

## Original Article

# MicroRNA-20b inhibits trophoblast cell migration and invasion by targeting MMP-2

Meiyuan Jin<sup>1\*</sup>, Haiyang Li<sup>2\*</sup>, Hongfeng Xu<sup>3</sup>, Gaoxiang Huo<sup>4</sup>, Yingyu Yao<sup>1</sup>

<sup>1</sup>Department of Obstetrics, Tongde Hospital of Zhejiang Province, Hangzhou, China; <sup>2</sup>Department of Gynaecology and Obstetrics, Zhoushan Municipal Women and Children Health Care Center, Zhoushan, China; <sup>3</sup>Department of Gynaecology and Obstetrics, Cixi Municipal Women and Children Health Care Center, Cixi, China; <sup>4</sup>Department of Obstetrics, The People's Hospital of Cangzhou City, Cangzhou, China. \*Equal contributors.

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**Abstract:** Insufficient trophoblast migration/invasion is associated with the preeclampsia (PE) development. Recently, microRNAs (miRNAs) have been confirmed to be involved in the pathogenesis of PE. The aim of the present study was to evaluate whether miRNAs is involved in the procession of PE by regulating the migration/invasion of trophoblast. First, we compared the expression profiles of miRNAs between normal and preeclamptic placentas using microarray. Validation analysis of miR-20b level in placentas and peripheral blood specimens was performed using quantitative reverse transcription PCR (qRT-PCR). Then, the effects of miR-20b on trophoblast cell migration and invasion were evaluated using wound healing assay and transwell migration assay. Further bioinformatics analysis, luciferase reporter assays and Western blot were performed to identify its target genes. The correlation between miR-20b and matrix metalloproteinase-2 (MMP-2) in placentas was determined by Pearson's correlation coefficient. Finally, HTR8/SVneo cells were co-transfected with miR-20b inhibitor and si-MMP-2 to explore the molecular mechanism by which miR-20b functions in the trophoblast migration/invasion. We found that miR-20b was elevated in placentas and peripheral blood specimens from preeclampsia patients. Further results show that overexpression of miR-20b significantly inhibited the invasiveness of human trophoblast cells, whereas miR-20b knockdown enhanced trophoblast cell invasion. Matrix metalloproteinase-2 (MMP-2), the most common enzymes in remodeling extracellular matrix components for metastasis, was proved to be a direct target of miR-20b. Inhibition of MMP-2 by siRNA could reverse the promoting effect of miR-20b inhibition on the invasion of trophoblast cells. Taken together, our study indicates that miR-20b inhibited trophoblastic invasion by targeting MMP2. The miR-20b/MMP-2 axis may provide novel insights into understanding the molecular pathogenesis of PE and may be a prognostic biomarker and therapeutic target for PE.

**Keywords:** microRNA-20b, pre-eclampsia, trophoblast invasion, HTR8/Svneo, matrix metalloproteinase 2

## Introduction

Preeclampsia (PE) is a pregnancy-specific syndrome characterized by hypertension and proteinuria, making it a major cause of maternal and perinatal mortality and morbidity [1, 2]. Limited invasion of trophoblast cells is essential for PE development, but clear studies on its mechanism are yet to emerge [3-5]. Therefore, the investigation into the molecular mechanisms related to insufficient trophoblastic invasion is of great significance.

MicroRNAs (miRNAs), a class of small non-coding RNAs, regulate gene expression at post-

transcription by binding to the 3'-untranslated regions of their target mRNAs for translational repression or mRNA cleavage [6, 7]. MiRNAs are reported to be involved in various cellular processes including proliferation, apoptosis, movement, and differentiation [8, 9]. It has been found that aberrant expression or dysfunction of miRNAs plays a key role in the development of PE [10-12]. For example, Zhang et al. found that miR-155 expression was upregulated in the placentas of women with PE and contributed to PE development by targeting and down-regulating angiogenic regulating factor CYR61 [13]. Another study revealed that miR-204 suppressed trophoblast-like cell invasion

by targeting matrix metalloproteinase-9 (MMP-9), suggesting miR-204 as a novel therapeutic target for preeclampsia [14]. Therefore, identification of novel PE-related miRNAs will offer insights into preeclampsia etiology.

In this study, we found that miR-20b expression was elevated in placentas and peripheral blood specimens. Functionally, we found that miR-20b could inhibit the migration and invasion of trophoblast cells. Furthermore, MMP-2 was identified to be a direct target of miR-20b and miR-20b exerted its effect on trophoblast cells by targeting MMP-2.

### Materials and methods

#### *Tissue samples*

Placentas and maternal blood samples tissues were obtained from 15 women with normal pregnancies and 15 women with PE at Department of Obstetrics, Tongde Hospital of Zhejiang Province. All experimental protocols were approved by the Ethics Committee of Tongde Hospital of Zhejiang Province. All studies were performed in accordance with the ethical guidelines of the Ethics Committee of the Tongde Hospital of Zhejiang Province. Informed consent was obtained from all patients.

#### *MicroRNA expression profiling*

miRNA profiling of placentas was performed with the 7th generation of miRCURY™ LNA Array (v.18.0, Exiqon) as previously described [15]. Expressed data were normalized using the median normalization. After normalization, significant differentially expressed miRNAs were identified through Volcano plot filtering. Hierarchical clustering was performed using MEV software (v4.6, TIGR). The miRNAs were considered to be significantly differentially expressed between the two groups (AD patient versus healthy control) if the fold changes (FC) was > 2.0 and the *P* value was < 0.05.

#### *Quantitative reverse transcription-PCR (qRT-PCR)*

Total RNA was isolated from placentas and peripheral blood specimens using the TRIzol reagents (Invitrogen, CA) following the manufacturer's instructions. For the detection of miR-20b, qRT-PCR assays were performed

using the TaqMan miRNA Assay (Life Technologies) following the manufacturer's instructions. For detection of the MMP-2 mRNA levels, 1 µg of total RNA was reverse transcribed using Script™ cDNA Synthesis Kit (Bio-Rad Laboratories), then performed on an Applied Biosystems 7900HT cyclor using SuperReal PreMix Plus (Tiangen, China). U6 and GAPDH functioned as normalization control in the expression analysis of miR-20b and MMP2, respectively. The relative expression of RNAs was calculated using the 2<sup>-ΔΔCt</sup> method. Each reaction was conducted in triplicate.

#### *Cell culture*

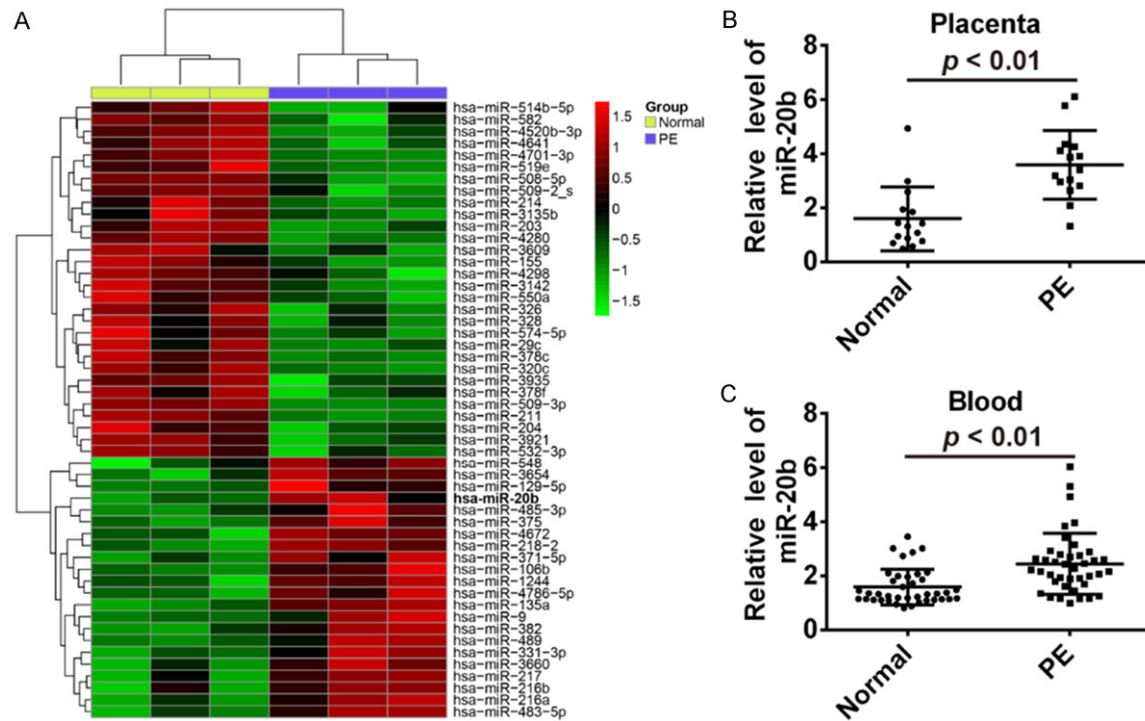
The extravillous trophoblast cell line HTR-8/SVneo was obtained from ATCC and maintained in RPMI 1640 medium (Life Technologies) supplemented with 10% fetal bovine serum (Life Technologies). Human 293T embryonic kidney cells were purchased from the American Type Culture Collection (Manassas, VA, USA) and maintained in Dulbecco's modified Eagle's medium (Invitrogen) containing 10% fetal bovine serum (Invitrogen) and 1/100 streptomycin-penicillin mix (Sigma). The cells were incubated at 37°C with 5% CO<sub>2</sub>.

#### *Transfection*

The miR-20b mimics, miR-20b inhibitor and negative control (NC) were synthesized by GenePharma (Shanghai, China). MMP-2 overexpression plasmid (pcDNA-MMP-2) and MMP-2 siRNA for silencing MMP-2 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Cell transfection was performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The cells were treated for further experiments 48 h after transfection.

#### *In vitro invasion and migration assay*

For wound healing assay, HTR-8/SVneo cells were transfected with miR-20b mimics, miR-20b inhibitor, miRNA negative control or si-MMP-2 and seeded in 6-well plates. After 48 h, when the cells reached 80% confluence, scratch wounds were wounded with a 10 µl pipette tip; cells were washed three times with PBS; and wound gaps were imaged and calculated by Image J software (NIH, Bethesda, MD, USA) at 24 h and 48 h.



**Figure 1.** miR-20b was upregulated in preeclamptic placentas and peripheral blood specimens. A. Heatmap of normalized expression levels of miRNAs in placentas samples from three patients with PE and three women with normal pregnancies. Green indicates low expression levels; red indicates high expression levels. B. Real-time PCR was performed to determine the expression levels of miR-20b in preeclamptic placentas (n = 15) and normal pregnancies (n = 15). C. Real-time PCR was performed to determine the expression levels of miR-20b in peripheral blood specimens from patients (n = 40) with PE compared with that from women (n = 40) with normal pregnancies.  $P < 0.01$  vs. Normal group.

For invasion assay, the invasive ability of trophoblasts was evaluated using the Transwell Matrigel invasion assay, as previously described [16]. The number of invaded cells was calculated by counting five random views under the microscope at 24 h and 48 h. The experiment was performed in triplicate and repeated for three times.

#### Western blot

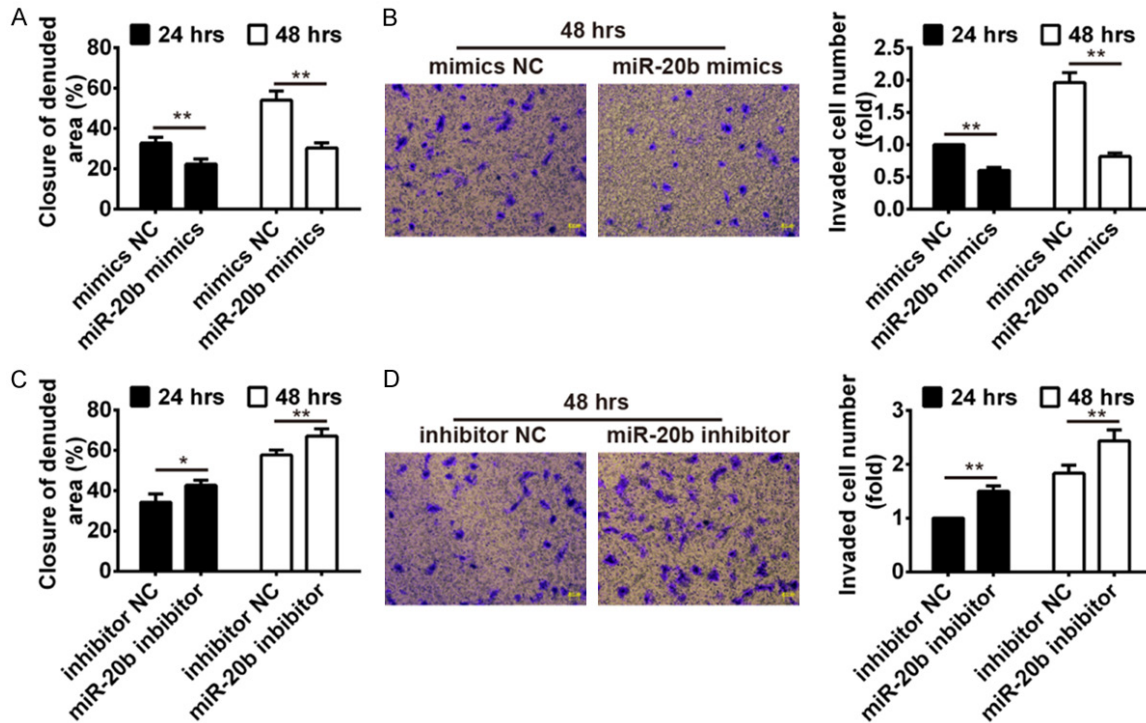
Cell lysates were prepared using RIPA buffer. Protein samples were subjected to SDS-polyacrylamide gel electrophoresis and transferred onto a PVDF membrane. The membrane was blocked with 5% nonfat milk. The membrane was then incubated with anti-MMP-2 antibody (1:1500, Abcam, Shanghai, China) or anti- $\beta$ -actin antibody (Abcam, Shanghai, China), followed by incubation with HRP-anti-rabbit secondary antibody at room temperature for 1 h. Signals were detected using an ECL Kit (Amersham Biosciences) according to the manufacturer's protocol.

#### Luciferase assays

A cDNA fragment of the MMP-2 3'-UTR mRNA containing the seed sequence of the miR-20b-binding site or a mutated binding site was cloned into the pmirGLO dual-luciferase vector (Promega, Madison, WI, USA). The constructed dual-luciferase vector was co-transfected with 10 pmol of miR-20b mimics, miR-20b inhibitor or NC into 293T cells. The cells were harvested and lysed 24 h later, and the luciferase activity was measured by the Dual-Luciferase Assay System (Promega) in accordance to the manufacturer's instructions.

#### Statistical analysis

Statistical analysis was performed using GraphPad Prism 5.0 (GraphPad Software, Inc., San Diego, CA, USA). Differences were analyzed with the Student's t-test between two groups or with one-way ANOVA among multiple groups. Correlation analyses were analyzed with a Pearson analysis. A  $p$ -value of less than 0.05 was considered statistically significant.



**Figure 2.** miR-20b inhibits the invasion and migration of trophoblast cells. HTR-8/SVneo cells were transfected with miR-20b mimics, miR-20b inhibitor or miRNA negative controls. A, C. Cell migration was measured at 24 h and 48 h using the wound healing assay. B, D. Cell invasion was measured at 24 h and 48 h using the transwell invasion assay. Data represent the mean  $\pm$  SD of three independent experiments. \*\* $P < 0.01$  compared with mimics NC or inhibitor NC.

## Results

### *miR-20b is up-regulated in preeclamptic placentae*

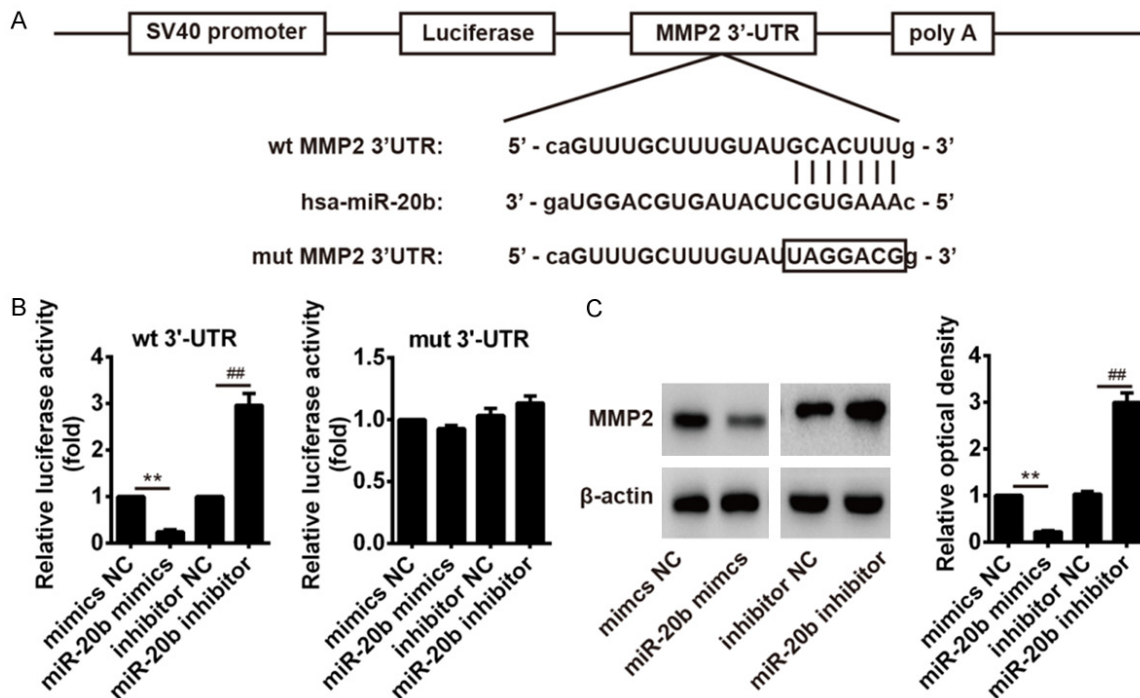
To determine the potential involvement of miRNAs in the pathogenesis of PE, we performed a miRNA microarray on placentas samples from three PE patients and three women with normal pregnancies. Our data revealed that compared with the normal group, 22 miRNAs were up-regulated and 30 miRNAs were downregulated in the PE group (Figure 1A). Among the aberrantly expressed miRNAs, miR-20b was one of the miRNAs being most significantly upregulated. In a previous study, miR-20b has been also found to be upregulated in PE placenta samples relative to normal placenta samples [17]. Moreover, miR-20b has been shown to inhibit tumor cell invasion in several types of human cancers [18-20]. Therefore, we chose miR-20b for further research.

To validate the expression trend of miR-20b obtained from miRNA microarray assay, quanti-

tative real-time RT-PCR (qRT-PCR) was used to detect miR-20b in placentas and peripheral blood specimens. As shown in Figure 1B, miR-20b level was significantly higher in placentas from fifteen patients with PE than in that from fifteen women with normal pregnancies. We also observed that miR-20b was markedly upregulated in peripheral blood specimens from 40 patients with PE compared with that from 40 women with normal pregnancies (Figure 1C). Collectively, these results provided sufficient evidence that miR-20b was prominently up-regulated in PE, suggesting that miR-20b may as an effective biomarker for the prognosis of patients with PE.

### *miR-20b inhibited the invasion and migration of trophoblast cells*

Since limited invasion of trophoblast cells is essential for PE development, we assess the role of miR-20b on the invasion and migration of trophoblast cells. The HTR8/SVneo cells, a first-trimester human extravillous trophoblasts (EVTs)-derived cell line, were transfected with



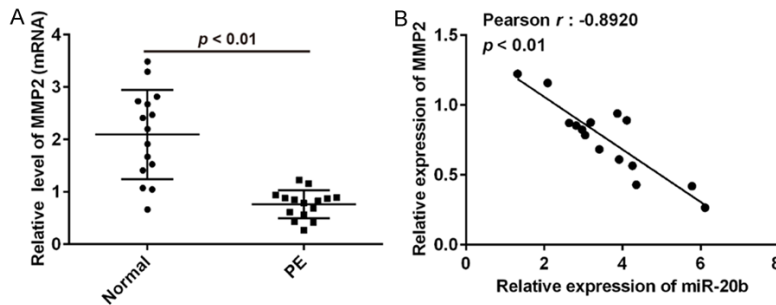
**Figure 3.** MMP-2 is a direct target of miR-20b. **A.** The putative binding site of miR-20b and MMP-2 is shown. **B.** Luciferase assay of HEK293 cells co-transfected with firefly luciferase constructs containing the MMP-2 wild-type or mutated 3'-UTRs and miR-20b mimics, mimics NC, miR-20b inhibitor or inhibitor NC, as indicated (n = 3). **C.** The expressions of MMP-2 protein after transfection with miR-20b mimic or miR-20b inhibitor were measured by Western Blot. Data represent the mean  $\pm$  SD of three independent experiments. \*\* $P < 0.01$  vs. mimics NC, ## $P < 0.01$  vs. inhibitor NC.

miR-20b mimics or miR-20b inhibitor and the corresponding scramble control, then the invasion and migration were measured by wound healing assay and transwell invasion assay at 24 and 48 h. The results of wound healing assay showed that wound closure of HTR8/SVneo cells with miR-20b mimics was slower than that in mimics NC group (**Figure 2A**) and the results of transwell invasion assay showed that numbers of invaded cells were obviously attenuated in the miR-20b mimics group compared with mimics NC group (**Figure 2B**). In contrast, the wound closure of HTR8/SVneo cells and numbers of invaded cells were significantly enhanced in miR-20b inhibitor group compared with inhibitor NC group (**Figure 2C, 2D**). These data indicated that miR-20b could inhibit invasion and migration of trophoblast cells and upregulation of miR-20b may be involved in the pathogenesis of PE.

#### MMP-2 was a direct target of miR-20b

To further elucidate the underlying molecular mechanisms by which miR-20b functions in

HTR8/SVneo cells, we used the bio-informatic tools to search for the potential targets. To our interest, MMP-2, which has been found to play an important role in the migration and invasion of trophoblast cells [21], could bind to miR-20b, suggesting a potential interaction between miR-20b and MMP-2 (**Figure 3A**). Thus, MMP-2 was selected for further investigation. To experimentally validate whether MMP-2 was a direct target of miR-20b, a dual-luciferase reporter assay was conducted. The results showed that overexpression of miR-20b significantly decreased the luciferase activity of wt-MMP-2-3'UTR, whereas knockdown of miR-20b increased luciferase activity. Likewise, cells co-transfected with miR-20b mimics, miR-20b inhibitor, and MMP-2-mut-3'UTR, showed no obvious change in their luciferase activity (**Figure 3B**). In addition, we explored whether miR-29b could modulate the expression of MMP-2. As shown in **Figure 3C**, the protein levels of MMP-2 was decreased after overexpression of miR-20b, whereas it increased after inhibition of miR-20b. Thus, miR-20b could



**Figure 4.** miR-20b negatively regulated the expression of MMP-2. A. The mRNA expression of MMP-2 was measured using qRT-PCR in placenta samples from fifteen patients with PE and fifteen women with normal pregnancies. Data represent the mean  $\pm$  SD of three independent experiments.  $P < 0.01$  vs. Normal group. B. The correlation between miR-20b and MMP-2 in placenta was determined by Pearson's correlation coefficient ( $r: -0.8920$ ,  $P < 0.01$ ).

negatively regulate MMP-2 expression in HTR8/SVneo cells.

#### *MiR-20b expression correlated inversely with MMP-2 mRNA level in PE*

To further elucidate the relationship between miR-20b and MMP-2 in PE, we detected the MMP-2 expression in 15 paired placentas from patients with PE and women with normal pregnancies using qRT-PCR. As shown in **Figure 4A**, the expression of MMP-2 in PE group was down-regulated when compared with normal group. More importantly, the expression levels of MMP-2 in placentas inversely correlated with the miR-20b levels (Pearson  $r: -0.8920$ ,  $P < 0.01$ , **Figure 4B**). These data suggested that miR-20b negatively regulated MMP-2 expression and their inversely correlation could be determined in clinical samples.

#### *MMP-2 was involved in miR-20b-mediated suppression of trophoblast cells migration and invasion*

Since MMP-2 is frequently down-regulated in PE and correlated with increased miR-20b expression, we sought to determine whether MMP-2 participated in miR-20b mediated suppressed invasion effects. To further address this issue, we used siRNA to knockdown the expression of MMP-2 after miR-20b inhibitor transfection. Wound healing and transwell assays indicated that the inhibition of MMP-2 significantly reduced the trophoblast cells migration and invasion that could be enhanced by miR-20b inhibitor (**Figure 5A, 5B**). Furthermore, to assessed that whether MMP2 overex-

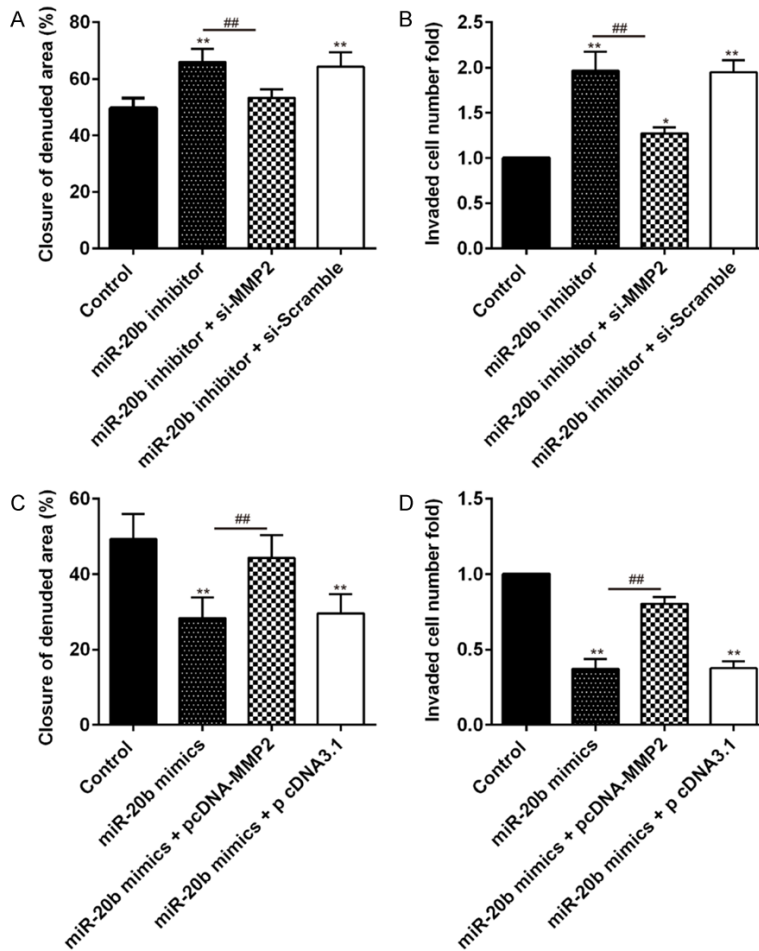
pression could compromise the inhibitory effects of miR-20b on cell migration and invasion, we transfected the pcDNA3.1-MMP-2 plasmid together with miR-120b mimics. As shown in **Figure 5C, 5D**, the overexpression of MMP-2 significantly attenuated the inhibitory effects of miR-20b mimics on the trophoblast cells migration and invasion. These data provided evidences that miR-20b suppressed cell invasion and migration through targeting MMP-2.

## Discussion

In the present study, we investigated the expression and the function of miR-20b in the development of PE. Our data showed that miR-20b expression was elevated in preeclamptic placentas and peripheral blood specimens. Moreover, we found that miR-20b inhibited the invasion and migration of trophoblast cells by decreasing MMP-2 expression. Our findings suggest that miR-20b is a novel miRNA that is involved in PE development.

A large body of evidence suggests that the expression of miRNAs is deregulated in preeclamptic placentas and the alternation of miRNAs plays an important role in the development and progression of PE [14, 22, 23]. For example, miR-141 was found to be upregulated in preeclamptic placentas [24]. Furthermore, reports have shown that circulating miRNAs are promising novel biomarkers for PE. For example, Wu et al. showed that miR-181a was increased in plasma from severe preeclamptic pregnancies compared with plasma from normal pregnancies [25]. In this study, we identified the miRNA expression profile in preeclamptic placentas and normal placentas, and miR-20b was found significantly up-regulated in preeclamptic placentas and peripheral blood specimens. Our data imply miR-20b may serve as a serum biomarker for PE.

MiR-20b is a member of miR-17 microRNA precursor family which includes miR-20a/b, miR-93, and miR-106a/b [26]. Recently, a study from Wang et al. showed that miR-20b is mark-



**Figure 5.** MMP-2 reversed the effect of miR-20b on cell invasion in HTR8/SVneo cells. HTR8/SVneo cells were co-transfected with si-MMP-2 and miR-20b inhibitor. A. Wound healing assay was performed to monitor cell migration after transfection with si-MMP-2 or miR-20b inhibitor. B. Transwell assay was performed to monitor cell invasiveness after transfection with si-MMP-2 or miR-20b inhibitor. C. Wound healing assay was performed to monitor cell migration after transfection with pcDNA-MMP-2 or miR-20b mimics. D. Transwell assay was performed to monitor cell invasiveness after transfection with pcDNA-MMP-2 or miR-20b mimics. Data represent the mean  $\pm$  SD of three independent experiments. \*\* $P < 0.01$  vs. control group, ## $P < 0.01$  vs. miR-20b inhibitor or miR-20b mimics.

edly upregulated in preeclamptic placentas, suggesting that miR-20b may involve in PE pathologic process [17]. However, so far, the role for miR-20b in trophoblastic invasion had not been reported. Interestingly, several studies have also been reported miR-20b plays important roles in the regulation of cell migration and invasion in many types of cancer cells, such as bladder cancer [27] and osteosarcoma [28]. Therefore, we investigate whether miR-20b mediated the invasion of trophoblast-like

cells. As expected, overexpression of miR-20b inhibited invasion and migration of trophoblast-like cells, whereas knockdown of miR-20b had an opposite result. These results suggest that miR-20b may play a role in regulating trophoblast invasion.

It is widely accepted that the insufficient trophoblast migration/invasion is correlated with the development of preeclampsia. Several studies have shown that the regulation of MMP activity is required for trophoblast invasion [29, 30]. For example, MMP2 is abundantly expressed in invading extravillous trophoblast (EVT) cells, and the expression of this gelatinase is highly related to trophoblast cell invasiveness [31, 32]. Moreover, MMP2 has been reported to participate in trophoblast cell invasion that is mediated by microRNAs, such as miR-29b [33]. However, whether MMP2 is implicated in miR-20b-mediated trophoblastic invasion remains unknown. In our study, using bioinformatics analysis and luciferase reporter assays, MMP-2 was proved to be a direct target of miR-20b and an inverse correlation was observed in preeclamptic placentas, suggesting that miR-20b

could negatively regulate MMP-2 expression in PE. In addition, our results also showed that inhibition of MMP-2 by siRNA could reverse the promoting effect of miR-20b knockdown on the invasion of trophoblast cells. Likewise, we also observed that overexpression of MMP-2 could attenuated the inhibitory effects of miR-20b overexpression on the invasion of trophoblast cells. These data indicate that miR-20b suppressed the trophoblastic invasion at least partly by targeting MMP-2.

In conclusion, we demonstrated that miR-20b is highly expressed in preeclamptic placentas and peripheral blood specimens. Furthermore, our study provides enough evidence that upregulation of miR-20b may contribute to PE by inhibiting the invasion of trophoblast cells via directly targeting MMP-2. Therefore, miR-20b/MMP-2 axis may be developed to be potential clinical predictive and therapeutic targets for PE.

## Disclosure of conflict of interest

None.

**Address correspondence to:** Yingyu Yao, Department of Obstetrics, Tongde Hospital of Zhejiang Province, No. 234, Gucui Road, Hangzhou 310012, China. Tel: +86-571-89972114; E-mail: yao\_yinyuy@sina.com

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