

## Original Article

# MiR-204 inhibits cell growth and induces cell apoptosis though targeting RAB13 in ovarian cancer cells

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**Abstract:** Deregulation of microRNA-204 has been frequently observed in cancer tumorigenesis, but its roles in ovarian cancer cells are not entirely clear. In this paper, we utilized real-time PCR to show that miR-204 is significantly down-regulated in ovarian cancer tissues and cell lines. In vitro assays show that over-expression of miR-204 inhibits cell growth, and induced cell apoptosis in ovarian cancer cells. Furthermore, we found that miR-204 can directly target RAB13. MiR-204 inversely correlates with RAB13 expression in human ovarian cancer tissues. These results may have implications for therapeutic strategies aiming to overcome ovarian cancer tumorigenesis.

**Keywords:** miR-204, RAB13, ovarian cancer, apoptosis

## Introduction

Ovarian cancer (OC) are a group of molecularly and etiologically heterogeneous cancers that keeps the fifth leading cause of cancer death in women and the leading cause of death from gynecological cancer [1]. The current standard therapy of ovarian cancer is surgical resection of visible tumors combined with chemotherapy such as paclitaxel, which are the conventional anticancer drugs with long-term clinical applications for cancer treatment with specific applications in ovarian cancer [2, 3]. Most patients with advanced ovarian cancer would eventually relapse following resistance to chemotherapy drugs. Due to the poor survival of patients with OC, it is crucial to identify novel therapy methods and more effective treatments of human ovarian cancer.

MicroRNAs (MiRs) are a group of small, noncoding RNAs of 22 nucleotides which regulate gene expression post-transcriptionally by affecting both the stability and translation of mRNAs. Numerous studies have indicated that the expression profiles of miRNAs are always changed significantly during the tumors development.

Further research revealed that miRNAs play crucial roles in the growth, invasion, differentiation and metastasis of tumor cells include ovarian tumor. MicroRNAs 125a and 125b inhibit ovarian cancer cells through post-transcriptional inactivation of EIF4EBP1 [4]. MiR-181c inhibits ovarian cancer metastasis and progression by targeting PRKCD expression [5]. MicroRNA-186 induces sensitivity of ovarian cancer cells to paclitaxel and cisplatin by targeting ABCB1 [6]. MicroRNA-133b inhibits proliferation and invasion of ovarian cancer cells through Akt and Erk1/2 inactivation by targeting epidermal growth factor receptor [7].

In the present study, we aimed to investigate the role miR-204 plays in ovarian cancer proliferation and invasion. Our results revealed that miR was downregulated in ovarian tumors and cell lines compared to normal tissues. Over-expression of miR-204 significantly inhibited the proliferation and colony formation, however, increased the apoptosis of ovarian cancer cells. Furthermore, we identified RAB3 as the target of miR-204, indicating that miR-204 may exert as a potential therapeutic target of ovarian cancer.

## Materials and methods

### *Human patient samples*

11 pairs of ovarian cancer tissues and adjacent normal tissues were provided by First Affiliated Hospital of University of South China. Approval was obtained from the Central South University's Ethics Committee on the Use of Human Samples. The samples were immediately quick frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$ .

### *Cell culture and transfection*

Ovarian cancer cell lines OVCAR-3, SKOV3 and HO-8910PM were obtained from. SKOV3 cells were maintained in Dulbecco's modified Eagle's (DMEM) medium (Invitrogen, CA, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco, Australia). Normal human ovarian surface epithelial (HOSE) cells were maintained in ovarian epithelial cell medium supplemented with  $1\times$  ovarian epithelial cell growth supplement 26260454. All cell lines were maintained at  $37^{\circ}\text{C}$  in a humidified incubator containing 5%  $\text{CO}_2$ . A total of  $2\times 10^5$  cells were seeded into 6-well plates and cultured overnight. MiR-204 mimics and miR-control transiently transfected using Lipofectamine 2000 transfection reagent (Invitrogen, CA, USA) Genepharma according to the manufacturer's instructions. The final concentration of miR-27a-3p mimics, miR-control was 50 nM.

### *MTT assay*

The cells ( $1\times 10^4$ ) were seeded into a 96-well plate and cultured overnight. The culture medium was removed and replaced with fresh medium containing 0.5 mg/ml MTT (Sigma-Aldrich, MO, USA) at various time-points (0, 12, 24, or 48 h) after transfection, followed by incubation at  $37^{\circ}\text{C}$  for 4 h. The medium was removed and 150  $\mu\text{l}$  dimethyl sulfoxide (DMSO), was added to solubilize the formazan crystals. The absorbance at 490 nm was detected with a Biotek Elx-800 plate reader.

### *Real-time-qPCR analysis*

RNA extraction of tissues and cell samples was performed with Trizol (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. RT-PCR was performed using 2  $\mu\text{g}$  of total RNA, AMV reverse transcriptase and random

primers (Takara, Kyoto, Japan). Real-time-PCR was performed according to the manufacturer's protocol using cDNA as templates and an SYBR Premix Ex Taq II kit (Takara, Kyoto, Japan) on a iQ5 real-time PCR detection system (Bio-Rad, USA). The relative gene expression level was calculated using  $2^{-\Delta\Delta\text{Ct}}$  method and was normalized by U6 or  $\beta$ -actin as an internal control for microRNA and mRNA respectively. All the qPCR assays were performed in triplicate.

### *Western blot*

Proteins were extracted using RIPA lysis buffer (containing 1% PMSF) then separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 10% acrylamide gels, and transferred into polyvinylidene difluoride (PVDF) (Millipore, USA) membrane. The membranes were blocked in 5% non-fat milk in Tris-buffered saline with Tween<sup>®</sup>-20 (TBST) for 2 hours and probed with primary antibodies at  $4^{\circ}\text{C}$  overnight followed by incubation with HRP conjugated secondary antibodies (Santa Cruz, CA, USA). Immunoreactivity was visualized using the enhanced chemiluminescence ECL kit (Pierce Biotechnology, IL, USA), and exposed to XAR-5 film (Kodak, USA). GAPDH was used as an internal control.

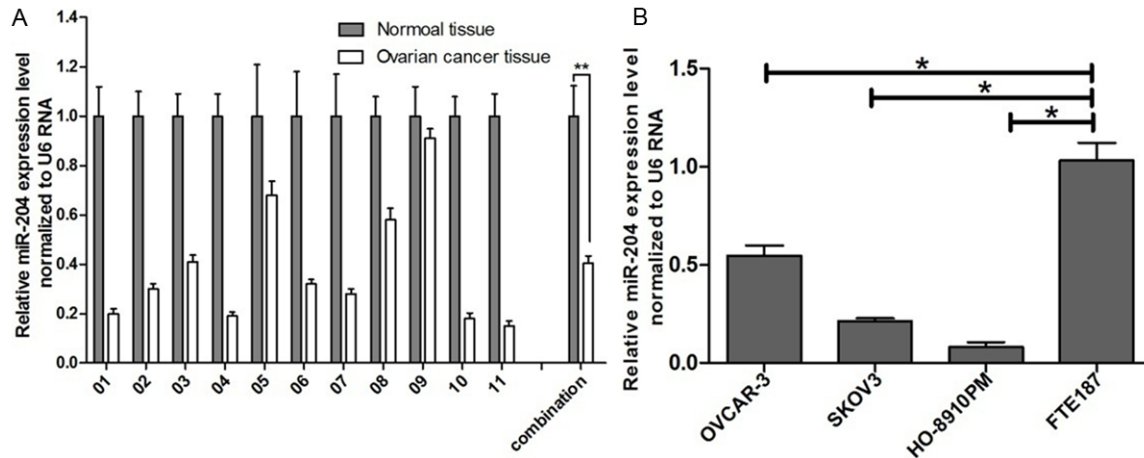
### *Colony formation assay*

500 cells were plated in a 6-well plate 48 h after transfection with miR-204 mimics or miR-control. The cells were cultured in an incubator with 5%  $\text{CO}_2$  at  $37^{\circ}\text{C}$  for 14 days and media was replaced every 3 days. At day 14, the media was removed and cells were washed twice with PBS. The colonies were fixed with 50% methanol for 15 min, dried and stained with 0.5% crystal violet solution for 30 min. Images of the stained plates were captured, and the colonies containing more than 50 cells were counted. Each assay was performed in triplicates.

### *Apoptosis analysis*

Cells ( $2\times 10^6/\text{ml}$ ) were seeded into 6-well plate, 48 h after transfection with miR-204 mimics or miR-control the cells were washed and resuspended at a concentration of  $1\times 10^6$  cells/ml. The cells were then stained with the Annexin V-FITC Apoptosis Detection Kit I (BD Biosciences, NJ, USA), followed by incubation at room temperature for 15 min in the dark. Cell apop-

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**Figure 1.** MiR-204 was downregulated in ovarian cancer tissues and cells. A. QPCR was used to evaluate the miR-204 expression in 11 pair of ovarian cancer tissues and normal tissues; miR-204 was significantly upregulated in ovarian cancer tissues. B. QPCR was used to evaluate the expression of miR-204 in ovarian cancer cells and FTE187 cell, miR-204 was significantly upregulated in ovarian cancer cells (\* $P < 0.05$ ).

tosis was analyzed with a FACS Calibur (BD Biosciences, Franklin Lakes, NJ, USA). Each experiment was performed for at least three times.

### Luciferase report assay

Cells ( $2 \times 10^5$ /ml) were seeded into 24-well plate and cultured overnight. The cells were cotransfected with 250 ng wild-type or mutant luciferase reporter constructs and 100 nM miR-204 mimics or miR-control mimics, in addition, 20 ng pRL-TK plasmid as control using Lipofectamine 2000 (Invitrogen, CA, USA). After 48 h, the luciferase was detected using the dual luciferase assay system (Promega, Madison, WI, USA) according to the manufacturer's instructions.

### Statistical analysis

Data are expressed as mean  $\pm$  standard deviation (SD). Statistical significance was determined using the one-way analysis of variance (ANOVA) with post-test Neuman-Keuls for more than two groups and Student's t test for two groups.  $P$  value of less than 0.05 indicates a statistically significant.

## Results

### MiR-204 is down-regulated in ovarian cancer tissues and cells

To explore the role of miR-204 in the development of ovarian tumors, we analyzed the miR-204 expression in 11 pair of ovarian tumor tis-

sues and adjacent normal tissues. Interestingly, miR-204 was significantly downregulated in all the ovarian tumor tissues in comparison with the normal tissues (Figure 1A). Furthermore, we analyzed the miR-204 expression in the ovarian cell lines such as OVCAR-3, SKOV3, HO-8910PM, and a normal human fallopian tube epithelia cell line FTE187 cells. As expected, miR-204 expression in ovarian cell lines were downregulated either compared to that in FTE187 (Figure 1B).

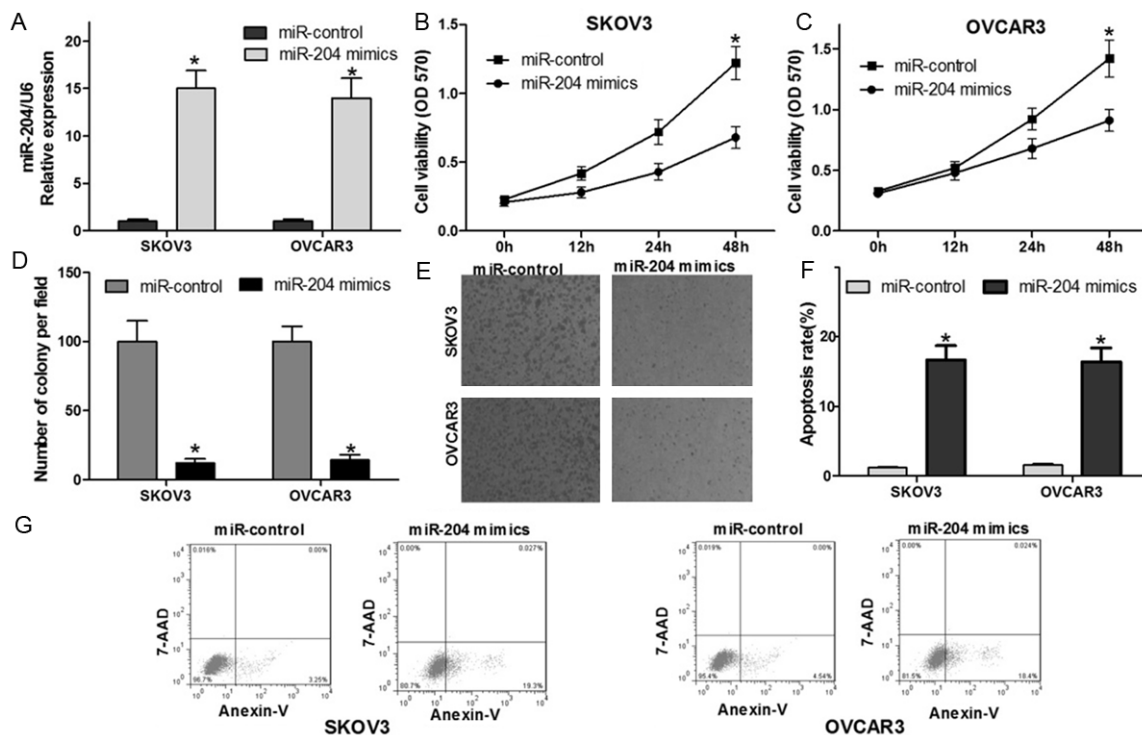
### MiR-204 overexpression inhibited the proliferation and induced the apoptosis in ovarian cancer cells

To confirm and further identify the role of miR-204 plays in ovarian, we transfected OVCAR-3 and SKOV3 cells with miR-204 mimics and miR-control. The miR-204 expression was significantly upregulated in OVCAR-3 and SKOV3 cells transfected with miR-204 mimics (Figure 2A). The MTT results revealed that overexpression of miR-204 notably inhibited the cell viability of both OVCAR-3 and SKOV3 cells (Figure 2B, 2C). In keeping with the cell viability, miR-204 also attenuated the colony formation of OVCAR-3 and SKOV3 cells (Figure 2D, 2E). Moreover, we indicated that miR-204 could significantly increase the apoptosis in OVCAR-3 and SKOV3 cells (Figure 2F, 2G).

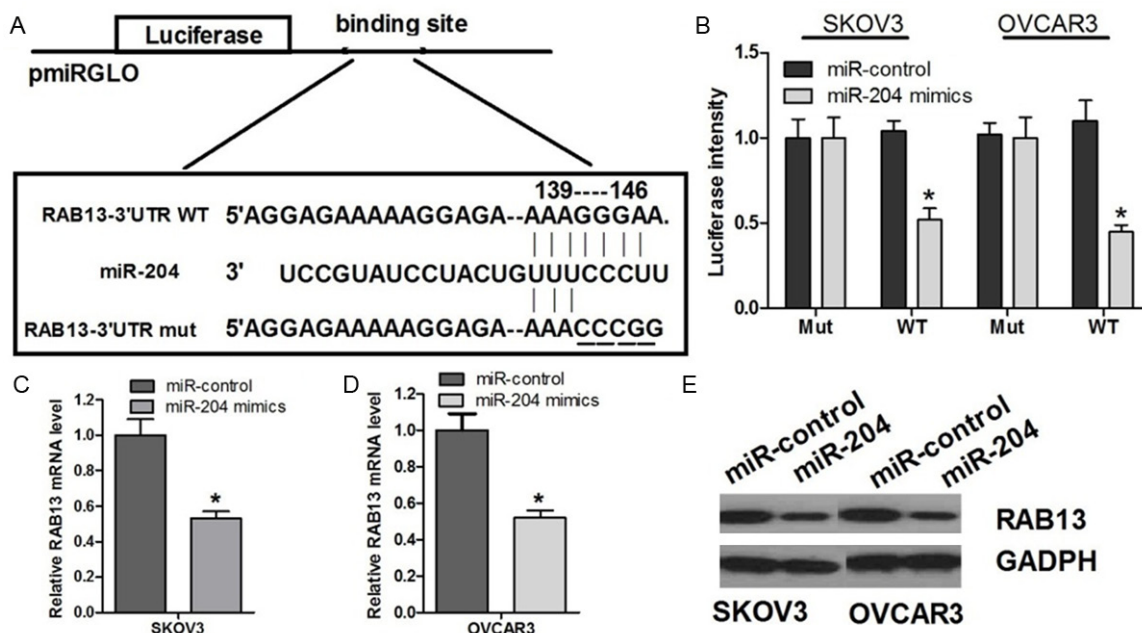
### RAB13 is a direct target gene of miR-204

Two algorithms of miRNA-PicTar and TargetScan were used to predict the candidate target gene

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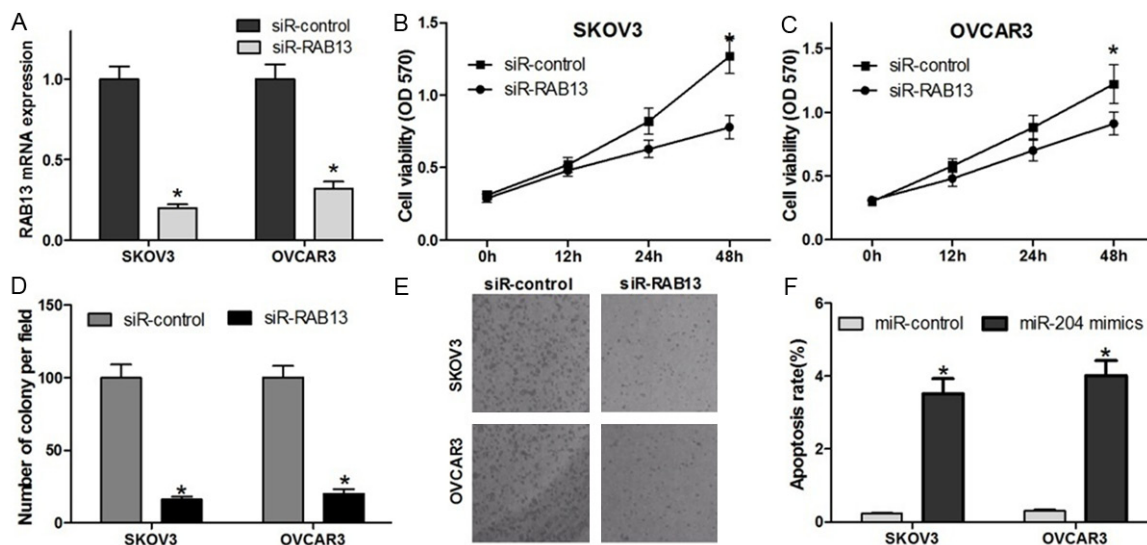
**Figure 2.** MiR-204 overexpression inhibited the proliferation and induced the apoptosis in ovarian cancer cells. A. The expression of miR-204 was significantly upregulated in SKOV3 and OVCAR-3 cells transfected with miR-240 mimics. B, C. Overexpression of miR-204 significantly inhibited the cell viability of SKOV3 and OVCAR-3 cells. D, E. Overexpression of miR-204 significantly inhibited the colony formation of SKOV3 and OVCAR-3 cells. F, G. Overexpression of miR-204 significantly increased the apoptosis of SKOV3 and OVCAR-3 cells (\*P < 0.05).



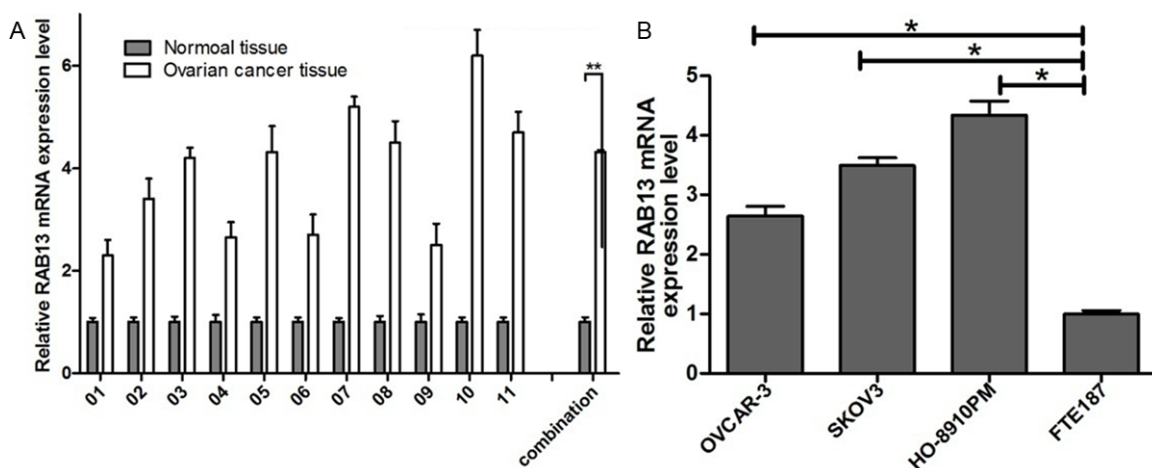
**Figure 3.** MiR-204 directly targeted RAB13 in SKOV3 and OVCAR-3 cells. A. The algorithms between miR-204 and the 3'UTR of RAB13 and also the mutant RAB13 3'UTR. B. Luciferase activity was measured by using a dual luciferase reporter assay, the Luciferase intensity in SKOV3 and OVCAR-3 cells transfected with mutant RAB13 3'UTR vector and miR-204 mimics was significantly attenuated. C, D. The mRNA expression of RAB13 was downregulated notably in SKOV3 and OVCAR-3 cells transfected with miR-204 mimics. E. The protein expression of RAB13 was downregulated notably in SKOV3 and OVCAR-3 cells transfected with miR-204 mimics (\*P < 0.05).



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**Figure 4.** Knockdown of RAB13 changes of ovarian cancer cellular characters. A. The mRNA expression of RAB13 was downregulated notably in SKOV3 and OVCAR-3 cells transfected with siRNA-RAN13. B, C. Knock down of RAB13 inhibited the cell viability of SKOV3 and OVCAR-3 cells. D, E. Knock down of RAB13 significantly inhibited the colony formation of SKOV3 and OVCAR-3 cells. F. Knock down of RAB13 significantly increased the apoptosis of SKOV3 and OVCAR-3 cells (\* $P < 0.05$ ).



**Figure 5.** RAB13 was upregulated in ovarian cancer tissues and cells. A. The mRNA expression of RAB13 was significantly upregulated in ovarian cancer tissues. B. The mRNA expression of RAB13 was significantly upregulated in ovarian cancer cells (\* $P < 0.05$ ).

of miR-204, among the mRNAs containing miR-204 recognition sites in their 3'UTRs; we focused on RAB13, a protein involved in tumorigenesis and progression. We located potential binding sites for miR-204 at the 3'UTR of RAB13 mRNAs (Figure 3A). To validate it, we performed luciferase reporter gene assay in OVCAR-3 and SKOV3 cells. The results showed that miR-204 significantly inhibited the luciferase activity of the reporter containing wild-type RAB13 3'UTR sequence, but failed to inhibit that of mutated RAB13 (Figure 3B). These data

suggested that miR-204 could directly target the 3'UTR of RAB13. In addition, we indicated that the overexpression of miR-204 by transfection of miR-204 mimics significantly inhibited the mRNA and protein expression of RAB13 (Figure 3C-E).

### Knockdown of RAB13 changes of ovarian cancer cellular characters

It has been demonstrated that RAB13 is a target of miR-204, accordingly, to identify whether

miR-204 attenuated the proliferation and increased the apoptosis of OVCAR-3 and SKOV3 cells through targeting RAB13, we constructed pSilencer/sh-RAB13 plasmids. The expression of RAB13 was significantly downregulated in OVCAR-3 and SKOV3 cells transfected with pSilencer/sh-RAB13 plasmids (**Figure 4A**). The knockdown of RAB13 significantly inhibited the cell viability and colony formation of both OVCAR-3 and SKOV3 cells, meanwhile, increased the apoptosis of them (**Figure 4B-F**).

*RAB13 was upregulated in ovarian cancer tissues and cells*

Finally, we evaluated the expression of RAB13 in patients' samples to further confirm our hypothesis. As expected, the expression of RAB13 was significantly upregulated in ovarian cancer tissues compared to normal tissues (**Figure 5A**). The expression of RAB13 in ovarian cell lines was in accordance with that in tissues, which was upregulated in ovarian cells in comparison with in normal cells (**Figure 5B**).

### Discussion

Recently, there have been accumulating deregulated miRNAs found in various cancer cells. These miRNAs offer potential therapeutic targets for cancer intervention and treatment. miR-204 is expressed from intron 6 of the gene encoding a transient receptor potential non-selective cation channel, subfamily M, member 3, TRPM3, that conducts Ca<sup>2+</sup> and Zn<sup>2+</sup> ions [8, 9]. As a tumor suppressor, miR-204 has been shown to be downregulated in human colon cancer, non-small-cell lung carcinoma, breast cancer, acute lymphoblastic leukemia, thyroid cancer, renal cell carcinoma [10-15]. The effect of miR-204 was carried out with multiple mechanisms, for instance, inhibiting the proliferation, migration, invasion and epithelial-mesenchymal transition (EMT) of cancer cells and induce apoptosis and autophages of them [16-19].

Ras-related proteins are involved in many human malignancies. Rab25 overexpression has been reported in colon, liver, and bladder cancers [20-22]. It is involved in regulating signal transduction and cellular pathways such as cell differentiation and proliferation, vesicle transportation, nuclear assembly, and adhesion and migration of cancer cells [23]. Another ras related protein rap1A, mediates thrombin-

stimulated, integrin-dependent glioblastoma cell Proliferation and tumor growth [24]. Previous studies have indicated that miR-451 and miR-338-3p could target RAB14 nasopharyngeal and non-small-cell lung carcinoma respectively [25, 26].

Although structurally similar, Ras-superfamily proteins are functionally diverse. Whereas some members exhibit oncogenic properties, others may serve as tumor suppressors. RAB13 is a member of the RAB family of GTPases and involved in neurite outgrowth through the process of filopodia formation [27]. It possess an array of functions in different cell types from regulation of tight junctions to neuronal plasticity, cell migration and glucose transporter trafficking. Rab13-dependent trafficking of RhoA is required for directional migration and angiogenesis [28]. Accordingly, RAB13 may possibly play a role in tumor progression. It has been demonstrated that rab13 regulates neurite outgrowth in PC12 cells through its effector protein, JRAB/MICAL-L2 [27]. However, more findings of RAB13 involved in human cancer remain limited.

In our work, we found miR-204 upregulated in ovarian cancer tissues and cells. Function experiments confirmed that miR-204 inhibited the cell viability and colony formation meanwhile induced apoptosis of ovarian cancer cells. Further, we validated that miR-204 targets RAB13 to exert biological effects by luciferase report assay. Owing to the vital role RAB13 plays in migration and angiogenesis, moreover, the suppress effect of miR-204 in cancers, these findings supplied a novel target that is potentially effective in tumor diagnose and treatment.

### Disclosure of conflict of interest

None.

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