-8/SVneo cells compared with cells in the control group and pcDNA control group (Fig. 4A). HTR-8/SVneo cell viability, apoptosis, migratory and invasive ability were subsequently analyzed using MTT, flow cytometry and Transwell assays, respectively. LncRNA MEG3 overexpression significantly inhibited HTR-8/SVneo cell viability (Fig. 4B), and prevented cell migration (Fig. 4C) and invasion (Fig. 4D), in addition to significantly inducing cell apoptosis (Fig. 4E and F) compared with cells in the control group and pcDNA control group. Supporting this, it was also found that LncRNA MEG3 overexpression significantly decreased protein levels of Bcl-2 whilst increasing levels of Bax compared with the control group and pcDNA control group (Fig. 4G-I).

**LncRNA MEG3 knockdown promotes the viability, migration and invasion of placental trophoblast cells, and reduces apoptosis.** The effects of LncRNA MEG3 knockdown on HTR-8/SVneo cells was next investigated. HTR-8/SVneo cells were transfected with sh-control, sh-MEG3, inhibitor control, miR-345-3p inhibitor or sh-MEG3 + miR-345-3p inhibitor for 48 h prior to RT-qPCR analysis to assess transfection efficiency. Compared with the control group and sh-control group, sh-MEG3 significantly reduced the levels of LncRNA MEG3 in HTR-8/SVneo cells (Fig. 5A), whereas the miR-345-3p inhibitor significantly reduced miR-345-3p levels in HTR-8/SVneo cells compared with the control group and inhibitor control group (Fig. 5B). sh-MEG3 significantly increased the levels of miR-345-3p in the HTR-8/SVneo cells compared with the control group and sh-control group, which was partially reversed by transfection with the miR-345-3p inhibitor (Fig. 5C).

Results from MTT assay, Transwell assay, and flow cytometry demonstrated that LncRNA MEG3 knockdown significantly promoted HTR-8/SVneo cell viability (Fig. 6A), enhanced cell migration and invasion (Fig. 6B and C) and reduced cell apoptosis (Fig. 6D and E) compared with the control group and sh-control group. In addition, compared with the control group and sh-control group, LncRNA MEG3 knockdown significantly increased Bcl-2 protein expression whilst reducing Bax protein expression (Fig. 6F-H). All the observed effects that LncRNA MEG3 knockdown exerted on HTR-8/SVneo cells were negated by transfection with the miR-345-3p inhibitor (Fig. 6).
Discussion

The present study demonstrated that the levels of lncRNA MEG3 in the blood and placental villous tissues from pregnant women with GDM was significantly upregulated compared with the pregnant women without GDM. miR-345-3p was found to be a target of lncRNA MEG3, which was downregulated in the blood and placental villous tissues from pregnant women with GDM. LncRNA MEG3 overexpression inhibited the cell viability, migration and invasion in placental trophoblast cells, and induced apoptosis, whilst suppression of lncRNA MEG3 expression exhibited the opposite effects. In
addition, all the effects of lncRNA MEG3 knockdown exerted on HTR-8/SVneo cells were reversed by the inhibition of miR-345-3p expression. These findings suggest that lncRNA MEG3 may be a novel diagnostic biomarker and therapeutic target for GDM.

Aberrant expression of lncRNAs is closely associated with the development of complex diseases, including cancer, cardiovascular diseases, nervous system diseases and diabetes (16-19). IncRNA MEG3 has been previously reported to participate in the development and progression of diseases, including retinopathy, osteoarthritis and cardiovascular disease (21-23). In particular, inhibition of DNA methyltransferase 3b (DNMT3B) has been shown to upregulate MEG3 expression in HCC cells (29). In recent years, a number of studies have suggested that lncRNAs participate in the occurrence and development of GDM (30,31). LncRNA MEG3, which is closely associated with insulin resistance in the body, is involved in the occurrence and development of GDM.
of diabetes (21-23). The pathogenesis of GDM is highly analogous to that of type 2 diabetes (32,33). Therefore, it was hypothesized in the present study that lncRNA MEG3 may serve an important role in the development of GDM.

Firstly, the expression of lncRNA MEG3 in the blood and placental tissues from pregnant women with GDM and non-GDM was examined. LncRNA MEG3 was demonstrated to be highly expressed in the blood and placental villous tissues from pregnant women with GDM. It was subsequently found that miR-345-3p was a target of lncRNA MEG3. miR-345-3p expression has been reported to be downregulated in diabetes mellitus (34) and diabetic cardiomyopathy (35). In the present study, it was found that miR-345-3p was downregulated in the blood and placental villous tissues from pregnant women with GDM. These observations suggested an important role for lncRNA MEG3 in GDM.

Placental trophoblasts serve an important role in the process of blastocyst implantation in early pregnancy (36,37). Trophoblast proliferation, apoptosis, invasion and migration are key to the establishment, maintenance and finally timely termination of physiological pregnancy (37,38). In particular, placental trophoblast function has become the focus of intense research on GDM pathogenesis (39). Therefore, the effect of lncRNA MEG3 on human chorionic trophoblast cell physiology was next investigated using the HTR-8/SVneo cell line as model. LncRNA MEG3 overexpression inhibited HTR-8/SVneo cell viability, migration and invasion in addition to inducing apoptosis; whilst the suppression of lncRNA MEG3 expression exerted the opposite effects. Notably, the effects lncRNA MEG3 knockdown exerted on HTR-8/SVneo cells were negated by miR-345-3p downregulation. However, a limitation of the present study was that a sh-MEG3 + inhibitor control group was not evaluated. Taken together, results from the present study suggest that the levels of lncRNA MEG3 was significantly upregulated in GDM, and it participated in the development and progression of GDM. This was possibly mediated via the regulation of human chorionic trophoblast cell physiology by targeting miR-345-3p expression. Therefore, lncRNA MEG3 may be a diagnostic and therapeutic target for GDM. However, the present study is only a preliminary study on the role of lncRNA MEG3 in GDM in vitro. To enhance the scientific significance of the results from the present study, the relationship between the levels of lncRNA MEG3 and blood glucose levels should be determined; in addition to in vivo studies on the role of lncRNA MEG3 in GDM development. These issues should be addressed further in any future research.

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Availability of data and materials

All data sets used and/or generated during the present study are available from the corresponding author on reasonable request.

Authors' contributions

HZ designed the study, performed all the experiments and sample collection, analyzed the data and prepared the manuscript.

Ethics approval and consent to participate

Written informed consent was obtained from each patient and this study was approved by the Ethics Committee of Weifang People's Hospital (Weifang, China). Written informed consent was obtained from each patient.

Patient consent for publication

Not applicable.

Competing interests

The author declares that they have no competing interests.

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