

FBXW7 inhibits invasion, migration and angiogenesis in ovarian cancer cells by suppressing VEGF expression through inactivation of β -catenin signaling

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Abstract. F-box and WD repeat domain containing 7 (FBXW7) is a tumor suppressor gene frequently inactivated in several human malignancies. The present study aimed to investigate the role of FBXW7 in the invasion, migration and angiogenesis of ovarian cancer (OC) cells, and to identify its potential molecular mechanisms. First, the expression levels of FBXW7 and vascular endothelial growth factor (VEGF) were detected in several human OC cell lines using western blotting. Subsequently, FBXW7 was overexpressed to determine VEGF expression in SKOV3 cells. Transwell, wound healing and tube formation assays were performed following transfection with FBXW7 and VEGF overexpression plasmids to assess invasion, migration and angiogenesis in SKOV3 cells, respectively. Western blot analysis was performed to detect the expression levels of epithelial-to-mesenchymal transition and angiogenesis-associated proteins. In addition, the expression levels of β -catenin and c-Myc were assessed, and lithium chloride (LiCl), an agonist of β -catenin signaling, was used to elucidate the molecular mechanisms by which FBXW7 mediates its antitumor activity in OC. The results demonstrated that FBXW7 expression was markedly down-regulated, whilst VEGF expression was markedly upregulated in OC cell lines compared with that in normal ovarian cells. Overexpression of FBXW7 significantly decreased VEGF expression in SKOV3 cells. Notably, overexpression of VEGF reversed the inhibitory effects of FBXW7 overexpression on the invasion, migration and angiogenesis of OC cells, accompanied by upregulated expression levels of N-cadherin, slug, CD31, VEGF receptor 1 (VEGFR1) and VEGFR2, and downregulated expression levels of E-cadherin. Furthermore,

overexpression of FBXW7 markedly suppressed β -catenin and c-Myc expression, whereas the decreased expression levels of VEGF, VEGFR1 and VEGFR2 following overexpression of FBXW7 were increased after treatment of SKOV3 cells with LiCl. Overall, the results of the present study suggested that FBXW7 inhibited invasion, migration and angiogenesis of OC cells by suppressing VEGF expression through inactivation of β -catenin signaling. Thus, FBXW7 may be used as a novel therapeutic target for the treatment of OC.

Introduction

Ovarian cancer (OC) is one of the deadliest gynecological malignancies, with high incidence and mortality rates worldwide (1,2). There were ~239,000 new cases and 152,000 deaths worldwide annually with regard to this disease in 2017 (3). Despite advancements in treatment options, the 5-year survival rate of patients with OC remains <35% globally due to distant metastasis and recurrence (4,5). Thus, it is urgent to obtain an improved understanding regarding the pathogenesis underlying OC to identify and develop more effective therapeutic targets for the treatment of this disease.

F-box and WD repeat domain containing 7 (FBXW7), a member of the F-box protein family, is an evolutionarily conserved F-box protein, containing two vital functional domains (F-box and WD), which are indispensable for its function (6). FBXW7 is a critical tumor suppressor gene, where mutations within this gene have been implicated in different types of human cancer (7,8). For instance, FBXW7 can target salt inducible kinase 2 for degradation, leading to the disruption of target of rapamycin 2-AKT signaling to inhibit pancreatic cancer cell proliferation and cell cycle progression (9). It has also been reported that FBXW7 suppresses oral squamous cell carcinoma proliferation and invasion regulated by miR-27a through the PI3K/AKT signaling pathway (10). Increasing evidence has suggested that FBXW7 serves as a key regulator in the proliferation, invasion, migration and apoptosis of human cancer cells through the degradation of oncoproteins, including c-Myc, in a proteasome-dependent manner (11,12). Previous studies have demonstrated that FBXW7 is deleted or methylated in epithelial OC, and its expression is negatively associated with the malignant potential of ovarian tumors (13,14). FBXW7

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has been reported to act as a positive regulator of angiogenesis in the endothelium of the growing vasculature (15). Increasing evidence has suggested that angiogenesis serves a crucial role in the invasion, migration and metastasis of OC, with a positive association between the rate and extent of angiogenesis and an unfavorable prognosis in patients with OC (16,17). Vascular endothelial growth factor (VEGF), a homodimeric glycoprotein, is the key mediator of angiogenesis, which binds two important VEGF receptors, VEGFR1 and VEGFR2 (18,19). However, the effects of FBXW7 on the angiogenesis of OC, and whether FBXW7 functions by regulating VEGF expression, remain unclear.

The present study aimed to investigate VEGF expression following overexpression of FBXW7 in OC cells. In addition, the role of FBXW7 on the invasion, migration and angiogenesis of OC cells, and its potential regulatory effects on VEGF expression were assessed.

Materials and methods

Cell culture. The normal human ovarian epithelial cell line (IOSE-80) and several OC cell lines (ES-2, SKOV3, PA1 and OVCAR3) were purchased from the American Type Culture Collection and maintained in DMEM (Invitrogen; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS (HyClone; Cytiva) at 37°C with 5% CO₂. Cells were treated with the β -catenin activator, lithium chloride (LiCl; 20 mM; Sigma-Aldrich; Merck KGaA) for 3 h at 37°C.

Human umbilical vein endothelial cells (HUVECs) were provided The Cell Bank of Type Culture Collection of the Chinese Academy of Sciences and cultured in a mixture containing RPMI-1640 medium (HyClone; Cytiva) and 10% FBS. The cells were incubated at 37°C in a humidified atmosphere which was maintained at 5% CO₂.

Cell transfection. SKOV3 cells were seeded into 6-well plates at a density of 2×10^5 cells/well and cultured at 37°C until they reached 80% confluence. pcDNA 3.1 containing FBXW7 (Ov-FBXW7) or VEGF (pc-VEGF), and the corresponding empty vectors used as negative controls (Ov-NC and pc-NC, respectively) were synthesized by Shanghai GenePharma Co., Ltd. SKOV3 cells were transfected with the respective plasmids (50 nM) using Lipofectamine[®] 2000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.) at 37°C for 24 h, according to the manufacturer's instructions. Cells were collected and the transfection efficiency was assessed via reverse transcription-quantitative (RT-q) PCR and western blot analyses 24 h post-transfection.

Invasion assay. Cell invasion was assessed using Transwell assay (pore size, 8.0 μ m; Corning Inc.) precoated with Matrigel (6.25 mg/l; BD Biosciences) overnight at 4°C. A total of 2×10^5 transfected SKOV3 cells were resuspended in 200 μ l serum-free DMEM and plated in the upper chambers of Transwell plates, whilst 600 μ l DMEM supplemented with 10% FBS was used as the chemoattractant and plated in the lower chambers. Following incubation for 24 h at 37°C, the invasive cells were fixed with 4% formaldehyde for 30 min at room temperature and subsequently stained with 0.1% crystal violet for 30 min at room temperature. Stained cells were counted in five randomly selected fields using an inverted light

microscope (Olympus Corporation; magnification, x100) and the results were analyzed using ImageJ software (version 1.52r; National Institutes of Health).

Wound healing assay. Cell migration was assessed using wound healing assays. Briefly, SKOV3 cells were seeded into 6-well plates at a density of 4×10^5 cells/well and cultured in DMEM supplemented with 10% FBS until they reached 80% confluence. The cell monolayers were scratched using sterile 200- μ l pipette tips. Cells were washed with PBS to elute the debris, and the medium was replaced with serum-free DMEM. Following incubation for 24 h at 37°C, the average distance of cells migrated into the wound surface was observed under an inverted light microscope (Olympus Corporation; magnification, x100). Quantitative analysis of the wound healing area was performed using ImageJ software (version 1.52r; National Institutes of Health).

Tube formation assay. A total of 1.5×10^4 HUVECs were seeded into 96-well plates precoated with Matrigel (10 mg/ml; BD Biosciences) at 4°C overnight and incubated for 6 h at 37°C in a 5% CO₂ incubator with the supernatants from SKOV3 cells transfected with Ov-FBXW7 or/and pc-VEGF. Tube formation was observed under an inverted light microscope (Olympus Corporation). The number of loops formed was counted in five randomly selected fields and analyzed using ImageJ software (version 1.52r; National Institutes of Health). A connection between two cells was counted as one capillary tube formation.

RT-qPCR. Total RNA was extracted from SKOV3 cells using TRIzol[®] reagent (Thermo Fisher Scientific, Inc.). Total RNA was reverse transcribed into cDNA using the First Strand cDNA synthesis kit (Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. qPCR was subsequently performed with 2 μ g cDNA using the SYBR Premix Ex Taq (Takara Bio, Inc.) and ABI 7500 equipment (Applied Biosystems; Thermo Fisher Scientific, Inc.). The following thermocycling conditions were used: Initial denaturation at 95°C for 10 min; followed by 40 cycles of denaturation at 95°C for 15 sec and annealing at 60°C for 1 min; and a final extension of 10 min at 72°C. The sequences of the gene-specific primers used in the present study were as follows FBXW7 forward, 5'-CACAGG CCTCAAGAGTGGC-3' and reverse, 5'-TTGCATCATATG CTTCACTTGTGT-3'; VEGF forward, 5'-GGGCAGAATCAT CACGAAGT-3' and reverse, 5'-AAATGCTTTCTCCGCTC TGA-3'; CD31 forward, 5'-TGCAGTGGTTATCATCGGA GTG-3' and reverse, 5'-CGTTGTTGGAGTTCAGAAAGTG-3'; VEGFR1 forward, 5'-TGCCTCAGAAGAGCTGAAAAC-3' and reverse, 5'-CACAGACTCCCTGCTTTTGCT-3'; VEGFR2 forward, 5'-GCACATTGGTGGTGGCTGAC-3' and reverse, 5'-CTCTCCTTCGGCTGGCATCT-3' and GAPDH forward, 5'-ACAACCTTGGTATCGTGGGAAGG-3' and reverse, 5'-GCCATCACGCCACAGTTTC-3'. Relative expression levels were calculated using the 2^{- $\Delta\Delta$ C_q} method and normalized to the internal reference gene GAPDH (20).

Western blotting. Total protein was extracted from SKOV3 cells using RIPA lysis buffer (Beyotime Institute of Biotechnology). Total protein was quantified using the bicinchoninic acid kit (Beyotime Institute of Biotechnology) and

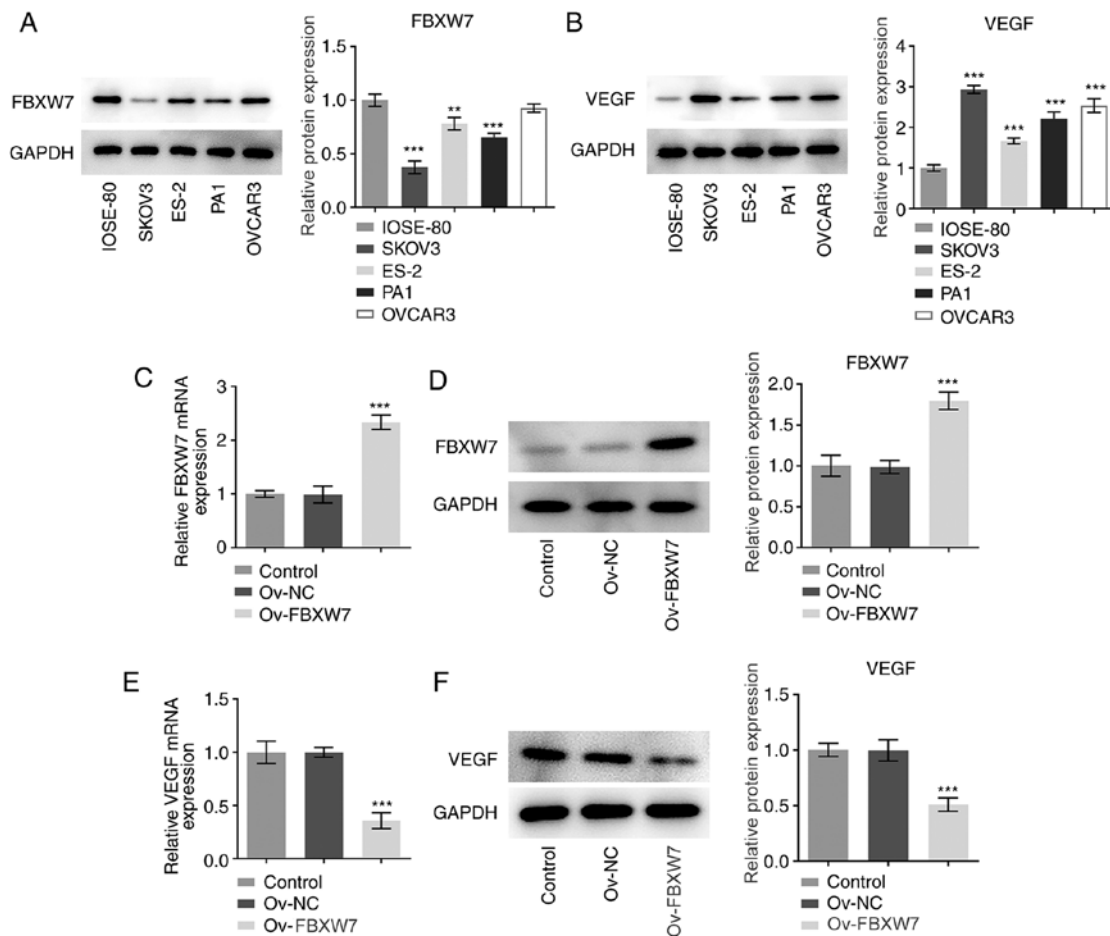


Figure 1. Overexpression of FBXW7 significantly decreases VEGF expression in SKOV3 cells. Protein expression levels of (A) FBXW7 and (B) VEGF in a normal human ovarian epithelial cell line (IOSE-80) and several OC cell lines (ES-2, SKOV3, PA1 and OVCAR3) were detected via western blot analysis. ** $P < 0.01$ and *** $P < 0.001$ vs. IOSE-80. (C) RT-qPCR and (D) western blot analyses were performed to detect FBXW7 expression following transfection with Ov-FBXW7. (E) RT-qPCR and (F) western blot analyses were performed to detect VEGF expression following overexpression of FBXW7. *** $P < 0.001$ vs. Ov-NC. FBXW7, F-box and WD repeat domain containing 7; VEGF, vascular endothelial growth factor; RT-qPCR, reverse transcription-quantitative PCR; Ov, overexpression; NC, negative control.

4 μ g protein/lane was separated by 10% SDS-PAGE. The separated proteins were subsequently transferred onto polyvinylidene fluoride membranes (EMD Millipore) and blocked with 5% skimmed milk for 1.5 h at room temperature. The membranes were incubated with corresponding primary antibodies at 4°C overnight. Following the primary antibody incubation, membranes were incubated with a goat anti-rabbit horseradish peroxidase (HRP)-conjugated secondary antibody (1:3,000; cat. no. 7074S; Cell Signaling Technology, Inc.) or horse anti-mouse HRP-conjugated secondary antibody (1:3,000; cat. no. 7076S; Cell Signaling Technology) for 1.5 h at room temperature. The immunoreactive protein bands on the membranes were visualized using an enhanced chemiluminescence assay (EMD Millipore). The relative intensity of target bands were semi-quantified using ImageJ software (version 1.52r; National Institutes of Health) and normalized by the intensity of GAPDH. Anti-FBXW7 (cat. no. ab109617; 1:1,000) antibody was provided by Abcam. Anti-VEGF (cat. no. 2463S; 1:1,000), anti-CD31 (cat. no. 3528S; 1:1,000), anti-VEGFR1 (cat. no. 64094S; 1:1,000), VEGFR2 (cat. no. 9698S; 1:1,000), anti-E-cadherin (E-cad; cat. no. 3195T; 1:1,000), anti-N-cadherin (N-cad; cat. no. 13116T; 1:1,000), anti-Slug (cat. no. 9585T; 1:1,000) and anti-GAPDH (cat.

no. 5174T; 1:1,000) antibodies were all purchased from Cell Signaling Technology, Inc.

Statistical analysis. All experiments were repeated three times independently. Statistical analysis was performed using GraphPad Prism 8.0 (GraphPad Software, Inc.). Data are presented as the mean \pm SD. An unpaired Student's t-test was used to compare differences between two groups, while one-way ANOVA followed by Tukey's post hoc test was used to compare differences among multiple groups. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Overexpression of FBXW7 significantly downregulates VEGF expression in OC cells. Firstly, the expression levels of FBXW7 and VEGF in a normal human ovarian epithelial cell line (IOSE-80) and several OC cell lines (ES-2, SKOV3, PA1 and OVCAR3) were detected via western blot analysis. As shown in Fig. 1A and B, FBXW7 expression was markedly downregulated, while VEGF expression was significantly upregulated in OC cell lines compared with in IOSE-80 cells, especially in SKOV3 cells, which were therefore used

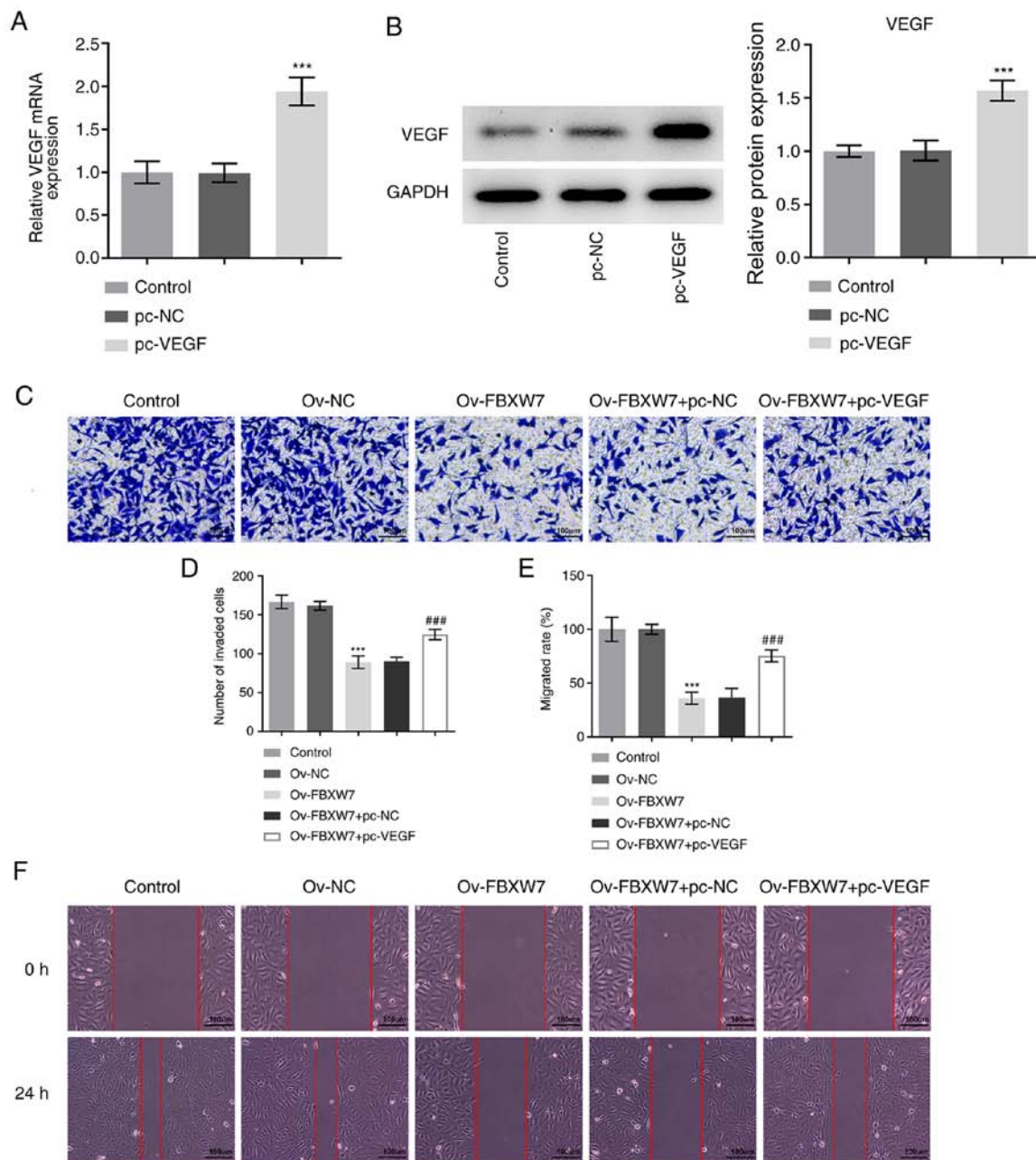


Figure 2. Overexpression of VEGF attenuates the inhibitory effects of FBXW7 overexpression on the invasion and migration of SKOV3 cells. (A) Reverse transcription-quantitative PCR and (B) western blot analyses were performed to detect VEGF expression following transfection with the VEGF plasmid. (C and D) Transwell assay was performed to assess the invasive ability of SKOV3 cells. (E and F) Wound healing assay was performed to assess the migratory ability of SKOV3 cells. Scale bar, 100 μ m. *** P <0.001 vs. pc-NC or Ov-NC; ### P <0.001 vs. Ov-FBXW7+pc-NC. VEGF, vascular endothelial growth factor; FBXW7, F-box and WD repeat domain containing 7; Ov, overexpression; NC, negative control.

in subsequent experiments. Subsequently, FBXW7 was overexpressed in SKOV3 cells. As presented in Fig. 1C and D, FBXW7 expression was significantly upregulated at both the transcriptional and protein levels following transfection with Ov-FBXW7 compared with those after transfection with Ov-NC. Notably, overexpression of FBXW7 significantly decreased VEGF mRNA and protein expression compared with the vector control group (Fig. 1E and F). Overall, these results suggested that overexpression of FBXW7 inhibited VEGF expression in OC cells.

Overexpression of VEGF restores the inhibitory effects of FBXW7 overexpression on the invasion, migration and

epithelial-to-mesenchymal transition (EMT) of OC cells. To determine whether FBXW7 regulates VEGF expression in OC, VEGF was overexpressed following transfection with a plasmid containing VEGF. As presented in Fig. 2A and B, VEGF mRNA and protein expression was significantly increased in the pc-VEGF group compared with in the pc-NC group.

Transwell and wound healing assays were performed to assess the invasive and migratory abilities of SKOV3 cells, respectively. As presented in Fig. 2C-F, overexpression of FBXW7 significantly inhibited the invasive and migratory abilities of SKOV3 cells compared with the Ov-NC group. Conversely, co-transfection with VEGF and FBXW7 overexpression plasmids significantly promoted the

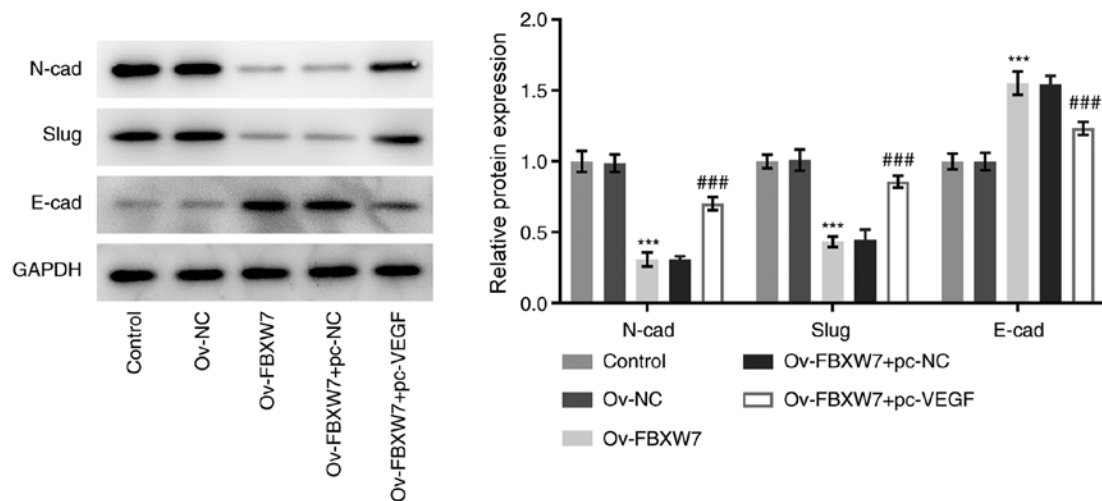


Figure 3. Overexpression of VEGF reverses the inhibitory effects of FBXW7 overexpression on the EMT process of SKOV3 cells. Western blot analysis was performed to detect the expression levels of EMT-associated proteins, including N-cad, slug and E-cad. ***P<0.001 vs. Ov-NC; ###P<0.001 vs. Ov-FBXW7+pc-NC. EMT, epithelial-to-mesenchymal transition; FBXW7, F-box and WD repeat domain containing 7; VEGF, vascular endothelial growth factor; Ov, overexpression; NC, negative control; N/E-cad, N/E-cadherin.

invasive and migratory abilities of SKOV3 cells compared with cells transfected with the FBXW7 overexpression plasmid alone (Fig. 2C-F). Additionally, overexpression of FBXW7 significantly decreased the expression levels of N-cadherin and slug, while significantly increasing E-cadherin expression, and these effects were reversed following co-transfection with the VEGF overexpression plasmid (Fig. 3). Collectively, these results indicated that overexpression of FBXW7 inhibited the invasion, migration and EMT process of OC cells by suppressing VEGF expression.

Overexpression of VEGF partially counteracts the impact of FBXW7 overexpression on the angiogenesis of OC cells. To investigate the effect of FBXW7 on the angiogenesis of OC cells, a tube formation assay was performed. As presented in Fig. 4A and B, there was no significant difference in the number of tubes formed between the control and the Ov-NC groups. However, a significantly decreased number of tubes was observed in the Ov-FBXW7 group compared with in the Ov-NC group (Fig. 4A and B). Notably, overexpression of VEGF enhanced the number of tubes formed compared with the Ov-FBXW7+pc-NC group (Fig. 4A and B). RT-qPCR and western blot analyses were performed to detect the expression levels of proteins associated with angiogenesis. The results demonstrated that overexpression of FBXW7 downregulated the expression levels of CD31, VEGFR1 and VEGFR, whereas co-transfection with FBXW7 and VEGF plasmids significantly increased their expression levels compared with the Ov-FBXW7+pc-NC group (Fig. 4C and D), which is consistent with the results of the tube formation assay. Overall, these results suggested that overexpression of FBXW7 suppressed the angiogenesis of OC cells by suppressing VEGF expression.

FBXW7 inhibits VEGF expression through inactivation of β -catenin signaling. To further elucidate the potential molecular mechanism by which FBXW7 mediates its antitumor effects in OC, western blot analysis was performed to detect the expression levels of key proteins in β -catenin signaling. As presented

in Fig. 5A, overexpression of FBXW7 significantly decreased the expression levels of β -catenin and c-Myc compared with the empty vector group. Furthermore, LiCl, an agonist of β -catenin signaling, was used to treat SKOV3 cells transfected with the FBXW7 plasmid, and the expression levels of angiogenesis-associated proteins were determined. As presented in Fig. 5B, treatment with LiCl significantly abrogated the inhibitory effects of FBXW7 overexpression on the expression levels of VEGF, VEGFR1 and VEGFR2. Collectively, these results suggested that FBXW7 may inhibit VEGF expression through inactivation of β -catenin signaling in SKOV3 cells.

Discussion

OC is one of the most common and lethal types of cancer in women, and has been a significant public health burden worldwide (21). Thus, it is of great importance to further understand the molecular mechanisms of OC tumorigenesis and progression to identify and develop effective therapeutic strategies. The results of the present study demonstrated that FBXW7 efficiently inhibited SKOV3 cell invasion and migration, as well as tube formation of HUVECs. Mechanistically, FBXW7 suppressed VEGF expression by inactivating β -catenin signaling.

Invasion and migration are two hallmarks of the malignant biological behavior of OC, and interdiction of these progresses is a critical factor to improve biomedical treatment worldwide (22,23). EMT, a process in which stationary epithelial cells attain a highly active mesenchymal phenotype, is an essential and important step in tumor cell invasion, migration and relocation (24-26). Downregulated expression levels of E-cadherin (a crucial epithelial marker) in epithelial cells, along with upregulated expression levels of mesenchymal proteins, including N-cadherin and slug, are common hallmarks of EMT (27,28). Angiogenesis refers to the generation of new blood vessels by the sprouting of endothelial cells from preexisting ones (29). It has been reported that tumors depend on the constant growth of new blood vessels, whereby interruption of the blood supply may eliminate the cancer (30).

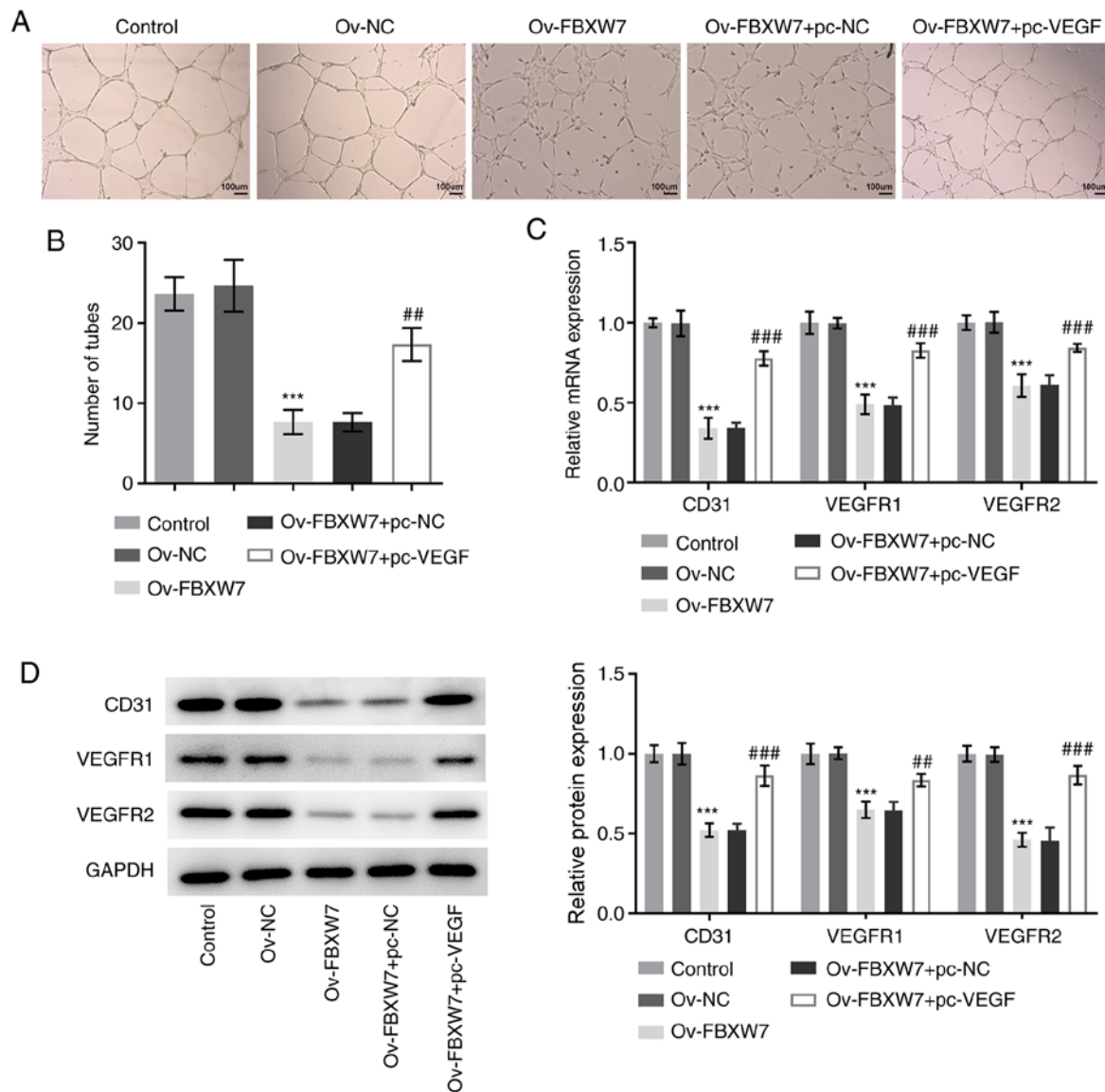


Figure 4. Overexpression of VEGF abrogates the effects of FBXW7 overexpression on the angiogenesis of SKOV3 cells. (A and B) Tube formation assay was performed to assess angiogenesis. (C) Reverse transcription-quantitative PCR and (D) western blot analyses were performed to detect the expression levels of angiogenesis-associated proteins. *** $P < 0.001$ vs. Ov-NC; ## $P < 0.01$, ### $P < 0.001$ vs. Ov-FBXW7+pc-NC. FBXW7, F-box and WD repeat domain containing 7; VEGF, vascular endothelial growth factor; Ov, overexpression; NC, negative control; VEGFR1/2, VEGF receptor 1/2.

Increasing evidence has suggested that angiogenesis is essential for cancer development by participating in the growth, invasion, migration and metastasis of cancer (31,32). FBXW7 is a vital tumor suppressor gene, and mutations in this gene have been implicated in different types of human cancer. For example, upregulated FBXW7 expression inhibits tumor invasion, migration, EMT and angiogenesis, including oral squamous cell carcinoma, breast cancer and non-small-cell lung cancer (10,33-35). Notably, FBXW7 has been reported to act as a potent positive regulator of angiogenesis in the endothelium of the growing vasculature (15). FBXW7 expression is downregulated in OC tissues, and low FBXW7 expression is negatively associated with the malignant potential of OC (14). Increasing evidence has suggested that FBXW7-knockdown can promote OC cell invasion and migration (36). The results of the present study demonstrated that overexpression of FBXW7 inhibited the invasion, migration, EMT and angiogenesis of OC cells. To the best of our knowledge, the present study was

the first to demonstrate the inhibitory effect of FBXW7 on the angiogenesis of OC.

VEGF, a homodimeric glycoprotein, is one of the most potent and specific angiogenic factors of tumor-induced angiogenesis and binds to VEGFR1 and VEGFR2 (18,19). Elevated VEGF expression has been observed in several OC cell lines and OC biopsies of different histological grades (37). A previous study has demonstrated that FBXW7 can block the effect of microRNA-182 on VEGF induction and angiogenesis in breast cancer cells (38). However, whether FBXW7 regulates VEGF expression in the progression of OC remains unclear.

The results of the present study demonstrated that overexpression of FBXW7 suppressed VEGF expression, while overexpression of VEGF partially counteracted the inhibitory effects of FBXW7 overexpression on the invasion, migration, EMT and angiogenesis of OC cells. To further elucidate the potential molecular mechanism by which FBXW7 mediates

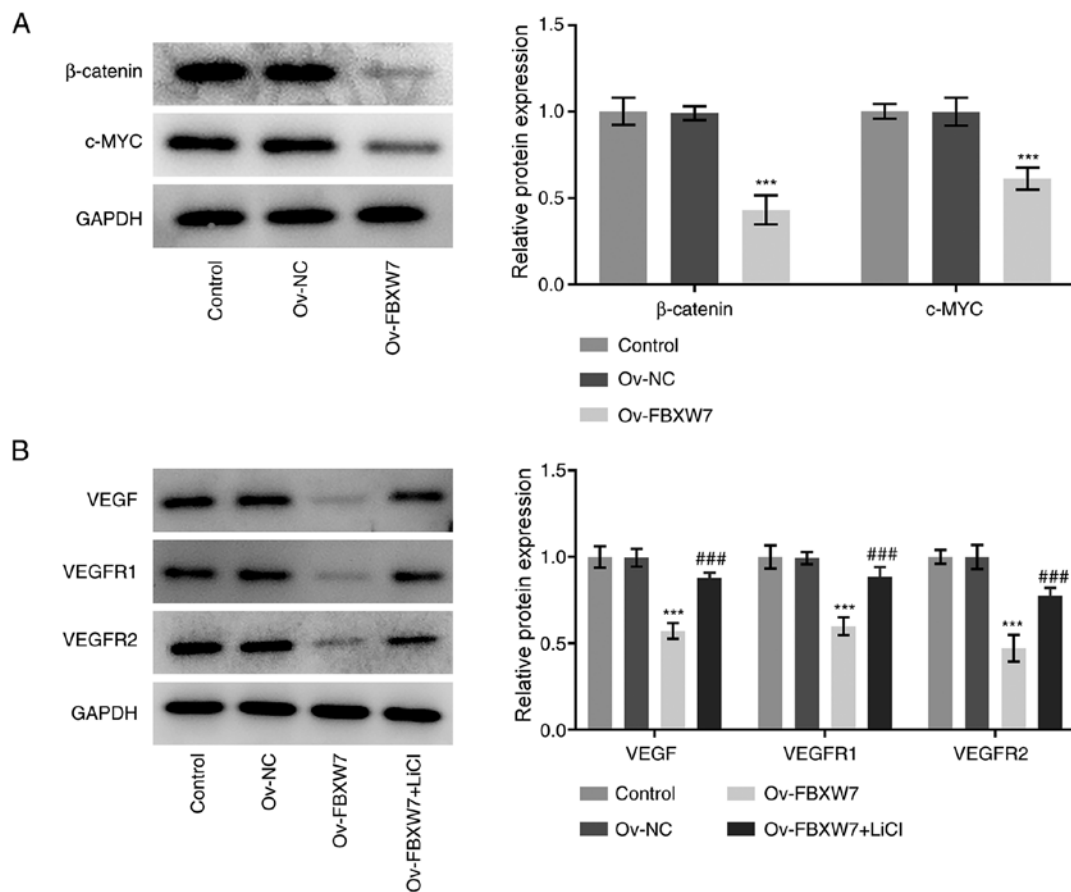


Figure 5. FBXW7 suppresses VEGF expression through inactivation of β -catenin signaling. Western blot analysis was performed to detect the protein expression levels of (A) β -catenin and c-Myc, and (B) VEGF, VEGFR1 and VEGFR2 following LiCl treatment. *** $P < 0.001$ vs. Ov-NC; ### $P < 0.001$ vs. Ov-FBXW7. FBXW7, F-box and WD repeat domain containing 7; VEGF, vascular endothelial growth factor; Ov, overexpression; NC, negative control; LiCl, lithium chloride.

its antitumor effects in OC, the expression levels of key proteins in β -catenin signaling were analyzed. Increasing evidence suggests that inhibition of the β -catenin signaling pathway restrains the proliferation, invasion, migration and angiogenesis of different types of cancer, including retinoblastoma, gastric cancer and OC (39-41). Previous studies have demonstrated that β -catenin signaling helps VEGF regulate angiogenesis, and that FBXW7 promotes the degradation of β -catenin (42,43). The results of the present study demonstrated that overexpression of FBXW7 inhibited the expression levels of VEGF, β -catenin and c-Myc. Notably, treatment with LiCl, an agonist of β -catenin signaling, increased the expression levels of VEGF, VEGFR1 and VEGFR2. Overall, the current results suggested that FBXW7 may inhibit VEGF expression through inactivation of β -catenin signaling in SKOV3 cells.

In conclusion, the results of the present study demonstrated that FBXW7 inhibited the invasion, migration and angiogenesis of OC cells. To the best of our knowledge, the present study was the first to provide insight into the anti-angiogenesis effects of FBXW7 on OC, and to suggest a promising therapeutic potential of FBXW7 in the targeted treatment of this disease. However, the use of a single OC cell line to analyze FBXW7 expression and its potential mechanisms in OC is a limitation of the present study. Therefore, future studies should confirm the results of the present study using multiple OC cell lines and *in vivo* models.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

LZ and YP searched the literature, designed the experiments and performed the experiments. YP and JS analyzed and interpreted the data. LZ wrote the manuscript. JS revised the manuscript. LZ and JS can authenticate the raw data in the present study. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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