TNFAIP8 promotes cell growth by regulating the Hippo pathway in epithelial ovarian cancer

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Abstract. Tumor necrosis factor-α-induced protein 8 (TNFAIP8) is an independent prognostic factor for cancer-specific and disease-free survival in patients with epithelial ovarian cancer (EOC). However, the exact mechanism of the biological role of TNFAIP8 in EOC remains unclear. In the present study, a siRNA specifically targeting TNFAIP8 was prepared to knock down TNFAIP8 in EOC cells. Cell growth, colony formation, apoptosis, and cell cycle distribution in TNFAIP8-deficient EOC cells were determined. In addition, the underlying molecular mechanisms were investigated by western blot analysis and reverse transcription quantitative polymerase chain reaction assays. It was demonstrated that the knockdown of TNFAIP8 inhibited EOC cell growth and colony formation, along with increased levels of apoptosis and cell cycle arrest. The results of the western blot analysis suggested that TNFAIP8 inhibited the expression of phosphorylated yes-associated protein 1 (YAP) while promoting total and nuclear YAP expression, followed by the regulation of apoptosis and cell cycle checkpoint protein expression in EOC. Overexpression of YAP in EOC cells efficiently attenuated cell growth inhibition in TNFAIP8-deficient EOC cells. In addition, knockdown of TNFAIP8 significantly impaired EOC tumor growth in vivo. Collectively, the data from the present study suggested that TNFAIP8 is an oncogene and a novel therapeutic target for EOC.

Introduction

Ovarian cancer is the fifth leading cause of cancer-associated mortality in women (1). In the United States of America, ovarian cancer is the second most common malignancy of the female genital tract, with an estimated 22,440 cases diagnosed and 14,080 mortalities in 2017 (1). It was demonstrated that in 2012 all over Asia, there were 110,526 diagnosed cases of ovarian cancer and China was the country with the highest number of cases (34,575 patients) (2). Ovarian cancer is classified based on the tissue of origin, for example epithelial, stromal endocrine or germ cells. Although certain types of cancer arise from cells that exist in histologically normal ovaries, the majority of ovarian cancer cases are derived from cells that typically reside in extra-ovarian tissue (3), including endometrioid carcinoma. Epithelial ovarian cancer (EOC) is the most prevalent type of ovarian cancer, accounting for 90% of all cases of ovarian cancer (4). The median age at diagnosis is 63 years. Although a clear etiological factor responsible for the development of ovarian cancer has not been identified at present, an increasing amount of evidence has been obtained on the initiation, progress, and treatment of ovarian cancer (5,6). Therefore, the development of novel diagnostic and therapeutic targets is necessary for EOC.

The Hippo pathway represents a novel tumor suppressor pathway, and dysregulation of Hippo signaling has been demonstrated to be a key regulator of tumor cell proliferation and survival (7). In mammalian systems, the Hippo pathway is composed of four core kinase complexes: Mammalian Ste2-like kinases 1/2 and large tumor suppressor kinases 1/2 (7,8). Activation of the Hippo tumor suppressor pathway increases the phosphorylation level of the transcription co-activator yes-associated protein 1 (YAP)/transcriptional co-activator with PDZ binding motif (TAZ), which results in the cytoplasmic retention of YAP/TAZ and protein degradation (9,10). High nuclear YAP expression was observed in ovarian cancer and associated with poor prognosis of patients with ovarian cancer (11,12). YAP expression was demonstrated to regulate ovarian cancer cell proliferation, chemo resistance, migration and pluripotency (13-17).

Tumor necrosis factor-α-induced protein 8 (TNFAIP8) belongs to the TNFAIP8 gene family, which consists of
TNFAIP8, TNFAIP8 like 1, TNFAIP8 like 2 and TNFAIP8 like 3. The TNFAIP8 gene was subsequently demonstrated to be involved in regulating human cancer cell proliferation, inflammation, metastasis and chemoresistance (18-21). TNFAIP8 messenger RNA and protein expression were upregulated in invasive ductal carcinoma tissues, and TNFAIP8 overexpression was markedly associated with axillary lymph node metastasis and a shorter survival time (22). Furthermore, the status of TNFAIP8 expression was an independent prognostic factor for cancer-specific and disease-free survival of patients with EOC (23). TNFAIP8 overexpression was associated with platinum resistance in EOCs with optimal cytoreduction, and was closely associated with residual tumor size (24). These data suggest that TNFAIP8 is an oncogene in human cancer development. However, the exact biological role of TNFAIP8 in ovarian cancer remains unclear.

The present study aimed to identify the functional role of TNFAIP8 in EOC through in vitro and in vivo experimental models. Additionally, the potential downstream targets of TNFAIP8 during regulating EOC growth were also investigated. The results of the current study suggested that TNFAIP8 was an oncogene in EOC, as supported by the decreased number of proliferative cells identified in TNFAIP8-knockdown EOC cells. Further, it demonstrated that the knockdown of TNFAIP8 inhibited EOC growth through regulation of Hippo signaling in vitro and in vivo. The results provided evidence to suggest the functional role and underlying mechanism of TNFAIP8 in regulating EOC.

Materials and methods

Cell culture and transfection. The ovarian cancer SKOV3, A2780s, A2780cp, PA-1 and CAOV3 cell lines were obtained from American Type Culture Collection (Manassas, VA, USA) and normal human epithelial cells (HOEC) were obtained from The Cell Bank of Type Culture Collection of Shanghai Chinese Academy of Sciences (Shanghai, China). All cells were cultured in Dulbecco's modified Eagle's medium (Hyclone; GE Healthcare Life Sciences, Logan, UT, USA) supplemented with 10% fetal bovine serum (EMD Millipore, Billerica, MA, USA) at 37°C with 5% CO₂.

Small interfering RNA (siRNA) targeting TNFAIP8 (siTNFAIP8, CTGGTGGTTCGTTTGTTTTA) and negative control siRNA (siNC, CGAGGGCCGACCTAACCTTGAG) were purchased from Guangzhou RiboBio Co., Ltd. (Guangzhou, China). A total of 5 nM siTNFAIP8 and siNC were transfected into A2780s and A2780cp cells using the transfection agent riboFECT CP (cat. no. C10511; Guangzhou RiboBio Co., Ltd) following the manufacturer's protocol. A pVax-based YAP overexpression system and blank vector pVax vectors were purchased from Shanghai GenePharma Co., Ltd. (Shanghai, China) and 2 µg/well were used to transfect the A2780s and A2780cp cells using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA), according to the manufacturer's protocol. At 48 h post transfection, transfected A2780s and A2790cp were collected for further experiments.

Cell viability assay. siRNA-transfected A2780s and A2780cp cells were seeded in a 96-well plate (1,500 cells/well), and the cell viability was measured daily using a Cell Counting kit-8 assay (Dojindo Molecular Technologies, Inc., Kumamoto, Japan) for 4 days, according to the manufacturer's protocol.

Colony formation assay. siRNA-transfected A2780s and A2780cp cells were seeded in a 6-well plate (1,000 cells/well), and the cells were cultured for 14 days. The cells fixed with 4% paraformaldehyde for 15 min at room temperature, followed by staining with crystal violet (cat. no. C0121; Beyotime Institute of Biotechnology, Haimen, China) at room temperature for 15 min. The number of colonies (>50 cells) in each well was counted and analyzed. A total of three independent experiments were performed.

Reverse transcription quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from cells using TRIzol® reagent (Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. A total of 1 µg RNA samples were subjected to RT-PCR (Takara Bio, Inc., Otsu, Japan), and resulting cDNA was analyzed in triplicate using SYBR-Green (Takara Bio, Inc.). qPCR conditions were as follows: Denaturation at 94°C for 2 min, amplification for 35 cycles at 94°C for 0.5 min, annealing at 60°C for 0.5 min and extension at 72°C for 1 min, followed by a terminal elongation step at 72°C for 10 min. The qPCR was performed with the CX-96 system (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Relative expression of TNFAIP8 were determined by 2^(-ΔΔCq), where Cq is the mean threshold cycle difference calculated following normalization to U6 values (25). The primer sequences were as follows: TINFAIP8 forward, 5'-GCC GTT AGA AGG AGA-3' and reverse, 5'-AGA GCC ATG GTA TTT-3' and U6 forward, 5' -TGC GTG ACA CAG GCA CAA AAGA-3' and reverse, 5' -GCA CCT CAC TAC CAG GCA CAA AAGA-3'.

Apoptosis detection. Flow cytometric analysis of apoptosis was performed using the Fluorescein isothiocyanate-Annexin V Apoptosis Detection kit I (BD Biosciences, San Jose, CA, USA), according to the manufacturer's protocol. The flow cytometry was performed on a Cytomics FC500 flow cytometer (Beckman Coulter, Inc., Brea, CA, USA). Flow cytometry data were analyzed using FlowJo 7.6 software (Tree Star Inc., Ashland, OR, USA).

Cell cycle analysis. For cell cycle analysis, cells were collected at the logarithmic stage of growth, centrifuged (4°C; 3 min; 1,200 x g) and then resuspended at 2x10⁶ cells/ml. Cells were fixed in 70% ethanol at 4°C for 15 min, washed in PBS, incubated in 50 µg/ml propidium iodide (PI; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) containing 0.1 mg/ml RNase A (Sigma-Aldrich; Merck KGaA) and 0.1% Triton X (Sigma-Aldrich; Merck KGaA) for 30 min and were analyzed on a Cytomics FC500 flow cytometer (Beckman Coulter, Inc.). Flow cytometry data were analyzed using FlowJo 7.6 software.

Western blot analysis. Cells were harvested in cell lysis buffer (Beyotime Institute of Biotechnology) for total protein extraction as described previously (26). The nuclear and cytoplasmic proteins were extracted with a subcellular protein extraction kit (EMD Millipore), according to the manufacturer's protocol.
The concentration was determined using a BCA kit (Beyotime Institute of Biotechnology). Equal quantities (10 µg) of denatured protein samples were resolved by 10% SDS-PAGE, and then transferred onto polyvinylidene fluoride membranes (EMD Millipore). Following blocking with 5% non-fat dry milk in TBS/0.05% Tween-20 at room temperature for 2 h, membranes were incubated with specific primary antibodies against TNFAIP8 (1:800; cat. no. 119-17060; RayBiotech Life, Norcross, GA, USA), cellular tumor protein p53 (p53; 1:1,000; cat. no. 2542; Cell Signaling Technology, Inc., Danvers, MA, USA), caspase-3 (1:600; cat. no. 9662; Cell Signaling Technology, Inc.), cyclin B1 (1:1,000; cat. no. 4138; Cell Signaling Technology, Inc.), cyclin D1 (1:1,000; cat. no. 2922; Cell Signaling Technology, Inc.), YAP (1:800; cat. no. 4932; Cell Signaling Technology, Inc.) and phosphorylated (p)-YAP (1:600; cat. no. 13008; Cell Signaling Technology, Inc.), followed by a horseradish peroxidase-conjugated secondary antibody (ZSGB-BIO; OriGene Technologies, Inc., Beijing, China). Proteins were visualized using enhanced chemiluminescent reagents (Pierce; Thermo Fisher Scientific, Inc.). GAPDH (1:5,000; cat. no. ABS16; EMD Millipore) was used as a loading control. Image-Pro Plus (version 6.0; Media Cybernetics, Inc., Rockville, MD, USA) was used to perform densitometry.

Animal model. The present study was approved by the Ethics Committee of Sichuan University (Chengdu, China) and complied with the animal guidelines of Sichuan University. A total of 10 6-week-old female BALB/c nude mice (weight, ~20 g) were purchased from Beijing HFK Bioscience Co. Ltd. (Beijing, China) and housed at specific-pathogen-free room (temperature, 22-25°C) with a 12 h dark/light cycle and ad libitum access to food and water. Short hairpin (sh)
RNA targeting TNFAIP8 (CTGCAGTGGTTTCAATGTTT AAACGTCGACAGTGT TTAA) and negative control (shNC, CGAGGGCGACTT AACCTTACGG) were purchased from Shanghai GenePharma Co., Ltd. and used to transfect the A2780s and A2780cp cells (2 µg/well) using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer’s protocol. At 48 h post transfection, puromycin (2 µg/ml) was added into the medium to select the transfected cells. Stably transfected cells were collected for TNFAIP8 detection by western blotting and further in vitro study. To establish the tumor model, 5x10⁶ A2780s cells that exhibited stable knockdown of TNFAIP8 (A2780s-shTNFAIP8) and negative control cells (A2780s-shNC) were subcutaneously injected into the right flank of n=5 per experimental group. Tumor length and width were measured every five days from the tenth day post-cell injection. Tumor volumes were calculated as ellipsoids (length x width²/2). At 25 days post-cell injection, the mice were sacrificed, and the tumors were removed and measured.

Statistical analysis. All values are presented as the means ± standard deviation, representative of 3-5 repeats. Significant differences were determined using GraphPad 5.0 software (GraphPad Software, Inc., La Jolla, CA, USA). The two-tailed Student’s t-test was used to evaluate the significance of differences between two groups of data, and one-way analysis of variance with Tukey’s post-hoc test was used to determine statistical differences between multiple groups. P<0.05 was considered to indicate a statistically significant difference.

Results

Knockdown of TNFAIP8 inhibits EOC cell growth. To investigate the biological function of TNFAIP8 in EOC, EOC cell lines (SKOV3, A2780s, A2780cp, PA-1 and CAOV3) and normal HOEC were cultured and collected for qPCR and western blot analysis. As demonstrated in Fig. 1A, TNFAIP8 mRNA was significantly upregulated in all EOC cell lines. The western blot analysis results confirmed the upregulation of TNFAIP8 in EOC cells (Fig. 1B). siRNA specifically targeting TNFAIP8 (siTNFAIP8) were used to transfect A2780s and A2780cp cells and were demonstrated to efficiently inhibit TNFAIP8 expression (Fig. 1C). Notably, knockdown of TNFAIP8 markedly inhibited the proliferation of A2780s and A2780cp cells by 58.4 and 61.3%, respectively (Fig. 1D and E). Furthermore, the colony formation ability of A2780s and A2780cp cells were also significantly downregulated in TNFAIP8-silenced cells (Fig. 1F and G). Collectively, TNFAIP8 was demonstrated to be an oncogene in EOC.

Knockdown of TNFAIP8 promotes EOC apoptosis. To additionally investigate the potential mechanism of TNFAIP8-based regulation of EOC growth, the levels of apoptosis were analyzed by flow cytometry. The results suggested that the knockdown of TNFAIP8 efficiently promoted apoptosis of A2780s [siNC 4.2±0.6% vs. (siTNFAIP8) 9.8±1.6%] and A2780cp cells [siNC 5.9±1.2% vs. (siTNFAIP8) 11.7±1.3%] (Fig. 2A). Furthermore, the transfected cells were collected for western blot analysis and a significant upregulation of p53 and caspase-3 expression levels was observed in TNFAIP8-silenced A2780s and A2780cp cells (Fig. 2B). These results demonstrated that siTNFAIP8 promoted apoptosis in EOC cells.

Knockdown of TNFAIP8 induces EOC cell cycle arrest. Next, the cell cycle in A2780s and A2780cp cells transfected with siNC or siTNFAIP8 were analyzed by PI staining and flow cytometry. As demonstrated in Fig. 3A, TNFAIP8 knockdown in A2780s and A2780cp cells increased the proportion of cells in the G0/G1 phase and decreased the proportion of cells in the S phase (Fig. 3A). In addition, the cell cycle checkpoint proteins cyclin B1 and cyclin D1 were markedly downregulated in TNFAIP8-knockdown A2780s and A2780cp cells (Fig. 3B). These results demonstrated the regulatory role of TNFAIP8 in the cell cycle.

TNFAIP8 promotes EOC growth through regulating Hippo signaling. To investigate the underlying mechanism of TNFAIP8-based regulation of EOC growth, the YAP expression was determined by western blot analysis. The whole proteins of A2780s and A2780cp cells transfected with siNC or siTNFAIP8 were collected. The results suggested that TNFAIP8 knockdown promoted p-YAP expression, but inhibited total YAP expression (Fig. 4A). Furthermore, TNFAIP8-knockdown decreased nuclear YAP expression levels, but increased cytoplasmic YAP expression levels (Fig. 4B). To additionally determine whether Hippo signaling served a crucial role in the TNFAIP8-based regulation of EOC growth, a plasmid-based YAP expression system was used to transfect A2780s cells. As indicated in Fig. 4C, siTNFAIP8-mediated total YAP expression downregulation was attenuated by pVax-YAP transfection. Notably, the cell growth inhibition in TNFAIP8-knockdown EOC cells was also abrogated by pVax-YAP transfection (Fig. 4D). Collectively, these results suggest that TNFAIP8 promoted EOC growth through regulating Hippo signaling.

Knockdown of TNFAIP8 impairs EOC tumor growth in vivo. To additionally investigate the functional role of TNFAIP8 on EOC growth in vivo, A2780s-shTNFAIP8 and A2780s-shNC cells were subcutaneously injected into the right flank of 6-week-old female BALB/c nude mice. At 30 days post-cell injection, the mice were sacrificed and the tumors were measured (Fig. 5A). The results revealed that the knockdown of TNFAIP8 significantly inhibited the tumor volume by 52.5% (final tumor volume of shNC vs. shTNFAIP8: 1376.2±187.2 mm³ vs. 654.3±97.2 mm³) (Fig. 5B) and tumor weight by 50.5% (tumor weight of shNC vs. shTNFAIP8: 1.05±0.09 g vs. 0.52±0.07 g) (Fig. 5C). These results suggested that the knockdown of TNFAIP8 inhibited the EOC tumor growth generated from A2780s cells.

Discussion

In the present study, TNFAIP8 was identified as an oncogene in EOC, as evidenced by the decreased number of proliferative cells identified in TNFAIP8-knockdown EOC cells. It was also demonstrated that the knockdown of TNFAIP8 inhibited EOC growth through Hippo signaling regulation in vitro and in vivo. A clearer understanding of the role and mechanisms of TNFAIP8 in EOC may provide novel therapeutic targets.
Figure 2. Knockdown of TNFAIP8 promotes epithelial ovarian cancer apoptosis. (A) Following siNC or siTNFAIP8 transfection for 48 h, A2780s or A2780cp cells were collected for PI and Annexin V staining, followed by flow cytometry analysis. The proportions of apoptotic cells were quantified. *P<0.05 vs. siNC group. (B) Western blot analysis of p53 and caspase-3 expression in A2780s and A2780cp cells transfected with siNC or siTNFAIP8 for 48 h. GAPDH was used as a loading control. The relative expression of p53 and caspase-3 was quantified. **P<0.01 vs. siNC group. si, small interfering; NC, negative control; TNFAIP8, tumor necrosis factor-α-induced protein 8; PI, propidium iodide; FITC, fluorescein isothiocyanate; p53, cellular tumor protein 53.

Figure 3. Knockdown of TNFAIP8 induces epithelial ovarian cancer cell cycle arrest. (A) Following siNC or siTNFAIP8 transfection for 48 h, A2780s or A2780cp cells were collected for propidium iodide staining, followed by flow cytometry analysis. The percentage of cells in each stage was analyzed. *P<0.05 and **P<0.01 vs. siNC group. (B) Western blot analysis of cyclin B1 and cyclin D1 expression in A2780s and A2780cp cells transfected with siNC or siTNFAIP8 for 48 h. GAPDH was used as a loading control. The relative expression of cyclin B1 and cyclin D1 was quantified. **P<0.01 vs. siNC group. si, small interfering; NC, negative control; TNFAIP8, tumor necrosis factor-α-induced protein 8.
TNFAIP8 was demonstrated to be a prognostic factor for cancer-specific and disease-free survival of patients with EOC (23). In the present study, it was demonstrated that the knockdown of TNFAIP8 inhibited EOC cell growth and colony formation, accompanied by increased levels of apoptosis and cell cycle arrest. Mechanistic studies indicated that the downregulation of TNFAIP8 promoted p-YAP expression while inhibiting nuclear and total YAP expression. To the best
of our knowledge, the present study provided, for the first time, biological evidence for the oncogenic role of TNFAIP8 in EOC, suggesting that TNFAIP8 may serve as a potential therapeutic target for EOC.

Various studies have demonstrated the upregulation of TNFAIP8 in a diverse range of cancer types: In esophageal squamous cell carcinoma, TNFAIP8 overexpression was identified in 73 (59.8%) tumor specimens and was significantly increased in patients with lymphatic recurrence (27). TNFAIP8 was also overexpressed in primary hepatocellular carcinoma (HCC) samples and was associated with Tumor Node Metastasis staging, recurrence and poor prognosis, whilst also serving as an independent favorable prognostic factor (28). In accordance with the upregulation of TNFAIP8 in EOC tissues from patients (23), the mRNA and protein expression levels of TNFAIP8 were upregulated in all EOC cell lines assessed in the present study. This result indicated that TNFAIP8 may function as an oncogene in EOC development.

TNFAIP8 was demonstrated to regulate the progression of various types of cancer. Dai et al (26) demonstrated that TNFAIP8 upregulated cell proliferation, migration, invasion, and xenograft tumor growth in HCC cells. TNFAIP8 also promoted cell proliferation and invasion in lung cancer (29). Concurrently, downregulated expression of TNFAIP8 via the overexpression of microRNA (miR)-9 markedly inhibited gastric cancer cell proliferation in vitro and tumor growth in vivo (20). miR-99a may induce osteosarcoma cell cycle progression and cell apoptosis by directly targeting TNFAIP8 (30). The results of the present study suggested that TNFAIP8 promoted cell growth and colony formation by inhibiting apoptosis and cell cycle arrest in EOC cells. Although additional in vivo studies are required, these data provide evidence to elucidate the functional role of TNFAIP8 in EOC development.

The Hippo pathway effector YAP increased cell proliferation, resistance to cisplatin-induced apoptosis, cell migration and anchorage-independent growth, and was associated with poor survival in ovarian cancer (31). Xia et al (14) demonstrated that the YAP/TEA domain transcription factors co-activator promoted ovarian cancer-initiated cell pluripotency and chemoresistance. YAP phosphorylation is regulated by its interactions with other proteins including serine/threonine-protein kinase LATS1, serine/threonine protein kinase 4/3 and angiomotin (32). Activation of the Hippo tumor suppressor pathway increases the phosphorylation level of the transcription co-activator YAP/TAZ, which results in the cytoplasmic retention of YAP/TAZ and protein degradation (9,10). The present study indicated that TNFAIP8 inhibited the expression of p-YAP while promoting total and nuclear YAP expression. Overexpression of YAP in EOC cells efficiently attenuated cell growth inhibition in TNFAIP8-deficient EOC cells. These data are consistent with those of previous studies that demonstrated the regulatory role of TNFAIP8 in Hippo signaling (28,29).

Additional experimental studies also suggest the involvement of TNFAIP8 in apoptosis and cell cycle checkpoint protein expression in EOC cells, which were identified as downstream targets of YAP (7,8). Collectively, the data from the present study provide experimental evidence that TNFAIP8 functions as an oncogene in EOC development and may be used as a therapeutic target for EOC. In future, additional studies are required to determine the direct targets of TNFAIP8 during the regulation of EOC growth.

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Availability of data and materials
All data generated or analyzed during this study are included within this published article.

Authors' contributions
XY and FZ were involved in acquisition of the data, YX was involved in analysis and interpretation of the data. XZ was involved in developing the study concept and design.

Ethics approval and consent to participate
The present study was approved by the Ethics Committee of Sichuan University and complied with the animal guidelines of Sichuan University.

Patient consent for publication
Not applicable.

Competing interests
All authors declare that they have no competing interests.

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