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miR-125b-1-3p inhibits trophoblast cell invasion by targeting sphingosine-1-phosphate receptor 1 in preeclampsia

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ABSTRACT

Preeclampsia (PE) is the leading cause of maternal and perinatal mortality and morbidity. Understanding the molecular mechanisms underlying placentation facilitates the development of better intervention of this disease. MicroRNAs are strongly implicated in the pathogenesis of this syndrome. In current study, we found that miR-125b-1-3p was elevated in placentas derived from preeclampsia patients. Transfection of miR-125b-1-3p mimics significantly inhibited the invasiveness of human trophoblast cells, whereas miR-125b-1-3p inhibitor enhanced trophoblast cell invasion. Luciferase assays identified that S1PR1 was a novel direct target of miR-125b-1-3p in the placenta. Overexpression of S1PR1 could reverse the inhibitory effect of miR-125b-1-3p on the invasion of trophoblast cells. These findings suggested that abnormal expression of miR-125b-1-3p might contribute to the pathogenesis of preeclampsia.

1. Introduction

Preeclampsia (PE), a pregnancy-specific syndrome characterized by *de-novo* onset of hypertension and proteinuria after 20 weeks of gestation, is the leading cause of maternal and perinatal mortality and morbidity [1]. It affects about 2–8% of pregnancies worldwide [2]. Although the etiology of preeclampsia is uncertain, abnormal placentation is believed to play an essential role in the development of this syndrome [3–5]. Poor trophoblast migration/invasion associated with impaired remodeling of the spiral arteries have been linked to preeclampsia [6–9]. Nevertheless, an understanding of the molecular mechanisms underlying the development of preeclampsia remains largely unknown.

MicroRNA (miRNA) are a class of 22- to 24-nucleotide small noncoding RNAs that regulate the expression of almost one third of all the human genes and involve in all fundamental cell events [10]. MicroRNA could silence gene expression by seed sequence pairing with the 3′-untranslated region (UTR) of target mRNAs [11], leading to repressed translation or induced mRNA cleavage of the target genes [12]. Several previously studies found about 600 miRNAs expressed in human placenta and profiled genome-wide expression of miRNAs in normal and preeclamptic placentas [13–19]. Although little is known about what roles of miRNAs play in human placenta development and functions, recent reports suggested that preeclampsia-associated aberrant expressed miRNAs play critical roles in human placentas development and function via regulating specific genes with various cell functions [20–29].

miR-125b-1-3p is one of the earliest discovered miRNAs that have been emphasized the importance in disease development and suppression on cancer and immunity [30]. Our unpublished microarray data indicated that miR-125b-1-3p was upregulated in placentas of preeclampsia. To date, the functions and pathways of miR-125b-1-3p in trophoblast cells remain unclear. These inspired us to investigate whether miR-125b-1-3p takes any roles in trophoblast cells.

In this study, we aim to elucidate the functional roles of miR-125b-1-3p in human trophoblast cells. We examined relative expression of miR-125b-1-3p and S1PR1 in the placenta derived from PE, and further studied the effect and regulatory mechanisms of miR-125b-1-3p on cell invasion. These findings emphasized the essential role of miR-125b-1-3p in the placentation and provide new insight into the pathology of the syndrome.

2. Materials and methods

2.1. Patients and samples collection

Placenta samples were collected from pregnant women who underwent perinatal medical care at the Department of Obstetrics

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and Gynecology, Affiliated Hospital of Weifang Medical University, China. Severe preeclampsia (SPE) was defined according to the definition in Williams Obstetrics (23rd edition). Briefly, patients had new-onset systolic blood pressure (SBP) ⩾ 160 mmHg or diastolic blood pressure (DBP) ⩾ 110 mmHg on two or more occasions, accompanying severe proteinuria (⩾3+ or ⩾2 g/24 h) at ⩾ 20 week of gestation. The blood pressure of all patients returned to normal, and symptoms of proteinuria disappeared 6 weeks postpartum. For the control group, women with renal disease, cardiovascular disease, transient hypertension in pregnancy, gestational diabetes mellitus, hepatitis, any evidence of spontaneous abortion, intrauterine fetal death, fetal chromosomal or other pregnancy complications were excluded from this study. The samples at the chorionic plate and basal plate were separately taken from the central part of placenta within 1 h of cesarean birth. Placenta samples were stored in liquid nitrogen until RNA extraction. Informed consent was obtained from women before sample collection. The Ethics Committee of Weifang Medical University approved the consent forms and the experiment to utilize the samples.

We collected data from 13 pregnancies complicated by SPE and 26 gestation-week-matched pregnant healthy controls. The detailed clinical characteristics of study subjects were summarized in Table 1.

2.2. Cell culture and transfection

The immortalized human trophoblast cell line, HTR8/SVneo cells, was a kind gift provided by Dr. Charles H. Graham at Queen's University, Canada [31]. Cells were cultured in RPMI1640 medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS), 100 IU/ml penicillin and 100 μg/ml streptomycin, and incubated under 5% CO_2 at 37 °C. All medium, FBS and enzymes were obtained from Invitrogen unless otherwise noted.

HTR8/SVneo cells were transfected with miR-125b-1-3p mimics, miR-125b-1-3p inhibitor or the scramble control (GenePharma, Shanghai, China) using Lipofectamine RNAiMAZ (Invitrogen, CA, USA). pcDNA4-S1PR1 (pS1PR1) or pcDNA 4 (pDNA4) using Lipofectamine 2000 reagent (Invitrogen, CA, USA). Cells were harvested for further assay at 48 h after transfection.

2.3. RNA extraction and real-time qPCR

Total RNA, including small RNA, was extracted from tissues and cells using TRIzol® reagent (Invitrogen, CA, USA) according to the manufacture's protocol. Reverse transcription was performed with oligo-dT or specific microRNA stem loop RT primers. To determine expression of miR-125b-1-3p, Real-time qPCR was performed using an miRcute miRNA qPCR Detection kit (Tiangen, Beijing, China) with U6 used as an endogenous control. To examine levels of S1PR1 expression, real-time qPCR was performed using the SYBR Premix Ex Taq kit (Applied Biosystems, CA, USA) according to the manufacturer's instructions, taking GAPDH as internal control. Relative expression levels of miR-125b-1-3p and S1PR1 were determined as previously described [32].

Table 1
Clinical characteristics of patients enrolled in the study.

	Normal (N = 25)	SPE (N = 13)	p-Value
Age (y)	29.56 ± 0.48	29.58 ± 0.68	0.9166
BMI (kg/m^2)	22.88 ± 0.42	22.62 ± 0.83	0.2069
SBP (mm Hg)	112.6 ± 1.9	161.2 ± 1.5	<0.0001
DBP (mm Hg)	77.56 ± 0.9	104.3 ± 1.0	<0.0001
Proteinuria (g/24 h)	NA	4.20 ± 0.28	NA
50 g GCT (mM)	6.51 ± 0.23	6.63 ± 0.36	0.2183
Nulliparous (%)	83.3	89.7	NA
Gestation day at delivery (d)	270.2 ± 0.8	240.3 ± 2.0	<0.0001
Infant birth weight (g)	3498 ± 121	2618 ± 178	<0.0001

Data are shown as mean ± SEM, and differences between normal and SPE patients were analyzed with Student-t test. BMI, body mass index; GCT, glucose challenge test; NA, not available.

2.4. Western blotting

Briefly, protein extracts were prepared using RIPA as previously reported [20]. Protein lysates were subjected to 10% SDS–PAGE and transferred to nitrocellulose membrane. The membranes were blocked with 5% defatted milk in PBST for 2 h, and were then incubated with primary antibodies overnight at 4 °C. The primary antibodies used included mouse anti-human S1PR1 (Santa Cruz, USA) and mouse anti-human GAPDH (Ambion, Austin, Texas, USA). Signals were detected using an Enhanced Chemiluminescence Plus kit (Thermo Scientific, Rockford, USA) and visualized after exposure to a Kodak film. The films were scanned and band intensities were analyzed by Image J (NIH, USA). Relative densities of S1PR1 were determined by normalization with GAPDH of the same blot.

2.5. In vitro tranwell insert invasion assay

In vitro invasion of HTR8/SVneo cells was performed in Matrigel-coated (Becton Dickinson; Franklin Lakes, NJ) transwell inserts (Costar, Cambridge, MA) containing polycarbonate filters with 8-μm pores as previously described [33]. Briefly, 1×10^5 cells per well were plated into the upper chamber in 200 μl RPMI 1640 medium without FBS. 800 μl of medium with 10% FBS was placed into the lower well of the chamber. After 24 h, the invaded cells were washed with PBS, fixed in methanol for 10 min, and stained with hematoxylin. The number of invaded cells was counted under a light microscope in 15 random-selected non-overlapping fields from each chamber at a magnification of 200×. Average cell numbers in each field were used for statistical analyses. All experiments were conducted in triplicate and the invasion index was expressed as the percentage of invaded cell number compared with the corresponding control.

2.6. Luciferase assays

The sequence in the 3′-UTR region of human S1PR1 gene targeted by miR-125b-1-3p was predicted with microRNA.org (http://www.microrna.org). 3′-UTR region of S1PR1 and a sequence with mutations of two nucleotides in the miR-125b-1-3p target site were cloned into pGL3 promoter vector to generate the recombinant constructs, pmir-S1PR1 and pmir-S1PR1-M respectively. For the luciferase assay, HTR8/SVneo cells were co-transfected with pmir-S1PR1 or pmir-S1PR1-M and miR-125b-1-3p mimics or scramble controls (NC). Luciferase activity was analyzed using the Dual-luciferase Reporter Assay System according to the manufacturer's instructions (Promega, WI, USA) at 48 h post transfection.

2.7. Statistical analysis

All experiments were repeated ⩾3 times independently. Results are presented as means ± SEM. Statistical comparisons were performed using Student t test and One- or Two-way ANOVA using SPSS statistics software (IBM, NY, USA), with $p < 0.05$ considered as significant. All cell experiments were performed in triplicates.

3. Results

3.1. Expressions of miR-125b-1-3p and S1PR1 were negatively correlated in preeclamptic patients

We examined expression levels of miR-125b-1-3p and S1PR1 between normal and severe preeclamptic placentas employing Real-time qPCR. Our data showed (Fig. 1A) that miR-125b-1-3p was significantly elevated in the basal plate of severe preeclamptic placentas than in that of the normal control placentas. However, we found no differences in its level in the chorionic plate between normal and severe preeclamptic placentas, as shown in Fig. 1B.

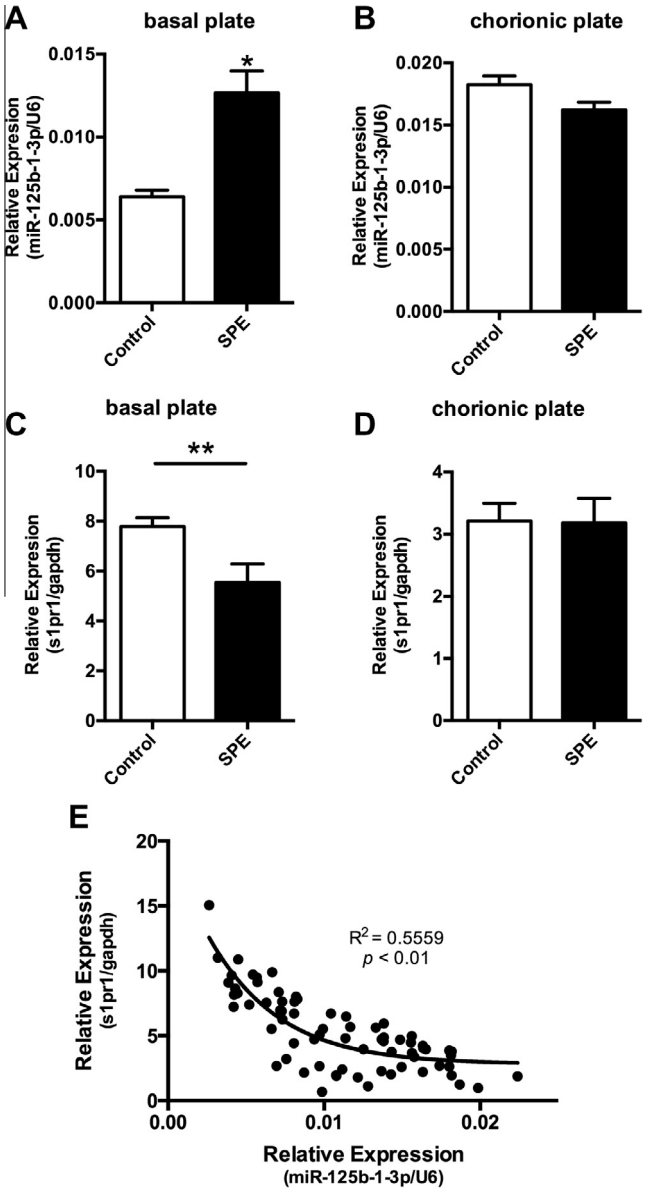

Fig. 1. Expression of miR-125b-1-3p and S1PR1 in placentas between severe preeclampsia patients (SPE) and their gestational week-matched normal control pregnancies (NC), as revealed by Real-time qPCR. The expression of miR-125b-1-3p was measured in the basal plate (A) and chorionic plate (B) of the placenta derived from preeclampsia patients and normal controls separately. Relative expression of miR-125b-1-3p was normalized by U6. Expression of S1PR1 was tested in the basal plate (C) and chorionic plate (D) of the placenta derived from preeclampsia patients and normal controls. Association of expression of S1PR1 and expression of miR-125b-1-3p in the placenta was analyzed using SPSS (E). Data was presented as mean ± SEM. Statistical comparison between SPE group and NC group was performed using Student-*t* test, with $p < 0.05$ considered as significant. $^{*}p < 0.05$.

We employed bioinformatics approaches to predict its target genes, including TargetScan, miRBase and miRNAMap. Sphingosine-1-phosphate receptor 1 (S1PR1) is one of the commonly predicted targets of miR-125b-1-3p. Then we tested relative expression of S1PR1 in the placenta. Expression of S1PR1 was significantly reduced in the basal plate of severe preeclamptic placentas than in that of the normal control placentas (Fig. 1C), whereas no difference in its level was observed in the chorionic plate (Fig. 1D). This result is interesting that expression of S1PR1 is negatively related to expression of miR-125b-1-3p in the placenta (Fig. 1E).

3.2. miR-125b-1-3p inhibited trophoblast invasion

To evaluate whether miR-125b-1-3p plays a role in placentation, we predicted target genes of miR-125b-1-3p using TargetScan, miRBase and miRNAMap, and then investigated the biological functions of the potential target genes using Gene Ontology. We observed genes enriched in important functions including invasion.

We transfected HTR8/SVneo cells with miR-125b-1-3p mimics or miR-125b-1-3p inhibitor and the corresponding scramble control. As shown in Fig. 2A and B, transfection of mimics or inhibitors for miR-125b-1-3p could significantly increase or reduce relative expression of miR-125b-1-3p in HTR8/SVneo cells.

As shown in Fig. 2C and D, in vitro transwell insert results showed that miR-125b-1-3p mimics transfection significantly increased the level of miR-125b-1-3p, thus inhibited the invasion of HTR8/SVneo cells compared with the scramble controls. In contrast, the invasion of cells that were transfected with miR-125b-1-3p inhibitor to reduce endogenous miR-125b-1-3p levels was enhanced. We also transfected HTR8/SVneo cells with S1PR1 siRNA to knockdown expression of S1PR1, efficiency of knockdown was reported in our previously study. We found that knockdown of S1PR1 was significantly reduced invasion of HTR8/SVneo cells. Together these results clearly suggested that miR-125b-1-3p significantly inhibited invasion of trophoblast cells.

3.3. miR-125b-1-3p directly targets S1PR1 in the placenta

We observed that both mRNA and protein levels of S1PR1 were significantly reduced in HTR8/SVneo cells transfected with miR-125b-1-3p mimics than the scramble controls in vitro, as shown in Fig. 3A and B, whereas inhibition of miR-125b-1-3p enhanced expression of S1PR1 (Fig. 3C and D).

The seed sequence of miR-125b-1-3p is complementary to the sequence of the 3′-UTR in S1PR1 mRNA. To verify the putative binding site of miR-125b-1-3p in the 3′-UTR in S1PR1 gene, we constructed two luciferase reporter vectors, one containing wild-type 3′-UTR in human S1PR1 mRNA downstream of firefly luciferase reporter gene (pmir-S1PR1) and another containing mutant 3′-UTR in the S1PR1 gene to generate mutant luciferase reporter vector (pmir-S1PR1-M) (Fig. 3E). As shown in Fig. 3F, miR-125b-1-3p mimics significantly reduced the relative luciferase activity of the pmir-S1PR1 vector compared with the scramble control but did not affect the luciferase activity of the pmir-S1PR1-M vector. These results demonstrated that S1PR1 could be directly targeted by miR-125b-1-3p in human placenta.

3.4. Overexpression of S1PR1 reversed the invasion inhibiting effect of miR-125b-1-3p in HTR8/SVneo cells

To figure out whether S1PR1 directly involved in the invasion-inhibiting effect of miR-125b-1-3p, we transfected HTR8/SVneo cells with miR-125b-1-3p together with a S1PR1 overexpressing vector (pS1PR1) to perform the rescue experiment. As shown in

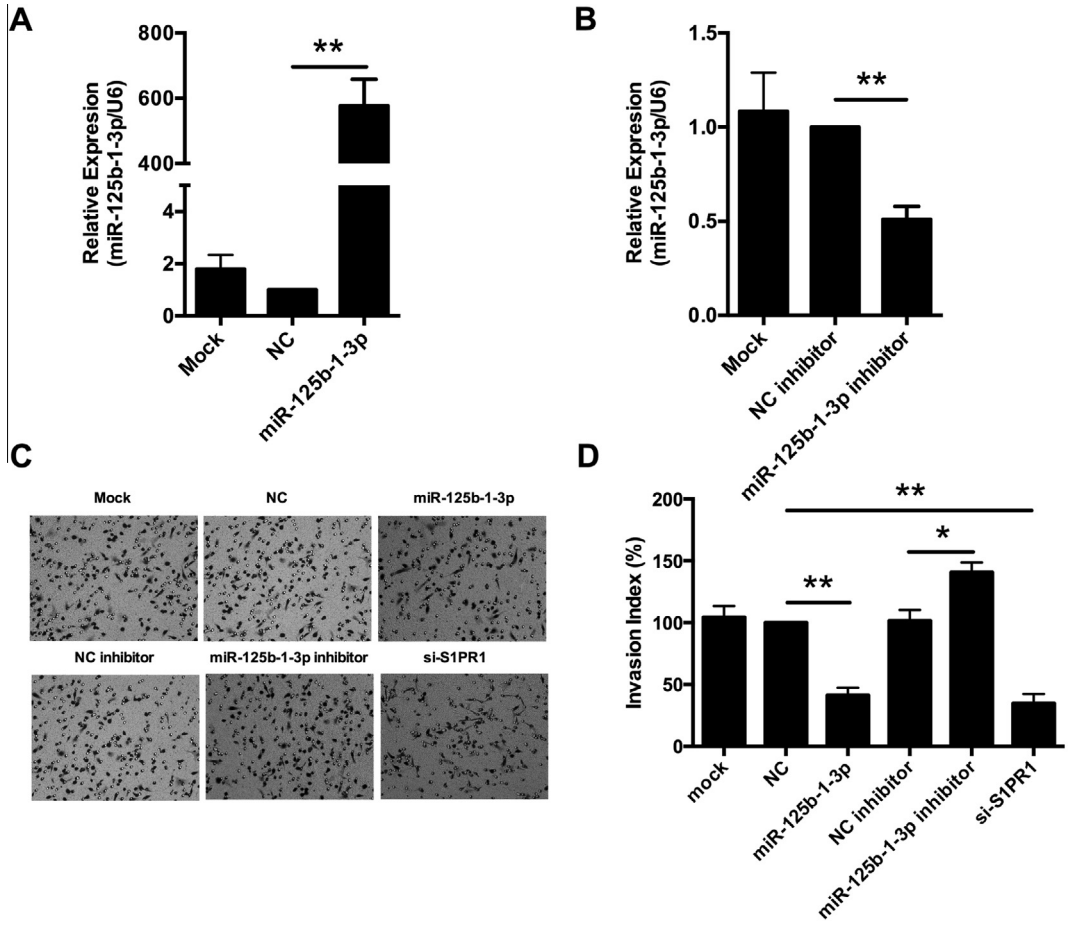

Fig. 2. Effect of miR-125b-1-3p on the invasion in HTR8/SVneo cells. (A) The expression of miR-125b-1-3p in HTR8/SVneo cells transfected with miR-125b-1-3p mimics (miR-125b-1-3p), scramble control (NC) or mock was revealed by real-time qPCR. (B) The expression of miR-125b-1-3p in HTR8/SVneo cells transfected with miR-125b-1-3p inhibitor, scramble inhibitor (NC inhibitor) or mock was revealed by real-time qPCR. Relative expression of miR-125b-1-3p was normalized by U6. The invasion of HTR8/SVneo cells transfected with miR-125b-1-3p, miR-125b-1-3p inhibitor and S1PR1 siRNA was tested using In vitro Transwell insert invasion assay, as shown the typical field in (C) and relative invasion index in (D). $N = 3$ performed in triplicates and data was presented as mean ± SEM. Statistical comparison between miR-125b-1-3p mimics (or inhibitor) and the corresponding NC was performed using Student-t test, with $p < 0.05$ considered as significant. $^{*}p < 0.05$, $^{**}p < 0.01$.

Fig. 4A and B, the invasion inhibiting effect of miR-125b-1-3p was rescued by overexpression of S1PR1. These results suggested that S1PR1 mediated the invasion-inhibiting effect of miR-125b-1-3p in trophoblast cells.

4. Discussion

Although it has been investigated for decades, the molecular mechanisms underlying the pathogenesis of preeclampsia remains largely unknown. It has been widely accepted that single molecule or signaling pathways can hardly account for the development of the complex syndrome, preeclampsia. miRNAs have been indicated to play essential roles in the development of many organs, including the placenta, by targeting a large amount of genes and involving in various biological processes. Therefore, the investigations of impaired miRNAs in preeclampsia placentas and genes targeted by those miRNAs are likely to be novel steps into understanding the development of the pregnancy-specific syndrome.

Several reports have revealed that expression profiles of miRNAs in the placenta of preeclampsia are deregulated compared with normal controls [13–19,21]. Some miRNAs are reported to be deregulated in the placenta of preeclampsia, further studies revealed that they were related to angiogenesis, inflammation and trophoblast cell migration/invasion [20,22–29]. Thus far, it remains to be clarified that how the deregulated miRNAs involve in the development of preeclampsia.

In current study, we observed aberrant miR-125b-1-3p expression in the placenta derived from preeclampsia patients. miR-125b-1-3p was significantly up-regulated in the basal plate, but not chorionic plate, of severe preeclamptic placentas than in that of the normal control placentas. This may due to the position where samples were taken in the placenta as previously reported [21].

Functional studies of miR-125b-1-3p have not been reported yet. We first predict its target genes using programs, including TargetScan, miRBase and miRNAMap. Using these bioinformatics approaches, we found about 580 commonly predicted miR-125b-1-3p target genes. We further analyzed the biological functions of these genes using Gene Ontology as previously reported [34,35]. We found that gene enriched in some important cellular functions, including proliferation, apoptosis and migration/invasion. It is generally accepted that poor trophoblast invasion and deficient trophoblast-mediated remodeling of the spiral arteries results in the onset of preeclampsia. Therefore, we examined the effect of miR-125b-1-3p on the invasion of trophoblast cells. Our results showed that transfection of miR-125b-1-3p mimics inhibit the invasion of HTR8/SVneo cells, whereas transfection of

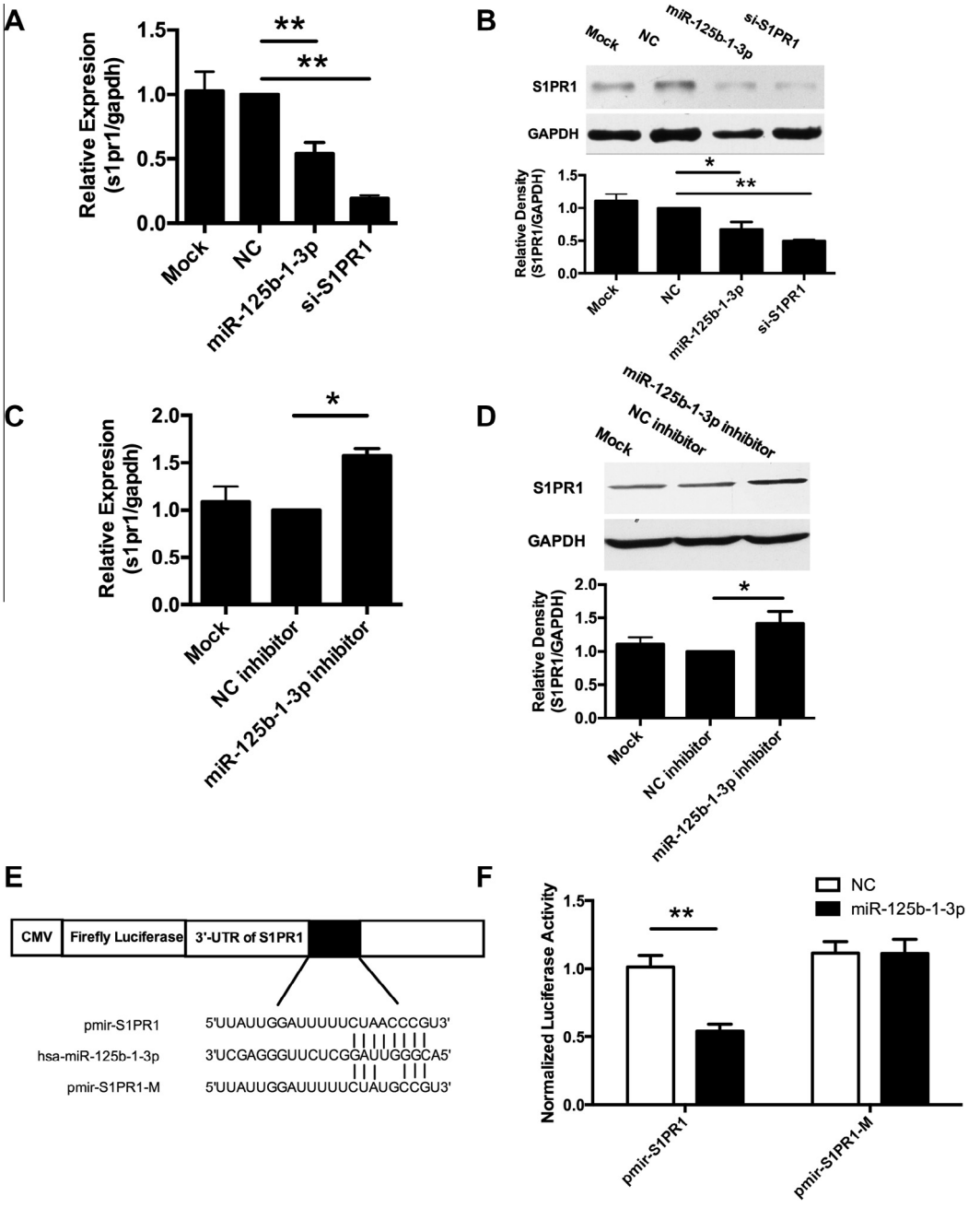

Fig. 3. Validation of sphingosine-1-phosphate receptor 1 (S1PR1) as the direct target of miR-125b-1-3p. Both mRNA (A) and protein (B) levels of S1PR1 in HTR8/SVneo cells transfected with scramble control (NC) and miR-125b-1-3p mimics (miR-125b-1-3p) were revealed by real-time qPCR and Western blot. Both mRNA (C) and protein (D) levels of S1PR1 in HTR8/SVneo cells transfected with scramble control inhibitor (NC inhibitor) and miR-125b-1-3p inhibitor (miR-125b-1-3p inhibitor) were revealed by real-time qPCR and Western blot. (E) Schematic map of luciferase reporter assay constructs. The miR-125b-1-3p target site within the 3′-UTR of S1PR1 was shown as black box. Sequences below indicated putative miR-125b-1-3p target site on wild-type (pmir-S1PR1) 3′-UTR, its mutated derivative (pmir-S1PR1-M), and the pairing region of miR-125b-1-3p. (F) Luciferase assay in HTR8/SVneo cells transfected with pmir-S1PR1 and pmir-S1PR1-M reporter together with miR-125b-1-3p or NC. $N = 3$ performed in triplicates and results were presented as mean ± SEM. Statistical comparison in separate groups between miR-210 and NC was performed using Student-t test, with $p < 0.05$ considered as significant. $^{*}p < 0.05$, $^{**}p < 0.01$.

miR-125b-1-3p inhibitor enhanced the invasion. These finding indicate that miR-125b-1-3p could suppress the invasiveness of trophoblast cells.

miRNAs usually play their essential roles by suppressing the expression of target gene mRNAs, target exploration is essential for clarifying the mechanisms of miRNAs. Several evidences in this study prove that S1PR1 is a direct functional target of miR-125b-1-3p in trophoblast cells. First, S1PR1 expression is negatively correlated with miR-125b-1-3p in the placenta. Second, S1PR1 expression could be reduced by transfection of miR-125b-1-3p mimics in HTR8/SVneo cells. Third, luciferase assay vector pmir-S1PR1, not pmir-S1PR1-M, was responsive to miR-125b-1-3p. Additionally, overexpression of S1PR1 could reverse the invasion-inhibiting effect of miR-125b-1-3p in trophoblast cells. These findings supported that S1PR1 is an essential target of miR-125b-1-3p, at least participating in mediating the inhibitory effect on the invasion of trophoblast cells.

S1PR1 is a G-protein-coupled receptor of bioactive lipid Sphingosine-1-phosphate implicated in the regulation of vascular and immune systems. S1PR1 is critical for inhibition of angiogenesis

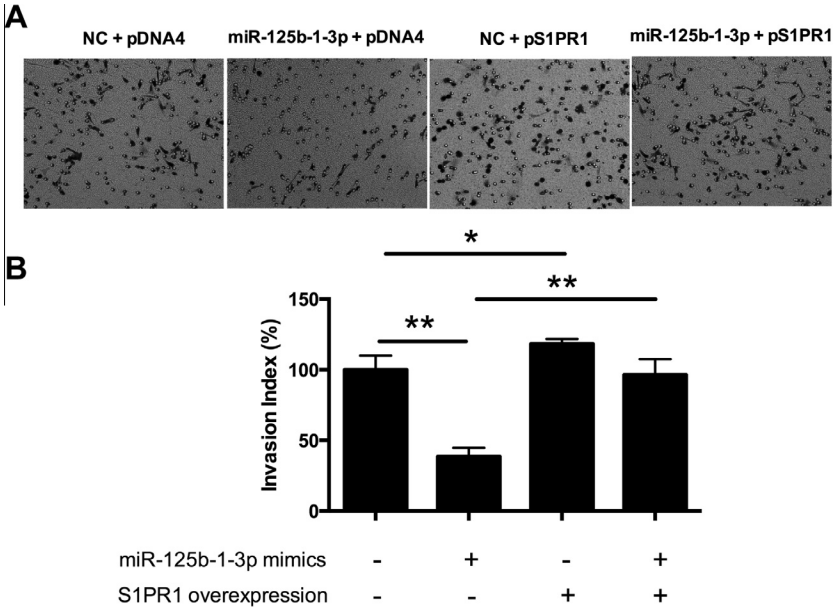

Fig. 4. Sphingosine-1-phosphate receptor 1 (S1PR1) rescued the effect of miR-125b-1-3p on the invasion in HTR8/SVneo cells. (A) A typical result of in vitro transwell insert assays to examine cell invasion in HTR8/SVneo cells transfected with miR-125b-1-3p and S1PR1 overexpressing pcDNA 4 vector (pS1PR1) alone or in combination, with scramble control (NC) or pcDNA 4 vector (pDNA4) as corresponding controls. (B) Relative invasion index based on 3 independent experiments. $N = 3$ performed in triplicates and data were presented as mean ± SEM. with $p < 0.05$ considered as significant. $^{*}p < 0.05$, $^{**}p < 0.01$.

and maintain of vascular stability [36], loss of S1PR1 leads to increased sprouting angiogenesis and vascular leak [37,38]. S1PR1 is also essential for lymphocyte recirculation and egress [39]. Recent evidence demonstrated that there are expressions of S1PR1 in human EVT cells [40,41], suggesting that S1PR1 may play roles in the regulation of EVT cells. Our previous reports demonstrated that activation of S1PR1 could promote invasion of trophoblast cells [42]. Here, we reported for the first time that invasion-inhibiting effect of miR-125b-1-3p is mediated, as least in part, by suppressing S1PR1.

In summary, our study provide new evidence that deregulated miR-125b-1-3p contribute to preeclampsia by inhibiting the invasion of trophoblast cells via directly targeting S1PR1. Therefore, miR-125b-1-3p and S1PR1 may be developed to be potential clinical predictive and therapeutic targets for preeclampsia.

Acknowledgments

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