Knockdown of BAG3 synergizes with olaparib to kill ovarian cancer cells via repressing autophagy

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ABSTRACT

This study aimed at expounding the synergistic effect of Bcl-2-associated athanogene 3 (BAG3) knockdown and poly ADP-ribose polymerase (PARP) inhibitor on ovarian cancer (OC) cells and the potential mechanism. Short hairpin RNA (shRNA) targeting BAG3 (sh-BAG3) was transfected into SK-OV-3 (SKOV-3 ;SKOV3) and A2780 cells, and western blot assay was used to detect transfection efficiency. Cell proliferation and apoptosis were detected by the cell counting kit-8 method, 5-Bromodeoxyuridine (BrdU) experiment and flow cytometry analysis, respectively. The expressions of apoptosis-related proteins Bax and Bcl-2, as well as the expressions of autophagy-related proteins LC3-I, LC3-II and Beclin-1, were examined by western blot assay. Additionally, the cells were treated with autophagy activator rapamycin to investigate whether the tumor-suppressive function of BAG3 knockdown+PARP inhibitor was dependent on autophagy. In this work, we demonstrated that BAG3 knockdown further sensitized OC cells to olaparib treatment, reducing cellular viability and promoting apoptosis. Both sh-BAG3 and olaparib decreased the expression of Beclin-1 and the LC3-II:LC3-I ratio, and their synergism further inhibited the process of autophagy. However, the aforementionede effects were reversed after the cells were treated with rapamycin. Based on these results, we concluded that BAG3 knockdown synergizes with olaparib to kill OC cells in vitro by repressing autophagy.

Significance of this study

What is already known about this subject?

- Bcl-2-associated athanogene 3 (BAG3) promotes cell proliferation and metastasis of ovarian cancer (OC) cells.
- BAG3 knockdown inhibits the autophagy of OC cells.
- Olaparib can treat patients with OC with metastasis and recurrence.

What are the new findings?

- BAG3 knockdown promotes the sensitivity of OC cells to olaparib.
- Olaparib inhibits the autophagy of OC cell lines SK-OV-3 and A2780.
- The combination of BAG3 knockdown and olaparib further impedes the process of autophagy.

How might these results change the focus of research or clinical practice?

- The combination of BAG3 inhibition and olaparib treatment may be a novel strategy for the treatment of OC.

INTRODUCTION

Due to the insidious symptoms, more than 70% of patients with ovarian cancer (OC) are in the advanced clinical stage when they are diagnosed, and OC has become the most deadly gynecological malignancy with a 5-year survival rate of about 15%–20%.[1 2] Progress has been made in the clinical application of standardized surgery, platinum-based chemotherapy, and targeted therapy such as poly ADP-ribose polymerase (PARP) inhibitors, and the survival time of patients with OC has been gradually improved in recent years,[3 4] whereas its recurrence rate is as high as 60%–80% due to the existence of multiple drug resistance.[5] Hence, exploring novel therapeutic targets of OC is currently the pivotal issue in the field of OC research.

Bcl-2-associated athanogene 3 (BAG3) is a member of the Bcl-2-associated athanogene (BAG) family.[6] BAG3 is considered as an anti-apoptosis and proautophagy factor, playing a prominent role in regulating cell apoptosis, cytoskeleton remodeling, autophagy, migration, invasion and stress responses of cancer cells.[7 8] In a previous report, BAG3 is a crucial oncogene in OC and modulates cancer cell proliferation, metastasis and drug resistance. For example, BAG3 is highly expressed in OC tissues and cells and promotes the expression of S-phase kinase-associated protein 2 by post-transcriptional regulation, thus promoting cell proliferation and metastasis of OC cells.[9 10] Importantly, BAG3 knockdown suppresses autophagy of OC cells, therefore facilitating the sensitivity of cells to cisplatin,[11] which suggests that inhibiting BAG3 is a promising strategy to sensitize OC cells to therapeutics.

The application of targeted therapies is a revolution for cancer treatment. PARP inhibitors exert a synergistic effect with mutations in DNA repair genes, inducing the apoptosis of cancer cells.[12] PARP inhibitors, such as olaparib, are approved by the Food and Drug Administration for the treatment of OC and patients with breast cancer with breast invasive

carcinoma mutations.[12 13] PARP inhibitors abrogate PARP function, resulting in the accumulation of single-strand breaks, which, in turn, can be converted into double-strand breaks (DSBs) that are unable to be repaired, leading to the apoptosis of cancer cells.[12] Olaparib significantly improves the survival time of patients with OC who have undergone three or more earlier lines of chemotherapy.[13] However, drug resistance limits its therapeutic effect.[14]

As a classical autophagy-related molecule, Beclin-1 forms a complex with Bcl-2 and is capable of inhibiting the activation of the classical autophagy pathway.[15] LC3-I and LC3-II are two forms of LC3, and the expression level of LC3-II reflects the degree of autophagy.[16] Increased autophagy is linked to enhanced tumor-suppressive functions of olaparib.[17] Besides, BAG3 knockdown can result in enhanced autophagy in OC cells.[11] Therefore, we supposed that, compared with OC cells treated with olaparib alone, inhibition of BAG3 would probably increase the sensitivity of OC cells to olaparib via repressing autophagy. This study was designed to validate our hypothesis.

MATERIALS AND METHODS

Cell culture

Both human OC cell lines SKOV3 (SKOV-3; SKOV3) and A2780 were purchased from the Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). All cells were cultured in Dulbecco's Modified Eagle's Medium (HyClone, Logan, Utah, USA) containing 10% inactivated fetal bovine serum (Thermo Fisher Scientific, Massachusetts, USA), 100 U/mL penicillin and 100 µg/mL streptomycin (HyClone) with 5% CO_2 at 37°C. Then, 0.25% trypsin (HyClone (Thermo Fisher Scientific, Logan, UT, USA.) was used to trypsinize and subculture the cells. Cells in the logarithmic growth phase were harvested for follow-up experiments.

Transfection

SKOV3 and A2780 cells in the logarithmic growth phase were inoculated into six-well plates at a density of 2×10^4/well and were cultured for 24 hours. After the medium was discarded, serum-free medium was added. Lipofectamine3000 (Invitrogen, Carlsbad, California, USA) was used to perform transfection, and western blot assay was used to detect the knockdown effect of short hairpin RNA (shRNA) targeting BAG3 (sh-BAG3) on OC cells. The sequence of sh-BAG3 was CCTTCTTCGTGGACCACAA, and the sequence of normal control shRNA (NC) was CCTGCTTAGGTACCCTCAA. sh-BAG3 and control shRNA were obtained from RiboBio (Guangzhou, China).

Western blot assay

Cells in the logarithmic growth phase were harvested and lysed with radio-immunoprecipitation assay buffer (Beyotime Biotechnology, Shanghai, China). After centrifugation, the supernatant was collected, and the protein in the supernatant was quantified using bicinchoninic acid method. Five per cent concentrated gel and 10% separation gel were selected, and protein samples (20 µL of protein sample/well) were separated by electrophoresis, and then the protein was transferred to polyvinylidene fluoride (PVDF) membrane (Millipore, Bedford, Massachusetts, USA). After that, the PVDF membrane was blocked with 5% skimmed milk for 2 hours and then incubated with the primary antibody at 4°C overnight. After being rinsed three times with tris buffered saline Tween (TBST) for 5 min each time, the membrane was incubated with secondary antibody for 2 hours at room temperature. After the membrane was washed three times with TBST, enhanced chemiluminescence (ECL) kit (Millipore) solutions A and B were mixed with a ratio of 1:1, by which the surface of the PVDF membrane was covered, and then the chemiluminescence signals were visualized by Bio-Rad ChemoDox XRS System. Antibodies used in this study included anti-BAG3 (Abcam, ab47124, 1:500), anti-Bax antibody (ab182733, 2000), anti-Bcl-2 antibody (ab32124, 1:2000), anti-LC3B (ab48394, 1:2000), anti-Beclin 1 (ab207612, 1:1000) and anti-glyceraldehyde-3 phosphate dehydrogenase (ab8245, 1:2000), all of which were purchased from Abcam (Shanghai, China).

Cell counting kit-8 (CCK-8) assay

SKOV3 and A2780 cells in the logarithmic growth phase were taken, followed by being trypsinized, centrifuged, counted, and then inoculated into 96-well plates with 100 µL/well at a density of 4×10^3 cells/well. After the cells were cultured for 24 hours, olaparib with different concentrations (1.0, 2.0, 4.0, 8.0 and 16.0 µM) was added, while the control group was added with the same amount of dimethyl sulfoxide. After 48 hours, the medium was discarded, and 200 µL of phosphate-buffered saline (PBS) was added to each well for washing the cells before PBS was discarded. After that, 10 µL of CCK-8 kit (Dojindo, Kumamoto, Japan) and 90 µL of fresh medium were added into each well, with which the cells were incubated for 1 hour. Finally, the absorbance (optical density) value of each well was measured at 450 nm wavelength with a microplate reader.

BrdU experiment

SKOV3 and A2780 cells in the logarithmic growth phase were harvested to prepare the single-cell suspension, and then it was inoculated into a 24-well plate at 1×10^5 cells/well. The cells were divided into four groups: control group, sh-BAG3 group, olaparib (4 µmol/L) group and sh-BAG3+olaparib (4 µmol/L) group. 5-Bromodeoxyuridine (BrdU) labeling reagent (Biocompare, San Francisco, California, USA) was added, and the plate was placed in an incubator for cell culture. After 12 hours of culture, the cells were fixed and anti-BrdU antibody was added to incubate the cells at room temperature for 2 hours. Subsequently, 4',6-diamidino-2-phenylindole, dihydrochloride (DAPI) solution was used to mark the nuclei. Finally, the number of BrdU-positive cells and the number of DAPI positive cells in three vision fields were counted under a fluorescence microscope (Olympus, Tokyo, China). Cell proliferation rate=the number of BrdU positive cells/the number of DAPI positive cells.

Flow cytometry

The cells were divided into four groups: control group NC, sh-BAG3 group, olaparib (4 µmol/L) group and sh-BAG3+olaparib (4 µmol/L) group. Cells in the logarithmic growth phase were selected and trypsinized with 0.25% trypsin. Afterwards, cells were collected into centrifuge tubes, washed with PBS twice and centrifuged at room

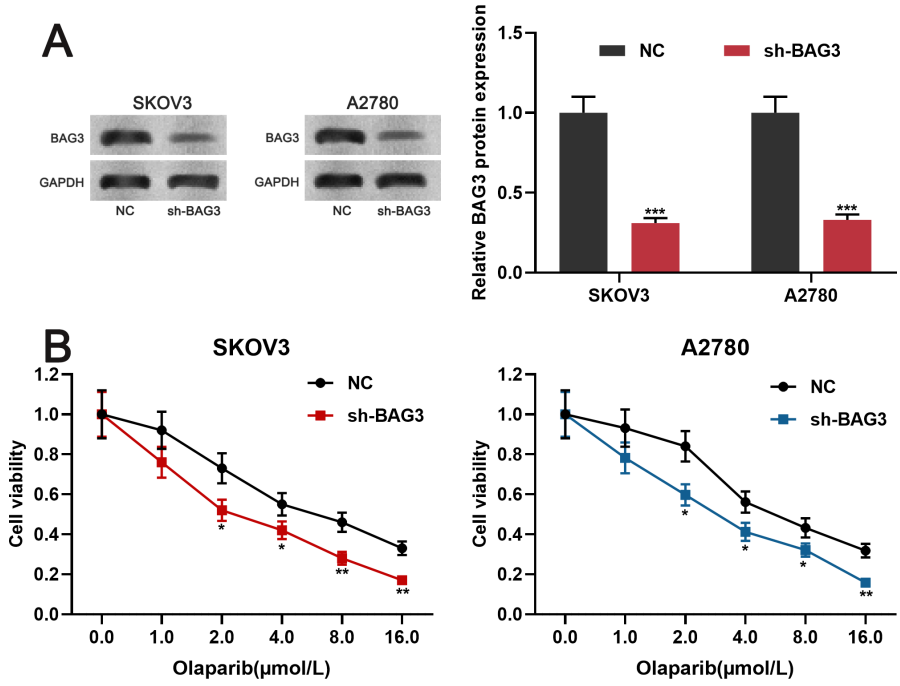

Figure 1 BAG3 knockdown and olaparib inhibited the viability of OC cells. (A) Western blot assay showed that the expression level of BAG3 protein was decreased after BAG3 shRNA was transfected into SKOV3 and A2780 cells. (B) Olaparib inhibited the viability of SKOV3 and A2780 cells in a concentration-dependent manner, and the inhibitory effect on the viability of BAG3 knockdown+olaparib treatment was more significant. BAG3, Bcl-2-associated athanogene 3; OC, ovarian cancer; sh-BAG3, BAG3 shRNA. SKOV3, SK-OV-3.

temperature for 5 min at 1500 revolution/min. After that, the supernatant was removed. Subsequently, annexin V-allophyclin (V-APC)/7-amino-actinomycin D (7-AAD) cell apoptosis detection kit (Yeasen Biotech Co., Shanghai, China) was used to detect the apoptosis of the cells. In brief, in 1× binding buffer, the cells were resuspended. Next, annexin V-APC solution and 7-AAD solution were added, with which the cells were incubated at room temperature in the dark for 30 min. Finally, the apoptosis of the cells was detected by flow cytometer.

Statistical analysis

All experiments were performed in triplicate. SPSS V.22.0 and GraphPad Prism V.8.0 (GraphPad Software, San Diego, California, USA) were used to analyze the data. The data were shown as mean±SD (x±s). t-Test was used for comparison between two groups, and the difference was statistically significant with $p<0.05$. In the figures, *, ** and *** indicated $p<0.05$, $p<0.01$ and $p<0.001$, respectively.

RESULTS

BAG3 knockdown synergized with PARP inhibitor to suppress the viability of OC cells

First of all, we transfected sh-BAG3 into SKOV3 and A2780 cells, and western blot assay showed that the transfection was successful (figure 1A). Subsequently, the CCK-8 method was used to detect the effect of different concentrations of olaparib (1, 2, 4, 8 and 16 μmol/L) on the cell viability of the NC group, the results of which displayed that the cell viability of SKOV3 and A2780 cells was significantly reduced with the increase in olaparib concentration, and the viability of cells in the sh-BAG3 group was lower than that of the NC group (figure 1B). These results supported that BAG3 knockdown and PARP inhibitor olaparib both inhibited the viability of OC cells, and BAG3 knockdown could promote the tumor-suppressive function of olaparib.

Combination of BAG3 knockdown and olaparib treatment could suppress the proliferation of SKOV3 and A2780 cells and promote their apoptosis

The aforementioned results indicated that the inhibitory effect of olaparib on OC cell viability was in a dose-dependent manner, and 4 μmol/L olaparib was chosen for the subsequent experiments. Through BrdU experiment and flow cytometry, we found that compared with the control group, the proliferation of OC cells in the sh-BAG3 group and the olaparib group was inhibited, while the apoptosis was promoted, and in sh-BAG3+olaparib group, BAG3 knockdown further sensitized OC cells to olaparib (figure 2A,B). Furthermore, western blot assay showed that BAG3 knockdown and olaparib treatment, respectively, promoted Bax expression and inhibited Bcl-2 expression, whereas the aforementioned effects were more obvious when BAG3 knockdown and olaparib treatment were combined, which indicated that the cytotoxicity was more significant (figure 2C).

Combination of BAG3 knockdown and olaparib treatment could inhibit the expression of Beclin-1 and suppress autophagy of OC cells

To further elaborate on the potential mechanism of the effects of combination of BAG3 knockdown and olaparib treatment on OC cells, we detected the expressions of

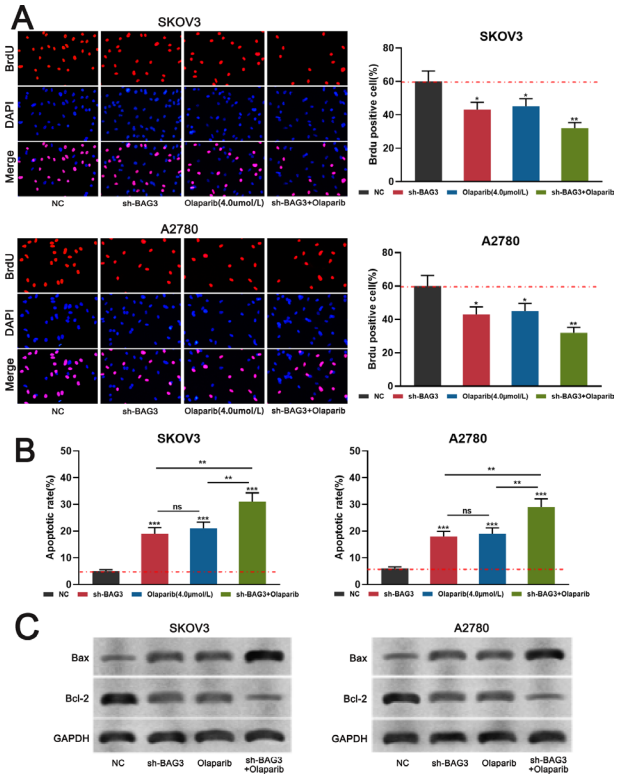

Figure 2 The combination of BAG3 knockdown and olaparib treatment could suppress the proliferation of SKOV3 and A2780 cells and promote their apoptosis. (A) BrdU experiment showed that, compared with the NC group, BAG3 knockdown and olaparib treatment (4.0 µmol/L) inhibited the proliferation of cells, and BAG3 knockdown+olaparib treatment (4.0 µmol/L) had a more significant inhibitory effect. (B) Compared with the NC group, BAG3 knockdown and olaparib treatment (4.0 µmol/L) promoted OC cell apoptosis, and BAG3 knockdown+olaparib treatment (4.0 µmol/L) promoted cell apoptosis more significantly. (C) Western blot assay showed that, compared with the NC group, the expression of Bax protein increased and the expression of Bcl-2 decreased in the sh-BAG3 group and the olaparib (4.0 µmol/L) group. In the sh-BAG3+olaparib (4.0 µmol/L) group, the aforementioned effects were more significant. BAG3, Bcl-2-associated athanogene 3; BrdU, 5-Bromodeoxyuridine; ns, not significant; OC, ovarian cancer; sh-BAG3, BAG3 shRNA.

autophagy-related proteins LC3-I, LC3-II and Beclin-1 by western blot assay, the results of which showed that BAG3 knockdown and olaparib, respectively, inhibited the expression of Beclin-1 in SKOV3 and A2780 cells and reduced the ratio of LC3-II:LC3-I, whereas the combination of BAG3 knockdown and olaparib treatment would further reduce these autophagy-related markers (figure 3A,B), indicating that BAG3 knockdown could probably sensitize OC cells to olaparib by suppressing autophagy.

Rapamycin could reverse the inhibitory effects of BAG3 knockdown and olaparib on autophagy

The aforementioned results hinted that the combination of BAG3 knockdown and olaparib treatment could inhibit the autophagy of SKOV3 and A2780 cells. Subsequently, we treated OC cells with autophagy activator rapamycin, and the expressions of LC3-I, LC3-II and Beclin-1 proteins were detected by western blot assay, the findings of which manifested that the combination of BAG3 knockdown and olaparib treatment could repress the expression of Beclin-1 and reduce the LC3-II:LC3-I ratio, whereas the aforementioned effects were partially abolished when rapamycin was added (figure 4A,B). To sum up, the combination of BAG3 knockdown and olaparib treatment played a tumor-suppressive role in OC cells by inhibiting autophagy.

DISCUSSION

Previous studies have reported that BAG3 is abnormally expressed in OC and plays tumor-promoting roles in OC cells via regulating proliferation, migration, invasion, apoptosis and drug resistance.[10 11 18–21] For example, BAG3 overexpression is correlated with poor prognosis of patients with OC, and BAG3 upregulates MMP2 expression and facilitates cell motility and invasiveness.[18] Besides, BAG3 can suppress the expression of miR-29b and, in turn, can increase the expression of Mcl-1, by which it induces the chemoresistance of OC cells.[19] Additionally, BAG3 cooperates with USP9X to stabilize the expression level of Mcl-1, thus promoting the drug resistance of OC cells to paclitaxel.[20] It is also reported that miR-340 inhibits PI3K and AKT pathways by inhibiting the expression of BAG3, thus inducing apoptosis of SKOV3 cells.[21] This study unmasked that BAG3 knockdown could suppress the viability of SKOV3 and A2780 cells, accelerate their apoptosis, inhibit the expression of Beclin-1, reduce the LC3-II:LC3-I ratio and inhibit the autophagy process. Our work confirms that BAG3 is a promising target for OC treatment.

The drug resistance of PARP inhibitors remains a crucial issue in clinical treatment of OC. Multiple strategies are employed to sensitize OC cells to PARP inhibitors. The natural compound alantolactone and olaparib have a significant synergistic effect, inducing DSB and thus killing OC cells.[22] Reportedly, PD-L1 expression of OC cells is upregulated after PARP inhibitor treatment, and targeting PD-L1 is able to counteract the inhibitory function of PARP inhibitor on CD8+ T cells and has synergistic antitumor effects with PARP inhibitor.[23] In this study, we found that BAG3 knockdown and olaparib treatment synergistically inhibited the viability and proliferation, and promoted the apoptosis of OC cells. These data suggested that targeting BAG3 could be beneficial to patients with OC resistant to PARP inhibitors, and this hypothesis requires more preclinical studies to be validated.

Autophagy refers to the process in which macromolecules and organelles in the cytoplasm of eukaryotic cells are transported to lysosomes for isolation and degradation, which is roughly divided into three stages, namely, autophagy induction, autophagy formation, and degradation/use. Chemotherapeutics can induce cellular apoptosis while triggering autophagy. Autophagy not only participates in the induction of cell death but also serves as a survival mechanism to protect tumor cells from apoptosis by degrading damaged macromolecules and organelles for recycling.[24] The drug resistance of multiple chemotherapeutics and targeted drugs is accompanied by enhanced autophagy. BAG3 is the sole member with the WW domain in the BAG family, which is quite important for inducing autophagy. For example,

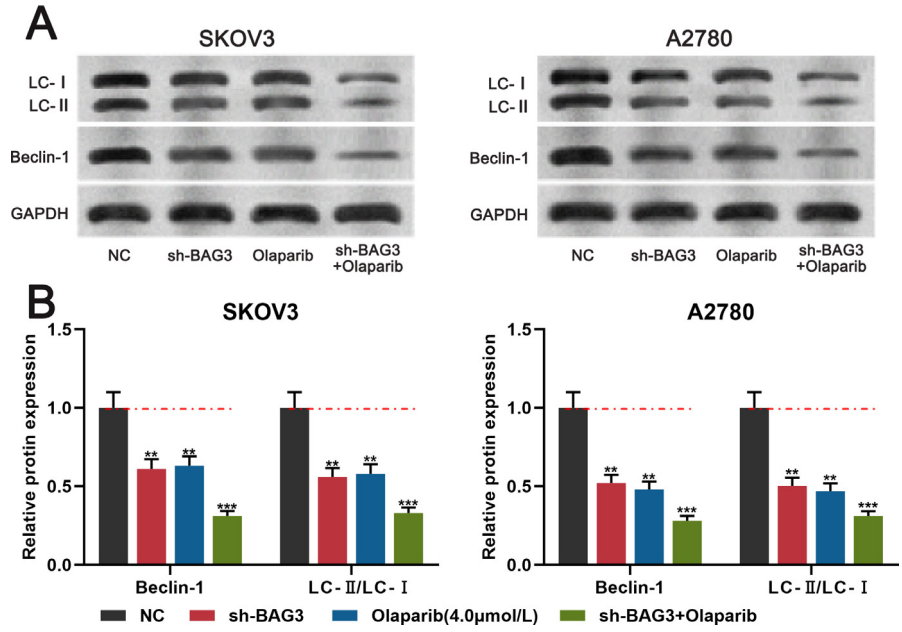

Figure 3 The combination of BAG3 knockdown and olaparib could inhibit the autophagy of SKOV3 and A2780 cells. (A,B) Western blot assay showed that Beclin-1 protein expression and LC-II:LC-I ratio decreased in the sh-BAG3 group and the olaparib (4.0 μmol/L) group compared with the NC group. However, in the sh-BAG3+olaparib (4.0 μmol/L) group, the aforementioned effects were more significant. BAG3, Bcl-2-associated athanogene 3; sh-BAG3, BAG3 shRNA.

BAG3 contributes to the phosphorylation of subunit α of translation initiation factor eIF2 independent of HSP70, eventually leading to translation stagnation and activation of autophagy.[25 26] Beclin-1 and LC3 are key proteins of autophagy. The main mechanism of Beclin-1 activating autophagy is to regulate the PI3K–Akt–mTOR pathway.[27] LC3 has two forms: LC3-I and LC3-II; after autophagy is initiated, a segment of polypeptide will be hydrolyzed and LC3-I transforms into LC3-II and aggregates onto the membrane of autophagosome.[28] The improved expression of LC3-II reflects the enhanced degree of autophagy.[28] The present study demonstrated that BAG3 knockdown combined with PARP inhibitor significantly inhibited the autophagy of cells, while the autophagy activator rapamycin

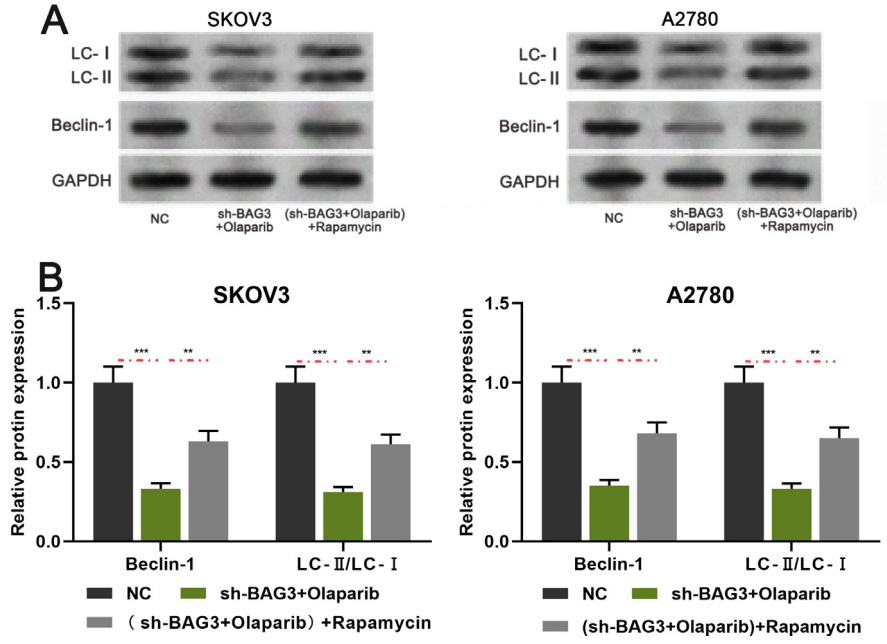

Figure 4 Rapamycin could reverse the inhibitory effects of BAG3 knockdown and olaparib treatment on autophagy of OC cells. (A,B) Western blot assay showed that, compared with the NC group, the expressions of Beclin-1 protein and LC-II:LC-I ratio decreased in the sh-BAG3+olaparib (4.0 μmol/L) group. However, the autophagy activator rapamycin partially reversed the aforementioned effects. BAG3, Bcl-2-associated athanogene 3; OC, ovarian cancer; sh-BAG3, BAG3 shRNA.

partially rescued the autophagy, indicating that the combination of BAG3 knockdown and PARP inhibition synergistically killed OC cells by inhibiting autophagy.

In summary, compared with the unilateral effect, the combination of BAG3 knockdown and olaparib can preferably suppress the proliferation of OC cells and accelerate apoptosis. Mechanistically, this strategy exerts its tumor-suppressive role by inhibiting autophagy. The findings of this study provide novel clues for the treatment of OC. It is worth noting that there are several limitations in this work. First, our conclusion is based only on in vitro experiments, lacking data from animal experiments. Additionally, no PARP inhibitor-resistant OC cell lines are established, and these models are necessary to investigate whether BAG3 knockdown can reverse the drug resistance of PARP inhibitors. These shortcomings remain to be solved in the following studies.

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