ORIGINAL ARTICLE

Whole transcriptome expression profiles in placenta samples from women with gestational diabetes mellitus

Lei Tang[1], Ping Li[1], Ling Li[1,2,*]

[1]Department of Endocrinology, Shengjing Hospital of China Medical University, Shenyang, China, and [2]Liaoning Province Key Laboratory of Endocrine Diseases, Shenyang, China

ABSTRACT

Aims/Introduction: Non-coding ribonucleic acids (ncRNAs) have recently been shown to be involved in various biological processes. However, most of these ncRNAs are of unknown function or without annotation. This study first investigated the whole transcriptome profiles of placentas to identify the potential functions that ncRNAs exerted in gestational diabetes mellitus (GDM).

Materials and Methods: Six placenta samples from healthy pregnant women ($n = 3$) and GDM ($n = 3$) were collected to analyze the whole transcriptome profiles by high-throughput sequencing. Differentially expressed ncRNAs were further validated by quantitative real-time polymerase chain reaction on an independent set of normal ($n = 20$) and GDM ($n = 20$) placenta samples.

Results: A total of 2,817 microRNAs (miRNAs), 23,339 long non-coding RNAs (lncRNAs) and 9,513 circular RNAs (circRNAs) were identified. There were 290 differentially expressed ncRNAs in GDM placentas compared with the placentas of healthy pregnant women. Two miRNAs, 86 lncRNAs and 55 circRNAs were upregulated, while two miRNAs, 86 lncRNAs and 59 circRNAs were downregulated in GDM. The expression of the selected ncRNAs, which were further validated by quantitative real-time polymerase chain reaction, was consistent with the sequencing results. Gene Ontology and Kyoto Encyclopedia of Genes and Genomes pathway analysis showed that the major targets of these ncRNAs were associated with insulin resistance, and abnormal glucose and lipid metabolism. A GDM-related competing endogenous RNA network suggested the interactions between lncRNAs, circRNAs, messenger RNAs and miRNAs.

Conclusions: The whole transcriptome profiles significantly differed in GDM placentas compared with the placentas of healthy pregnant women, which might be valuable for detecting novel ncRNAs, and providing new research insights into exploring the pathogenic mechanisms of GDM.

INTRODUCTION

Gestational diabetes mellitus (GDM), characterized by glucose intolerance with onset or first recognition during pregnancy, is one of the most common pregnancy complications. The incidence of GDM varies from countries to regions within a country, and it is presently approximately 17.5% in China[1]. Accumulating studies have shown that GDM could result in short-term and long-term adverse outcomes for both the mother and fetus[2–4]. The risks of perinatal complications, such as pre-eclampsia and type 2 diabetes in later life, were significantly increased in pregnant women with GDM. The risks to the fetus include premature birth, stillbirth, macrosomia, respiratory distress syndrome and neonatal hypoglycemia, and increased risks of metabolic diseases in offspring, including obesity, type 2 diabetes and cardiovascular disease.

The etiology of GDM is still unclear; however, numerous evidence has shown that GDM is a multifactorial disease, and its pathogenesis might be related to genetic factors, lifestyle, chronic inflammation and adipokines[5,6]. As a temporary organ formed during pregnancy, the placenta is not just the only

interface connecting the mother and fetus, participating in the process of nutrient transport, gas exchange and blood circulation, but also has important endocrine functions. Placenta-derived hormones antagonize insulin and increase insulin resistance, which in turn leads to GDM[7]. Furthermore, the origin of most GDM-related adverse pregnancy outcomes could be traced to the placenta[8]. Therefore, the placenta has become an important organ for researching the pathogenesis of GDM.

Initially, non-coding ribonucleic acids (ncRNAs) were regarded as "transcriptional noises," but accumulating evidence has shown that these ncRNAs have a series of crucial regulatory potentials both in transcription and post-transcription, which participate in many biological processes[9]. These ncRNAs are divided into short non-coding RNAs and long non-coding RNAs (lncRNAs) in accordance with their lengths[10,11]. Micro-RNA (miRNA), a class of single-stranded RNA of 19–24 nucleotides in length, is recognized as a post-transcriptional regulator of gene expression by binding to the target sites to influence messenger RNAs (mRNAs) degradation or translational inhibition[12]. LncRNA, defined as ncRNAs longer than 200 nucleotides, is usually divided into exonic, intronic, intergenic and overlapping lncRNA according to their locations relative to the protein-coding transcripts[13]. Circular RNA (circRNA), an emerging class of ncRNAs with covalently closed loop structures, has highly conserved sequences, stable expression and high degrees of tissue specificity[14,15]. Most of the known circRNAs are produced from the back-splicing of exons through lariat-driven and intron pairing-driven circularization, and intron self-circularization[16]. Studies have reported that some circRNAs have miRNAs binding sites that competitively interact with miRNAs, functioning as a miRNA sponge to regulate the transcription of miRNA-targeted genes[17]. Studies have shown that these ncRNAs were involved in various biological processes, including cell proliferation, differentiation, invasion, apoptosis and other physiological functions. Dysregulated expression of ncRNAs has been found to be associated with many human diseases, such as cancer, diabetes, cardiovascular and cerebrovascular disorders, and pregnancy-related complications[18,19].

To date, the function of most ncRNAs has been unknown and without annotation, especially lncRNAs and circRNAs, and the potential molecular mechanisms underlying the ncRNAs in GDM remain unclear. Therefore, we first investigated the whole transcriptome profiles of placentas from GDM and healthy pregnant women by high-throughput sequencing to identify the potential functions that ncRNAs exerted in GDM and provide novel insights into the pathological mechanisms of GDM in the present study.

METHODS

Study participants

Between December 2017 and May 2018, singleton pregnant women who underwent cesarean section with close follow-up to postpartum in Shengjing Hospital of China Medical University, Shenyang, China, were recruited. Participants were divided into two groups according to the 75-g oral glucose tolerance test during pregnancy: the normal glucose tolerance (NGT) group ($n = 23$) and the GDM group ($n = 23$). Exclusion criteria for all participants included previous type 1 and type 2 diabetes; any other diseases that affected blood glucose levels, including hyperthyroidism, Cushing syndrome and pancreatitis; pregnancy-related complications, such as pre-eclampsia; and severe heart, liver or kidney diseases. This study protocol was approved by the ethics committee of Shengjing Hospital of China Medical University. Written informed consent was obtained from all enrolled participants. Data collection included maternal age, height, pre-gestational weight, pre-gestational body mass index, delivery time, and sex and birthweight of newborns. Characteristics of all participants are summarized in Table 1.

Diagnostic criteria of GDM

According to the diagnostic criteria of the International Association of Diabetes and Pregnancy Study Groups[20], GDM was diagnosed if one or more of the following criteria were fulfilled: fasting plasma glucose ≥5.1 mmol/L, 1-h plasma glucose ≥10.0 mmol/L or 2-h plasma glucose ≥8.5 mmol/L following the 75-g oral glucose tolerance test.

Table 1 | Characteristics of participants

Individuals for sequencing	NGT group $n = 3$	GDM group $n = 3$	P
Maternal age (years)	29.3 ± 0.6	29.0 ± 0.0	0.423
Pre-gestational BMI (kg/m^2)	28.2 ± 0.7	29.0 ± 0.0	0.128
Fasting plasma glucose (mmol/L)	4.5 ± 0.2	5.7 ± 0.2**	0.001
1-h plasma glucose (mmol/L)	8.3 ± 0.3	10.7 ± 0.1**	<0.001
2-h plasma glucose (mmol/L)	6.4 ± 0.5	8.8 ± 0.2**	0.001
GA (%)	11.3 ± 0.1	11.8 ± 0.1**	0.002
HbA1c (%)	4.9 ± 0.1	5.8 ± 0.2**	0.001
Delivery time (weeks)	39.3 ± 0.6	38.7 ± 0.6	0.230
Birthweight of newborns (g)	3,333 ± 58	3,300 ± 100	0.643
Individuals for validation	$n = 20$	$n = 20$	
Maternal age (years)	29.6 ± 0.4	29.4 ± 1.2	0.768
Pre-gestational BMI (kg/m^2)	28.0 ± 1.8	29.0 ± 1.9	0.106
Fasting plasma glucose (mmol/L)	4.7 ± 0.3	5.6 ± 0.5 **	<0.001
1-h plasma glucose (mmol/L)	8.1 ± 1.2	10.5 ± 0.9 **	0.002
2-h plasma glucose (mmol/L)	6.7 ± 1.0	8.5 ± 1.7 **	0.003
GA (%)	11.4 ± 1.6	12.0 ± 1.6	0.407
HbA1c (%)	5.0 ± 0.2	5.6 ± 0.5 **	0.002
Delivery time (weeks)	39.0 ± 1.0	38.7 ± 0.6	0.454
Birthweight of newborns (g)	3,337 ± 183	3,332 ± 233	0.954

Data are presented as the mean ± standard deviation. Compared with the normal glucose tolerance (NGT) group. BMI, body mass index; GA, glycated albumin; GDM, gestational diabetes mellitus; HbA1c, hemoglobin A1c. *$P < 0.05$, **$P < 0.01$.

Sample collection and preparation

The maternal surfaces of fresh placentas from pregnant women were collected immediately after delivery. With the center of the placenta as the center point, maternal surfaces of placenta samples were collected at the three apex positions of the equilateral triangle, which were 3.0–5.0 cm from the center. The cut depth was 0.5–1.0 cm, and the sample size was $1.0 \times 1.0 \times 1.0$ cm. The tissues were washed with phosphate-buffered saline and immediately placed in liquid nitrogen to be snap-frozen. Subsequently, all samples were stored at $-80°C$ until RNA extraction.

High-throughput sequencing

A total of six placenta samples (three NGT and three GDM) were selected for whole transcriptome analysis by high-throughput sequencing.

RNA extraction and quality control

Total RNA was extracted using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. RNA degradation and contamination were monitored on 1% agarose gels. RNA purity and concentration were measured using a NanoDrop 2000 (Thermo Fisher Scientific, Wilmington, DE, USA) and a Qubit 3.0 Fluorometer (Life Technologies, Carlsbad, CA, USA). RNA integrity was assessed using the RNA 6000 Nano Kit with the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA).

RNA library preparation and sequencing

Whole transcriptome analysis by high-throughput sequencing required the construction of two sequencing libraries, a small RNA library and a strand-specific library for ribosomal RNA removal. The former library can obtain miRNAs sequence information, and the latter library can obtain sequence information of mRNAs, lncRNAs and circRNAs. The small RNA library was constructed in accordance with the NEBNext® Multiplex Small RNA Library Prep Set for Illumina® (NEB, Ipswich, MA, USA). The strand-specific library for ribosomal RNA removal was generated using the NEBNext® Ultra™ II Directional RNA Library Prep Kit for Illumina® (NEB) with the Ribo-Zero rRNA Removal Kit Reference (Illumina, Madison, MI, USA). Sequencing was carried out on an Illumina HiSeq X Ten (Illumina).

Quantitative real-time polymerase chain reaction

A total of 40 placenta samples (20 NGT and 20 GDM) were selected for validation of differentially expressed ncRNAs by quantitative real-time polymerase chain reaction (qRT–PCR).

Total RNA extraction and concentration measurement were the same as the previous method. Complementary deoxyribonucleic acid (cDNA) was synthesized by using M-MLV Reverse Transcriptase (Invitrogen) according to the manufacturer's instructions. Subsequently, qRT–PCR (BIONEER, Daejeon, Korea) was performed to measure the expression levels of miRNAs, lncRNAs and circRNAs according to the manufacturer's instructions. qRT–PCR was performed in a 20-μL reaction volume, including 10 μL 2×Power Taq PCR Master Mix (BioTeke, Beijing, China), 0.3 μL SYBR Green (Solarbio, Beijing, China), 2 μL cDNA, 0.5 μL PCR Forward Primer (10 μmol/L), 0.5 μL PCR Reverse Primer (10 μmol/L) and 6.7 μL nuclease-free water. The protocol was initiated at 94°C for 5 min, followed by 94°C (10 s), 60°C (20 s) and 72°C (30 s) for a total of 40 cycles. U6 and β-actin were used as internal control for miRNAs, lncRNAs and circRNAs, respectively. All reactions were performed in three independent wells. The relative expression levels of differentially expressed ncRNAs were determined using the $2^{-\triangle\triangle Ct}$ method[21]. The primer sequences a listed in Table S1.

Gene ontology and Kyoto encyclopedia of genes and genomes analysis

Gene Ontology (GO; http://geneontology.org/) analysis was performed to describe gene annotations of differentially expressed ncRNAs in biological process, cellular component and molecular function[22].

Kyoto Encyclopedia of Genes and Genomes (KEGG; http://www.kegg.jp/) analysis was performed to analyze the functions and related biological pathways. The enrichment factor was the value ratio between the sequenced genes and all annotated genes enriched in the pathway[23].

Competitive endogenous RNA network

Competitive endogenous RNA (ceRNA), a new mechanism of transcriptional regulation, suggested that lncRNAs or circRNAs might participate in the pathogenesis of GDM by competitively binding to miRNAs through a miRNA response element. miRanda and microRNA.org (http://www.microrna.org/microrna/home.do) were used to predict the interactions between the possible targeted miRNAs of differentially expressed lncRNAs or circRNAs and mRNAs.

Statistical analysis

All data processing and statistical analyses were performed using SPSS 22.0 software (IBM Corporation, Armonk, NY, USA) and GraphPad Prism 6.0 (GraphPad Software Inc., San Diego, CA, USA). Variables were expressed as the mean ± standard deviation. The differences between the groups were analyzed using Student's t-test. $P < 0.05$ was considered statistically significant.

RESULTS

Characteristics of ncRNAs

A total of 109,562,576 raw reads were obtained in the small RNA library, with 109,535,915 clean reads remaining after screening. A total of 405,557,836 raw reads were obtained in the strand-specific library for ribosomal RNA removal, and 402,195,361 clean reads were remained after screening for the following analysis. Each sample was subjected to compare with

the reference genome and predict of miRNAs, lncRNAs and circRNAs. Finally, 96.21% of clean reads mapped to the human reference genome, and 35,669 ncRNAs were identified, including 2,817 miRNAs, 23,339 lncRNAs and 9,513 circRNAs (Figure 1, Tables S2,S3).

A total of 2,817 miRNAs were identified, including 1,710 known miRNAs in miRbase and 1,107 new miRNAs predicted by miRDeep2[24]. A total of 23,339 lncRNAs were identified, including 8,816 new lncRNAs predicted by the intersection of Coding Potential Calculator[25], Coding-Non-Coding Index[26], Coding Potential Assessment Tool[27] and the protein families database (Pfam)[28] protein domain analysis. Of the 8,816 new lncRNAs, there were 7,007 lincRNAs, 1,189 sense-lncRNAs, 285 antisense-lncRNAs and 298 intronic-lncRNAs. A total of 9,513 circRNAs were identified, including 4,493 known circRNAs in circBase and 5,020 new circRNAs predicted by the intersection of Circular RNA Identification[29] and find_circ[30]. These genes were widely scattered on almost all human chromosomes, and there were 8,326 exonic, 666 intronic and 521 intergenic regions.

Identification of differentially expressed ncRNAs
In total, 290 differentially expressed ncRNAs were determined to have a fold change ≥2.0 and $P < 0.05$; 143 ncRNAs were upregulated and 147 ncRNAs were downregulated (Table S4). Compared with the NGT group, four miRNAs, 172 lncRNAs and 114 circRNAs were differentially expressed in the GDM group. Two miRNAs, 86 lncRNAs and 55 circRNAs were upregulated, while two miRNAs, 86 lncRNAs and 59 circRNAs were downregulated in the GDM group (Figure 2). The significant differences in ncRNAs expression between the NGT and GDM groups were represented by Volcano Plot (Figure 3).

Validation of differentially expressed ncRNAs by qRT–PCR
Among the aforementioned differentially expressed ncRNAs, three miRNAs (two upregulated miRNAs and one
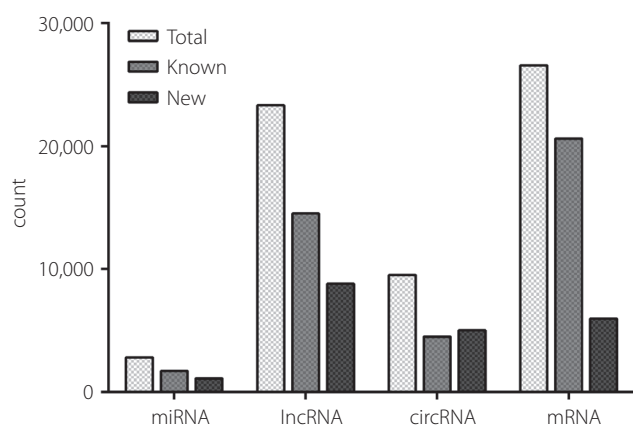

Figure 2 | Characteristics of differentially expressed transcripts. circRNA, circular ribonucleic acid; lncRNA, long non-coding ribonucleic acid; mRNA, messenger ribonucleic acid; miRNA, micro-ribonucleic acid.

downregulated miRNA), seven lncRNAs (five upregulated lncRNAs and two downregulated lncRNAs) and five circRNAs (two upregulated circRNAs and three downregulated circRNAs) were selected for validation by qRT–PCR. The relative expression levels of the selected ncRNAs were consistent with the sequencing results (Figure 4; Table S5).

GO and KEGG pathway analysis
We performed functional annotation and enrichment analysis on genes of differentially expressed lncRNAs and circRNAs (Figure 5; Tables S6,S7).

GO analysis on genes of differentially expressed lncRNAs showed that the term with the most genes and the most significantly enriched term in biological process was nucleic acid phosphodiester bond hydrolysis activity (GO: 0090305); the term with the most genes was protein complex (GO: 0043234) and the most significantly enriched term was microvillus (GO: 0005902) in cellular component; the term with the most genes and the most significantly enriched term in molecular function was RNA-directed DNA polymerase activity (GO: 0003964). Of the top 10 enrichment pathways in the KEGG analysis, the most enriched pathway was glycosaminoglycan degradation (ko00531).

Figure 1 | Characteristics of different transcripts. circRNA, circular ribonucleic acid; lncRNA, long non-coding ribonucleic acid; miRNA, micro-ribonucleic acid; mRNA, messenger ribonucleic acid.
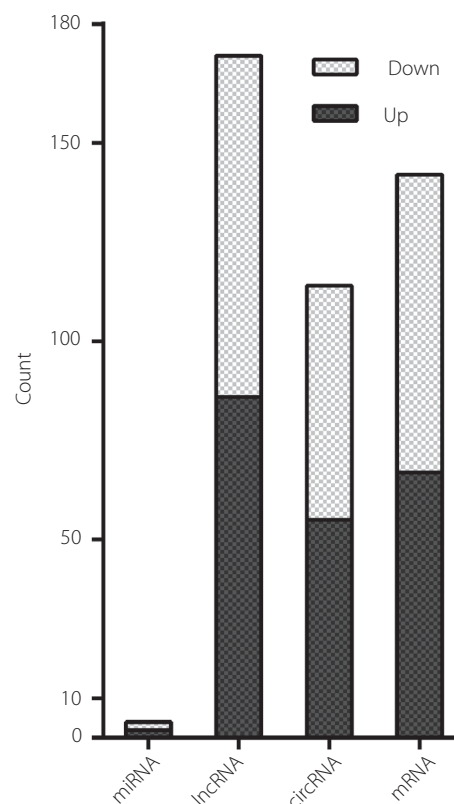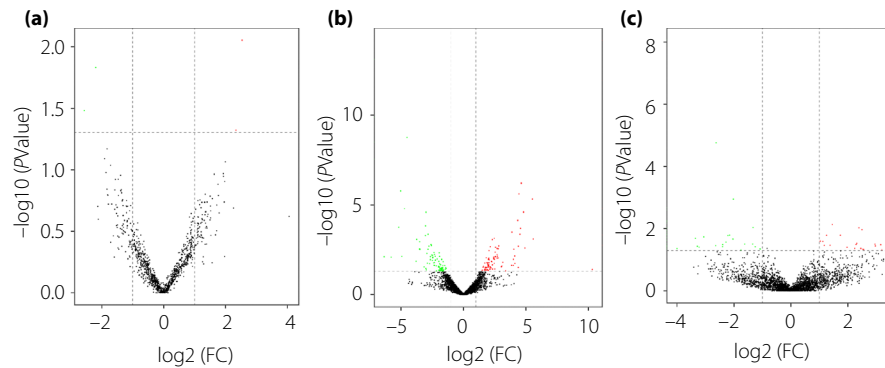
Figure 3 | Volcano plots of differentially expressed non-coding ribonucleic acids (RNAs). Volcano plots showing significantly different expression of (a) microRNAs, (b) long non-coding RNAs and (c) circular RNAs between the two groups. Red points, upregulated non-coding RNAs; green points, downregulated non-coding RNAs. FC, fold changes.
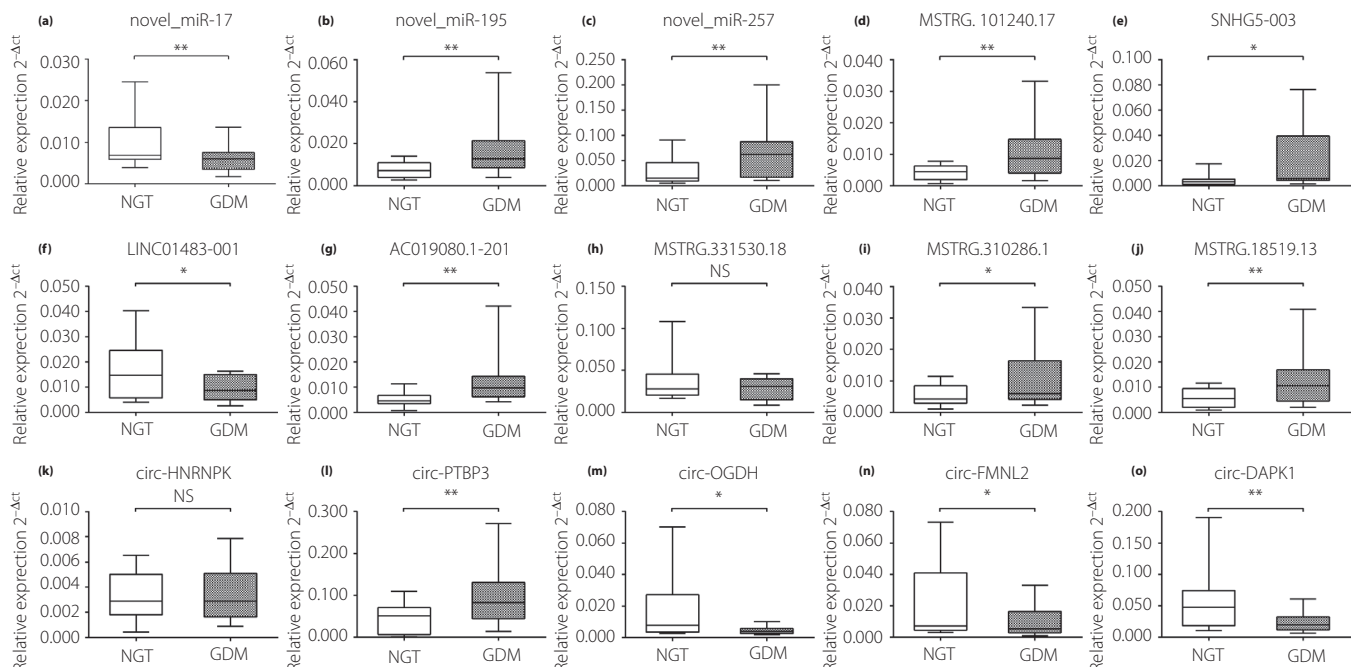

Figure 4 | Quantitative real-time polymerase chain reaction validation for the non-coding ribonucleic acids (RNAs). The relative expression levels of selected (a–c) microRNAs, (d–j) long non-coding RNAs and (k–o) circular RNAs were validated by quantitative real-time polymerase chain reaction. Compared with the normal glucose tolerance (NGT) group, *$P < 0.05$, **$P < 0.01$. GDM, gestational diabetes mellitus; NS, not significant.

GO analysis on genes of differentially expressed circRNAs showed that the term with the most genes was small molecule metabolic process (GO: 0044281), and the most significantly enriched term was activation of phospholipase C activity (GO: 0007202) in biological process; the term with the most genes was cell junction (GO: 0030054), and the most significantly enriched term was phosphatidylinositol 3-kinase complex (GO: 0005942) in cellular component; the term with the most genes was phospholipid binding (GO: 0005543), and the most significantly enriched term was phosphatidylinositol binding (GO: 0035091) in molecular function. Of the top 10 enrichment pathways in the KEGG analysis, the most enriched pathway was the Rap1 signaling pathway (ko04015).

ceRNA network

The ceRNA network can reveal the modes and functions of different ncRNAs, and a regulatory relationship among various RNAs, which lncRNAs or circRNAs regulate gene expression
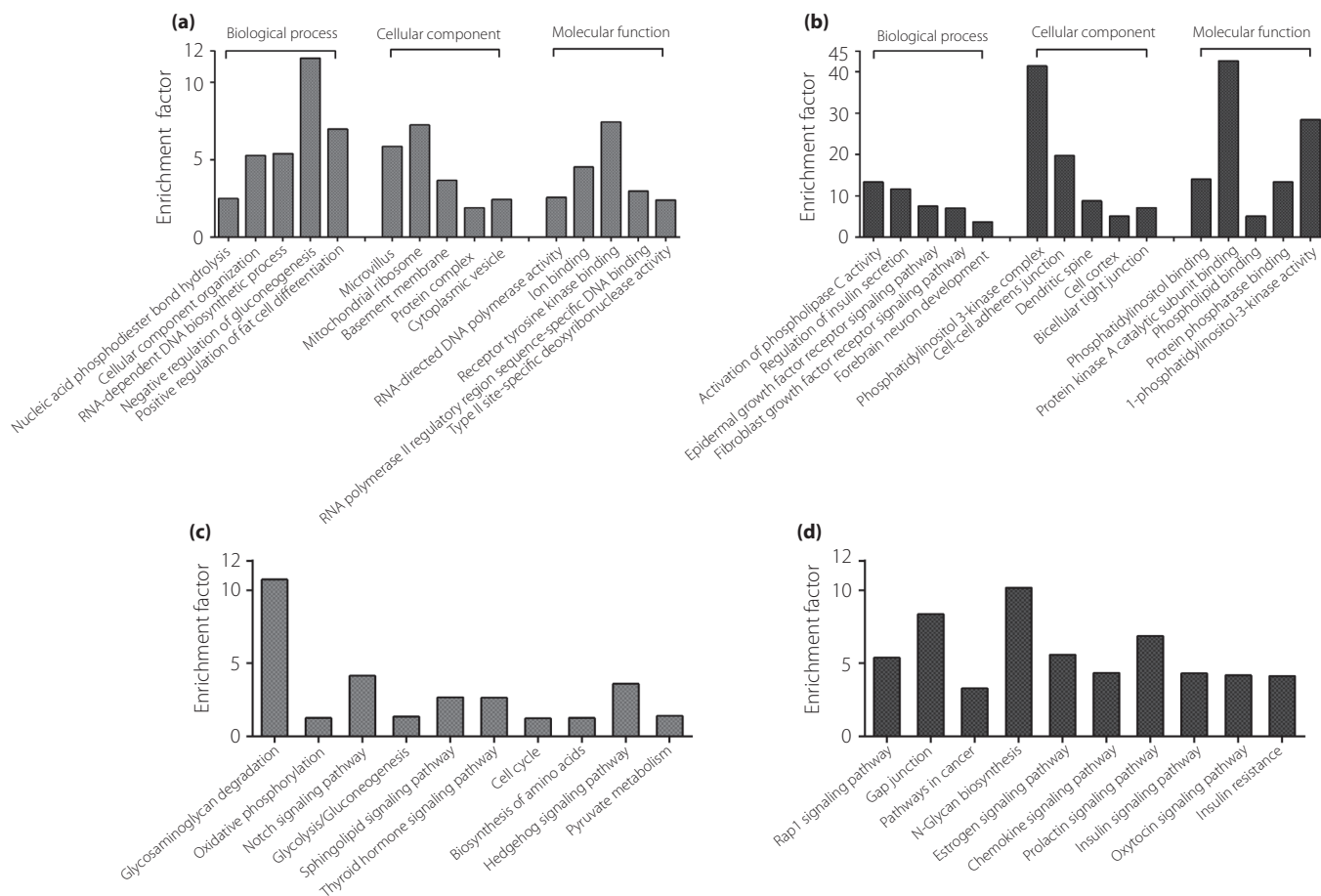
Figure 5 | Gene Ontology and Kyoto Encyclopedia of Genes and Genomes pathway analysis of differentially expressed genes. Gene Ontology annotation of (a) long non-coding ribonucleic acids (RNAs) and (b) circular RNAs. Kyoto Encyclopedia of Genes and Genomes pathway analysis of (c) long non-coding RNAs and (d) circular RNAs. The *x*-axis represents terms in biological process, cellular component and molecular function. DNA, deoxyribonucleic acid.
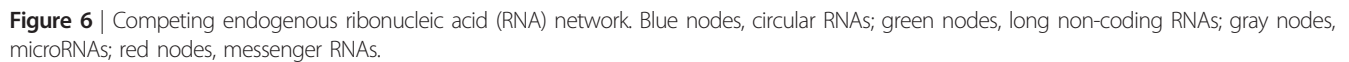
through a miRNA sponge mechanism by binding to the common miRNA-binding sites. Therefore, we constructed a GDM-related ceRNA network to investigate the interactions between lncRNAs, circRNAs, mRNAs and miRNAs (Figure 6). We found that a lncRNA or circRNA could sponge several miR-NAs, and a miRNA could also interact with multiple ncRNAs. These results highlighted the important role of lncRNAs or cir-cRNAs in pathogenesis of GDM by interacting with miRNAs.

DISCUSSION

With the development of molecular biotechnology, ncRNAs have received increasing attention in recent years. Numerous studies have shown that ncRNAs played a crucial role in various biological processes and differentially expressed in many placenta-related diseases, such as GDM[18,19,31]. Thus, investigating known ncRNAs and discovering novel unannotated ncRNAs could provide a significant promotion for researching GDM. The present study first investigated the whole

transcriptome profiles of placentas from GDM and healthy pregnant women by high-throughput sequencing, a more comprehensive identification of ncRNAs, to identify the differentially expressed ncRNAs, and to explore the potential biological functions and the relationship between these ncRNAs and GDM.

A total of 35,669 ncRNAs were identified, including 2,817 miRNAs, 23,339 lncRNAs and 9,513 circRNAs. In addition to most ncRNAs located in the miRBase, RefSeq and circBase database, we also found many novel transcripts in our sequencing data. There were 290 differentially expressed ncRNAs in GDM placentas; 143 ncRNAs were upregulated and 147 ncRNAs were downregulated, including four miRNAs, 172 lncRNAs and 114 circRNAs. We performed qRT–PCR on some of the differentially expressed ncRNAs based on our predicted sequences. The results showed that the expression of the selected ncRNAs was in line with the expression in sequencing. Compared with healthy pregnant women, these detected

Figure 6 | Competing endogenous ribonucleic acid (RNA) network. Blue nodes, circular RNAs; green nodes, long non-coding RNAs; gray nodes, microRNAs; red nodes, messenger RNAs.

ncRNAs expression patterns in GDM placentas were significantly different, speculating that they might play an important role in the pathogenesis of GDM, and are expected to become new molecular biomarkers of GDM.

We performed GO and KEGG pathway analysis to explore the biological functions and potential pathways on genes of differentially expressed lncRNAs and circRNAs. In the GO analysis on genes of differentially expressed lncRNAs, the most significant biological processes were nucleic acid phosphodiester bond hydrolysis and cellular component organization, and the most significant cellular components were microvillus and mitochondrial ribosome. KEGG pathway analysis showed several significantly enrich pathways that correlated with glucose and lipid metabolism. The sphingolipid signaling pathway was involved in the regulation of various biological processes, such as cell proliferation, adhesion, migration survival and apoptosis. It also participated in the progression of diabetes, including insulin sensitivity and secretion, β-cell apoptosis, and the development of diabetic inflammatory states[32]. The Notch signaling

pathway was associated with the regulation of physiological insulin[33]. Thyroid hormone[34] and Hedgehog signaling pathways[35] have also been found to be involved in the improvement of insulin sensitivity in skeletal muscle and insulin resistance in adipose tissue. Genes encoding cell cycle regulators influenced gestational glucose tolerance and regulation[36]. Furthermore, researchers found that the levels of several metabolites (serine, proline, leucine/isoleucine, glutamic acid, tyrosine ornithine, adipate and pyruvate) were significantly different in GDM, highlighting the importance of altered amino acid, fatty acid and carbohydrate metabolism, and suggesting that these metabolites might have contributed to the occurrence and progression of GDM[37,38]. In the GO analysis on genes of differentially expressed circRNAs, the most significant biological processes were activation of phospholipase C activity and regulation of insulin secretion. KEGG pathway analysis also showed several pathways that correlated with GDM, including the prolactin signaling pathway, insulin signaling pathway and insulin resistance. Prolactin and placental lactogen signaled through the

prolactin receptor to promote the proliferation and expansion of β-cells, which was indispensable for maintaining maternal blood glucose. Deletion of the prolactin signaling pathway in β-cells during pregnancy led to the development of GDM, decreased β-cell proliferation and failure to expand β-cell mass. These results revealed the molecular role of this pathway in coordinating the physiological β-cell responses during pregnancy[39]. Loss of the estrogen signaling pathway resulted in glucose intolerance and excessive weight gain, accompanied by reduced physical activity and impaired adaptive heat production[40]. Deregulation of the Rap1 signaling pathway also led to multiple systemic metabolic disorders characterized by excessive fat accumulation, dyslipidemia, hepatic steatosis and glucose intolerance[41]. Additionally, the chemokine–receptor axis could affect hepatic inflammation and fibrosis by mediating hepatic infiltration[42]. Cellular and animal studies have established that G protein-coupled estrogen receptor (GPER) was involved in the regulation of feeding behavior and inflammation, as well as glucose and lipid homeostasis. GPER manifested pleiotropic effects in metabolically active tissues, such as adipose, liver, pancreas and skeletal muscle. GPER deficiency led to increased adiposity, insulin resistance and metabolic dysfunction in mice[43]. Six-month-old GPER knockout (KO) male and female mice displayed increased body weight compared with wild-type littermates, and GPER KO female mice exhibited glucose intolerance at this age. Insulin resistance was evident in GPER KO male mice from 6 months, and glucose intolerance was pronounced at 18 months. One-year-old GPER KO male mice displayed an abnormal lipid profile with higher cholesterol and triglyceride levels[44]. Previous studies have demonstrated that angiopoietin 2 positively correlated with waist circumference, body mass index, systolic blood pressure, fasting plasma glucose, hemoglobin A1c, homeostasis model assessment for insulin resistance and triglycerides in diabetes patients. Angiopoietin 2 was independently associated with cardiovascular risk factors[45]. Meanwhile, angiopoietin 2 was also found to be independently related to type 2 diabetes mellitus with angiopathy[46]. Correlation analysis showed that lipoprotein lipase gene polymorphism in placental tissue was positively related to insulin resistance. It was determined to be associated with GDM[47]. In addition, placental lipoprotein lipase activity was positively correlated with newborn birthweight and percentage fat, and was also found to be related to insulin resistance of their offspring[48]. In line with these studies, the present data showed that ANGPT2 and lipoprotein lipase were higher expressed, whereas GPER1 was lower expressed in the GDM group compared with the NGT group. Furthermore, BCL2 and BCL2L1 were upregulated in GDM placentas, speculating that placental apoptosis abnormally affects placental growth and function, as well as nutrient transport, and altered placental morphology subsequent to apoptosis might account for functional adaptations in GDM placentas[49]. The expression of BCL2 and BCL2L1 were also higher in the GDM group compared with the NGT group in the present study. In addition to

participating in cell proliferation, activation of extracellular signal-regulated kinase also mediated insulin resistance in skeletal muscle of obese individuals[50]. It has been reported that extracellular signal-regulated kinase was increased in patients with GDM, and was associated to placental fatty acid translocase and fatty acid-binding protein, which might affect lipid metabolism. As aforementioned, the present results are consistent with previous studies (Figure S1). Although the etiology of GDM was unclear, our study demonstrated that insulin resistance and insufficient insulin secretion were vitally fundamental pathophysiological mechanisms for GDM, which was consistent with previous molecular mechanism studies and genomic analysis[31,33]. The transcripts in each term were valuable and meaningful for further mechanism research, which might provide ideas and insights into the pathogenesis of GDM, and the theoretical basis for new therapeutic strategies in GDM.

Based on high-throughput sequencing, researchers screened a large number of differentially expressed ncRNAs in different tissues or blood samples. These differentially expressed ncRNAs have aroused general interest in mechanism research, but most studies of GDM focused on miRNAs. Downregulation of miR-143 mediated the metabolic transition from oxidative phosphorylation to aerobic glycolysis in GDM placentas, whereas overexpression of miR-143 increased mitochondrial respiration and mitochondrial complex expression, and decreased the expression of glycolytic enzymes[52]. Furthermore, miR-143 might play a role in the mitogen-activated protein kinase signaling pathway associated with macrosomia[53]. The expression level of miR-518d in GDM placentas was significantly higher, and high expression of miR-518d was negatively correlated with peroxisome proliferator-activated receptor-α, which might participate in the occurrence of GDM by regulating expression[54]. MiR-410 could induce human embryonic stem cells to differentiate into islet endoderm, promote β-cell proliferation, increase insulin secretion and greatly improve glucose metabolism in mice with GDM by direct targeting lactate dehydrogenase A[55]. In addition, miR-185 has also been involved in the insulin signaling pathway[56]. MiRNAs are important post-transcriptional regulators of gene expression and key elements in ceRNAs by direct base pairing to target genes to regulate mRNA expression, and influence the occurrence and development of diseases[17,31]. The ceRNA network is a novel mechanism of interaction between RNAs that reveals the patterns and a wide range of the regulatory relationships among different RNAs. Therefore, we constructed a GDM-related ceRNA network to explore the functions and the potential interaction mechanisms of ncRNAs based on our sequencing data. In the present study, we observed that a lncRNA or circRNA could sponge several miRNAs, and a miRNA could also interact with multiple ncRNAs, suggesting that these lncRNAs or circRNAs harbored one or more miRNA-binding sites through a sponge mechanism to participant in GDM. However, the interaction between the lncRNA/circRNA-miRNA-mRNA ceRNA network requires further mechanism studies.

Nowadays, there are more than 1.9 million women with diabetes in the world, and this number is expected to increase to 313 million by 2040. Among them, the number of young women with diabetes will reach 60 million[57]. As one of the most common complications during pregnancy, GDM seriously harms maternal and infant health. Non-coding RNA, which participates in a variety of biological processes, has gradually attracted attention in the etiology studies of GDM. In summary, our whole transcriptome expression profiles identified differentially expressed ncRNAs between GDM and healthy pregnant women, and bioinformatics analysis further helped us fully understand and predict the potential interactions between these dysregulated circRNAs, lncRNAs and miRNAs. This might contribute to the diagnosis and treatment of GDM, and provides new research insights into exploring the pathogenesis of GDM. However, exploration has only just begun, and further molecular mechanism researches should be performed for a more comprehensive understanding of these differentially expressed ncRNAs and their interactions in GDM.

ACKNOWLEDGMENTS

DISCLOSURE
The authors declare no conflict of interest.

REFERENCES

1. Zhu W, Yang H, Wei Y, et al. Comparing the diagnostic criteria for gestational diabetes mellitus of World Health Organization 2013 with 1999 in Chinese population. *Chin Med J (Engl)* 2015; 128: 125–127.
2. Feig DS, Zinman B, Wang X, et al. Risk of development of diabetes mellitus after diagnosis of gestational diabetes. *CMAJ* 2008; 179: 229–234.
3. Farrar D, Simmonds M, Bryant M, et al. Hyperglycaemia and risk of adverse perinatal outcomes: systematic review and meta-analysis. *BMJ* 2016; 354: i4694.
4. Dickens LT, Thomas CC. Updates in gestational diabetes prevalence, treatment, and health policy. *Curr Diab Rep* 2019; 19: 33.
5. de Gennaro G, Palla G, Battini L, et al. The role of adipokines in the pathogenesis of gestational diabetes mellitus. *Gynecol Endocrinol* 2019; 35: 737–751.
6. Bawah AT, Seini MM, Abaka-Yawason A, et al. Leptin, resistin and visfatin as useful predictors of gestational diabetes mellitus. *Lipids Health Dis* 2019; 18: 221.
7. Ryan EA, Enns L. Role of gestational hormones in the induction of insulin resistance. *J Clin Endocrinol Metab* 1988; 67: 341–347.
8. Ilekis JV, Tsilou E, Fisher S, et al. Placental origins of adverse pregnancy outcomes: potential molecular targets: an Executive Workshop Summary of the Eunice Kennedy Shriver National Institute of Child Health and Human Development. *Am J Obstet Gynecol* 2016; 215: S1–S46.
9. Chowdhury D, Choi YE, Brault ME. Charity begins at home: non-coding RNA functions in DNA repair. *Nat Rev Mol Cell Biol* 2013; 14: 181–189.
10. Costa FF. Non-coding RNAs: meet thy masters. *BioEssays* 2010; 32: 599–608.
11. Jia H, Osak M, Bogu GK, et al. Genome-wide computational identification and manual annotation of human long noncoding RNA genes. *RNA* 2010; 16: 1478–1487.
12. Bartel DP. MicroRNAs: target recognition and regulatory functions. *Cell* 2009; 136: 215–233.
13. Derrien T, Johnson R, Bussotti G, et al. The GENCODE v7 catalog of human long noncoding RNAs: analysis of their gene structure, evolution, and expression. *Genome Res* 2012; 22: 1775–1789.
14. Jeck WR, Sorrentino JA, Wang K, et al. Circular RNAs are abundant, conserved, and associated with ALU repeats. *RNA* 2013; 19: 141–157.
15. Qu S, Yang X, Li X, et al. Circular RNA: a new star of noncoding RNAs. *Cancer Lett* 2015; 365: 141–148.
16. Vicens Q, Westhof E. Biogenesis of circular RNAs. *Cell* 2014; 159: 13–14.
17. Hansen TB, Jensen TI, Clausen BH, et al. Natural RNA circles function as efficient microRNA sponges. *Nature* 2013; 495: 384–388.
18. Chen H, Zhang Z, Feng D. Prediction and interpretation of miRNA-disease associations based on miRNA target genes using canonical correlation analysis. *BMC Bioinformatics* 2019; 20: 404.
19. Bao Z, Yang Z, Huang Z, et al. LncRNADisease 2.0: an updated database of long non-coding RNA-associated diseases. *Nucleic Acids Res* 2019; 47: D1034–D1037.
20. International Association of Diabetes and Pregnancy Study Groups Consensus Panel, Metzger BE, Gabbe SG et al. International association of diabetes and pregnancy study groups recommendations on the diagnosis and classification of hyperglycemia in pregnancy. *Diabetes Care* 2010; 33: 676–682.
21. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. *Methods* 2001; 25: 402–408.
22. Ashburner M, Ball CA, Blake JA, et al. Gene ontology: tool for the unification of biology. The Gene Ontology Consortium. *Nat Genet* 2000; 25: 25–29.
23. Kanehisa M, Araki M, Goto S, et al. KEGG for linking genomes to life and the environment. *Nucleic Acids Res* 2008; 36: D480–D484.
24. Friedländer MR, Mackowiak SD, Li N, et al. miRDeep2 accurately identifies known and hundreds of novel microRNA genes in seven animal clades. *Nucleic Acids Res* 2012; 40: 37–52.
25. Kong L, Zhang Y, Ye ZQ, et al. CPC: assess the protein-coding potential of transcripts using sequence features and

support vector machine. *Nucleic Acids Res* 2007; 35: W345–W349.

26. Sun L, Luo H, Bu D, *et al*. Utilizing sequence intrinsic composition to classify protein-coding and long non-coding transcripts. *Nucleic Acids Res* 2013; 41: e166.

27. Wang L, Park HJ, Dasari S, *et al*. CPAT: Coding-Potential Assessment Tool using an alignment-free logistic regression model. *Nucleic Acids Res* 2013; 41: e74.

28. Finn RD, Bateman A, Clements J, *et al*. Pfam: the protein families database. *Nucleic Acids Res* 2014; 42: D222–D230.

29. Gao Y, Wang J, Zhao F. CIRI: an efficient and unbiased algorithm for de novo circular RNA identification. *Genome Biol* 2015; 16: 4.

30. Westholm JO, Miura P, Olson S, *et al*. Genome-wide analysis of drosophila circular RNAs reveals their structural and sequence properties and age-dependent neural accumulation. *Cell Rep* 2014; 9: 1966–1980.

31. Leng L, Zhang C, Ren L, *et al*. Construction of a long non-coding RNA-mediated competitive endogenous RNA network reveals global patterns and regulatory markers in gestational diabetes. *Int J Mol Med* 2019; 43: 927–935.

32. Ng ML, Wadham C, Sukocheva OA. The role of sphingolipid signaling in diabetes-associated pathologies (Review). *Int J Mol Med* 2017; 39: 243–252.

33. Batista TM, Garcia-Martin R, Cai W, *et al*. Multi-dimensional transcriptional remodeling by physiological insulin in vivo. *Cell Rep* 2019; 26: 3429–3443.e3.

34. Moon MK, Kang GH, Kim HH, *et al*. Thyroid-stimulating hormone improves insulin sensitivity in skeletal muscle cells via cAMP/PKA/CREB pathway-dependent upregulation of insulin receptor substrate-1 expression. *Mol Cell Endocrinol* 2016; 436: 50–58.

35. Yao Q, Liu J, Xiao L, *et al*. Sonic hedgehog signaling instigates high-fat diet-induced insulin resistance by targeting PPARγ stability. *J Biol Chem* 2019; 294: 3284–3293.

36. Liu S, Liu Y, Liao S. Heterogeneous impact of type 2 diabetes mellitus-related genetic variants on gestational glycemic traits: review and future research needs. *Mol Genet Genomics* 2019; 294: 811–847.

37. Zhao H, Li H, Chung ACK, *et al*. Large-scale longitudinal metabolomics study reveals different trimester-specific alterations of metabolites in relation to gestational diabetes mellitus. *J Proteome Res* 2019; 18: 292–300.

38. Qiu C, Enquobahrie DA, Frederick IO, *et al*. Early pregnancy urinary biomarkers of fatty acid and carbohydrate metabolism in pregnancies complicated by gestational diabetes. *Diabetes Res Clin Pract* 2014; 104: 393–400.

39. Banerjee RR, Cyphert HA, Walker EM, *et al*. Gestational diabetes mellitus from inactivation of prolactin receptor and MafB in Islet β-Cells. *Diabetes* 2016; 65: 2331–2341.

40. Ueda K, Takimoto E, Lu Q, *et al*. Membrane-initiated estrogen receptor signaling mediates metabolic homeostasis via central activation of protein phosphatase 2A. *Diabetes* 2018; 67: 1524–1537.

41. Yeung F, Ramírez CM, Mateos-Gomez PA, *et al*. Nontelomeric role for Rap1 in regulating metabolism and protecting against obesity. *Cell Rep* 2013; 3: 1847–1856.

42. Parker R, Weston CJ, Miao Z, *et al*. CC chemokine receptor 2 promotes recruitment of myeloid cells associated with insulin resistance in nonalcoholic fatty liver disease. *Am J Physiol Gastrointest Liver Physiol* 2018; 314: G483–G493.

43. Sharma G, Prossnitz ER. G-protein-coupled estrogen receptor (GPER) and sex-specific metabolic homeostasis. *Adv Exp Med Biol* 2017; 1043: 427–453.

44. Sharma G, Hu C, Brigman JL, *et al*. GPER deficiency in male mice results in insulin resistance, dyslipidemia, and a proinflammatory state. *Endocrinology* 2013; 154: 4136–4145.

45. Siddiqui K, Joy SS, Nawaz SS, *et al*. Angiopoietin-2 level as a tool for cardiovascular risk stratification in hypertensive type 2 diabetic subjects. *Postgrad Med* 2018; 130: 402–408.

46. Li L, Qian L. Serum angiopoietin-2 is associated with angiopathy in type 2 diabetes mellitus. *J Diabetes Complications* 2015; 29: 568–571.

47. Li DD, Su DY, Xue L, *et al*. Relationship between a lipoprotein lipase gene polymorphism in placental tissue and insulin resistance in patients with gestational diabetes mellitus. *Genet Mol Res* 2015; 14: 7751–7758.

48. Heerwagen MJR, Gumina DL, Hernandez TL, *et al*. Placental lipoprotein lipase activity is positively associated with newborn adiposity. *Placenta* 2018; 64: 53–60.

49. Magee TR, Ross MG, Wedekind L, *et al*. Gestational diabetes mellitus alters apoptotic and inflammatory gene expression of trophobasts from human term placenta. *J Diabetes Complications* 2014; 28: 448–459.

50. Ruiz-Alcaraz AJ, Lipina C, Petrie JR, *et al*. Obesity-induced insulin resistance in human skeletal muscle is characterised by defective activation of p42/p44 MAP kinase. *PLoS ONE* 2013; 8: e56928.

51. Ruiz-Palacios M, Prieto-Sánchez MT, Ruiz-Alcaraz AJ, *et al*. Insulin treatment may alter fatty acid carriers in placentas from gestational diabetes subjects. *Int J Mol Sci* 2017; 18: pii: E1203.

52. Muralimanoharan S, Maloyan A, Myatt L. Mitochondrial function and glucose metabolism in the placenta with gestational diabetes mellitus: role of miR-143. *Clin Sci (Lond)* 2016; 130: 931–941.

53. Zhang JT, Cai QY, Ji SS, *et al*. Decreased miR-143 and increased miR-21 placental expression levels are associated with macrosomia. *Mol Med Rep* 2016; 13: 3273–3280.

54. Zhao C, Zhang T, Shi Z, *et al*. MicroRNA-518d regulates PPARα protein expression in the placentas of females with gestational diabetes mellitus. *Mol Med Rep* 2014; 9: 2085–2090.

55. Mi Y, Guo N, He T, *et al*. miR-410 enhanced hESC-derived pancreatic endoderm transplant to alleviate gestational diabetes mellitus. *J Mol Endocrinol* 2015; 55: 219–229.

56. Carreras-Badosa G, Bonmatí A, Ortega FJ, *et al.* Dysregulation of placental miRNA in maternal obesity is associated with pre- and postnatal growth. *J Clin Endocrinol Metab* 2017; 102: 2584–2594.

57. International Diabetes Federation. World Diabetes Day 2017 to focus on women and diabetes. 2017. Available from: https://www.idf.org/news/2:world-diabetes-day-2017-to-focus-on-women-and-diabetes.html.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1 | The relative expression levels of target genes.

Table S1 | Primer sequences

Table S2 | List of differentially expressed messenger ribonucleic acids

Table S3 | Gene Ontology analysis of differentially expressed messenger ribonucleic acids

Table S4 | List of differentially expressed non-coding ribonucleic acids

Table S5 | Expression levels of differentially expressed non-coding ribonucleic acids

Table S6 | Gene Ontology and Kyoto Encyclopedia of Genes and Genomes analysis of differentially expressed long non-coding ribonucleic acids

Table S7 | Gene Ontology and Kyoto Encyclopedia of Genes and Genomes analysis of differentially expressed circular ribonucleic acids