Abstract. In the present study, the tumor-suppressive role of microRNA-127-3p (miR-127-3p) in epithelial ovarian cancer (EOC) was elucidated. Expression of miR-127-3p was examined by quantitative RT-PCR (qRT-PCR) in 9 EOC cell lines and clinical samples from 13 EOC patients. EOC cell lines, OVCAR-3 and Caov-3, were transduced with a lentivirus to overexpress endogenous miR-127-3p. The tumor-suppressive effects of miR-127-3p on EOC proliferation, bufalin sensitivity, invasion and in vivo growth were investigated through proliferation, bufalin sensitivity wound-closure and in vivo tumorigenicity assays, respectively. In addition, luciferase reporter assay and qRT-PCR were conducted to verify whether the Bcl-2-associated athanogene 5 (BAG5) gene was the downstream target of miR-127-3p in EOC. BAG5 was subsequently upregulated in the OVCAR-3 and Caov-3 cells to examine its functional correlation with miR-127-3p regulation in EOC. The results revealed that in both EOC cell lines and EOC tumor tissues, miR-127-3p was downregulated. Lentiviral-mediated miR-127-3p overexpression exerted tumor-suppressive effects in OVCAR-3 and Caov-3 cells by reducing in vitro proliferation and invasion, increasing bufalin sensitivity, and inhibiting in vivo tumor growth. miR-127-3p directly regulated the BAG5 gene in EOC. Subsequent BAG5 upregulation ameliorated the tumor-suppressive effects of miR-127-3p overexpression in EOC. In conclusion, miR-127-3p functions as a tumor suppressor in EOC, and its influence on EOC is directly through regulation of BAG5.

Introduction

Epithelial ovarian cancer (EOC) is one of the most malignant gynecological cancers worldwide (1,2). In the United States alone, there were more than 20,000 estimated new cases, and more than 14,000 deaths due to EOC (3). Both genetic and epigenetic factors may contribute significantly to the origin of EOC (2). Despite numerous efforts, the exact mechanisms of EOC pathogenesis are fundamentally unknown, and methods for early diagnosis and novel therapies are largely lacking. Thus, it is critical to explore the molecular mechanisms underlying EOC carcinogenesis, development and metastasis to seek novel therapeutic targets for patients with EOC.

MicroRNAs (miRNAs) are a group of small-length (18-22 nucleotides long) non-coding RNAs that bind the complementary sites of the 3'-untranslated region (3'-UTR) of target genes to induce gene inhibition and protein degradation (4,5). miRNAs have been shown to play critical roles in human cancer, acting as either oncogenes or tumor suppressors (6,7). In human EOC, both upregulated and downregulated miRNAs have been discovered (8), suggesting that the regulation of miRNAs in EOC may be complex, as well as their associated signaling pathways. MicroRNA-127-3p (miR-127-3p), also referred to as miR-127, was often found to be downregulated in human tumors and acts as a tumor suppressor in breast and gastric cancer (9,10). miR-127-3p was also found to be downregulated in clinical samples from EOC patients (11,12). Yet, the functional mechanisms of miR-127-3p have never been elucidated in EOC.

In the present study, we evaluated the expression pattern of miR-127-3p in both EOC cell lines and EOC tumor samples. Then, we hypothesized that miR-127-3p may act as a tumor suppressor in EOC as in other human cancers, and tested this hypothesis by endogenously overexpressing miR-127-3p in EOC cell lines, OVCAR-3 and Caov-3, through lentiviral transduction. The functional effects of miR-127-3p overexpression on EOC proliferation, drug (bufalin) sensitivity, invasion and in vivo tumorigenicity were then carefully evaluated. Furthermore, we hypothesized that the
Bcl-2-associated athanogene 5 (BAG5) gene is the downstream target of miR-127-3p in EOC. We then tested this hypothesis through dual-luciferase reporter assay and qRT-PCR. Subsequently, BAG5 was upregulated in OVCAR-3 and Caov-3 cells, and its interaction with miR-127-3p overexpression on regulating EOC was further investigated.

Materials and methods

Statement of ethics. All experimental protocols were approved by the ethics committees at the participating hospitals. All procedures were conducted in accordance to the principles of the Declaration of Helsinki, and Local and National Medical Practice Laws. Consent forms were signed by all participating patients.

Cell lines and patients. In this study, five EOC cell lines, SKOV-3, OVCAR-3, Caov-3, ES-2, PA-1 and a non-tumorigenic human-derivative ovarian cell line, HS-832, were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). Four EOC cell lines, MCAS, OVC4A32, OVC4A29 and PEO4 were obtained from the Cell Bank of the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). All cells were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) (both from Thermo Fisher Scientific, USA) in a tissue culture chamber supplemented with 95% CO2 and 5% CO2. Thermo Fisher Scientific, USA) in a tissue culture chamber supplemented with 10% fetal bovine serum (FBS) (both from China). All cells were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) (both from China). All cells were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) (both from China). All cells were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) (both from China).

Materials and methods

RNA isolation and quantitative RT-PCR. Total RNA was isolated from the EOC cell lines or EOC patient clinical samples using an RNeasy Mini kit (Qiagen, USA) according to the manufacturer's protocol. Using 100 ng RNA from each sample, cDNA was reversely synthesized with a TaqMan Reverse Transcription kit (Applied Biosystems, USA). Quantitative RT-PCR (qRT-PCR) was conducted on an ABI Prism 7900 Sequence detection system (Applied Biosystems). For BAG5 gene detection, a SYBR Green PCR Master Mix kit (Applied Biosystems) was conducted with the following reaction conditions, 95°C for 15 min; 38 cycles of 95°C for 30 sec, 58°C for 40 sec, and 70°C for 30 sec. 18S was used as a loading control. For miR-127-3p detection, a TaqMan miRNA assay (Applied Biosystems) was conducted with the following reaction conditions, 95°C for 15 min; and 38 cycles of 95°C for 30 sec and 62°C for 40 sec. U6 snRNA was used as a loading control. All reactions were conducted in biological triplicates. Relative gene expression levels were measured as fold changes using the 2^ΔΔCt method.

miR-127-3p overexpression. A lentiviral vector expressing miR-127-3p mimics (L-miR127-Mimic) and a control lentivirus (L-C) were obtained from Ribobio Biotech (China). To overexpress hsa-miR-127-3p in EOC cell lines, 5x10^5 OVCAR-3 or Caov-3 cells were transduced with L-miR127-Mimic, along with a multiplicity of infection of 10 and 5 µg/ml Polybrene for 4 h. The control EOC cells were transduced with L-C. After 3 washes (5 min each time), the EOC cells were replenished with fresh culture medium and maintained for 48 h. After that, floating cells were aspirated. Healthy cells were suspended, re-seeded and readied for the next experiments.

Cancer proliferation assay. Five hundred OVCAR-3 or Caov-3 cells were seeded in a 96-well plate. The growth of EOC cells was measured by a CellTiter 96® Aqueous One cell proliferation assay (Promega, Madison, WI, USA) according to the manufacturer's protocol. Briefly, proliferation solution (20 µl) was added into the cell culture for 1 h at 0, 1, 2, 3 and 4 days. The absorbance was measured at 490 nm.

 Bufalin sensitivity assay. OVCAR-3 or Caov-3 cells were incubated with bufalin at various concentrations (0, 0.1, 0.5, 1, 10 and 100 ng/ml) for 48 h. The sensitivity of the EOC cells to bufalin was estimated by relative cell viability, which was measured by proliferation assay and then normalizing the absorbance values to the ones under control conditions (no bufalin treatment).

Invasion assay. In vitro invasive capability was measured by a wound closure assay. Briefly, 5x10^4 OVCAR-3 or Caov-3 cells were seeded in 96-well plates. Once confluent, the cells were incubated with 10 µg/ml mitomycin (Sigma-Aldrich, USA) for 2 h to stop proliferation. Then, a 96-pin wound-maker (Essen Biosciences, UK) was used to create defined wound areas. Phase-contrast images were captured immediately (0 h) and 24 h after wound creation. The invasive capability was determined by measuring the reduction on the wound area between 0 and 24 h using ImageJ software (NIH, Bethesda, MA, USA).

Tumorigenicity assay. OVCAR-3 cells transduced with L-miR127-Mimic or L-C were subcutaneously injected into both flanks of four adult athymic nude mice (1x10^6/injection in 8-week old mice). Tumor volume (mm^3) was monitored weekly for 5 weeks, using the formula L x W^2/2, in which L and W are the longest and shortest diameters respectively of the tumor. After 5 weeks, the mice were sacrificed. Tumors from both flanks were extracted from 4 mice (M1-M4) for visual examination.

Luciferase reporter assay. Wild-type and mutant 3’-UTRs of the human BAG5 gene were cloned into the psiCHECK2 luciferase vector (Promega). HEK293T cells were seeded in 6-well plates and co-transfected with BAG5-WildType, BAG5-Mutant or Renilla luciferase vectors, and L-C or L-miR-Mimic lentiviruses. Forty-eight hours after co-transfection, a dual-luciferase reporter assay (Promega) was conducted according to the manufacturer's protocol. Relative luciferase activities (against Renilla activity) were normalized to the activity with L-C transfection.

BAG5 upregulation assay. The whole sequence of the human BAG5 gene was cloned into a eukaryotic expression vector pcDNA3.1 (Life Technologies, USA) to generate a BAG5 overexpressing vector (pcDNA/BAG5). An empty pcDNA3.1 vector (pcDNA+/+) was used as the control. OVCAR-3 and Caov-3 cells were then transfected with pcDNA/BAG5 or pcDNA+/+ using the Massachusetts Institute of Technology (MIT) transfection method.

Subsequently, BAG5 was upregulated in OVCAR-3 and Caov-3 cells, and its interaction with miR-127-3p overexpression on regulating EOC was further investigated.

Materials and methods

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pcDNA/+ using Lipofectamine 2000 reagent (Thermo Fisher Scientific). Forty-eight hours after transfection, qRT-PCR was conducted to verify upregulation efficiency.

**Statistical analysis.** All assays were repeated at least three times. Results are presented as means ± standard errors. Statistical analyses were conducted using the Student's t-test on Windows-based SPSS software (SPSS 11.0; SPSS, Inc., USA). Statistical differences were significant at $P<0.05$.

**Results**

**miR-127-3p is downregulated in EOC cell lines and EOC tumors.** The expression of miR-127-3p was compared among nine EOC cell lines, SK-OV-3, OVCAR-3, Caov-3, ES-2, PA-1, MCAS, OVCA432, OVCA429, PEO4, and a non-tumorigenic human-derived ovarian cell line, HS-832 by qRT-PCR. It showed that miR-127-3p was markedly downregulated in all examined EOC cell lines, when compared with its level in the HS-832 cells (Fig. 1A, P<0.05). The expression of miR-127-3p was also compared, by qRT-PCR, between paired EOC tumors (T) and adjacent normal ovarian epithelial tissues (normal) in 13 patients with EOC. The result demonstrated that miR-127-3p was downregulated in the EOC tumors compared to the level in the normal ovarian tissues (Fig. 1B, P<0.05).

**miR-127-3p overexpression inhibits cancer growth and increases bufalin sensitivity in EOC.** Two of the EOC cell lines, OVCAR-3 and Caov-3, were transduced with L-miR127-Mimic to overexpress endogenous miR-127-3p. The control EOC cells were transduced with an empty control lentivirus, L-C. After transduction, qRT-PCR was conducted to compare miR-127-3p expression between the EOC cells transduced with L-miR127-mimic or L-C. Transduction of L-miR127-Mimic, as compared to L-C, markedly upregulated endogenous miR-127-3p in both the OVCAR-3 and Caov-3 cells (Fig. 2A, P<0.05).

We then compared cancer cell growth in the lentiviral-transduced EOC cells. The proliferation assay showed that miR-127-3p overexpression significantly inhibited cancer growth in the OVCAR-3 cells 2 days after the onset of the proliferation assay, and in Caov-3 cells 24 h after the onset of the proliferation assay (Fig. 2B, P<0.05). We also assessed the effect of miR-127-3p on EOC bufalin sensitivity. miR-127-3p overexpression significantly increased bufalin sensitivity by reducing cell viability in both the OVCAR-3 and Caov-3 cells (Fig. 2C, P<0.05).

**miR-127-3p overexpression reduces cancer cell invasive capability in EOC.** In the lentiviral-transduced OVCAR-3 and Caov-3 cells, a wound-closure assay was conducted.
to assess the effect of miR-127-3p overexpression on EOC invasion. Images are shown for the EOC cells immediately after wound creation (0 h), and 24 h later. A reduced degree of wound closure was observed in the OVCAR-3 and Caov-3 cells transduced with L-miR127-Mimic, than that in the cells transduced with L-C (Fig. 3, left). Subsequent quantification confirmed that miR-127-3p overexpression markedly reduced the invasive capabilities in both the OVCAR-3 and Caov-3 cells (Fig. 3, right, P<0.05).

**miR-127-3p overexpression inhibits in vivo tumorigenicity of EOC.** We then investigated whether miR-127-3p overexpression had an effect on the in vivo growth of EOC. L-miR127-Mimic-transduced OVCAR-3 cells (1x10^6) were subcutaneously injected into the left flanks of 8-week-old athymic nude mice (n=4). L-C-transduced OVCAR-3 cells (1x10^6) were subcutaneously injected into the right flanks of the nude mice. The in vivo growth curves were monitored weekly, for 5 consecutive weeks, by measuring tumor volume (mm^3). The result showed that miR-127-3p overexpression markedly inhibited in vivo EOC tumor growth (Fig. 4A, P<0.05). At the end of the tumorigenicity assay, tumors were extracted from both flanks of the mice, and compared. L-miR-127-Mimic-transduced EOC tumors were significantly smaller than the L-C-transduced tumors in all 4 mice (Fig. 4B).
BAG5 is directly associated with miR-127-3p overexpression in EOC. Since we demonstrated that miR-127-3p overexpression has a tumor-suppressive effect on EOC, we then explored the molecular target of miR-127-3p. Through web-search on miRNA targets, including miRDB (www.mirdb.org) and TargetScan Human (www.targetscan.org), we found that the BAG5 gene is a candidate downstream target gene of miR-127-3p (Fig. 5A). We then used a dual-luciferase reporter assay to verify this hypothesis. HEK293T cells were co-transfected with BAG5-wild-type, BAG5-Mutant or Renilla luciferase vectors, and L-C or L-miR127-Mimic lentiviruses. Forty-eight hours after co-transfection, quantification demonstrated that the relative luciferase activities between L-C and L-miR127-Mimic transfections significantly differed in cells transfected with BAG5-wildType luciferase reporter, but in cells transfected with BAG5-Mutant luciferase reporter (Fig. 5B, *P<0.05; P>0.05), thus confirming that BAG5 is the target gene of miR-127-3p. In addition, we examined the mRNA levels of BAG5 in miR-127-3p overexpressed OVCAR-3 and Caov-3 cells. The result of qRT-PCR demonstrated that miR-127-3p overexpression significantly downregulated endogenous BAG5 in the EOC cells (Fig. 5C, *P<0.05).

BAG5 upregulation induces opposite effects opposite to those of miR-127-3p overexpression in EOC. In order to elucidate the function of the BAG5 gene in miR-127-3p-mediated EOC regulation, we transfected L-miR127-Mimic-transduced OVCAR-3 and Caov-3 cells with a BAG5-overexpressing vector pcDNA/BAG5, or a control vector pcDNA/+. Forty-

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Figure 3. miR-127-3p overexpression reduces cancer invasion in EOC. After lentiviral transduction, OVCAR-3 (A) and Caov-3 cells (B) were seeded in 96-well plates and a wound-closure assay was performed. Phase-contrast images were captured at 0 and 24 h after wound creation (left). The invasive capability was determined by measuring the reduction in wound area between 0 and 24 h in EOC cells (right, *P<0.05).

Figure 4. miR-127-3p overexpression reduces EOC growth *in vivo*. (A) Lentiviral-transduced OVCAR-3 cells were subcutaneously injected into both flanks of four athymic null mice (1x10^6 cells per flank per mouse). For five consecutive weeks, tumor volumes (mm^3) were measured weekly using the formula, L x W^2/2, in which L and W stand for the longest and shortest diameters of the tumor. Tumor volume was compared between tumors transduced with L-miR127-Mimic and tumors transduced with L-C (*P<0.05). (B) At the end of the tumorigenicity assay, tumors were extracted from 4 mice (M1-M4) and compared.
eight hours after transfection, qRT-PCR demonstrated that endogenous BAG5 mRNAs were significantly upregulated in the EOC cells transfected with pcDNA/BAG5 than the expression level in the EOC cells transfected with pcDNA/+ (Fig. 6A, P<0.05).

We then examined the effect of BAG5 upregulation on cancer growth, bufalin sensitivity and invasive capabilities in those double-transfected EOC cells. The proliferation assay showed that BAG5 upregulation revitalized cancer growth in the miR-127-3p-overexpressing EOC cells (Fig. 6B, P<0.05). The bufalin sensitivity assay demonstrated that BAG5 upregulation ameliorated bufalin sensitivity in the miR-127-3p-overexpressing EOC cells (Fig. 6C, P<0.05). Furthermore, wound-closure assay showed that BAG5 upregulation also significantly restored invasive capability in the miR-127-3p-overexpressing EOC cells (Fig. 7, P<0.05).

Thus, our experiments using the double-transfected EOC cells clearly demonstrated that BAG5 upregulation induced effects opposite those of miR-127-3p overexpression on EOC growth, bufalin sensitivity and invasion.

**Discussion**

MicroRNAs have been shown to play important roles in EOC regulation, acting as either oncogenes or tumor suppressors (8,12). MicroRNA-127-3p has been identified as a tumor suppressor in other human cancers (9,10,13), and has been shown to be downregulated in EOC tumors (11,12). Yet, the exact functional roles of miR-127-3p in EOC are elusive. In our study, we first used quantitative method, qRT-PCR, to assess the expression pattern of miR-127-3p. We verified that miR-127-3p was downregulated in EOC cell lines in vitro, as well as in vivo clinical samples of EOC tumors.

Since all evidence points to a downregulated expression pattern of miR-127-3p in EOC, we took further step to explore the functional mechanism of miR-127-3p. We thus created two EOC cell lines, OVCAR-3 and Caov-3, with stable miR-127-3p overexpression. Subsequent functional experiments demonstrated that miR-127-3p acted as a tumor suppressor in EOC, as overexpression of miR-127-3p inhibited EOC proliferation, drug (bufalin) sensitivity, invasion and **in vivo** tumor growth.
development. Therefore, collectively (9,10,13), it may well demonstrate that miR-127-3p predominantly acts as a tumor suppressor across various cancer types.

Through investigations on breast and bladder cancer, it was found that the major molecular target of tumor-suppressive miR-127-3p was a zinc-finger repressor gene BCL6 (9,14). However, the results of our study revealed that the BAG5 gene, a novel candidate, was likely the target gene of miR-127-3p in ovarian cancer. Our dual-luciferase reporter assay demonstrated that has-miR-127-3p did bind to the complimentary sites on the human BAG5 gene. Our qRT-PCR analysis also showed that the BAG5 gene was directly downregulated by miR-127-3p overexpression in the EOC cell lines, OVCAR-3 and Caov-3. Moreover, our functional experiments provided unambiguous evidence showing that upregulation of the BAG5 gene inversely regulated the tumor-suppressive effects of miR-127-3p overexpression in EOC. Notably, BAG5 belongs to the gene family of BCL2, which was reported to be involved in chromosomal translocations of the BCL6 gene (15,16). Therefore, future investigations focusing on trans-locational evidence of BCL2/BCL6 in patients with EOC may help to identify whether BAG5 and BCL6 are interactively involved in the microRNA regulation in EOC.

Figure 6. BAG5 upregulation reverses the effects of miR-127-3p overexpression on proliferation and bufalin sensitivity in EOC. (A) In the L-miR127-Mimic-transduced OVCAR-3 and Caov-3 cells, second transfection of the BAG5-overexpressing vector (pcDNA/BAG5) or an empty vector (pcDNA/+ ) was performed for 48 h. qRT-PCR was conducted to compare endogenous BAG5 mRNA expression ( *P<0.05). (B) After second-transfection of the miR-127-3p-overexpressing EOC cells, a proliferation assay was conducted to compare cancer cell growth between the cells transfected with pcDNA/BAG5 and cells transfected with pcDNA/+ ( *P<0.05). (C) A bufalin sensitivity assay was conducted to compare the cell viability between miR-127-3p-overexpressing EOC cells which were transfected with pcDNA/BAG5 and those which were transfected with pcDNA/+ ( *P<0.05).
In conclusion, we discovered novel regulatory mechanisms of miR-27-3p acting as a tumor suppressor in EOC. Moreover, we discovered a novel molecular target, BAG5 gene, to be the downstream target of miR-127-3p in regulating EOC development. Our results further the knowledge of epigenetic regulations in EOC, as well as help to develop new targets for early diagnosis and optimal therapies for patients with EOC.

References