# Knockdown of ARHGAP30 inhibits ovarian cancer cell proliferation, migration, and invasiveness by suppressing the PI3K/AKT/mTOR signaling pathway

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ABSTRACT

The mortality and morbidity rates of ovarian cancer (OC) are high, but the underlying mechanisms of OC have not been characterized. In this study, we determined the role of Rho GTPase Activating Protein 30 (ARHGAP30) in OC progression. We measured ARHGAP30 abundance in OC tissue samples and cells using immunohistochemistry (IHC) and RT-qPCR. EdU, transwell, and annexin V/PI apoptosis assays were used to evaluate proliferation, invasiveness, and apoptosis of OC cells, respectively. The results showed that ARHGAP30 was overexpressed in OC tissue samples and cells. Inhibition of ARHGAP30 suppressed growth and metastasis of OC cells, and enhanced apoptosis. Knockdown of ARHGAP30 in OC cells significantly inhibited the PI3K/AKT/mTOR pathway. Treatment with the PI3K/AKT/mTOR pathway inhibitor buparlisib simulated the effects of ARHGAP30 knockdown on growth, invasiveness, and apoptosis of OC cells. Following buparlisib treatment, the expression levels of p-PI3K, p-AKT, and p-mTOR were significantly decreased. Furthermore, buparlisib inhibited the effects of ARHGAP30 upregulation on OC cell growth and invasiveness. In conclusion, ARHGAP30 regulated the PI3K/AKT/mTOR pathway to promote progression of OC.

**Key words:** ARHGAP30; ovarian cancer; cell invasiveness; PI3K/AKT/mTOR pathway.

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**Contributions:** OH, study concept and design; XC, JL, literature search, data extraction; YY, LZ, data analysis; XC, manuscript drafting. All the authors have read and approved the final version of the manuscript and agreed to be accountable for all aspects of the work.

**Conflict of interest:** the authors declare no potential conflicts of interest for the research, authorship, and/or publication of this article.

**Ethics approval:** the current study was approved by the Medical Ethics Committee of Jiangxi Cancer Hospital (no. 2022.023, date 2022.10.10).

**Availability of data and materials:** the data of this study are available from the corresponding author upon request.

## Introduction

Ovarian cancer (OC) is a common cancer in the reproductive system.[1] The 5-year survival rate for patients with advanced OC is only 29%.[2] For women at high risk for development of OC, screening methods include transvaginal ultrasound and testing for the tumor biomarker CA125.[3] However, these screening strategies do not reduce the overall rate of OC-related mortality. Therefore, there is an urgent need for identification of molecular biomarkers that can facilitate early detection of OC. Rho GTPases are GTP-binding proteins.[4] Recent studies have shown that Rho GTPases are overexpressed in many human cancers and are linked to tumorigenesis, invasion, and metastasis.[5,6] Rho GTPases bind with inactive GDP or with active GTP.[7] ARHGAP30 is a Rho GTPase activating protein (GAP) specific to Rac1 and RhoA that controls cell adhesion and cytoskeleton formation.[8] A previous study showed that ARHGAP30 is a crucial regulator of p53 acetylation and may be a prognostic indicator of colon cancer.[9] In addition, ARHGAP30 has been shown to suppress lung cancer growth through inhibition of the Wnt/β-catenin pathway.[10] The GEPIA database (http://gepia.cancer-pku.cn/index.html) showed that ARHGAP30 was highly expressed in OC.[11] Predictive analysis using the STRING database (https://cn.string-db.org/) showed that ARHGAP30 interacted with PI3K/AKT/mTOR signaling pathway-related proteins (AKT1, PIK3CD and MTOR).[12] However, the roles and molecular mechanisms of ARHGAP30 in OC have not been previously reported.

The PI3K/AKT pathway is important in OC. Studies have shown that the PI3K/AKT/mTOR pathway may be a potential target for treatment of OC,[13] and activation of this pathway could promote tumor angiogenesis and expansion.[14] Inhibitors of the PI3K/AKT/mTOR pathway have been shown to dramatically slow the growth of OC.[15] In roughly 70% of OC cases the PI3K/AKT/mTOR pathway is upregulated, which results in increased angiogenesis, cell survival, and metabolism.[16] Several clinical studies have shown that the PI3K pathway is a promising target for the treatment of OC.[17-19] T-Complex 1 (TCP1) is significantly upregulated in OC and promotes OC cell expansion through regulation of PI3K/AKT/mTOR signaling.[20] Knockdown of METTL3 has been shown to inhibit development of OC through inhibition of the PI3K/Akt/mTOR pathway.[21]

We hypothesized that ARHGAP30 may promote OC development through activation of PI3K/AKT/mTOR signaling. We characterized the mechanisms by which ARHGAP30 and PI3K/AKT/mTOR signaling modulate OC cell proliferation, migration, invasion, and apoptosis in clinical samples and *in vitro*.

## Materials and Methods

### Patients' recruitment

Twenty-five OC tissues and adjacent normal tissues (2 cm away from OC tissues) were collected from female OC patients (30-50 years old) after tumor resection from March 2020 to March 2022. The inclusion criteria were as follows: i) age ≥18 years; ii) complete surgical removal with OC confirmed histologically. The exclusion criteria were as follows: i) patients with cardiovascular disease, diabetes mellitus, acute inflammation, hematological disease, renal disease, or other cancer; ii) patients with a documented transfusion within the past 3 months. This research protocol was approved by the Medical Ethics Committee of Jiangxi Cancer Hospital (no. 2022.023). All participants provided written informed consent. Information on the clinicopathological characteristics of the patients is presented in Table 1.

### Cell culture and transfection

A human normal ovarian surface epithelial cell line (IOSE-80) and three human OC cell lines (Skov3, Ovcar3, and A2780) were obtained from American Type Culture Collection (ATCC, Gaithersburg, MD, USA). IOSE-80, Skov3, Ovcar3, and A2780 cells were cultured in DMEM supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. All cells were maintained as monolayer cultures in an incubator with a 5% $CO_2$ atmosphere at 37°C. At 70% confluence, OC cells were transfected with si-ARHGAP30 using a Lipo 3000 Transfection Kit (Invitrogen, Shanghai, China). Si-NC and si-ARHGAP30 were obtained from Invitrogen. Cells were collected 48 h after transfection for further experiments.

### Immunohistochemical staining

Tissue samples were fixed in 4% paraformaldehyde (Sigma-Aldrich, St. Louis, MO, USA) for 48 h at room temperature, then embedded in paraffin. Tissues were cut into 4-μm sections and then incubated with anti-ARHGAP30 antibody (PA5-57604, 1:200, Invitrogen) at 4°C overnight. The sections were then incubated with secondary antibody (Goat anti-Rabbit IgG H&L, ab6721, 1:5000; Abcam, Cambridge, UK) for 60 min. The slides were washed three times with PBS. Then, 50 μL of DAB solution was added for 3-5 min. Following incubation with DAB, the slides were washed. After washing the slides were stained with hematoxylin for 5 min at room temperature, then washed again. The slides were then incubated with 1% HCl in alcohol for 2 s to stain

**Table 1. Correlation between ARHGAP30 expression and clinicopathological characteristics in ovarian cancer patients.**

| Clinical parameters | n | ARHGAP30 expression | | P |
|---|---|---|---|---|
| | | Low expression | High expression | |
| Age (years) | | | | 0.09175 |
| >40 | 11 | 5 | 6 | |
| ≤40 | 14 | 5 | 9 | |
| Tumor size | | | | 0.03709 |
| >2 cm | 14 | 6 | 8 | |
| ≤2 cm | 11 | 5 | 6 | |
| TNM stage | | | | 0.26430 |
| I-II | 13 | 6 | 7 | |
| III-IV | 12 | 4 | 8 | |
| Distant metastasis | | | | 0.09175 |
| Positive | 10 | 3 | 7 | |
| Negative | 15 | 8 | 7 | |

the tissue blue, post-transparent dehydration, and neutral gums to seal the sections. Positive (human cell line A-431) and negative (PBS) controls were included in all immunohistochemistry (IHC) analyses. Brown staining represents positive areas. ImageJ software (NIH, Bethesda, MD, USA) was used for the quantification of IHC staining.

## RT-qPCR

Cellular RNA was isolated using TRIzol. Then, cDNA was generated utilizing an RT kit (TOYOBO, Osaka, Japan). Reverse transcription qPCR was performed using SYBR Green Master (Roche, Basel, Switzerland) with primers for ARHGAP30 (Forward: 5'-GGAGGAGTATGGAGTGGTGGATGG-3'; Reverse: 5'-AGGTCTGGCTTCCGCTCTGAC-3'). Ct values were determined using a 7500 RT PCR system. Data were analyzed by the $2^{-\Delta\Delta CT}$ method.

## Western blot

OC cells were divided into three groups: si-NC, si-ARHGAP30, and buparlisib (a PI3K/AKT/mTOR pathway inhibitor). OC cells were lysed using RIPA buffer (RIPA; Beyotime Institute of Biotechnology, Jiangsu, China) for total protein isolation. Total protein concentration was calculated, and proteins were separated by SDS-PAGE. Primary antibodies against Ki67 (ab92742, 1:5,000;Abcam), MMP9 (ab76003, 1:5,000; Abcam), Bax (ab32503, 1:5,000; Abcam), p-PI3K (ab278545; 1:1,000;Abcam), PI3K (ab140307; 1:1,000; Abcam), p-AKT (ab38449; 1:1,000; Abcam), AKT (ab8805; 1:1,000; Abcam), p-mTOR (ab109268, 1:5,000;Abcam), mTOR (ab134903, 1:10,000; Abcam), and GAPDH (ab8245, 1:5,000; Abcam) were incubated with membranes for 15 h at 4 °C. Then, the membranes were incubated with secondary antibodies (goat anti-rabbit IgG H&L, ab6721, 1:5,000; Abcam) for 70 min at 26°C. Proteins were visualized using ECL solution (Applygen Technology, Beijing, China). Quantification was performed using ImageJ software (NIH).

## EdU assay

Cell growth was determined using an EdU Proliferation Kit (Abbkine, Wuhan, China). Cells ($1\times10^4$ cells) were cultured in 24-well plates at 37 ˚C for 24 h. After incubation with 50 μM EdU at 37°C for 2 h, the cells were fixed with 4% paraformaldehyde at 37°C for 30 min, then neutralized with glycine. The cells were permeabilized using 0.5% TritonX-100 at room temperature for 10 min, then incubated with 500 uL Click-iT mixture for 30 min. Then, the cells were incubated with DAPI for nuclear staining. Cells were visualized using a fluorescence microscope. The proliferation index was evaluated by counting the proportion of green fluorescent-positive cells.

## Analysis of apoptosis

Apoptosis was determined using an Annexin V-FITC kit (BD Bioscience, Franklin Lakes, NJ, USA) according to the manufacturer's instructions. OC cells ($2.5\times10^5$ cells per well) were allowed to adhere in 6-well plates for 24 h. The cells were harvested via 0.25% pancreatin without ethylene diamine tetraacetic acid (EDTA) (Yubo Biotech Co., Ltd., Shanghai, China). Cells were

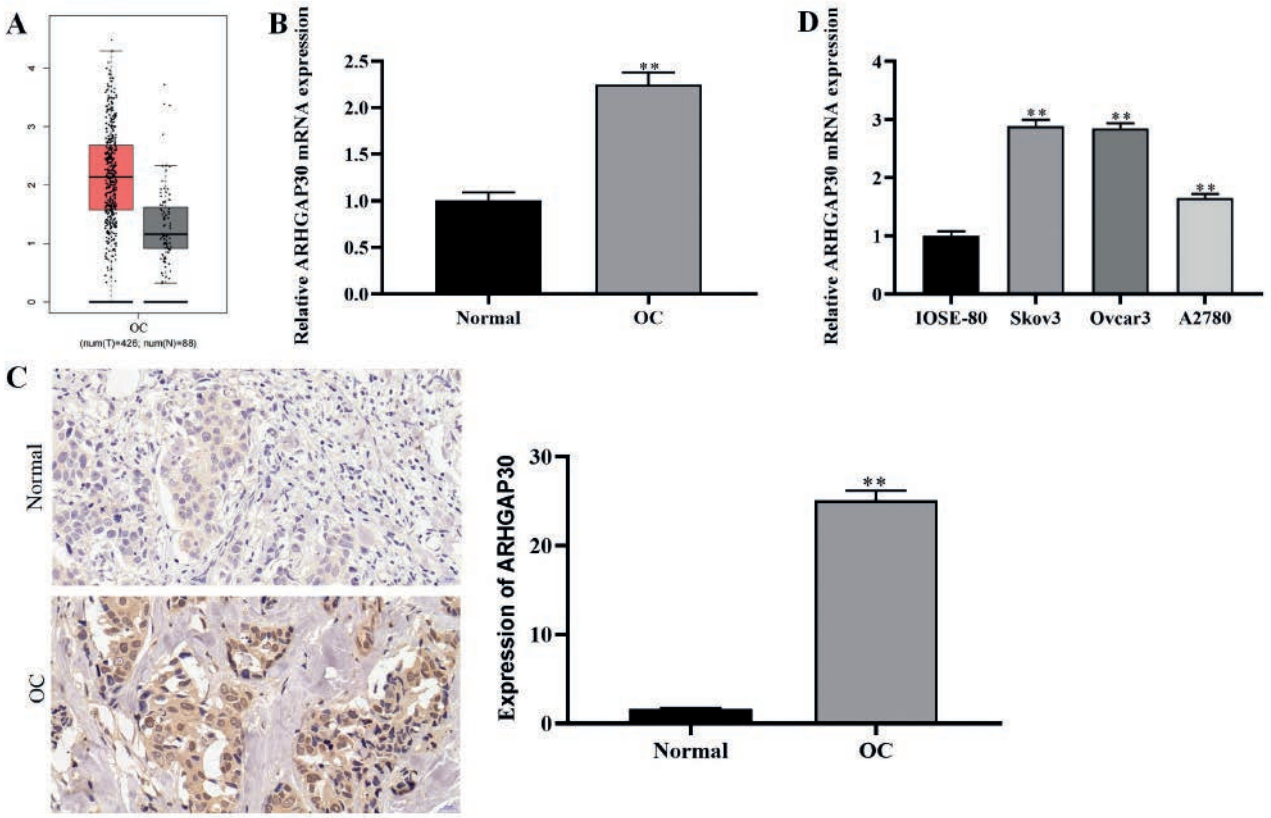


**Figure 1. The abundance of ARHGAP30 in ovarian cancer. A) The abundance of ARHGAP30 in healthy individuals and patients with OC. B) ARHGAP30 mRNA levels in OC tissues. C) Immunohistochemistry analysis of the abundance of ARHGAP30 in OC tissues and healthy tissues (×200, scale bar: 20 μm). D) ARHGAP30 mRNA was measured in OC cells and ovarian epithelial cells.**

labeled with FITC-conjugated annexin V and 5 μL of PI. After incubation in the dark at room temperature for 15 min, the samples were immediately analyzed using ADP (Beckman Coulter) flow cytometry. The data were processed using Cellquest software (Becton Dickinson). Apoptotic cells included early apoptotic cells and late apoptotic cells. Experiments were performed in triplicate and repeated twice to assess the consistency of the reaction.

### Transwell assay

In FBS-free medium, $1\times10^5$ cells were seeded on the upper layer of a transwell system to evaluate migration. The lower wells were filled with the medium containing FBS (Sigma-Aldrich). After one day, the cells were fixed with 4% paraformaldehyde (Sigma-Aldrich) at room temperature for 12 min. After incubation for 48 h, the cells were stained with 0.1% crystal violet solution (Sigma-Aldrich) at room temperature for 5 min. Migrated cells were visualized using a microscope. To evaluate invasion, cells were added to the upper layer containing Matrigel. A solution containing DMEM (Sigma-Aldrich) and FBS (Sigma-Aldrich) was added to the lower layer. After one day cells that invaded the Matrigel were analyzed using the procedure described for transwell assay above.

### Statistical analysis

GraphPad Prism (GraphPad Prism 8; GraphPad Software, Inc., San Diego, CA, USA) was used for data analysis. Data are presented as the mean ± SD. Analysis between two groups was performed using Student's *t*-test. Differences among three or more groups were analyzed using one-way ANOVA.

## Results

### Increased abundance of ARHGAP30 in OC cells and tissues

The abundance of ARHGAP30 in patients with OC was evaluated using the GEPIA database (http://gepia.cancer-pku.cn/). The results showed that patients suffering from OC had higher expression of ARHGAP30 than healthy individuals (Figure 1A). Then, we determined the abundance of ARHGAP30 in clinical OC tissues and showed that ARHGAP30 mRNA was upregulated in OC tissues (Figure 1B). Immunohistochemistry analysis showed that OC tissues had higher ARHGAP30 expression than normal tissues (Figure 1C). All OC cell lines had higher expression of ARHGAP30 than IOSE-80 cells (Figure 1D). These results indicated that ARHGAP30 might be involved in development of OC.

### ARHGAP30 knockdown inhibited OC cell proliferation and migration

We created stable ARHGAP30 knockdown OC cell lines. Figure 2A shows successful knockdown of ARHGAP30 expres-

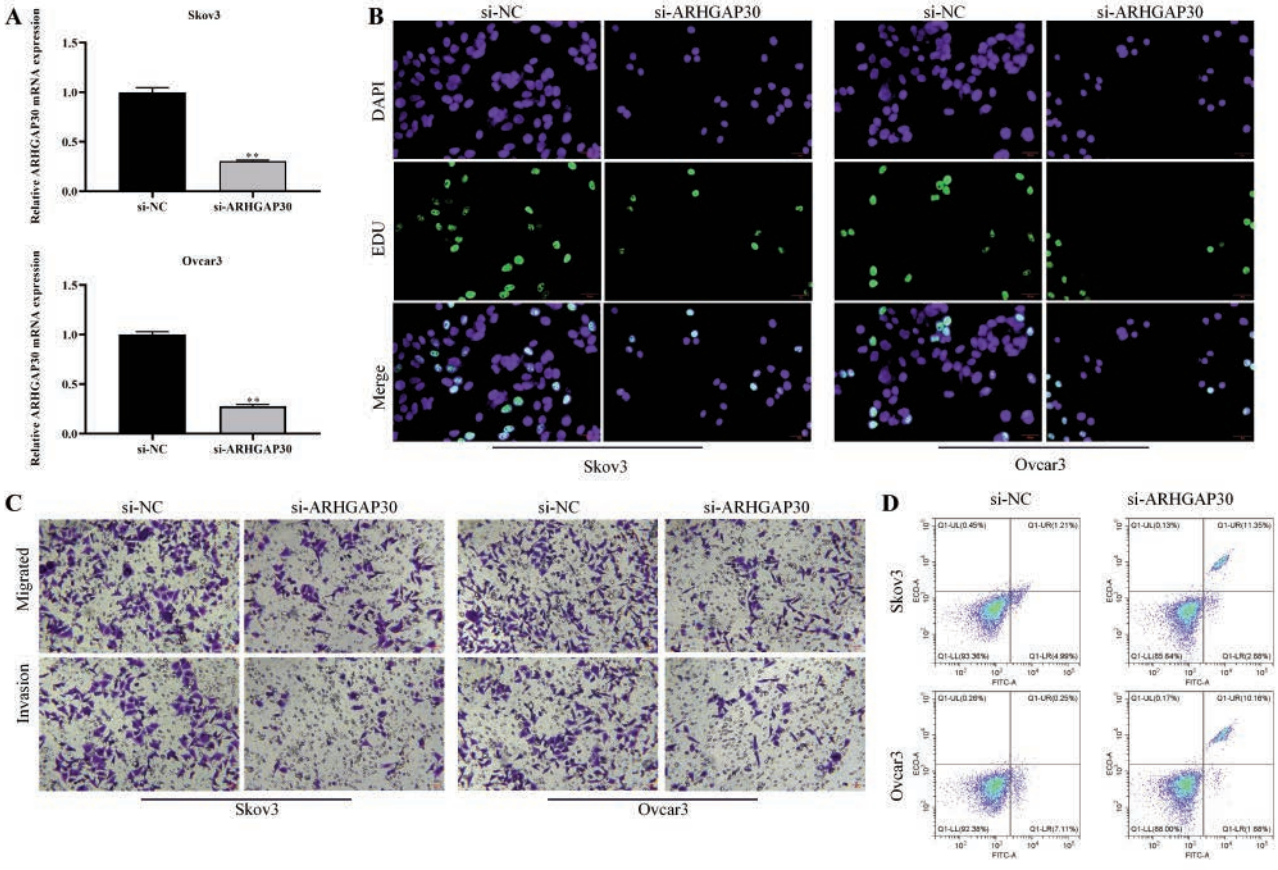


**Figure 2. ARHGAP30 downregulation inhibited OC cell growth and spread and promoted apoptosis. A) Suppression of ARHGAP30. B) Ovarian cancer cell proliferation was detected after the transfection with si-ARHGAP30 (×200, scale bar: 20 μm). C) Migrative and invasion of OC cells following ARHGAP30 knockdown (×200, scale bar: 50 μm). D) Apoptosis of OC cells was measured using flow cytometry after ARHGAP30 knockdown.**

sion in Skov3 and Ovcar3 cells. We then evaluated proliferation of Skov3 and Ovcar3 cells to determine the effects of ARHGAP30 knockdown on OC cell progression. Knockdown of ARHGAP30 inhibited Skov3 and Ovcar3 cell growth, according to EdU assay. Transfection with si-ARHGAP30 significantly decreased OC cell proliferation compared to that observed in cells transfected with si-NC (Figure 2B). Transwell assay showed that ARHGAP30 down-regulation significantly reduced Skov3 and Ovcar3 migration and invasion (Figure 2C). Furthermore, si-ARHGAP30 significantly increased the proportion of apoptotic OC cells (Figure 2D).

### ARHGAP30 regulated OC cell progression through the PI3K/AKT/mTOR pathway

ARHGAP30 was found to interact with PI3K/AKT/mTOR according to the STRING database (https://cn.string-db.org/) (Figure 3A). The expression levels of activated PI3K (p-PI3K), activated AKT (p-AKT), and activated mTOR (p-mTOR) were significantly decreased after knockdown of ARHGAP30 in Skov3 cells (Figure 3B), which indicated that the PI3K/AKT/mTOR pathway may modulate ARHGAP30 regulation. Therefore, we treated Skov3 cells with the PI3K/AKT pathway inhibitor buparlisib. The results showed that buparlisib exerted effects similar to those observed in response to ARHGAP30 knockdown (Figure 3B). Moreover, buparlisib induced effects on proliferation, metastasis, and apoptosis markers in Skov3 cells similar to those observed in response to ARHGAP30 knockdown. The expression levels of Ki67, MMP9, and Bax were reduced after buparlisib treatment (Figure 3C). Ovcar3 cells were used to further confirm these findings. Both si-ARHGAP30 and Buparlisib reduced the expression levels of p-PI3K, p-AKT, and p-mTOR (Figure 4A). The expression levels of Ki67, MMP9, and Bax were also decreased by si-ARHGAP30 and buparlisib (Figure 4B).

## Discussion

RhoGAPs are RAS superfamily proteins that regulate vital physiological activities in cancer development.[22] RhoGAPs have been shown to increase RhoGTPase activity and increase GTP hydrolysis.[4] Furthermore, RhoGAPs are commonly dysregulated in tumors.[22,23] ARHGAP30 is a RhoGAP that regulates Rac1 and RhoA activity.[8] ARHGAP30 was previously thought to be a Wrch-1-interacting protein that governs cell adhesion and actin dynamics.[24] However, a study showed that p53 acetylation and activation in colon cancer were modulated by ARHGAP30.[9] Furthermore, ARHGAP30 has been shown to slow the spread of pancreatic and lung cancers through inhibition of Wnt/β-catenin signal transduction.[10,25] The role of ARHGAP30 in OC development remains unknown. Analysis using the GEPIA database, IHC, and RT-qPCR showed that OC tissue samples and cells had high levels of ARHGAP30 expression, which indicated that ARHGAP30 was important in tumor survival. Furthermore, ARHGAP30 knockdown promoted OC cell apoptosis and inhibited metastasis. These findings suggest that ARHGAP30 may promote the development of OC through regulation of proliferation, migration, and invasion, and inhibition of apoptosis.

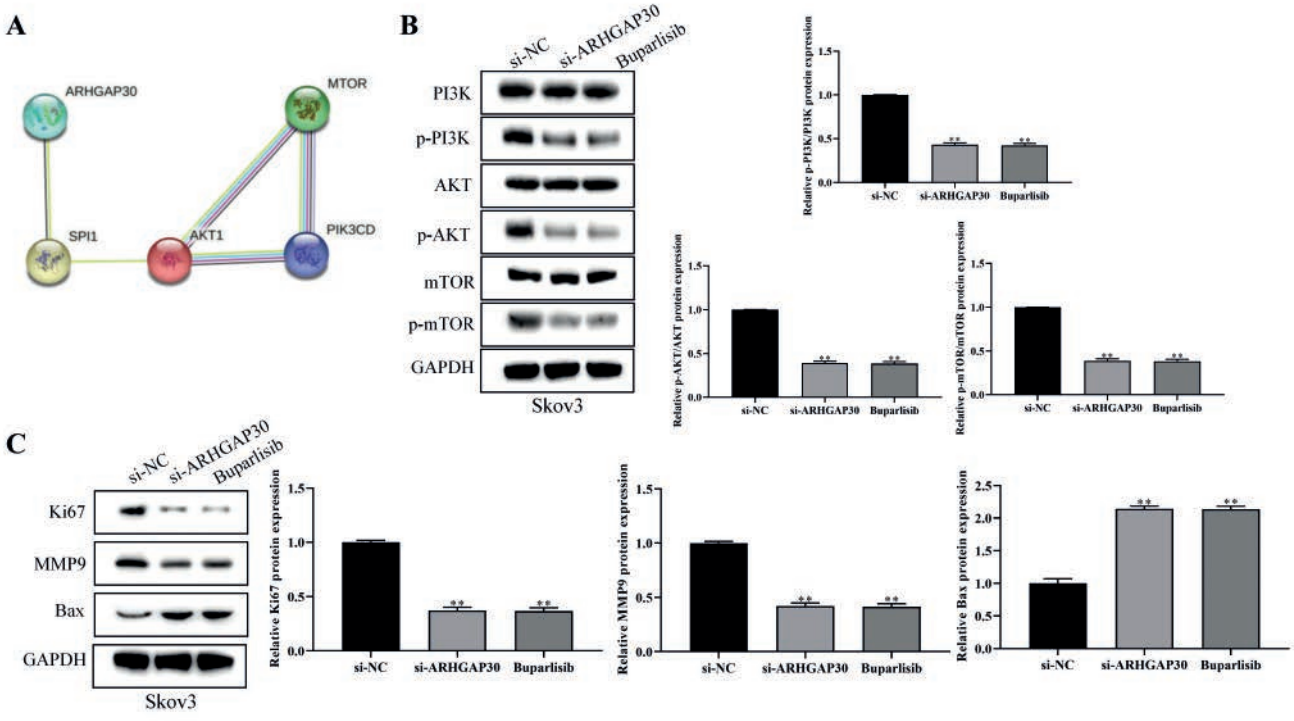


**Figure 3. ARHGAP30 regulated OC cell progression through the PI3K/AKT/mTOR pathway. A) The interaction between ARHGAP30 and the PI3K/AKT/mTOR pathway was predicted by the STRING database. B) Both si-ARHGAP30 and buparlisib reduced the abundance of p-PI3K, p-AKT, and p-mTOR in Skov3 cells. C) The expression levels of Ki67, MMP9, and Bax were also decreased by si-ARHGAP30 and buparlisib in Skov3 cells.**

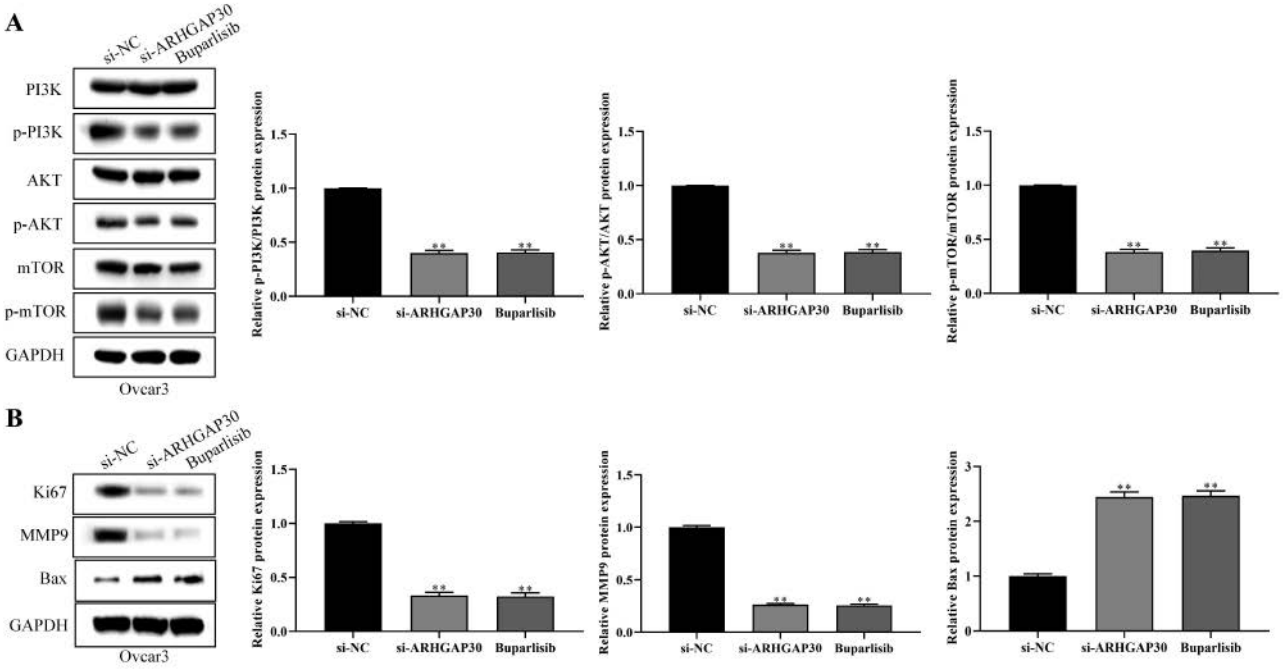


**Figure 4. The association between ARHGAP30 and the PI3K/AKT/mTOR pathway was verified in Ovcar3 cells. A) Both si-ARHGAP30 and buparlisib reduced the abundance of p-PI3K, p-AKT, and p-mTOR in Ovcar3 cells. B) The expression levels of Ki67, MMP9, and Bax were decreased by si-ARHGAP30 and buparlisib in Ovcar3 cells.**

Signaling through the PI3K/AKT/mTOR pathway is blocked under hypoxic conditions and in response to nutrient insufficiency resulting in tumor survival.[16,26] Furthermore, increased expression of nuclear transcription factors such as Slug and ZEB, amplification of the PI3K/AKT signaling can successfully promote EMT, and hence reducing the amount of E-cadherin; or provoking the abundance of MMPs to breakdown E-cadherin.[27] Previous studies have found that steroid receptor coactivator 3 (SRC-3) and tumor necrosis factor receptor-associated factor 4 (TRAF4) promote OC cell growth, migration, invasion, and stemness by activating the PI3K/Akt pathway.[28] Compound C passed PI3K/AKT/mTOR/NF-κB pathway has been shown to inhibit OC progression.[15] Furthermore, CXCR4 has been shown to modulate PI3K/Akt/mTOR signaling in epithelial OC (EOC). In addition, the CXCR4-PI3K/Akt/mTOR axis promotes the formation of cancer stem cells (CSCs) and epithelial-mesenchymal transition (EMT), resulting in resistance to chemotherapy.[14]

In this study, we found that ARHGAP30 interacted with PI3K/AKT/mTOR signaling pathway-related proteins. Furthermore, we found that ARHGAP30 knockdown inhibited proliferation, migration, and invasion of OC cells and promoted cell apoptosis through inhibition of the PI3K/AKT/mTOR signaling pathway. These results were consistent with those from previous studies.

Our study was subject to the following limitations. Future *in vivo* should focus on the mechanisms by which ARHGAP30 modulates OC. Furthermore, studies with larger sample sizes are needed to evaluate the clinical relevance of ARHGAP30. We showed that ARHGAP30 was overexpressed in OC. By controlling the PI3K/AKT/mTOR pathway, ARHGAP30 downregulation effectively inhibited the spread and growth of OC cells. These results indicated that ARHGAP30 may be a promising candidate for OC therapy.

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Received for publication: 21 January 2023. Accepted for publication: 22 April 2023.

*Licensee PAGEPress, Italy*
*European Journal of Histochemistry 2023; 67:3653*
*doi:10.4081/ejh.2023.3653*