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miR-486-5p inhibits invasion and migration of HTR8/SVneo trophoblast cells by down-regulating ARHGAP5

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ABSTRACT

Introduction: Appropriate implantation of trophoblast cells is necessary for successful pregnancy outcome. This process requires proper migration and invasion of trophoblast cells into the maternal endometrium and the myometrium. Dysregulation of circulating microRNAs in preeclampsia has been reported in several studies. Furthermore, miR-486-5p was reportedly increased within exosomes derived from maternal circulation in preeclamptic pregnancy. However, the roles of elevated miR-486-5p in preeclampsia has not yet been clarified.

Methods: HTR8/SVneo trophoblast cells were transfected with miR-486-5p, and the ARHGAP5 expression was examined by quantitative reverse transcription polymerase chain reaction (RT-qPCR) and Western blotting. A reporter assay using a luciferase construct containing the ARHGAP5 3′-untranslated region (3′UTR) was performed to determine whether or not ARHGAP5 is a direct target of miR-486-5p. Changes in migration and invasion abilities were examined by a wound healing assay and invasion assay, respectively.

Results: The ARHGAP5 expression was significantly decreased in miR-486-5p-transfected cells according to RT-qPCR and Western blotting. A dual luciferase reporter gene assay showed that miR-486-5p acts directly on the 3′UTR of ARHGAP5 mRNA. The migration and invasion abilities were suppressed in miR-486-5p-transfected cells. Downregulation of ARHGAP5 by small interfering RNA transfection inhibited trophoblast cell migration and invasion, resembling that of miR-486-5p transfection.

Discussion: The migration and invasion abilities of HTR8/SVneo cells were suppressed by miR-486-5p at least partly through inhibiting the ARHGAP5 expression. These data suggest that miR-486-5p is involved in the pathogenesis of preeclampsia and that miR-486-5p is a viable potential biomarker for predicting the onset risk of preeclampsia.

1. Introduction

Implantation is a highly regulated process wherein trophoblast cells invade and migrate into the maternal endometrium and the myometrium. Trophoblast cells penetrate the uterine spiral arteries and remodel them to increase blood flow towards the placenta. Failure of these processes has been indicated in the pathogenesis of preeclampsia [1,2]. Preeclampsia is a multisystemic pregnancy-associated disorder characterized by hypertension and proteinuria. It affects 2%–8% of all pregnancis, and causes maternal, fetal and neonatal morbidity and mortality [3]. At present, no effective treatment for this disease has yet been developed.

Rho GTPase-activating protein 5 (ARHGAP5; also known as RhoGAPp190-B, p190-B), is the most abundant GTPase-activating protein (GAP) for the Rho family of small GTPases. It plays a role in the regulation of actin cytoskeleton-based mechanisms, thus influencing cell migration and invasion [4,5]. Previous studies have shown that ARHGAP5 can play an oncogenic role by enhancing the migration and invasion activity of tumor cells, such as breast cancer [6], nasopharyngeal cancer [7], non-small cell lung cancer [8], colorectal carcinoma [9], hepatocellular carcinoma [10] and gastric cancer [11].

Micro RNAs (miRNAs) play critical roles in developmental and physiological processes by regulating the expression of their target genes. Increasing evidence suggests that miRNAs affect several aspects of trophoblast cells, such as migration and invasion [12–16]. Furthermore, the downregulation of miR-486-5p reportedly contributes to tumor progression and metastasis by targeting ARHGAP5 in lung cancer [8]. However, a previous study found that miR-486-5p in exosomes

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secreted by placenta is higher in preeclampsia pregnancy than in normal pregnancy [17]. In addition, our preliminary studies revealed that miR-486-5p in plasma was significantly elevated in women with preeclampsia compared with normal pregnant women. However, the relationship between the miR-486-5p and ARHGAP5 expression in preeclampsia has not been examined.

We herein report our investigation of the function of miR-486-5p in preeclampsia pregnancy.

2. Materials and methods

2.1. Cell and cell culture

Cells from the immortalized human trophoblast cell line HTR-8/SVneo were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured in RPMI-1640 media (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; Gibco). The cells were cultured in a humid incubator buffered with 5% CO_2 and 95% air at 37 °C.

2.2. Transfection of precursor miRNA or siRNA

Pre-miR miRNA precursor molecules (hsa-miR-486-5p and Negative Control miRNA) were obtained from Ambion (Waltham, CA, USA). Small interfering RNA (siRNA) against ARHGAP5 (sc-44077) was obtained from Santa Cruz Biotechnology (Dallas, TX, USA). Negative Control siRNA (sc-37007) was also obtained from Santa Cruz Biotechnology. HTR-8/SVneo cells were transfected using Lipofectamine RNAiMAX transfection reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Transfected cells were cultured at 37 °C with 5% CO_2. Forty-eight hours later, cells were collected for the subsequent study.

2.3. Real-time polymerase chain reaction (PCR)

Real-time quantitative PCR (qPCR) was performed as described previously [18]. In brief, total RNA was extracted from cells using the RNeasy kit (Qiagen, Germantown, MD, USA). The Super Script II Reverse Transcriptase (Invitrogen) was used to synthesize cDNA using random primers. Real-time qPCR was performed in triplicate using the StepOne Real-Time PCR System (Applied Biosystems, Carlsbad, USA). GAPDH was used as endogenous internal control. Micro RNA extraction was performed using the mirVana miRNA isolation kit (Invitrogen) according to the manufacturer's instructions. RNA containing miRNA was then reverse transcribed using the MicroRNA reverse transcription kit (Applied Biosystems) in combination with the stem-loop primer for miR-486-5p and the endogenous control RNU48 (Applied Biosystems) and served as a template for the quantification of the expression of mature miRNA. qPCR of miRNA was performed according to the manufacturer's instructions (Applied Biosystems). Data analyses were performed using the ΔΔCq method [19].

2.4. Western blot analysis

Whole proteins of cells were extracted with Pierce RIPA buffer (Thermo Fisher Scientific, Waltham, CA, USA). The protein concentrations were determined using a Pierce BCA Protein Assay Kit (Thermo Fisher Scientific). Equal amounts of lysate proteins from each sample were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; Bio-Rad Laboratories, Hercules, CA, USA) and transfected onto PVDF membranes. After blocking with 10% bovine serum albumin (New England BioLabs, Ipswich, MA, USA) for 1 h at room temperature, the membranes were incubated with primary antibodies at 4 °C overnight, followed by incubation with secondary antibodies at 37 °C for 1 h. Antibodies including anti-Rho GAP p190-B (sc-393241, 1:1000), horseradish peroxidase conjugated anti-rabbit IgG (sc-2357, 1:2000) and horseradish peroxidase conjugated anti-mouse IgG (sc-2031, 1:1000) were obtained from Santa Cruz Biotechnology. Anti-β-actin (#4970, 1:1000) was obtained from Cell Signaling Technology (Boston, MA, USA). The blots were visualized using enhanced chemiluminescence (ECL Plus; GE Healthcare Life Sciences, Pittsburgh, PA, USA).

2.5. Wound healing assay

To determine cell migration, transfected cells were placed in six-well plates and incubated to reach confluence. The monolayer was scratched using a 200micropipette tip. The cells were rinsed with phosphate-buffered saline to remove detached cells and then incubated in medium without FBS. Time-lapse images were captured at 0 h and 24 h. The wound width was measured using the ImageJ software program (NIH). The wound migration rate was calculated according to the article by Grada et al. [20] as follows: $\mu m/h = (A_0\text{-}A_{24})/24$, where A_0 represents the width of the initial wound, and A_{24} represents the remaining width of the wound at 24 h after injury.

2.6. Cell invasion assay

Migratory and invasive abilities were assessed using Corning BioCoat Matrigel Invasion Chamber, (Corning, NY, USA), according to the manufacturer's instructions. In brief, transfected HTR-8/SVneo cells containing serum-free medium were seeded in the upper chambers. Medium containing 10% FBS was added to the lower chambers. After incubation for 24 h, non-invading cells were removed with a cotton swab from the upper side of the membrane. Cells that penetrated the membrane were fixed in 4% methanol for 10 min and stained with 0.1% crystal violet. In each insert, three microscopic fields were photographed randomly and cell numbers were counted manually.

2.7. Luciferase reporter assay

Dual-Luciferase miRNA Target Expression vector containing 3′UTR of ARHGAP5 (HmiT010578-MT05) and pEZX-MT05 control vector (CmiT000001-MT05) were purchased form GeneCopoeia (Rockville, MD, USA). HTR8/SVneo seeded in 24-well plates were co-transfected with Lipofectamine 2000 (Invitrogen) complexed with the 3′UTR of ARHGAP5 vector or pEZX-MT05 control vector and Pre-miR-486-5p or pre-miR-negative control according to the manufacturer's instructions. The luciferase activity was assayed at 48 h after transfection, using the Secrete-Pair Dual Luminescence Assay kit (GeneCopoeia) according to the manufacturer's instructions.

2.8. Sample collection

Placental tissues were obtained immediately at birth. The collection of placental tissues was performed with the approval of the local ethical committee in the Osaka Medical and Pharmaceutical University, and written informed consent was obtained from all patients enrolled in this study. Classification of PE was according to the American College of Obstetricians and Gynecologist (ACOG) guidelines.

2.9. Immunohistochemistry

The tissue samples were fixed in formalin and embedded in paraffin. Deparaffinized and rehydrated sections (4 μm) were heated by microwave in 0.01 mol/l citrate buffer with a pH of 6.0 for 15 min for antigen retrieval. After blocking, the sections were incubated at 4 °C overnight with anti-ARHGAP5 antibodies (Sigma-Aldrich, HPA046993, 1:10). Then, the samples were incubated with biotinylated second antibodies for 1 h. Finally, the slides were washed with PBS and incubated with H_2O_2/diaminobenzidine substrate solution for 5 min.

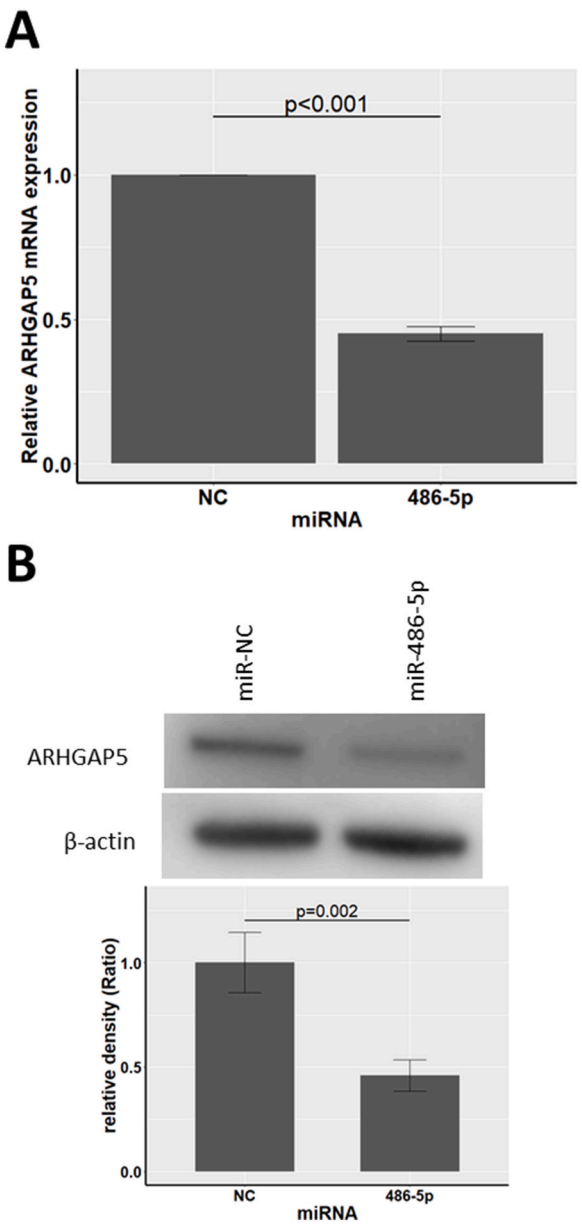

Fig. 1. Effects of transfection of miR-486-5p on the ARHGAP5 expression in HTR8/SVneo cells.
HTR-8/SVneo cells were transfected with either miR-486-5p or negative control (NC) microRNA. The ARHGAP5 expression in HTR8/SVneo cells was analyzed by RT-qPCR (A) and a Western blot analysis (B) at the mRNA and protein levels, respectively.

2.10. Statistical analyses

Data were analyzed using the R software program. The difference between two groups was examined by Welch's two-sample *t*-test at a significance level of $p < 0.05$.

3. Results

3.1. The overexpression of miR-486-5p suppresses the ARHGAP5 expression in HTR8/SVneo trophoblast cells

The overexpression of miR-486-5p was induced by the transfection of Pre-miR-486-5p, and we investigated whether or not the ARHGAP5 expression could be altered by the overexpression of miR-486-5p.

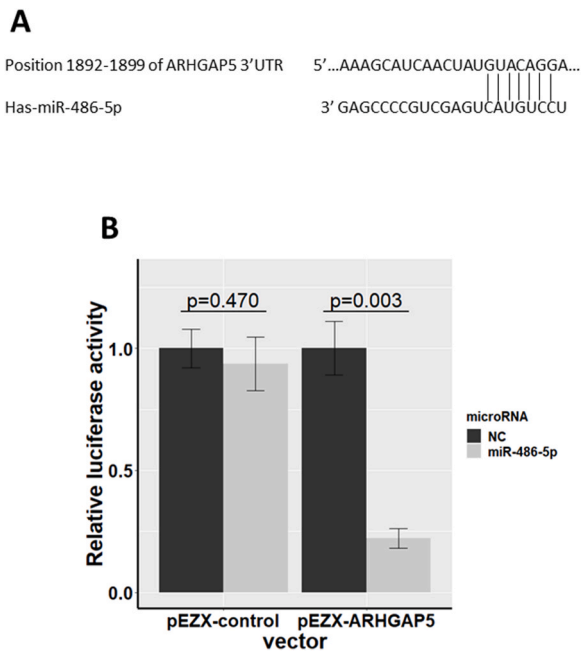

Fig. 2. ARHGAP5 is a direct target of miR-486-5p in HTR8/SVneo cells.
The bioinformatic algorithm predicted the 3′-UTR of ARHGAP5 to have a target site of miR-486-5p (A). HTR8/SVneo cells were co-transfected with the ARHGAP5 3′UTR reporter construct (pEZX-ARHGAP5) or control vector (pEZX-control) and with miR-486-5p or negative control (NC) micro RNA. A dual-luciferase reporter gene assay was performed to detect the luciferase activity (B).

Reverse transcription qPCR (RT-qPCR) revealed that the mRNA level of ARHGAP5 was decreased in Pre-miR-486-5p-transfected cells compared with negative control miRNA (Pre-miR–NC)–transfected control cells (Fig. 1A). The protein level of ARHGAP5 was also decreased by miR-486-5p overexpression compared with Pre-miR-NC (Fig. 1B).

3.2. ARHGAP5 is a direct target of miR-486-5p

The bioinformatic tool TargetScan Human 7.2 (http://www.targetscan.org/) showed that the 3′UTR of the ARHGAP5 gene contains a putative miR-486-5p target site (Fig. 2A). To investigate whether or not miR-486-5p directly targets the ARHGAP5 3′UTR, we performed a dual luciferase reporter assay. The luciferase activity of ARHGAP5-3′UTR was significantly reduced in cells co-transfected with miR-486-5p and a plasmid containing a luciferase reporter driven by the wild-type human ARHGAP5 3′UTR (pEZX-ARHGAP5) compared with complexes of negative control miRNA (NC) and pEZX-ARHGAP5 (Fig. 2B). These results indicated that ARHGAP5 is a direct target of miR-486-5p.

3.3. The migration and invasion of HTR8/SVneo cells were suppressed by the overexpression of miR-486-5p

To explore the role of miR-486-5p in the biological functions of trophoblast cells, we performed a wound healing assay and invasion assay with HTR8/SVneo cells transfected with Pre-miR-486-5p or negative control miRNA. As shown in Fig. 3A and B, the migration ability was significantly inhibited in Pre-miR-486-5p-transfected cells compared with negative control cells. Transfection of Pre-miR-486-5p also inhibited HTR8/SVneo cell invasion (Fig. 4A and B). These findings suggest that the overexpression of miR-486-5p can suppress the migrative and invasive capacity of HTR8/SVneo trophoblast cells.

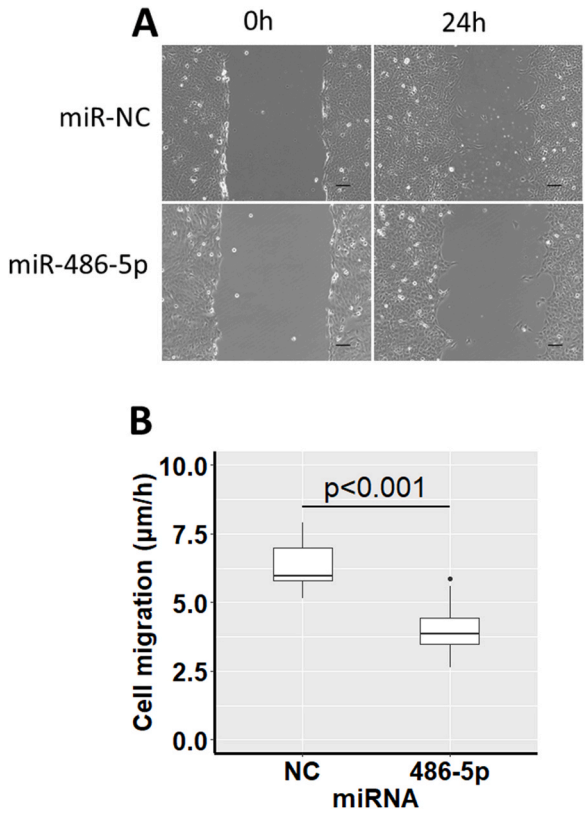

Fig. 3. Migration ability of HTR8/SVneo cells after overexpression of miR-486-5p.
Representative images of the wound healing assay (A) and quantification (B). Transfection of miR-486-5p suppressed the migration compared with the transfection of negative control (NC) micro RNA. Scale bars: 100 μm.

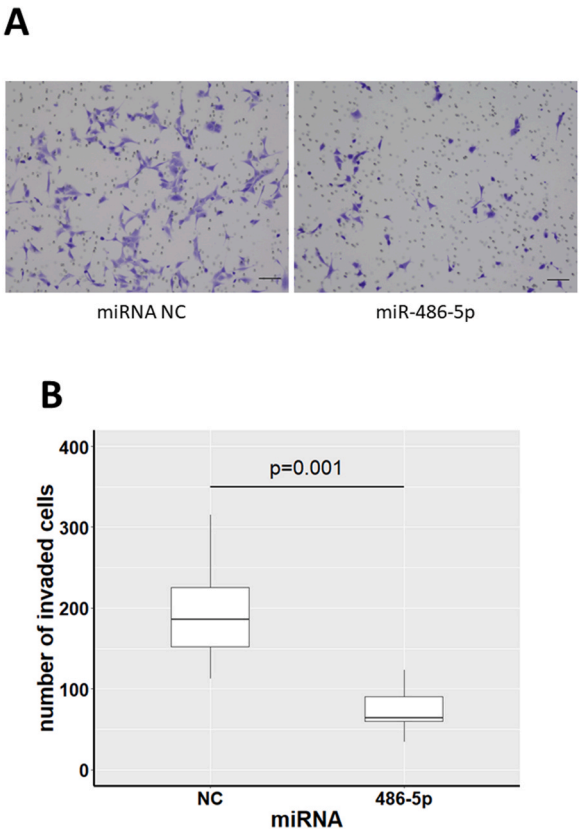

Fig. 4. Invasion ability of HTR8/SVneo cells after overexpression of miR-486-5p.
Representative images of the invasion assay (A) and quantification (B). Transfection of miR-486-5p suppressed the invasion compared with the transfection of negative control (NC) micro RNA. Scale bars: 100 μm.

3.4. Knockdown of ARHGAP5 can mimic the result of miR-486-5p overexpression

To confirm whether or not the decreased migrative and invasive capacity in miR-486-5p-transfected trophoblast cells was dependent on the suppression of ARHGAP5 expression, ARHGAP5 siRNA and negative control siRNA were transfected in HTR8/SVneo cells. We first confirmed that the ARHGAP5 expression was suppressed in ARHGAP5 siRNA-transfected cells compared with negative control siRNA-transfected cells at both the mRNA and protein levels (Fig. 5A and B). We then performed a wound healing assay and invasion assay to evaluate the effect of ARHGAP5 silencing on the migration and invasion abilities of HTR8/SVneo cells. As shown in Fig. 5C and D, knockdown of ARHGAP5 significantly suppressed the migration ability of HTR8/SVneo cells. The number of invaded cells was also significantly decreased in ARHGAP5 siRNA-transfected cells compared with control siRNA-transfected cells (Fig. 5E and F). These results collectively suggested that increased the expression of miR-486-5p in serum of preeclampsia pregnant women was involved in the suppression of migrative and invasive ability of trophoblast cells through inhibiting the ARHGAP5 expression.

3.5. miR-486-5p and ARHGAP5 expression in the placenta

To confirm whether or not placental miR-486-5p level was increased in preeclampsia patients compared with normal pregnancy, RT-qPCR was conducted on preeclampsia-complicated placenta (n = 14) and normal pregnant controls (n = 8). As shown in Fig. 6A, miR-486-5p expression in the placenta was significantly higher in preeclampsia compared with normal pregnancy. We further assessed the expression of ARHGAP5 in the placental samples. As shown in Fig. 6B, ARHGAP5 in the placental tissue was highly expressed in normal pregnancy compared with preeclampsia. These observations support our *in vitro* data demonstrating downregulation of ARHGAP5 via increased miR-486-5p in preeclampsia.

4. Discussion

Previous studies have shown that the miRNA expression in the placenta and maternal plasma differs between preeclampsia and normal pregnancy [21–25]. Maternal circulating miRNAs are thus considered to have potential utility as biomarkers for diagnosing or predicting preeclampsia because of their stable values and capability of being sampled non-invasively [26,27].

Our preliminary study showed that miR-486-5p levels were elevated in plasma of preeclampsia patients compared with normal pregnant patients. This result is consistent with that of a previous study showing that miR-486-5p was the most significantly elevated miRNA in preeclampsia pregnancies compared with normal pregnancies in maternal plasma placenta-derived exosomes [17].

Preeclampsia has been recognized as related to poor placental implantation. In the present study, we demonstrated that the migrative and invasive abilities of trophoblast cells were significantly inhibited by miR-486-5p. We also revealed a possible pathogenesis of preeclampsia by which dysfunction of trophoblast cells partly depends on the increased miR-486-5p, which modulates the ARHGAP5 expression of trophoblast cells.

miR-486-5p is located on chromosome 8p11.21, and originally identified as a tumor suppressor in lung cancer. Pim-1 kinase oncogene was the target for miR-486-5p in lung cancer [28]. A growing body of

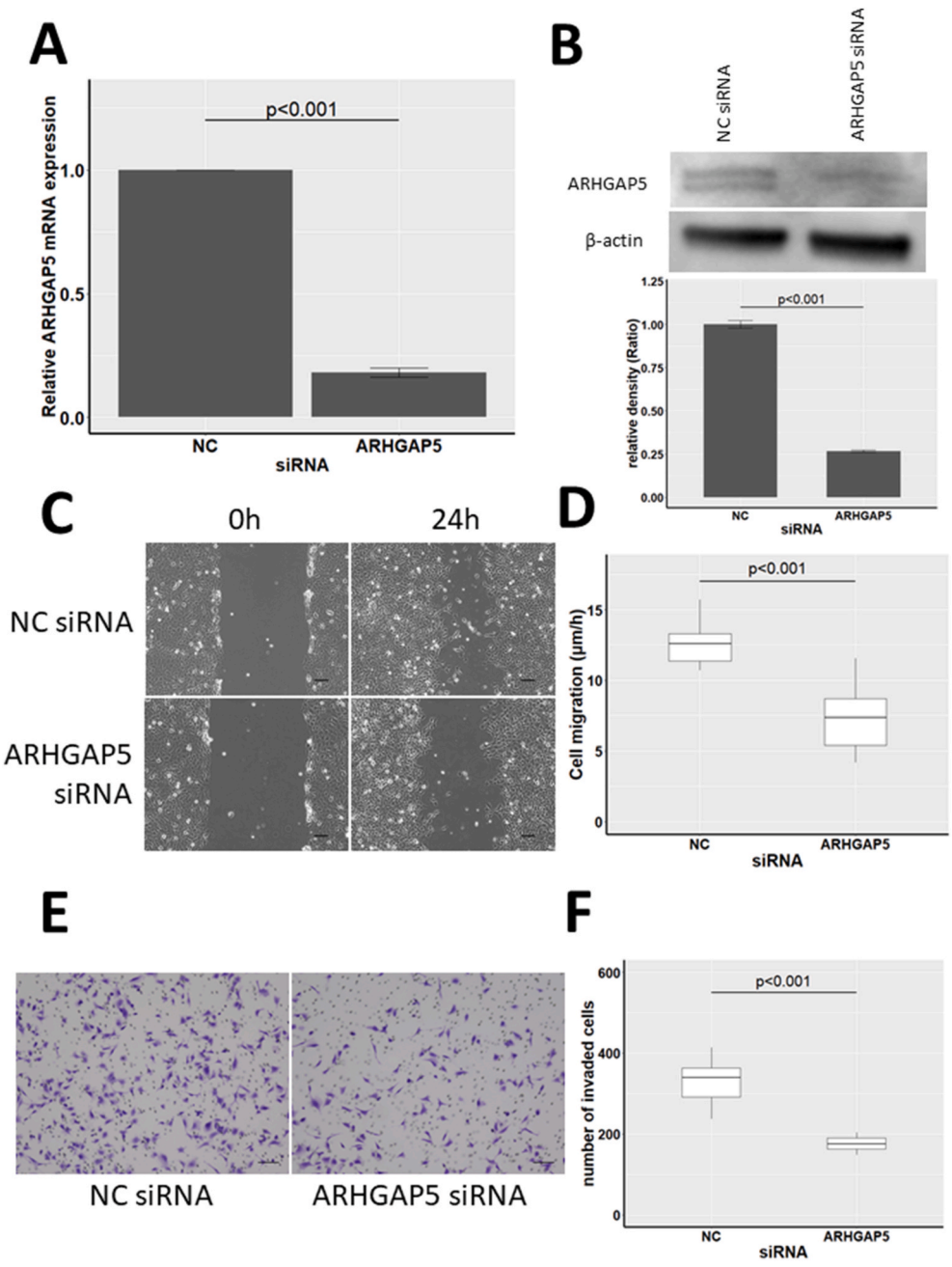

Fig. 5. Effects of silencing ARHGAP5 in HTR8/SVneo cells. HTR-8/SVneo cells were transfected with ARHGAP5 siRNA or negative control (NC) siRNA. The ARHGAP5 expression in HTR8/SVneo cells was analyzed by RT-qPCR (A) and a Western blot analysis (B) at the mRNA and protein levels, respectively. Representative images of the wound healing assay (C) and quantification (D). Transfection of ARHGAP5 siRNA suppressed the migration compared with the transfection of negative control (NC) siRNA. Scale bars: 100 μm. Representative images of the invasion assay (E) and quantification (F). Transfection of ARHGAP5 siRNA suppressed the invasion compared with the transfection of negative control (NC) siRNA. Scale bars: 100 μm.

reports show that miR-486-5p plays a tumor suppressive role in various cancers. For example, lung cancer cell proliferation and migration were attenuated by miR486-5p via the inhibition of insulin-like growth factor 1 (IGF1) and IGF1 receptor (IGF1R) [29]. Liu et al. reported a negative correlation between the miR-486-5p expression and the invasive potential in osteosarcoma cells by inhibiting PIM1 which mediates epithelial-mesenchymal transition [30]. In gastric cancer, miR-486-5p suppressed proliferation and migration through targeting fibroblast growth factor (FGF) 9 [31]. In addition, a recent report indicated that the cell migration and invasion ability of lung cancer cells was inhibited by miR486-5p through reducing the ARHGAP5 expression [8]. These results underscore the potential utility of miR-486-5p as a therapeutic target in cancer.

The ARHGAP5 gene encodes Rho GAP5, which is a member of the Rho GAP family. ARHGAP5 plays an important role in tumor progression, particularly with regard to cellular adhesion and motility [32]. Recent studies have suggested that upregulation of ARHGAP5 contributes to the invasive and metastatic behavior of a variety of cancers. However, the functions of ARHGAP5 in trophoblast cells have not yet been elucidated. Consistent with the previous findings concerning cancer, the results obtained from our current study showed that a reduced ARHGAP5 expression suppressed the migration and invasion ability of HTR8/SVneo human trophoblast cells. In addition, our functional analysis showed that trophoblast cell migration and invasion was also inhibited by small interfering RNA-mediated knockdown of ARHGAP5. Consequently, our findings suggested that the downregulation of ARHGAP5 might be involved in incomplete trophoblast cell motility, which is widely recognized as a pathogenesis of preeclampsia, and that miR-486-5p may serve as a potential predictor of preeclampsia and a target for therapeutic intervention against preeclampsia.

A number of nanoparticle formulations have proven to be successful platforms for delivering miRNA mimics and anti-miRNA. Polyethylene

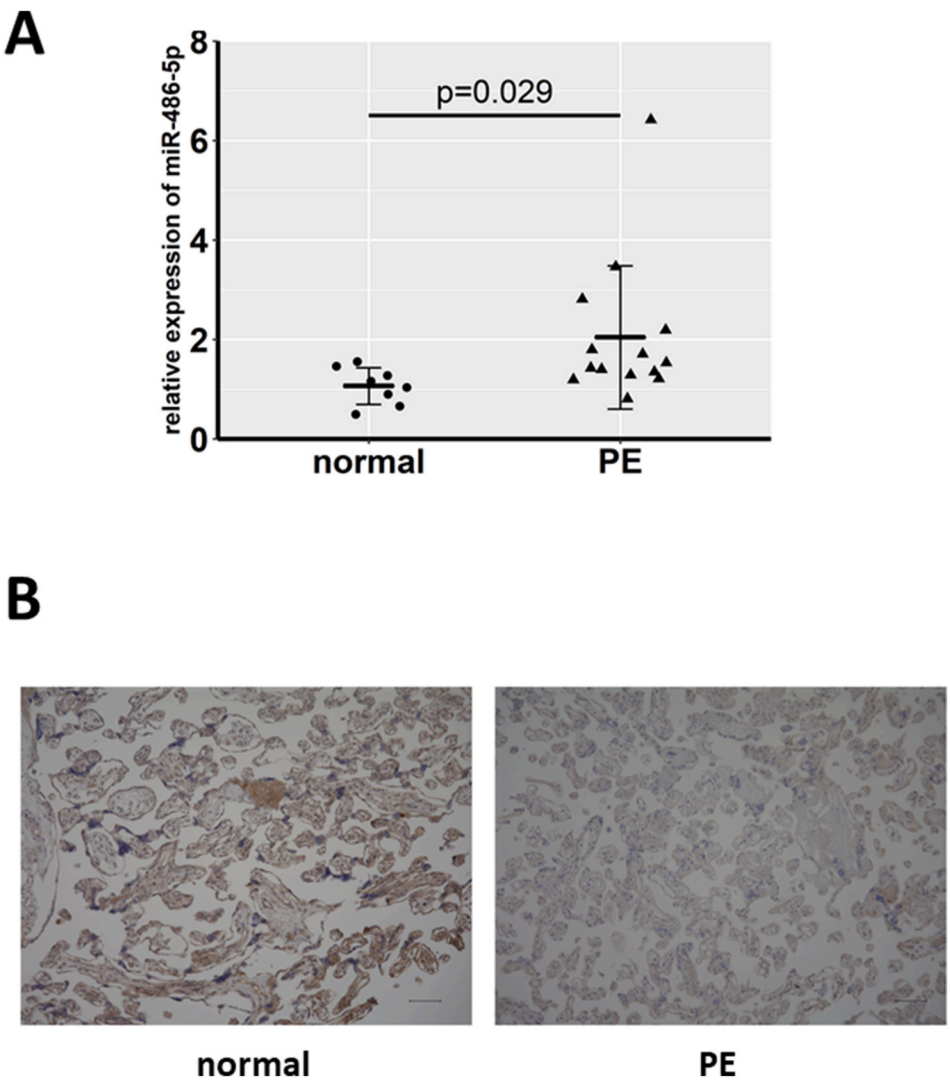

Fig. 6. miR-486-5p and ARHGAP5 expression in the placenta. The expression of miR-486-5p (A) and ARHGAP5 (B) was examined by RT-qPCR and immunohistochemistry, respectively. The relative expression of miR-486-5p (A) in the placenta from preeclampsia (PE) (median, n = 14) was higher than that of normal pregnancy (median, n = 8). Representative immunohistochemical staining of ARHGAP5 in the placentas from normal pregnancy and preeclampsia pregnancy (B). Scale bars: 100 μm.

glycol (PEG)-coated liposomes encapsulated with miR-134 inhibited growth of skin squamous cell carcinoma in a xenograft mouse model through the downregulation of the Forkhead Box M1 [33]. Preloading with anti-miR-21 and anti-miR-10b encapsulated in PEG-polyactide-co-glycolide (PLGA) increased chemosensitivity in glioblastoma cell xenografts in mice [34]. Nanomedicine has become a hot topic in reproductive medicine. In human placenta, PEGylated gold nanoparticles 10–30 nm in diameter were found to reach the trophoblast cell layer, but not the fetus [35]. Recently, nanomedicine has offered the potential to stably encapsulate siRNA and transfer it to trophoblast cells. Yu et al. reported that sFlt1 siRNA conjugated to poly-amidoamine significantly reduced sFlt1 secretion from cultured HTR-8/SVneo trophoblast cells as well as circulating sFlt1 levels, mean arterial pressure and the urine protein level in preeclampsia rat model. This early report suggests a possible therapeutic model of delivering siRNAs in preeclampsia pregnancy [36]. These nanoparticles might be able to be adapted to transport anti-miR-486-5p in order to treat cases of compromised placental development in preeclampsia pregnancy.

Several limitations associated with the present study warrant mention. Our study only involved a cell model analysis, and whether or not these findings are also relevant *in vivo*, including the notion of nanoparticles encapsulated with anti-miR486-5p as therapy in a preeclampsia mouse model, remains to be confirmed.

In conclusion, the present findings suggest that miR-486-5p may play a pathogenic role in the HTR8/SVneo human trophoblastic cell line by downregulating ARHGAP5 expression. Although further *in vivo* functional and pathological studies are needed to clarify its function, miR-486-5p may be useful as a predictive biomarker and therapeutic target in the treatment of preeclampsia.

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Declarations of interest

None.

Author contributions

M.H. designed the research. S.T. and M.H. wrote the manuscript. S. T., M.H., M.N., N.N. and M.O. were involved in data analysis and interpretation. All authors critically revised the report, commented on drafts of the manuscript, and approved the final report.

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