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Elevated levels of hypoxia-inducible microRNA-210 in pre-eclampsia: new insights into molecular mechanisms for the disease

Yi Zhang [a, #], Mingyu Fei [a, #], Geng Xue [a], Qi Zhou [a], Yin Jia [a], Li Li [b], Hong Xin [c], Shuhan Sun [a, *]

[a] *Department of Medical Genetics, Second Military Medical University, Shanghai, China*
[b] *Department of Obstetrics and Gynecology, First Affiliated Hospital of Anhui Medical University, Hefei, China*
[c] *Department of Obstetrics and Gynecology, Second Hospital of Hebei Medical University, Shijiazhuang, China*

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Abstract

Pre-eclampsia is a leading cause of maternal and foetal morbidity and mortality worldwide. Insufficient uteroplacental oxygenation is believed to be responsible for the disease. However, what molecular events involve in hypoxic responses and how they affect placental development remain unclear. Recently, miRNAs have emerged as a new class of molecules in response to hypoxia. We show here that the expression of microRNA-210 (mir-210) is up-regulated in patients with pre-eclampsia, as well as in trophoblast cells cultured under hypoxic conditions. Ectopic expression of mir-210 inhibited the migration and invasion capability of trophoblast cells. Ephrin-A3 and Homeobox-A9, which related with cell migration and vascular remodelling, were then experimentally validated as the functional targets of mir-210 both *in vivo* and *in vitro*. Using luciferase reporter, chromatin immunoprecipitation (ChIP) and small interfering RNA (siRNA) experiments, we finally identified a new transcriptional mechanism that the overexpression of mir-210 under hypoxia was regulated by NF-κB transcriptional factor p50, apart from the well-known HIF 1α. Taken together, our study implicates an important role for mir-210 in the molecular mechanism of pre-eclampsia.

Keywords: pre-eclampsia • hypoxia • microRNA • trophoblast cell • transcriptional regulation

Introduction

Pre-eclampsia is a multi-system disorder that is unique to human pregnancy, affecting 5–7% of pregnancies worldwide. The clinical features of pre-eclampsia can manifest as either a maternal syndrome (hypertension and proteinuria with or without other multi-system dysfunction occurring during the third trimester of pregnancy) or foetal syndrome (foetal growth restriction, perinatal deaths, preterm birth and abnormal oxygenation). Although the aetiology of pre-eclampsia is largely unknown, the placenta in general and trophoblast in particular is a prerequisite for the development of the disease [1, 2]. It is characterized by incomplete trophoblast invasion and aberranted spiral arterial remodelling leading to decreased uteroplacental perfusion and, therefore, is thought to give rise to placental ischaemia and hypoxia. This theory has been further strengthened by clinical observations that in women destined to develop pre-eclampsia, uteroplacental blood flow is reduced by 50–70% [3, 4]. In some studies, the hypoxia-inducible factor (HIF) protein levels are significantly elevated in the pre-eclamptic placenta [5, 6]. Furthermore, restriction of placental perfusion in several animal species has resulted in a pre-eclampsia-like illness [7], and experimental hypoxia is also correlated with pre-eclampsia features [8]. These data lend credence that placental hypoxia may contribute to the pathogenesis of pre-eclampsia and lead to the syndrome. To understand the dysregulated molecular events characteristic of pre-eclampsia associated with placental hypoxia is therefore very important.

Recently, endogenous microRNA (miRNA) molecules have been emerged as essential factors that are involved in numerous cellular processes including differentiation, proliferation, apoptosis and the stress response [9, 10]. miRNA are highly conserved,

[#]Both the authors contributed equally to this work.
*Correspondence to: Shuhan SUN,
Department of Medical Genetics,
Second Military Medical University, Shanghai 200433, China
Tel.: +86-21-81871053
Fax: +86-21-81871053
E-mail: shsun@vip.sina.com

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small non-coding RNAs ~22 nucleotides in length, and it can negatively regulate gene expression typically by interacting with the 3′ untranslated regions (3′-UTR) of specific mRNA targets to affect mRNA stability and translation. It is estimated that great than 30% of all mRNA transcripts are regulated by miRNA in some context [11], but the functions of specific miRNA molecules are still being determined. Over the past 3 years, several independent studies have reported that some specific miRNAs are highly up-regulated in hypoxic cells and demonstrated their importance for cell response to hypoxia. Screening to identify hypoxia-induced miRNAs has initially focused on culturing a variety of tumour cells under hypoxic conditions, given that hypoxia is a typical characteristic of solid tumours [12–15]. The reported hypoxia-induced miRNAs from these studies are not completely identical maybe because of the technical difference, screened range and cellular context. However, mir-210 is identified as a unique hypoxia-induced miRNA that is ubiquitously induced in various cell types. Furthermore, recent research suggests that mir-210 is specifically regulated in some human disease states. Using miRNA microarray or TaqMan-based low-density array assay, it was found that mir-210 is overexpressed in placental tissue derived from patients with pre-eclampsia [16, 17]. However, the underlying mechanism was not elucidated.

In this study, we investigated whether mir-210 contributes to the pathogenesis of pre-eclampsia, a complex disorder widely believed to be associated with placental hypoxia. Our results show that mir-210 is induced in patients with pre-eclampsia and in hypoxia-treated trophoblast cells. The up-regulated mir-210 attenuates trophoblast cell migration and invasion by repressing expression of Homeobox-A9 (HOXA9) and Ephrin-A3 (EFNA3). Using luciferase reporter, chromatin immunoprecipitation (ChIP) and small interfering RNA (siRNA) experiments, we provide evidence that apart from the well-known HIF regulator, hypoxia-induced NF-κB p50 could also regulate mir-210 expression.

Materials and methods

Patient enrollment and sample processing

A total of 45 pregnant Chinese women who visited the Department of Obstetrics and Gynecology, the Second Hospital of Hebei Medical University (Hebei, China) and the First Affiliated Hospital of Anhui Medical University (Anhui, China), were included in this study. Of these 45 women, 15 had mild pre-eclampsia (mPE), 15 had severe pre-eclampsia (sPE) and 15 were healthy pregnant controls matched for age, gestational age, parity and body mass index (BMI) at the time of blood sampling (Table S1). The healthy pregnant women had normal blood pressure with the absence of medical and obstetrical complications. Pre-eclampsia was defined according to the guideline of National Institutes of Health publication No. 00-3029 [18]. mPE was defined as maternal systolic blood pressure ≥140 mm Hg and/or diastolic blood pressure ≥90 mm Hg on two occasions, separated by 6 hrs, and proteinuria ≥300 mg in a 24-hr period after 20-week gestation. sPE was considered as either sever hypertension (maternal blood pressure >160/110 mm Hg on two separate readings at least 6 hrs apart) or sever proteinuria (>2 g protein in a 24-h period). None of the patients with pre-eclampsia had any prior history of essential hypertension or renal disorder, and none of them received treatments prior to blood sampling. Samples of maternal peripheral blood (4 ml) were collected into tubes containing EDTA. To obtain plasma, we centrifuged the samples twice at 4°C as described previously [19]. After the first centrifugation at 1600 × *g* for 10 min, the supernatants were carefully transferred into a new tube, and centrifuged again at 16,000 × *g* for 10 min. TRIzol LS reagent (Invitrogen, Carlsbad, CA, USA) in volumetric ratios of 3:1 was then added to the harvested maternal plasma. Placental samples were obtained and preserved in RNAlater (Ambion, Austin, TX, USA) immediately after delivery. The placental samples used in this study were randomly chosen from the mPE, sPE and control groups, and the criterion used for selection was that the controls were matched in gestational age to the individuals with pre-eclampsia. The study was approved by the institutional review board of each participating institution, and all patients gave informed consent.

Isolation and culture of cytotrophoblast cells, culture of JAR cells and cell treatments

The procedures to isolated cytotrophoblast cells (CT) were performed as described previously [20]. Briefly, the first trimester placenta tissues (5- to 10-week gestation) were obtained from the cases of legal abortion with informed consent and institutional review board approval. Dissected villous tissues were digested in phosphate-buffered saline (PBS) supplemented with 0.125% trypsin (Gibco, Grand Island, NY, USA), 0.42 mM $MgSO_4$, and 20 U/ml DNase type 1 (Invitrogen) at 37°C with gentle incubation for 15 min. After pushing through a mesh of 100-μm size, the collected cells were purified by Percoll density gradient (Sigma, St. Louis, MO, USA) centrifugation. CT cells were recovered in the density of the gradient between 1.048 and 1.062 g/ml. The isolated cells were incubated with lysis buffer containing 155 mM NH_4Cl, 10 mM $KHCO_3$ and 0.14 mM EDTA (pH 7.2) on ice for 3–5 min to eliminate contaminated red blood cells. Cell purity and viability was examined by anticytokeratin staining and trypan blue exclusion, respectively. The present culture method yielded over 90% purity and 95% viability for CT cells. Isolated CT cells were placed in six-well plates with a density of 5×10^6 cells per well in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal bovine serum (FBS). After overnight culture under regular culture conditions (5% CO_2 in air, 21% O_2) to equilibrate the cultures, cells were cultured in lowered oxygen conditions (5% CO_2–2% O_2–93% N_2) at 37°C, with the oxygen concentration being correctly regulated in an InVivo200 hypoxia work station (Biotrace International, Cincinnati, OH, USA) for different periods.

The human choricarcinoma cell line JAR was obtained from American Type Culture Collection. Cells were grown in RPMI 1640 medium supplemented with 10% FBS, 2 mM glutamine and 2 mM penicillin/streptomycin. Similar to trophoblast cells, JAR cells were also exposed to hypoxic environments for different time periods.

In separate experiments involving overexpression or down-modulation of mir-210 in CT or JAR cells, double-stranded mir-210 mimics, single-stranded mir-210 inhibitor or their relative control scramble sequence (GenePharma, Shanghai, China) at a final concentration of 50 nM was

introduced into cells. Transfections were performed using a Lipofectamine 2000 kit (Invitrogen) according to the manufacturer's instructions. After 6 hrs transfection, cells were re-fed with fresh medium and were immediately used in the following experiments.

In separate experiments involving silencing of NFκB p50 or HIF 1α, small interfering RNA (siRNAs) targeting NFκB p50, HIF1α, or a control siRNA of a scramble sequence (Santa Cruz Biotechnology, CA, USA) were transfected into JAR cells according to the manufacture's instructions. The medium was changed 6 hrs after transfection, and cells were immediately exposed to normoxic or hypoxic conditions for 48 hrs before harvesting.

RNA extraction and real-time quantitative RT-PCR

Total RNA was extracted from tissues and cells with TRIzol reagent (Invitrogen). Total RNA containing small RNA was extracted from 500 μl of plasma using TRIzol LS reagent (Invitrogen) and miRNeasy Mini Kit (Qiagen, Hilden, Germany). After the chloroform-addition step and phase separation, 1.5 volumes of absolute ethanol were added to the aqueous phase and the mixture was loaded into miRNeasy column according to the manufacture's protocol. To minimize DNA contamination, the eluted RNA preparation was treated with DNase I (Invitrogen). The concentration of all RNA samples were quantified by NanoDrop 1000 (NanoDrop, Wilmington, DE, USA).

Real-time quantitative PCR (QPCR) was performed with a standard SYBR-Green PCR kit protocol on a Step One Plus real-time PCR system (Applied Biosystems, Foster City, CA, USA). Stem-loop reverse transcription for mature mir-210 and U6 RNA was designed as previously described [21]. U6 RNA was used as an miRNA internal control. β-Actin was used as an endogenous control to normalize the amount of total mRNA in each sample. All reactions were run in triplicate. Fold changes in the relative expression was calculated by the $2^{-\Delta\Delta Ct}$ method. PCR primers are listed in Table S2.

Plasmid constructs and luciferase activity assays

The 3′-UTRs of the predicted target gene EFNA3 or HOXA9 containing potential mir-210-binding sites were amplified by PCR from human genomic DNA. The PCR product was digested with *Xba*I and cloned into a pGL3-promoter vector (Promega, Madison, WI, USA) immediately downstream of the luciferase gene. A 894 and 200 bp of promoter sequence upstream of mir-210 stem-loop structure and relevant sequenced-deleted mutants were generated from human genomic DNA by PCR amplification. The PCR products were digested with *Hand*III and *Xho*I and cloned into the promoterless pGL3-Basic vector (Promega). The mutants in the HIF 1α- and p50-biding sites were generated by overlap extension method. All the construct sequences were verified by automated DNA sequencing (Applied Biosystems). PCR primers are listed in Table S2.

For experimental validation of selected miRNA targets, JAR cells were cotransfected with 500 ng/well of pGL3-EFNA3 or pGL3-HOXA9 constructs with mir-210 mimics or a negative control. For determination of promoter activity, cells were transfected with 500 ng/well of the pGL3 promoter reporter. Each well was cotransfected with 50 ng of pRL-TK vector expressing renilla luciferase to control for transfection efficiency. Luciferase assays were performed 48 hrs after transfection using the dual-luciferase reporter assay system (Promega). The relative firefly luciferase activity was normalized with renilla luciferase activity.

Western blot analysis

Protein lysates from each sample were prepared, quantified with the Bradford assay, run on 12% SDS-PAGE and transferred electrophoretically to a polyvinylidene fluoride membrane using standard methods. After incubation with antibodies (all from Santa Cruz Biotechnology, Santa Cruz, CA, USA) specific for either HOXA9, EFNA3, NF-κB p50 or HIF 1α, the blots were incubated with IRdye800-conjugated rabbit anti-goat and goat anti-mouse secondary antibody (LI-COR Biosciences, Lincoln, NE, USA) and visualized using an Odyssey infrared scanner (LI-COR Biosciences).

Transwell migration and matrigel invasion assay

Cell migration and invasion assays were performed as described previously [22]. Cells (5×10^5) transfected with mir-210 mimics, anti-mir-210 or the relative negative controls were plated in medium without serum in the upper chamber of a transwell (24-well plate; 8.0-μm pore sizes; Corning, Somerset, NJ, USA) coated with or without matrigel (BD Biosciences, Bedford, MA, USA). Growth medium was added to the lower chambers. Plates were then either incubated in standard culture conditions (21% O_2) or hypoxic conditions (2% O_2) for 72 hrs. Cells that did not migrate or invade through the pores were removed with a cotton swab and cells on the underside of the culture insert were stained with haematoxylin (sigma) and quantified.

Chromatin immunoprecipitation assay

ChIP assay was performed with JAR cells cultured under normoxia or hypoxic conditions for 48 hrs using a ChIP kit (Upstate Biotechnology, Lake Placid, NY, USA) according to the manufacturer's recommended procedure. For each ChIP, anti-NFκB p50 (Santa Cruz Biotechnology) and non-specific immunoglobulin G (IgG, Sigma) were used. Primers are listed in Table S2. Amplified PCR products were electrophoresed on 4% agarose gel and visualized by ethidium bromide staining.

Evaluation of NF-κB p50 activity

Nuclear extracts from JAR cells cultured under normoxic or hypoxic conditions were prepared using a Nuclear Extraction Kit (Active Motif, Carlsbad, CA, USA) according to the manufacturer's instructions. NF-κB p50 activation was quantified by way of enzyme-linked immunosorbent assay using a TransAm NF-κB p50 transcription factor assay kits (Active Motif) according to the manufacturer's instructions.

Statistical analysis

Values are expressed as means and standard deviations from at least three independent experiments. The Mann–Whitney test or Student's t-test was used to compare two independent samples where appropriate. The Kruskal–Wallis test was used to assess differences among groups and the Nemenyi test was used to further compare in two related groups when significant differences were detected. Two-tailed *P* values of less than 0.05 were considered as statistically significant. All statistical analyses were performed using SAS software (version 9.1.3).

Results

Plasma mir-210 level is increased in patients with pre-eclampsia

The expression level of mir-210 is dramatically increased in placental tissues from patients with pre-eclampsia [16, 17], but its presence in circulation has not yet been explored. To investigate the potential of circulation mir-210 as a biomarker for pre-eclampsia, 15 patients with mPE, 15 with sPE were recruited, the level of mir-210 in their plasma was detected by QPCR and compared to the levels found in 15 matched healthy pregnancy controls. The clinical characteristics of the pregnant woman groups are shown in Table S1. There were no significant differences between the three groups in terms of age, body mass index and gestational age at blood collection. Maximum blood pressure and maximum proteinuria were significantly increased in the pre-eclampsia group compared to the normal pregnant group. All QPCR values were normalized to U6 and expressed as fold changes. As shown in Figure 1, The median change of mir-210 levels in patients with mild pre-eclampsia and severe pre-eclampsia were 3.27-fold [interquartile range (IQR) 2.08- to 4.47-fold] and 9.49-fold change (IQR, 7.10- to 13.93-fold), respectively, which were significantly higher when compared with that of the gestational age-matched healthy controls (IQR, 0.65- to 1.14-fold) ($P < 0.001$).

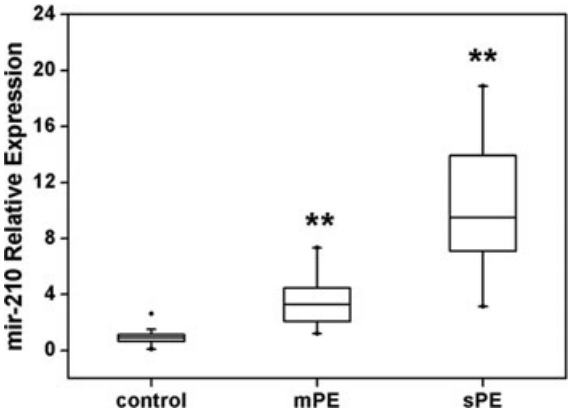

Fig. 1 Plasma mir-210 level increases in patients with pre-eclampsia. Box plots compare the fold change in mir-210 levels in the plasma of 15 patients with mPE, 15 patients with sPE and 15 healthy pregnancy controls. The box represents the interquartile range and the line across the box indicates the median value. $**P < 0.001$ compared to the healthy pregnancy control group.

Expression of mir-210 is up-regulated in hypoxia-treated trophoblast cells

Placental hypoxia likely plays an important role in the pathogenesis of pre-eclampsia. However, it remains uncertain what dysregulated molecular events involved in hypoxic responses, and little is known concerning the regulation of miRNA expression during hypoxia associated with pre-eclampsia. We therefore investigated the effect of hypoxia on the expression of mir-210 in pre-eclampsia. Accordingly, an *in vitro* method mimicking the effects of hypoxia in the human placenta is essential in understanding of the molecular events characteristic of pre-eclampsia. Several models are commonly used, including primary isolated cytotrophoblast cells, human trophoblast cell lines and organ culture [8]. In this study, CTs isolated from the first trimester placenta tissues and trophoblast-derived human cell line (JAR) were used and cultured under hypoxic conditions (2% oxygen) for 0, 12, 24, 48 and 72 hrs. RNA was isolated from the cell cultures, and the expression levels of mir-210 at each time point were determined in triplicate by QPCR. All PCR values were normalized to U6, which previous experiments showed was not regulated by hypoxia [14]. Our data showed that mir-210 levels were strongly up-regulated in hypoxia-treated trophoblast cells. After hypoxia induction, mir-210 levels in CT cells increased about 8.0- and 13.0-fold higher than normoxic control at 24 and 48 hrs, respectively, and the up-regulation was maintained for the next 72 hrs. The similar results were achieved in hypoxia-treated JAR cells (Fig. 2). In view that JAR cells can provide a large number of uniform cells, and more importantly, they share many characteristics of early placental trophoblasts [23, 24], we chose JAR cells as a model for human trophoblasts in some of the following experiments.

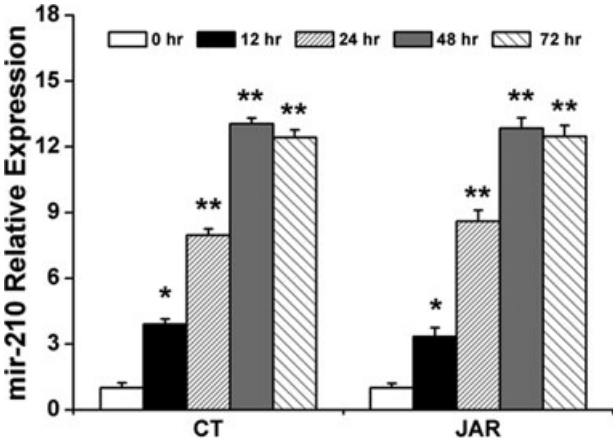

Fig. 2 Up-regulation of mir-210 in hypoxia-treated trophoblast cells. CT and JAR cells were treated with 2% oxygen for 0, 12, 24, 48 and 72 hrs, respectively. The relative expression levels of mir-210 were measured by QPCR and normalized to U6. Values expressed as fold change compared to normoxic controls. Data are shown as means and standard deviations from triplicate experiments. $*P < 0.05$, $**P < 0.001$.

Ectopic expression of mir-210 in trophoblast cell attenuates cell migration and invasion

Because shallow trophoblast invasion and impaired spiral arterial remodelling are considered as initial pathological steps in pre-

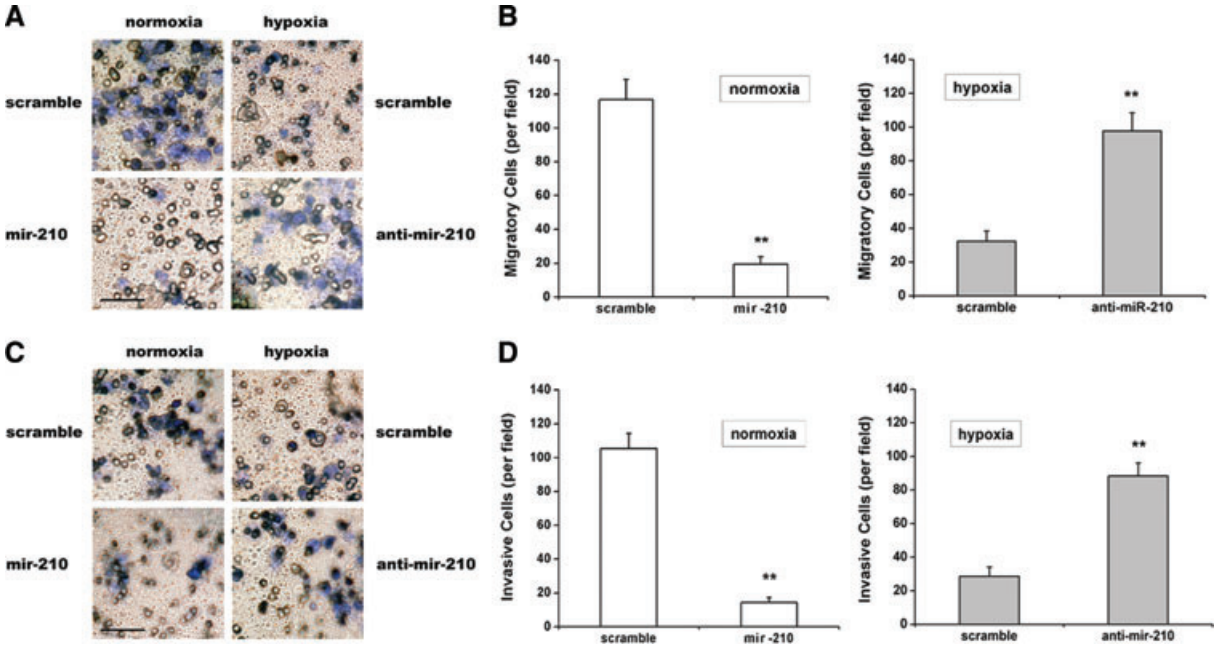

Fig. 3 Determination of mir-210 involvement in trophoblast cell migration and invasion by transwell assays. Representative migration (**A**) or invasion (**C**) experiment of CT cells transfected with mir-210 mimics (under normoxic conditions), anti-mir-210 (under hypoxic conditions) or negative controls. Size bar = 50 μm. The percentage of migrated cells (**B**) or invaded cells (**D**) is shown as means and standard deviations based on at least three independent experiments. $^{**}P < 0.001$.

eclampsia, the study was then designed to determine whether ectopic expression of mir-210 affects the capability of trophoblast cell migration and invasion. CT cells isolated from the first trimester placenta tissues were transfected with mir-210 mimics and after 72 hrs induction, the migration and invasion of cells were assessed by transwell assays. Comparing with control group, mir-210 induction greatly decreased cell migration (Fig. 3A and B) and invasion (Fig. 3C and D) capacity. To demonstrate whether mir-210 involved in the suppressed invasion capability is elicited by hypoxia, CT cells were then exposed to 72 hrs of hypoxia. As expected, hypoxia treatment inhibited cell migration (Fig. 3A and B) and invasion (Fig. 3C and D), yielding a similar result to that observed in cells overexpressing mir-210 (Fig. 3). These hypoxic cells were transfected with anti-mir-210 that binds with high affinity and specificity to the complementary miRNA, inactivating its function. Remarkably, compared with control group, the migration and invasion capacity of CT cells were greatly increased by 72 hrs of mir-210 blocking. These results strongly suggest that hypoxia suppresses migration and invasion of the CT cells, at least in part by increasing the level of mir-210.

mir-210 represses ephrin-A3 and homeobox-A9 expression

To elucidate the mechanisms by which mir-210 attenuates trophoblast cell migration and invasion, we used three different prediction algorithms: TargetScan (http://www.targetscan.org), mirBase (http://microrna.sanger.ac.uk) and PicTar (http://pictar.mdc-berlin.de) to identify mir-210 targets. Among the candidate genes, ephrin-A3 (EFNA3) and homeobox-A9 (HOXA9) are two high-scoring candidates. The ephrin ligands binding the Eph receptor members are known to responsible for diverse biological functions, including cell migration, vascular remodelling and vascular development during embryogenesis [25], whereas the homeoboxs encoding for transcription factors are responsible for biological functions, also including cell migration and vascular development [26]. These two targeted candidates, therefore, attracted our interest. As shown in Figure S1A and B, the EFNA3 or HOXA9-encoded mRNA contains a 3′-UTR element that is partially complementary to mir-210, indicating that mir-210 would directly target these sites. To validate this prediction, the EFNA3 or HOXA9 complementary sites were cloned into the 3′-UTR of the firefly luciferase vector and cotransfected with mir-210 mimics or negative controls in JAR cells. As shown in Figure S1C and D, mir-210 significantly reduced the luciferase activity of pGL3-EFNA3 and pGL3-HOXA9, whereas such inhibition was not observed in cells cotransfected with a control scramble sequence or mock. Afterwards, EFNA3 or HOXA9 mRNA and protein levels in JARs, with or without mir-210 mimics transfection, were then detected using QPCR and Western blot to determine whether mir-210 acts through mRNA degradation or translational repression. Our results showed that after 48 hrs of transfection, enforced mir-210 expression led to a significant reduction of EFNA3 protein levels but not mRNA levels (Fig. S1E),

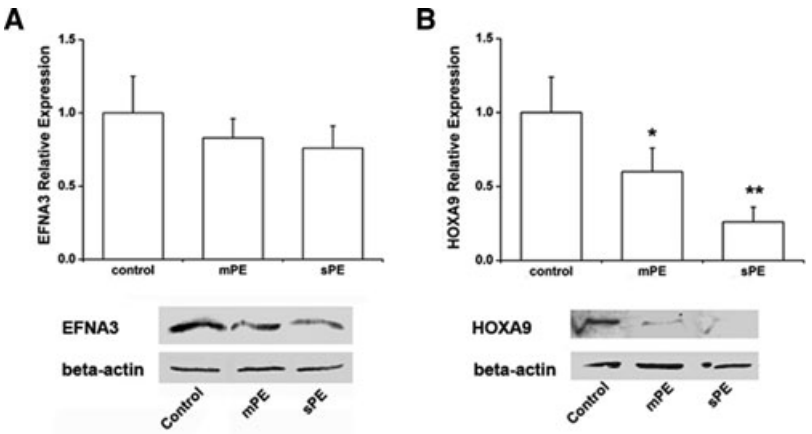

Fig. 4 Expression levels of EFNA3 and HOXA9 in placentas from patients with pre-eclampsia. Post-transcript levels of EFNA3 and HOXA9 in placental samples from patients with mPE ($n = 6$), patients with sPE ($n = 6$) and gestational age-matched healthy controls ($n = 6$) were analysed by QPCR and Western blot, respectively. PCR values were expressed as fold changes and normalized to β-actin. (**A**) Down-regulation of EFNA3 expression in pre-eclampsia placentas *versus* paired healthy placentas was due to decreased protein levels, but not mRNA levels. (**B**) Down-modulation of HOXA9, both at mRNA levels and protein levels were investigated in pre-eclampsia placentas compared to healthy control tissues. Data are shown as means and standard deviations. $*P < 0.05$, $**P < 0.001$ compared to the healthy control tissues.

whereas the reduction of HOXA9 was observed at both the mRNA and protein levels in comparison with negative controls in JAR cells (Fig. S1F). Above results strongly indicate that with mir-210, EFNA3 expression is suppressed by translational repression whereas HOXA9 expression is suppressed by both mRNA degradation and translational repression.

Ephrin-A3 and homeobox-A9 expression are down-regulated in placentas from patients with pre-eclampsia

The up-regulation expression of mir-210 in placental tissues from patients with pre-eclampsia has been shown [16, 17]. We next sought to investigate whether EFNA3 and HOXA9 expressions were inversely correlated with the levels of mir-210 in pre-eclampsia placentas. Placental samples from six patients with mPE, six patients with sPE and six gestational age-matched healthy controls were analysed for the levels of EFNA3 and HOXA9 mRNA by QPCR, and protein by Western blot. A clear down-regulation of HOXA9, both at mRNA levels and protein levels was seen in pre-eclampsia placentas compared to healthy control tissues (Fig. 4B). The EFNA3 expression in placental tissues was similar to that observed above for JAR cells, that is, EFNA3 mRNA levels were not significantly affected, whereas protein levels decreased substantially (Fig. 4A).

mir-210 is regulated by the transcription factor NF-κB p50

Since the hypoxia regulation of mir-210 was first identified by miRNA microarray in 2007 [15], several studies have reported that mir-210 was regulated by HIF 1α [12, 14, 27] or HIF 2α [28]. Indeed, HIF is central to the cellular response to hypoxia, but it is not alone in displaying sensitivity to oxygen levels. There are several other transcription factors have been reported to display hypoxia sensitivity [29], principal among which is the transcription factor NF-κB. In contrast to HIF being activated by hypoxia, NF-κB has been long considered to be activated by oxidative stress, recent study provided evidence that both transcription factors are responsive to both conditions [30]. More importantly, NF-κB transcriptional activity was found significantly enhanced in patients with pre-eclampsia [31–33]. In our experimental set-up, NF-κB p50 transcriptional activity dramatically increased when JAR cells cultured under hypoxic conditions (Fig. 5A), which was consistent with the variation of mir-210 in the same conditions. Furthermore, JAR cells treated with the tumour-necrosis factor (TNF), a commonly used activator of NF-κB, resulting in significantly increased mir-210 levels (Fig. 5B). These findings promoted us to explore whether mir-210 expression is also NF-κB regulated. In previous studies, authors have characterized the mir-210 promoter through searching a 2.3 kb promoter sequence upstream of mir-210 stem-loop structure, and identified that a functional HIF 1α-binding site was located about 400 bp upstream of the structure [14]. In this study, we search the potential mir-210 regulator in a 200-bp core promoter region immediately upstream of mir-210 stem-loop structure. Serial deletion constructs were made and reporter assays were performed to determine the effect of the deletions on reporter gene activity (Fig. 5C). We investigated that a sequence, extending from 110 to 140 bp upstream of the stem-loop structure, is responsible for the robust induction of mir-210 under hypoxia. We then performed a computational screen (http://mapper.chip.org) [34] and found that NF-κB subunit p50 localizes within this promoter region (Fig. 5D). We next wanted to determine if NF-κB regulates mir-210 expression by binding to the mir-210 promoter directly. Two sets of primers were designed for ChIP assay. One set flanks the binding site of NF-κB p50 identified by serial deletion, the other as a control is a non-specific set located approximately 300 bp downstream of this p50-binding site. As shown in Figure 5E, NF-κB p50 specifically binds to the functional binding site, which means that mir-210 can be directly regulated by specific binding of NF-κB p50 to its putative promoter.

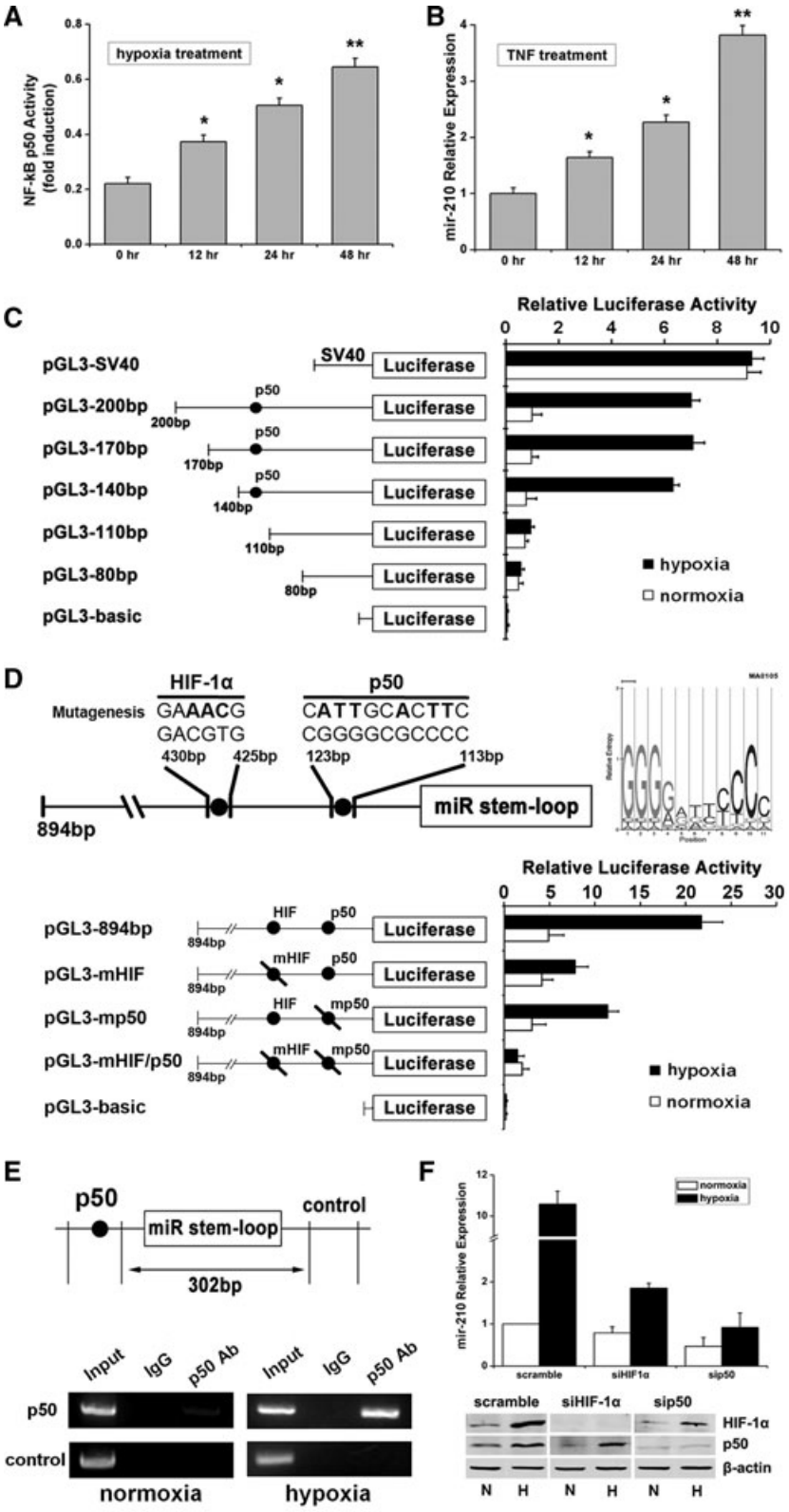

Fig. 5 mir-210 is transcribed by the transcription factor NF-κB p50. (**A**) NF-κB p50 activity was up-regulated in nuclear extracts of JAR cells cultured under hypoxic conditions. $*P <$ 0.05, $**P < 0.001$. (**B**) JAR cells treated with TNF resulted in significantly increased levels of mir-210. $*P < 0.05$, $**P < 0.001$. (**C**) Analysis of the mir-210 promoter. Promoter fragments were cloned into the pGL3-Basic vector, a pGL3-simian virus 40 construct (pGL3-SV40) and a promoter-less vector (pGL3-Basic) were used as positive and negative references for promoter activities, respectively. The cells were exposed to normoxia or hypoxic conditions for 48 hrs. Serial deletion constructs are represented in the bar diagram on the left, whereas their relative promoter activities are displayed on the right of the figure. The data shown as means and standard deviations of three independent experiments. (**D**) Bioinformatics analysis disclosed the potential binding site of NF-κB p50. Mutation of the p50-binding site partially inhibited the hypoxia-induced transcription. Simultaneous mutation of HIF 1α- and p50-binding sites completely inhibited this response. (**E**) ChIP assay. JAR cells were cultured under normoxia or hypoxic conditions for 48 hrs, respectively. PCR was performed with primers specific to the functional mir-210 site and negative site, respectively. Upper: Relative locations of ChIP primers on mir-210 promoter. Lower: ChIP results. (**F**) The upper panel shows mir-210 expression levels in JAR cells with siRNA knockdown of NF-κB p50, HIF 1α or a scramble control siRNA with or without exposure to hypoxic conditions for 48 hrs. The lower panel shows siRNA knockdown and hypoxia induction of NF-κB p50 and HIF 1α in these cells.

An about 1 kb promoter sequence upstream of mir-210 stem-loop structure that includes HIF1α and p50 response elements was cloned into the pGL3-Basic vector to assessed whether the luciferase activity was induced by both of the two elements. The HIF 1α- and p50-binding sites were then mutated and the transcriptional activities of the mutated promoters were determined. As shown in Figure 5D, independent mutation of the HIF 1α- or p50-binding site partially inhibited the hypoxia-induced transcription, and simultaneous mutation of the two sites accumulate this inhibitory effect, indicating that mir-210 could be regulated by both HIF 1α and p50. To further address the role of p50 *versus* HIF 1α in mir-210 expression, siRNAs against NF-κB p50, HIF 1α or scramble control siRNAs were transfected into JAR cells. As analysed by QPCR, JARs transfected with scramble control siRNA had significant induction of mir-210 expression under 2% oxygen, whereas this induction was inhibited in cells transfected with siRNAs against either NF-κB p50 or HIF 1α (Fig. 5F). Interestingly, the down-regulation of mir-210 expression in cells transfected with p50 siRNA seemed more prominent than that in cells transfected with HIF 1α siRNA, indicating that there might be a cross-talk between NF-κB p50 and HIF 1α in regulating the transcriptional expression of mir-210.

Discussion

Despite advances in perinatal care, frequency of pre-eclampsia has not changed over these years. Currently, women who develop pre-eclampsia can only be identified by the appearance of clinical symptoms such as hypertension and proteinuria. No known biomarkers or predictive tests are available for early detection of the disorder. The opposite with this is the higher prevalence of pre-eclampsia associated with a higher mortality of mother and foetus. Therefore, it has been a subject of intensive clinical and laboratory investigations aimed at identifying new and effective biomarkers for the disorder. During the past decade, several markers have been identified as potential serum/plasma biomarkers for pre-eclampsia (including, for example, soluble fms-like tyrosine kinase 1 and vascular endothelial growth factor). However, recent studies indicated that pre-eclampsia would not develop in all women with changed levels of these protein markers [35, 36]. This suggests that other factors are also likely to affect the occurrence and expression of the disease.

The discovery of nucleic acids circulating in the peripheral blood has created a new approach for the non-invasive diagnosis of clinical diseases [37, 38]. Circulating mRNA molecules have been investigated as potential markers for disease, and recent studies on miRNAs offer the possibility of developing yet another class of molecular markers [19, 39]. Unlike screening for the expression of large numbers of mRNAs, a modest number of miRNAs or even one specific miRNA might be sufficient to differentiate patients from healthy individuals. In this study, we determined if mir-210 can be detected in the circulation and initial evaluated the feasibility of using it as a non-invasive biomarker for pre-eclampsia. We observed that mir-210 levels in plasma from pre-eclampsia patients were significantly higher than those in normal pregnancy, and seemed to correlate well with disease severity, providing an exciting proof-of-principle that circulating mir-210 may serve as a potential diagnostic marker for pre-eclampsia. Of cause, further validation of mir-210 as a biomarker in a larger sample set, and prospective studies to evaluate its value in both normal and high-risk women are necessary.

Hypoxia is the condition of insufficient oxygen supply to tissues, which results from physiological or pathological changes such as high altitude, anaemia or abnormal and insufficient vasculature. Hypoxia plays a causal role in the pathogenesis of pre-eclampsia. Placentation at high altitude has been proved as a natural *in vivo* model for studying the hypoxia-related events in pre-eclampsia. Our results showed that mir-210, the most consistently and robustly induced miRNA under hypoxia, was strongly up-regulated in hypoxia-treated trophoblast cells. Although much progress has been made since the first report of hypoxia affecting miRNA 3 years ago [15], tremendous challenges still lie ahead. As pointed out in the latest review paper [40]: 'what role does mir-210 play in other diseases where hypoxia plays an important role (*i.e.* pre-eclampsia)?', 'what are the other transcriptional factors regulating mir-210 expression', and so on. Our study could help to answer these questions.

It is known that insufficient trophoblast invasion and aberranted spiral arterial remodelling lead to placental ischaemia and hypoxia, and consequently contribute to the development of pre-eclampsia. Histological examination of placental bed biopsies from pre-eclamptic women demonstrates the limited migration into superficial decidua and reduced invasion into the myometrial portions of the spiral arteries by cytotrophoblast cells [2]. Meanwhile, it is worth noting that placental hypoxia can itself directly block the invasion and differentiation of cytotrophoblasts in primary culture studies, indicating that there is a positive feedback loop between hypoxia and abnormal placentation [41, 42]. However, the details of this feedback mechanism remain unclear. We found that high mir-210 levels could inhibit migration and invasion of trophoblast cells, and hypoxia altered these cell behaviours at least in part by up-regulating mir-210 expression. Fasanaro *et al.* found that hypoxia and mir-210 stimulated endothelial cell migration [43]. The discrepancy with our study might be because of the differences in the cell lines, cell culture and stimulation conditions. Considering the relationship between miRNA regulation and its targets expression may show various patterns in different cellular contexts, experimental validation of selected miRNA targets in trophoblast cells was still performed in this study although mir-210 targeting EFNA3 and/or HOXA9 has ever been described in previous studies [14, 43, 44]. EFNA3 is a member of the ephrin ligand family, which function during the development of diverse organ systems including axon guidance and cell migration [25]. It is found that EFNA1 expression is limited to the invasive trophoblast lineage throughout pregnancy, suggesting that this receptor ligand system may be responsible for targeting of the trophoblasts to the uterine tissue (interstitial invasion) and spiral arteries (endovascular invasion) [45]. However, in their study, the authors were puzzled that in pre-eclampsia placenta, which is often characterized by defective

shallow trophoblast migration, EFNA1 mRNA levels were similar with that of normal placenta. Interestingly, it is seemingly in accordance with the results of our present study. We also found that EFNA3 mRNA levels were not significantly affected in patients with pre-eclampsia, whereas protein levels decreased substantially, indicating that EFNA3 expression is suppressed with mir-210 by translational repression. The other mir-210 target, HOXA9, is a member of homeobox gene family and has been shown to play a crucial role for angiogenesis. HOXA9 ablation in endothelial cells inhibits *in vitro* sprout formation and cell migration [26]. We reported here that a significant down-modulation of HOXA9, both at mRNA and protein levels appeared in pre-eclampsia placentas compared to healthy control tissues, which is consistent with the role of HOXA9 reported in the previous study [26].

Because of the critical role of the HIF in hypoxic response, so far almost all studies about the signaling pathway involved in mir-210 regulation are performed around the HIF transcription factor [12, 14, 27, 28]. However, it is clear that a number of other transcription factors are also activated by hypoxia either directly or indirectly, such as activator protein-1, NF-κB and CREB [29]. Interestingly, NF-κB p50 transcriptional activity could be significantly induced in patient with pre-eclampsia [31–33], as well as in trophoblast cells cultured under hypoxia conditions. By constructing mir-210 promoter deletion/point mutants and ChIP analysis, we identified a functional NF-κB p50-binding site and demonstrated its role in regulating mir-210 expression under hypoxia. Although the signaling molecular events linking cellular hypoxia to transcriptional activation are less well known for NF-κB than for HIF, it has been reported that the same oxygen-sensing hydroxylases responsible for conferring hypoxia sensitivity to the HIF pathway may also regulate important components of the NF-κB pathway [30]. In addition, recent studies have demonstrated that a high degree of cross-talk exists between the NF-κB and HIF signaling pathways. NF-κB has been found to play a role in hypoxia-induced HIF 1α mRNA expression [46], and in basal levels of HIF 1α gene expression [47, 48]. More importantly, the above found has been proved to occur *in vivo* [47]. The HIF 1α promoter contains a canonical NF-κB-binding site at a position-197/-188 upstream of the transcriptional start site, the mutation of which leads to a loss of HIF 1α up-regulation by hypoxia [47, 48]. We might also investigate the cross-talk effect in the present study when we tried to address the role of NF-κB and HIF in mir-210 expression. It was shown that in cells transfected with p50 siRNA under hypoxia, the down-regulation of mir-210 expression seemed more marked than that in cells transfected with HIF 1α siRNA.

In summary, we report here that higher levels of mir-210 are found in patients with pre-eclampsia and hypoxia-treated trophoblast cells. We further identify a new pathway, hypoxia/NF-κB/mir-210/EFNA3 and HOXA9, that is likely to play an important role in the pathogenesis of pre-eclampsia and therefore provides an opportunity for therapeutic intervention in pre-eclampsia. In addition, this work is a necessary and beneficial complement for elucidating the biological function of mir-210, the micromanager of the hypoxia pathway.

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Conflict of interest

The authors confirm that there are no conflicts of interest.

Supporting information

Additional Supporting Information may be found in the online version of this article:

Fig. S1 mir-210 targets EFNA3 and HOXA9.

Table S1 Clinical characteristics in the pregnant woman groups

Table S2 Primers and oligonucleotide sequences in the study

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