MicroRNA-206 Contributes to the Progression of Preeclampsia by Suppressing the Viability and Mobility of Trophocytes via the Inhibition of AGTR1

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Summary

The development of preeclampsia (PE) is associated with the impaired trophoblast motility. MicroRNAs (miRs) contribute to the modulation of trophoblast invasion. In the current study, the role of miR-206/AGTR1 in the TNF-α-induced invasion defect of trophoblasts was explored. The levels of miR-206 and ATGR1 in clinical placenta tissues were investigated. Trophoblasts were treated with TNF-α, and the levels of miR-206 and ATGR1 were modulated. Changes in cell viability, invasion, and inflammation in trophoblasts were detected. The level of miR-206 was induced, while the level of AGTR1 was suppressed in placenta tissues. In *in vitro* assays, TNF-α suppressed viability, induced inflammatory response, inhibited invasion, upregulated miR-206, and down-regulated AGTR1. The inhibited expression of miR-206 or the overexpression of AGTR1 counteracted the effects of TNF-α, indicating the key role of the miR-206/AGTR1 in progression of PE. Collectively, miR-206 suppressed viability, induced inflammatory response, and decreased invasion of trophoblasts by inhibiting AGTR1.

Key words

AGTR1 • Inflammation • Invasion • miR-206 • Preeclampsia

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Introduction

Preeclampsia (PE) is a term used to describe the presence of proteinuria and hypertension during the 20-week gestation [1,2]. As a multisystemic disorder, PE can further progress to widespread microangiopathy that affects multiple organs, including kidney, liver, and brain, and thus is now a leading causable factor contributing to the morbidity and mortality of pregnancies worldwide [3]. Currently, the only effective treatment strategy for PE is still premature delivery/termination of pregnancy. However, new manifestation strategies for PE based on the target of angiogenic factors are being developed and are reported to have significantly improved effects on the prognosis of both the mother and the fetus [4-7]. Additionally, the increasing attention paid to the pathogenesis of PE has introduced more potential targets for the future handling of the disorder.

PE is classified as a late-pregnancy illness, yet the molecular changes that lead to its onset happen earlier in pregnancy. In general, it is acknowledged that the placenta, and more particularly, the trophoblasts, play a significant role in the formation of PE because multinucleated trophoblasts, which are a hallmark of embryonic implantation, penetrate the uterine epithelium [8]. Thus, the disorder in trophoblast activities, especially defects in the invasion into the uterus will cause the failure in the maternal spiral arteries remodeling and now is conceived as the central mechanism underlying the initiation of PE [9,10]. Given the key role of trophoblasts in the progression of PE, elucidating the detail mechanism mediating trophoblast cell differentiation, especially differentiation toward the invasive pathway, will provide valuable information for explanation of the pathogenesis of PE and identify novel diagnostic

biomarkers for PE. Additionally, the restoration of the regular function of trophoblasts may become an attractive potential approach for the management of PE. The differentiation of trophoblasts is regulated by multiple factors such as oxygen tension within the maternal-fetal interface, various hormones, and growth factors [8]. In the recent years, microRNAs (miRs) are also reported to be involved in placental development and differentiation of trophoblasts, which elicits emerging interests in exploring the role of specific miRs as potential diagnostic and treatment targets against PE.

With the aid of high throughput sequencing technology, multiple aberrant placental miRs associated with PE have been identified [11-14]. For instance, miR-210 and miR-377 are upregulated in PE placenta based on the study by Enquobahrie *et al.* [12], while in other studies, the authors report that miR-144 and miR-223 are downregulated in PE patients [15-17], indicating that different miRs play distinct roles in the development of PE. However, even regarding the same miR, conflicting data exists among studies. For example, the expression status of miR-210 and miR-377 reported by Mayor-Lynn *et al.* [14] is exactly opposite to the data by Enquobahrie *et al.* [12]. Therefore, careful validation of differential miRs expression using appropriate specimens as well as the comprehensive exploration of the downstream effectors mediated by miRs is crucial.

MiR-206 is previously reported to be up-regulated in PE patients based a microarray detection [18]. Further studies also confirm that the miR may participant in the development of PE in multi-pronged mechanisms including the regulation of trophoblast invasion ability [19-21], which infers the potential of the miR as a further treatment target for PE. In the current study, we performed additional work to support the involvement of miR-206 in the progression of PE. The clinical expression of miR-206 as well as its downstream effector AGTR1 in placenta tissues of PE patients was determined, and the correlation with the clinico-pathological feature of the patients was analyzed. Moreover, the expression status of miR-206 was modulated in HTR-8/SVneo trophoblasts to verify the mechanism by which miR-206 was involved in the modulation of trophoblast invasion.

Materials and Methods

Statement of ethics

The study was approved by the ethic committee of the First People's Hospital of Wenling for the related screening, inspection, and data collection (No. KY-2023-2011-01). All the patients had signed a written informed consent form. All the experiment procedures were performed in accordance with the Declaration of Helsinki.

Patients and specimen collection

Placenta samples were collected from 65 pregnancies from Nov 2019 to June 2020 in the First People's Hospital of Wenling, and were divided into Control group and PE group. Samples in Control group were collected from placenta tissues of pregnancies underwent cesarean delivery due to issues such as scarred uterus and cephalopelvic disproportion. For samples in PE group, placenta tissues were collected from PE patients diagnosed based on the criteria that systolic pressure was higher than 140 mm Hg, diastolic pressure higher than 90 mm Hg, and urine protein higher than 0.3 g/24 h. Additionally, the clinicopathological parameters included age, body weight, gestational weeks, systolic pressure, and diastolic pressure of all the participants were collected and analyzed according. The study was approved by the ethic committee of the First People's Hospital of Wenling for the related screening, inspection, and data collection (No. KY-2023-2011-01). All the patients had signed a written informed consent form. All the experiment procedures were performed in accordance with the Declaration of Helsinki.

Reverse transcription quantitative PCR (RT-qPCR)

Total RNA in placenta tissues was extracted using a RNA simple Total RNA Kit (DP419, TIANGEN, China), and cDNA templates were achieved *via* the reverse transcription of RNA using Super M-MLV reverse transcriptase (RP6502, BioTeke, China). The qPCR reaction mixture contained 10 μl of 2×Power Taq PCR MasterMix (PR1702, BioTeke, China), 0.5 μl of miR-206 primers (Table S1), 1 μl of the cDNA template, and 8 μl of RNAse free H_2O, and the amplification was performed following thermal cycling parameters as follows: 95 °C for 10 min for denaturation, 95 °C for 10 s by 40 cycles, 60 °C for 20 s, and 72 °C for 30 s. The relative expression level of miR-206 was calculated using Data Assist Software version 3.0 (Applied Biosystems/Life Technologies) with GAPDH as the internal reference gene according to the formula of $2^{-\triangle\triangle ct}$.

Immunochemical detection

The expression level and distribution of AGTR1 in clinical placenta samples were determined with

immunochemical (IHC) detection. Tissues were sectioned, dewaxed with dimethylbenzene, and hydrated with alcohol. Then the primary antibody against AGTR1 (1:1000) (ab124734, Abcam, China) was incubated with the tissues at 4 °C overnight, followed by the incubation by the secondary antibody (1:2000) (ab288151, Abcam, China) at 37 °C for 30 min. After re-stained with haematoxylin and dehydrated, the images were scanned and captured using an Aperio ScanScope GL (Aperio Technologies, Vista, CA, USA) at 400× magnification. The expression status of AGTR1 was further scored and compared between the two groups.

Dual luciferase assay

The direct binding by miR-206 to AGTR1 in trophoblasts was determined using a dual luciferase assay. A pmirGLO vector (Promega Corporation) containing 3'UTR region of AGTR1, and mimics of miR-206 (5'-TGGAATGTAAGGAAGTGTGTGG-3'; Shanghai GenePharma Co., Ltd.) were transfected into trophoblasts using Lipofectamine™ 3000 according to the manufacturer's instruction. The relative luciferase activity was detected using a microplate reader (GloMax, Promega) 48 h after the transfection with Renilla luciferase (psi-CHECK2) as the internal control.

Modulation of miR-206 and AGTR1 in HTR-8/SVneo trophoblasts

To determine the role of miR-206/AGTR1 axis in the progression of PE, the expression status of miR-206 and AGTR1 was modulated in human villous trophoblast HTR-8/SVneo (Procell, China). Cells were firstly incubated in RPMI 1640 medium (Nacalai Tesque, Kyoto, Japan) containing 5 % fetal bovine serum (FBS) (Sigma-Aldrich) at 37 °C in an atmosphere consisting of 5 % CO_2 and 95 % air and then subjected to different treatments: Control group, healthy HTR-8/SVneo cells; TNF-α group, cells were exposed to 1 μg/ml TNF-α for 72 h for mimicking PE symptoms *in vitro* [22] in that TNF-α plays an essential role in accelerating trophoblast cell death; Inhibitor group, cells were transfected with miR-206 inhibitor (5'-CCACACACUUCCUUA-UCCACAU-3') synthesized by Sango Biotech, Co. Ltd, Shanghai, China) and then exposed to TNF-α treatment; AGTR1 group, cells were transfected with AGTR1 expression vector (synthesized by Sango Biotech, Co. Ltd, Shanghai, China) and then exposed to TNF-α treatment. After TNF-α treatments, the cells were collected. The expression levels of miR-206 and AGTR1, the production of cytokines, cell viability, and cell mobility were then determined.

Enzyme-linked immunosorbent assay (ELISA)

The TNF-α-induced inflammatory responses was determined by measuring the production of IL-6 (ab234570, Abcam, USA), IL-1β (ab255730, Abcam, USA), and TNF-α (ab236712, Abcam, USA) with corresponding ELISA kits according to the manufacturer's instruction: briefly, 2×10^5 cells was ground into homogenate and centrifuged at 3000× g for 10 min. The supernatant was then retained, and the concentrations of cytokines were measured using corresponding kits, and the levels were recorded using a microplate reader (SpectraMax M5, Molecular Devices) at 450 nm wavelength.

Cell viability detection

Cell viability of HTR-8/SVneo cells was determined using a CCK-8 assay: 5×10^3 cells were incubated in one well of a 96-well plate for 48 h, which was followed by an incubation with 100 μl of CCK-8 medium (DOJINDO, Japan) for another 2 h. Cell viability was recorded by measuring the OD_{450} value using a microplate reader (SpectraMax M5, Molecular Devices).

Transwell assay

The transwell assay was conducted to measure the invasion ability of HTR-8/SVneo cells: 2×10^4 cells were inoculated into the upper chamber of a transwell system (Corning star, Cambridge, MA) previously coated with 40 μl matrigel (BD Biosciences, San Jose, CA, USA). After the formation of a reconstituted basement membrane, cells were allowed to penetrate through the porous membrane for 4 h. After the penetration, cells on the upper surface of the membrane were completely removed, and cells on the lower surface were stained in a solution containing 1 % (w/v) crystal violet. The invasion ability of trophoblasts was assessed by determining the penetrating number of cells using the Image-Pro Plus 6.0 software at 200× magnification (Nikon).

Western blotting assay

Total protein product was extracted using a Total Protein Extraction Kit (WLA019, Wanleibio, China) and was quantified using the BCA method. Afterwards, 40 μg of protein was subject to the sodium dodecylsulfate

polyacrylamide gel electrophoresis (SDS-PAGE), transferred to the PVDF membrane, and incubated with the skim milk powder solution for 1 h. Then the membrane was firstly incubated with primary antibodies against AGTR1 (1:1000) (ab124734, Abcam, China), MMP-2 (1:1000) (ab92536, Abcam, China), MMP-9 (1:1000) (ab76003, Abcam, China) and GAPDH (1:1000) (ab8245, Abcam, China) overnight at 4 °C, and then with secondary HRP IgG antibodies (1:5000) for 45 min at 37 °C. The protein blots were developed using Beyo ECL Plus reagent and the images were captured in the Gel Imaging System. The relative expression of target proteins was calculated with GAPDH as the internal reference protein.

Statistical analysis

Continuous data was expressed as mean ± standard deviation (SD), and the immunohistochemical score was represented as the number for each score. The difference in continuous data was analyzed using ANOVA followed by *post hoc* Tukey test, and the difference in the immunohistochemical score was analyzed using Wilcoxon rank sum test. Difference between two groups was analyzed using Student's *t*-test for normal distribution data or Mann-Whitney U test for abnormal distribution data. The correlation between miR-206 level or ATGR1 level and blood pressure was analyzed using Pearson or Spearman correlation analysis. Significance was accepted when the two-tailed P value was smaller than 0.05. All the statistical analyses and graph plotting were conducted using GraphPad Prism version 8.0.0 for Windows (GraphPad Software, San Diego, California USA, www.graphpad.com).

Results

Clinicopathological information of participants

The current study collected placenta tissues from 65 participants, which included 29 healthy controls and 36 PE patients. The clinicopathological information of participants was compared between controls and patients. As shown in Table 1, no significant difference regarding age (P=0.073) and body weight (P=0.588) was detected between healthy controls and PE patients. However, the gestational week of controls was significantly longer than of PE patients (Table 1; P<0.001), while the systolic pressure and diastolic pressure of controls were significantly lower (Table 1; P<0.001).

Table 1. Clinicopathological information.

	Health control (n=29)	PE patients (n=36)	P value
Age (year)	30.03 ± 1.95	30.73 ± 1.62	0.073
Body weight (kg)	79.66 ± 3.32	80.60 ± 2.53	0.588
Gestational week (wk)	38.27 ± 0.70	35.30 ± 0.67	<0.001
Systolic pressure (mm Hg)	120.17 ± 4.07	170.57 ± 9.73	<0.001
Diastolic pressure (mm Hg)	67.17 ± 3.8	94.87 ± 2.42	<0.001

Expression of miR-206 and AGTR1 in placenta tissues

To preliminary assess the potential interaction between miR-206/AGTR1 axis and the progression of PE, the expression status of the two factors was determined in placenta samples. Based on the analysis, the expression level of miR-206 in PE patients was much higher than that in healthy controls (Fig. 1A; P<0.01). To the contrary, the level and distribution of AGTR1 was lower in health controls than in PE patients (Fig. 1B; P<0.01). Moreover, the correlation between miR-206/AGTR1 level and blood pressure was also analyzed. The data showed the expression level of miR-206 was positive with both systolic pressure (R=0.986, P<0.0001) and diastolic pressure (R=0.88, P<0.0001), while the level of AGTR1 showed a negative correlation with the two parameters (systolic pressure: R=-0.922, P<0.0001; diastolic pressure: R=-0.794, P<0.0001).

MiR-206 directly bind to the 3'UTR region of AGTR1

The potential interaction was predicated by TargetScan and the expression status of the two factors was negative correlated in placenta tissues (Fig. 2A; R=-0.995, P<0.0001). However, whether the direct regulation of miR-206 on ATGR1 existed in placenta needed verification. Thus, a dual luciferase assay was performed with human trophoblasts. The data showed that only the co-transfection with miR-206 mimics and

wild type of 3'UTR sequence would influence the activity of luciferase (Fig. 2B), indicating that miR-206 could directly bind to the 3'UTR of AGTR1 and the binding was specific.

Modulation of miR-206/AGTR1 axis influenced the inflammatory response and cell viability in HTR-8/SVneo cells

The key role of miR-206/AGTR1 axis in the progression of PE was further explored *in vitro* with HTR-8/SVneo cells. PE symptoms was mimicked by incubating the cells with TNF-α. The incubation induced the production of cytokines including IL-6, IL-1β, and TNF-α in trophoblasts (Fig. 3A-3C), which was associated with the suppressed cell viability (Fig. 3D). At molecular level, the expression of miR-206 was up-regulated (Fig. 3E), while the expression of AGTR1 was down-regulated by TNF-α (Fig. 3F). Nevertheless, the changes in inflammation and cell viability were restored by miR-206 inhibitor or AGTR1 expression vector: the production of cytokines was suppressed and the cell viability was restored by the two treatments (Fig. 3A-3D). Regarding changes in miR-206/AGTR1 axis, the inhibition of miR-206 increased the level of AGTR1, while the overexpression of AGTR1 had little influence on the expression of miR-206 (Fig. 3E and 3F), further confirming the regulation of AGTR1 by miR-206 in trophoblasts.

Modulation of miR-206/AGTR1 axis influenced the invasion ability in HTR-8/SVneo cells

The impairment on the invasion ability of trophoblasts was a key factor contributing to the progression of PE. In *in vitro* model, the incubation with TNF-α substantially suppressed the invasion ability of trophoblasts (Fig. 4A). Moreover, the levels of MMP2 and MMP9 were also significantly inhibited by TNF-α (Fig. 4B), further confirming the impaired invasion ability of trophoblasts. However, the inhibition of miR-206 or the overexpression of AGTR1 increased the penetrating cell number in the transwell assay and restored the levels of MMP2 and MMP9, maintaining the invasion ability of trophoblasts even under the treatment of TNF-α (Fig. 4).

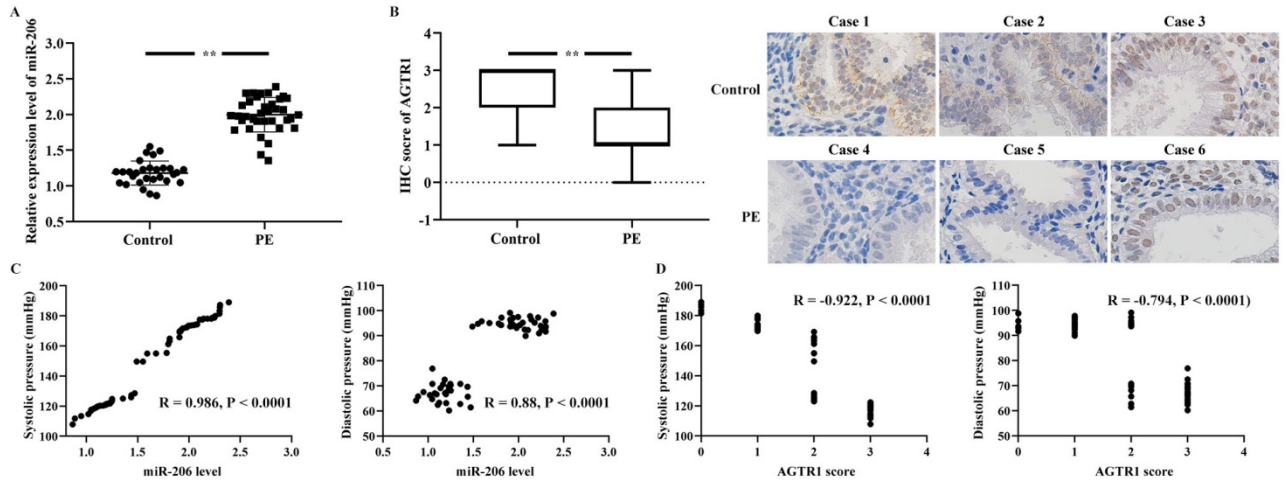

Fig. 1. Expression status of miR-206/AGTR1 in clinical placenta samples and relation with blood pressure (n=29 for Control group and n=36 for PE group). (**A**), expression status of miR-206 detected by RT-qPCR detections. (**B**), expression status of ATGR1 detected by immunochemical detections. (**C**), relation between miR-206 level and blood pressure. (**D**), relation between ATGR1 level and blood pressure. ** P<0.01 vs. Control samples.

Discussion

PE seriously affects the normal functions of multiple organs and systems, causing a variety of adverse consequences and even threating the life of maternal and fetus. Currently, the pathogenesis of PE is partially revealed and the only effective treatment strategy for the disorder depends on premature delivery/termination of pregnancy. Thus, the comprehensive understanding of the mechanism initiating PE has become the prerequisite for the early diagnosis and clinical management of PE. Based on the current studies, the impaired development of placenta is a key factor contributing to the onset and progression of PE [23]. In the early development stage of the embryo, invasive trophoblasts can reduce the internal resistance of blood vessels, expand the capacity, and then

ensure reperfusion the placenta. However, once the invasion ability of trophoblasts is reduced, the spiral arteries will not be fully redeemed and the placental reperfusion decreases, which induces the progression of PE and initiation of the systemic inflammatory response of multiple organs [24]. Thus, the restoration of the regular invasion ability of trophoblasts has now become a promising strategy for handling PE.

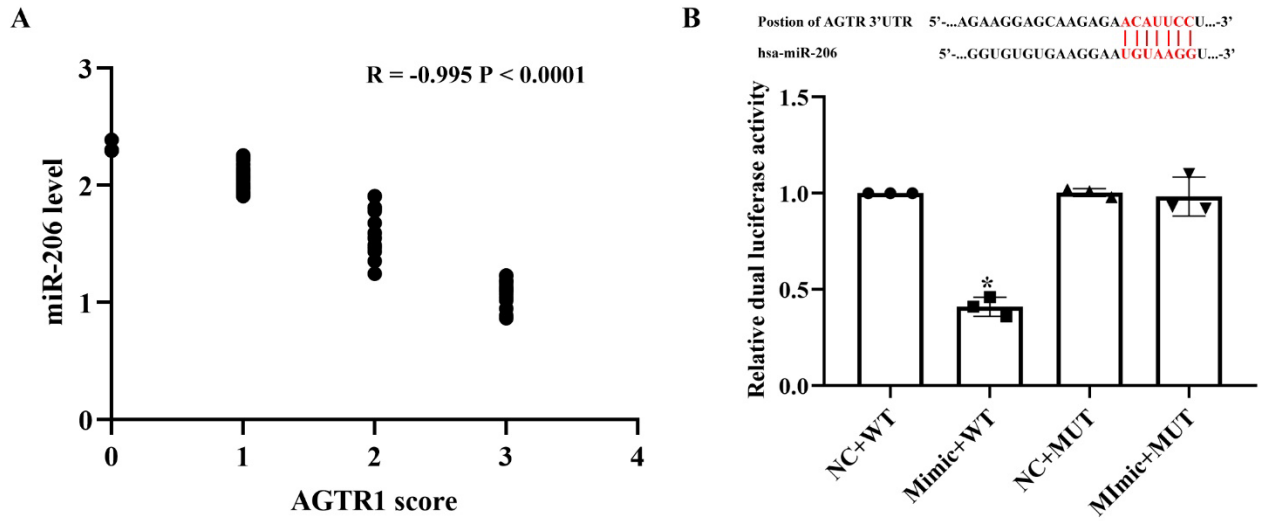

Fig. 2. MiR-206 directly regulated the activity of AGTR1 by binding to the 3'UTR sequence. (**A**), relation between miR-206 level and AGTR1 level in clinical placenta samples (n=29 for Control group and n=36 for PE group). (**B**), interaction between miR-206 and AGTR1 detected by a dual luciferase assay (n=3). * P<0.05 vs. NC+WT group.

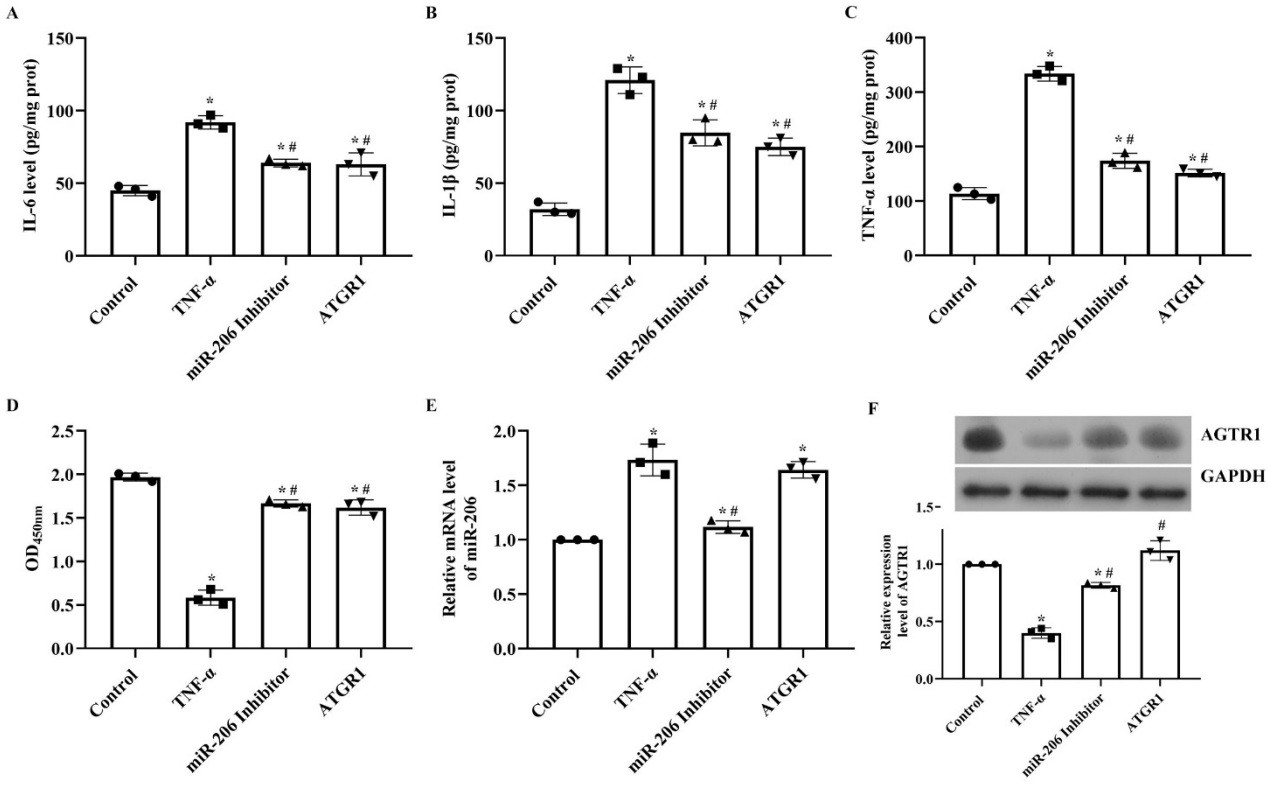

Fig. 3. Inhibition of miR-206 or overexpression of ATGR1 suppressed inflammatory response, increased cell viability in TNF-α treated HTR-8/SVneo trophoblasts (n=3). (**A**), IL-6 level detected by ELISA. (**B**), IL-1β level detected by ELISA. (**C**), TNF-α level detected by ELISA. (**D**), cell viability detected by CCK-8 assays. (**E**), expression status of miR-206 detected by RT-qPCR detections. (**F**), expression status of ATGR1 detected by western blotting detections. * P<0.05 vs. Control group. # P<0.05 vs. TNF-α group.

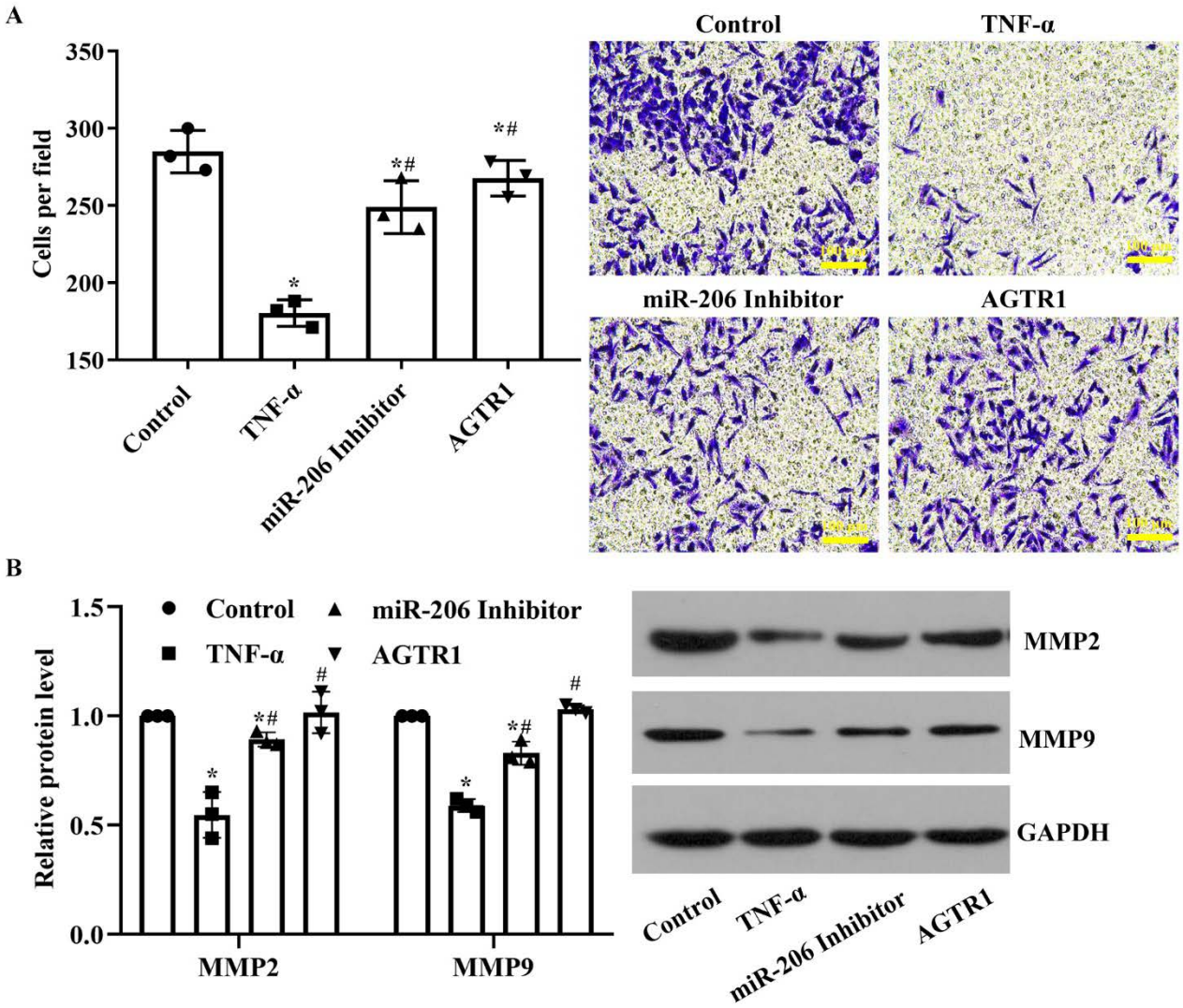

Fig. 4. Inhibition of miR-206 or overexpression of ATGR1 increased invasion ability and expression of MMPs in TNF-α treated HTR-8/SVneo trophoblasts (n=3). (**A**), quantitative analysis results and representative images of invasive ability detected by transwell assays. (**B**), expression status of MMPs detected by western blotting detections. * P<0.05 vs. Control group. # P<0.05 vs. TNF-α group. Scale bar, 200 μm.

In the current study, the potential mechanism mediating the reduced invasion ability of trophoblasts was further explored by focusing on miR-206/AGTR1 axis. The investigation with clinical placenta samples showed that the level of miR-206 was significantly up-regulated in PE patients, while the level of AGTR1 was significantly down-regulated. As a class of important non-coding RNA, miRs are small RNA molecules with a length of 19-24 nucleotides and participant in the regulation of important biological processes such as cell growth, differentiation, and apoptosis by binding to the 3'UTR of targets [25]. According to the previous reports, certain miRs such as miR-210, miR-155, miR-196, miR-195, miR-26 and miR-181a are induced in PE patients and might play pro-PE roles [26], while other miRs such as miR-144 and miR-223 are suppressed in PE patients and might function as suppressors against PE [26]. Additionally, some miRs showed opposite effects among different studies. For instance, the expression levels of miR-210 and miR-377 are different between the studies by Mayor-Lynn *et al.* [14] and by Enquobahrie *et al.* [12]. Regarding miR-206, the miR is reported to be induced in PE patients [18] and participants in the development of PE by suppressing the invasion of trophoblasts [19,20]. In the current study, the data with TNF-α-treated trophoblasts showed that the level of miR-206 was negatively correlated with viability and invasion ability of trophoblasts, while was positively related with the production of inflammatory cytokines, further confirming the pro-PE role of miR-206 based on clinical investigation.

The downstream effector of miR-206, AGTR1,

was shown to be down-regulated both in PE patients and in TNF-α-treated trophoblasts. The factor is involved in the regulation of angiotensin II that is the major signaling molecule of the RAS. AGTR1 is majorly distributed in brain, lung, heart, kidney, and vascular system, and the dysfunction of the factor will lead to the disrupted equilibrium of vasoconstriction, vascular growth, aldosterone synthesis, and renin synthesis and release [27]. Regarding PE, molecular evidence has shown that AGTR1 autoantibodies are potential contributors to PE [28]. However, there are also studies indicating that certain mutation in AGTR1 gene may not have relation with the onset of PE [29]. In the current study, our data showed a negative relation between AGTR1 and the progression of PE. Furthermore, the direct interaction between miR-206 and AGTR1 was also verified with a dual luciferase assay. To confirm the indispensable role of miR-206/AGTR1 axis in the pathogenesis of PE, the levels of the two factors were modulated in TNF-α-treated trophoblasts. The data showed both miR-206 inhibition and AGTR1 overexpression would attenuate impairments on trophoblasts induced by TNF-α. Combined with the clinical investigation, it was concluded that the induced miR-206 level contributed to the inhibition of AGTR1, which further induced the disequilibrium in AGTR1-mediated maternal renin-angiotensin system [30] and finally resulted in the onset of PE.

Collectively, the miR-206/AGTR1 was a key signaling transduction in PE development: the induced expression of miR-206 would induce inflammatory response, and reduce viability and invasion ability of trophoblasts, which depended on the inhibition of AGTR1. The findings outlined in the current study highlighted the potential of miR-206/AGTR1 axis as promising targets for the treatment of PE. The development of future anti-PE strategies can take the signaling transduction into consideration.

Conflict of Interest

There is no conflict of interest.

Acknowledgements

Not applicable.

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Supplementary Table 1. Primer information.

	Direction	Sequence (5'→3')
miR-206	Forward	GTTATGGAATGTAAGGAAGTGTGTGG
	Reverse	CCATCATGCTCTCGACCTGTC
U6	Forward	GCTTCGGCACATATACTAAAAT
	Reverse	CGCTTCACGAATTTGCGTGTCAT