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High expression of an unknown long noncoding RNA RP11-290L1.3 from GDM macrosomia and its effect on preadipocyte differentiation

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Abstract

Aims: Gestational diabetes mellitus (GDM)-induced macrosomia is predominantly characterized by fat accumulation, which is closely related to adipocyte differentiation. An unknown long noncoding RNA RP11-290L1.3, referred to as RP11, was identified to be dramatically upregulated in the umbilical cord blood of women with GDM-induced macrosomia in our previous study. We conducted this study to identify the function of RP11 in GDM-induced macrosomia.

Methods: The effects of RP11 gain- and loss-of-function on HPA-v (human preadipocytes-visceral) adipogenesis were determined with lentivirus mediated cell transduction. The mRNA and protein expression levels of adipogenesis makers were evaluated by qPCR/Western blot. Then, we performed the microarray and pathway analysis to explore the possible mechanisms by which RP11 regulates adipogenesis.

Results: Overexpression of RP11 significantly enhanced adipocyte differentiation and increased the mRNA and protein expression levels of adipogenesis makers, such as PPARγ, SREBP1c, and FASN by qPCR/Western blot. Knockdown of RP11 showed opposite effects. Microarray and pathway analysis showed, after RP11 knockdown, 1612 genes were upregulated, and 583 genes were down-regulated which were found to be mainly involved in metabolic pathways, insulin signaling pathway and MAPK signaling pathway.

Conclusion: In conclusion, the unknown IncRNA RP11 serves as a positive factor on preadipocyte differentiation which could shed light on fetal fat accumulation in GDM.

Key Words
- long noncoding RNA
- GDM
- preadipocyte differentiation
- macrosomia
- fat accumulation

Introduction

Fetal overgrowth is the predominant adverse outcome of GDM. Fetal macrosomia, defined as growth beyond an absolute birth weight, typically 4000 g (1), is not only closely associated with high incidence of maternal complications, but also with increased susceptibility to obesity and type 2 diabetes in adults (2). GDM-induced macrosomia is mainly characterized by size increase of insulin-sensitive tissues and organs, especially the fat tissue (3, 4). Adipogenesis, during which preadipocytes develop into mature adipocytes, is a critical process for fat mass accumulation and obesity development. Although fetal hyperinsulinemia caused by maternal hyperglycemia has been considered as an important cause of GDM macrosomia, the precise pathogenesis by which GDM mother affects fetal fat accumulation though umbilical cord blood remains poorly understood.
Long noncoding RNAs (lncRNAs) are a class of endogenous RNA molecules with a length of more than 200 bases. Significant associations between altered lncRNA profiles and GDM-related complications, such as macrosomia or trophoblast dysfunction, have been observed (5). There is increasing evidence that lncRNAs play important roles in the physiological and pathological development of adipogenesis (6, 7), stem cell pluripotency (8), cancer (9), cell development and differentiation (10, 11), etc. Cui et al. (12) demonstrated that the transcribed ultraconserved IncRNA, uc.417, could serve as a negative regulator of brown adipose tissue thermogenesis through the p38 MAPK pathway. Recently, Sun et al. (6) identified 175 lncRNAs that are specifically regulated during adipogenesis. These lncRNAs were shown to be strongly involved in adipogenesis under the control of key transcription factors, such as peroxisome proliferator-activated receptor γ (PPARγ) and CCAAT/enhancer-binding protein α (C/EBPα).

With an aim to explore whether lncRNAs are involved in GDM-induced fat accumulation, we hybridized the umbilical cord vein blood from normal pregnancy and GDM-induced macrosomia groups to a microarray. The results were deposited in NCBI’s Gene Expression Omnibus (accession number GSE65737) (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE65737) (13). Of the 1151 differentially expressed IncRNAs, the expression of IncRNA RP11-290L1.3 (RP11; ENST00000552367) was identified to be dramatically upregulated in the umbilical cord blood of women with GDM-induced macrosomia (fold change of 14.1039315); however, its function remains unknown.

In this study, we analyzed the clinical correlation between the expression of RP11 in the umbilical cord blood and neonatal fat mass (FM), F% (percent of body fat), and neonatal birth weight. The role of RP11 in adipogenesis was investigated by performing knockdown and overexpression experiments in human preadipocytes-visceral (HPA-v). Furthermore, the possible mechanisms by which RP11 regulates adipogenesis were primarily explored by microarray analysis of RP11-silenced HPA-v.

**Materials and methods**

**Ethics statement**

This study was conducted in accordance with the Declaration of Helsinki and was approved by the Ethics Committee of the Women’s Hospital of Nanjing Medical University. Written informed consent was obtained from all subjects prior to enrolment.

**Subjects**

All subjects enrolled in this study attended regular prenatal check-ups and delivered their babies at the Women’s Hospital of Nanjing Medical University in 2017. GDM was diagnosed using a standardized 75 g oral glucose tolerance test (OGTT) at 24–28 weeks of pregnancy. Based on the International Association of Diabetes and Pregnancy Study Group criteria (14), GDM was diagnosed if one or more of the following abnormality were met: glucose level equal to or greater than fasting 5.1 mmol/L, 1-h post load 10.0 mmol/L, or 2-h post load 8.5 mmol/L. Strict diet and exercise control or insulin treatment was administrated to achieve the goal of fasting glucose level of less than 5.3 mmol/L and 2-h postprandial level of less than 6.7 mmol/L.

The exclusion criteria for mothers were as follows: multiple pregnancies, hypertensive disorders, history of diabetes prior to conception, polycystic ovary syndrome, Cushing’s syndrome, pheochromocytoma, acromegaly, history of smoking, chemical dependency, use of assisted reproductive technology, multiple gestation, and any other confounding pathologies, including hyperthyroidism and hypothyroidism. The exclusion criteria for infants were as follows: preterm birth, fetal growth restriction, fetal congenital anomalies, and other confounding pathologies, such as neonatal infection. In this study, 32 GDM subjects with neonatal birth weights equal to or exceeding 4 kg (macrosomia group) and 47 non-GDM controls with neonatal birth weights less than 4 kg (control group) were enrolled. Both maternal and neonatal characteristics are presented in Table 1.

**Sample collection**

Umbilical cord blood samples from the macrosomia and control groups were collected at the time of cesarean or vaginal delivery. Whole blood was separated into serum and cellular fractions by centrifugation at 1574 g for 10 min, followed by centrifugation at 14,167 g and 1574 g for 10 min to completely remove any cell debris. The plasma sample (2 mL) from each woman was filtered through a 0.22 μm filter (MILLEXGV; Millipore) and then mixed with 2 mL TRIZol reagent and 0.4 mL chloroform. Following this, the aqueous layer was transferred to a new tube and 1.5 mL of 70% ethanol was added. This mixture was then applied to the RNeasy mini column (Qiagen) according to the manufacturer’s recommendations. RNA quantity and quality were measured using the NanoDrop ND-1000 spectrophotometer. Total RNA was eluted with
20 μL RNase-free water after DNase digestion and then stored in liquid nitrogen (15).

**Neonatal body composition analysis**

Anthropometric measurements, including bicipital, tricipital, subscapular, and suprailliacal skinfold thicknesses were performed for 79 infants within 12 h after childbirth. All measurements were performed by a well-trained neonatologist. Each measurement was repeated three times, and the results were averaged. Neonatal body FM and fat percentage (F%) were calculated according to the Weststrate method (16), as follows: SFT4 = the sum of bicipital, tricipital, subscapular, and suprailliacal skinfold thickness; body density (D) = 1.1235 - 0.0719 × log (SFT4); F% = (585/D - 550) × 100%; FM = F% × body weight. The neonatal characteristics are presented in Table 1.

**Table 1** Maternal and neonatal characteristics of control group and GDM group.

|                         | Control group (n = 47) | GDM (n = 32) | P value |
|-------------------------|------------------------|--------------|---------|
| **Maternal characteristics** |                        |              |         |
| Age (years)             | 28.1 ± 0.85            | 28.0 ± 2.70  | 0.85    |
| Gravidity               | 1.43 ± 0.74            | 1.69 ± 0.85  | 0.17    |
| Parity                  | 1.17 ± 0.38            | 1.22 ± 0.42  | 0.60    |
| Pre-pregnancy BMI (kg/m²)| 23.2 ± 1.80            | 22.2 ± 1.85  | 1.25    |
| HbA1C (%)               | 5.31 ± 0.64            | 5.40 ± 0.59  | 0.02    |
| OGTT, fasting (mmol/L)  | 4.68 ± 0.38            | 4.93 ± 0.38  | 0.01    |
| OGTT, 1 h (mmol/L)      | 9.11 ± 0.56            | 9.82 ± 0.62  | 3.3E-06 |
| OGTT, 2 h (mmol/L)      | 7.74 ± 0.53            | 8.12 ± 0.52  | 0.003   |
| Gestational week        | 39.3 ± 0.83            | 39.3 ± 0.54  | 0.70    |
| Delivery mode (vaginal delivery) | 35 (47.5%)             | 18 (56.3%)   | 0.15    |
| Triglycerides (mmol/L)  | 3.89 ± 1.25            | 4.72 ± 1.71  | 0.03    |
| Cholesterol (mmol/L)    | 6.94 ± 1.47            | 7.66 ± 1.53  | 0.04    |
| Insulin therapy         | 0 (0%)                 | 6 (18.8%)    |         |
| **Neonatal characteristics** |                        |              |         |
| Birth weight (kg)       | 3.40 ± 0.31            | 4.20 ± 0.11  | 1.7E-23 |
| Gender (male)           | 29 (61.7%)             | 19 (59.4%)   | 1.00    |
| Bicipital (mm)          | 3.9 ± 0.3              | 4.0 ± 0.3    | 0.26    |
| Tricipital (mm)         | 5.8 ± 1.4              | 7.6 ± 0.4    | 8E-11   |
| Subscapular (mm)        | 5.7 ± 0.3              | 7.5 ± 0.3    | 3.4E-31 |
| Skinfold thicknesses (mm)| 5.8 ± 0.3              | 7.5 ± 0.3    | 4.87E-35|
| Neonatal body fat mass (FM) (g) | 648 ± 111              | 960 ± 52.3   | 7E-26   |
| Fat percentage (F%)     | 18.9 ± 1.60            | 22.9 ± 0.68  | 5.4273E-23|
| SFT4 (mm)               | 21.2 ± 2.01            | 26.5 ± 1.03  | 2.83E-24|
| Body density (D)        | 1.03 ± 0.002           | 1.02 ± 0.001 | 6.7E-23 |

SFT4 equals to the sum of bicipital, tricipital, subscapular, and suprailliacal skinfold thicknesses; body density (D) = 1.1235 - 0.0719 × log (SFT4); FM = F% × body weight; F% = (585/D - 550) × 100%.

Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Total RNA was isolated using TRIzol reagent (Invitrogen) and then reverse transcribed using the PrimeScript® RT reagent kit with gDNA Eraser (Perfect Real Time) (Takara) according to the manufacturer’s instructions. The expression of RP11 in the blood samples and HPA-v cells was measured by qRT-PCR. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the internal control. The RP11 primer sequences used were as follows: sense, TTCAGGCAGAGTTGGAGGTG and antisense, GGCAAGGAGATCGACTTTCG. The relative expression of RP11 was determined using the 2ΔΔCt method and normalized to GAPDH expression. Information on the primers used is presented in Table 2.

Cell culture and preadipocyte differentiation

HPA-v (ScienCell Research Laboratories, San Diego, CA, USA) were cultured in preadipocyte medium (PAM; ScienCell Research Laboratories) supplemented with 5% fetal bovine serum (Gibco), 1% preadipocyte growth supplement (ScienCell Research Laboratories), and 1% penicillin/streptomycin at 37°C in 5% CO₂.

To induce differentiation, confluent HPA-v (day 0) were cultured in differentiation medium containing serum-free PAM supplemented with 250 nM insulin, 1 mM dexamethasone, 0.5 mM 3-isobutyl-1-methylxanthine, and 1 μM rosiglitazone. The culture medium was
replaced after 4 days. Cells were then cultured in serum-free Dulbecco’s modified Eagle’s medium containing 250 nM insulin, which was replaced every 2 days until lipid accumulation was observed in the cells (day 10). HPA-v were harvested on days 0, 1, 4, 8, and 10 for further experiments.

**Lentivirus-mediated overexpression of IncRNA RP11**

The viral vector we used contains an open reading frame for GFP. IncRNA RP11 was overexpressed in HPA-v using recombinant lentiviral vectors (GenePharma Co., Ltd, Shanghai, China) containing either pre-IncRNA RP11 or negative control precursor IncRNA. For lentiviral infection, preadipocytes were plated overnight in 6-well plates (Corning) at a concentration of 3 × 10^5 cells per well. On reaching about 40% confluency, the stage at which the transfection efficiency is optimal, preadipocytes were transfected with either IncRNA RP11 overexpression lentiviral vector or negative control in 5 μg/mL polybrene (Sigma–Aldrich). At 24 h post transfection, fresh medium was added to the preadipocyte culture. Green fluorescent protein (GFP) levels were measured at 48 and 72 h post transfection, and the expression of IncRNA RP11 was verified using qRT-PCR. After the transfected cells reached confluence, differentiation was induced as described previously.

**Lentivirus-mediated knockdown of IncRNA RP11**

IncRNA RP11 was knocked down in HPA-v using recombinant lentiviral vectors (GenePharma Co., Ltd, Shanghai, China) containing either pre-IncRNA RP11 or negative control precursor IncRNA and two anti-IncRNAs against IncRNA RP11 (SH1 and SH2). The sequences of SH1 and SH2 were as follows: ACCGAAAGTGCTCGATCAGTTCCTGCG and GTGCAGTTCCTGCGATTCAGTTCCTGCG, respectively. For lentiviral infection, preadipocytes were processed as described previously. The lentivirus-mediated expression of IncRNA RP11 was verified using qRT-PCR.

**Oil red O staining**

In vitro differentiated cells (day 8) were washed with PBS, followed by fixation with 4% paraformaldehyde for 30 min at room temperature. Next, cells were washed twice with 60% isopropanol and stained with 0.6% (w/v) filtered oil red O solution (Sigma–Aldrich; stock solution, 3 mg/mL in isopropanol; working solution, 60% oil red O stock solution and 40% distilled water) for 30 min at 37°C. Cells were then washed with PBS to remove unbound dye, and visualized and photographed under a microscope (Axio–Home; Carl Zeiss) at ×200 magnification. The intracellular triglyceride content relative to total protein content was measured using the Triglyceride Assay kit (Applygen Technologies Inc., Beijing, China) and BCA Protein Assay kit (Pierce) according to the manufacturer’s instructions. At the indicated time points, mature adipocytes were treated with lysis buffer, and the cell lysates were harvested and homogenized. Subsequently, the cell suspension was retained to detect the triglyceride and protein concentrations.

**Proliferation assay**

Cell proliferation assay was performed using the CellTrace™ Far Red Cell Proliferation kit (Invitrogen) for flow cytometry. Briefly, human preadipocytes were first transfected with either the IncRNA RP11 overexpression/knockdown or negative control lentiviral vector. At 48 h post transfection, isolated cells were plated in 6-well plates at a density of 1 × 10^5 cells per well, until they reached 50% confluency. CellTrace™ medium was added to each well of the plate according to the manufacturer’s instructions. Next, the dyed cells were cultured for 1 h at
37°C, and then imaged and counted by flow cytometry (MoFlo XDP; Beckman Coulter, USA) at a 72 h time point. The cell proliferation assay was performed independently for three times.

**Western blotting**

Cells were lysed in lysis buffer containing 20 mM Tris-HCl (pH 8.0), 150 mM NaCl, 10% glycerol, 20 mM beta-glycerophosphate, 1% NP-40, 5 mM EDTA, and 0.5 mM EGTA supplemented with protease inhibitors (Roche Applied Science). The cell extracts were boiled in sodium dodecyl sulfate (SDS) loading buffer (150 mM Tris–HCl (pH 6.8), 12% SDS, 30% glycerol, 0.02% bromophenol blue, and 6% 2-mercaptoethanol) at 95°C for 10 min. Protein content was measured using the Pierce BCA Protein Assay kit (Thermo Fisher Scientific), according to the manufacturer’s instructions. Next, proteins (30 g) were separated on 10% SDS-polyacrylamide gels and transferred to a 0.2 μm PVDF membrane (Millipore). After blocking with 5% skimmed milk, the membranes were incubated overnight at 4°C with appropriate primary antibodies against SREBP1c (Abcam), peroxisome proliferator-activated receptor gamma (PPARγ; Abcam), C/EBPα (Abcam), FABP4 (Abcam), LPL (Abcam), FASN (Abcam), and GAPDH (Abcam). Subsequently, the membranes were hybridized with a secondary antibody conjugated with peroxidase (Santa Cruz Biotechnology). The protein levels were quantitated using the ChemiDoc system (Bio–Rad).

**Microarray analysis**

lncRNA RP11 expression was knocked down in HPA-v using anti-lncRNA against lncRNA RP11 (SH1 and SH2). After differentiation was induced as previously described, total RNA was isolated from differentiated cells using an RNeasy kit (Qiagen). After RNA quality determination and RT, microarray analysis for detection of gene expression was performed using a Whole Human Genome Microarray (Agilent Technologies) according to the manufacturer’s protocol. Data were extracted using the Agilent Feature Extraction software (version 9.5). Differentially expressed genes between the SH1/SH2 and control groups were identified, and an intersection was made. Differentially intersecting genes were identified via analyzing the fold change and the Student’s t-test P value. For fold change analysis, a cutoff value of 2 was used. Next, all candidate genes were mapped to gene ontology (GO) terms in the database (http://www.geneontology.org/).

Pathway analysis is a functional analysis that maps genes to the Kyoto Encyclopedia of Genes and Genomes (KEGG; http://www.genome.jp/kegg) pathways.

**Statistical analysis**

Means and standard deviations were calculated for demographic data. The differential expression of RP11 between normal control and GDM women complicated with macrosomia was analyzed by Student’s t-test using SPSS software (version 17.0; SPSS). P < 0.05 was considered statistically significant.

Pearson’s correlation coefficient analysis was used to analyze the correlation between the relative expression of RP11 (normalized to GAPDH) and neonatal birth weight, F%, or FM in the umbilical cord blood of 79 subjects. P < 0.05 was considered statistically significant.

**Results**

**RP11 is a highly conserved lncRNA**

lncRNA RP11 is an intergenic lncRNA located on chromosome 12q21.2 in the human genome. Multiple alignment analysis of the RP11 locus showed that it is highly conserved in the rhesus, human, mouse, dog, and elephant genomes using the University of California Santa Cruz (UCSC) genome browser (Santa Cruz; http://genome.ucsc.edu). The RP11 sequence in the human and mouse genomes showed 81.37% identity.

**Maternal and neonatal demographic and anthropometric parameters**

Analysis of maternal and neonatal demographic and anthropometric characteristics showed that there were no significant differences in maternal age, gestational age, gravidity, parity, nulliparous and neonatal gender between the GDM macrosomia and normal control groups (P > 0.05). However, the pre-pregnancy BMI and OGTT results of women in the macrosomia group were higher than that of women in the control group (P < 0.05), and the neonatal anthropometric parameters, including birth weight, tricipital SF, subcapular SF, suprailiacal SF, SFT4, neonatal body FM, and F%, but excluding bicipital SF were dramatically higher in the macrosomia group (P < 0.01; Table 1).
Correlation between RP11 expression and neonatal body composition

Pearson’s correlation coefficient analysis suggested a significant positive correlation between RP11 expression and neonatal birth weight ($r=0.8003$, $P < 0.01$), F% ($r=0.7999$, $P<0.05$) and FM ($r=0.8150$, $P<0.001$) (Fig. 1).

Effect of RP11 overexpression on preadipocyte proliferation and differentiation

RP11 was overexpressed in HPA-v using recombinant lentiviral vectors containing pre-RP11 and negative control precursor. The preadipocytes did not show significant morphological changes after RP11 overexpression (Fig. 2A). qRT-PCR results confirmed that RP11 was significantly overexpressed in the RP11 overexpression group compared to the control group ($P<0.001$; Fig. 2B). Results of cell proliferation assay showed that human preadipocytes transfected with RP11 overexpression or negative control lentivirus exhibited proliferation rates of 50.6 and 46.8%, respectively. There was no significant difference in proliferation rate between the two groups ($P > 0.05$; Fig. 2E). Differentiation of human preadipocytes stably transfected with RP11 overexpression or negative control lentivirus was then induced. Measurement of the triacylglycerol content showed that lipid accumulation was significantly increased in RP11-overexpressing cells on day 7 of adipogenesis (Fig. 3A). The morphology of the cells and the size of the lipid droplets were analyzed by oil red O staining. More than 80% of the cells in the control group had differentiated into mature adipocytes, and lipid droplets accumulation was more obvious after RP11 overexpression (Fig. 3B). Subsequently, the effects of RP11 overexpression on several important factors involved in the differentiation of preadipocytes were evaluated. Results showed that the mRNA and protein expression levels of PPARγ, SREBP1c, and FASN were dramatically increased in the RP11 overexpression group, whereas those of LPL and C/EBPα were not significantly altered (Fig. 3C and D).

Effect of RP11 silencing on preadipocyte proliferation and differentiation

RP11 expression was silenced in HPA-v using two lentiviral vectors: SH1 (ACCGAAAGTCGATCTCCTTGC) and SH2 (GTGCAGTTCCTTGAGCTTGAC). The preadipocytes did not show significant morphological changes after RP11 silencing (Fig. 2C). qRT-PCR results confirmed that RP11 expression was dramatically down-regulated in the SH1 and SH2 groups compared with the control group (Fig. 2D). SH1 ($P < 0.01$) and SH2 ($P < 0.001$) lentiviral vectors were chosen for further experiments. Results of cell proliferation assay indicated that preadipocyte
Figure 2
Effects of RP11 overexpression or silence on preadipocyte proliferation by flow cytometry. Fluorescence microscopy to detect preadipocytes morphology changes after RP11 overexpression (A) or silence (C). (B) The preadipocytes did not show significant morphological changes in all groups. The relative expression of RP11 normalized to GAPDH expression in control and RP11 overexpression groups. (D) The relative expression of RP11 normalized to GAPDH expression in control and RP11 silence groups. RP11 was dramatically down-regulated in SH1 (P < 0.01) and SH2 (P < 0.001) group. (E) Effects of RP11 overexpression on preadipocyte proliferation by flow cytometry (P < 0.001). Left: 0 h in negative control group. Middle: 72 h incubation after RP11 overexpression in negative control. Right: 72 h incubation after RP11 overexpression in RP11 overexpression group (P > 0.05). (F) Effects of RP11 silence on preadipocyte proliferation by flow cytometry. Left: 0 h in negative control group. Middle: 72 h incubation after RP11 silence by SH1 (P > 0.05). Right: 72 h incubation after RP11 silence by SH2 (P > 0.05).
proliferation was not significantly altered after RP11 silencing ($P > 0.05$; Fig. 2F).

Measurement of the triacylglycerol content showed that lipid accumulation was significantly decreased in the SH1 and SH2 groups on day 7 of adipogenesis (Fig. 4A). Oil red O staining results for intracellular lipid accumulation during differentiation showed significantly less fat droplet accumulation after RP11 silencing (SH1: $P < 0.001$, SH2: $P < 0.001$) (Fig. 4B). Further, our results showed that the mRNA expression of PPARγ (SH1: $P < 0.001$, SH2: $P < 0.001$), SREBP1c (SH1: $P < 0.05$, SH2: $P < 0.01$), FASN (SH1: $P < 0.01$, SH2: $P < 0.001$), and LPL (SH1: $P < 0.001$, SH2: $P < 0.001$) was significantly decreased in the RP11-silenced groups, while that of C/EBPα was not significantly altered ($P > 0.05$) (Fig. 4C). The protein expression of the four important factors involved in preadipocyte differentiation was then analyzed. The results showed that the protein expression of PPARγ (SH1: $P < 0.001$, SH2: $P < 0.001$), SREBP1c (SH1: $P < 0.001$, SH2: $P < 0.01$), FASN (SH1: $P < 0.001$, SH2: $P < 0.001$), and LPL (SH1: $P > 0.05$, SH2: $P < 0.05$) was significantly decreased after RP11 silencing (Fig. 4D).

**Differentially expressed mRNAs after RP11 silencing**

lncRNAs can modulate expression of genes that are positioned in the vicinity of their transcription sites in a cis-acting manner (17). To identify molecular mediator response to RP11, we used a transcription position bioinformatic analysis and found RP11 sites located...
Figure 4
Efficiency of RP11 silence in preadipocyte. (A) Triacylglycerol content detected lipid accumulation at day 7 during adipogenesis (SH1: \( P < 0.05 \), SH2: \( P < 0.01 \)). (B) Intracellular lipid accumulation during differentiation stained with oil red. The results showed significantly less fat droplets accumulation after RP11 silence (SH1: \( P < 0.001 \), SH2: \( P < 0.001 \)). (C) mRNA expressions of five important factors involved in preadipocytes differentiation. The results showed that mRNA expressions of PPAR\( \gamma \) (SH1: \( P < 0.001 \), SH2: \( P < 0.001 \)), SREBP1c (SH1: \( P < 0.05 \), SH2: \( P < 0.01 \)), FASN (SH1: \( P < 0.01 \), SH2: \( P < 0.001 \)) and LPL (SH1: \( P < 0.001 \), SH2: \( P < 0.001 \)) were significantly decreased in RP11 silence groups, whereas the expressions of C/EBP\( \alpha \) were not obviously changed (\( P > 0.05 \)). (D) Protein expressions of four important factors involved in preadipocytes differentiation. The results showed that protein expressions of PPAR\( \gamma \) (SH1: \( P < 0.001 \), SH2: \( P < 0.001 \)), SREBP1c (SH1: \( P < 0.001 \), SH2: \( P < 0.05 \)), FASN (SH1: \( P < 0.001 \), SH2: \( P < 0.01 \)) and LPL (SH1: \( P > 0.05 \), SH2: \( P < 0.05 \)) were significantly decreased after RP11 was silenced.

Figure 5
Study on the relationship between RP11 and PHLDA1. (A) The transcriptional position analysis of RP11 in the mouse genome. PHLDA1 is located upstream of RP11. (B) The transcriptional of PHLDA1 was unaffected by RP11 overexpression.
within pleckstrin homology-like domain, family A, member 1 (PHLDA1) (Fig. 5A). We also examined the expression of PHLDA1 in RP11-overexpressed adipocytes. However, the expression of PHLDA1 was unaffected (Fig. 5B), providing the evidence that RP11 does not act in cis to regulate expression of its neighbor.

To further understand the mechanism by which RP11 regulates the adipocyte thermogenic program, we performed gene chip analysis of differentiated RP11 silenced adipocytes. RP11 expression was silenced by transfection with the SH1 or SH2 lentiviral vector. Using microarray analysis, a total of 4765 differentially expressed mRNAs were identified between the SH1 and control groups, and a total of 3833 mRNAs were identified between the SH2 and control groups (fold change ≥ 2, P < 0.05). Intersection analysis showed that 1612 genes were upregulated in both the SH1 and SH2 groups as compared with the control group, whereas 583 genes were down-regulated in both the SH1 and SH2 groups (Fig. 6A and B). The top 20 up- and down-regulated mRNAs between the RP11-silenced and control groups are presented in Table 3.

**GO and pathway analysis**

Intersection of differentially expressed genes was analyzed by GO and pathway analyses to determine the genes attributes in biological process, cellular components, and molecular functions (Supplementary Figures 1 and 2, see section on supplementary materials given at the end of this article). Among them, 1612 upregulated genes were found to be involved in cytokine–cytokine receptor interaction, sphingolipid metabolism, NOD-like receptor signaling pathway, etc. Interestingly, 583 down-regulated genes were found to be involved in metabolic pathways, insulin signaling pathway, and triglyceride metabolic process, which have been reported to be closely related with preadipocyte differentiation (Fig. 6C and D).

**Discussion**

GDM is a common disease during pregnancy, which may lead to fetal metabolic disorders, such as macrosomia, hyperglycemia, diabetes, obesity, and cardiovascular disease (18). GDM-induced macrosomia also can cause shoulder dystocia, postpartum hemorrhage, and an increase in the rate of cesarean sections. As mentioned previously, adipogenesis is a critical process involved in fat accumulation and obesity development, and RP11 is highly expressed in the umbilical cord blood of women with GDM-induced macrosomia, suggesting the need to further explore the association between RP11 and GDM-induced fat accumulation.

According to our research, we found that RP11 expression may be an important factor that regulates neonatal body FM and fat percentage. Following RP11 overexpression, more number of lipid droplets were accumulated in the cells, indicating the more active process of lipid uptake and TG synthesis. Moreover, the expression of certain crucial transcription factors, like PPARγ was obviously increased in the RP11 overexpression group, which was consistent with the results of oil red O staining. Basseri et al. (19) demonstrated that loss of PHLDA1 results in mature-onset obesity and insulin resistance by regulating lipogenesis. We also examined the expression of PHLDA1 in RP11-overexpressed adipocytes. However, the expression of PHLDA1 was unaffected. Furthermore, RP11 silencing decreased preadipocyte differentiation. For a comprehensive understanding of RP11 function, we performed microarray analysis after RP11 silencing, and found that the differ or upregulated downstream genes were enriched in some biological processes related to interferon (IFN) responses, type I IFN signaling, and IFN-inducing pathways, such as the TLR pathway, while the down-regulated downstream genes were involved in triglyceride metabolic process, energy reserve metabolic process, insulin signaling pathway, MAPK signaling, and other processes.

It is well known that obesity is caused due to an imbalance between food intake and energy consumption. Because of extra nutrient uptake, excess fatty acids and glucose are used to be the substrates of the synthesis for triglyceride in adipocytes, which eventually results in obesity (20). Fat accumulation is determined by the balance between triglyceride synthesis and breakdown. After the uptake of carbohydrates, the circulating glucose is taken up by adipocytes and converted into pyruvate through glycolysis. Pyruvate oxidation plays a vital role in the tricarboxylic acid (TCA) cycle. Citrate, an intermediate in the TCA cycle, is an important substrate for de novo lipogenesis (21). Results of microarray analysis showed that pathways associated with pyruvate metabolism and TCA cycle were down-regulated, meaning that the de novo lipogenesis is suppressed, which in turn leads to the decrease of fat deposition. Thus we can suppose that dysfunction of metabolic pathways contributes to the development of obesity.

The insulin signaling pathway also plays a critical role in glucose and lipid metabolism. Normally, secreted
insulin first binds to the tyrosine-autophosphorylated insulin receptor, then activate insulin receptor substrate 1 (IRS1), and finally stimulates glucose transport via activating GLUT-4 (22). Increased insulin levels and GLUT-4 translocation promote glucose uptake and energy storage in the adipose tissue, which inhibits lipolysis and prevents the adipose tissue from supplying energy to other organs (23). In addition, insulin has been shown to stimulate lipid synthesis and suppress lipid degradation through increasing the level of the transcription factor SREBP1c (24). Furthermore, it was shown that IR-deficient (insulin receptors-deficient) mice whose insulin receptors were knocked out displayed lower levels of plasma triglyceride and decreased FM, suggesting that the insulin signaling pathway may promote obesity through regulating glucose and lipid metabolism (25).

The adipose tissue expands by two main mechanisms: hypertrophy or hyperplasia. Obesity is associated with abnormal adipocyte growth, resulting from the induction of preadipocyte differentiation (26). Among the various transcription factors related to adipocyte differentiation, PPARγ is the most critical factor (27). PPARγ, highly expressed in the adipose tissue, has two main isoforms: PPARγ1 and PPARγ2. The former is present in the bulk of the body tissues, while the latter is primarily found in the fat tissue. PPARγ binds another nuclear hormone receptor called retinoid X receptor alpha to form a heterodimer, and regulates the transcription of genes involved in energy and lipid metabolism. Besides, it also activates adipocyte differentiation and promotes fat accumulation. Studies have shown that PPARγ−/− cells may not display adipogenic action due to lack of activation of genes involved in adipogenesis (28, 29). PPARγ signaling is closely linked with the MAPK signaling pathway. Four MAPK cascades have been identified in eukaryotic cells: ERK, Janus tyrosine kinase (JNK)/stress-activated...
Table 3  Top 20 up-regulated and down-regulated mRNAs between RP11 silence and control groups.

| Name          | SH1/control | Fold change | Name          | SH2/control | Fold change |
|---------------|-------------|-------------|---------------|-------------|-------------|
| RSAD2         | 162         |            | SH1/control   | 68.3        |
| CXCL11        | 104         | 60.1       | CXCL10        | 91.6        | 52.5        |
| CMPK2         | 83.8        | 49.2       | MX1           | 78.3        | 48.4        |
| IFI44L        | 78.0        | 46.7       | GBP4          | 74.1        | 46.7        |
| LGALS9        | 73.7        | 45.3       | BST2          | 72.2        | 44.3        |
| CCL5          | 70.3        | 40.8       | IFITM1        | 65.6        | 39.7        |
| IFIT2         | 63.3        | 39.5       | HERC6         | 61.1        | 37.9        |
| USP41         | 60.4        | 36.6       | RAB2B7        | 59.4        | 36.2        |
| CH25H         | 59.4        | 35.7       | MX2           | 58.8        | 35.1        |
| ISG15         | 58.1        | 34.7       | IFI27         | 58.1        | 33.0        |
| IFIT3         | 56.7        | 32.4       | RSAD2         | 162         |            |
| Top 20 up-regulated mRNAs | | | Top 20 down-regulated mRNAs | | |
| Name          | SH1/control | Fold change | Name          | SH2/control | Fold change |
| RSAD2         | 162         |            | SH1/control   | 68.3        |
| CXCL11        | 104         | 60.1       | CXCL10        | 91.6        | 52.5        |
| CMPK2         | 83.8        | 49.2       | MX1           | 78.3        | 48.4        |
| IFI44L        | 78.0        | 46.7       | GBP4          | 74.1        | 46.7        |
| LGALS9        | 73.7        | 45.3       | BST2          | 72.2        | 44.3        |
| CCL5          | 70.3        | 40.8       | IFITM1        | 65.6        | 39.7        |
| IFIT2         | 63.3        | 39.5       | HERC6         | 61.1        | 37.9        |
| USP41         | 60.4        | 36.6       | RAB2B7        | 59.4        | 36.2        |
| CH25H         | 59.4        | 35.7       | MX2           | 58.8        | 35.1        |
| ISG15         | 58.1        | 34.7       | IFI27         | 58.1        | 33.0        |
| IFIT3         | 56.7        | 32.4       | RSAD2         | 162         |            |

protein kinase, p38 MAPK, and ERK5 signal transduction pathways (30). Previous evidence suggests that PPARγ can be phosphorylated and regulated by MAPK members, including ERK and p38. The expression and transcription of PPARγ were shown to be increased after ERK and p38 MAPK activation, which promoted the differentiation of adipocytes. Another study conducted by Jung Hwan Oh reported that artemisia princeps suppressed adipocyte differentiation via downregulation of the PPARγ and MAPK pathways (31, 32).

To explore the underlying mechanism of obesity, a previous study focused on the interaction between immune and metabolic systems and proposed a phenomenon called infectobesity (33). Human studies also have shown that intrinsic and proper IFN responses are favorable for antiviral and anti-obesity effects (34). Similarly, the neonates of pregnant women with GDM tend to have more mastadenovirus and papillomavirus in their intestinal flora (35). Mastadenoviruses have been identified as the strongest infectious agents contributing to obesity, and a series of animal studies have confirmed this finding (36).

Among the IFN family, type I IFNs, including IFN-α and IFN-β have been largely researched with regards to their role in the immune system and effects on metabolism. A study conducted by Ariel D Quiroga and colleagues demonstrated that IFN-α-2b, belonging to the type I IFN class, exhibits potent anti-obesity effects related to increased fatty acid oxidation and inhibition of the master cholesterogenic transcription factor, SREBP-2, which influences cholesterol synthesis (37). Kyoungran Lee et al. also reported that IFN-α particularly suppresses lipid accumulation during the early phase of adipogenesis. Further, type I IFNs can activate STATs, which have been shown to modulate PPAR-γ signaling through JAK (38). In a previous study, animal experiments showed that IFN-β suppressed inflammatory cell infiltration into adipose tissue as well as production of inflammatory mediators potentially via the inhibition of the nuclear factor kappa B pathway, which subsequently resulted in prevention of adipose hypertrophy and weight gain (39). Additionally, interferon-regulatory factors (IRFs) involved in IFN signaling have also been shown to play a role in the development of adipocytes, and several IRF proteins have been revealed to possess the ability to repress adipogenesis (27). Based on these findings, we speculate that IFNs may play a vital role in the inhibition of adipogenesis, and thus exert anti-obesity effects.

Our study has some limitations. First, we only focus on the expression of RP11 in the umbilical cord blood. Future studies should assess whether the RP11 is differentially expressed after neonatal period, its expression change during the course of pregnancy or it is associated with metabolic controls during pregnancy. Secondly, although we have identified the differentially expressed downstream genes which involved in the adipocyte differentiation, the target gene and the specific mechanism are still unknown. Thirdly, the study were performed in experimental models, additional animal experiments may be needed to confirm our conclusion.
In conclusion, in our study we found that RP11 is highly expressed in the umbilical cord blood of women with GDM macrosomia, and overexpression or silencing of RP11 can significantly promote or inhibit the differentiation of preadipocytes into mature adipocytes, respectively. Furthermore, the differentially expressed downstream genes identified after RP11 silencing may be its target genes, which were found to be closely involved in biological processes related to adipocyte differentiation, such as the insulin signaling pathway, metabolic pathways, MAPK signaling pathway, etc. Although the underlying mechanism and the specific target genes of RP11 remain unclear, our findings can be applied for the future screening of RP11 downstream targets, which will help to further enhance our understanding of GDM-induced fat accumulation.

Supplementary materials
This is linked to the online version of the paper at https://doi.org/10.1530/EC-20-0584.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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