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CCAAT enhancer-binding protein α suppresses proliferation, metastasis, and epithelial-mesenchymal transition of ovarian cancer cells via suppressing the Wnt/β-catenin signaling

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CCAAT enhancer-binding protein alpha (CEBPA, also known as C/EBPα) is a transcription factor that plays an essential role in regulating terminal differentiation and cell proliferation of many tissues. The objective of this study was to explore the potential function of CEBPA in ovarian cancer. The expression of CEBPA in ovarian cancer samples and adjacent normal tissues was evaluated by qRT-PCR. The putative role of CEBPA in ovarian cancer cells was evaluated by immunohistochemistry, western blot, cell viability assay, BrdU incorporation assay, soft agar colony formation assay, Transwell cell migration and invasion assay, tumor xenograft formation, and lung metastasis model. We found that CEBPA was downregulated in ovarian cancer samples and predicted a poor prognosis. CRISPR/Cas9-mediated CEBPA knockout promoted proliferation, anchorage-independent growth, migration, invasion, and EMT of ovarian cancer cells, while enforced CEPBA expression suppressed proliferation, anchorage-independent growth, migration, invasion, EMT, tumor xenograft growth, and lung metastasis of ovarian cancer cells. Furthermore, we found that the knockout of CEBPA activated Wnt/β-catenin signaling in ovarian cancer cells, while CEBPA overexpression suppressed Wnt/β-catenin activation. Our data indicated that CEBPA acted as a tumor suppressor in ovarian cancer, and might be a potential prognostic marker for ovarian cancer treatment.

Key words: CCAAT enhancer-binding protein alpha (CEBPA), ovarian cancer, epithelial-mesenchymal transition (EMT), Wnt/β-catenin

Ovarian cancer is the deadliest gynecologic cancer in women due to the late diagnosis and intrinsic or acquired chemoresistance. It is a heterogeneous disease and can be divided into different histological subtypes with different cells of origin and genetic variations. Among these subtypes, epithelial ovarian cancer takes up nearly 90% of all ovarian cancers, thus ovarian cancer is mainly referring to epithelial ovarian cancer in this article. Ovarian cancer is the seventh most common cancer and the eighth most common cause of cancer-related death in women [1]. It is estimated that there are 239,000 new cases (3.6% of all cancer cases) and 152,000 deaths (4.3% of all cancer deaths) of ovarian cancer every year all over the world [1]. The mortality-to-incidence rate of ovarian cancer is greater than 0.6 and varies between countries [2]. The prognosis of ovarian cancer has not changed for decades, and the 5-year survival for all ovarian cancer patients is only 45.6% [3]. Genetic factors, age, postmenopausal hormonal therapy use, infertility, and nulliparity are risk factors for ovarian cancer [3]. The standard-of-care therapy for ovarian cancer patients includes cytoreductive surgery and platinum-based chemotherapy [4]. Despite these aggressive treatments, recurrence and resistance to chemotherapy are frequently observed in ovarian cancer patients due to a high degree of inter- and intratumoral heterogeneity. Thus, understanding the molecular mechanism of tumorigenesis is important for improving the outcomes of ovarian cancer.

CCAAT enhancer-binding protein alpha (CEBPA, also known as C/EBPα), the founding member of a family of basic region leucine zipper (bZIP) transcriptional factors, plays a key role in regulating tissue-specific gene expression, proliferation, and terminal differentiation [5]. The CEBPA gene is located at chromosome 19q13.1 and is intron-less. There are two protein isoforms of CEBPA, p42 and p30. The p42 is the full-length isoform, while the p30 lacks the TE-I and TE-II transactivation domains. The p30 isoform is a dominant-negative isoform of CEBPA, thus competes with the p42 isoform and inhibits downstream

transcriptions [6]. CEBPA has been demonstrated to act as a bona fide tumor suppressor in a variety of cancer types, such as acute myeloid leukemia (AML) and lung cancer [7]. CEBPA is highly expressed in hematopoietic stem cells, myeloid progenitors, and granulocytes, and regulates genes controlling myeloid differentiation [8]. CEBPA is frequently mutated in nearly 10% of AML, and biallelic CEBPA mutated AML has been recognized as a distinct genetic entity by World Health Organization classification recently [9]. CEBPA is also highly expressed in airway epithelial cells and acts as a master regulator of airway epithelial differentiation. Knockout of CEBPA in lung epithelium dramatically increased the number of lung adenocarcinoma and adenomas [10]. CEBPA is inducing expressed in ovarian theca and interstitial cells by hormones and is essential for ovulation, luteinization, and expression of key target genes [11, 12]. In addition, the expression level of CEBPA is associated with ovarian cancer prognosis, but the exact role of CEBPA in ovarian cancer is unknown [13].

In the present study, we explored the function of CEBPA in ovarian cancer and potential underlying mechanisms. We found that CEBPA was downregulated in ovarian cancer samples and predicted a poor prognosis. In addition, CEBPA was involved in the regulation of proliferation, anchorage-independent growth, migration, invasion, and EMT of ovarian cancer cells, and this might be due to the inhibiting Wnt/β-catenin activation. Our results indicated that CEBPA acted as a tumor suppressor in ovarian cancer and might be a potential prognostic marker for ovarian cancer treatment.

Patients and methods

Patient samples. All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Informed consent was signed by all patients enrolled in our study. The collection and use of human ovarian tissue samples were reviewed and approved by the ethics committee of the Affiliated Hospital of BeiHua University. A total of 97 pairs of ovarian cancer samples and paired adjacent normal tissues were obtained from the Affiliated Hospital of BeiHua University from March 2014 to May 2017. Tissue samples were fresh frozen and store at –80 °C. The tumor stage was judged according to the Federation International of Gynecology and Obstetrics stage system (FIGO stage). The demographic and clinicopathological features of enrolled patients were retrieved from the hospital database. To evaluate overall survival, follow-up was performed 48 months post-surgery.

Cell culture. Ovarian cancer cell lines OV-90, A2780, SK-OV-3, Caov-3, Caov-4, and ES-2 were purchased from the American Type Culture Collection (ATCC). Ovarian cancer cell lines JHOS-2, JHOS-4, and OVK18 were obtained from the RIkagaku KENkyusho/Institute of Physical and Chemical Research (RIKEN). Human HEK293T cell line was purchased from ATCC. All cell lines were maintained in RPMI-1640 medium (Invitrogen, USA) supplemented with 10% fetal bovine serum (Hyclone, USA) and 1% PenStrep (100 U/ml penicillin and 100 μg/ml streptomycin) in a humid atmosphere containing 5% CO_2 at 37 °C.

Quantitative real-time polymerase chain reaction (qRT-PCR). TRIzol (Invitrogen, USA) was used to extract total RNA from tissue samples and culture cells. Then, total RNA was reversely transcribed into cDNA using the cDNA Reverse Transcription Kit (Takara, Japan). RT-qPCR was conducted according to the standard protocol of SYBR Premix Ex Taq kit (Takara, Japan) on a Roche Light Cycler 480 RealTime PCR System (Roche Diagnostics, Basel, Switzerland). GAPDH was used as an internal control. Data were analyzed by the $2^{-\Delta\Delta Ct}$ method. The primer sequences used in qRT-PCR are listed below. CEBPA, forward, 5'-AGTTCCTGGCCGACCTGTTC-3', and reverse, 5'-GCTGCTTGGCTTCATCCTCCT-3'. GAPDH, forward, 5'-TGACAACTTTGGTATCGTGGAAGG-3', and reverse, 5'-AGGCAGGGATGATGTTCTGGAGAG-3'. Each sample was done in triplicates.

Plasmids and transfection. CEBPA knockout was realized by designing two short guide RNAs (sg-CEBPA-1 and sg-CEBPA-2) targeting human CEBPA using the Optimized CRISPR Design web tool (http://crispr.mit.edu/). Then, these short guide RNAs were cloned into lentiCRISPRv2 vector (Addgene, plasmid #52961). A non-targeting sequence was cloned into lentiCRISPRv2 vector as sg-NC control. CEBPA expression vector was constructed by cloning the full length of CEPBA into the pCDH-CMV-MCS-EF1-Puro (System Biosciences #CD510B-1) lentiviral vector. Empty pCDH-CMV-MCS-EF1-Puro vector was used as EV control. Lentivirus particles were generated by co-transfecting with helper plasmids pCMV-VSV-G, pRSV-REV, and pMDL in HEK293T cells using Lipofectamine 3000 (Invitrogen, USA). Virus-containing medium was collected at 24, 48, and 72 h post-transfection. For virus infection, cells (1×10^6) were incubated with 1 ml virus-containing medium and 8 μg/ml polybrene overnight. Lipofectamine 3000 was used for transient transfection according to standard protocol. The DNA sequence for short guide RNAs are listed below: sg-CEBPA-1, 5'-GCGCGAGCGCAACAACATCG-3', sg-CEBPA-2, 5'-GGAGTCGGCCGACTTCTACG-3', sg-NC, 5'-ACGGAGGCTAAGCGTCGCAA-3'.

Immunohistochemistry (IHC). The paraffin-embedded tissue samples were cut into 20 μm thick sections. Then sections were deparaffinized by xylene and rehydrated by ethanol gradients (100%, 95%, 70%, and 50%) and deionized water. Antigen retrieval was conducted by microwave-heating in sodium citrate buffer. Then the slides were incubated with C/EBPα Rabbit mAb (Cell Signaling #8178, 1:200) at 4 °C overnight. Antibody staining was visualized using the ChemMate Envision detection system (Dako

Cytomation). Sections were then stained with hematoxylin for 5 min at room temperature.

Western blot. The nucleus and cytoplasmic fractions of ovarian cancer cells were prepared as previously described [14]. Protein lysates were collected by digesting with RIPA buffer containing protease and phosphatase inhibitors. Protein concentration was determined by BCA kit (Thermo Fisher, USA). A total of 30 μg protein was loaded on 10% SDS-PAGE. Then protein was transferred to nitrocellulose membranes. Then membranes were incubated with specific primary antibodies at 4 °C overnight and corresponding secondary antibodies for 1 h at room temperature. The specific primary antibodies are listed below. ZEB1 #70512, E-Cadherin #3195, Vimentin #5741 Phospho-β-Catenin (Ser33/37/Thr41) #9561, Phospho-GSK-3β (Ser9) #5558, GSK-3β#12456, Lamin B1 #12586 and GAPDH #5174 were obtained from Cell Signaling and used at 1:1,000 dilution. Anti-CEBPA antibody #ab40761 was purchased from Abcam and used at 1:1,000 dilution. Goat anti-rabbit IgG HRPlinked antibody (Cell Signaling #7074; 1:4,000) was used as a secondary antibody.

Cell viability assay. CellTiter-Glo Luminescent Cell Viability Assay kit (Promega #G7572) was used to measure the viability of cultured cells. In brief, cells and CellTiter-Glo reagents were balanced to room temperature and then mixed thoroughly on an orbital shaker. Incubated the plates in a dark place for 15 min to stable the luminescence signal, then recorded the luminescence signal on a microplate reader. Each sample was done in triplicates.

BrdU incorporation assay. Cells were incubated with BrdU (10 μmol/l) for 3 h. Then, the cells were fixed with 4% paraformaldehyde for 10 min and washed with PBS. Cells were incubated with BrdU mouse mAb (Cell Signaling #5292, 1:1,000) at room temperature for 1 h, followed by the staining with anti-mouse IgG (Alexa Fluor 488 Conjugate) (Cell Signaling #4408, 1:500). DAPI (Invitrogen, USA) was used to stain the nuclei. BrdU incorporated cells were visualized under a fluorescence microscope.

Soft agar colony formation assay. Soft agar colony formation assay was conducted as previously described [15]. In brief, cells (5000/well) were seeded in 4% top agar and cultured for 3 weeks. Then the colonies were stained with 0.5 mg/ml thiazolyl blue tetrazolium bromide (MTT, Sigma-Aldrich, USA) for 4 h. Images were obtained by a scanner. Each sample was done in triplicates.

Transwell cell migration and invasion assay. Cells (1×10^5) were seeded in 500 μl serum-free medium into the upper chamber of Transwell chambers (Costar Corp, USA). The lower chamber was filled with 500 μl medium containing 20% fetal bovine serum. To evaluate cell migration, cells were allowed to migrate towards lower chamber for 48 h, then fixed by 4% paraformaldehyde for 15 min and stained with crystal violet. To evaluate cell invasion, the upper chamber was pre-coated with Matrigel (BD Biosciences, USA). Then cells were allowed to invade through the Matrigel for 48 h, and fixed by 4% paraformaldehyde for 15 min and stained with Giemsa dye. All experiments were carried out in triplicates.

Tumor xenograft formation and lung metastasis. All animal experiments in accordance with the ethical standards and were approved by the Animal Care and Experimental Committee of Affiliated Hospital of BeiHua University. SK-OV-3 cells (2×10^6) transduced with CEBPA expression lentivirus or EV control were subcutaneously injected into nude mice (n=4). Tumor xenografts were allowed to grow for 4 weeks. Tumor volume was monitored every four days and calculated by the formula (length × width^2)/2. To evaluate lung metastasis, SK-OV-3 cells (1×10^6) transduced with CEBPA expression lentivirus or EV control were injected into the tail vein of nude mice (n=5) and the tumors formed in the lung were allowed to grow for 4 weeks. All mice were sacrificed at the end of the experiment. Tumor xenografts were dissected out and weighed. For lung metastasis, the lungs were dissected out and the number of tumors formed in the lung was calculated.

Statistical analysis. GraphPad Prism 8.0 software was used for statistical analysis. Overall survival was plot using the Kaplan-Meier method and analyzed by the Log rank test. A two-tailed Student's t test was used to calculate statistical significance between two comparator groups. The difference of multiple groups was analyzed by one-way analysis. All data were shown as mean ± standard deviation (x ± SD) with at least three replicates. A p-value ≤0.05 was considered statistically significant.

Results

CEBPA is downregulated in ovarian cancer patients and predicts poor prognosis. To explore the potential function of CEBPA in ovarian cancer, we evaluated the expression of CEBPA in 97 pairs of ovarian cancer samples and adjacent normal tissues by qRT-PCR. We found that CEBPA expression was downregulated or lost in nearly 40% of all ovarian cancer patients (Figure 1A). Downregulation of CEBPA in ovarian cancer patients was further validated by IHC staining (Figure 1B). We could observe that CEBPA positive cells were dramatically reduced in tumor samples compared with normal tissues. Then, we performed a correlation analysis between CEBPA expression and clinicopathological features of ovarian cancer patients. Ovarian cancer patients were divided into CEBPA high expression group and low expression group using the median of CEBPA expression as cut-off value. We found that low CEBPA expression was positively correlated with advanced FIGO stage and distant metastasis (Table 1). In the Kaplan-Meier survival analysis, ovarian cancer patients with low CEBPA expression had shorter overall survival (Figure 1C). The expression of CEBPA in several frequently used ovarian cancer cell lines was detected by qRT-PCR (Figure 1D). We found that CEBPA had low expression in Caov-4, ES-2, JHOS-4, and SK-OV-3 cells, and high expression in A2780, Caov-3,

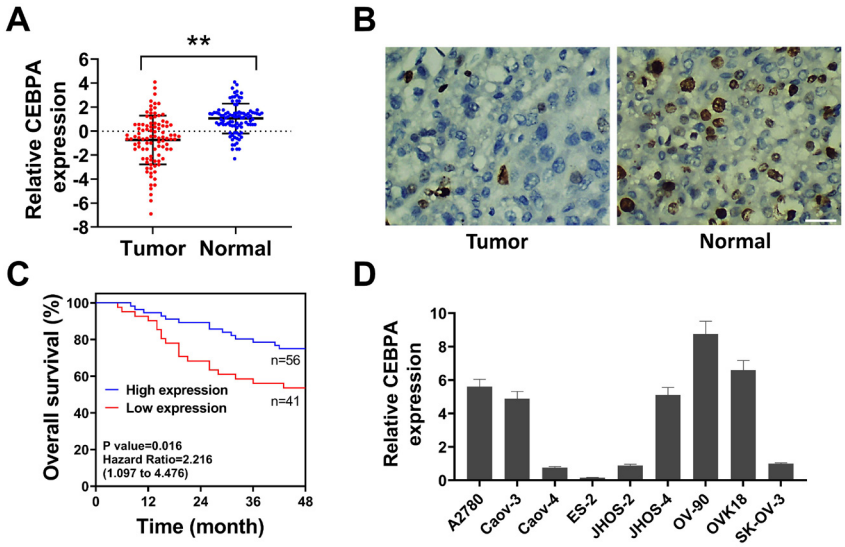

Figure 1. CEBPA is downregulated in ovarian cancer patients and predicts a poor prognosis. A) Relative expression of CEBPA in 97 pairs of ovarian cancer samples (Tumor) and adjacent normal tissues (Normal) was evaluated by qRT-PCR. B) Expression of CEBPA in ovarian cancer samples (Tumor) and adjacent normal tissues (Normal) was evaluated by IHC staining. Scale bar = 50 μm. C) Kaplan-Meier survival analysis of ovarian cancer patients according to CEBPA expression. D) Relative expression of CEBPA in ovarian cancer cell lines was evaluated by qRT-PCR. **p≤0.001

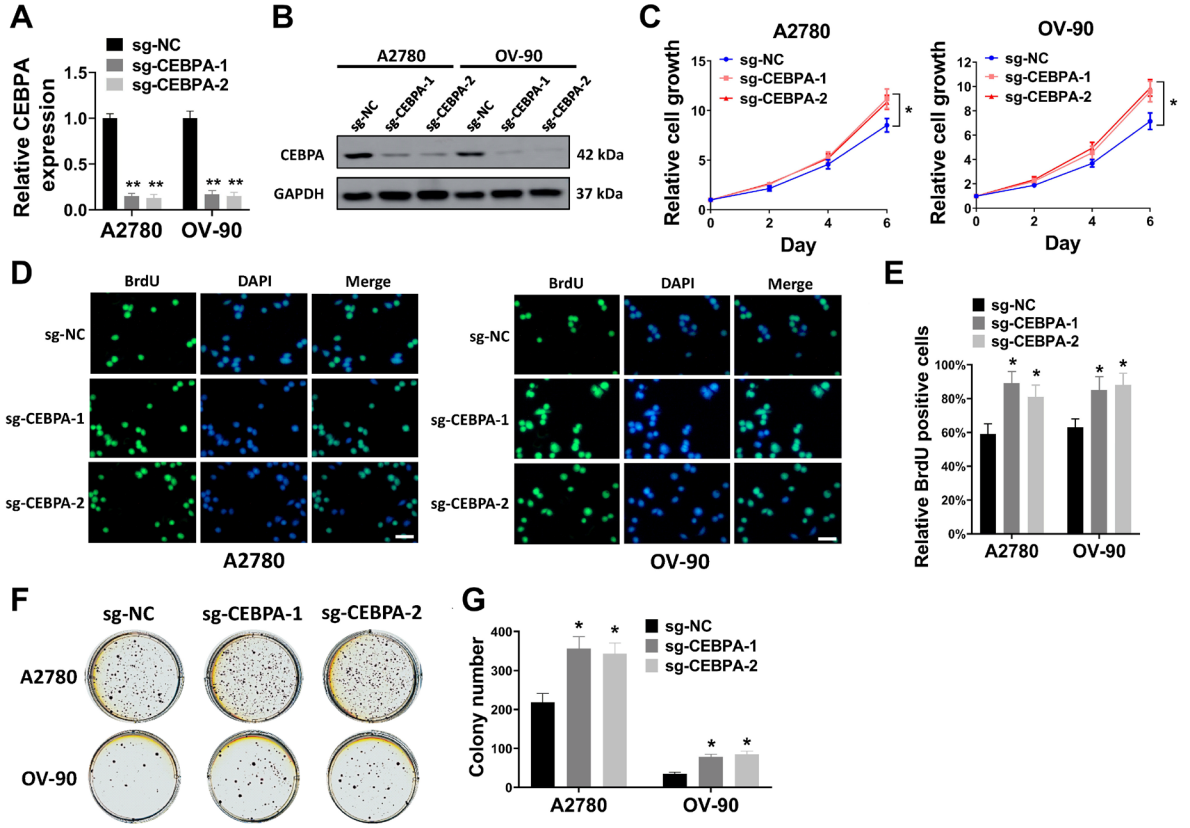

Figure 2. CRISPR/Cas9-mediated CEBPA knockout promotes proliferation and anchorage-independent growth of ovarian cancer cells. A, B) A2780 and OV-90 cells were transduced with sg-CEBPA-1, sg-CEBPA-2, or sg-NC, then relative CEBPA expression was evaluated by qRT-PCR (A) or western blot (B). C) A2780 and OV-90 cells were transduced with sg-CEBPA-1, sg-CEBPA-2, or sg-NC, and then cells were seeded in 96-well plates (2500/well) for cell viability assay. D, E) A2780 and OV-90 cells transduced with sg-CEBPA-1, sg-CEBPA-2, or sg-NC were used for the BrdU incorporation assay. Representative images (D) and percentage of BrdU positive cells (E) are shown. Scale bar = 50 μm. F, G) A2780 and OV-90 cells transduced with sg-CEBPA-1, sg-CEBPA-2, or sg-NC were used for soft agar colony formation assay. Representative plates (F) and the average number of colonies (G) are shown. *p≤0.05, **p≤0.001

JHOS-3, OV-90, and OVK18 cells. Taken together, we found that CEBPA was downregulated in ovarian cancer patients and predicted a poor prognosis.

CRISPR/Cas9-mediated CEBPA knockout promotes proliferation and anchorage-independent growth of ovarian cancer cells. To elucidate the potential role of CEBPA in ovarian cancer, we knocked out CEBPA in ovarian cancer cells by CRISPR/Cas9 system. A2780 and OV-90 cells were selected in our study for their high levels of CEBPA expression. Two short guide RNAs (sgRNAs) specifically targeting CEBPA (sg-CEBPA-1 and sg-CEBPA-2) were designed and transduced into A2780 and OV-90 cells by lentivirus. A non-targeting short guide RNA was used as sg-NC control. We found that the mRNA and protein expression of CEBPA was significantly reduced by both two sgRNAs, indicating that we successfully knocked out CEBPA in those cells (Figures 2A, 2B). As CEBPA possesses the ability to suppress cell proliferation [16], we evaluated the influence of CEBPA knockout on cell growth of A2780 and OV-90 cells. As expected, knockout of CEBPA increased the growth of A2780 and OV-90 cells (Figure 2C). In the BrdU incorporation assay, we found that knockout of CEBPA apparently enlarged the number of the BrdU positive cells (Figures 2D, 2E). Anchorage-independent growth is the ability of transformed cells to grow independently on a solid surface. It is a hallmark of carcinogenesis and can be evaluated by soft agar colony formation assay. In our study, we found that CEBPA knockout increased the number of colonies formed in soft agar, suggesting that CEBPA depletion facilitated the malignant transformation of ovarian cancer cells (Figures 2F, 2G). Above all, we found that CRISPR/Cas9-mediated CEBPA knockout promoted proliferation and anchorage-independent growth of ovarian cancer cells, suggesting that CEBPA acted as a tumor suppressor in ovarian cancer.

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

Characteristics	n	CEBPA expression		p-value
		Low	High	
Age (Years)				0.963
≤60	52	25	27	
>60	45	22	23	
Tumor size (mm)				0.215
≥30	41	24	17	
<30	56	23	33	
FIGO* stage				**0.013**
I–II	28	8	20	
III–IV	69	39	30	
Pathologic type				0.898
Serous	73	39	34	
Mucous and others**	24	8	16	
Lymph node metastasis				0.168
Positive	73	36	37	
Negative	24	11	13	
Distant metastasis				**0.038**
Positive	36	27	9	
Negative	61	20	41	

Notes: *FIGO-Federation International of Gynecology and Obstetrics; **others-others include the endometrioid, clear cell, and undifferentiated ovarian cancers

CRISPR/Cas9-mediated CEBPA knockout facilitates migration, invasion, and EMT of ovarian cancer cells. CEBPA is an important transcriptional factor that regulates terminal differentiation and maintains tissue homeostasis. Indeed, CEBPA is crucial for epithelial maintenance by suppressing EMT of breast cancer cells [17]. In other studies, CEBPA was reported to associate with the homeostasis of the gastric epithelium [18], and suppress migration and/or invasion of cervical squamous cell carcinoma and lung adenocarcinoma [19, 20]. As nearly 90% of ovarian cancers are of epithelial origin, we speculated that CEBPA knockout might affect the EMT of ovarian cancer cells. Thus, we knocked out CEBPA in A2780 and OV-90 cells, then evaluated for cell migration and invasion by Transwell cell migration or invasion assay. In the Transwell cell migration assay, we found that the knockout of CEBPA raised the number of migration cells compared with sg-NC control (Figures 3A, 3B). In the Transwell cell invasion assay, knockout of CEBPA dramatically increased the number of invasion cells of A2780 and OV-90 (Figures 3C, 3D). Next, we evaluated the expression of EMT markers (ZEB1, E-cadherin, and Vimentin) by western blot. We found that knockout of CEBPA increased the expression of mesenchymal markers ZEB1 and Vimentin, and decreased the expression of epithelial marker E-cadherin in A2780 and OV-90 cells (Figures 3E, 3F). These results indicated that CRISPR/Cas9-mediated CEBPA knockout facilitated migration, invasion, and EMT of ovarian cancer cells.

Enforced CEBPA expression suppresses proliferation, anchorage-independent growth, and tumor xenograft growth of ovarian cancer cells. In order to validate the function of CEBPA in ovarian cancer cells, we also forced CEBPA expression in two ovarian cancer cell lines, which had low endogenous CEBPA expression (SK-OV-3 and ES-2), and evaluated cell proliferation, anchorage-independent growth, and tumor xenograft growth in nude mice. The full-length CEBPA was cloned into a lentiviral expression vector and the empty lentivirus vector (EV) was used as a control. The expression of CEBPA was verified by qRT-PCR and western blot and we found that CEBPA expression was significantly overexpressed in SK-OV-3 and ES-2 cells (Figures 4A, 4B). Then, cell growth of SK-OV-3 and ES-2 cells was evaluated by cell viability assay. We found that enforced CEBPA expression apparently repressed cell growth of SK-OV-3 and ES-2 cells (Figure 4C). In soft agar colony formation assay, CEBPA overexpression reduced the colony numbers of SK-OV-3 and ES-2 cells formed in soft agar (Figures 4D, 4E). SK-OV-3 cells (2×10^6) were transduced with CEBPA expression lentivirus or EV control,

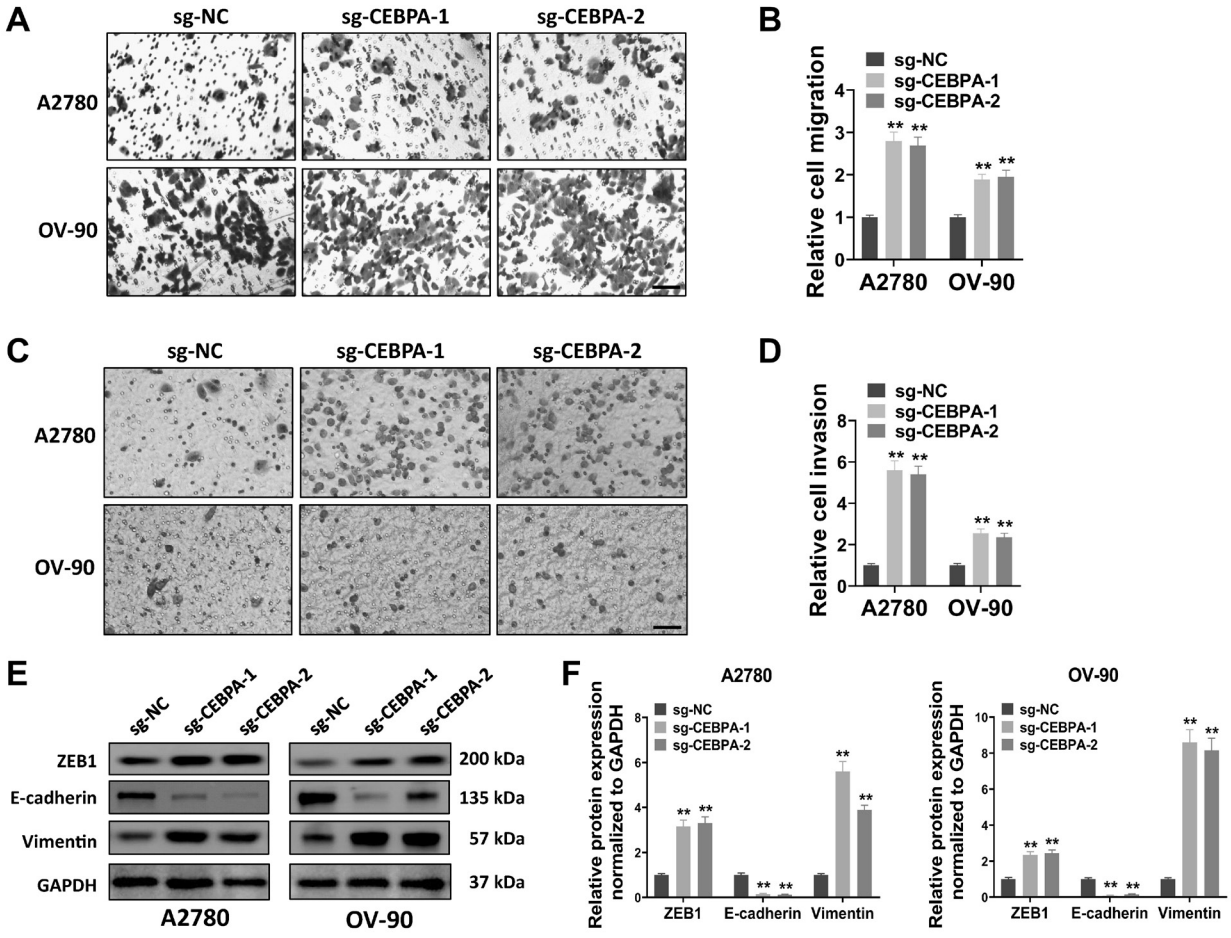

Figure 3. CRISPR/Cas9-mediated CEBPA knockout facilitates migration, invasion, and EMT of ovarian cancer cells. A, B) A2780 and OV-90 cells were transduced with sg-CEBPA-1, sg-CEBPA-2, or sg-NC, and then cells were used for the Transwell cell migration assay. Representative images (A) and relative migration cells (B) are shown. Scale bar = 50 μm. C, D) A2780 and OV-90 cells were transduced with sg-CEBPA-1, sg-CEBPA-2, or sg-NC, and then cells were used for the Transwell cell invasion assay. Representative images (C) and relative invasion cells (D) are shown. Scale bar = 50 μm. E, F) A2780 and OV-90 cells were transduced with sg-CEBPA-1, sg-CEBPA-2, or sg-NC, and then cell lysates were collected for western blot (E). Relative protein expression normalized to GAPDH is shown (F). **p≤0.001

then subcutaneously injected into nude mice. We found that forced CEBPA expression suppressed tumor xenograft growth of SK-OV-3 cells, with decreased tumor volume and weight (Figures 4F–4H). Besides, forced expression of CEBPA in SK-OV-3 xenografts was verified by qRT-PCR (Figure 4I). Collectively, our results indicated that enforced CEBPA expression suppressed proliferation, anchorage-independent growth, and tumor xenograft growth of ovarian cancer cells.

Enforced CEBPA expression inhibits migration, invasion, EMT, and lung metastasis of ovarian cancer cells. To validate the potential function of CEBPA in the regulation of EMT of ovarian cancer cells, we forced CEBPA expression in SK-OV-3 and ES-2 cells, then evaluated for cell migration, invasion, EMT, and lung metastasis. In the Transwell cell migration and invasion assay, CEBPA overexpression decreased the number of migration and invasion cells in SK-OV-3 and ES-2 (Figures 5A, 5B). The expression of ZEB1, E-cadherin, and Vimentin was evaluated by western blot. We found that forced CEBPA expression suppressed the expression of ZEB1 and Vimentin, but increased the expression of E-cadherin (Figures 5C, 5D). To explore the influence of CEBPA overexpression on migration and invasion of ovarian cancer cells *in vivo*, we established a lung metastasis model by injecting SK-OV-3 cells (1×10^6) into the tail vein of nude mice. We found that forced CEBPA expression dramatically reduced the number of tumors formed in the lung, indicating that CEBPA suppressed the metastasis of ovarian cancer cells *in vivo* (Figures 5E, 5F). In addition, overexpression of CEBPA in tumors formed in the lungs was validated by qRT-PCR (Figure 5G). Above all, our results indicated that enforced CEBPA expression inhibited migration, invasion, EMT, and lung metastasis of ovarian cancer cells, corresponding with our previous results.

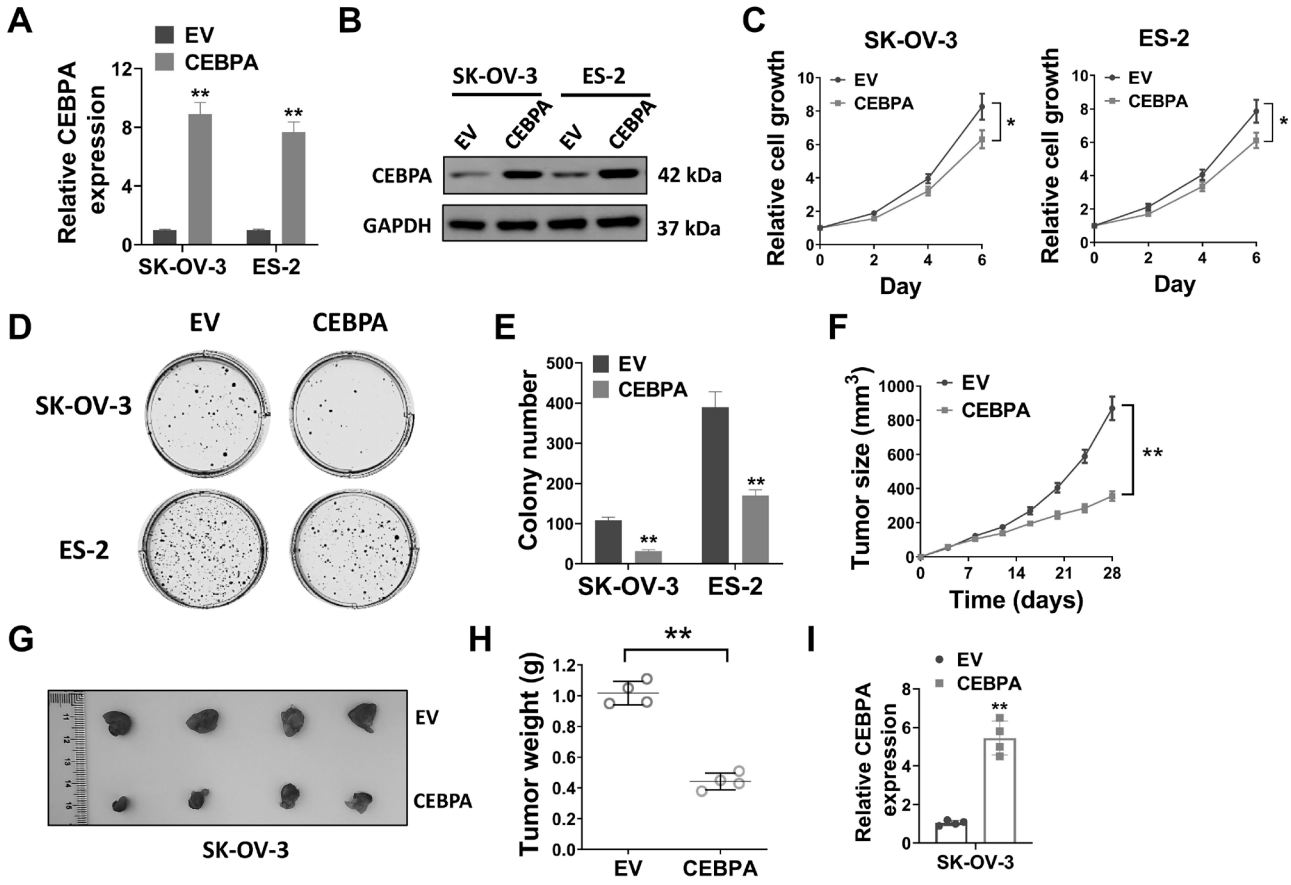

Figure 4. Enforced CEBPA expression suppresses proliferation, anchorage-independent growth, and tumor xenograft growth of ovarian cancer cells. A, B) SK-OV-3 and ES-2 cells were transduced with CEBPA expression lentivirus or EV control, and then the relative CEPBA expression was evaluated by qRT-PCR (A) and western blot (B). C) SK-OV-3 and ES-2 cells were transduced with CEBPA expression lentivirus or EV control, then cells were seeded in 96-well plates (2500/well) for cell viability assay. D, E) SK-OV-3 and ES-2 cells transduced with CEBPA expression lentivirus or EV control were used for soft agar colony formation assay. Representative plates (D) and the average number of colonies (E) are shown. F, H) SK-OV-3 (2×10^6) transduced with CEBPA expression lentivirus or EV control were subcutaneously injected into nude mice and allowed to grow for 4 weeks. Tumor growth curves (F), representative images (G), and tumor weight (H) are shown. I) Relative expression of CEBPA in SK-OV-3 xenografts was evaluated by qRT-PCR. *p≤0.05, **p≤0.001

Wnt/β-catenin activation is repressed by CEBPA in ovarian cancer cells. To elucidate the underlying molecular mechanism of CEBPA in ovarian cancer, we evaluated the activation of several important signaling pathways in cancer cells, such as PI3K/Akt, MAPK, and Wnt/β-catenin pathways. Accumulated studies indicate that Wnt/β-catenin is activated in ovarian cancer cells and plays a role in ovarian cancer stemness, EMT, and therapy resistance [21, 22]. In our study, we found that CEBPA knockout increased the nuclear translocation of β-Catenin and phosphorylation of GSK-3β, and decreased the phosphorylation of β-Catenin of A2780 and OV-90 cells, indicating the activation of Wnt/β-catenin by CEBPA depletion (Figures 6A, 6B). Moreover, forced expression of CEBPA reduced the nuclear translocation of β-Catenin and phosphorylation of GSK-3β, and increased the phosphorylation of β-Catenin of SK-OV-3 and ES-2 cells, suggesting that CEBPA suppressed Wnt/β-catenin activation in ovarian cancer cells (Figures 6C, 6D). Thus, we speculated that CEBPA regulated proliferation, migration, invasion, and EMT of ovarian cancer cells via the Wnt/β-catenin signaling.

Discussion

Though CEBPA mutations are almost confined to AML and high-risk myelodysplastic syndrome, the tumor suppressor functions of CEBPA have been demonstrated in a variety of cancer types. CEBPA is expressed in the foveolar epithelium of normal gastric mucosa but lost in nearly 30% of gastric cancer cases. Transducing of full length CEBPA suppresses the proliferation of gastric cancer cells by decreasing Cyclin D1 and regulating Ras/MAPK signaling [18]. CEBPA is expressed in alveolar type II cells, alveolar macrophages, and Clara cells in the lung, but downregulated or lost in human non-small-cell lung cancer. Condi-

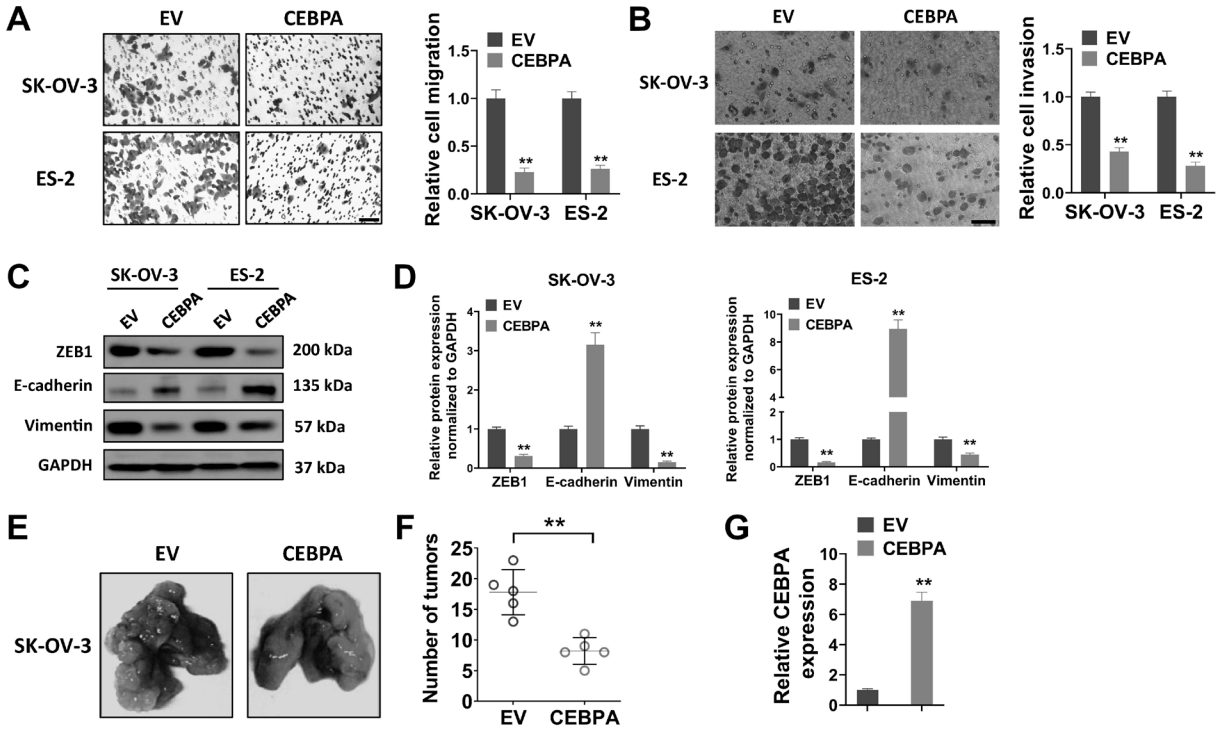

Figure 5. Enforced CEBPA expression inhibits migration, invasion, EMT, and lung metastasis of ovarian cancer cells. A) SK-OV-3 and ES-2 cells were transduced with CEBPA expression lentivirus or EV control, then the cells were used for the Transwell cell migration assay. Representative images and relative migration cells are shown. Scale bar = 50 μm. B) A2780 and OV-90 cells were transduced with sg-CEBPA-1, sg-CEBPA-2, or sg-NC, then the cells were used for the Transwell cell invasion assay. Representative images and relative invasion cells are shown. Scale bar = 50 μm. C, D) SK-OV-3 and ES-2 cells were transduced with CEBPA expression lentivirus or EV control, then cell lysates were collected for western blot (C). Relative protein expression normalized to GAPDH is shown (D). E, F) SK-OV-3 (1×10^6) transduced with CEBPA expression lentivirus or EV control were injected into the tail vein of nude mice and allowed to grow for 4 weeks. Representative images of the lung (E) and the number of tumors formed in the lung (F) are shown. G) Relative expression of CEBPA in tumors formed in the lung was evaluated by qRT-PCR. **p≤0.001

tional deletion of CEBPA in transgenic mice significantly increases the number and size of tumors formed in the lung and is associated with the downregulation of p38α MAP kinase [23]. CEBPA is highly expressed in terminally differentiated, mature liver hepatocytes, but is virtually absent in hepatomas. Overexpression of CEBPA impairs proliferation and tumorigenicity in human hepatoma cell lines [24]. The expression of CEBPA can be induced by luteinizing hormones in ovarian theca and interstitial cells [11]. In addition, CEBPA is essential for ovulation, luteinization, and expression of key target genes in the ovary [12]. In our study, we found that CEBPA was downregulated in ovarian cancer patients and predicted poor prognosis. Knockout of CEBPA facilitated proliferation, anchorage-independent growth, migration, invasion, and EMT of ovarian cancer cells, while the overexpression of CEBPA showed contrary effects. Our results indicated that CEBPA acted as a tumor suppressor in ovarian cancer.

CEBPA possesses the ability to arrest cell proliferation by several mechanisms, including interacting with CDK2 and CDK4, inducing the expression of p21 waf1/cip1, and interacting with the SWI/SNF complex [25–27]. Virtually, CEBPA overexpression suppresses proliferation in squamous cell carcinoma [28], hepatoma [24], gastric cancer [18], and cervical squamous cell carcinoma [19]. In our study, we found that CEBPA overexpression suppressed the growth of ovarian cancer cells, while knockout of CEBPA promoted proliferation of ovarian cancer cells. Our results indicated that as a well-characterized tumor suppressor, CEBPA had the ability to inhibit the proliferation of ovarian cancer cells. Moreover, CEBPA is important for regulating terminal differentiation and maintaining tissue homeostasis. In breast cancer, CEBPA is required to maintaining epithelial homeostasis by repressing the expression of key mesenchymal markers and preventing EMT [17]. CEBPA is involved in the homeostasis of the gastric epithelium [18] and represses migration and/or invasion of cervical squamous cell carcinoma and lung adenocarcinoma [19, 20]. In our study, we found that CEBPA knockout facilitated migration, invasion, and EMT of ovarian cancer cells, while the overexpression of CEBPA exhibited opposite effects. These results demonstrated that CEBPA was involved in maintaining ovarian epithelium

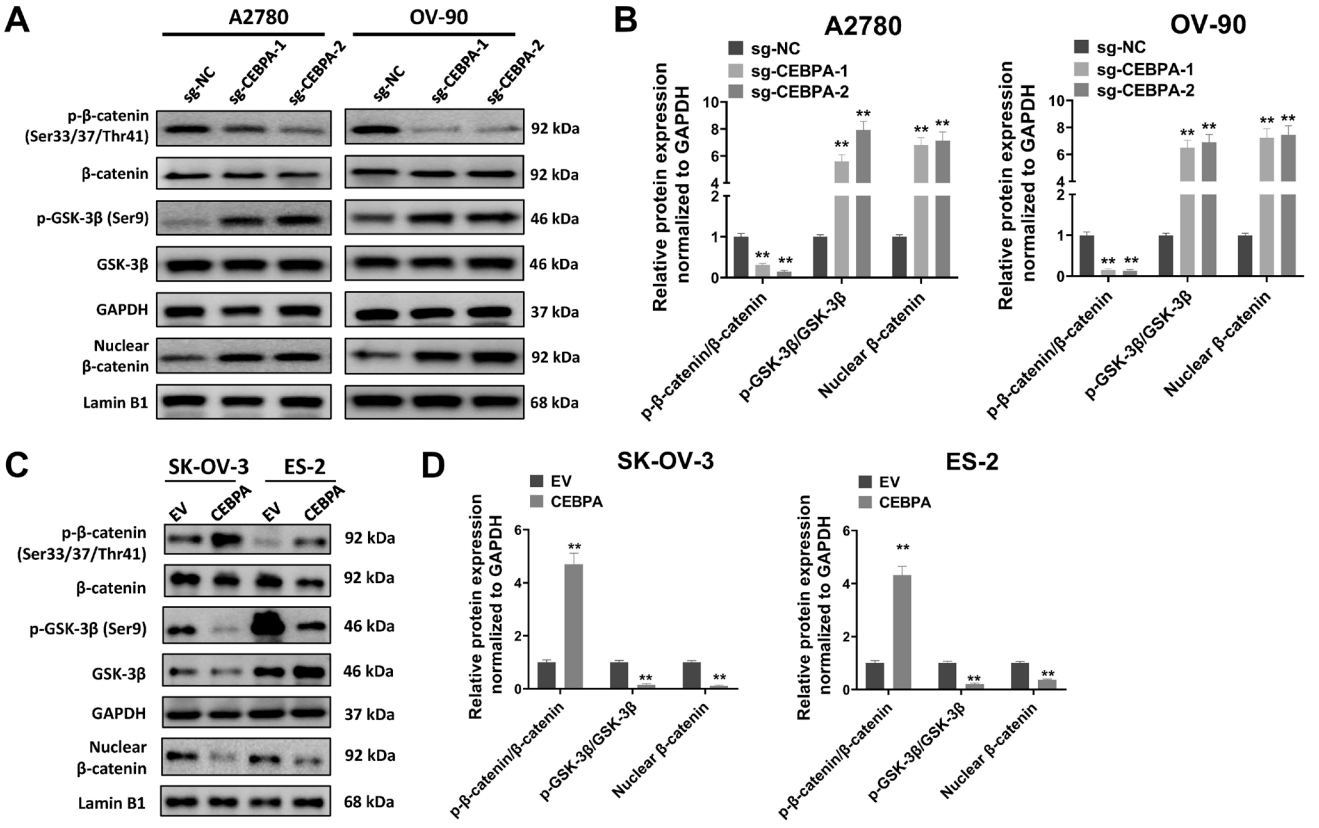

Figure 6. Wnt/β-catenin activation is repressed by CEBPA in ovarian cancer cells. A, B) A2780 and OV-90 cells were transduced with sg-CEBPA-1, sg-CEBPA-2, or sg-NC, then cell lysates were collected for western blot (A). Relative protein expression normalized to GAPDH is shown (B). C, D) SK-OV-3 and ES-2 cells were transduced with CEBPA expression lentivirus or EV control, then cell lysates were collected for western blot (C). Relative protein expression normalized to GAPDH is shown (D). **p≤0.001

homeostasis. Downregulation of CEBPA might promote EMT of ovarian epithelial cells, thus promoted progression of ovarian cancer by increasing metastasis. Indeed, we found that CEBPA overexpression dramatically reduced lung metastasis of ovarian cancer cells.

Numerous studies indicate that the Wnt/β-catenin pathway plays an important role in cancer stemness, metastasis, EMT, and chemoresistance of all subtypes of epithelial ovarian cancer [29]. Notably, members of the C/EBP family are also reported to be involved in Wnt/β-catenin activation. CCAAT/enhancer-binding protein-β (C/EBP-β) is found to negatively regulate Wnt/β-catenin signaling by mediating Axin1 expression and Wnt10b promoter activity [30, 31]. In colon cancer, a negative correlation of CEBPA and Wnt/β-catenin activation has been found [32]. In addition, CEBPA-mediated upregulation of PLIN2 promotes chronic myelocytic leukemia progression by regulating the GSK3 and Wnt/β-catenin signaling pathways [33]. In lung adenocarcinoma, CEBPA suppresses migration, invasion, and EMT of lung adenocarcinoma cells by inhibiting the transcription of β-catenin [20]. In our study, we found that knockout of CEBPA activated Wnt/β-catenin signaling in ovarian cancer cells, while CEBPA overexpression suppressed Wnt/β-catenin activation. As Wnt/β-catenin plays an important role in regulating metastasis and EMT of ovarian cancer, we speculated that CEBPA might regulate proliferation, migration, invasion, and EMT of ovarian cancer cells via inhibiting the Wnt/β-catenin signaling. However, more experiments are needed to further prove this.

In summary, we found that CEBPA was downregulated in ovarian cancer patients and predicted a poor prognosis. Knockout of CEBPA promoted proliferation, anchorage-independent growth, migration, invasion, and EMT of ovarian cancer cells, while enforced CEBPA expression suppressed proliferation, anchorage-independent growth, tumor xenograft growth, migration, invasion, EMT, and lung metastasis of ovarian cancer cells. Furthermore, Wnt/β-catenin signaling was activated by CEBPA knockout, while CEBPA overexpression exhibited opposite effects. Our data indicated that CEBPA acted as a tumor suppressor in ovarian cancer and might be a potential prognostic marker for ovarian cancer treatment.

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