Original Article

MiR-195 participates in the placental disorder of preeclampsia via targeting activin receptor type-2B in trophoblastic cells

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Objective: Preeclampsia is a pregnancy-related syndrome. Shallow invasion of uterine wall by trophoblast cells has been generally accepted as the major pathological change of this disorder. We previously found downregulation of miR-195 in preeclamptic placentas. Bioinformatic analysis predicted a type II activin receptor, activin receptor type-2B (ActRIIB), as one of the potential targets of miR-195. Considering the key function of activin A on trophoblast cell behaviors and placenta development, we proposed miR-195 may affect trophoblast cell invasion by repressing the expression of ActRIIB.

Methods: The colocalization of ActRIIB and miR-195 in human placenta was measured by in-situ hybridization and immunohistochemistry. Western blotting, real-time PCR and dual luciferase assay were performed in human trophoblast cell line, HTR8/SVneo cells, to validate the targeting of ActRIIB by miR-195. Cell invasiveness was analyzed using transwell insert invasion assay in HTR8/SVneo cells.

Results: In human placenta, ActRIIB and miR-195 exhibited similar localization in various subtypes of trophoblast cells, including villous and extravillous trophoblasts. The protein expressions of ActRIIB in preeclamptic placenta were significantly higher as compared with the normal controls, which was opposite to the changing pattern of miR-195. In HTR8/SVneo cells, miR-195 could directly target and suppress the expression of ActRIIB. Meanwhile, the invasion-promoting effect of miR-195 on trophoblast cells could be largely impeded by ActRIIB overexpression.

Conclusion: In human trophoblast cells, miR-195 could promote cell invasion via directly targeting ActRIIB. The impaired miR-195 expression may contribute to the occurrence or development of preeclampsia through interfering with activin/nodal signaling in the placenta.

Keywords: activin receptor type-2B, invasion, miR-195, preeclampsia, trophoblast cells

Abbreviations: 3′UTR, 3′ untranslated region; ActRIIA, activin receptor type-2A; ActRIIB, activin receptor type-2B; CTB, cytotrophoblast; EVT, extravillous trophoblast; miRNA, microRNA; PE, preeclampsia; RT-PCR, real-time PCR; STB, syncytiotrophoblast

INTRODUCTION

MicroRNAs (miRNAs) are small single-stranded noncoding RNAs with 19–24 nucleotides [1,2]. They modulate gene expression by pairing with complementary mRNA sequence of target genes, usually in the 3′ untranslated region (3′UTR) and forming the RNA-induced silencing complex to degrade the mRNA or repress protein translation [1,3,4]. It is known that approximately one third of human genes can be regulated by miRNAs, and these small RNAs can participate in diverse biological progresses such as cell proliferation, differentiation, apoptosis, angiogenesis and immune response [5–8].

Preeclampsia is a serious pregnancy-related syndrome that affects 2–7% pregnancies worldwide. It has been one of the major contributors to maternal and fetal mortality and morbidity [9]. This syndrome is generally defined as new onset of hypertension in company with proteinuria or multiple organ complications at or after the 20th week of gestation [10]. The cause of preeclampsia is complex and remains largely unknown, but placenta deficiency at early gestation has been well accepted to be strongly associated with the occurrence of the disease. The poor placentation and insufficient maternal blood supply are believed to participate in the onset of maternal signs, including hypertension and proteinuria [11]. Recently, more and more evidences indicated the differential miRNA profiles in

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TABLE 1. Clinical characteristics of the pregnant women recruited in this study

	Normal pregnancy ($n = 39$)	Preeclampsia ($n = 19$)	*P* value
Maternal age (years)	31.4 ± 0.7	29.9 ± 1.1	0.233
BMI (kg/m^2)	22.1 ± 0.3	21.6 ± 0.5	0.462
SBP (mmHg)	116.6 ± 0.7	162.6 ± 6.5[a]	0.001
DBP (mmHg)	76.7 ± 0.7	104.1 ± 3.9[a]	0.001
50 g GCT (mmol/l)	9.4 ± 2.1	6.9 ± 0.3	0.415
24-h urine protein (g)	0.09 ± 0.06	4.09 ± 0.82[a]	0.001
Primiparous percentage (%)	77.8	73.3	NA.
Gestational day at delivery (day)	267 ± 2	245 ± 5[a]	0.001
Infant birth weight (g)	3284 ± 84.6	2206 ± 158.9[a]	0.001

Data are presented as mean ± SEM, and significant difference between groups was analyzed by one-way ANOVA. GCT, glucose challenge test.
[a]Compared with normal pregnancy, $P < 0.05$.

placenta and serum of preeclamptic patients [12–14], and the participation of the altered miRNAs expression in the occurrence and development of preeclampsia has been suggested.

MiR-195 belongs to miR-15/107 family [15,16]. Members of this family, including miR-195 and miR-497, share the sequence AGCAGC near the 5′-end, which controls the target specificity, and they modulate overlapping list of targets [17,18]. We previously identified miR-195, but not other miR-15/107 family members, as one of the down-regulated small RNAs in preeclamptic placenta [19,20], and demonstrated ActRIIA, a type II receptor of activin A, was one of its functional targets in human placenta, which could mediate the invasion-promoting effect of miR-195 in human trophoblast cells [19]. Interestingly, with bioinformatics analysis using TargetScan, we found activin receptor type-2B (ActRIIB), the shared type II receptor of activin A and nodal, was also among the predicted targets list of miR-195. Ours and studies of many others have revealed the crucial roles of activin/nodal signaling in human placenta development, especially the differentiation of trophoblastic cells. Overactivation of this signaling pathway in preeclamptic placenta has been suggested on the basis of the overexpression of activin A [21], nodal and ALK7 [22], ActRIIA [19] and Smad2 [23], strongly indicating the involvement of the disturbed activin/nodal signaling in the development of preeclampsia [24–26]. We therefore proposed that miR-195 may take considerable roles by ActRIIB, apart from ActRIIA in trophoblast cells.

To address the hypothesis, we determined the colocalization of miR-195 with ActRIIB in human placenta, and analyzed the association of miR-195 expression with that of ActRIIB in preeclamptic placenta. In-vitro studies, using human trophoblastic cell line and HTR8/SVneo cells, were performed to verify the direct binding of miR-195 to 3′UTR of *ACVR2B* mRNA, the repression on ActRIIB expression by miR-195, as well as the participation of ActRIIB on the invasion-modulating effect of miR-195 in trophoblast cells.

MATERIALS AND METHODS

Study participants

Placenta tissues from normal pregnant individuals and preeclamptic patients were collected at the Department of Obstetrics and Gynaecology, Peking University Third Hospital, China. The use of human placenta tissues in this study was approved by the Ethic Committees of the Institute of Zoology, Chinese Academy of Sciences and Peking University Third Hospital. Written consents were obtained from all the study participants. A total of 39 normal pregnant and 19 severe preeclamptic study participants were enrolled in this study. Normal pregnancy was defined as a uniparous gestation in a previously normotensive woman who did not exhibit any gestational complication and delivered a healthy newborn with an adequate weight for gestational age after 37 weeks of pregnancy. Preeclampsia was characterized according to the International Society for the Study of Hypertension in Pregnancy. Briefly, the patients had not suffered from preexisting or chronic hypertension before gestation, but showed SBP at least 160 mmHg or DBP at least 110 mmHg on no less than two occasions, accompanied by significant proteinuria (>2 g/24 h or 3+ by dipstick in two random samples obtained at >4 h interval) or multiple organ damage after 20 weeks of gestation. All the enrolled pregnant women were subjected to cesarean deliveries. Those who developed transient hypertension in pregnancy, gestational diabetes, cardiovascular disease, renal disease, intrauterine fetal death, fetal congenital or chromosomal abnormalities or pregnancies conceived by in-vitro fertilization were excluded from this study. The clinical characteristics of the enrolled women were listed in Table 1.

Tissues of human chorionic villi and decidua at early gestation (weeks 7–9) were obtained at Beijing Haidian Hospital (Beijing, China) from the patients who underwent therapeutic termination of pregnancy. The patients accepted no special medical treatment before termination of pregnancy, and the gestational week was determined by the morphological observation and pathological examination of the villi, with the record of menstrual cycles as a reference.

Immunohistochemistry

Human early pregnant villi and term placentas were fixed at 4% PFA before embedded in paraffin wax. Sections of 5 μm were collected to dewaxed, rehydrated, retrieved in 0.01 mol/l citrate buffer (pH = 6.0) for 15 min and immersed in 1% H_2O_2 for 10 min, then incubated with the primary antibody against ActRIIB (anti-ActRIIB, 1 : 100, Abcam, Cambridge, UK) or cytokeratin 8 (anti-CK8, 1 : 500, Novus Biologicals, Littleton, Colorado, USA) in 1% BSA at 4°C overnight. After washed in PBS, the sections were incubated with HRP-conjugated secondary antibodies (Zhongshan Goldenbridge, Beijing, China) for 1 h at room

temperature and visualized with DAB (Zhongshan Goldenbridge) as a substrate. Slides were counterstained with hematoxylin.

In-situ hybridization

Fresh human early pregnant villi and term placentas were fixed at 4% PFA, incubated through serial concentrations of sucrose solution and embedded in Tissue-Tek O.C.T. compound (Sakura Finetek, Torrance, California, USA). Eight-micron sections were preheated, fixed, then hybridized with the miRCURY LNA miRNA Detection probe (RiboBio, Guangzhou, China) at 55°C overnight. After washing in a series of saline sodium citrate (SSC) buffer, the sections were incubated with AP-conjugated anti-DIG antibody at 37°C for 2 h (Roche, Indianapolis, Indiana, USA) and visualized with BCIP/NBT (Promega, Madison, Wisconsin, USA) as substrate. Slides were counterstained with Nuclear Fast Red (Dingguo Changsheng, Beijing, China).

Sequences and constructs

The mimics for miR-195 and the scramble negative control were designed and purchased from Genepharma (Shanghai, China). To generate ActRIIB expression plasmid (pActRIIB), the coding sequence of *ACVR2B* (gene encoding ActRIIB) was inserted into pcDNA4.0 vector (Invitrogen, Carlsbad, California, USA) at the restriction sites of Hind III and BamH I. To construct luciferase report plasmids, we used bioinformatics prediction and found three conserved binding sites of miR-195 in 3′UTR region of *ACVR2B* at position 109–115, 1279–1285 and 9650–9659, respectively. For each binding site, about 200 bp fragment containing the potential binding site sequence in the middle was amplified and inserted into 3′UTR of firefly luciferase gene on the pMIR-REPORT luciferase plasmid backbone to construct wild type reporter plasmid, which was named as ActRIIB-BD1, ActRIIB-BD2 and ActRIIB-BD3. The reporter plasmids were point-mutated at the binding sites of miR-195 to generate mutated report plasmids (MUT) using PrimeSTAR HS DNA Polymerase (Takara, Dalian, China) according to the manufacturer's instruction. All the constructs were confirmed by DNA sequencing.

Cell culture and treatment

The immortalized human trophoblast cell line HTR8/SVneo was kindly gifted by Dr CH Graham at Queen's University, Canada [27]. The cells were cultured in RPMI 1640 medium (Invitrogen, Carlsbad, California, USA) supplemented with 10% fetal bovine serum (FBS), and were passaged every 3 days. Transient transfection of miRNA duplexes mimics or plasmids was performed using lipofectamine2000 reagent, according to the manufacturer's instruction (Invitrogen). The cells were subjected to further analysis 48 h after transfection.

RNA extraction and real-time PCR

Total RNA were extracted using TRIzol reagent (Invitrogen) according to the manufacturer's instruction. Two micrograms of total RNA were subjected to reverse-transcription into cDNA by oligo (dT) primer and M-MLV reverse transcriptase (Promega). MiRcute miRNA First-strand cDNA Synthesis Kit (Tiangen Biotech, Beijing, China) was used for reverse transcription of miRNA. Real-time PCR (RT-PCR) was performed using LightCycler 480II system (Roche), and the quantification of mRNA or miRNA expressions were performed according to the instruction of SYBR Premix ER Taq II (Takara, Dalian, China) or miRcute miRNA Premix (Tiangen Biotech, Beijing, China) detection kits. The relative mRNA/miRNA level was calculated by the $2^{-\Delta CT}$ method [28], and ΔCT indicated the subtraction of the cycle threshold (CT) value for GAPDH or U6 from the CT value for the interest mRNA or miRNA.

Protein extraction and western blotting analysis

Tissue samples or cultured cells were lysed by RIPA buffer containing 150 mmol/l NaCl, 10 mmol/l Tris (pH7.6), 1% NP-40, 0.5% Na deoxycholate, 0.1% SDS, 1 mmol/l NaF, Na_3VO_4 and protease inhibitor cocktail (Sigma Aldrich, St. Louis, Missouri, USA). Following incubation for 30 min on ice, whole-cell lysates were centrifuged at 12 000 rpm for 15 min at 4°C to remove debris. Protein concentration was measured using the BCA Protein Assay Kit (BOSTER, Wuhan, China). The protein extracts were subjected to SDS-PAGE and transferred to 0.45 μm nitrocellulose membrane (GE Healthcare, Marlborough, Connecticut, USA). The membranes were blocked by 5% nonfat milk in 0.1% phosphate buffered saline with Tween-20 for 2 h, incubated with primary antibodies overnight at 4°C. The antibodies included rabbit anti-human ActRIIB (Abcam) and mouse anti-human GAPDH. Horseradish peroxidase-conjugated secondary antibody (Jackson, Mississippi, USA) was added to the membranes for another 2 h at room temperature. Signals were visualized using Super-Signal West Pico Chemiluminescent Substrate (Thermo Scientific, Waltham, Massachusetts, USA) and detected with GeneGnome XRQ (Syngene, Frederick, Maryland, USA). The images were analyzed with ImageJ software. The relative density of ActRIIB was measured by comparing its densitometry values with that of GAPDH in the same blot.

Dual luciferase reporter assay

HTR8/SVneo cells were seeded into 24-well plates, and transfected with 80 ng of pMIR-REPORT plasmid construct, 8 ng of pRL-TK vector encoding renilla luciferase and 50 nM of miRNA mimics. The cells were cultured for 48 h after transfection, and activities of firefly and renilla luciferase were measured using Dual-Glo Luciferase Assay System (Promega) according to the manufacturer's instructions. Triplicates in each group were included.

Transwell insert invasion assay

HTR8/SVneo cells were plated into 6-well plates and transfected with 50 nM of miR-195 mimics or negative control, together with 2 μg of pActRIIB or pcDNA4.0. Forty-eight hours later, cells were trypsinized and seeded into 24-well fitted Transwell inserts (8 μm pore size; Costar, Cambridge, Massachusetts, San Diego, California, USA) which were precoated with matrigel (BD Biosciences, USA). RPMI 1640 culture media with 1% FBS were added into the insert,

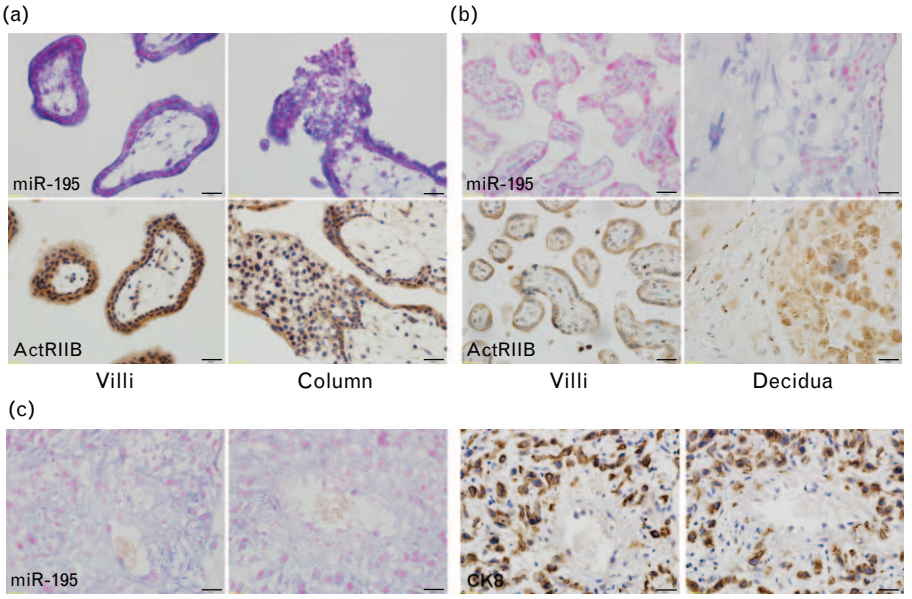

FIGURE 1 Localization of activin receptor type-2B and miR-195 in human placenta. (a) In-situ hybridization for miR-195 and immunohistochemical staining for activin receptor type-2B in human placental villi at early pregnancy. (b) In-situ hybridization for miR-195 and immunohistochemical staining for activin receptor type-2B in human term placenta. (c) Localization of miR-195 and CK8 in human decidual tissue at early pregnancy. The bar indicates 20 μm.

and the lower chambers were loaded with RPMI 1640 medium with 10% FBS. Twenty-eight hours later, the cells were fixed with glutaraldehyde and stained with hematoxylin. Noninvaded cells on the upper surface of the insert membrane were removed by cotton swab, whereas the invaded cells at the lower surface were counted under light microscope. The invasion index was presented as the percentage of invaded cell number compared with the corresponding control.

Statistical analysis

All statistical analyses were conducted by SPSS 17.0 software, and the data of western blotting, RT-PCR, dual-luciferase assay and invasion analysis were reported as mean ± SEM, according to the results of at least three repeated experiments. Comparisons between groups were performed using one-way analysis of variance, and P less than 0.05 was considered significant difference.

RESULTS

Colocalization of miR-195 and activin receptor type-2B in human placental trophoblast cells

The distribution of ActRIIB and miR-195 in human placenta was detected by immunohistochemistry and in-situ hybridization. At early gestation, the in-situ hybridization of miR-195 was observed in villous cytotrophoblast, syncytiotrophoblast and invasive trophoblast cell column (Fig. 1a), as well as the invasive extravillous trophoblasts within the decidual stroma (Fig. 1c). Staining for CK8 was performed in the adjacent section to identify the epithelial trophoblasts. The distribution of miR-195 in late gestational placenta was principally observed in various trophoblast cell types (Fig. 1b). Immunoreactivity of ActRIIB demonstrated similar expression pattern with that of miR-195, being primarily in various subtypes of trophoblasts (Fig. 1a and b).

Expressions of activin receptor type-2B in preeclamptic placenta

We previously found the downregulation of miR-195 in preeclamptic placentas, primarily in the chorionic plate but not the basal plate [20]. Here we measured the expression of ActRIIB with western blotting, and found the protein level of ActRIIB was significantly higher in chorionic plate of preeclamptic placentas than that in the normal pregnant ones (Fig. 2a and b). The differential expression of ActRIIB was not observed in the basal plate. The mRNA expression of ActRIIB had little difference between the normal and preeclamptic placentas (Fig. 2c).

Validation of activin receptor type-2B as a direct target of miR-195 in human trophoblast cells

The targeting of ActRIIB by miR-195 was predicted using bioinformatics methods. Here we validated it in human trophoblast cell line, HTR8/SVneo. As shown in Fig. 3a, transfection of double-stranded miR-195 mimics into HTR8/SVneo cells led to 180-fold increase in miR-195 expression. The mRNA level of ActRIIB in these cells was significantly downregulated to 73% of that in the negative control cells that were transfected with scramble small RNA (Fig. 3b), whereas the protein expression of ActRIIB was decreased to 70% (Fig. 3c).

Furthermore, we validated whether ActRIIB was a direct target of miR-195 using dual luciferase reporter assay. We generated wild type of luciferase reporter constructs ActRIIB-BD1, ActRIIB-BD2 and ActRIIB-BD3 which containing each of the three predicted binding sites for miR-195 in 3′UTR of *ACVR2B* (gene encoding ActRIIB) and MUT plasmid carrying each mutated seed sequence. MiR-195 mimics were transfected into HTR8/SVneo cells together with one of the generated reporter plasmid, whereas

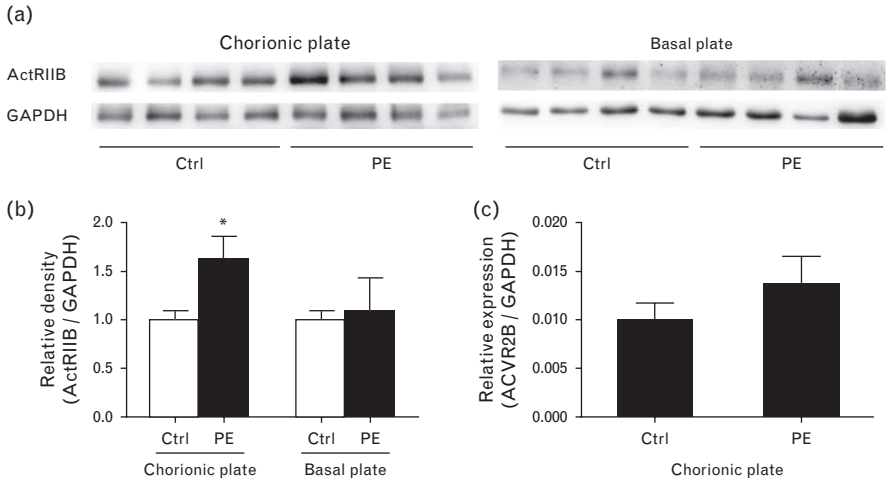

FIGURE 2 Protein expression of activin receptor type-2B was upregulated in preeclamptic placenta. (a) and (b) Protein levels of activin receptor type-2B in normal and preeclamptic placentas were measured by western blotting. The relative expression was normalized by GAPDH. (c) Real-time PCR to reveal the mRNA expression of activin receptor type-2B in the chorionic plate of the placentas derived from normal and preeclamptic pregnancies.

pRL-TK control plasmid encoding a renilla luciferase was co-transfected as internal reference. Compared with scramble control (negative control), miR-195 mimics significantly reduced the relative luciferase activity of wild type of ActRIIB-BD1, ActRIIB-BD2 or ActRIIB-BD3 constructs by about 50–70% (Fig. 3d). The effect of miR-195 mimics on the relative luciferase activity of mutated BD1 or BD3 was disappeared, whereas the relative luciferase activity of mutated BD2 remained to be depressed by miR-195. These data indicated ActRIIB as a direct target of miR-195 in trophoblast cells and demonstrated the 109–115 nucleotides and 9650–9659 nucleotides in *ACVR2B* 3′UTR UTR regions as the binding sites for miR-195.

Overexpression of activin receptor type-2B impeded the invasion-promoting effect of miR-195 on trophoblast cells

Our previous report demonstrated that miR-195 could enhance the invasive ability of trophoblast cells via suppressing ActRIIA expression [19]. As ActRIIB and ActRIIA are common type II receptors for activin A, we tested

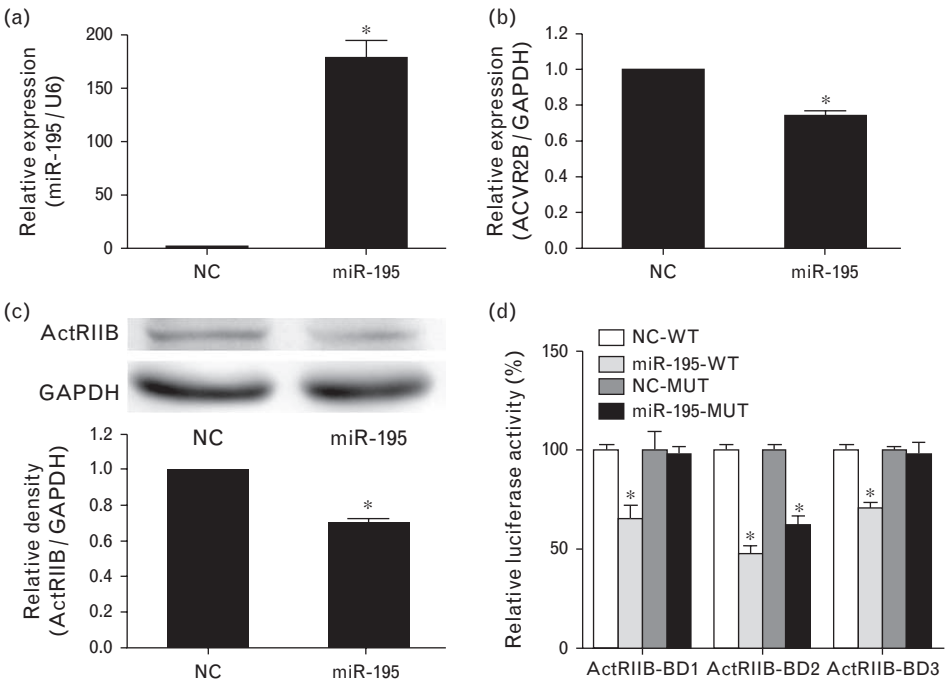

FIGURE 3 Activin receptor type-2B was the direct target of miR-195. (a) and (b) real-time PCR analysis of miR-195 and activin receptor type-2B mRNA in HTR8/SVneo cells transfected with miR-195 mimics or negative control after 48 h. The results were normalized by U6 or GAPDH. (c) Protein levels of activin receptor type-2B in HTR8/SVneo cells transfected with miR-195 mimics or negative control after 48 h were measured by western blotting. The data was normalized by GAPDH. (d) Luciferase activity in HTR8/SVneo cells 48 h after cotransfection of report plasmid, pRL-TK and miR-195 or negative control. For each predicted binding site, mutation plasmid had the same sequence with wild type plasmid except for 3-nucleotide mutation in the seed sequences.

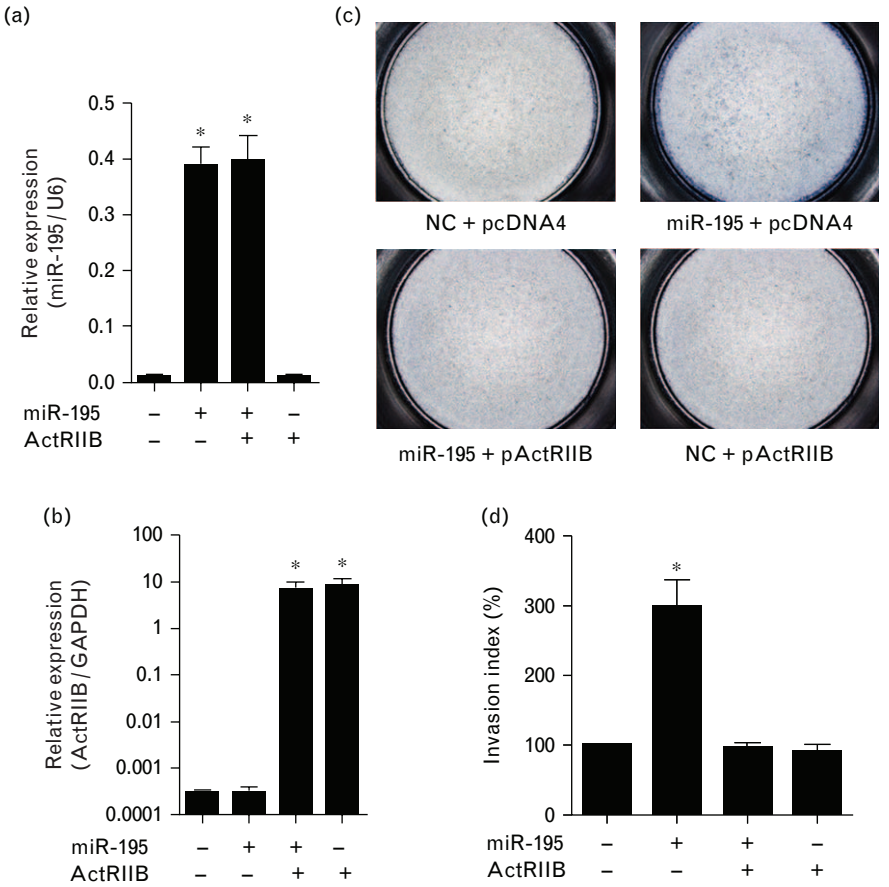

FIGURE 4 Activin receptor type-2B abolished the invasion-promoting effect of miR-195 on HTR8/SVneo cells. (a) and (b) Transfection efficiency detection. The overexpression of miR-195 (a) and activin receptor type-2B mRNA (b) was measured by real-time PCR after miR-195 and pActRIIB transfection. (c) and (d) Transwell insert invasion analysis. The invaded cells in (c) were counted and statistically represented in (d).

whether ActRIIB could also mediate the effect of miR-195 on cell invasion. The plasmid carrying the full length of ActRIIB cDNA was constructed and named as pActRIIB. MiR-195 mimics was transfected with or without pActRIIB into HTR8/SVneo cells, and invasion assay were performed 48 h after transfection. The transfection efficiency was determined by RT-PCR for miR-195 or ActRIIB (Fig. 4a and b). As shown in Fig. 4c and d, miR-195 could evidently promote cell invasion. Overexpression of ActRIIB alone had no effect on invasive behavior of HTR8/SVneo cells, whereas it could largely abrogate the invasion-promoting effect of miR-195 mimics. The data demonstrated that the function of miR-195 on trophoblast cell invasion was mediated, at least in part, by ActRIIB.

DISCUSSION

In recent years, more and more differentially expressed miRNAs have been identified in the placentas from preeclamptic patients, and functional studies have demonstrated multiple roles of miRNAs in modulating trophoblast cell behaviors. For instance, miR-210, the most accepted upregulated miRNA in preeclamptic placenta, could be stimulated by hypoxia [29] and may contribute to preeclampsia through inhibiting oxygen consumption [30] and cell invasion [31,32] in trophoblastic cells. MiR-34a could target Notch1 and Jagged1 to inhibit cell invasion [33]. In JEG3 cells, MiR-152 had no change on cell invasion, but promoted NK cell-mediated cytolysis [34]. Accumulating evidences are indicating the important roles of miRNAs in placental development and the occurrence of preeclampsia.

MiR-195 is one of the downregulated miRNAs in preeclamptic placenta. Function of this small RNA has been reported in various kinds of tumor. For instance, it induced cell cycle arrest and inhibited the proliferation in cervical cancer cells via cyclin D1 [35]. By negatively regulating CCND1, miR-195 restrained the invasion and migration of osteosarcomal cells [36]. In hepatocellular carcinoma, miR-195 reduced cell growth and induced apoptosis via modulating Wnt3a [37] and SRC-3 [38]. Cell proliferation, migration and invasion could be suppressed by miR-195 through CHEK1 [39], MYB [40], HDGF [41] and IFG1R [42] in nonsmall cell lung cancer. Human placenta trophoblast cells share properties of active proliferation and invasion with tumor cells. However, these tumor-like behaviors in trophoblast cells are tightly organized in unique temporal and spatial manners during pregnancy, implying different microenvironment and regulatory network between trophoblast cells and tumor cells. Unlike the report in tumor cells, miR-195 substantially improved cell invasion, but had no influence on cell growth in trophoblast cells, and its

invasion-regulating effect was not in association with CCND1 expression [19]. These observations indicated the unique working mechanism of this small molecule in placental trophoblast cells.

In the present study, ActRIIB was proved to be a functional target of miR-195, which participated in the invasion-regulating effect of the small RNA. The similar expression patterns of miR-195 and ActRIIB in human placenta trophoblasts as well as their opposite changing property in preeclamptic placentas indicated their physiological association. Results from the luciferase report assay and site mutation in the putative binding sites demonstrated the direct binding of miR-195 to the 3′UTR region of *ACVR2B* gene in human trophoblast cells. In addition, ActRIIB could significantly abolish the promoting effect of miR-195 on trophoblast cell invasion. These evidences demonstrated that miR-195 could regulate trophoblast cell invasion by targeting ActRIIB.

We previously identified ActRIIA as a target of miR-195 in human trophoblast cells. Both ActRIIA and ActRIIB are transmembrane serine/threonine kinase receptors for activin A and nodal, two members of transforming growth factor beta (TGF-β) superfamily. Ligands of this large family initiate signal transduction by interacting with varied combination of type I and type II receptors to form a multimeric receptor ligand complex [43,44]. The type II receptor then recruits and cross-phosphorylates specific type I receptor and triggers a downstream signaling activation [45–48]. TGF-β signaling participates in numerous physiological and pathological events involving different cell types. Aberrant expressions of many members in this superfamily have been demonstrated in preeclampsia pregnancies. For instance, productions of activin A and nodal, as well as their receptors and downstream Smad2, were significantly enhanced in preeclamptic placentas, which may lead to over activated signaling and excessive cell apoptosis and less invasion [21–23]. Activin A can bind to ActRIIA or ActRIIB, whereas Nodal only binds to ActRIIB. In human placenta, activin A primarily regulates trophoblast cell invasion [49] and Nodal promotes cell apoptosis [50]. High level of activin A may enhance Nodal expression and facilitate Nodal-induced cell apoptosis. We found miR-195 basically increased cell invasiveness, but did not influence cell growth or viability. Therefore, we propose that this small RNA may control activin A signaling by targeting ActRIIA and ActRIIB, but may not intervene Nodal signaling in which ActRIIB is involved. However, our evidences regarding the role of miR-195 were so far obtained in an in-vitro trophoblast cell line, which may not well reflect the complex in-vivo conditions. On the other hand, there is a substantial time gap between early trophoblast invasion and later development of preeclampsia, which must be taken into account when exploring the pathogenesis of the pregnancy disorder. Considering that the differentiation status and properties of trophoblast cells highly vary along gestation, the physiological roles of miR-195 should be tightly associated with placental developmental stages. Further evidences, especially the results of in-vivo study, are needed in this issue.

The fact that miRNAs in one gene cluster usually work in similar ways is notable. MiR-195 belongs to miR-15/107 family which consists of several paralogous and evolutionarily conserved miRNAs, miR-15a, miR-15b, miR-16, miR-103, miR-107, miR-195, miR-424, miR-497, miR-503 and miR-646 [51]. They share an AGCAGC sequence near the 5′-end, which means they may have overlapping target genes [17,18]. As a matter of fact, miR-195, miR-16, miR-103 and miR-107 were reported to share GRN as the same targets in H4 glioneuronal cells [16]. MiR-195 and miR-497 also shared the same direct targets including CCNE1, CCND3, CDC25A, CDK4 and BTRC in HCC cells [52]. In the miR-15/107 family, some members have been demonstrated to express differentially in preeclamptic placenta. For instance, the level of miR-16 and miR-103 were increased in preeclamptic placentas compared with normal ones [53,54], whereas miR-15b were downregulated [55]. It is yet to be determined whether miR-195 and these differential miRNAs work in a synergistic, antagonistic or redundant way during the placenta development and how they participate in the pathological process of preeclampsia.

Another interesting point is the reason for miR-195 downregulation in preeclamptic placenta. So far, the expression regulation of miR-195 in human placenta remains largely unknown. It was reported that the expression of miR-195 was inhibited by NF-κB in myoblasts and skeletal muscle tissue [56]. The NF-κB signaling has been found to be involved in the regulation of trophoblast behaviors, and the expression and activity of NF-κB in preeclamptic placentas were increased compared with the normal controls [57,58]. As a transcription factor involved in various cellular events, NF-κB could be activated by TNF-α [58] and angiotensin II type 1 receptor autoantibodies [59], which were reported to be upregulated in preeclamptic patients. It's therefore likely that over-activated NF-κB signaling might be involved in the reduced miR-195 in preeclamptic placenta. Interestingly, miR-195 could directly target several downstream effectors of NF-κB like IKKα and TAB3 in hepatocellular carcinoma [60], and activin A could increase the nuclear translocation of NF-κB [61]. It seems indicating a regulatory loop among activin A, miR-195 and NF-κB signaling. Further studies based on these evidence are needed to eventually elaborate the reason of miR-195 downregulation in preeclampsia placenta.

In general, the present study demonstrated that the elevated level of ActRIIB in preeclamptic placenta was the result of the repressive regulation by miR-195 that was downregulated in the patients, and ActRIIB could mediate the modulation of trophoblast cell invasion by miR-195 in human trophoblast cells. Taking this study and the previous reports together, the aberrant expression of miR-195 may account for the overactivation of Activin A/Nodal signaling in preeclamptic placenta by targeting the two type II receptors. Further investigation, especially the in-vivo studies, will deepen our understanding on the fine-tune regulation of trophoblast behaviors by miRNAs, which may contribute to revealing the cause of this complex disorder.

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Authors' contribution: H.W. and H.W. performed the experiments and drafting the manuscript; M.L. and Y.B. help to performed immunohistochemistry and in-situ hybridization; Y.-X.L. and L.J. contributed in sample collection; C.P. participated partly in study design. Y.Y. and Y.-L.W. designed the study, revised the manuscript and coordinated the working team. All authors approved for the publication of the draft.

Conflicts of interest

There are no conflicts of interest.

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Reviewers' Summary Evaluations

Reviewer 1

This complex piece of molecular biology, which many readers may find heavy going, is of significance for three main reasons. First it further develops the concept that micro RNAs modulate gene expression. Second, it links miR-195 selectively with ActRIIB – a trophoblastic cell surface receptor for transforming growth factor beta. But perhaps most importantly it provides yet one further piece of evidence to suggest that the origins of preeclamplia lie in dysregulation of the process of trophoblastic invasion in early pregnancy. The jigsaw pieces are slowly coming together.

Reviewer 2

Strengths: Translational in-vitro experiment, covering not only expression of biomarkers, but also the functional effects on expression and cell function.

Weakness: Third trimester placentas may express something else than first trimester placentas. Therefore, the supposed relation between shallow trophoblast invasion and miR195 is plausible in vitro, but not proven *in vivo*.