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MiR-222-5p promotes the growth and migration of trophoblasts by targeting AHNAK

X. DONG[1], J. ZHAO[2], J. HAN[1], X.-J. HAN[1], C.-M. ZHAO[3], A.-X. ZOU[3], H.-G. QI[3]

[1]Department of Obstetrics and Gynecology Emergency, The Second Women's Insurance Hospital of Jinan City, Jinan, China
[2]Department of Reproductive Center, The Second Women's Insurance Hospital of Jinan City, Jinan, China
[3]Department of Obstetrics and Gynecology, The Second Women's Insurance Hospital of Jinan City, Jinan, China

Xue Dong and Jing Zhao contributed equally to this work

Abstract. – OBJECTIVE: The purpose of this study was to detect microRNA-222-5p (miR-222-5p) levels in placental tissues of preeclampsia (PE) pregnancies, and to explore the role of miR-222-5p in the proliferative and migratory potentials of trophoblast cell line HTR-8/SVneo.

PATIENTS AND METHODS: Expression levels of miR-222-5p and AHNAK in placental tissues of PE pregnancies (n=24) and healthy pregnancies (n=24) were detected by quantitative real-time polymerase chain reaction (qRT-PCR). Potential influences of miR-222-5p and AHNAK on proliferative, migratory and apoptotic potentials in HTR-8/SVneo cells were examined. At last, Luciferase assay was conducted to illustrate the interaction between miR-222-5p and AHNAK in trophoblasts.

RESULTS: It was found that miR-222-5p was downregulated in placental tissues of PE pregnancies. Overexpression of miR-222-5p stimulated proliferative and migratory potentials, and inhibited apoptosis in HTR-8/SVneo cells. Moreover, AHNAK was the target gene binding to miR-222-5p, and overexpression of AHNAK inhibited proliferative and migratory potentials and promoted apoptosis in HTR-8/SVneo cells.

CONCLUSIONS: MiR-222-5p stimulates proliferative and migratory potentials and inhibits apoptosis in HTR-8/SVneo cells by negatively regulating AHNAK.

Key Words:
MiR-222-5p, AHNAK, Trophoblasts, Preeclampsia.

Introduction

Preeclampsia (PE) is featured by hypertension, urinary protein, and edema in pregnancies, with an incidence of about 2-8%. As a severe complication during pregnancy, PE seriously endangers lives of both pregnancies and fetuses[1-3]. Insufficient trophoblast infiltration and angiogenesis disorders in the placenta are responsible for the pathogenesis of PE. Expression levels of various molecules associated with invasion and angiogenesis are significantly changed during the disease course of PE[4].

Differentially expressed miRNAs have been discovered in placenta and peripheral blood of PE pregnancies, and they potentially influence the development of PE[5-7]. In addition, miRNAs are vital regulators in trophoblast phenotypes[8]. Trophoblasts are the main components of placenta, which are of significance to the formation of placenta[9]. Of note, trophoblasts maintain pregnancy in the first trimester, and dysfunctional trophoblasts are suggested to result in insufficient remodeling of spiral arteries, as well as ischemia and hypoxia of placenta. Zhu et al[10] proposed that miRNAs are able to influence biological functions of trophoblasts through targeting downstream genes. Of note, miR-29b inhibits angiogenesis and stimulates apoptosis in trophoblasts, thereafter, regulating the development of PE[11].

Li et al[12] showed that miR-222-5p is abnormally expressed in epithelial ovarian cancer, serving as a potential biomarker. MiR-222-5p is also involved in atherosclerosis by mediating lipid metabolism[13]. In this paper, it was found that miR-222-5p was downregulated in placental tissues of PE pregnancies, and its regulatory effects on trophoblast phenotypes were mainly explored.

Corresponding Author: Xue Dong, BM; e-mail: jnsdefybjybgs@jn.shandong.cn

Patients and Methods

Sample Collection

Placental tissues were collected from PE pregnancies (n=24) and healthy pregnancies (n=24) in The Second Women's Insurance Hospital of Jinan City. Pregnancies with gestational diabetes, placenta previa, placental abruption or other complications during pregnancy were excluded, and all the maternal sources of preeclampsia placental tissue met the diagnostic criteria (ACOG) for the disease. This investigation was approved by the Ethics Committee of The Second Women's Insurance Hospital of Jinan City and conducted after informed consent of each subject was obtained.

Cell Culture

Human trophoblast cell line HTR-8/SVneo was purchased from American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured in Roswell Park Memorial Institute-1640 (RPMI-1640, HyClone, South Logan, UT, USA) containing 10% fetal bovine serum (FBS) (Gibco, Rockville, MD, USA) in a 5% CO_2 incubator at 37°C.

Cell Transfection

Transfection plasmids were synthesized by GenePharma (Shanghai, China). Cells were inoculated in a 6-well plate one day prior to transfection and then transfected with 50-100 nM plasmids using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). The original medium was replaced with a fresh one at 6 h.

Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

Total RNAs in cells and placental tissues were isolated and reversely transcribed into complementary deoxyribose nucleic acids (cDNAs), followed by qRT-PCR (TaKaRa, Otsu, Shiga, Japan). Relative level of the target was calculated using $2^{-\Delta\Delta Ct}$ method. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) served as the internal reference. Primer sequences are listed as follows: miR-222-5p: 5′-ACACTCCAGCTGGG CTCAGTAGCCAGTGTA-3′ (forward), 5′-CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGAGGAUCUA-3′ (reverse), U6: 5′-CTCGCTTCGGCAGCACA-3′ (forward), 5′-AACGCTTCACGAATTTGCGT-3′ (reverse), Bax: 5′-CCCGAGAGGTCTTTTTCCGAG-3′ (forward), 5′-CCAGCCCATGATGGTTCTGAT-3′ (reverse), Bcl-2: 5′-GGTGGGGTCATGTGTGTGG-3′ (forward), 5′-CGGTTCAGGTACTCAGTCATCC-3′ (reverse), GAPDH: 5′-GCAAGGATACTGAGAGCAAGAG-3′ (forward), 5′-GGATGGAATTGTGAGGGAGATG-3′ (reverse), AHNAK: 5′-CCACCCCAACTGGGACTTTG-3′ (forward), 5′-CACTCCCCTGTAACTTGCCTG-3′ (reverse).

Enzyme-Linked Immunosorbent Assay (ELISA)

Culture medium was collected for detecting expression levels of Bax and Bcl-2 using the commercial ELISA kit (Sigma-Aldrich, St. Louis, MO, USA).

Cell Proliferation Assay

The cells were inoculated in a 96-well plate with 3×10^3 cells per well. At the appointed time points, absorbance value at 450 nm of each sample was recorded using the cell counting kit-8 (CCK-8) kit (Dojindo Laboratories, Kumamoto, Japan) for plotting the viability curves.

Luciferase Assay

Wild-type and mutant Luciferase vectors targeting AHNAK were constructed based on the predicted binding sequences in the 3′ untranslated region (3′ UTR) of miR-222-5p and AHNAK. Next, cells were co-transfected with wild-type/mutant-type AHNAK vectors and miR-222-5p mimics/NC, respectively using Lipofectamine 2000. 24 hours later, cells were lysed and subjected to Luciferase activity measurement (Promega, Madison, WI, USA).

Transwell Migration Assay

A total of 200 μL of suspension (5×10^5 cells/mL) were inoculated in the upper transwell chamber (Millipore, Billerica, MA, USA) inserted in a 24-well plate with 500 μL of medium containing 10% FBS in the bottom. After 48 h incubation, bottom cells were reacted with 15-min methanol, 20-min crystal violet and captured using a microscope (Olympus, Tokyo, Japan). Finally, migratory cells were counted in 10 random fields per sample.

Statistical Analysis

GraphPad software Version 6.0 (GraphPad Software, Inc. La Jolla, CA, USA) was used for data analysis. Data were expressed as mean ± SD (standard deviation). Differences between groups were compared using the *t*-test, and Pearson

correlation test was conducted to assess the relationship between two gene expressions. $p<0.05$ showed that the difference was statistically significant.

Results

MiR-222-5p was Downregulated in PE Pregnancies and it Stimulated Proliferative and Migratory Potentials of Trophoblasts

Placental tissues were collected from healthy pregnancies and PE pregnancies. Compared with healthy pregnancies, miR-222-5p was downregulated in placental tissues of PE pregnancies (Figure 1A), suggesting that miR-222-5p may be involved in PE. Subsequently, miR-222-5p mimics was constructed and its transfection efficacy in HTR-8/SVneo cells was tested (Figure 1B). It was found that overexpression of miR-222-5p markedly increased viability (Figure 1C) and migratory cell number (Figure 1D) in trophoblasts.

MiR-222-5p Inhibited Apoptosis in Trophoblasts

Influences of miR-222-5p on expression levels of Bax and Bcl-2 were examined. It was shown that overexpression of miR-222-5p downregulated Bax and upregulated Bcl-2 in HTR-8/SVneo cells, while knockdown of miR-222-5p yielded the opposite results (Figure 2A, 2B). In addition, expression levels of Bax and Bcl-2 in culture medium of HTR-8/SVneo cells transfected with miR-222-5p mimics or inhibitor showed the same trends as their mRNA levels (Figure 2C, 2D).

AHNAK was the Target Gene of MiR-222-5p

Binding sequences in the 3'UTR of miR-222-5p and AHNAK were identified through bioinformatics method (Figure 3A). Subsequently, Luciferase assay revealed that overexpression of miR-222-5p markedly decreased the Luciferase activity in wild-type AHNAK vector, and knockdown of miR-222-5p obtained the opposite result (Figure 3B, 3C). As a result, it was detected that

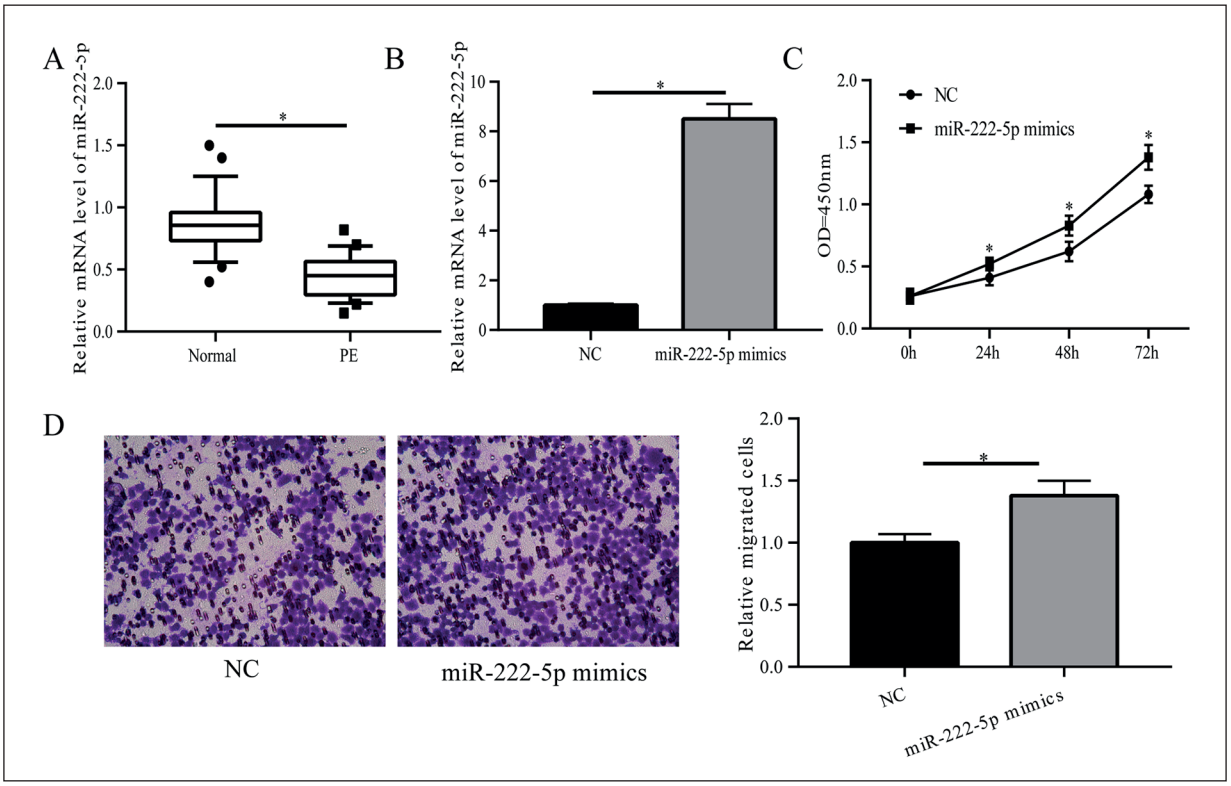

Figure 1. MiR-222-5p is downregulated in PE pregnancies and stimulates proliferative and migratory potentials of trophoblasts. **A,** MiR-222-5p levels in placental tissues of healthy pregnancies (n=24) and PE pregnancies (n=24). **B,** Transfection efficacy of miR-222-5p mimics in HTR-8/SVneo cells. **C,** Viability in HTR-8/SVneo cells transfected with miR-222-5p mimics or NC. **D,** Migration in HTR-8/SVneo cells transfected with miR-222-5p mimics or NC (magnification: 200×).

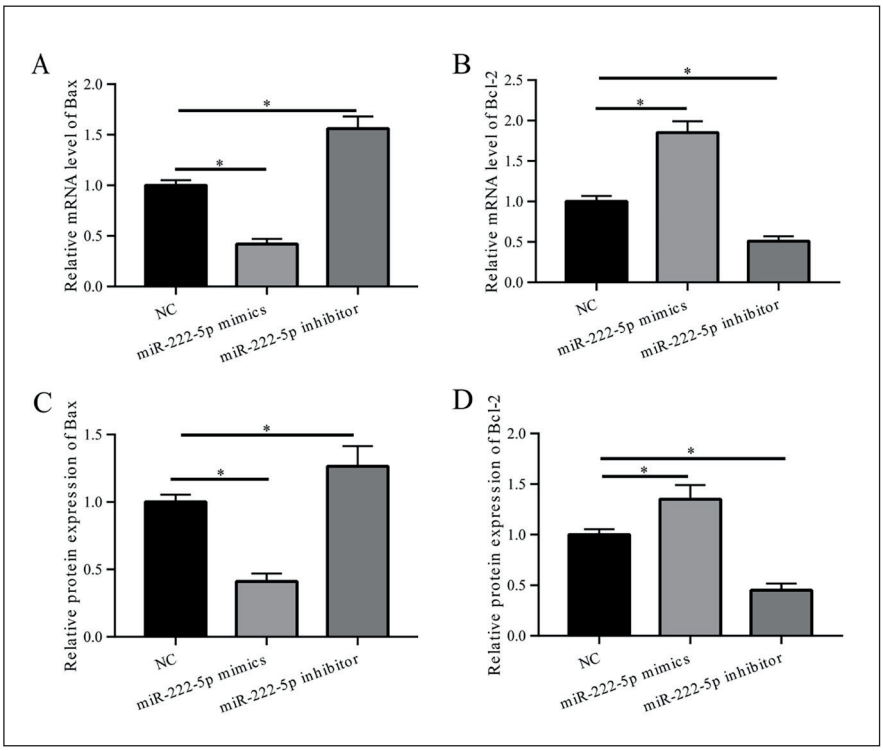

Figure 2. MiR-222-5p inhibits apoptosis in trophoblasts. **A,** Relative mRNA level of Bax in HTR-8/SVneo cells transfected with NC, miR-222-5p mimics or miR-222-5p inhibitor. **B,** Relative mRNA level of Bcl-2 in HTR-8/SVneo cells transfected with NC, miR-222-5p mimics or miR-222-5p inhibitor. **C,** Relative level of Bax in culture medium of HTR-8/SVneo cells transfected with NC, miR-222-5p mimics or miR-222-5p inhibitor. **D,** Relative level of Bcl-2 in culture medium of HTR-8/SVneo cells transfected with NC, miR-222-5p mimics or miR-222-5p inhibitor.

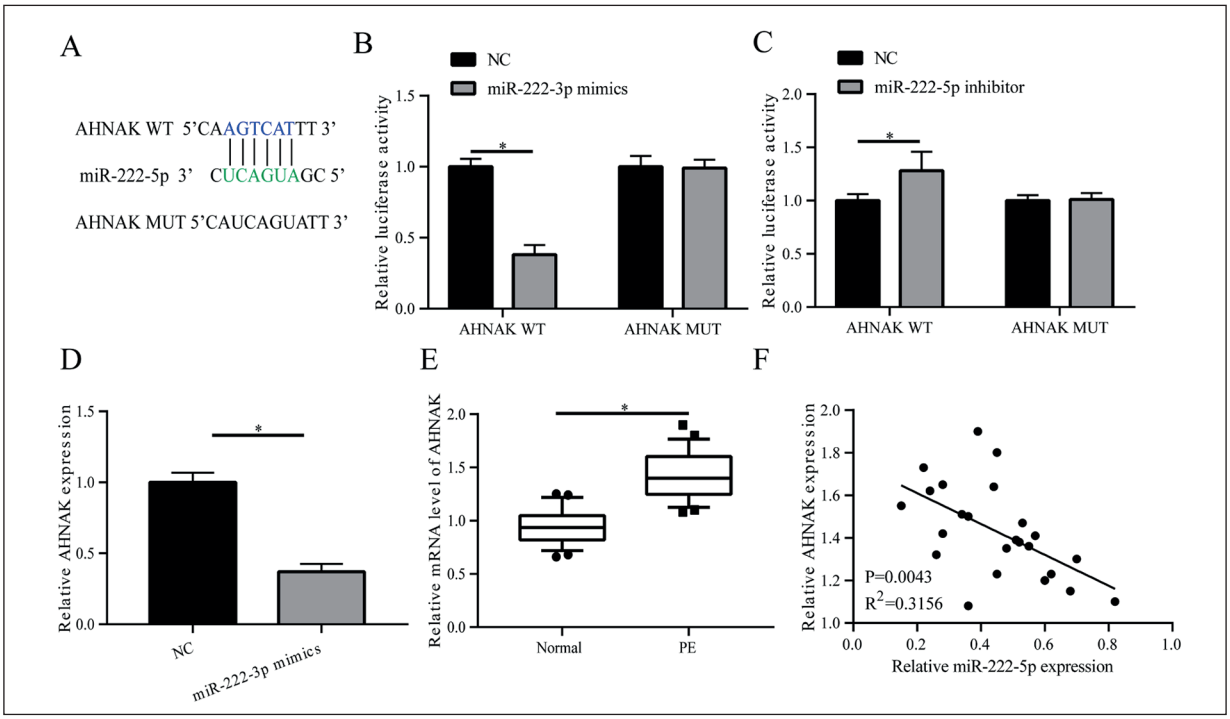

Figure 3. AHNAK is the target gene of miR-222-5p. **A,** Binding sequences in the 3'UTR of miR-222-5p and AHNAK. **B,** Luciferase activity in HTR-8/SVneo cells co-transfected with NC/miR-222-5p mimics and AHNAK WT/AHNAK MUT. **C,** Luciferase activity in HTR-8/SVneo cells co-transfected with NC/miR-222-5p inhibitor and AHNAK WT/AHNAK MUT.**D,** AHNAK level in in HTR-8/SVneo cells transfected with NC or miR-222-5p mimics. **E,** AHNAK levels in placental tissues of healthy pregnancies (n=24) and PE pregnancies (n=24). **F,** A negative correlation between expression levels of miR-222-5p and AHNAK in placental tissues of PE pregnancies.

AHNAK was the target gene binding to miR-222-5p. In HTR-8/SVneo cells overexpressing miR-222-5p, AHNAK was remarkably downregulated (Figure 3D). Moreover, AHNAK was upregulated in placental tissues of PE pregnancies (Figure 3E), and negatively correlated to miR-222-5p level (Figure 3F).

Influences of AHNAK on Trophoblast Phenotypes

Potential influences of AHNAK on trophoblast phenotypes were explored. It was shown that overexpression of AHNAK decreased viability (Figure 4A) and migratory HTR-8/SVneo cell number (Figure 4B). Furthermore, overexpression of AHNAK upregulated Bax (Figure 4C) and downregulated Bcl-2 (Figure 4D) in trophoblasts. Expression changes of Bax and Bcl-2 in culture medium of HTR-8/SVneo cells overexpressing AHNAK showed similar trends (Figure 4E, 4F).

Discussion

Multiple factors are responsible for the pathogenesis of PE, including chronic inflammation, oxidative stress, placental dysplasia, insufficient immune tolerance, genetic factors, imbalance of anti-angiogenic and pro-angiogenic factors, and placental ischemia and hypoxia. The placenta is the organ of substance exchange between mother and fetus during pregnancy. Dysfunctional placenta is a risk factor for the onset of PE. At present, biological functions of miRNAs in regulating trophoblast behaviors and neovascularization during placental development have been well concerned[14]. In this paper, miR-222-5p was downregulated in the placental tissues of PE pregnancies, suggesting that miR-222-5p may be involved in the development of PE.

The trophoblast is the fundamental organ during embryo implantation and placenta formation[15]. Functional trophoblasts contribute to the maintenance and development of normal pregnancy[16]. Suppressed proliferation and metastasis, as well as excessive apoptosis of trophoblasts result in PE[17-19]. We showed that overexpression of miR-222-5p stimulated proliferative and migratory potentials in trophoblasts, and inhibited apoptosis.

Calmodulin ANHAK, also known as desmoyokin, is abnormally expressed in many types of cells and diseases. Upregulation of ANHAK is associated with poor prognosis of pancreatic duc-

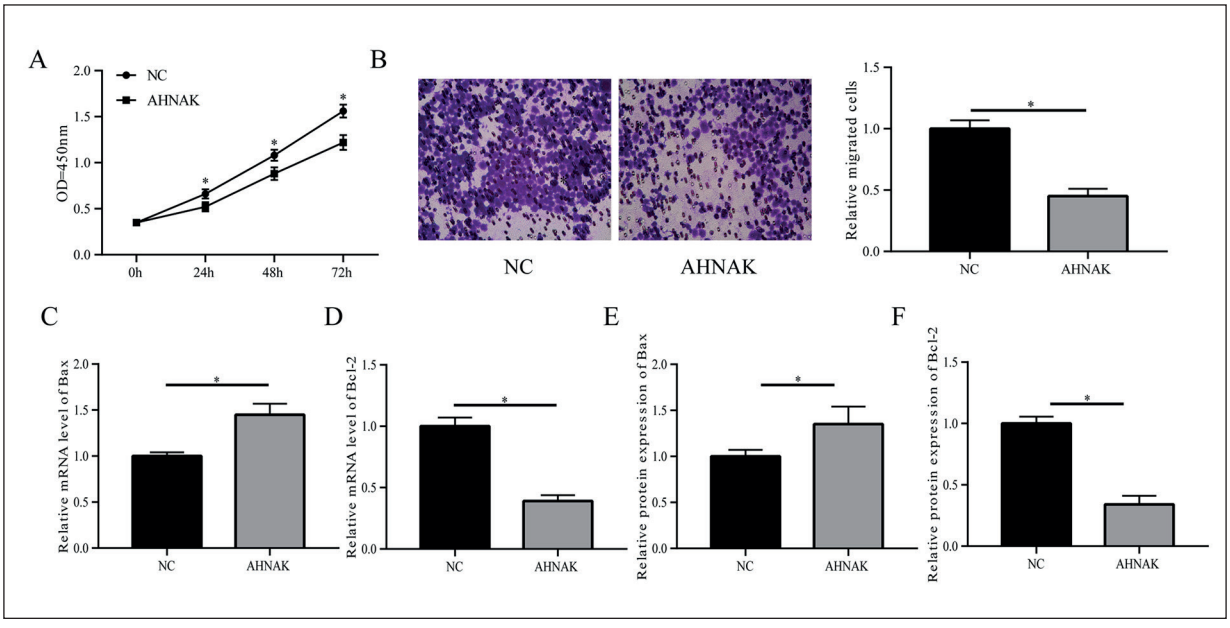

Figure 4. Influences of AHNAK on trophoblast phenotypes. **A,** Viability in HTR-8/SVneo cells transfected with pcDNA-AHNAK or NC. **B,** Migration in HTR-8/SVneo cells transfected with pcDNA-AHNAK or NC (magnification: 200×). **C,** Relative mRNA level of Bax in HTR-8/SVneo cells transfected with pcDNA-AHNAK or NC. **D,** Relative mRNA level of Bcl-2 in HTR-8/SVneo cells transfected with pcDNA-AHNAK or NC. **E,** Relative level of Bax in culture medium of HTR-8/SVneo cells transfected with pcDNA-AHNAK or NC. **F,** Relative level of Bcl-2 in culture medium of HTR-8/SVneo cells transfected with pcDNA-AHNAK or NC.

tal adenocarcinoma by mediating epithelial-mesenchymal transition[20]. Overexpression of RNF38 promotes TGF-β signaling by ubiquitinating and degrading ANHAK in hepatocellular carcinoma. In addition, ANHAK inhibits the proliferative and invasive capacities of triple-negative breast cancer cells[21]. Through bioinformatics prediction and experimental verification, ANHAK was proven to be the downstream gene targeting miR-222-5p. In trophoblasts and placental tissues of PE pregnancies, its level was negatively regulated by miR-222-5p. Contrary to miR-222-5p, ANHAK inhibited proliferative and migratory capacities, and stimulated apoptosis in trophoblasts.

Conclusions

To sum up, miR-222-5p promotes proliferative and migratory potentials in trophoblasts through negatively regulating ANHAK. Our findings provide novel targets in prevention and early intervention of PE. It is noteworthy that our conclusion should be validated in *in vivo* models in the future.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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