

## Original Article

# MicroRNA-409-3p inhibits cell proliferation, migration and invasion by targeting RAB10 in endometrial carcinoma

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**Abstract:** MicroRNAs (miRs) are small non-coding RNAs that involved in the development and progression of human cancer by negatively regulating its target genes. As one of them, miR-409-3p has been recently shown to be dysregulated in some malignancies, but its expression and biological function in endometrial carcinoma (EC) remain elusive. In this study, we found miR-409-3p was significantly downregulated in human EC tissues compared with normal endometrial tissues by quantitative real-time PCR (qRT-PCR) analysis. Functional analyses *in vitro* indicated overexpression of miR-409-3p remarkably suppressed EC cell proliferation, induced cell cycle arrest at G0/G1 phase and apoptosis, and reduced migratory activity, as revealed by CCK-8 assay, colony formation assay, flow cytometry and Transwell assays, respectively. Using bioinformatics and luciferase reporter assay, we identified RAB10 was a target of miR-409-3p. Furthermore, overexpression of miR-409-3p increased the expression of PARP cleavage and E-cadherin and decreased the expression of RAB10, CDK4, Cyclin D1, N-cadherin and Vimentin using Western blot analysis. These findings suggest that miR-409-3p could be a potential tumor suppressor in EC through the direct targeting of RAB10.

**Keywords:** Endometrial carcinoma, miR-409-3p, RAB10, cell proliferation, migration

## Introduction

Endometrial carcinoma (EC) is one of the most common female malignancies worldwide [1]. Over the past decades, its incidence and mortality rates have alarmingly increased mainly ascribed to a list of risk factors, including obesity [2], excess estrogen syndrome [3] and metal oestrogens [4]. Currently, surgery plus radiotherapy is the standard treatment for early stage disease, but is difficult for patients in advanced stage because of the unclear pathological mechanisms [5, 6]. Therefore, a deeper understanding of the molecular mechanisms responsible for EC pathogenesis is critical for developing new therapeutic strategies to improve the outcomes of patients.

MicroRNAs (miRs) are a class of short non-coding RNAs with a length of ~22 nucleotides and widely expressed in many species and tissues

[7]. They could significantly affect multiple biological processes including cell proliferation, apoptosis, metastasis and development by regulating gene expression at post-transcriptional level via directly binding to the 3'-untranslated region (UTR) of their target mRNAs [8-10]. On average, each miR could target approximately 200 mRNAs in human [11]. Accumulating evidences have revealed that aberrantly altered miRs can promote tumorigenesis by modulating expression of oncogenes or tumor suppressor genes during cancer progression [12]. In addition, miRs themselves also act as either oncogenes or tumor suppressors. Among these miRs, miR-409-3p was firstly reported to be as a tumor suppressor in gastric cancer by inducing cell growth inhibition and apoptosis by targeting PHF10 [13]. Subsequently, miR-409-3p has been demonstrated to play a suppressive role in numerous types of cancer, such as bladder cancer [14], lung adenocarcinoma [15],

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osteosarcoma [16] and breast cancer [17]. On contrary, Sajni Josson *et al.* reported that miR-409-3p was elevated in the tissues of human prostate cancer patients with higher Gleason scores and capable of promoting the malignant transformation of prostate epithelium in mice [18]. Nevertheless, little is known about the biological function of miR-409-3p and its target genes in EC.

Therefore, the present study aimed to investigate the expression of miR-409-3p in EC tissues and its biological functions, as well as the underlying mechanisms. Our data revealed a suppressive role of miR-409-3p in EC by down-regulating the level of its target gene RAB10.

### Materials and methods

#### *Tissue samples*

Total 20 paired of EC tissue specimens and adjacent normal tissue specimens were obtained from patients undergoing surgical resection from January 2013 to December 2015 at First Affiliated Hospital of Suzhou University. Tissue samples were trimmed and snap frozen in liquid nitrogen until use.

#### *Cell lines and transfection*

The human EC cell lines, RL95-2 and HEC-1B were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China) and maintained in Dulbecco's Modified Eagle's Medium (DMEM) with 10% fetal bovine serum and 1% penicillin and streptomycin in a humidified atmosphere containing 5% CO<sub>2</sub> at 37°C. MiR-409-3p mimics and scrambled mimics were purchased from GenePharma (China). For functional analysis, RL95-2 and KLE cells were each transfected with miR-409-3p mimics using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, USA) according to the manufacturer's instruction. Meanwhile, cells were transfected with scrambled mimics as negative control (NC).

#### *Quantitative real-time PCR (qRT-PCR)*

Total RNA was isolated from human tissues and the treated cells using Trizol (Invitrogen) and 2 µl RNA was reverse-transcribed to cDNA using TaqMan® MicroRNA Reverse Transcription Kit (Applied Biosystems). The expression

level of miR-409-3p was quantified using ABI PRISM® 7900HT Sequence Detection System (Applied Biosystems) following the PCR conditions: 95°C for 5 min, and 40 cycles of denaturation at 95°C for 10 sec and annealing/elongation at 60°C for 20 sec. The relative miR-409-3p expression was normalized to the expression level of U6 small nuclear RNA by the 2<sup>-ΔΔCt</sup> method.

#### *Cell viability assay*

CCK-8 assay was performed to measure the cell viability. Briefly, cells were seeded into 96-well plates after transfection with miR-409-3p mimics or scrambled mimics at a density of 2000 cells per well and incubated until adherent. Each well was added 10 µL CCK-8 solution and incubated for 4 h. Cell viability was determined every 24 h (up to 72 h). The absorbance value at 450 nm was measured using a microplate reader.

#### *Colony formation assay*

EC cells stably transfected with miR-409-3p mimics or miR-NC were trypsinized to single cell suspensions and seeded 6-well plates at 500 cell per well. After 14 days culture, the cells were fixed with methanol for 10 min and then stained with 0.5% crystal violet in 20% methanol. The representative images of colonies were observed under the microscope.

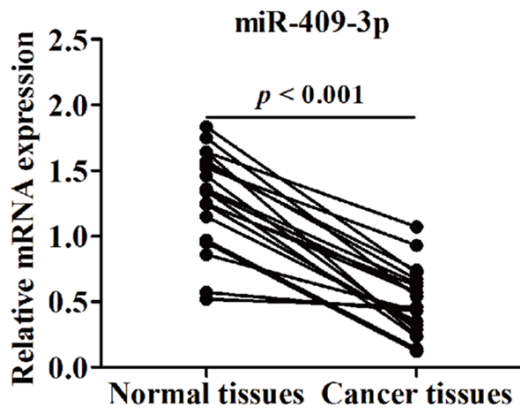
#### *Flow cytometry analysis for cell cycle and apoptosis*

Flow cytometry was used to analyze cell cycle distribution and apoptosis. For cell cycle assay, cells were reseeded in 6-cm dishes and washed twice with PBS. Then cells were trypsinized and harvested by centrifugation. Pre-cooled 70% ice-cold ethanol was added to fix cells. Samples were kept at -20°C overnight. After washing with PBS, cells were treated with PI and RNAase (BD Biosciences, New Jersey, USA) following the manufacturer's protocol. The PI signal was examined by a flow cytometry. For cell apoptotic assay, cells were collected and subjected to Annexin V-APC/7-AAD double staining according to the manufacturer's instruction.

#### *Trans-well cell migration assay*

The migration capacity of cells was evaluated using 8.0 µm pore size transwell chamber

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**Figure 1.** The relative expression levels of miR-409-3p in 20 paired endometrial carcinoma tissues and adjacent non-cancerous tissues were assessed by qRT-PCR.

(Costar Corp., Cambridge, MA, USA). In brief, cells were suspended in 100  $\mu$ l of serum-free medium and seeded in the upper chambers. The lower chamber was added DMEM medium. After 24 h, cells on the upper surface were removed, and migrated cells on the lower surface were fixed in 4% paraformaldehyde, followed by staining with 0.1% crystal violet. The number of migrating cells was counted under a microscope and the means for each chamber in 5 fields were calculated.

### Dual-luciferase reporter assay

Luciferase assays were performed to verify the regulation of RAB10 by miR-409-3p. In brief, cells were cultured in a 24-well plate (approximately  $1 \times 10^5$  cells per well) for 1 day. When they reached 70-80% confluence, cells were cotransfected with 50 nM miR-409-3p mimic or scramble mimic and 100 ng luciferase reporter plasmid (GenePharma, Shanghai, China) expressing wild type or mutant 3'-UTR RAB10 sequences using Lipofectamine 2000 (Invitrogen). After 48 h transfection, cells were harvested and the relative luciferase activity was measured by the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions.

### Western blotting

Tissues and cells were lysed in ice-cold lysis buffer (Cell Signaling Technology) and the supernatant protein concentrations were determined using Bradford (Beyotime, China). Equal

amount of protein samples (60  $\mu$ g) were separated by 10% SDS-PAGE and transferred onto PVDF membranes. After blocking for 1 h with 5% fat-free milk, the membranes were incubated with primary antibodies against RAB10, CDK, Cyclin D1, PARP, E-cadherin, N-cadherin, Vimentin (1:1000, Cell Signaling Technology, Boston, USA) and GAPDH (1:2000, Proteintech, Chicago, USA) overnight at 4°C. Then the membranes washed and incubated with appropriate HRP-conjugated secondary antibodies (1:5000; Dako, Carpinteria, CA, USA) for 1 h. The immunoreactive bands were detected using ECL Plus detection reagent (Santa Cruz Biotechnology). GAPDH was used as the internal control.

### Statistical analysis

Data were analyzed using GraphPad Prism version 5 and expressed as the mean  $\pm$  SD. The two-tailed Student's *t*-test was used to evaluate the difference between NC and miR-409-3p mimics groups. All *p*-value < 0.05 was considered statistically significant.

## Results

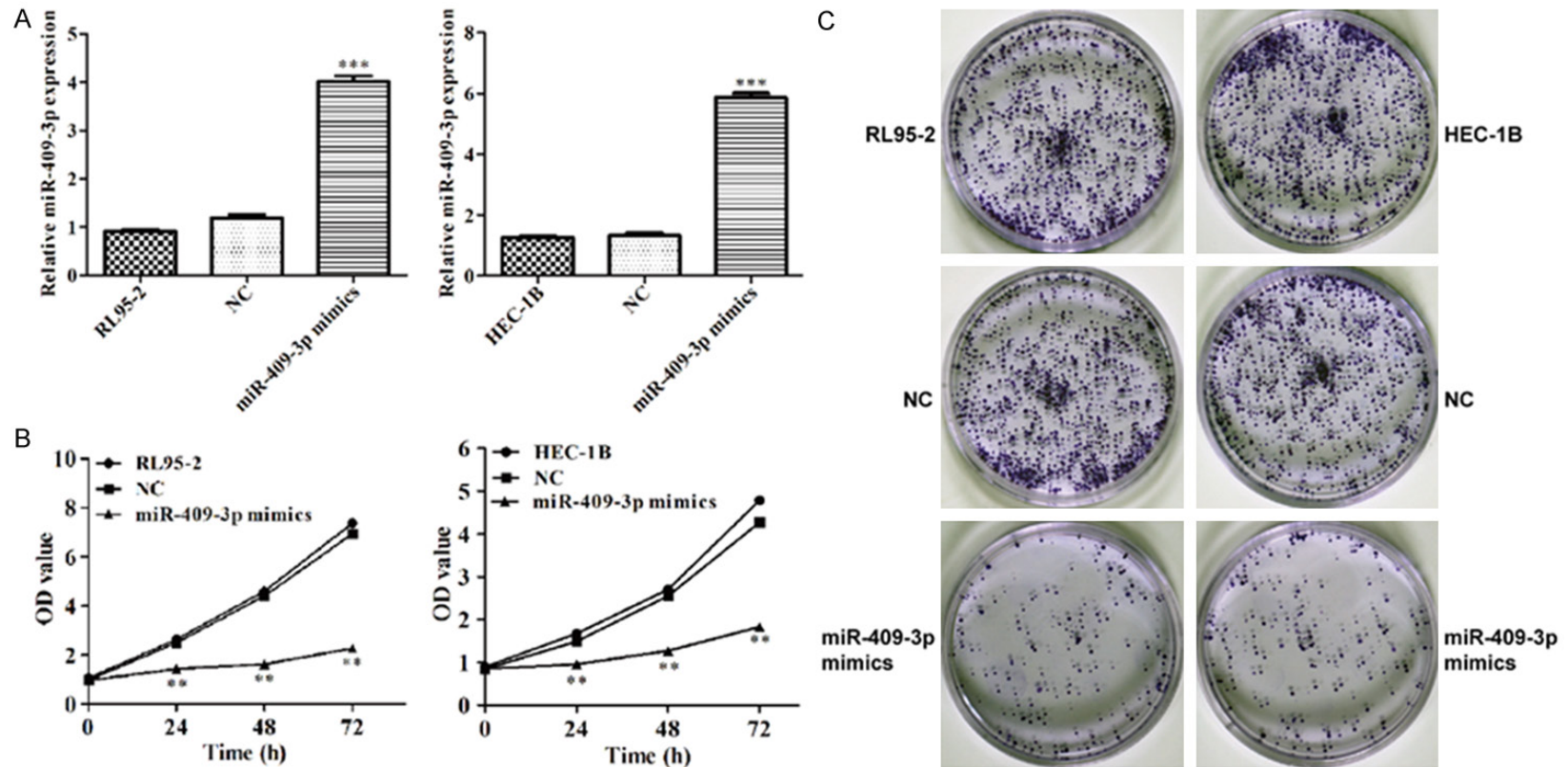
### The expression of miR-409-3p in EC tissues

To determine the role of miR-409-3p in EC, qRT-PCR was performed on RNA extracted from 20 pairs of EC tissues and patient-matched non-cancerous tissues. **Figure 1** showed that miR-409-3p expression level was significantly reduced in EC tissues compared with in patient-matched noncancerous tissues. Based on the findings, we considered that miR-409-3p was decreased in the development of EC and then focused on miR-409-3p for further studies to evaluate it induced tumor biology in EC.

### Overexpression of miR-409-3p inhibits cell viability and proliferation in EC cells

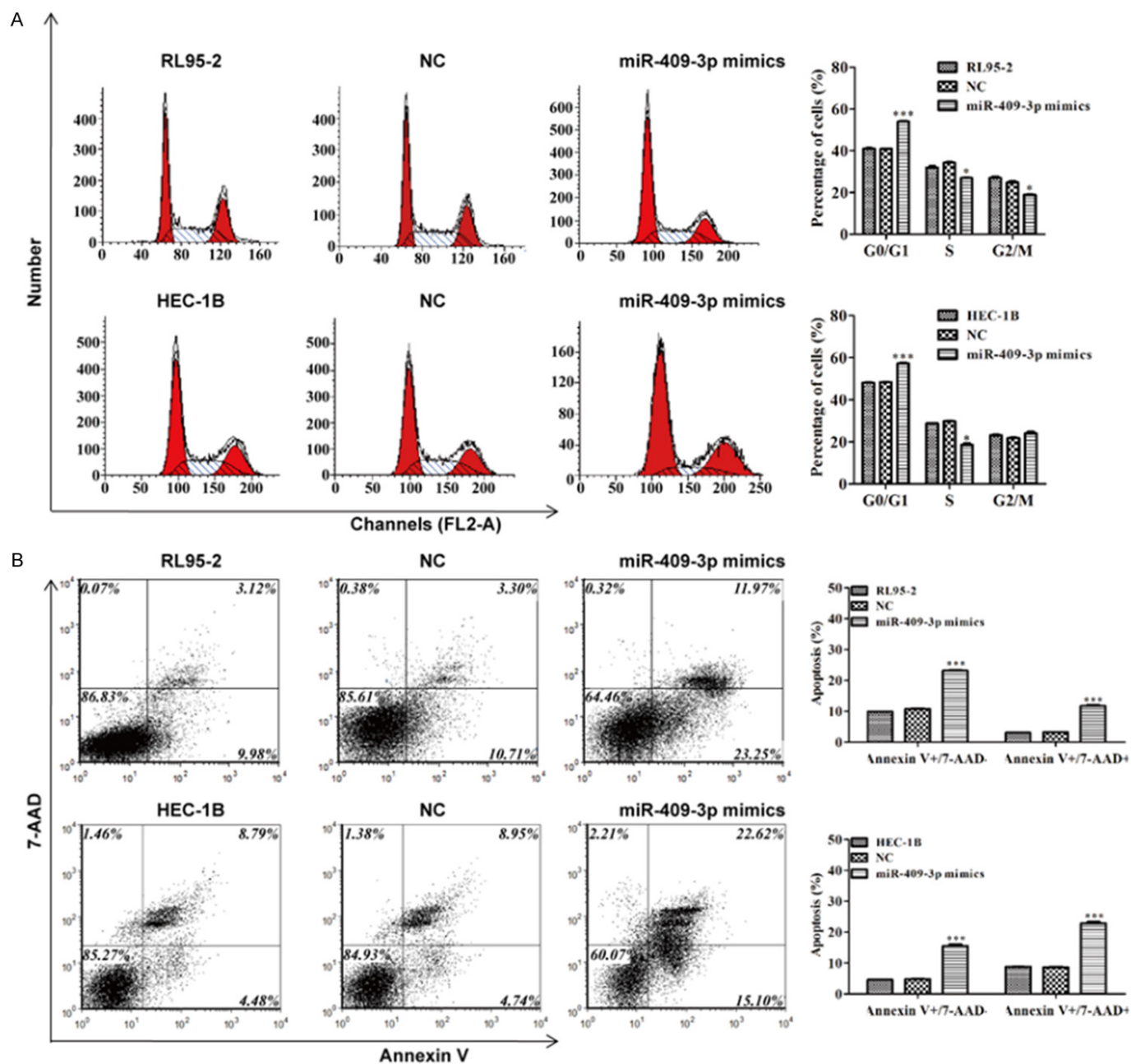
To better understand the biological functions of miR-409-3p in EC, two EC cell lines, RL95-2 and KLE were stably transfected with miR-409-3p mimics or negative control mimics. The ectopic expression of miR-409-3p was detected by qRT-PCR analysis. As shown in **Figure 2A**, the expression of miR-409-3p was significantly increased in miR-409-3p mimics-transfected RL95-2 and KLE cells compared with the corresponding NC-transfected cells or blank control ( $P < 0.001$ ). The CCK-8 assay showed that

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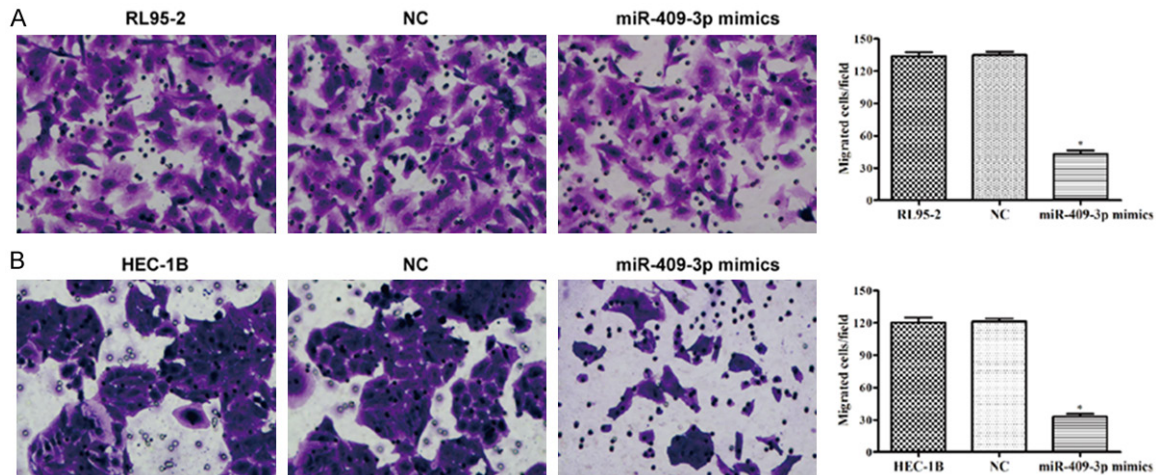
**Figure 2.** Overexpression of miR-409-3p suppresses endometrial carcinoma cell viability and colony formation ability *in vitro*. A: The expression of miR-409-3p was significantly elevated in by miR-409-3p mimics transfection. B: CCK-8 assay was carried out to evaluate the cell viability of endometrial carcinoma cell lines, RL95-2 and HEC-1B after transfected with miR-409-3p mimics or negative control mimics. C: Representative micrographs of colony formation of RL95-2 and HEC-1B after transfected with miR-409-3p mimics or negative control mimics.

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**Figure 3.** Overexpression of miR-409-3p induced G0/G1 phase arrest and promoted apoptosis of endometrial carcinoma cells. A: Flow cytometry with PI staining was used to analyze cell cycle distribution in RL95-2 and HEC-1B cells after transfected with miR-409-3p mimics or negative control mimics. B: Flow cytometry with Annexin V/7-AAD double staining was used to analyze cell apoptosis in RL95-2 and HEC-1B cells after transfected with miR-409-3p mimics or negative control mimics. Results are representative of three separate experiments; data are expressed as the mean  $\pm$  standard deviation, \* $P < 0.05$ ; \*\*\* $P < 0.001$ .



**Figure 4.** Overexpression of miR-409-3p suppressed cell migration ability in endometrial carcinoma. Trans-well assay was used to evaluate cell migration ability in RL95-2 (A) and HEC-1B (B) cells after transfected with miR-409-3p mimics or negative control mimics. Results are representative of three separate experiments; data are expressed as the mean  $\pm$  standard deviation, \* $P < 0.05$ .

transfection of miR-409-3p mimics could significantly suppress cell viability in both cell lines compared with the negative controls (Figure 2B,  $P < 0.01$ ). Furthermore, colony formation assay demonstrated that miR-409-3p overexpression remarkably reduced the ability of colony formation, as revealed by smaller and fewer colonies formed in miR-409-3p mimics-transfected RL95-2 and KLE cells (Figure 2C).

### Overexpression of miR-409-3p induces cell cycle arrest and apoptosis in EC cells

Next, we investigated the effect of miR-409-3p expression on cell cycle progression and apoptosis of EC cells. As shown in Figure 3A, miR-409-3p mimics-transfected RL95-2 and KLE cells presented a higher G0/G1 phase population ( $P < 0.001$ ) and a lower S phase population ( $P < 0.05$ ), compared with NC-treated cells, indicating an arrest of cell cycle at G0/G1 phase. In addition, flow cytometry with Annexin V/7-AAD double staining further indicated overexpression of miR-409-3p significantly increased overall apoptotic cells, including early apoptosis (Annexin V+/7-AAD-) and late apoptosis (Annexin V+/7-AAD+) by nearly 2.5-fold and

2.75-fold in RL95-2 and KLE cells, respectively (Figure 3B,  $P < 0.001$ ). These results demonstrated that overexpression of miR-409-3p plays an important role in cell cycle regulation and apoptosis in EC.

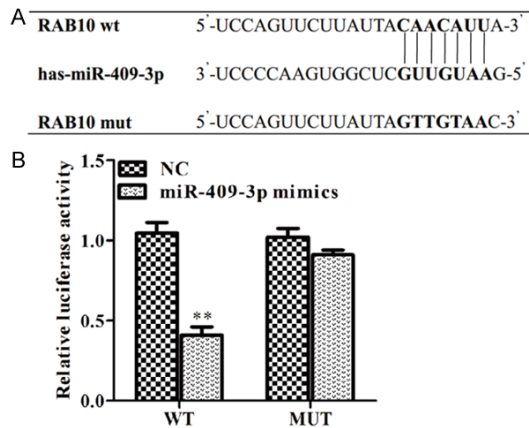
### Overexpression of miR-409-3p suppresses EC cell migration

To study whether miR-409-3p is involved in EC metastasis, we determined the effects of miR-409-3p on the migration of EC cells. The results showed that the cell migration ability was dramatically suppressed in RL95-2 (Figure 4A,  $P < 0.05$ ) and KLE (Figure 4B,  $P < 0.05$ ) transfected with miR-409-3p mimics in comparison with negative control mimics or blank control. These results suggest that miR-409-3p significantly inhibits *in vitro* migration of EC cells.

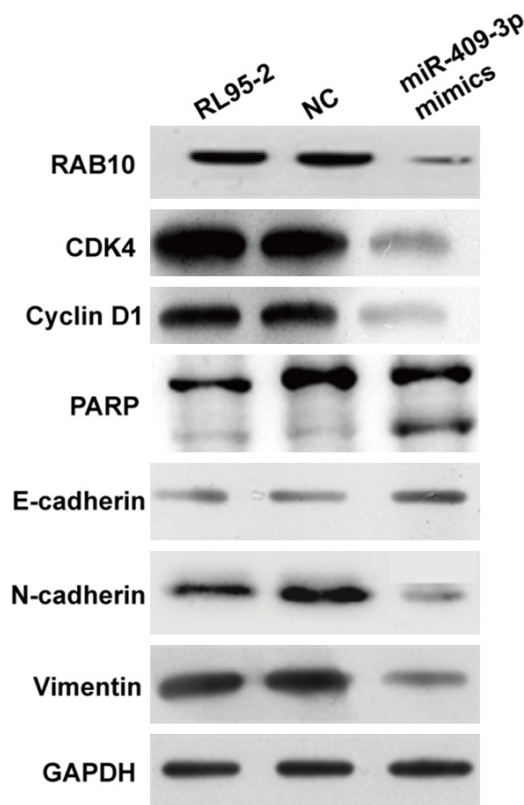
### MiR-409-3p targets RAB10 via binding to its 3'UTR

To elucidate the underlying mechanisms of miR-409-3p in EC, we analyzed the target gene of miR-409-3p and found RAB10 exhibited miR-409-3p-binding sequences in its 3'-UTR

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**Figure 5.** miR-409-3p directly targets RAB10. A: miR-409-3p and its predicted binding sequence in the 3'-UTR of RAB10; B: Relative luciferase activity is determined 48 h after transfection. Results are representative of three separate experiments; data are expressed as the mean  $\pm$  standard deviation,  $**P < 0.01$ .



**Figure 6.** Overexpression of miR-409-3p reduced RAB10, CDK4, Cyclin D1, N-cadherin and Vimentin, while increased cleaved PARP and E-cadherin protein expression by Western blotting.

regions using the miRNA target-detecting software (Figure 5A). We then performed dual-lucif-

erase reporter assay to further confirmed RAB10 is a target for miR-409-3p. As shown in Figure 5B, the luciferase activity of wide-type RAB10 3'-UTR was significantly reduced by miR-409-3p mimics ( $P < 0.01$ ), but the luciferase activity of RAB10 mutated binding sites was unaffected by the expression of miR-409-3p mimics. These results strongly support that miR-409-3p can bind to the 3'-UTR of RAB10.

*Overexpression of miR-409-3p directly affects RAB10 expression and the downstream molecules in EC cells*

Next, we analyzed the effect of miR-409-3p expression on the expression of RAB10 in EC cells. As shown in Figure 6, transfection of miR-409-3p mimics could decrease the expression of RAB10 protein in RL95-2 cells, which further indicates that RAB10 is a direct target of miR-409-3p in EC. Furthermore, Western blotting demonstrated that overexpression of miR-409-3p downregulated the protein expression of CDK4, Cyclin D1, N-cadherin and Vimentin, while upregulated PARP cleavage and E-cadherin expression in RL95-2 cells.

### Discussion

In this study, we investigated the function of miR-409-3p in the progression of EC and showed for the first time that miR-409-3p was significantly downregulated in EC tissues compared with corresponding non-tumorous tissues. Functional analyses *in vitro* further indicated that overexpression of miR-409-3p decreased cell proliferation and migration, as well as induced cell cycle arrest and apoptosis. These data suggests that miR-409-3p functions as a tumor suppressor in EC cells, which is consistent with the previous studies that showed that miR-409-3p could inhibit tumor cell growth and metastasis in gastric cancer [13], bladder cancer [14] and lung cancer [15].

To further elucidate the underlying mechanisms, we firstly confirmed RAB10 is a promising target for miR-409-3p using predicting software and dual-luciferase reporter assay. In confirmation of functional interaction, overexpression of miR-409-3p reduced RAB10 protein expression in EC cells. The small GTPase RAB10 is a member of the RAS oncogene superfamily, which has been demonstrated to promote tumor cell proliferation in osteosarcoma [19]. We thus infer that upregulation of miR-

409-3p may inhibit proliferation, migration and promote apoptosis in EC by directly decreasing expression of RAB10.

Next, we addressed the molecular mechanism of miR-409-3p in suppressing cell proliferation and migration. As our best knowledge, cell proliferation is mainly regulated by cell cycle progression and phases of cell cycle are controlled by different CDK/cyclin complexes [20, 21]. As a key checkpoint of cell cycle, G1/S transition is responsible for initiation and completion of DNA replication, in which CDK4/Cyclin D1 complex plays an important role in S phase progression [22, 23]. In addition to cell cycle regulation, apoptosis also plays a crucial role in cell proliferation by maintaining cellular homeostasis between cell division and cell death. Poly ADP-ribose polymerase (PARP) participates in DNA repair and can induce apoptosis [24, 25]. In this study, overexpression of miR-409-3p reduced CDK4, Cyclin D1 protein expression and increasing PARP cleavage. These results further demonstrate upregulation of miR-409-3p induces cell cycle arrest at G0/G1 phase via downregulation of CDK4/Cyclin D1 and promotes apoptosis by upregulating PARP. Moreover, we assessed the levels of E-cadherin, N-cadherin and Vimentin, which serve crucial functions in migration and invasion. As epithelial-type marker, E-cadherin is adhesion molecule belongs to calcium-dependent family and its removal promotes cell migration and invasion [26, 27]. N-cadherin and Vimentin are both mesenchymal markers play an important role in the recovery of cell polarity and their downregulation could result in the inhibition of tumor cell migration and invasion [28, 29]. In the present study, we found that overexpression of miR-409-3p increased E-cadherin expression and decreased N-cadherin and Vimentin expression, providing further evidence that miR-409-3p may inhibit cell migration in EC.

In summary, the present study presented some evidences that miR-409-3p is a potential tumor suppressor gene in EC by exerting its inhibitory effect on the proliferation and migration of EC cells at least partially via inhibiting the protein expression of its target RAB10. These findings will provide the basis for further investigations to develop more promising diagnostic method and gene therapy target for EC.

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### Disclosure of conflict of interest

None.

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