Differential Expression of MicroRNAs in Omental Adipose Tissue From Gestational Diabetes Mellitus Subjects Reveals miR-222 as a Regulator of ERα Expression in Estrogen-Induced Insulin Resistance

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Omental adipose tissue plays a central role in insulin resistance in gestational diabetes mellitus (GDM), and the molecular mechanisms leading to GDM remains vague. Evidence demonstrates that maternal hormones, such as estradiol, contribute to insulin resistance in GDM. In this study we determined the differential expression patterns of microRNAs (miRNAs) in omental adipose tissues from GDM patients and pregnant women with normal glucose tolerance using AFFX miRNA expression chips. MiR-222, 1 of 17 identified differentially expressed miRNAs, was found to be significantly up-regulated in GDM by quantitative real-time PCR ($P < .01$), and its expression was closely related with serum estradiol level ($P < .05$). Furthermore, miR-222 expression was significantly increased in 3T3-L1 adipocytes with a high concentration of 17β-estradiol stimulation ($P < .01$), whereas the expressions of estrogen receptor (ER)-α protein and insulin-sensitive membrane transporter glucose transporter 4 (GLUT4) protein ($P < .01$) were markedly reduced. In addition, ERα was shown to be a direct target of miR-222 in 3T3-L1 adipocytes by using the luciferase assay. Finally, antisense oligonucleotides of miR-222 transfection was used to silence miR-222 in 3T3-L1 adipocytes. The results showed that the expressions of ERα and GLUT4, the insulin-stimulated translocation of GLUT4 from the cytoplasm to the cell membrane and glucose uptake in mature adipocytes were dramatically increased ($P < .01$). In conclusion, miR-222 is a potential regulator of ERα expression in estrogen-induced insulin resistance in GDM and might be a candidate biomarker and therapeutic target for GDM. **(*Endocrinology* 155: 1982–1990, 2014)**

Gestational diabetes mellitus (GDM), defined as glucose intolerance that is first recognized during pregnancy, is one of the most common pregnancy complications and affects approximately 1%-14% of all pregnancies (1). Women with GDM have a high risk of other pregnancy complications, including gestational hypertension, macrosomia, and diabetic ketoacidosis, and are more likely to suffer from type 2 diabetes after the pregnancy. The offspring of GDM mothers are at a higher risk of metabolic diseases, such as diabetes, obesity, and cardiovascular disease, in their adult lives (2, 3).

GDM is characterized by peripheral (adipose tissue, skeletal muscle, and liver) insulin resistance (4). It has been shown that omental adipose tissue is more strongly associated with an adverse metabolic risk profile, such as the presence of insulin resistance and type 2 diabetes, than sc adipose tissue (5). Although the molecular mechanisms of insulin resistance in GDM have not been completely elucidated, rising levels of maternal hormones, including es-

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Abbreviations: ASO, antisense oligonucleotides; BMI, body mass index; CT, cycle threshold; ER, estrogen receptor; FC, fold change; GDM, gestational diabetes mellitus; GLUT4, glucose transporter 4; miRNA, microRNA; NGT, normal glucose tolerance; OGTT, oral glucose tolerance test; qRT-PCR, quantitative RT-PCR; UTR, untranslated region.

trogen, prolactin, placental lactogens, and progesterone, coincide with the development of maternal insulin resistance (6–8). Estradiol and estrogen receptor (ER)-α are involved in regulating pancreatic insulin levels at physiological concentrations and are critical in the development of obesity and insulin resistance (9–11). After binding to the ER, estradiol reduces the expression of the insulin-sensitive membrane transporter, glucose transporter 4 (GLUT4), in adipose tissue and muscle, thus decreasing insulin sensitivity (11, 12). In addition, the ER itself may also regulate GLUT4 levels (13, 14). These proteins may also be involved in pregnancy-induced insulin resistance and GDM.

MicroRNAs (miRNAs) are small 22- to 25-nt-long noncoding RNA molecules that regulate the translation of target mRNAs by binding to their 3′-untranslated regions (UTRs) leading to translation inhibition and/or mRNA degradation (15). miRNAs are known to be involved in many biological functions, including cell growth and proliferation, cell differentiation, organogenesis, metabolism, and immunity and in diseases such as cancer, cardiovascular diseases, and type 2 diabetes (16). In 2011, our group first reported that some differentially expressed serum miRNAs, miR-29a, miR-222, and miR-132, could become candidate biomarkers to predict GDM (17), but the roles of miRNAs in insulin resistance in GDM are currently unknown.

Because omental adipose tissue has a central role in insulin resistance, we performed a global miRNA gene expression array using AFFX miRNA expression chips in paired women omental adipose tissue samples (using three samples from GDM patients and three from pregnant women with normal glucose tolerance). This procedure was followed by individual quantitative RT-PCR (PCR) (qRT-PCR) assays to investigate the associations between miRNA expression and insulin resistance in omental adipose tissue in GDM. miR-222, one of the identified 17 differentially expressed miRNAs, was chosen to further study the relationship between its expression and insulin resistance in GDM.

Materials and Methods

Ethics statement

The study was approved by the Ethics Committee of Nanjing Medical University. All subjects gave written informed consent before taking part in the study.

Subjects and samples

Patients registered at the Department of Obstetrics and Gynecology, Nanjing Maternity and Child Health Care Hospital, were enrolled in this study. The diagnosis of GDM was based on American Diabetes Association criteria (18). Omental adipose tissue samples were obtained at the time of cesarean delivery from GDM patients (n = 13) and healthy pregnant women who had had normal glucose tolerance (NGT) during pregnancy (n = 13). Samples of omental adipose tissue were immediately (within 1 min) frozen in liquid nitrogen after explantation. The indications for cesarean delivery of both the GDM group and the NGT group (at 38–39 wk of gestation) were either breech presentation or fetal macrosomia. The ages of the subjects ranged from 23 to 33 years and their body mass index (BMI) from 23.2 to 27.2 kg/m^2. The exclusion criteria for both groups included hypertensive disorders, history of type 2 diabetes, smoking, chemical dependency, assisted reproductive technology treatment, multiple pregnancies, fetal congenital anomalies, and any other confounding pathologies (including intrahepatic cholestasis of pregnancy, hyperthyroidism and hypothyroidism). All GDM patients improved their glycemic control though dietary management, without using insulin or metformin. Three paired omental adipose tissue samples from the GDM and NGT groups were used to perform the global miRNA gene expression array using AFFX miRNA expression chips. The other 10 paired samples were used for further validation.

Omental adipose tissue total RNA extraction

Total RNA from omental adipose tissue samples was extracted using Trizol and analyzed using the UV2800 UV spectrophotometer (UNIC) with an A260 to A280 ratio of between 1.8 and 2.0 to ensure highly quantity and quality RNA samples. In addition, an Agilent 2100 Bioanalyzer (Agilent Technologies) was used to determine the integrity of the RNA in the samples by calculating the RNA integrity numbers and measuring 28S to 18S rRNA ratio in each sample. The miRNAs were isolated from the total RNA and purified using the mirVana miRNA isolation kit (Ambion).

miRNA expression profiles

AFFX miRNA expression chips were used to perform the miRNA expression assay. Total RNA from six omental adipose tissue samples (three from GDM patients and three from the NGT group) was isolated, and miRNAs were labeled using the RNA labeling kit. The labeled miRNA hybridization solution was added to the chip for hybridization for 17 hours at 45°C, 60 rpm. After being washed and stained using the GeneChip Fluidics Station 450 (Affymetrix), the chip was inserted into the Affymetrix autoloader carousel using an appropriate Fluidics Script and scanned with the GeneChip Scanner 3000 using GeneChip operating software.

qRT-PCR of miR-222 in omental adipose tissue samples

Validation of the miRNA expression data for miR-222 by qRT-PCR was performed on total RNA extracted from the 13 paired omental adipose tissue samples from the GDM and NGT groups, using TaqMan microRNA assays (Life Technologies Inc) in the 7900 HT real-time PCR system (Life Technologies), according to the manufacturer's protocols. MiR-16 was used as an endogenous control due to its stable expression in all samples in the profiling experiments. The relative expression values of miR-222 vs the control were obtained using the $\delta\delta$cycle threshold (CT) method (relative quantification = $2^{-\Delta\Delta CT}$) using SDS

version 2.3 and RQ Manager 1.2 software (Life Technologies). Real-time RT-PCR of miR-222 was performed using gene-specific primers (forward: TATCCAGTGCAGGGTCCGAGGTAT and reverse: TAATAGAAAGCTACATCTGGCTACTGGGT). Real-time RT-PCR of miR-16 was performed using gene-specific primers (forward: TATCCAGTGCAGGGTCCGAGGTAT and reverse: CGGCGGTAGCAGCACGTAAATAT).

Cell culture of mouse 3T3-L1 adipocytes

Mouse 3T3-L1 adipocytes (5×10^4 cells; American Type Culture Collection) were cultured as described previously (19). The effects of 17β-estradiol (Sigma) on the adipocytes were tested at a range of doses (10^{-2} to 1 nmol/L). The effects of 17β-estradiol on miR-222 expression were also determined. All the experiments were repeated three or four times.

Detection of serum estradiol

Serum specimens were collected at the time of cesarean delivery. The estradiol levels in the GDM and NGT groups (n = 13 for both) were immediately (within 30 min) determined using a Modular E170 platform electrochemiluminescence immunoassays (Roche Diagnostics).

Synthesis of antisense oligonucleotides of mmu-miR-222

Oligonucleotides were prepared using conventional phosphoramidite chemistry and DNA synthesis equipment (ABI). The 2′-O-methoxyethyl phosphoramidites and succinate-linked solid supports were prepared as described previously (20). Synthesis of antisense oligonucleotides (ASOs) for the treatment of adipocytes was as described previously (21). The ASOs used were a mmu-miR-222 ASO (5′-ACCCAGUAGCCAGAUGUAGCU-3′) or the negative control ASO (5′-CAGUACUUUUGUGUAGUACAA-3′).

Transfection of 3T3-L1 adipocytes

A total of 10^4 3T3-L1 cells were seeded into each well of a six-well cell culture plate (Costar). When the cells reached 30%-50% confluence, they were transfected with 250 nM oligonucleotides (either miR-222 ASO or negative control ASO) using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. At 5 hours after transfection, the transfection solution was replaced with complete culture medium. The cultures were supplemented with adipocyte-inducing medium (AIM) when the cells reached 100% confluence to induce adipocyte differentiation. Fully differentiated 3T3-L1 adipocytes were lysed and inhibition of miR-222 expression was verified by qRT-PCR as described above.

Luciferase reporter expression assays using luciferase genes with mutated or wild-type ERα-3′ UTR in 3T3-L1cells cotransfected with miR-222 or miR-SCR

The wild-type ERα-3′-UTR was amplified by PCR from human cDNA using the following PCR primers: forward, 5′-CCAAGCTTAACCCTATTGAGAGGTGATGT-3′ and reverse, 5′-CGAGCTCGCTACATAAGATTGTCTGTCAT-3′. The ERα 3′UTR mutated reporter construct was prepared by mutagenizing the miR-222 seed match region in ERα 3′UTR. The mutated ERα 3′-UTR was amplified using the following primers: forward, 5′-CCAAGCTTAACCCTATTGAGAGGTGATGT-3′ and reverse, 5′-CGAGCTCGGTTCTTAAGATTGTCTGTCAT-3′. The DNA segments produced from these amplifications were inserted into the pMIR-REPORT miRNA expression reporter vector (Ambion) using the *Sac*I and *Hin*dIII sites.

3T3-L1 cells were plated at an initial density of 1×10^4 cells per well into 96-well plates. Differentiated 3T3-L1 cells (day 7 of differentiation) were cotransfected with pMIR-REPORT vectors containing either the wild-type or mutated ERα 3′-UTR segments along with the control vector, *Renilla* luciferase reporter vector (pRL-TK). Cells were transfected with the precursor microRNAs for miR-222 (pre-miR-222) and the miR-scrambled control (miR-SCR) at 24 hours after transfection with reporter constructs and incubated for an additional 24 hours. Assays were performed to determine the level of gene expression using the dual luciferase reporter assay kit (Promega). To normalize transfection efficiency, the *Renilla* luciferase-expressing plasmid was used as an internal control. Three independent experiments were performed in triplicate. The mean values of the normalized ratios were compared.

Immunoblotting of ERα and GLUT4

Total protein was extracted as described previously. Plasma membrane proteins were extracted using the eukaryotic membrane protein extraction reagent (Pierce). Western blot analysis was performed with primary antibodies against ERα, GLUT4, and β-tubulin (Abcam) (19).

Glucose uptake assays

2-Deoxy-d-[^{3}H]glucose (CIC) uptake was assayed as described previously (22). The experiments were repeated three times.

Data processing and statistical analysis

miRNA expression levels were summarized based on the raw probe intensities using the Robust Multichip Average procedure in the APT package (Affymetrix). Quantile normalization was done on both the GDM and the normal data sets. Only miRNAs with an at least 1.5-fold change between the GDM and normal tissues, along with a value of $P < .05$ for the detected changes, were considered for further analyses. Statistical significance was analyzed using the unpaired Student's *t* test, and a value of $P \leq .05$ was considered to be statistically significant.

Results

Clinical characteristics of the study population

Clinical and laboratory data were compared between pregnant women with GDM and those with NGT. As shown in Table 1, there were no statistically significant differences between the GDM and NGT groups for maternal age, parity, BMI, gestational age, and placental weight. Women from the GDM group had higher glucose levels at each oral glucose tolerance test (OGTT) time

Table 1. Clinical Data for the GDM Patients and the NGT Controls

	GDM Group (n = 13)	NGT Group (n = 13)	*P* Value
Age, y	27.62 ± 3.10	27.85 ± 3.36	.857
Gravidity	1.38 ± 0.51	1.46 ± 0.52	.705
Parity	1	1	
BMI, kg/m^2	25.51 ± 0.93	24.91 ± 1.23	.178
OGTT, fast, mmol/L	5.14 ± 0.43	4.36 ± 0.31	.000
OGTT, 1 h, mmol/L	11.05 ± 0.97	9.03 ± 0.79	.000
OGTT, 2 h, mmol/L	10.15 ± 1.03	9.03 ± 0.79	.004
HbA1c, %	5.74 ± 0.32	5.09 ± 0.31	.000
Birth weight, kg	3.63 ± 0.36	3.33 ± 0.27	.025
Placental weight, kg	0.54 ± 0.03	0.53 ± 0.02	.328

Data are presented as means ± SEM.

point and had a higher glycated hemoglobin level than women from the NGT group. The GDM mothers had heavier fetuses than did NGT group mothers.

Differential expression of miRNAs in omental adipose tissue between GDM patients and normal pregnant women

We identified 17 differentially expressed miRNAs in omental adipose tissue between the GDM and NGT groups, by using AFFX miRNA expression chips (**?**/suppl_file/**-13-.pdf**"Supplemental Table 1). We performed hierarchical clustering using the techniques of average linkage clustering and Euclidian distance. The cluster image for miRNA expression is shown in Supplemental Figure 1.

miR-222 is up-regulated in GDM omental adipose tissue and negatively correlated with the expression of ERα protein and GLUT4 protein

miR-222 expression was examined using qRT-PCR in further omental adipose tissue samples from the GDM and NGT groups (n = 13 for both). The relative expression levels of miR-222 are shown in Figure 1. miR-222 expression levels were significantly up-regulated in omental adipose tissue from GDM patients compared with tissue from the NGT group ($P < .01$). The relationships of miRNA-222 expression levels with ERα and GLUT4 protein levels in omental adipose tissue were analyzed using Spearman's correlation analysis. miR-222 expression levels were negatively correlated with the protein levels of both ERα ($R^2 = 0.6771$, $P < .05$) and GLUT4 ($R^2 = 0.6414$, $P < .05$) in omental adipose tissue (Figure 1).

Serum estradiol levels in the GDM group and the NGT controls

Maternal serum estradiol levels were measured at the time of cesarean delivery in GDM women and NGT controls (n = 13 for both). A significant increase in serum estradiol levels was noted in the GDM group as compared with NGT group (Figure 2; *, $P < .05$).

High estradiol concentrations in adipocytes increase miR-222 expression and decrease ERα and GLUT4 expression

Exposure of 3T3-L1 adipocytes to increasing concentrations of 17β-estradiol (10^{-2}, 10^{-1}, and 1 nmol/L) resulted in an overall overexpression of miR-222 when compared with the baseline expression level (10^{-2} nmol/L). miR-222 was initially overexpressed at 10^{-1} [fold change (FC) = 1.64] and 1 (FC = 2.63) nmol/L of estrogen. ERα was down-regulated at 10^{-1} (FC = 1.58) and 1 (FC = 3.28) nmol/L of estrogen, and GLUT4 was down-regulated at 10^{-1} (FC = 1.24) and 1 (FC = 2.22) nmol/L of estrogen (Figure 3).

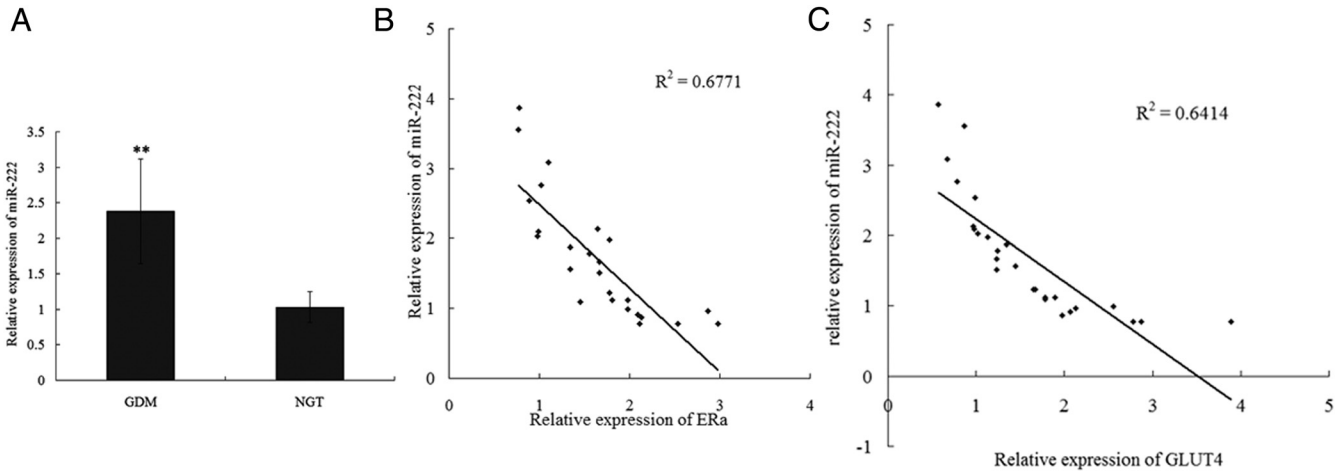

Figure 1. The relative expression levels of miR-222 and the proteins ERα and GLUT4 in omental tissue samples from the GDM and NGT groups (n = 13 for each group). A, Representative quantitative densitometry analysis of miR-222 normalized to miR-16 calculated according to the $2^{-\delta\delta Ct}$ method. **, $P < .01$; *, $P < .05$. B, Spearman's correlation analysis of ERα protein expression levels ($R^2 = 0.6771$, $P < .05$). C, Spearman's correlation analysis of GLUT4 protein expression level ($R^2 = 0.6414$, $P < .05$).

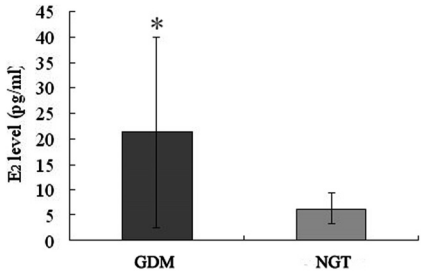

Figure 2. Maternal serum estradiol levels in the GDM group and the NGT group determined using electrochemiluminescence. The bars represent mean ± SD. A significant increase was noted in the GDM group. E_2, 17β-estradiol. *, $P < .05$.

ERα protein expression is up-regulated in miR-222-silenced differentiated 3T3-L1 adipocytes

Cultured differentiated 3T3-L1 adipocytes were infected either with the miR-222 ASO or the negative control ASO for 48 hours. Using qRT-PCR and Western blot, significant decreases in miR-222 levels in the miR-222 ASO group were observed (Figure 4A, $P < .01$). In the miR-222 ASO group, there was a 3-fold increase in ERα levels (Figure 4, B and C).

Expression of luciferase gene with mutated or wild-type ERα 3′-UTR in 3T3-L1 cells cotransfected with miR-222 or miR-SCR

We performed expression assays using the luciferase reporter gene system using the wild-type ERα 3′-UTR or a mutated version to validate the miR-222 target prediction. The vector constructed by inserting either the wild-type sequence of the ERα 3′-UTR ERα mRNA (ERα-3′-UTR) or a mutated seed sequence of the miR-222-binding site (ERα-3′-UTR-mut) into the 3′-UTR of the pMIR-REPORT luciferase reporter (Figure 5A). We found that cotransfection of the vector with the wild-type ERα-3′-UTR and the miR-222 precursor, pre-miR-222, inhibited luciferase activity, whereas cotransfection of the vector with ERα-3′-UTR-mut and pre-miR-222 caused no inhibition of luciferase activity (Figure 5B). These results validated the hypothesis that miR-222 is able to bind of to the 3′-UTR of ERα mRNA. We then investigated the impact of miR-222 on the expression of ERα and GLUT4. qRT-PCR revealed that ERα and GLUT4 mRNA decreased significantly 48 hours after 3T3-L1 cells were transfected with pre-miR-222(Figure 5, C and D).

Effect of miR-222 on basal and insulin-stimulated glucose uptake in 3T3-L1 adipocytes

To determine whether miR-222 affects insulin sensitivity, we assessed glucose uptake in differentiated 3T3-L1 adipocytes. In miR-222-silenced cells, basal glucose uptake was similar to that observed in controls. However, insulin-stimulated glucose uptake was approximately 40% higher in these cells than in the controls (Figure 6).

Effects of miR-222 ASO on basal and insulin-stimulated GLUT4 translocation

In adipocytes, insulin-stimulated glucose uptake is dependent on the translocation of the insulin responsive glucose transporter GLUT4 from the intracellular storage compartments to the plasma membrane. Therefore, using miR-222-silenced differentiated 3T3-L1 adipocytes, we examined GLUT4 translocation to the plasma membrane in response to insulin. The results demonstrated that both total GLUT4 protein content and insulin-stimulated GLUT4 translocation to the plasma membrane were increased when miR-222 was inhibited in differentiated 3T3-L1 adipocytes (Figure 7).

Discussion

There is increasing evidence that miRNAs play an important role in many aspects of metabolism and glucose ho-

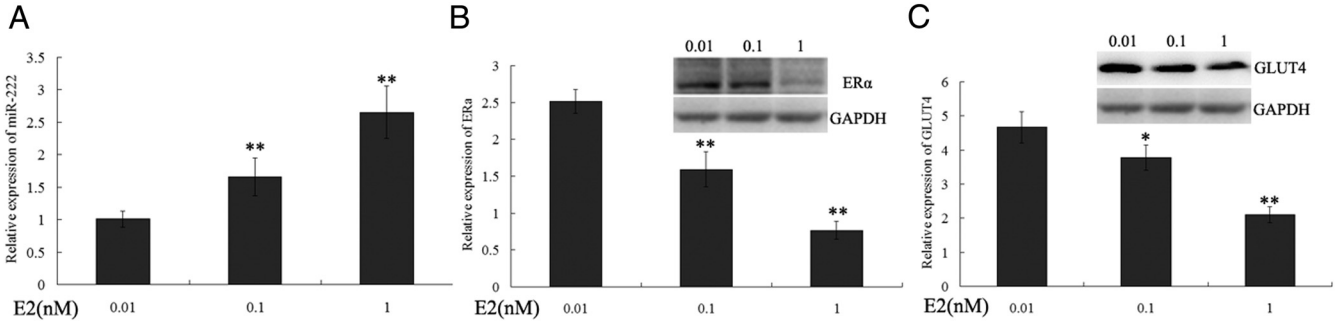

Figure 3. Effects of stimulation with a range of estradiol concentrations (10^{-2}, 10^{-1}, and 1 nmol/L) on the expression levels of miR-222, ERα protein, and GLUT4 protein. A, Quantitative real-time PCR expression analysis of miR-222 in 3T3-L1 adipocytes after stimulation with a range of estradiol concentrations. miR-222 expression levels were up-regulated with increasing concentrations of exogenous 17β-estradiol. The *miR-16* gene was used as a control. E_2, 17β-estradiol. B and C, Expressions of ERα protein (Figure 4 B) and GLUT4 protein (Figure 4 C) in 3T3-L1 adipocytes after stimulation with a range of estradiol concentrations. Western blot analysis showed that both ERα and GLUT4 protein expressions were significantly down-regulated with increasing concentrations of exogenous estrogen. GAPDH protein was used as a control. The bars represent ERα or GLUT4 to GAPDH ratios ± SD. GAPDH, glyceraldehyde-3-phosphate dehydrogenase *, $P < .05$; **, $P < .01$. The experiments were repeated three times.

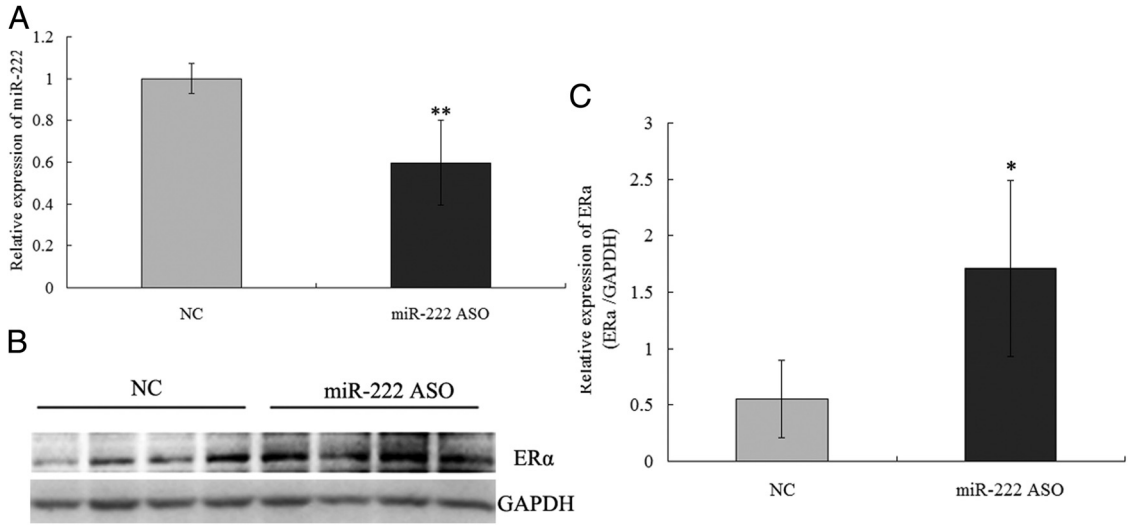

Figure 4. Effects of miR-222 ASO transfection on ERα protein expression in 3T3-L1 adipocytes. A, The relative expression of miR-222 was reduced 40% when the miR-222 ASO was transfected in 3T3-L1 cells. NC, negative control. B, Western blots showed that ERα protein levels were increased in the miR-222 ASO group. C, Bar paragraph showed that the relative expression of ERα protein was up-regulated about 3-fold in the miR-222 ASO group compared with the negative control group. Value represents the mean ± SD of four experiments. *, $P < .05$; **, $P < .01$.

meostasis and hence may be involved in the pathogenesis of disorders such as type 2 diabetes. However, it is the first time that we constructed a miRNA expression profile of pregnant women omental adipose tissue and identified differentially expressed miRNAs that are potentially involved in insulin resistance in GDM, by using a global miRNA gene expression array in paired human omental adipose tissue samples from GDM and NGT subjects.

In our microarray screening, we found that the expression levels of nine miRNAs were significantly lower in GDM omental tissue, whereas the expression levels of eight miRNAs were significantly higher in GDM omental tissue than in normal tissue (Supplemental Table 1). Although the relationships between many of these differentially expressed miRNAs and GDM remain vague, several of them have been identified as components of pathways triggered by, or contributing to, the pathology of insulin resistance. miR-222 is up-regulated in the adipose tissue of diabetic rats (23) and has also been reported to regulate estradiol concentrations of patients with polycystic ovarian syndrome characterized by intrinsic insulin resistance and dysfunctional adipose tissue glucose metabolism (24, 25). miR-29a/b/c can be induced by high glucose and high insulin, and overexpression of miR-29a/b/c leads to insulin resistance (26). miR-143 participates in adipocyte differentiation, is induced in adipogenesis, and is down-regulated in obesity (27).

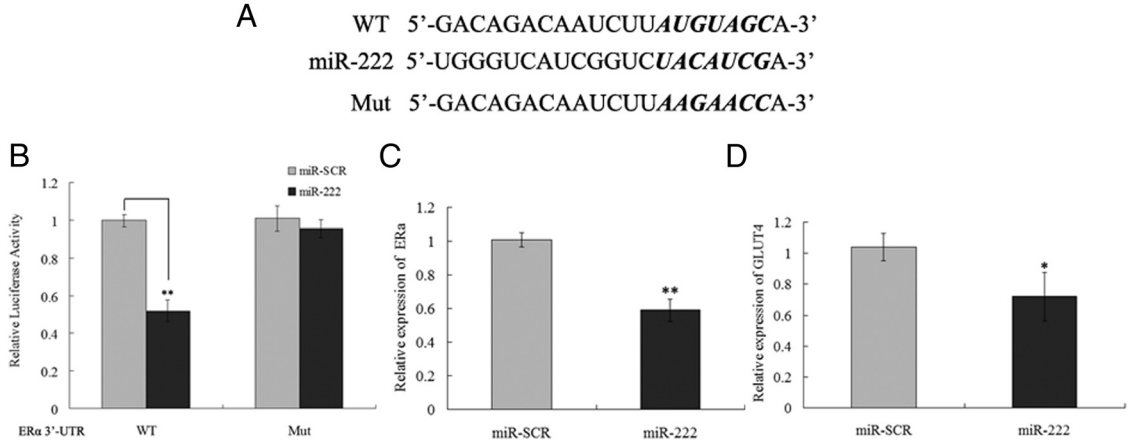

Figure 5. miR-222 directly targets ERα in 3T3-L1 cells. A, The 3′-UTR fragment of wild-type (WT) ERα and mutant (Mut) ERα were PCR amplified cloned. B, Relative luciferase expression levels obtained from pMIR-REPORT miRNA expression vectors containing either WT or Mut ERα 3′-UTR regions, when cotransfected with precursors of scrambled control miRNA (miR-SCR) or miR-222, and the *Renilla* control vector was calculated as the ratio of firefly/renilla luciferase activities in the cells and normalized to those of the control. The results are presented as means ± SD from three independent experiments with each experiment performed in triplicate. **, Significant difference ($P < .01$). C and D, Overexpression of miR-222 inhibited ERα and GLUT4 mRNA expression. *, Significant difference ($P < .05$).

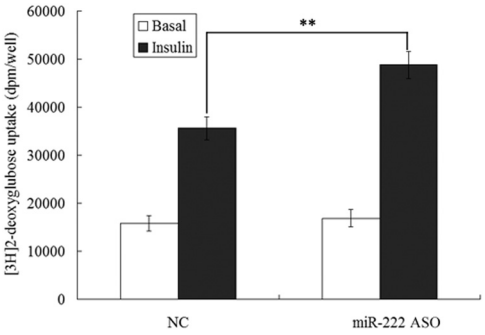

Figure 6. Effects of miR-222 ASO transfection on basal and insulin-stimulated glucose uptake in 3T3-L1 adipocytes. In miR-222-silenced cells, basal glucose uptake was similar to that observed in control cells, but in insulin-stimulated cells, glucose uptake was approximately 34% higher than in controls. NC, negative control. *, $P < .05$; **, $P < .01$.

Although genetic and behavioral risk factors have been demonstrated to be related to GDM, changes in the hormonal environment of pregnancy, which affect insulin sensitivity, seem to be involved in the development of GDM. Different gestational hormones secreted by the placenta may shed light on the differences between the pathogenesis of GDM and type 2 diabetes. Previous findings have demonstrated that estrogen, androgen, and human placenta lactogen as well as other hormones may play an important role in insulin sensitivity (28, 29).

It has been reported that high estrogen concentration can reduce insulin sensitivity in the late stages of pregnancy (30). The action of estrogen is mediated by the ER and its isoforms ERα and ERβ (31). In animals lacking ERα, insulin resistance is associated with reduced GLUT4 protein levels and decreased glucose uptake in skeletal muscle (32) and in vivo and in vitro studies indicate that a high concentration of estrogen can inhibit the expression of the insulin-sensitive transporter GLUT4 in adipose tissue, muscle, and liver (33). Directly activation of ERα positively mediates GLUT4 medicated glucose uptake and insulin action in skeletal muscle (34). Estrogen could increase the expression of GLUT4 and the ERα antagonist could reverse the estrogen-induced increase in the expression of GLUT4 (35). Recent study also showed that estrogen enhanced the glucose uptake capacity and led to translocation of GLUT4 to the plasma membrane in an ERα-dependent manner (36). Because ERα is a direct target of miR-222 in breast cancer cells, with a specific binding site at the seed sequence of miR-222 (37, 38), our results indicated that miR-222 was up-regulated in GDM omental tissue, and ERα and GLUT4 were both down-regulated in GDM omental tissue and the expression level of miR-222 in omental tissue were significantly correlated serum estradiol levels, we hypothesize that miR-222 may be involved in estrogen-induced insulin resistance possibly via regulatory effects on ERα and GLUT4.

In present study, we further demonstrated that in 3T3-L1 adipocytes, stimulation with a relatively high concentration of estrogen significantly increased miR-222 expression. High levels of estrogen suppressed ERα expression and reduced the expression and translocation of GLUT4 protein. Our luciferase reporter expression assays also strengthen the possibility that 3′-UTR of ERα mRNA is a direct target of miR-222 in 3T3-L1 adipocytes and ERα mRNA decreased significantly after 3T3-L1 cells were transfected with miR-222. We also found miR-222 silencing in 3T3-L1 adipocytes dramatically increased the expression of both ERα and GLUT4 and increased the insulin-stimulated translocation of GLUT4 from the cytoplasm to the cell membrane and the glucose uptake of mature adipocytes. Therefore, miR-222 could be an important regulator of ERα expression in estrogen-induced GDM insulin resistance. Indeed, the conclusions would be strengthened by further overexpression of miR-222 to determine whether this molecule directly alters ERα and GLUT4 expression and glucose transport, independent of the estradiol levels.

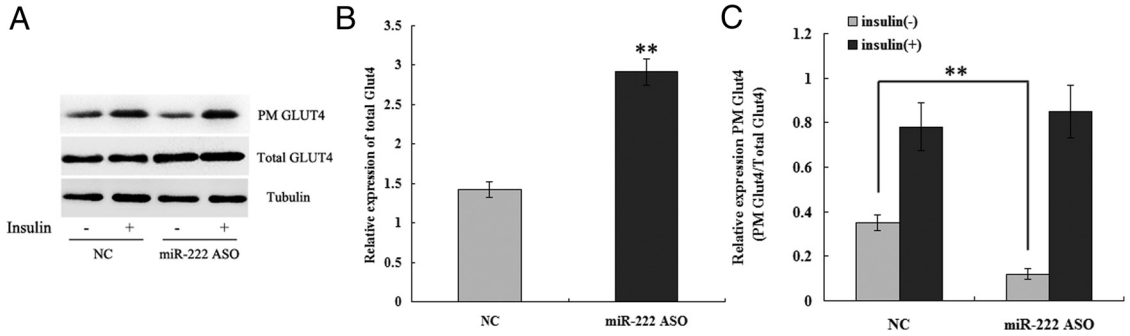

Figure 7. Effects of miR-222 ASO transfection on basal and insulin-stimulated GLUT4 translocation. A, Western blots showed that down-regulation of miR-222 increased insulin-stimulated GLUT4 translocation to the plasma membrane (PM) and increased total GLUT4 protein content. NC, negative control. B, Bar paragraph showed that the total GLUT4 protein content was increased when the miR-222 ASO was transfected. C, Bar graph showed that transfection of miR-222 ASO increased insulin-stimulated GLUT4 translocation to the plasma membrane (PM). *, $P < .05$; **, $P < .01$.

In conclusion, we constructed a miRNA expression profile of pregnant woman omental adipose tissue and identified a number of differentially expressed miRNAs potentially involved in insulin resistance in GDM by performing a global miRNA gene expression array in paired human omental adipose tissue samples. We conclude that miR-222 may be involved in insulin resistance, possibly via regulatory effects on ERα and GLUT4, and might be a candidate biomarker and therapeutic target for GDM.

Acknowledgments

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