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Research Article

A positive feedback self-regulatory loop between miR-210 and HIF-1α mediated by CPEB2 is involved in trophoblast syncytialization: implication of trophoblast malfunction in preeclampsia†

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Abstract

The pregnancy complication preeclampsia is directly associated with hypoxic stress and insufficient trophoblast cell differentiation. The hypoxia-inducible microRNA (miRNA), miR-210, has been identified as a significantly up-regulated miRNA in preeclamptic placenta, and evidence in other cell types has indicated a feedback regulation between miR-210 and hypoxia-inducible factor-1α (HIF-1α) under hypoxic condition. It remains unclear whether and how the feedback loop between miR-210 and HIF-1α may contribute to trophoblast dysfunction in preeclampsia. Here, we proved that cytoplasmic polyadenylation element-binding 2 (CPEB2) was a direct target of miR-210 in human trophoblast. CPEB2 could inhibit the translation of hypoxia-induced HIF-1α via directly binding the cytoplasmic polyadenylation element (CPE) site in the 3′-untranslated region (UTR) of HIF-1α mRNA. The increase in the HIF-1α level upon hypoxia treatment could be efficiently reversed by miR-210 inhibitor. In addition, CPEB2 was primarily expressed in villous syncytiotrophoblasts, and the suppression of trophoblast cell syncytialization by miR-210 could be significantly rescued by CPEB2 overexpression. In preeclamptic placenta, the expression of CPEB2 was evidently lower than normal pregnant control, and the miR-210 level was aberrantly higher and trophoblast syncytialization was limited. The findings revealed a positive feedback loop between miR-210 and HIF-1α that is mediated by CPEB2 in human trophoblasts, and demonstrated a mechanism underlying the insufficient trophoblast syncytialization in preeclampsia under hypoxic stress.

Summary sentence

A positive feedback loop between miR-210 and HIF-1α that is mediated by CPEB2 in human trophoblast demonstrates the mechanism of insufficient syncytialization in preeclampsia under hypoxic stress.

Key words: CPEB2, HIF-1α, miR-210, preeclampsia, trophoblast syncytialization

Introduction

Preeclampsia is a pregnancy complication that is characterized by newly onset hypertension and proteinuria occurring after the 20th week of gestation. It affects 3–5% of pregnant women worldwide, leading to restricted fetal growth, multiple organ injury in the mother, and even death of the fetus or mother [1]. Histological observations of preeclamptic placentas demonstrate shallow trophoblast invasion, limited uterine blood vessel remodeling, and insufficient trophoblast syncytialization, indicating that the reduced blood perfusion and excessive hypoxia at the feto-maternal interface at early gestation may be the predominant cause of preeclampsia [2].

Accumulating evidence has revealed the differential expression of several microRNAs (miRNAs) in preeclamptic placenta, and their roles in multiple cell events during pregnancy are under studying. miRNA belongs to non-coding small RNA that is composed of 19–25 nucleotide residues. It can bind the 3′-untranslated region (3′-UTR) of target genes and post-transcriptionally suppress their expression [3]. Previous studies have demonstrated miR-210 as a robustly up-regulated miRNA in preeclamptic placenta, and this miRNA can inhibit trophoblast migration and invasion by suppressing several targets including iron-sulfur cluster protein (ISCU), Ephrin-A3, Homeobox-A9, KCMF1, and THSD7A [4–7]. Although miR-210 is widely expressed in various subtypes of trophoblasts [8], functional studies of this small RNA have been largely focused on the regulation of extravillous trophoblast cells. The significance of miR-210 in trophoblast syncytialization remains unknown.

It is well-known that miR-210 is a hypoxia-responsive miRNA, whose transcription is enhanced by hypoxia-inducible factor-1α (HIF-1α) [9]. Interestingly, there has been evidence in some cell types indicating a positive feedback loop between miR-210 and HIF-1α. The mechanisms involve inhibition of HIF-1α [10], repression of Runt-related transcription factor-3 (RUNX3) [11] or glycerol-3-phosphate dehydrogenase 1-like (GPD1L) [12] and a subsequent enhancement of the stability of HIF-1α. We analyzed the predictive targets of miR-210 using multiple databases including TargetScan Human 7.1, miRDB, microRNA.org, and TargetMiner, and found cytoplasmic polyadenylation element-binding 2 (CPEB2) as one of the potential targets with high predictive score. Members of the CPEB family can bind the uracil-enriched cytoplasmic polyadenylation element (CPE) at the 3′-UTRs of certain mRNAs and block their polyA tail elongation to inhibit mRNA translation [13]. Report in neuroblastoma cells demonstrated CPEB2 could suppress HIF-1α translation [14]. All the evidences lead us to propose that miR-210, CPEB2, and HIF-1α may form a regulatory loop to reciprocally mediate the effect of hypoxia on trophoblast cell differentiation.

To address the hypothesis, we examine whether miR-210 can regulate HIF-1α translation via targeting CPEB2, and whether miR-210/CPEB2 can modulate trophoblast cell differentiation toward syncytialization pathway. The study deepens the understanding of the mechanism by which hypoxia regulates trophoblast cell differentiation, and provides new evidence to demonstrate the pathology of extensive hypoxia causing compromised trophoblast cell function in preeclampsia.

Materials and methods

Study subjects

The use of human placental specimen used in this study was approved by the Ethics Committee at Institute of Zoology, Chinese Academy of Sciences (No. IOZ16039) and Peking University Third Hospital (No. 2016-145-03). Written consents were achieved from all the enrolled pregnant women.

The enrolled placental specimens were obtained from Chinese Han pregnant women who underwent perinatal care in the Department of Obstetrics and Gynaecology, Peking University Third Hospital, China. The pregnancy outcomes were defined according to the International Society for the Study of Hypertension in Pregnancy guidelines [15]. Briefly, preeclampsia was defined as a pregnancy, which has no preexisting hypertension or chronic hypertension history, but displayed a diastolic blood pressure $\geq$ 110 mmHg or systolic blood pressure $\geq$ 160 mmHg on at least two occasions after the 20th week of gestation, and accompanied with significant proteinuria (>2 g/24 h in two random samples collected no <4 h apart) or multiple organ damage. Pregnant women were excluded from this study if they suffered from transient hypertension during pregnancy, intrauterine fetal death, cardiovascular disease, renal disease, gestational diabetes, fetal congenital or chromosomal abnormalities, or pregnancies conceived by in vitro fertilization. To obtain the gestational-age-matched control placental specimen for preeclampsia, women with unexplained preterm labor (PTL) were enrolled. PTL control was defined as a uniparous gestation in a previously normotensive woman who exhibited no any other gestational complications before 37 weeks of pregnancy. The placental specimens were obtained after Cesarean section. Near the umbilical cord insertion point on the placental disk, the specimens from chorionic and basal plates were separately obtained, snap-frozen, and preserved in liquid nitrogen. The clinical characteristics of the chosen pregnant women are summarized in Table S1.

Human chorionic villi at early pregnancy were obtained from healthy pregnant women at gestational weeks 7–9 (n = 4; gestational age = 7.75 $\pm$ 0.96 weeks; maternal age = 26.75 $\pm$ 3.77 years) who underwent therapeutic pregnancy termination without prior special medical treatment at Peking University Third Hospital, China. Gestational weeks of the chorionic villi were determined by morphological observation and pathological examination, with the menstrual cycle record as a reference.

Sequences and constructs

To generate a plasmid expressing human CPEB2 (ENST00000538197.1), the full-length coding sequence was amplified and inserted into pcDNA4.0 vectors (Invitrogen, CA,

USA) between the HindIII and EcoRI restriction sites and named pCPEB2. To perform luciferase reporter assays, an approximately 200-bp 3′UTR segment in human CPEB2 mRNA containing the predicted miR-210 binding site was cloned into a pMIR-REPORT luciferase plasmid (Promega), constructing a wild-type reporter plasmid (CPEB2-WT). Two of the six nucleotides in the predicted miR-210 binding sequence were point-mutated by PrimeSTAR HS (Takara, Dalian, China) to generate a mutated reporter plasmid (CPEB2-MUT). To generate in situ hybridization probes of CPEB2, an approximately 600-bp segment of CPEB2 cDNA was cloned into a pGEM-T-EASY plasmid (Promega). The mRNA probes were acquired by using DIG RNA Labeling Kit (SP6/T7) (Roche) according to the manufacturer's instructions. All of constructs were confirmed by sequencing. The primers for plasmid construction were listed in Table S2. miR-210 mimics, miR-210 inhibitors (In210), CPEB2 siRNA, and respective negative controls (NC and inhibitor negative control – InNC) were purchased from GenePharma (Shanghai, China). The sequences are: siCPEB2, 5′-GGAACUAUGAAUCAGAUAUTT-3′ (sense) and 5′-AUAUCUGAUUCAUAGUUCCTT-3′ (anti-sense); miR-210 mimics, 5′-CUGUGCGUGUGACAGCGGCUGA-3′ (sense), and 5′- AGCCGCUGUCACACGCACAGUU-3′ (anti-sense); scramble NC, 5′-UUCUCCGAACGUGUCACGUTT-3′ (sense) and 5′-ACGUGACACGUUCGGAGAATT-3′ (anti-sense); miR-210 inhibitor, 5′-UCAGCCGCUGUCACACGCACAG-3′ (sense); inhibitor NC (InNC), 5′-CAGUACUUUUGUGUAGUACAA-3′ (sense).

In situ hybridization

Fresh human villous tissues were fixed in 4% paraformaldehyde (PFA) at 4 °C for 4 h. The fixed villi were incubated in 10% and 25% sucrose solution, and embedded in Tissue-Tek O.C.T compound (Sakura Finetek, Torrance, CA, USA). About 8-μm-thick sections were cut, mounted on polylysine coated slides, and stored at −80 °C. For in situ hybridization, sections were rehydrated in PBS, fixed in 4% PFA for 15 min, digested by proteinase K (10 μg/ml, Roche, Indianapolis, IN, USA) for 3 min at room temperature, and hybridized with the miRCURY LNA microRNA detection probe (RiboBio, Guangzhou, China) or mRNA probes for CPEB2 at 55 °C in a moisture chamber overnight. Sections were washed in a series of saline sodium citrate buffer, blocked in blocking reagent (Roche) for 1 h, and incubated with AP-conjugated anti-DIG antibody (Roche) at 37 °C for 2 h. Positive signals were visualized with NBT/BCIP substrate (Promega, WI, USA). Slides were dehydrated in a serial concentration of ethanol, cleared in xylene, and mounted in neutral balsam.

Cell culture and transfection

The BeWo cells (purchased from ATCC) were cultured in high glucose Dulbecco modified Eagle medium (DMEM, Invitrogen) supplemented with 10% fetal bovine serum (Gibco) in a humidified atmosphere containing 5% CO_2 at 37 °C. The cells were passaged every 4 days at a ratio of 1:2. Transient transfection of plasmid or miRNA mimics was performed using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions.

RNA immunoprecipitation

RNA immunoprecipitation (RIP) analysis was performed according to the procedure described by Keene et al. [16]. Briefly, BeWo cells were lysed by snap-freezing after suspended with RIP lysis buffer containing protease cocktail (Sigma Aldrich, MO, USA) and RNase inhibitor (NEB). After centrifugation at 15 000 *g* at 4 °C, the supernatant was collected and incubated with pre-bound protein A/G agarose bead-antibody mixture at 4 °C for 4 h. The beads were then pelleted by centrifugation, and TRIzol reagent (Invitrogen) was used to isolate the CPEB2-bound RNA.

RNA extraction and real-time PCR

Total RNA was extracted using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. For mRNA, total RNA (2 μg) was reverse-transcribed into cDNA with M-MLV reverse transcriptase (Promega) by using oligo (dT) primer. MiRcute miRNA First-strand cDNA synthesis kit (Tiangen Biotech Co., Beijing, China) was used for miRNA reverse-transcription according to the instruction. Quantitative real-time PCR (qPCR) was performed with Roche LightCycler480II detection system (Roche). For cDNA detection, the experiment was carried out following the instructions of the SYBR Premix ER Taq II (Takara). The reaction for each sample was at 94 °C for 2 min, followed by 40 cycles of 94 °C for 20 s, 60 °C for 34 s. For miRNA detection, the experiment was carried out following the instructions of the MiRcute MiRNA Premix for miRNA (Tiangen Biotech Co.). Reactions were at 95 °C for 30 s, followed by for 40 cycles of 95 °C for 5 s, 60 °C for 30 s. The relative expression level of specific gene was adjusted to human GAPDH (for mRNA) or U6 (for miRNA) levels using the $2^{-\Delta CT}$ method. The primers for real-time PCR were listed in Table S2.

Western blot analysis

The proteins from placental tissues and cultured cells were extracted by RIPA lysis buffer (150 mM NaCl, 10 mM Tris, 1% NP-40, 0.5% Na deoxylcholate, 0.1% SDS, 1 mM NaF, pH = 7.6) containing 1% protease cocktail. Lysates were incubated on ice for 0.5 h. The supernatants were collected after centrifuging, and the protein concentration was detected by a BCA protein assay (Boster Biological Technology, Wuhan, China). About 30 μg protein was subjected to SDS-PAGE, then transferred to a 0.45-μm nitrocellulose membrane (GE Healthcare, Marlborough, CT, USA). The membrane was blocked with 5% BSA in PBS with 0.1% Tween 20 (0.1% PBST) for 2 h and then incubated with antibody against CPEB2 (ab51069, Abcam, Shanghai, China), HIF-1α (ab16066, Abcam, Shanghai, China), or β-actin (#4790, Cell Signaling Technology, Shanghai, China) at 4 °C overnight. The membrane was washed in 0.1% PBST and incubated with horseradish peroxidase-conjugated secondary antibody (Jackson, MS, USA). Signals were visualized by SuperSignal West Pico chemiluminescent substrate (Thermo Scientific, Waltham, MA, USA) and detected by a GeneGnome XRQ system (Syngene, Frederick, MD, USA).

Dual-luciferase report assay

BeWo cells were planted into 24-well plates (20 000 per well) 12 h before transfection. For each well, cells were transfected with 80 ng of reporter plasmid encoding firefly luciferase, 8 ng of the Renilla luciferase vector (pRL-TK) encoding Renilla luciferase, and 100 nM miR-210 mimics/scramble miRNA. Following 48 h, the cells were lysed by 100 μL passive lysis buffer (Promega, Madison, WI, USA), and luciferase activity was detected by Dual-Glo luciferase assay system (Promega) according to manufacturer's instructions.

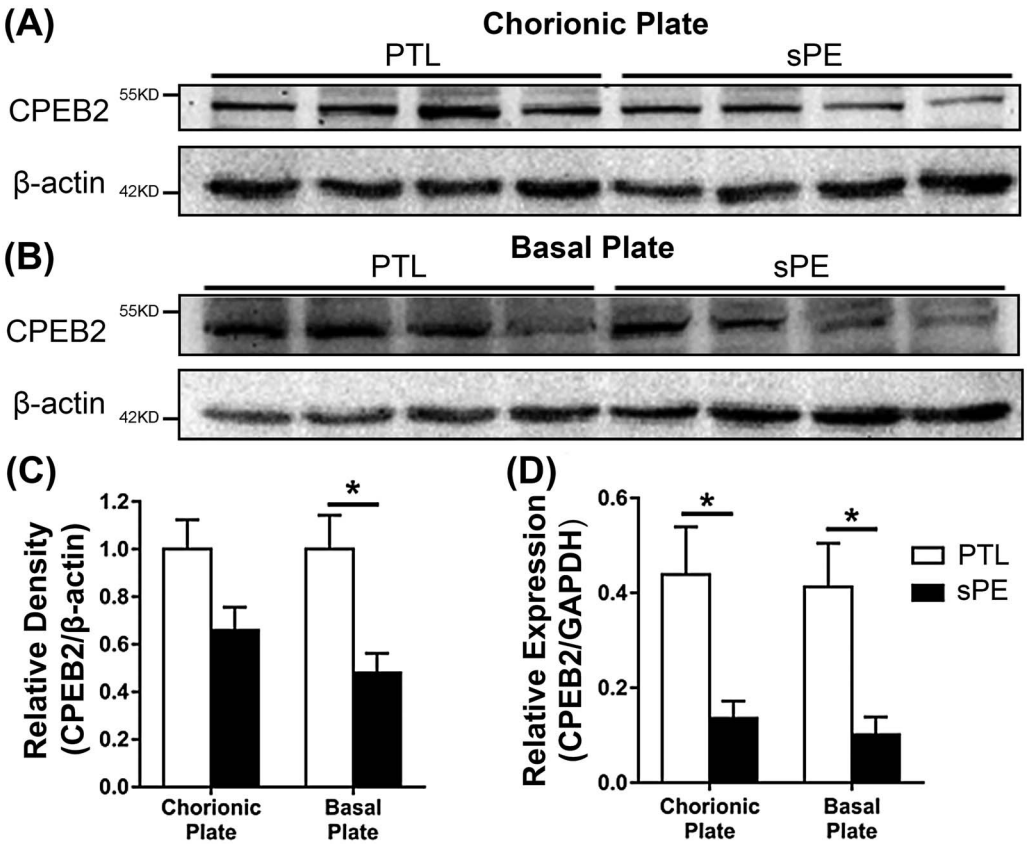

Figure 1. Expression of CPEB2 in the placentas from patients with severe preeclampsia. (A, B) Typical results of Western blotting showing CPEB2 protein level in the chorionic (A) and basal plate (B) of the placentas derived from women with sPE and gestational age-matched PTL. (C) Bar chart showing the statistical results of Western blotting. (D) Results of real-time PCR revealed CPEB2 expression in the placentas from sPE and PTL. Data in (C) and (D) are presented as means ± SEM, and comparison between groups is carried out with Student *t*-test. $^{*}P < 0.05$.

Statistical analysis

All statistical analyses were performed with SPSS 17.0 software. Data are presented as Means ± SEM according to the results of at least three independently repeated experiments. Comparisons among groups were evaluated by one-way ANOVA with Games-Howell post hoc test. For the characteristics of the patients involved in this study, data are presented as the means ± SD, and Mann–Whitney U test was used to evaluate the differences. $P < 0.05$ was considered as significant difference.

Results

CPEB2 was down-regulated in placentas from preeclamptic patients

An increasing number of studies including ours have shown that the miR-210 level is increased in placentas and plasma from preeclamptic patients [8]. Bioinformatic analysis using database including TargetScan Human 7.1, miRDB, microRNA.org, and TargetMiner revealed CPEB2 as one of miR-210 target candidates with high predicting score. To validate the prediction, we first measured CPEB2 expression in the placentas derived from severe preeclamptic (sPEs) patients and gestational age-matched PTL patients, in which the level of miR-210 have been detected in our previous study [8]. Western blotting showed that CPEB2 protein expression was down-regulated in both the chorionic (Figure 1A) and basal plate (Figure 1B) of sPE placentas compared to the corresponding PTL control. Statistical analysis revealed a 40–50% reduction in CPEB2 protein level in the sPE placenta (Figure 1C). Results of real-time PCR showed that CPEB2 mRNA level in the sPE placentas decreased to approximately 30% of PTL controls (Figure 1D). These data suggested an inverse trend of CPEB2 and miR-210 expression in preeclamptic placentas.

Co-localization of miR-210 and CPEB2 in placental syncytiotrophoblasts

The localization of miR-210 and CPEB2 in the placenta was detected by in situ hybridization in adjacent sections of chorionic villi at early pregnancy. Strong miR-210 signal was observed in villous cytotrophoblasts (CTBs), syncytiotrophoblasts (STBs) (Figure 2A and C), and column trophoblasts (Figure 2E). CPEB2 expression was strong in STBs, moderate in CTBs, and weak in column trophoblasts (Figure 2B, D, and F). Co-localization of miR-210 and CPEB2 was observed in villous STBs and CTBs.

Validation of CPEB2 as a direct target of miR-210 in human trophoblasts

We validated the regulation of CPEB2 by miR-210 in trophoblasts by using a human trophoblast cell line, BeWo, as an in vitro model. Transfection of miR-210 mimics in BeWo cells increased the miR-210 level by 10-folds (Figure 3A), and reduced the CPEB2 mRNA level and protein level by approximately 50% and 70%, respectively, when compared to the NC cells that were transfected with scramble

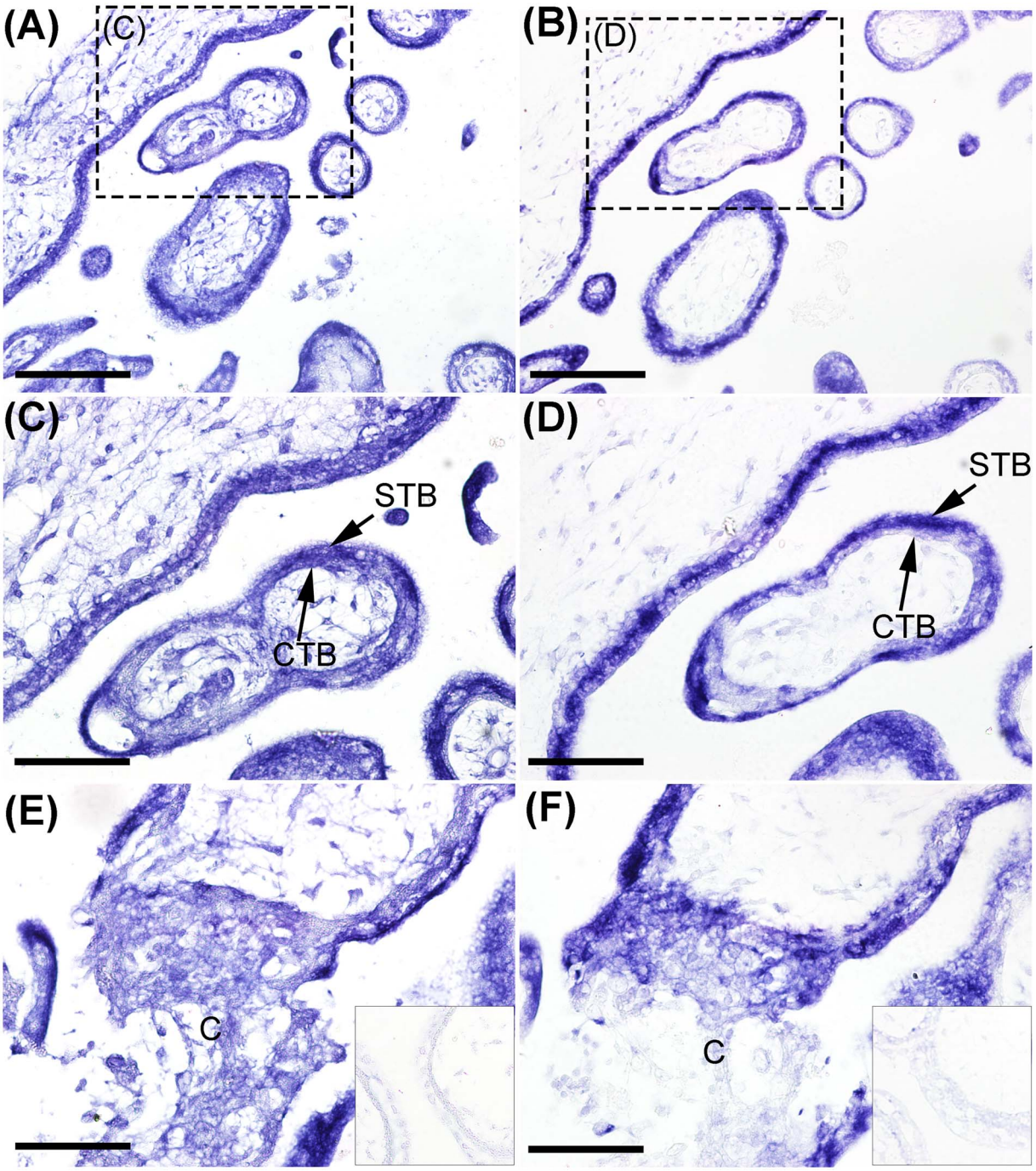

Figure 2. In situ hybridization to show the expression of miR-210 (A, C, E) and CPEB2 (B, D, F) in the adjacent sections of human placental villi at gestational weeks 7–8. (C) and (D) The magnification of the indicated area in (A) and (B), respectively. The inserted area in (E) and (F) shows result of NC for miR-210 and CPEB2, respectively. STB: syncytiotrophoblast; CTB: cytotrophoblast; col: column trophoblast. Scale bars represent 100 μm in panels (A, B), and 50 μm in panels (C–F).

small RNA (Figure 3B–D). On the contrary, inhibition of miR-210 expression by specific inhibitor in BeWo cells elevated CPEB2 expression by approximately 25% (Figure S1).

Dual-luciferase reporter assay was carried out to determine whether miR-210 could directly target CPEB2. Based on the bioinformatic analysis, the predicted miR-210 binding site locates on nucleotides 3405–3411 of the 3′-UTR in CPEB2 mRNA. We constructed a luciferase reporter plasmid that carried a 200 bp fragment of the CPEB2 3′-UTR containing miR-210 binding site (CPEB2-WT), and generated a mutated construct with point-mutations in the miR-210 binding site (CPEB2-MUT) (Figure 3E). BeWo cells were co-transfected with reporter plasmid (CPEB2-WT or CPEB2-MUT), miR-210 mimics or scramble NC, and a renila reference control plasmid, pRL-TK. It was shown that miR-210 mimics significantly suppressed the luciferase activity of CPEB2-WT plasmid (Figure 3F), while it had little effect on the activity of

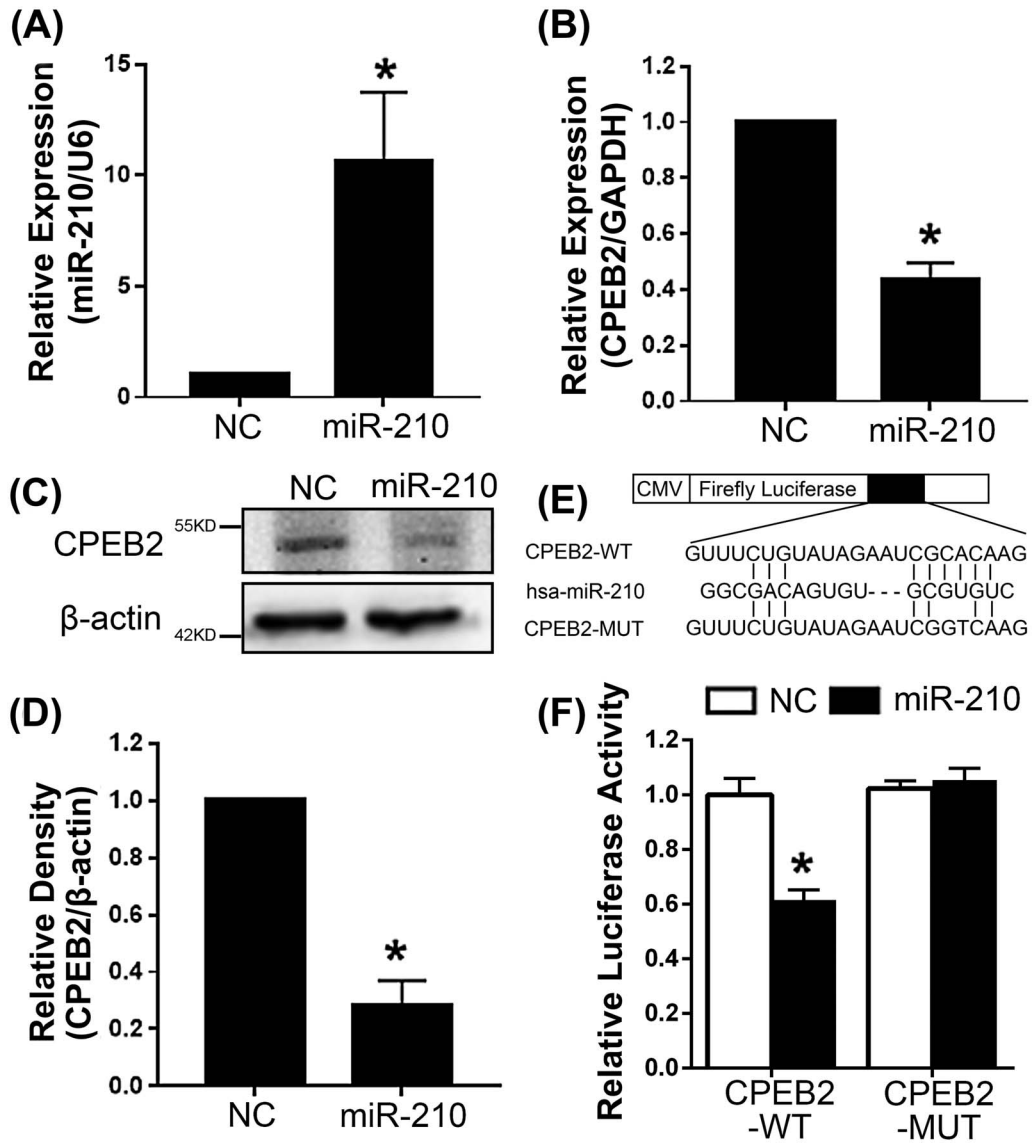

Figure 3. Validation of CPEB2 as a direct target of miR-210 in human trophoblast cell line, BeWo. (A–D) Transfection of miR-210 in BeWo cells altered CPEB2 expression. The cells were transfected with scramble NC or miR-210 mimics (miR-210). The miR-210 level (A), the CPEB2 mRNA level (B), and protein level (C, D) were measured by real-time PCR (A, B) and Western blotting (C, D). (E) Schematic diagram of the plasmid construction for dual luciferase report assay. (F) Dual-luciferase reporter assays in BeWo cells that were co-transfected with wild-type (CPEB2-WT) or mutated (CPEB2-MUT) reporter plasmids, and miR-210 mimics or scramble NC. Data are presented as the means ± SEM according to three independently-repeated experiments, and statistical comparison between groups was carried out by one-way ANOVA with Games-Howell post hoc test. *, compared with NC, $P < 0.05$.

CPEB2-MUT reporter plasmid (Figure 3F). The data proved that miR-210 could directly bind the 3′-UTR of CPEB2 mRNA and inhibit CPEB2 expression.

Hypoxia-induced increase in HIF-1α is blocked by inhibiting miR-210

To examine whether there exists a reciprocal feedback between miR-210 and HIF-1α in human trophoblast cells, BeWo cells were cultured under hypoxic condition (2% O_2). At 48 h, the miR-210 level was up-regulated by 80% (Figure 4A), and CPEB2 expression was decreased by approximately 40–60% at the mRNA and protein levels, respectively (Figure 4B–D), when comparing to the normoxic control (20% O_2). Meanwhile, hypoxia treatment led to a 40% increase in the HIF-1α protein level, while transfection with the miR-210 inhibitor (In210) could totally block the hypoxia-induced HIF-1α increase (Figure 4E and F).

CPEB2 suppressed HIF-1α translation under hypoxic condition.

The common function of CPEB proteins is binding the CPE at the 3′-UTRs of specific mRNA and blocking its polyA tail elongation to inhibit mRNA translation. We examined whether CPEB2 was involved in the translation of HIF-1α, in order to clarify how miR-210 regulates HIF-1α expression. Results of RIP in BeWo cells revealed a direct binding of CPEB2 protein to the 3′-UTR of HIF1A mRNA (Figure 5A), indicating the possibility of CPEB2 to regulate HIF-1α translation in human trophoblasts.

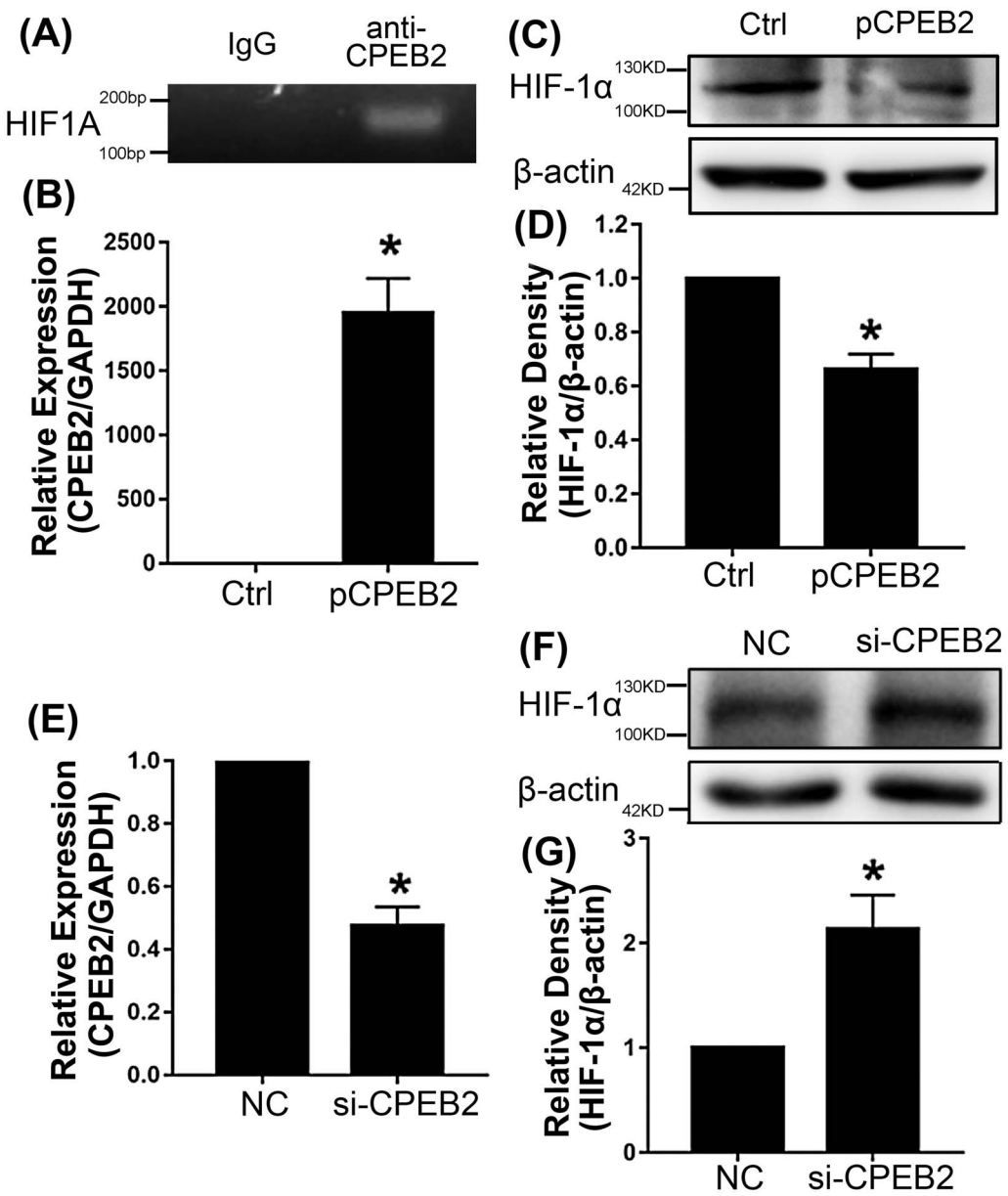

Figure 4. Hypoxia-induced increase in HIF-1α is blocked by inhibiting miR-210. (A) Real-time PCR showing the miR-210 level in BeWo cells cultured in 20% or 2% O_2 conditions for 48 h. (B–D) Measurement of CPEB2 mRNA (B) or protein expression (C, D) in BeWo cells cultured in 20% or 2% O_2 conditions for 48 h. (E, F) Results of Western blotting showing the HIF-1α expression in BeWo cells transfected with miR-210 inhibitors (In210) or controls (InNC) and treated with 20% or 2% O_2. Data are presented as the means ± SEM according to three independently-repeated experiments, and statistical comparison between groups was carried out by one-way ANOVA with Games-Howell post hoc test. *, compared with NC, $P < 0.05$.

Subsequently, we transfected BeWo cells with a CPEB2 overexpression plasmid (pCPEB2) and maintained the cells at hypoxic conditions (2% O_2) for 6 h. The exogenous overexpression of CPEB2 (Figure 5B) significantly lowered down the protein level of HIF-1α to approximately 60% of that in control cells that were transfected with blank vector plasmid (Figure 5C and D). Such effect was similar to that of miR-210 inhibitor, as shown in Figure 4E and F. On the contrary, specific siRNA of CPEB2 (si-CPEB2) that caused 60% down-regulation of CPEB2 in BeWo cells could increase HIF-1α level to approximately 2-fold of control (Figure 5E–G).

miR-210 inhibited BeWo cell syncytialization via CPEB2

Considering the co-localization of miR-210 and CPEB2 in villous STBs, we investigated the participation of miR-210 and CPEB2 in trophoblast syncytialization. Forskolin (FSK, an adenylate cyclase activator)-induced BeWo cell is a well-accepted syncytialization model. Here, we found obvious upregulation of the syncytialization markers, hCGβ and syncytin 2, in BeWo cells following treatment with 20 μM FSK for 48 h (Figure 6A–C), indicating the enhanced syncytialization in BeWo cells. In these cells, miR-210 overexpression could largely abolish the FSK-induced increase in hCGβ and syncytin 2 expression (Figure 6A–C). We transfected BeWo cells

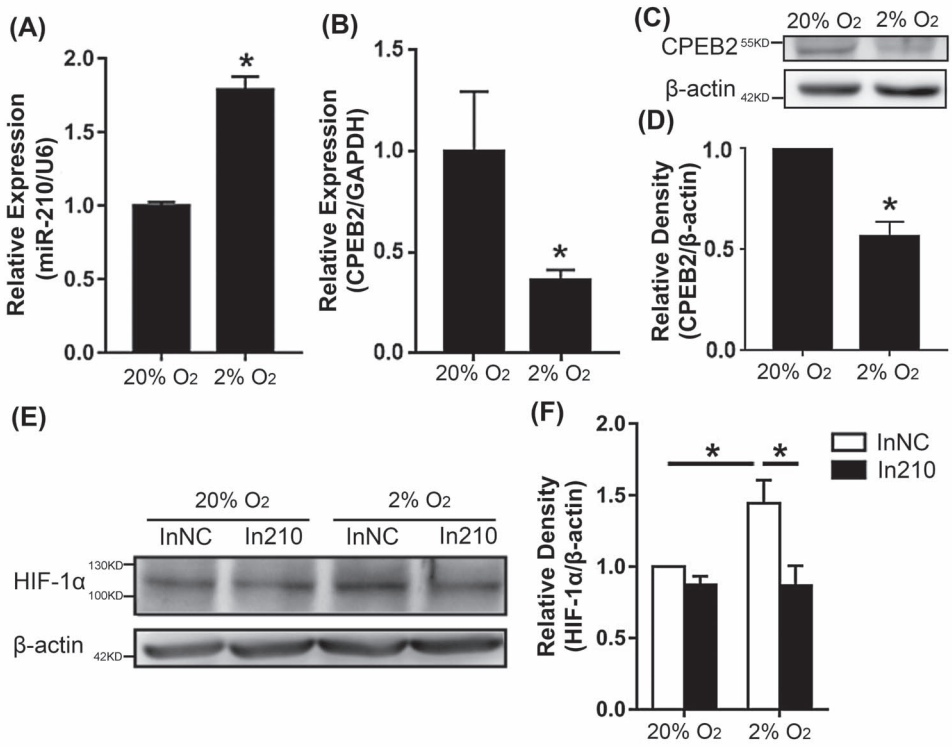

Figure 5. Influence of HIF-1α by CPEB2 in human trophoblasts. (A) RIP was performed using CPEB2 antibody (anti-CPEB2) or isotype control (IgG), and HIF1A enrichment was detected by specific primers. (B–G) Change in the HIF-1α protein level following CPEB2 knockdown or overexpression in BeWo cells. Cells were transfected with plasmid carrying CPEB2 cDNA (pCPEB2) (B–D) or specific siRNA for CPEB2 (si-CPEB2) (E–G), and were cultured in 2% O_2 condition for 6 h. Efficiency of CPEB2 overexpression or knocking down are presented in (B) and (E). Typical results of Western blotting showing the HIF-1α protein level in (C) and (F), and the statistical results according to three independently repeated experiments are presented in (D) and (G). Data are presented as means ± SEM. *, compared with corresponding control, $P < 0.05$.

with pCPEB2, and then subjected the cells to FSK treatment as well as miR-210 overexpression. The inhibitory effect of miR-210 on the expressions of hCGβ and syncytin 2 could be well reversed by pCPEB2 transfection (Figure 6D and E). The data indicated that miR-210 inhibited trophoblast cell syncytialization via repressing CPEB2.

Discussion

Our present study revealed a positive feedback loop among miR-210, CPEB2, and HIF-1α in human placental trophoblasts, which is involved in regulating trophoblast cell syncytialization. Under hypoxic condition, HIF-1α induces miR-210 expression, which targets and represses CPEB2. CPEB2 acts as a negative regulator of HIF-1α translation through binding to the CPE site of the 3′-UTR in HIF-1α mRNA. HIF-1α-induced miR-210 can therefore exaggerate the hypoxic effect in placental trophoblasts, for instance, inhibiting trophoblasts syncytialization. This feedback loop may help to understand the mechanism of trophoblast dysfunction upon hypoxic stress in preeclamptic placentas (Figure 6F).

The up-regulation of miR-210 in preeclamptic placenta has been well described. Our previous study and the data shown here demonstrated the expression of this small RNA in villous STBs, while the knowledge of its function in trophoblast syncytialization are largely lacking. The findings in the present study clarify an inhibitory effect of miR-210 on trophoblast syncytialization via its target CPEB2. Insufficient trophoblast syncytialization has been found in preeclamptic placenta, as indicated by lowered expression of hCG and Syncytin 2, increased number of intermediate trophoblasts indicating villous immaturity [17, 18]. It remains unclear whether the defects in syncytialization are causes of preeclampsia. Considering the critical roles of syncytiatrophoblasts in feto-maternal nutrition exchange and placental endocrine, insufficient syncytialization leads to restriction in fetal growth and abnormal release of placental factors, which are predominant pathology of preeclamspia. Pathological hypoxia has been proved a predominant factor in the suppression of trophoblast cell syncytialization by decreasing the expression of Syncytin and its receptors [19], and the key transcriptional factor GCM1 in human trophoblast cells [17, 20, 21]. In the present study, we illustrate a positive feedback loop among HIF-1α/miR-210/CPEB2, which leads to the exaggeration of hypoxic effect on the suppression of trophoblast cell syncytialization. It is therefore most likely that the increase in miR-210 in preeclamptic placenta is not only the consequence of the limited oxygen supply to the placenta, but also participate in the exacerbation of the hypoxic stress on trophoblast cell differentiation toward syncytial pathway.

The HIF-1α/miR-210/CPEB2 positive feedback loop seems not commonly existing in various subtypes of trophoblasts. Our in situ hybridization proved that co-localization of miR-210 and CPEB2 is primarily in villous trophoblasts, indicating the HIF-1α/miR-210/CPEB2 loop may predominantly participate in modulating villous trophoblast cell behaviors, but not extravillous trophoblast migration or invasion. Further study is needed to investigate the possible role of this loop in CTB cell growth. The mechanisms

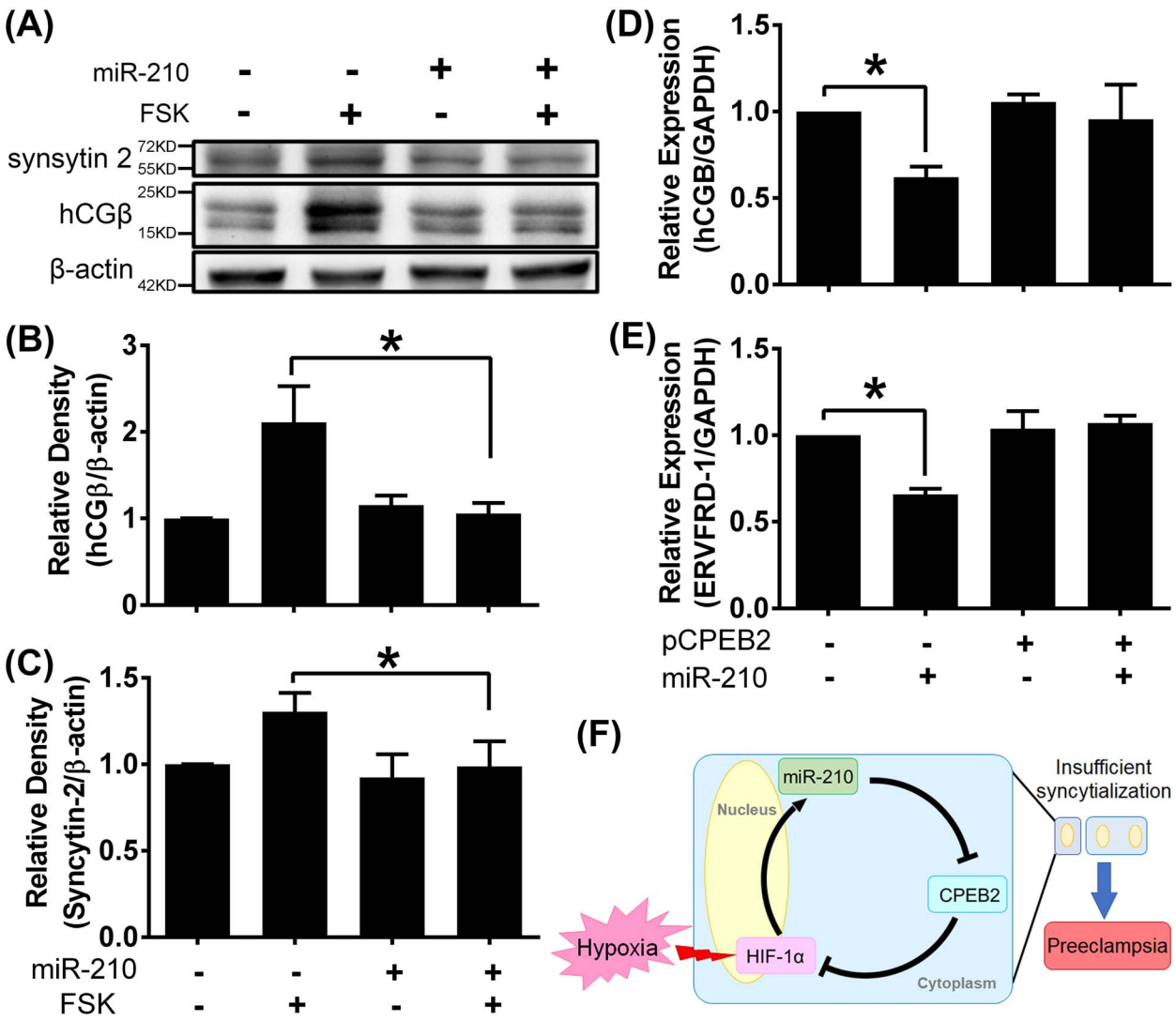

Figure 6. miR-210 inhibits BeWo cell syncytialization via CPEB2. (A–C) Western blotting to measure hCGβ and syncytin 2 expression in BeWo cells that were transfected with miR-210 mimics (miR-210) or scramble NC and treated with 20 μM FSK or vehicle. Typical blots are shown in (A), and statistical results based on three independently repeated experiments are shown in (B) and (C). (D, E) Results of real-time PCR showed hCGβ (hCGB) and syncytin 2 (ERVFRD1) in BeWo cells transfected with CPEB2-encoding plasmid (pCPEB2) or control plasmid (Ctrl) and miR-210 mimics (miR-210) or scramble NC and under 20 μM FSK treatment. Data in (B–E) are presented as means ± SEM according to three independently-repeated experiments, and statistical comparison between groups was carried out by one-way ANOVA with Games-Howell post hoc test. $^*P < 0.05$. (F) Schematic diagram showing the positive feedback loop among miR-210, CPEB2, and HIF-1α. Under hypoxic condition, the loop regulation further enlarges the hypoxic response in trophoblast cells, impairing trophoblast cell syncytialization, which may contribute to the occurrence of preeclampsia.

underlying the positive feedback loop between miR-210 and HIF-1α are complex, as reported in multiple cell types. ISCU [4, 22], RUNX3, GPD1L, and HIF-3α [11, 12, 23] are involved in mediating the feedback regulation. The up-regulation of miR-210 has been suggested as an indicator of hypoxia in the occurrence of tumor [24, 25]. As a matter of fact, increasing evidence are indicating the functions of miR-210 in regulating trophoblast cell growth, migration and invasion, hormone production, as well as syncytialization as shown here. We assume the factors including CPEB2, ISCU, RUNX3, GPD1L, HIF-3α, etc. may be involved in the regulatory loop of HIF-1α/miR-210 and lead to the hypoxic stress to harm multiple trophoblast cell behaviors in preeclamptic placenta. The methods of targeting these factors in the placenta may help to develop therapeutic strategies for preeclampsia.

Although we clearly proved the function of HIF-1α/miR-210/CPEB2 positive feedback loop in regulating trophoblast syncytialization, miR-210 was not the only up-stream regulator for CPEB2. For instance, it has been demonstrated that NPGPx modulated CPEB2 and HIF1a translation in response to oxidative stress [26]. NPGPx bound to CPEB2, and compromised CPEB2-mediated suppression of HIF-1α. CPEB2 was found to be relevant to prognosis of ER^+ and ER^- breast cancer [27], indicating estrogen may be a potent regulator for CPEB2. It is therefore interesting to study whether NPGPx-CPEB2-HIF-1α pathway or estrogen signaling are involved in the hypoxic response in the placenta.

The fact that little dysfunction was observed in miR-210 knockout mice seems paradoxical to the in vitro studies. It is most likely that there exist redundant mechanisms in cells to mediate the hypoxic

response. For instance, CPEB2 is target of miR-885-5p and miR-181a in tumor cells, which increases tumor metastasis and decreases paclitaxel resistance, respectively [28, 29]. Interestingly, elevation in miR-181a was also found in preeclamptic placenta, and it suppressed trophoblast cell invasion and cell cycle progression [30, 31]. Thus, the down-regulation of CEPB2 in preeclamptic placenta may derived from various pathological factors in addition to increased miR-210, which leads to attenuation of the suppression in HIF-1α translation. Although miR-210 is a typical hypoxia-response gene, the deficiency in this one gene may not influence the complex regulation of hypoxic response in the placenta. Therefore, further studies are needed to identify reliable targets that can correct the hypoxic stress in preeclamptic placenta.

In general, here we demonstrated a HIF-1α/miR-210/CPEB2 cascade that is involved in regulating trophoblast cell response to hypoxia and cell differentiation toward syncytial pathway. The finding highlights a mechanism of the adverse effect of excessive hypoxia on trophoblast development and its role in the etiology of preeclampsia.

Supplementary data

Supplementary data are available at *BIOLRE* online.

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Authors' contributions

Hao Wang, Yangyu Zhao, Ming Liu, Xuan Shao, and Xiaotao Bian conducted experiments, including cell culture, cell transfection, plasmid construction, real-time PCR, Western blotting, in situ hybridization, dual-luciferase report assay, and RIP. Rongcan Luo analyzed variation of CPEB2 in placental tissues. Yangyu Zhao, Yongqing Wang, and Yu-xia Li helped the collection of placental tissues. Yan-Ling Wang and Ming Liu designed the study, and drafted the manuscript. All authors had final approval of the submitted versions.

Conflict of interest

The authors have declared that no conflict of interest exists.

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