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REPRODUCTIVE PHYSIOLOGY AND DISEASE

miR-181d-5p, which is upregulated in fetal growth restriction placentas, inhibits trophoblast fusion via CREBRF

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Abstract

Purpose Fetal growth restriction (FGR) is a common complication characterized by impaired placental function and unfavorable pregnancy outcomes. This study aims to elucidate the expression pattern of miR-181d-5p in FGR placentas and explore its effects on trophoblast fusion.

Methods The expression pattern of miR-181d-5p in human FGR placentas were evaluated using qRT-PCR. Western blot, qRT-PCR, and Immunofluorescence analysis were performed in a Forskolin (FSK)-induced BeWo cell fusion model following the transfection of miR-181d-5p mimic or inhibitor. Potential target genes for miR-181d-5p were identified by screening miRNA databases. The interaction between miR-181d-5p and Luman/CREB3 Recruitment Factor (CREBRF) was determined through a luciferase assay. Moreover, the effect of CREBRF on BeWo cell fusion was examined under hypoxic conditions.

Results Aberrant up-regulation of miR-181d-5p and altered expression of trophoblast fusion makers, including glial cell missing 1 (GCM1), Syncytin1 (Syn1), and E-cadherin (ECAD), were found in human FGR placentas. A down-regulation of miR-181d-5p expression was observed in the FSK-induced BeWo cell fusion model. Transfection of the miR-181d-5p mimic resulted in the inhibition of BeWo cell fusion, characterized by a down-regulation of GCM1 and Syn1, accompanied by an up-regulation of ECAD. Conversely, the miR-181d-5p inhibitor promoted BeWo cell fusion. Furthermore, miR-181d-5p exhibited negative regulation of CREBRF, which was significantly down-regulated in the hypoxia-induced BeWo cell model. The overexpression of CREBRF was effectively ameliorated the impaired BeWo cell fusion induced by hypoxia.

Conclusions Our study demonstrated that miR-181d-5p, which is elevated in FGR placenta, inhibited the BeWo cell fusion through negatively regulating the expression of CREBRF.

Keywords FGR · Placenta · Trophoblast cell · miR-181d-5p · CREBRF · Hypoxia

Introduction

Fetal growth restriction (FGR) is a prevalent pregnancy complication, affecting approximately 5–10% of all pregnancies. Recent guidelines from the American College of Obstetricians and Gynecologists (ACOG) have emphasized the significant risks associated with FGR, including premature birth, intrauterine demise, and neonatal death [1]. Epidemiological studies have consistently shown an increased likelihood of adverse developmental outcomes

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Table 1 Sequence of primers for qRT-PCR

Gene	Forward Primer (5' → 3')	Reverse Primer (5' → 3')
CREBRF	ACCCACTTCAAGCACACAAAT	GGGTTGATCTTTACCTTTGCCT
Syn1	CCTCAAACCTCACCTGTGTAAAAT	AGAGCCATTCAAACAACGATAGG
GCM1	GCCAAGCAAGAGCAGCAAA	TCATCTCAAAGGACACAGGTTCA
ECAD	AAGGTGACAGAGCCTCTGGAT	ACTCTCTCGGTCCAGCCCA
GAPDH	AGATCATCAGCAATGCCTCCT	TGGTCATGAGTCCTTCCACG
miR-181d-5p	CCAACATTCATTGTTGTCGGTGGGT	R

in fetuses with FGR, such as cognitive disorders in childhood, Bronchopulmonary Dysplasia, and metabolic disorders [2, 3]. Despite these risks, effective interventions for FGR remain elusive in clinical practice.

The etiology of FGR is multifactorial, with placental insufficiency being identified as the predominant cause [4]. FGR placentas commonly exhibit distinctive characteristics, including inadequate placental vascular perfusion and compromised trophoblast cell invasion [5, 6]. Furthermore, impaired trophoblast fusion is thought to contribute to the pathogenesis of FGR [7]. Recent studies have highlighted significant down-regulation of trophoblast fusion markers, such as syncytin-1 and syncytin-2, in FGR placentas [8]. Additionally, the down-regulation of Signal Transducer and Activator of Transcription 3 (STAT3) in FGR placentas has been implicated in abnormal trophoblast fusion [9]. Moreover, decreased levels of p45 NF-E2 in FGR placentas contribute to the excessive formation of syncytiotrophoblast, thereby exacerbating placental insufficiency [10].

MicroRNAs (miRNAs) have emerged as crucial regulators in the development of FGR by modulating translational repression or mRNA degradation. For instance, the upregulation of miR-141 in placentas has been demonstrated to promote FGR pathogenesis by regulating the expression of pleomorphic adenoma gene 1 (PLAG1) [11]. Additionally, the overexpression of miR-424 in FGR placentas has been shown to significantly inhibit trophoblast cell invasion and proliferation through targeting estrogen-related receptor γ (ERRγ) [12]. In other contexts, miR-181d-5p has been implicated in the regulation of Epithelial-Mesenchymal Transition in breast and pancreatic cancer cells [13, 14], as well as the modulation of blood-tumor barrier (BTB) permeability by targeting SRY-box transcription factor 5 (SOX5) [15]. Moreover, miR-181d-5p has been found to be highly expressed in gestational trophoblastic neoplasia (GTN) [16]. However, the role of miR-181d-5p in placental function remains elusive. Therefore, in this study, we aimed to investigate

Table 2 Clinical characteristics of maternal and neonatal in normal and FGR group

Characteristic	Control group ($n=7$)	FGR group ($n=7$)	*P*-value
Maternal age (year)	26 ± 3	28 ± 1.46	0.1137
Maternal height (cm)	160 ± 5	162 ± 3.20	0.2181
Pregestational weight (kg)	53 ± 7	53.71 ± 7.52	0.9148
Pregestational BMI (kg/cm^2)	20.85 ± 1.83	20.38 ± 2.72	0.7119
Placental weight (g)	543 ± 40	498.57 ± 53.98	0.1064
Birth weight (g)	3395 ± 556	1813.57 ± 491.72	0.0001
Fetal body length (cm)	52 ± 4	40.85 ± 4.18	0.0005
Fetal head circumference (mm)	330 ± 13	293.57 ± 21.76	0.0024
Fetal abdominal circumference (mm)	334 ± 16	269.71 ± 27.02	0.0002
Gestational age at delivery	39 ± 0.76	34 ± 1.83	< 0.0001
Parity			
Median	1	1	-
Range	0–2	0–2	-
Mode of delivery			
Vaginal	4 (57.1%)	1 (14.3%)	-
Caesarean section, CS	3 (42.9%)	6 (85.7%)	-
Fetal sex			
Boys	3 (42.8%)	2 (28.6%)	-
Girls	4 (57.2%)	5 (71.4%)	-
Mode of conceive			
Natural	7 (100%)	7 (100%)	
Assisted pregnancies	0 (0%)	0 (0%)	

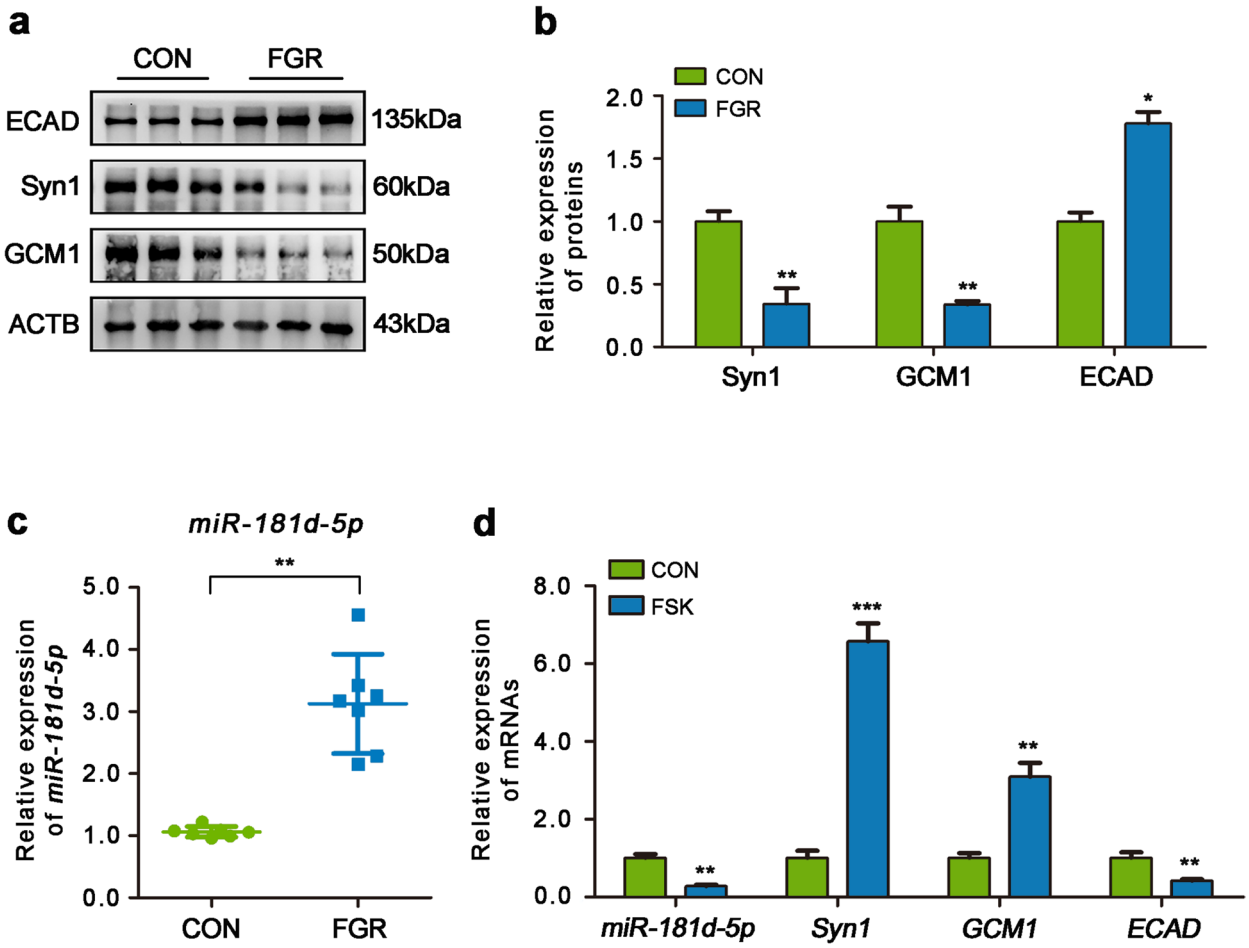


Fig. 1 Expression pattern of miR-181d-5p in the placenta and FSK-induced BeWo cell fusion model. **a-b** Western blot analysis was performed to detect the protein levels of trophoblast fusion markers, GCM1, Syn1, and ECAD in human placenta with or without FGR ($n=3$ lanes per group). **c** The relative expression levels of *miR-181d-5p* in human placentas were verified by qRT-PCR ($n=7$ samples per group). **d** qRT-PCR was used to detect the levels of *miR-181d-5p* and the mRNA levels of *GCM1*, *Syn1*, and *ECAD* in BeWo cell fusion model with or without FSK treatment ($n=3$ lanes per group). The results are presented as mean ± standard deviation. Statistical significance between two groups was analyzed using Student's *t*-test. * indicates a significant difference compared to the control group. * $P<0.05$, ** $P<0.01$, *** $P<0.001$

the expression pattern of miR-181d-5p in FGR placentas and evaluate its effect on trophoblast fusion.

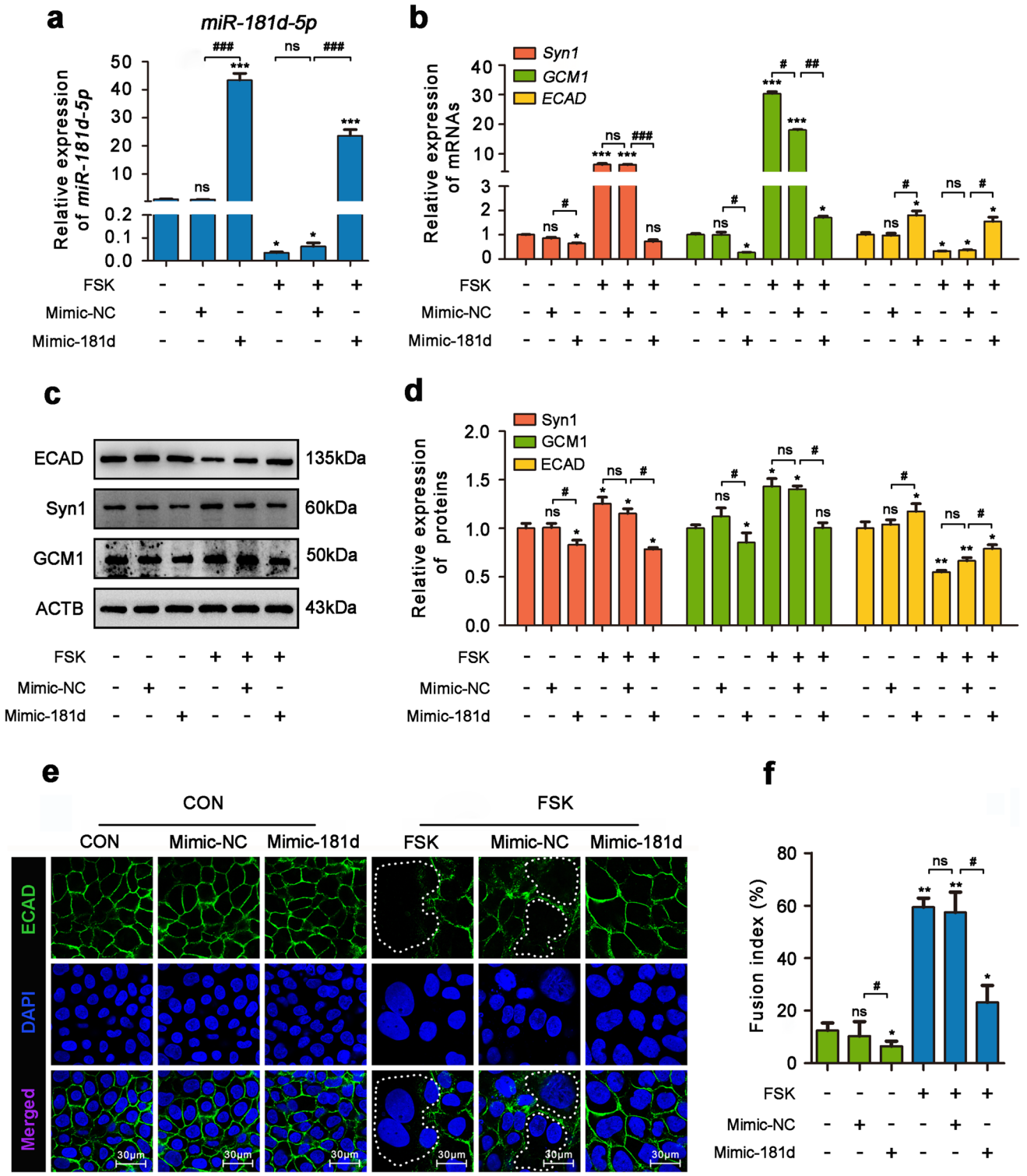
Materials and methods

Clinical samples collection

Placental samples were obtained from *the First Affiliated Hospital of Chongqing Medical University*, encompassing both individuals with FGR and those without the condition. Individuals of this study were divided into two groups: the normal group ($n=7$) and the FGR group ($n=7$). Inclusion and exclusion criteria were strictly followed for participant selection. The criteria were as follows: 1) pregnant women aged between 25 to 35 years; 2) no history of abnormal pregnancy or childbirth, non-smokers, no alcohol consumption, and no prolonged exposure to medications; 3) all placental tissues collected after term delivery, 4) exclusion of patients with gestational diabetes mellitus (GDM), antiphospholipid antibody syndrome (AAS) and other diseases. This project was approved by the *Biomedical Research Ethics Committee of Chongqing Medical University*. Informed consent was obtained from all participating pregnant women.

Cell culture and hypoxia induction

The BeWo cell line and HEK293T cell line were purchased from the American Type Culture Collection (ATCC). BeWo cells were cultured in Ham's F-12K medium (Boster, Wuhan, China) supplemented with 15% (v/v) fetal bovine



serum (FBS; Lonsera, Shanghai, China), and 100 μg/mL penicillin–streptomycin (Beyotime, Shanghai, China). HEK293T cells were cultured in DMEM-high glucose medium (Sigma-Aldrich, MO, USA) supplemented with 10% (v/v) fetal bovine serum (FBS; Lonsera). To induce BeWo cell fusion, cells were treated with 25 μM Forskolin (FSK; Sigma-Aldrich) for 72 h, which was dissolved in DMSO (Sigma-Aldrich,). All cultured cells were maintained at 37 °C incubator containing 5% (v/v) CO_2 and 20% (v/v) O_2. For the hypoxia induction, BeWo cells were cultured in a 37 °C incubator containing 1% (v/v) O_2, 5% (v/v) CO_2, 94% (v/v) N_2.

◂**Fig. 2** miR-181d-5p mimic inhibited BeWo cell fusion. **a-b** BeWo cells were transfected with miR-181d-5p mimic in the presence of FSK for 72 h. The relative expression levels of *miR-181d-5p*, *GCM1*, *Syn1*, and *ECAD* were analyzed by qRT-PCR ($n=3$ samples per group). **c-d** Expressions of GCM1, Syn1, and ECAD in the FSK-induced BeWo cell fusion model with or without miR-181d-5p mimic were analyzed by Western blot ($n=3$ lanes per group). **e** Immunofluorescence detection of ECAD (green) in the BeWo cell fusion model with or without miR-181d-5p mimic ($n=3$ lanes per group). Nucleus were stained with Hoechst (blue). Scale bars = 30 μm. **f** Graphical representation of the ratio of fused BeWo cells. The results are presented as mean ± standard deviation. Statistical significance among multiple groups was analyzed using one-way ANOVA. * indicates a significant difference compared to the control group. # denotes a significant difference between the groups indicated by the crossbar. * $P<0.05$, ** $P<0.01$, *** $P<0.001$. # $P<0.05$, ## $P<0.01$, ### $P<0.001$

Cell transfection

BeWo cells were seeded in a 24-well plate and transfected with a final concentration of 50nM miR-181d-5p mimic (Ribobio, Guangzhou, China) or 100nM miR-181d-5p inhibitor (Ribobio), along with their respective negative controls (mimic-NC and inhibitor-NC). The transfection procedure was carried out using the riboFECT CP Transfection Kit (Ribobio). Additionally, the BeWo cells were treated with 25 μM FSK for 72 h.

The overexpression plasmid containing the Luman/CREB3 Recruitment Factor (CREBRF) gene was obtained from GeneCopoeia (Guangzhou, China) and transfected into BeWo cells using *TransIntro*™ EL Transfection Reagent (TransGen Biotech, Beijing, China) as per the manufacturer's instructions. For each transfection in a 24-well plate, a total of 0.8μg of plasmid was utilized, while 4.0μg of plasmid was used for a 6-well plate format. Following a 6-h transfection, cells were cultured with medium supplemented with 25 μM FSK and incubated for an additional 72 h.

RNA extraction and quantitative real-time PCR

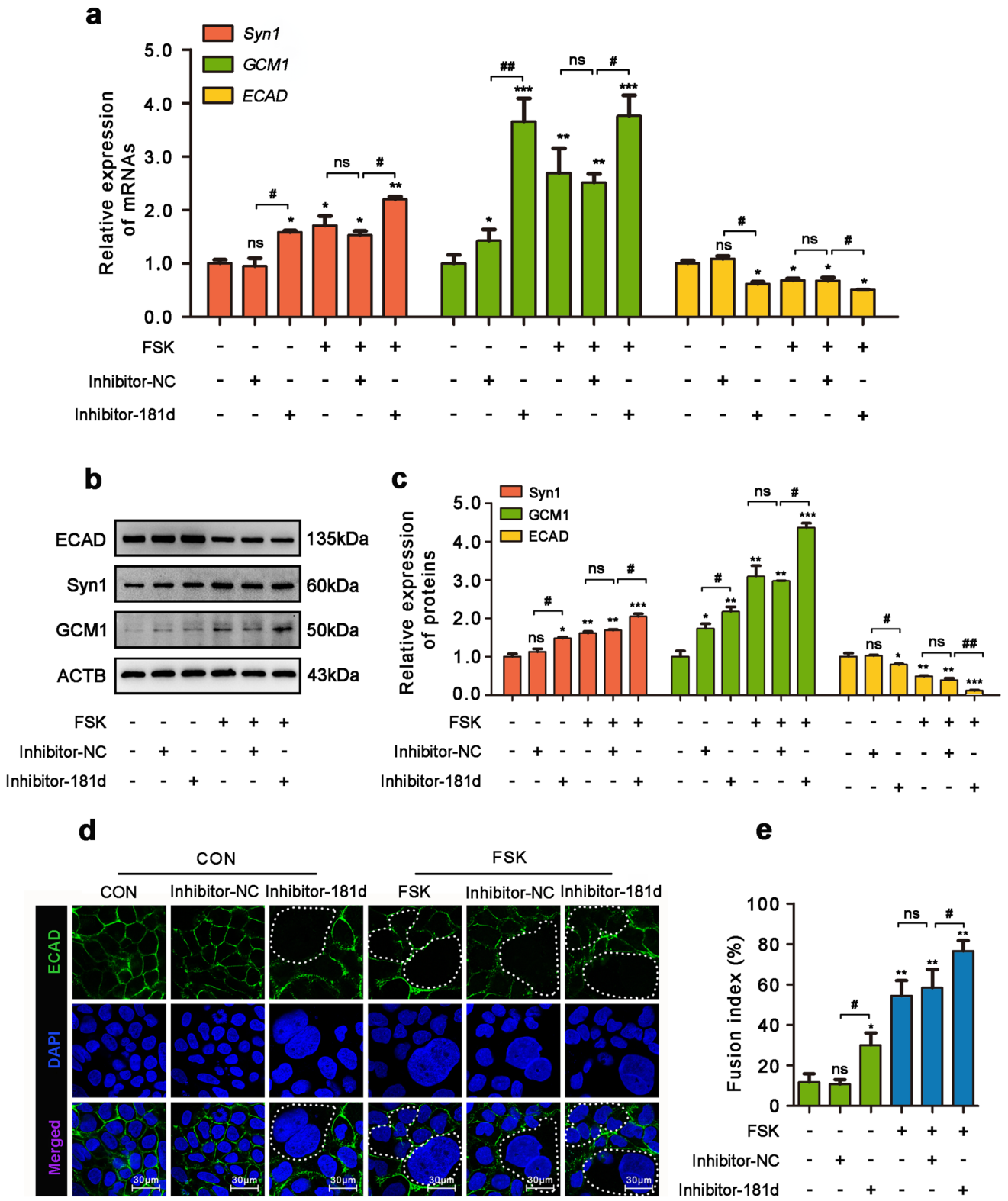
Total RNA was extracted from placental tissues and BeWo cells using the Trizol reagent (Takara, Dalian, China). The concentration of the obtained total RNA was determined using absorbance spectroscopy (Nanodrop, Thermo Fisher Scientific Life Sciences). Subsequently, cDNA synthesis was performed using PrimeScript™ RT Master Mix (Takara), followed by analysis with 2 × SYBR Green qPCR Master Mix (Bimake, Shanghai, China) on a Real-Time PCR System (Bio-Rad Laboratories, CA, USA). For miRNA analysis, first-strand cDNA was synthesized using the miRNA First Strand cDNA Synthesis Kit (Sangon Biotech, Shanghai, China). The expression levels of miR-181d-5p were assessed using the MicroRNAs qPCR Kit (Sangon Biotech) and a Real-Time PCR System (Bio-Rad). *GAPDH* and *U6* served as internal reference markers for genes and miRNAs, respectively. The primer concentration used in this study was 10nM per primer. All primers of genes and miRNAs were synthesized and purified by the BGI Corporation (Beijing, China) and Sangon Biotech (Table 1).

Western blot analysis

BeWo cells and placenta tissues were lysed using pre-cooled RIPA buffer (Beyotime) supplemented with a complete protease inhibitor cocktail (PMSF, Beyotime). The resulting protein lysates were separated by 8% (v/v) SDS-PAGE gel, then transferred to 0.45 μm PVDF membrane (Bio-Rad), and blocked with 5% (w/v) fat-free milk powder (Boster) at 37 °C for 1 h. Subsequently, the membranes were incubated with primary antibodies overnight at 4 °C. The primary antibodies used in this study were as follows: anti-CREBRF (1:1000, Thermo Fisher, MA, USA, PA5-68552); anti-ECAD (1:1000, Cell Signaling Technology, CST, MA, USA, 14472); anti-GCM1 (1:500, Sigma-Aldrich, DR1123); anti-Syncytin 1 (Syn1; 1:1000, Bioss, Beijing, China, bs-2962-R); anti-β-actin (1:1000, Boster, BM3873). The membranes were then washed three times with 0.5% (v/v) PBST (Phosphate buffered saline (PBS) with 0.5% (v/v) Tween-20) the following day, followed by incubation with HRP-labeled secondary antibodies (goat anti-rabbit or goat anti-mouse IgG) (1:1000, Boster, BA1041, BA1038) at 37 °C for 1 h. Chemiluminescent signals were detected using an ECL reagent (NCM Biotech, Suzhou, China). β-actin was utilized as an internal reference marker. Immunoblot quantification was performed using ImageJ.

Immunofluorescence

Cells were washed three times with PBS, and fixed with 4% (w/v) paraformaldehyde (PHA) for 10 min at room temperature. Subsequently, the cells were permeabilized using 0.1% (v/v) Triton X-100 for 10 min and blocked with 3% (v/v) goat serum and 5% (w/v) BSA at 37 ℃ for 1 h. The cells were incubated with anti-ECAD (1:200, CST) overnight at 4 ℃. On the following day, the cells were washed three times with PBS and incubated with fluorescent secondary antibody (1:1000, Thermo Fisher, Ashland, OR, USA, A-11029) at 37 ℃ for 1 h. Finally, the cells were incubated with Hoechst (1:10000, Thermo Fisher, H21486) for 10 min. Images were acquired using a confocal microscope (C2si, Nikon, Japan).



Luciferase assay

The potential binding sites between miR-181d-5p and CREBRF were predicted using TargetScan7 (http://www.targetscan.org/vert_72/). The wild-type (WT) CREBRF sequences containing the predicted miR-181d-5p binding sites were amplified from genomic DNA and inserted into the psi-CHECK2 dual-luciferase vector. Additionally,

◂**Fig. 3** miR-181d-5p inhibitor promoted BeWo cell fusion. **a** BeWo cells were transfected with miR-181d-5p inhibitor in the presence of FSK for 72 h. The relative mRNA levels of *GCM1*, *Syn1*, and *ECAD* in FSK-induced BeWo cell fusion model with or without miR-181d-5p inhibitor were verified by qRT-PCR ($n=3$ lanes per group). **b-c** Protein expressions of GCM1, Syn1, and ECAD were detected by Western blot ($n=3$ lanes per group). **d** Immunofluorescence detection of ECAD (green) in FSK-induced BeWo cell fusion model with or without miR-181d-5P inhibitor ($n=3$ lanes per group). Nucleus were stained with Hoechst (blue). Scale bars = 30 μm. **e** Graphical representation of the ratio of fused BeWo cells. The results are presented as mean ± standard deviation. Statistical significance among multiple groups was analyzed using one-way ANOVA. * indicates a significant difference compared to the control group. # denotes a significant difference between the groups indicated by the crossbar. * $P<0.05$, ** $P<0.01$, *** $P<0.001$. # $P<0.05$, ## $P<0.01$, ### $P<0.001$

mutated versions of the predicted miR-181d-5p binding sites within the 3' untranslated region (UTR) of CREBRF were also generated and inserted into the psi-CHECK2 dual-luciferase vector. HEK293T cells were seeded in a 12-well plate and allowed to attach for 12 h prior to transfection. The plasmids containing psi-CHECK2, psiCHECK2-CREBRF, and psiCHECK2-CREBRF-Mut, along with miR-181d-5p mimic-NC or miR-181d-5p mimic, were co-transfected into the HEK293T cells and incubated for 36 h. For each transfection in a 12-well plate, a mixture of 1 μg plasmid and miR-181d-5p mimic (or mimic-NC) was used. Luciferase activity was measured using Trans Detect Double-Luciferase Reporter Assay Kit (Transgene, Beijing, China) according to the manufacturer's instructions.

Statistical analysis

Statistical analysis was conducted using Graphpad Prism 5.01 or IBM SPSS Statistics 24 software. Each experiment was repeated at least three times, and the results are presented as mean ± standard deviation. The normality of the data distribution was evaluated using the Shapiro–Wilk test. For normally distributed data, the student's *t*-test was used to determine the statistical differences between two groups. Multiple group comparisons were performed using one-way ANOVA, followed by Tukey's post hoc test. Significant significance between different groups and the control group is indicated by an asterisk (*), while significant differences within two groups are denoted by a number sign (#). In both cases, a single symbol signifies a p-value <0.05, two symbols indicate a p-value <0.01, and three symbols denote a p-value <0.001.

Results

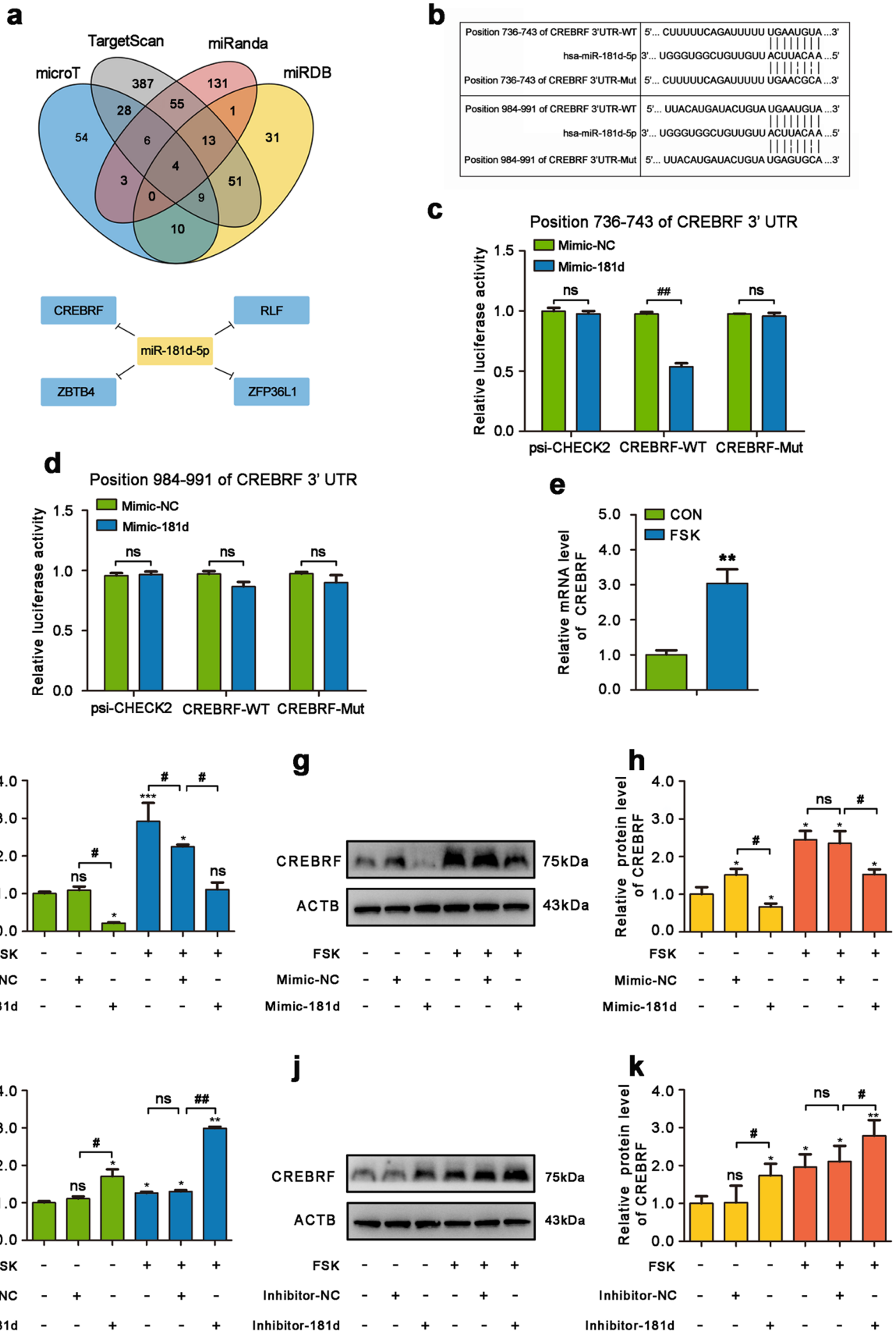
The expression pattern of miR-181d-5p in placenta and BeWo cells

The analysis of clinical characteristics of mothers and neonates in the normal and FGR groups revealed that neonates in the FGR group exhibited significantly lower birth weight, head circumference, abdominal circumference, body length, and gestational age at delivery compared to those in the normal group (Table 2). Conversely, no significant differences were observed in maternal age, weight, height, pregestational BMI, and placental weight between the normal and FGR groups (Table 2).

The down-regulation of trophoblast fusion markers, including GCM1, Syn1, and ECAD, suggests impaired syncytialization in FGR placentas. Western blot analysis revealed that GCM1 and Syn1 were down-regulated, while ECAD was up-regulated in the FGR placentas compared to normal controls (Fig. 1a-b). Additionally, qRT-PCR results demonstrated significantly higher expression of *miR-181d-5p* in human FGR placentas than in normal placentas (Fig. 1c). To investigate the involvement of miR-181d-5p in trophoblast fusion, we employed the FSK-induced BeWo cell fusion model. The up-regulation of *GCM1* and *Syn1*, along with the down-regulation of *ECAD* indicated the successful induction of the BeWo cell fusion model (Fig. 1d). Furthermore, qRT-PCR results revealed the down-regulation of *miR-181d-5p* in the BeWo cell fusion model (Fig. 1d).

The effect of miR-181d-5p mimic on the fusion of BeWo cells

Subsequently, the FSK-induced BeWo cell fusion model was transfected with the miR-181d-5p mimic. The qRT-PCR results revealed a significant up-regulation of *miR-181d-5p*, with a 43-fold increase in the mimic-181d group without FSK treatment, and a 22-fold increase in the mimic-181d group with FSK treatment (Fig. 2a). The introduction of the miR-181d-5p mimic reduced the mRNA levels of *GCM1* and *Syn1*, while inducing the expression of *ECAD* in the BeWo cell model with or without FSK treatment (Fig. 2b). The Western blot analysis results were consistent with the qRT-PCR results (Fig. 2c-d). Furthermore, the immunofluorescence results provided additional evidence that the miR-181d-5p mimic impaired BeWo cell fusion (Fig. 2e).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◂**Fig. 4** miR-181d-5p negatively regulated the expression of CREBRF. **a** Venn analysis of potential target genes of miR-181d-5p using TargetScan, miRDB, microT, and miRanda. **b** Predicted binding sites between miR-181d-5p and CREBRF in TargetScan7. **c-d** Luciferase activities were measured in HEK293T cells co-transfected with either miR-181d-5p mimic or minic-NC and luciferase reporters containing psi-CHECK2, CREBRF-WT or CREBRF-Mut. The data are presented as the ratio of renilla luciferase activity to firefly luciferase activity. **e** qRT-PCR analysis of *CREBRF* mRNA levels in the FSK-induced BeWo cell fusion model ($n=3$ lanes per group). **f-k** Western blot and qRT-PCR detection of CREBRF in the FSK-induced BeWo cell fusion model with or without the transfection of miR-181d-5p mimic or inhibitor ($n=3$ lanes per group). The results are presented as mean ± standard deviation. Statistical significance between two groups was analyzed using Student's *t*-test, while statistical significance among multiple groups was analyzed using one-way ANOVA. * indicates a significant difference compared to the control group. # denotes a significant difference between the groups indicated by the crossbar. * $P<0.05$, ** $P<0.01$, *** $P<0.001$. # $P<0.05$, ## $P<0.01$, ### $P<0.001$

The effect of miR-181d-5p inhibitor on the fusion of BeWo cells

In addition, the FSK-induced BeWo cell fusion model was transfected with the miR-181d-5p inhibitor. Both qRT-PCR and western blot consistently demonstrated that the expression levels of GCM1 and Syn1 were up-regulated in BeWo cells with the transfection of miR-181d-5p inhibitor, while ECAD was down-regulated (Fig. 3a-c). The promotion of BeWo cell fusion by the miR-181d-5p inhibitor was further confirmed by ECAD immunofluorescence staining (Fig. 3d).

Identification of the CREBRF as a target gene of miR-181d-5p

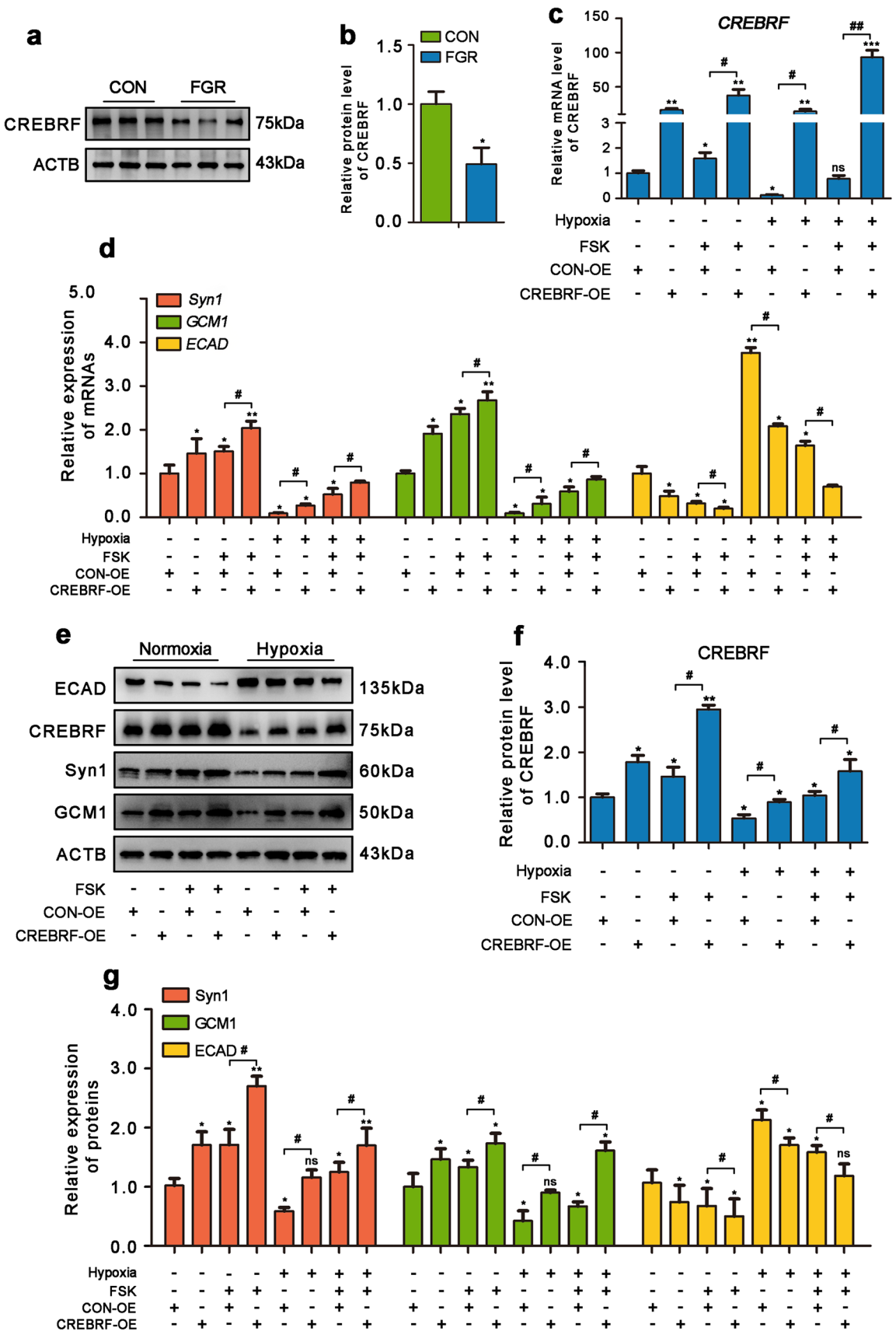
Four miRNA databases, namely TargetScan, miRDB, microT, and miRanda, were utilized to screen potential target genes of miR-181d-5p. The Venn plot results revealed that CREBRF, RLF zinc finger (RLF), zinc finger and BTB domain containing 4 (ZBTB4), and ZFP36 ring finger protein like 1 (ZFP36L1) were identified as potential targets regulated by miR-181d-5p (Fig. 4a). Among these candidates, CREBRF was selected as the focal point of this study due to its potential involvement in the cAMP pathway, known to regulate trophoblast cell fusion. Subsequent analysis using TargetScan7 identified two potential interaction sites between miR-181d-5p and CREBRF (Fig. 4b). To confirm the specific interaction, a luciferase assay was performed, confirming the presence of the interaction site between miR-181d-5p and CREBRF at position 736–743 within the CREBRF 3'UTR (Fig. 4c-d). Additionally, qRT-PCR analysis demonstrated an up-regulation of *CREBRF* in BeWo cells with FSK-induction (Fig. 4e). Further investigation was carried out to evaluate the effect of miR-181d-5p on CREBRF expression by transfecting cells with a miR-181d-5p mimic or inhibitor. The results demonstrated that overexpression of miR-181d-5p significantly inhibited CREBRF expression, while the inhibition of miR-181d-5p led to an elevation of CREBRF expression. (Fig. 4f-k).

Overexpression of CREBRF improved the trophoblast fusion impaired by hypoxia

The expression of CREBRF was detected in human placentas from both the FGR group and the control group. Western blot analysis revealed a significant decrease of approximately 50% in CREBRF levels in the placentas of the FGR group compared to the control group (Fig. 5a-b). The hypoxia cell model has been widely utilized to investigate placental dysfunction in FGR [17]. To investigate the role of CREBRF in trophoblast fusion, we performed transfection using an overexpression plasmid containing CREBRF in a hypoxic BeWo cell model. qRT-PCR analysis demonstrated a significant down-regulation of *CREBRF* and trophoblast markers, including *Syn1* and *GCM1*, accompanied by up-regulation of *ECAD* under hypoxic conditions (Fig. 5c-d). Moreover, the overexpression of CREBRF positively influenced BeWo cell fusion by regulating the expression of trophoblast fusion markers, *GCM1*, *Syn1*, and *ECAD* (Fig. 5c-d). The Western blot analysis results were consistent with the qRT-PCR findings (Fig. 5e-g).

Discussion

The placenta plays a critical role in facilitating the exchange of materials between the mother and fetus, thereby supporting optimal fetal development throughout pregnancy [18]. Dysfunction of the placenta is a prominent contributor to the development of FGR [19]. Previous studies have indicated that the trophoblast cell fusion is impaired in FGR placentas [20]. Consistent with these findings, our research revealed a significant down-regulation of protein expression levels of trophoblast fusion markers, including Syn1 and GCM1, along with a significant up-regulation of ECAD in FGR placentas compared to normal placentas, providing further evidence of compromised syncytialization in FGR placentas. However, the precise underlying mechanisms responsible for impaired syncytialization in FGR remain to be elucidated.


a
CON
FGR
CREBRF
75kDa
ACTB
43kDa
b
CON
FGR
Relative protein level of CREBRF
c
CREBRF
Relative mRNA level of CREBRF
Hypoxia
FSK
CON-OE
CREBRF-OE
d
Syn1
GCM1
ECAD
Relative expression of mRNAs
e
Normoxia
Hypoxia
ECAD
135kDa
CREBRF
75kDa
Syn1
60kDa
GCM1
50kDa
ACTB
43kDa
f
CREBRF
Relative protein level of CREBRF
g
Syn1
GCM1
ECAD
Relative expression of proteins

◂**Fig. 5** Overexpression of CREBRF rescued hypoxia-induced BeWo cell fusion impairment. **a-b** Western blot analysis of CREBRF expression in human placenta ($n=3$ lanes per group). **c-d** qRT-PCR detection of relative mRNA levels of *CREBRF*, *GCM1*, *Syn1* and *ECAD* in the hypoxic FSK-induced BeWo cell fusion model under transfection of miR-181d-5p mimic or inhibitor were detected by qRT-PCR. Normoxia group was maintained at 20% O2, while the hypoxia group was exposed to 1% O2 ($n=3$ lanes per group). **e–g** Western blot analysis of protein levels of CREBRF, GCM1, Syn1 and ECAD ($n=3$ lanes per group). The results are presented as mean ± standard deviation. Statistical significance between two groups was analyzed using Student's t-test, while statistical significance among multiple groups was analyzed using one-way ANOVA. * indicates a significant difference compared to the control group. # denotes a significant difference between the groups indicated by the crossbar. * $P<0.05$, ** $P<0.01$, *** $P<0.001$. # $P<0.05$, ## $P<0.01$, ### $P<0.001$

MicroRNAs (miRNAs) are small, non-coding RNA molecules that play a crucial role in the post-transcriptional regulation of target genes, contributing to various biological processes [21]. Accumulating evidence indicates the involvement of miRNAs in the development of pregnancy complications, such as FGR, through their regulatory effects on trophoblast function [22, 23]. For instance, miR-526b-5p has been shown to inhibit trophoblast proliferation by downregulating Foxp1 expression [24]. Up-regulation of miR-378a-5p inhibits cell cycle progression by targeting cyclin G2, thereby impeding the differentiation and fusion of BeWo cells [25]. Additionally, miR-92a-1-5p inhibits BeWo cell fusion by down-regulating the expression of trophoblast cell fusion-related genes, dysferlin (DYSF) and protein kinase cAMP-activated catalytic subunit alpha (PRCAKA) [26]. miR-212-3p promotes trophoblast proliferation and migration in FGR by targeting placental growth factor [27]. In this study, we observed a significant up-regulation of miR-181d-5p in FGR placentas, suggesting its potential as a promising biomarker for early FGR diagnosis and as a predictive tool for pregnancy outcomes, benefiting both clinicians and patients.

miR-181d-5p has been reported to participate in the epithelial-mesenchymal transition process by negatively regulating the expression of Caudal-related homeobox transcription factor 2 (CDX2) and homeobox A5 (HOXA5) [13]. Furthermore, down-regulation of miR-181d-5p has been shown to enhance the proangiogenic capacity of Human Umbilical Vein Endothelial Cells by increasing the expression of cyclin-dependent kinase inhibitor 3 (CDKN3) [28]. Studies have confirmed the abnormally high expression of miR-181d-5p in gestational trophoblastic disease [16]. However, the precise role of miR-181d-5p in trophoblasts, placental development, and associated disorders remains to be fully elucidated. In this study, we demonstrated that elevated levels of miR-181d-5p inhibit trophoblast cell fusion, suggesting that up-regulation of miR-181d-5p may contribute to impaired trophoblast fusion and subsequent placental dysfunction observed in FGR. Furthermore, using four miRNA databases (TargetScan, miRDB, microT, and miRanda), we identified CREBRF, RLF, ZFP36L1, and ZBTB4 as putative target genes of miR-181d-5p.

CREBRF has been identified as a regulator of CREB3, a crucial component of the cAMP signaling pathway that drives trophoblast fusion [29]. CREB and CREB3 are important members of the ATF/CREB family, known for their role in regulating cellular processes such as glucose and lipid metabolism, cell growth, and proliferation [30]. Previous studies have demonstrated that CREB, activated by the cAMP/PKA signaling pathway, promotes trophoblast cell fusion by regulating the expression of Furin and GCM1 [31, 32]. In our study, we observed a decrease in CREBRF expression in FGR placentas, while its expression increased in the FSK-induced BeWo cell fusion model. These finding suggest that potential involvement of CREBRF in the trophoblast cell fusion may be mediated through the activation of the CAMP signaling pathway. However, it remains unclear whether CREBRF contributes to placental dysfunction via other pathways.

Maintaining oxygen homeostasis during pregnancy is vital for optimal fetal and placental growth and development. Previous studies have indicated that early pregnancy hypoxia may have beneficial effects on placental development [33]. However, it is important to note that prolonged exposure to hypoxia throughout pregnancy can lead to unfavorable outcomes, such as fetal growth restriction (FGR) and preeclampsia (PE) [19]. In our investigation, we observed a notable down-regulation of CREBRF expression under hypoxic conditions. Futrthermore, the overexpression of CREBRF improved trophoblast fusion impaired by hypoxia. Nevertheless, the precise regulatory mechanisms governing the modulation of CREBRF expression by hypoxia warrant further investigation.

Conclusion

In summary, our study demonstrated the elevated expression of miR-181d-5p in the FGR placenta and its inhibitory effect on trophoblast cell fusion through negative regulation of CREBRF. These results suggest that the abnormally high expression of miR-181d-5p in the FGR placenta may contribute to dysregulated CREBRF expression and the previously reported defects in trophoblast fusion. The identification of miR-181d-5p and CREBRF as potential targets opens provides new opportunities for therapeutic interventions in the management of FGR.

Limitation

We confirmed that miR-181d-5p inhibits trophoblast fusion by targeting CREBRF. However, the functional experiments were only conducted in BeWo cells. Although we observed the down-regulation of miR-181d-5p in FGR placentas, we can only hypothesize that the signaling pathways regulated by miR-181d-5p are functionally similar in FGR placentas.

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Author's contributions Tai-Hang Liu and Li-Juan Fu conceived and designed the study. Zhi-Hong Wu conducted most of the experiments and wrote the manuscript. Fang-Fang Li and Ling-Ling Ruan contributed to data analysis. Qian Feng, Shuang Zhang, Zhuo-Hang Li, Antonia Otoo, and Jing Tang revised the manuscript. Yu-Bin Ding designed, coordinated, and revised the manuscript. All authors read and approved the final manuscript.

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Data availability All data in this study are included in this manuscript.

Declarations

Ethics approval and consent to participate This project was approved by the Biomedical Research Ethics Committee of Chongqing Medical University. Informed consent was obtained from pregnant women that participated in this study.

Consent for publication Written consent has been obtained from each patient or subject after full explanation of the purpose and nature of all procedures used.

Competing interests There is no conflict of interest that could have appeared to influence the work reported in this paper.

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