

RESEARCH PAPER



## Wilms' tumor 1 (*WT1*) promotes ovarian cancer progression by regulating E-cadherin and ERK1/2 signaling

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### ABSTRACT

Wilms' tumor 1 (*WT1*) is reported to play an important role in tumor invasion and metastasis, two hallmarks of ovarian cancer (OC) that influence treatment efficacy and prognosis. However, the specific roles and underlying mechanisms of *WT1* in OC have not been fully understood. Here, we investigated the potential function and signaling pathways of *WT1* in OC cells. We showed that *WT1* was significantly upregulated in human OC tissues and closely associated with OC type, grade and FIGO stage. In cultured cells and xenograft mouse models, *WT1* depletion significantly inhibited cell migration and invasion, reversed epithelial–mesenchymal transition (EMT), and prevented metastasis of OC cells. We further demonstrated that *WT1* inhibited E-cadherin expression via targeting *E-cadherin* gene promoter by chromatin immunoprecipitation and luciferase reporter assay. Moreover, ERK1/2 activation was suppressed upon *WT1* silencing. Inhibiting ERK1/2 phosphorylation increased E-cadherin expression and suppressed *WT1*-induced OC cell migration and invasion. Taken together, our study reveals *WT1* exerts a tumor-promoting role in OC, enhancing EMT through negative modulation of E-cadherin expression via ERK1/2 signaling. *WT1* may represent a novel therapeutic target that may improve the prognosis of OC.

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### KEYWORDS

Ovarian cancer; *WT1*; migration and invasion; EMT; ERK1/2 signaling

## 1. Introduction

Ovarian cancer (OC) is a major gynecologic malignancy and the leading cause of cancer-related death among women [1]. It behaves more aggressively and has a worse prognosis than any other cancer involving the female genital tract [2]. Approximately 70% of patients with OC are diagnosed at advanced stages due to a lack of specific initial symptoms and limited approaches for early detection [3]. The 5-year survival rate for ovarian cancer shows a ~ 90% survival rate at early stages, but decreased to 30% in those at stage III and IV [4,5]. Although great efforts have been made toward improving effective treatment, postoperative locoregional recurrence and distant metastasis still occur frequently and confer high risks of morbidity and mortality.

The Wilms' Tumor 1 (*WT1*) gene encodes a zinc finger transcription factor and RNA-binding protein that regulates transcription of numerous target genes and manifests both tumor suppressor and oncogenic activities [6]. It was initially discovered as a tumor suppressor in Wilms' tumor of the kidneys [7]. Subsequent studies revealed that *WT1* can also act as an oncogene in multiple tumors, including leukemia, lung cancer, breast cancer, and glioblastoma [8]. In OC, *WT1* is overexpressed and positively associated with epithelial ovarian cancer [9–11]. Additionally, it is most prevalent in advanced stage tumors (stage III/IV) as well as in 100% of metastatic lesions, suggesting that *WT1* expression is enhanced during OC progression to metastasis [12]. *WT1* can promote the tumorigenesis through various pathways, and plays an important role in tumor invasion and metastasis. Some recent studies have investigated the link

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between *WT1* and its potential role in OC progression [13,14]. For example, Gupta *et al.* found *WT1* was necessary for transcriptional upregulation of *SHMT1*, which mediates the cancer-promoting activity of *WT1* [13]. However, metastasis is the most lethal attribute of OC with highly complicated molecular mechanisms. Considering *WT1* has been evaluated as a potential novel therapeutic target for the metastatic serous ovarian carcinoma [12,15], it is necessary for us to further understand its detailed molecular mechanism in OC metastasis.

OC metastasis is closely associated with epithelial–mesenchymal transition (EMT), a biologic process during which epithelial cells acquire new features of mesenchyme [16], which plays an important role in upregulating the expression of cell motility-related proteins, enhancing cell migration and invasion and promoting cancer cell metastasis [17,18]. Recent studies have shown that E-cadherin represents a downstream target of *WT1* in the regulation of EMT [19–21]. The *WT1*-driven EMT via the downregulation of E-cadherin may be one of the mechanisms for shedding light on the OC metastatic progression.

Given the emerging importance of *WT1* in OC and EMT, we examined the association between *WT1* and OC, and further explored the molecular mechanisms by which *WT1* mediates OC invasion and metastasis.

## 2. Material and methods

### 2.1 Tissue specimens and cell culture

This study was approved by the Ethics Committee of Second Affiliated Hospital of Nanjing Medical University. Written informed consent was obtained from each patient. Human primary OC samples and the corresponding adjacent normal ovarian tissues were collected from 49 patients with OC during primary surgery or before neoadjuvant chemotherapy in the Second Affiliated Hospital of Nanjing Medical University from 2014 to 2017. Ovarian cancers were classified based on International Federation of Gynecology and Obstetrics (FIGO) staging system and WHO grading system. The clinicopathological features are summarized in Table 1. Normal human ovarian epithelial cell line IOSE386, epithelial OC cell

**Table 1.** Association of *WT1* with the clinicopathological characteristics of ovarian cancer.

Variables	Cases (49)	WT1 expression		P
		Low/Negative	High	
Age(years)				NS
≤55	14	4	10	
>55	35	12	23	
Histological type				< 0.05
Serous	37	5	32	
Non-serous	12	9	3	
Mucinous	9	6	3	
Clear cell	2	2	0	
Endometrioid	1	1	0	
FIGO stage				< 0.05
Stage I–II	12	8	4	
Stage III–IV	37	8	29	
Histological grade				< 0.05
Grade 1–2	31	14	17	
Grade 3	18	2	16	

NS; Not statistically significant

lines A2780, HO8910, and HO8910PM, were obtained from the Chinese Academy of Sciences Cell Bank (Shanghai, China). Epithelial OC SKOV3 cells and HEK 293 T cells were from ATCC. HEK 293 T were maintained in Dulbecco's Modified Eagle Medium (DMEM). IOSE386, A2780, HO8910, and HO8910PM and SKOV3 were cultured in RPMI-1640. Each medium was supplemented with 10% fetal bovine serum (Gibco, Mexico). Cells were cultured at 37°C in a humidified 5% CO<sub>2</sub> atmosphere. For cell viability assays, cells were seeded into 96-cell plates at a density of 4,000 cells/well and assessed using cell counting kit-8 (CCK-8) reagent (Yeasen, Shanghai, China) on day 1, 2, 3 post cell seeding. Absorbance values were detected at the wavelength of 450 nm using a platereader (Thermo Scientific Multiskan GO, Finland).

### 2.2 shRNA-based stable *WT1* knockdown and transient *WT1* overexpression

The shRNA vectors LV3-GFP-*WT1*-shRNA (*WT1*-shRNA) and LV3-shNC (negative control) were from Genechem (Shanghai, China). The target sequences were as follows: LV3-*WT1*-#2:CTC ACT CCT TCA TCA AAC AGG; LV3-*WT1*-#3:

GCC CTA CAG CAG TGA CAA TTT; LV3-WT1-#4:GCT TAC CCA GGC TGC AAT AAG; LV3-WT1-#5:GGA GAC ATA CAG GTG TAA AC; LV3-shNC control #1: GTT CTC CGA ACG TGT CAC GT. SKOV3 and HO8910PM cells were infected by recombinant lentivirus expressing above shRNAs generated from 293 T cells. Stable cells were selected by 1  $\mu\text{g}/\text{mL}$  puromycin. The plasmids pCMV3-WT1-Flag (pCMV3-WT1, WT1 overexpression) and pCMV3-C-Flag (pCMV3, negative control) were from Sino Biological (Beijing, China). A2780 cells were transiently transfected with above plasmids using Lipofectamine 2000 (Invitrogen).

### 2.3 Immunohistochemistry (IHC)

The clinical samples were sectioned into 5  $\mu\text{m}$  sections. After deparaffination and dehydration, slides were treated with hydrogen peroxide at room temperature and then heated to retrieve the antigen. Immunohistochemical analyses were performed using a Histostain Kit (Invitrogen, USA) with antibodies against WT1 (Ab89901, Abcam). Diaminobenzidine (DAB) was used as the chromogenic reaction, and hematoxylin was used to stain nuclei. Scoring for WT1 expression was based on the proportion of cells in a given tissue sample exhibiting distinct nuclear immunopositivity (percentage scale: 0, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90, and 100). The WT1 scoring results were transformed into a two-tiered scale (level 1 (negative): less than 10%, level 2 (positive): equal or more than 10%) [22].

### 2.4 Wound healing assay

Cells were seeded in 6-well plates and grown to full confluence in complete medium. The monolayer of cells was scratched with a sterile 100  $\mu\text{L}$  pipette tip. The wounded areas were observed and imaged under microscope at 0, 24, and 48 h after scratches.

### 2.5 Transwell invasion assay

The invasive ability of the cells was performed in 24-well plates with 8.0- $\mu\text{m}$  pore size chamber inserts (Corning, USA). The upper surface of the

filter was coated with 50  $\mu\text{L}$  of Matrigel (BD Biosciences, San Jose, CA) diluted 1:6 in serum-free medium. Approximately  $5 \times 10^4$  cells were placed in the upper chamber and cultured in serum-free medium, and 500  $\mu\text{L}$  medium with 10% FBS was added to the lower chambers. After 48 h, cells on the lower surface were fixed with 4% paraformaldehyde, stained with 0.1% crystal violet, and counted under an inverted microscope.

### 2.6 Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from tissues and cells using TRIzol (15,596,026, Invitrogen). A High Capacity RNA-to-cDNA Kit (Applied Biosystems) was used to synthesize the first strand of cDNA. The total cDNA was used as starting materials for RT-PCR with SYBR Green PCR Master Mix (Yeasen, Shanghai, China). All primer sequences are listed in Supplementary Table S1. Conditions for detecting mRNAs were used: 95°C for 10 min, 40 cycles of 95°C for 15 s, and 60°C for 1 min. All target gene transcripts were normalized to GAPDH using the  $2^{-\Delta\Delta\text{CT}}$  method.

### 2.7 Western blotting analysis

The tissues and cultured cells were homogenized with lysis buffer with protease inhibitors. Proteins were separated by SDS-PAGE on a 10% gel and were transferred to nitrocellulose (NC) membranes. After blocking with 5% nonfat milk, the membranes were incubated with primary antibodies against WT1 (ab89901, Abcam), E-cadherin (ab1416, Abcam), Vimentin (sc-6260, Santa Cruz Biotechnology), p-AKT (4060, CST), AKT (4691, CST), p-RPS6 (Ser235/236) (2211,CST), RPS6 (2217,CST), p-ERK1/2 (Thr202/204) (4370, CST), ERK1/2 (4695, CST), and anti-GAPDH (sc-47,724, Santa Cruz Biotechnology) overnight at 4°C, followed by secondary antibodies for 2 h at room temperature. The protein bands were visualized in the membranes using an Enhanced Chemiluminescence Detection Kit (Pierce Biotechnology, USA), and Image J software (National Institutes of Health, USA) was used to measure the band intensities. GAPDH was used as the internal control.

## 2.8. Flow cytometry analysis

Cells were digested by 0.25% trypsin-EDTA, harvested and fixed in 70% ethanol overnight at 4°C. Then, cells were resuspended in PBS, followed by incubation with propidium (PI), RNase A and staining solution (Cat# 40,310, Yeasen, Shanghai, China) in the dark. After filtration through 37 µm mesh, 10,000 cells were analyzed by flow cytometry (BD FACSVerser, BD Company) with ModFit software (Verity Software House, Inc.).

## 2.9 Luciferase reporter assay

The -178 bp to +92 bp of *E-cadherin* promoter fragment cloned in pGL3 vector was gifted by Prof. Antonio García de Herreros (Institut Municipal d'Investigació Mèdica, Universitat Pompeu Fabra, Spain). The *E-cadherin* promoter with the mutated binding site (GGTGGG at -146 bp was mutated to TTTGTT) was cloned into the luciferase reporter vector and named mutant. SKOV3 cells were seeded into 12-well plates and cotransfected with pMCV3-WT1, pMCV3 as control, and luciferase reporter vectors using Lipofectamine 2000 (Invitrogen). After 48 hours, cells were harvested and the luciferase activity was measured by the dual-luciferase reporter assay system (Cat#: E2920, Promega).

## 2.10 Chromatin immunoprecipitation (ChIP)

Cells were crosslinked with 1% formaldehyde for 10 min at 37°C and then quenched in 125 mM glycine on ice for 5 min. Cell lysates were sonicated under conditions yielding fragments ranging from 200 bp to 1000 bp. The material was clarified, and pre-cleared with protein A-agarose beads. The supernatant was used in immunoprecipitation reactions with antibodies against WT1, or rabbit IgG (Upstate 12-370) as a control. Eluted chromatin was amplified using specific *E-cadherin* primers (-180 Forward AACTCCAGGCTAGAGGGTCA; +31 Reverse TCACAGGTGCTTTGCAGTTC) that flanked three potential *WT1* binding sites in the promoter region of *E-cadherin*. All ChIP experiments were repeated three times with different chromatin preparations.

## 2.11 Animal xenograft tumor model

Female BALB/c nude mice (6 wk old) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). All animal experiments were conducted according to the standard institutional guidelines of Nanjing Medical University (Nanjing, China), and the current experiments were approved by the Use Committee for Animal Care. *WT1*-depleted SKOV3 cells or control cells ( $3 \times 10^7$ ) in 0.1 mL Matrigel (1:1) were injected subcutaneously into the right upper flank of the nude mice. Sixteen mice were randomly divided into two groups. Eight mice were used for *WT1*-depleted cell injection and eight for control cell injection. The tumor volume in mm<sup>3</sup> was calculated as (length × width<sup>2</sup>) / 2. Mice were sacrificed 8 wk post cell injection and tumors were isolated for western blot.

## 2.12 Hematoxylin-eosin staining

The mouse samples were obtained and fixed in 10% formalin for 24 h at room temperature. Then, the samples were embedded in paraffin and stained with hematoxylin-eosin (H&E) staining to obtain histological samples of the tumor. Five random areas of interest were examined in each section and images were obtained using a microscope.

## 2.11 Statistical analysis

All statistical analysis was performed with SPSS 11.0. Statistical data were presented as mean ± SD and compared using Student's t test or one-way ANOVA. The comparison of categorical data was done using Chi-square test.  $P < 0.05$  was considered statistically significant.

## 3. Results

### 3.1 *WT1* expression is upregulated in OC tissues and negatively related to *E-cadherin* expression

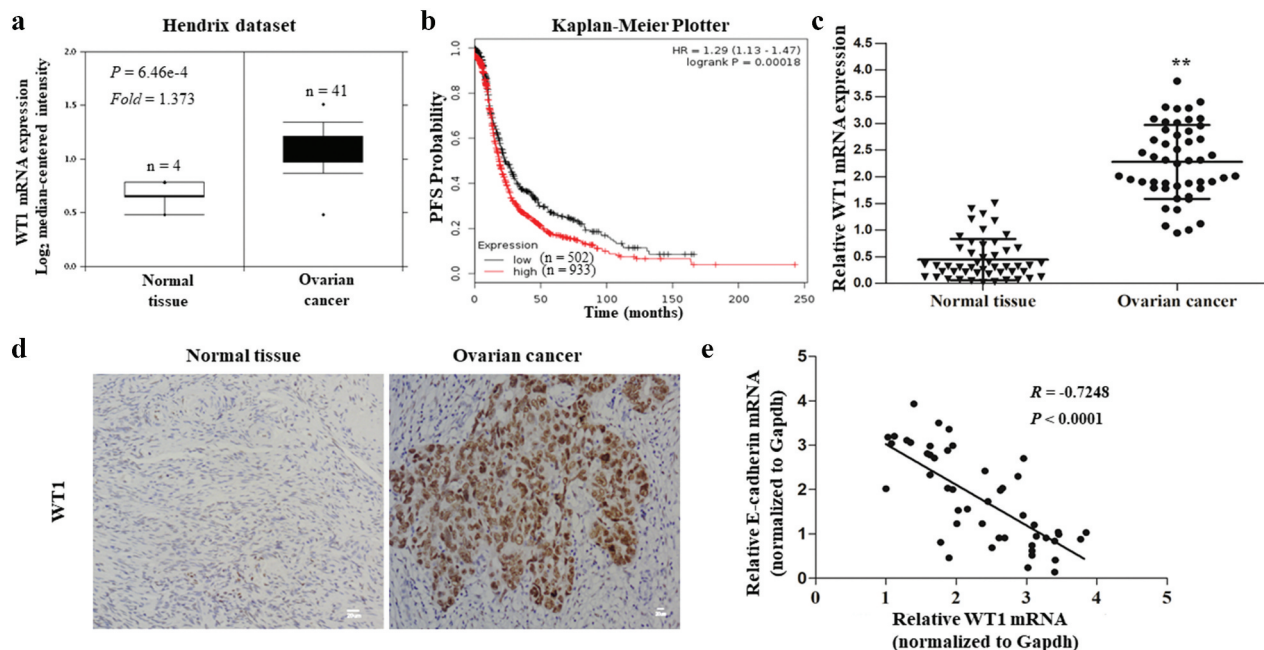
To determine the clinical significance of *WT1* in OC, we first examined *WT1* mRNA levels in human OC using Oncomine cancer microarray database [23] (<https://www.oncomine.org/resource>). According to the Hendrix dataset

( $P$  value =  $6.46 \times 10^{-4}$ , fold change = 1.373), *WT1* transcript levels were significantly elevated in clinical human OC tissues compared to the normal tissues (Figure 1(a)). We next assessed the effect of *WT1* on the survival of patients with OC in the Kaplan–Meier Plotter database [24] (<http://www.kmplot.com/>) and observed that *WT1* expression was closely associated with the progression-free survival of patients with OC [Log-rank  $P = 0.00018$ , HR = 1.29 (1.13–1.47)] (Figure 1(b)). Then, a panel of 49-paired tissue specimens of OC and adjacent normal tissues were tested by qRT-PCR and immunohistochemistry. We found that *WT1* mRNA expression was upregulated in OC tissues (Figure 1(c)) and *WT1* staining was positive in 33 out of 49 OC tumor sections, while none of the 49 adjacent normal tissue sections stained positive for *WT1* (representative images shown in Figure 1(d)). Moreover, we found patients with high *WT1* expression also had low expression of E-cadherin; thus, a linear correlation test was performed using the mRNA expression of *WT1* and E-cadherin, showing *WT1* expression was negatively related to E-cadherin expression

( $R = -0.7248$ ,  $P < 0.0001$ ) (Figure 1(e)). In addition, we further assessed the relationship between *WT1* mRNA expression and clinicopathological features in the 49 patients. As shown in Table 1, the *WT1* expression level was higher in serous OC tumors than in non-serous histology (ovarian mucinous cystadenocarcinoma, ovarian clear cell carcinoma, and ovarian endometrioid carcinomas). The expression of *WT1* was also higher in tumors at an advanced FIGO stage and high grade. These data confirm that *WT1* is upregulated in human OC and may play an important role in the progression of OC.

### 3.2 Knockdown of *WT1* may not affect OC cell proliferation

To strengthen the hypothesis that *WT1* is primarily expressed in human OC cells, we compared *WT1* expression levels in OC cell lines (SKOV3, A2780, HO8910 and HO8910PM) and normal human ovarian cell line (IOSE386) by using qRT-PCR and western blot assays. We found that the expression of *WT1* mRNA and protein was similar

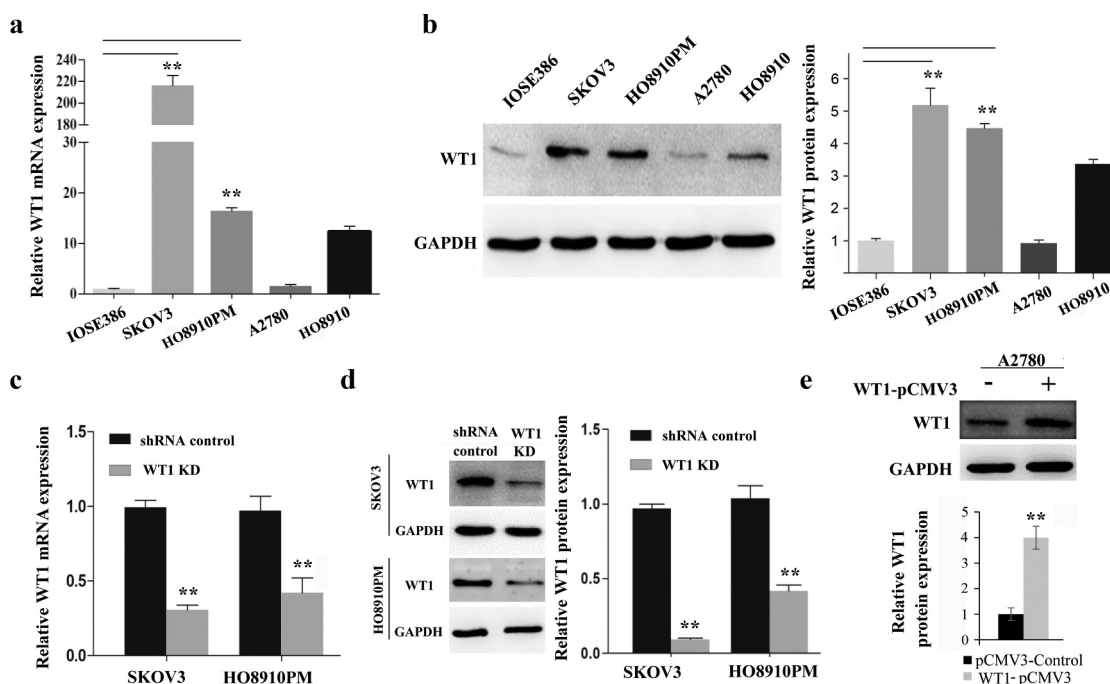


**Figure 1.** Clinical significance of *WT1* in human ovarian cancer. A, Box plots of *WT1* mRNA levels in OC tissues and normal ovarian tissues in the Oncomine cancer microarray database. B, Kaplan–Meier curves of progression-free survival (PFS) probability in OC patients with high levels of *WT1* expression (red) as compared to those with low *WT1* expression levels (black). C, The mRNA expression of *WT1* in OC tissues and their matched non-tumor counterparts in the 49 patients. \*\*  $P < 0.01$ . D, Representative images of immunohistochemistry staining for *WT1* in OC tissues and their paired non-tumor tissues. Magnification  $\times 10$ . Bar = 20  $\mu\text{m}$ . E, A linear correlation study was performed between mRNA expression of *WT1* vs. *E-cadherin* in OC tissues of the 49 patients.

in IOSE386 and A2780 cells, but significantly higher in SKOV3 and HO8910PM cells (Figure 2(a,b)) (Note: HO8910PM OC cells are more metastatic than the parental H8910 human OC cells) [25,26]. Considering 93.22% of the contaminants in cell lines established in laboratories of China were HeLa cells or a possible hybrid of HeLa with an unknown cell line [27], the HPV18 E6 expression was examined in OC cell lines HO8910PM, SKOV3 and A2780, compared with HeLa cells using qRT-PCR. As shown in Supplementary Figure S1, these OC cells did not express HPV18 E6, indicating our cell lines can be used for the following studies. To study the role of *WT1* in OC carcinogenesis, we then generated *WT1*-depleted SKOV3 and HO8910PM cell lines using a lentiviral approach. Among the four LV3-*WT1* shRNAs tested in SKOV3 cells, LV3-*WT1*-#4 construct showed the most significant inhibitory effect on *WT1* expression and therefore was selected for further analysis (Supplementary Figure S2). The *WT1* knockdown efficiency was confirmed via

qRT-PCR and western blotting in both SKOV3 and HO8910PM cells ( $P < 0.01$ ) (Figure 2(c,d)). Conversely, to evaluate the effect of *WT1* overexpression on OC, we transfected A2780 cells with pCMV3 or pCMV3-*WT1* vector and showed an increase of *WT1* expression by western blotting (Figure 2(e)).

To test whether *WT1* affects OC cell proliferation, we first performed the cell cycle analysis by flow cytometry. Results showed no significant change in the percentage of cells in the G0/G1 phase and G2/M phase after *WT1* knockdown on SKOV3 and HO8910PM cells in contrast with their controls (Supplementary Figure S3A, B). Then, the fold increase in cell proliferation was tested using CCK-8at 24-h intervals. As shown in Supplementary Figure S3C, *WT1* depletion as well as *WT1* overexpression did not show a significantly different growth rate compared with the controls for up to 3 d. Taken together, these results reveal that *WT1* may not affect the proliferation of OC cells.



**Figure 2.** *WT1* knockdown and overexpression in ovarian cancer cell lines. (a,b) *WT1* expression in four ovarian cancer cell lines (SKOV3, A2780, HO8910, HO8910PM) and normal human ovarian cell line (IOSE386) was examined using qRT-PCR and western blot assay. GAPDH used as loading control. \*\*  $P < 0.01$  represents difference in SKOV3 and HO8910PM cell lines compared with IOSE386, respectively. (c,d) The knockdown efficiency of *WT1* was detected by qRT-PCR and western blotting in SKOV3 and HO8910PM cells. GAPDH served as loading control. (e) The overexpression efficiency of *WT1* was examined by western blotting in A2780 cells. The graph on the right of figures B and D, or lower panel of figure E showed the quantification of the results. The data were presented as the mean  $\pm$  SD from three experimental replicates. \*\* $P < 0.01$  compared with the compared group.

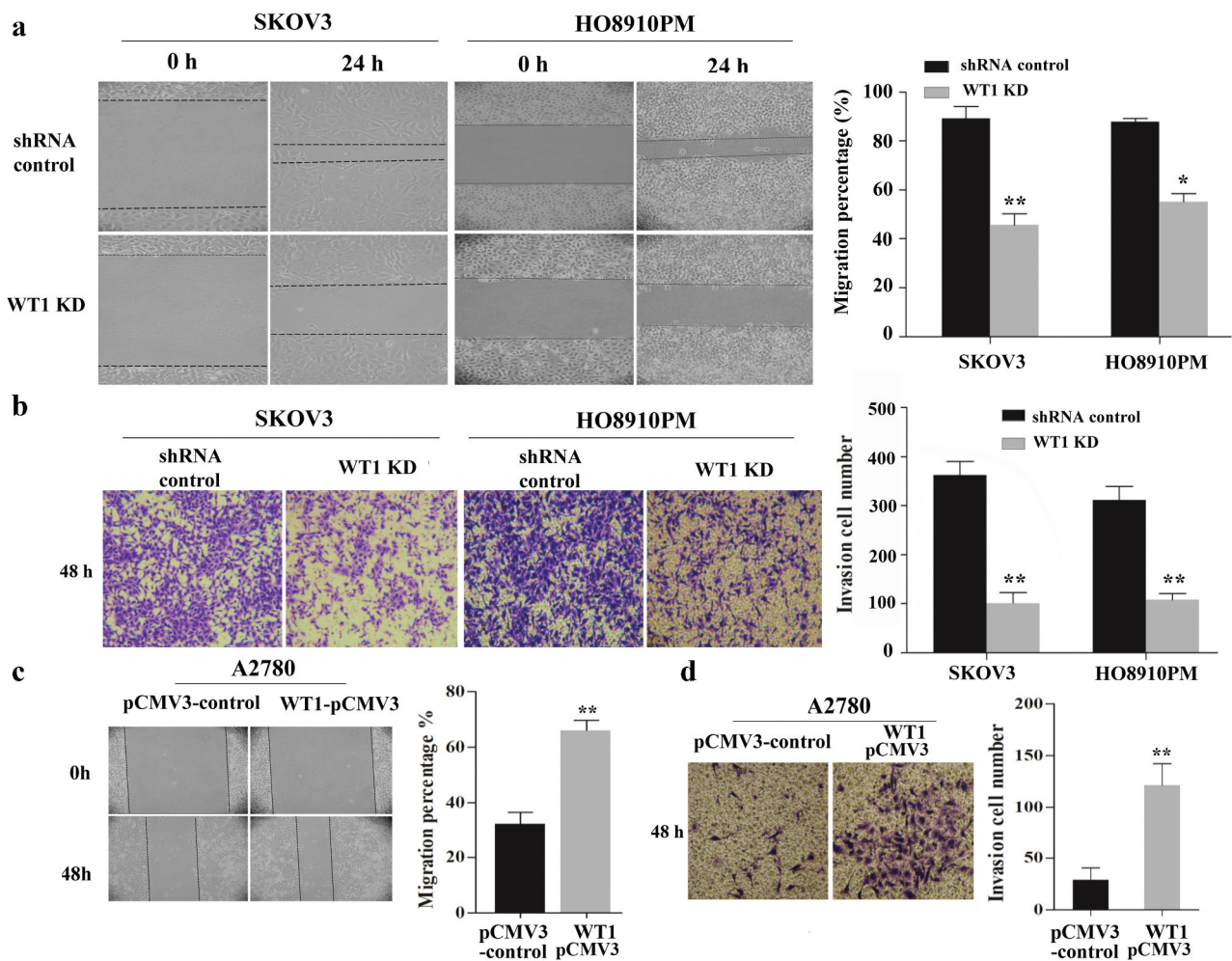
### 3.3 WT1 promotes OC cell migration and invasion

We next explored the function of *WT1* in OC cell migration and invasion. The effect of *WT1* knockdown on OC cell migration was tested using wound-healing assay. Results showed that *WT1* depletion significantly reduced the ability of SKOV3 and HO8910PM cells to migrate into the wound compared to the controls (Figure 3(a)). Then, we performed transwell invasion assay across a Matrigel membrane and found *WT1* knockdown significantly weakened cell invasion capabilities of SKOV3 and HO8910PM cells compared with their controls (Figure 3(b)). Moreover, we examined the effect of *WT1* overexpression on OC cell migration and invasion. The wound

healing and transwell assays demonstrated that exogenous expression of *WT1* markedly enhanced the migration and invasion capacity of A2780 cells compared with the controls (Figure 3(c,d)). These data indicate *WT1* may promote OC cell invasion and migration.

### 3.4 Silencing *WT1* reverses EMT via upregulation of E-cadherin in OC cells

EMT is a biological phenomenon in which cells lose the characteristics of epithelial cells and acquire the characteristics of mesenchymal cells under certain conditions, and is closely related to *in situ* infiltration and distant metastasis of multiple tumors [17,18]. To identify whether *WT1* can



**Figure 3.** *WT1* enhances cell migration and invasion in ovarian cancer. (a,c) Representative images of wound-healing assay with *WT1* knockdown in SKOV3 and HO8910PM cells or *WT1* overexpression in A2780 cells. (b,d) Transwell assay showing that the invasive ability of SKOV3 and HO8910PM cells was significantly suppressed after *WT1* silencing and *WT1* overexpression promoted the invasion capacity of A2780 cells. The right panel showed the quantitative results. The data were presented as the mean  $\pm$  SD from three experimental replicates. \* $P < 0.05$ , \*\* $P < 0.01$ .

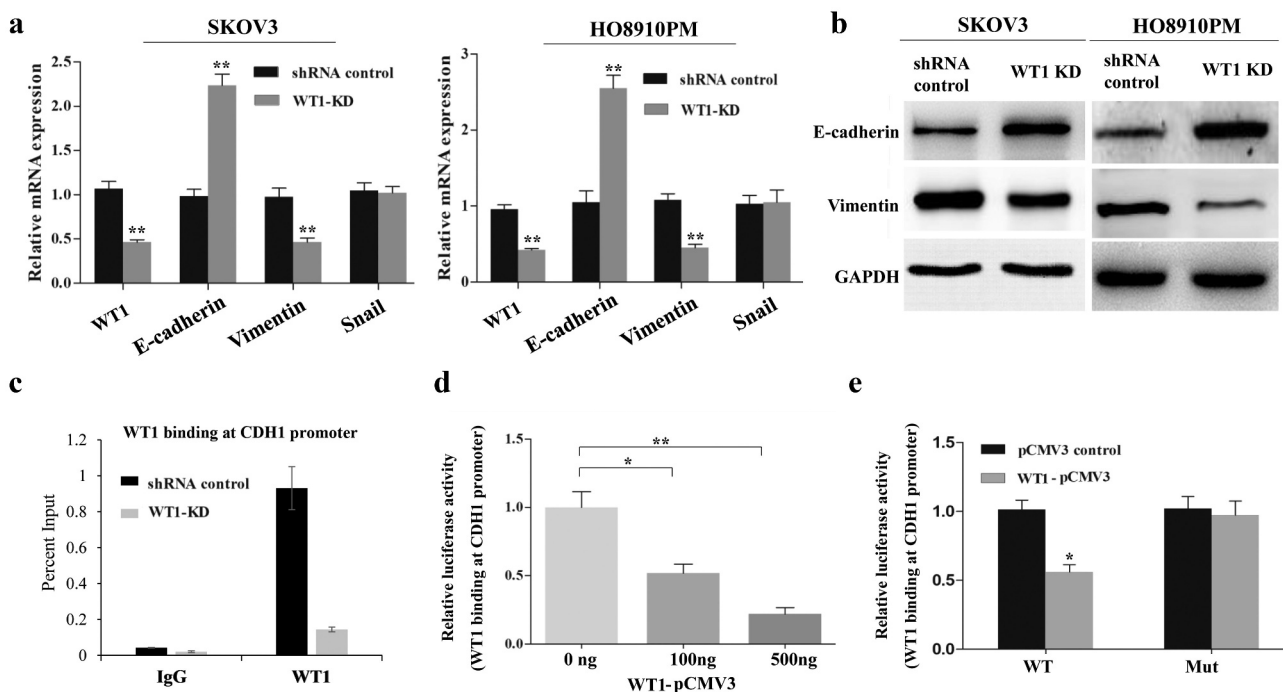
promote OC cell EMT, we evaluated the expression of E-cadherin (an epithelial marker) and Vimentin (a mesenchymal marker) in SKOV3 and HO8910PM cells by qRT-PCR and western blot analyses. As shown in Figure 4(a,b), *WT1* silencing resulted in an increase in the expression of E-cadherin and a decrease in Vimentin at both mRNA and protein levels. We also found decreased *WT1* slightly inhibited the expression of ZEB1 and Twist, while no difference was observed in Snail expression after *WT1* knock-down (Supplementary Figure S4).

Previous studies in prostate cancer cells have shown that *WT1* can target *E-cadherin* proximal promoter at -146 bp upstream of the transcription start site and mediate transcriptional repression of *E-cadherin* [20]. To confirm that *WT1* could also target this proximal promoter region in OC cells, we performed *WT1* ChIP in SKOV3 cells. Results showed endogenous *WT1* to be enriched at the proximal promoter region of *E-cadherin* gene

(Figure 4(c)). To further determine whether *WT1* transcriptionally regulates *E-cadherin* promoter activity in SKOV3 cells, the wild type or mutated (*WT1* binding site at -146bp) *E-cadherin* reporter construct was co-transfected along with increasing doses of pCMV3-*WT1* in SKOV3 cells. The following luciferase reporter assay showed that *WT1* overexpression significantly inhibited luciferase activity of the construct containing the wild type *E-cadherin* promoter, but not that of mutated one (Figure 4(d,e)), indicating that *WT1* could promote EMT by downregulation of *E-cadherin* via direct binding to its promoter in OC cells.

### 3.5 *WT1* may suppresses *E-cadherin* expression via activation of ERK signaling

Accumulated evidences have confirmed that *E-cadherin* expression can be down-regulated in cancer cells in response to extracellular stimuli through activation of the AKT/mTOR and MEK/ERK

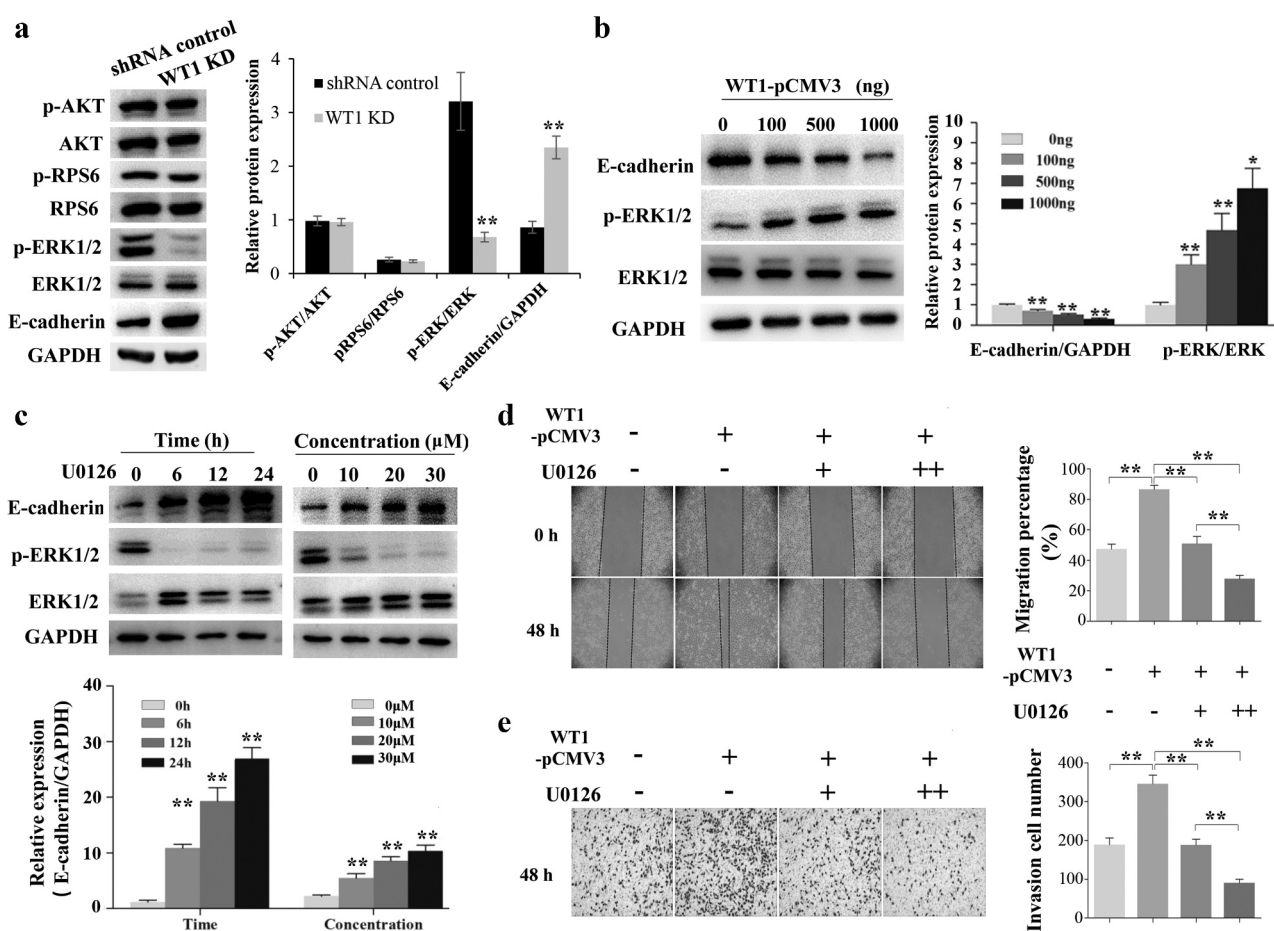


**Figure 4.** Silencing *WT1* reverses EMT in ovarian cancer cells and correlates with an increase in *E-cadherin* expression. (a,b) qRT-PCR and western blot were used to detect changes in the mRNA and protein expression of EMT-associated genes in *WT1* knockdown SKOV3 and HO8910PM cells. (c) ChIP-qPCR analysis of *WT1* binding at *E-cadherin* proximal promoter region at the -146 bp upstream of the transcription start site in SKOV3 cells. Data were normalized to the percent of Input. (d) Luciferase activity of reporter construct carrying *E-cadherin* promoter in SKOV3 cells in the presence of indicated amounts of *WT1* expression vector to test the effect of *WT1* on *E-cadherin* promoter. E, Luciferase activity of wild type or mutant *E-cadherin* proximal promoter containing a mutated -146 *WT1* binding site with pCMV3-*WT1* or pCMV3. Luciferase activity was measured and normalized to protein concentration. Data are reported relative to luciferase activity of pCMV3 transfected cells. The data were presented as the mean  $\pm$  SD from three experimental replicates. \* indicated  $P < 0.05$ ; \*\* indicated  $P < 0.01$ .



signaling pathways [28,29]. In the present study, we explored whether WT1 could regulate the EMT of OC cells via AKT and ERK signaling pathways. As shown in Figure 5(a), WT1 knockdown did not affect the activation of AKT, or RPS6 in SKOV3 cells. However, silencing WT1 led to a significant decrease of ERK1/2 phosphorylation. Moreover, overexpression of WT1 could promote the phosphorylation level of ERK1/2 and decrease the E-cadherin expression in a dose-dependent manner (Figure 5(b)). To further explore the potential role of WT1 in ERK1/2 regulated E-cadherin downregulation, we treated WT1-overexpressed A2780 cells with the MEK1/2 inhibitor

U0126 and then tested the E-cadherin expression levels. Results showed that U0126 inhibited ERK1/2 phosphorylation and upregulated E-cadherin expression (Figure 5(c)), suggesting that WT1 may also regulate E-cadherin expression via ERK1/2 signaling. We next treated WT1-overexpressed A2780 cells with U0126 and then tested cell migration and invasion. Results showed that inhibiting ERK1/2 phosphorylation significantly decreased WT1-induced OC migration and invasion (Figure 5(d,e)). Collectively, these results demonstrate that WT1 may also have the ability to promote EMT via activation of ERK1/2 signaling in OC cells.



**Figure 5.** WT1 promotes EMT through ERK1/2 phosphorylation. (a) Western blotting to detect p-Akt, Akt, p-RPS6, RPS6, p-ERK1/2, ERK1/2 and E-cadherin expression in WT1 knockdown and shRNA control SKOV3 cells. (b) Overexpression of WT1 obviously changed the expression levels of E-cadherin and p-ERK1/2 in a dose-dependent manner. (c) The protein expression of E-cadherin and p-ERK1/2 was determined in WT1-overexpressed A2780 cells treated with U0126. (d,f) Wound healing and transwell assays were performed to detect the migration and invasion capacity of A2780 cells treated with pCMV3 vector and different concentrations (0  $\mu$ M, 10  $\mu$ M, 20  $\mu$ M) of U0126 after the *de novo* expression of WT1 separately. The panel on the right of figures A, B, D, E or lower panel of figure C showed the quantification of the results. The data were presented as the mean  $\pm$ SD from three experimental replicates. \* $P < 0.05$ ; \*\*  $P < 0.01$ .

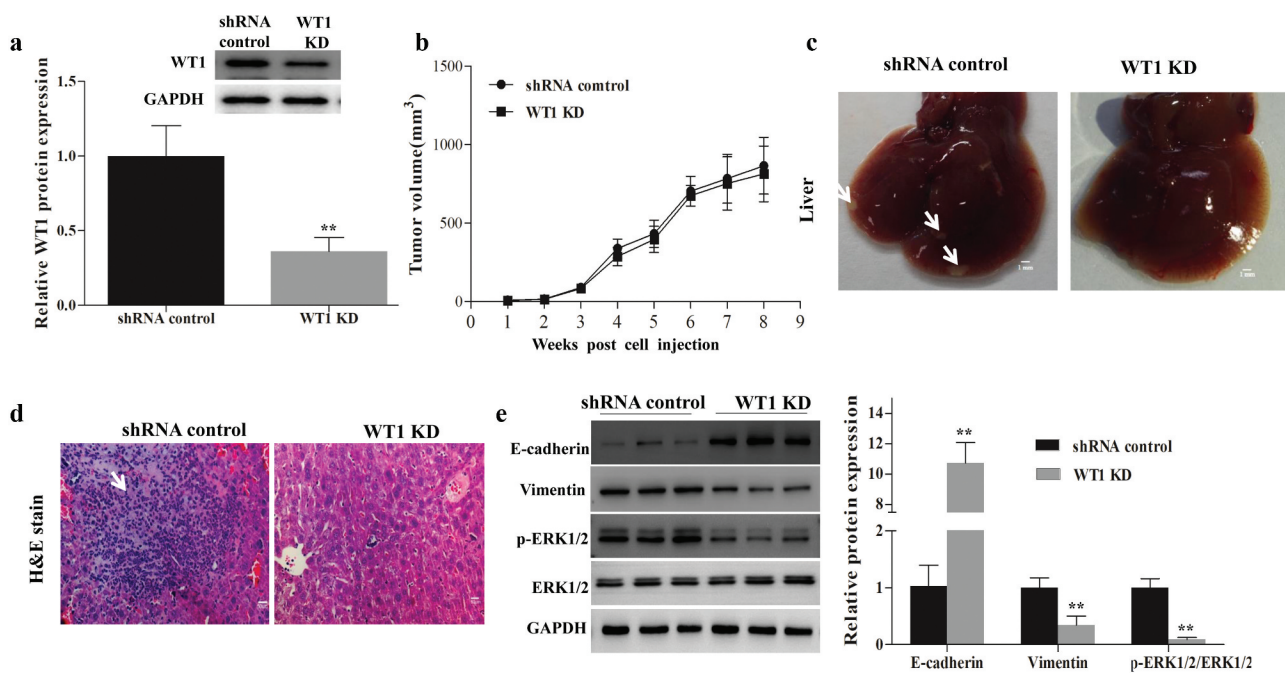
### 3.6 WT1 is essential for tumor metastasis of OC in mice

To investigate the role of *WT1* in promoting migration and invasion of OC cells, an *in vivo* assay was performed by injecting *WT1* knock-down or shRNA control tumor cells subcutaneously into the right flank of nude mice, separately. First, we confirmed the knockdown efficiency of *WT1* in established tumors by western blotting. As expected, the tumors in mice inoculated with *WT1*-depleted cell lines had diminished expression of *WT1* (Figure 6(a)). Next, the tumor volume of each group was determined, and no significant difference was found (Figure 6(b)). However, the tumor metastases in livers with small hepatic nodules (white arrow) spreading throughout the liver surface, developed in 5 out of 8 mice (62.5%) injected with the control SKOV3 cells, whereas no such metastasis was observed in 8 mice (0%) injected with *WT1* KD cells (Figure 6(c)). Histological analysis on the livers confirmed the metastasis foci in the shRNA

control tumors (Figure 6(d)). Moreover, to test whether the difference of tumor metastasis is dependent on EMT and ERK1/2 activity, we compared the expression of EMT-related indicators and ERK1/2 activation in the xenograft mouse tumor samples. Results showed that the tumors from the mice inoculated with *WT1* KD cell lines had an up-regulated level of E-cadherin and diminished expression of Vimentin and p-ERK1/2 (Figure 6(e)), suggesting that *WT1*-ERK1/2-EMT pathway may also be involved in the regulation of metastatic process of OC cells *in vivo*.

## 4. Discussion

Numerous studies report that *WT1* exerts an oncogenic role rather than a tumor-suppressive role in the development and progression of different types of cancers [30]. For instance, higher *WT1* expression is associated with higher histological stage and worse prognosis in breast cancer [31], or advanced tumor stage of head and neck



**Figure 6.** *WT1* depletion reduces SKOV3 cells metastasis *in vivo*. (a) Western blotting was performed to confirm the knockdown efficiency of *WT1* in established tumors. (b) Tumor growth curves were summarized in the line chart. (c) Image of metastatic liver nodules spreading throughout the live tissues in mice injected with shRNA control SKOV3 cells. *WT1* knockdown clearly abolished the metastasis. White arrows indicated tumor foci. Bar = 1 mm. (d) Hematoxylin and eosin (H&E) staining was used to visualize the metastatic nodules in tumors from mice injected with shRNA control SKOV3 cells. The arrow indicated tumor foci. Magnification x 20. Bar = 50  $\mu$ m. E, Western blot was performed to detect changes in the protein expression of EMT-associated genes proteins and ERK1/2 activation in the xenograft mouse primary tumors from the mice inoculated with *WT1* KD cells compared to the controls. The lower panel of figure A or the panel on the right of figure E showed the quantification of the results. \*\*  $P < 0.01$ .

squamous cell carcinoma [32]. In the current study, we found WT1 was highly expressed in OC tissues compared with non-tumor ovaries. We also noticed WT1 expression in OC was more associated with adverse prognostic factors including advanced stage, high grade and progression-free survival. These findings suggest that WT1 may be involved in regulating OC progression. Then, we investigated the role of *WT1* on OC cell malignant behavior and found that the depletion of *WT1* suppressed the ability of OC cells (SKOV3 and HO8910PM) to migrate and invade *in vitro*, while overexpression of WT1 promoted the invasion and migration of OC cell line A2780. Besides, *WT1* knockdown also suppressed the ability of SKOV3 tumors to metastasize *in vivo*. These findings suggest that WT1 may promote tumor metastasis in OC. It is also worth noting that WT1 expression is characteristic of the serous phenotype, especially high-grade serous ovarian cancer (HGSOC), with much lesser degrees of staining of other morphological subtypes of ovarian carcinoma [33,34]. Given that various histological types of OC cause different biological behavior, and that OC cell lines used in this study (e.g. SKOV3, A2780) do not represent a good approximation of the HGSOC genotype [35], we will further explore the mechanisms of *WT1* on different subtypes of OC with representative cell models in future studies.

Apart from the analysis in aspects of cell invasiveness and migration capability, our investigation also focused on the influence of *WT1* on EMT. Recent researches have displayed that *WT1* is implicated in the regulation of mesenchymal cell maintenance and EMT during the development of mesodermal tissues [36,37]. Downregulation of E-cadherin is thought to promote metastasis during early carcinogenesis progression [38] and has been reported to occur by many transcription factors such as *Snail*, *WT1* and *ZEB1/2* [19,39]. Many studies have characterized the relationship between *WT1* and its downstream molecule E-cadherin. *WT1* controlled epicardial EMT and the subsequent generation of vasculature progenitors through direct transcriptional regulation of *Snail* and *E-cadherin*, two of the major mediators of EMT [19]. *WT1* transcriptionally repressing E-cadherin in prostate cancer cells may contribute

to migration and metastasis [20]. WT1 expression was correlated with clinical stage, metastasis, and survival rate in non-small-cell lung cancer patients by suppressing E-cadherin via direct binding to the promoter [21]. Here, we provided evidence demonstrating, in OC cells, WT1's functions on EMT are also through transcriptionally repressing E-cadherin expression via targeting its proximal promoter region. Our study indicated that direct suppression of E-cadherin by *WT1* may demonstrate an evident link between EMT and the oncogenic function of *WT1*, enhancing migration and metastasis of OC cells. Though Vimentin expression is also affected upon *WT1* depletion in OC cells, we did not observe the direct binding of *WT1* on *Vimentin* promoter. It could be the reason, to the best of our knowledge, that *Vimentin* promoter does not contain *WT1* binding site. Alternatively, *Vimentin* promoter contains an NF- $\kappa$ B site, a PEA3-binding site, tandem AP-1 binding sites, and a  $\beta$ -catenin/TCF binding site [39–41]. *WT1* may indirectly target to these regions through interacting with such transcription factors. We will test these hypotheses in our future studies.

*WT1* also appeared to be involved in cell signaling and has different effects on the activation of the PI3K/AKT and ERK1/2 pathways in cancer cells. Graziano *et al.* found *WT1* could act as a tumor promoter in osteosarcoma via PI3K/AKT pathway. *WT1* was also involved in the autophagy in osteosarcoma cells and proliferation of malignant peripheral nerve sheath tumors through the PI3K/AKT pathway [42,43]. Moreover, accumulative evidence showed that *WT1* could mediate the development of Wilms' tumors and the activation of the *KRAS* proto-oncogene through ERK1/2 phosphorylation [44–46]. In the present study, we found *WT1* did not affect the activation of AKT, but significantly modulate ERK1/2 activation in OC cells. Inhibiting ERK1/2 phosphorylation could upregulate E-cadherin expression and reverse *WT1*-induced migration and invasion of OC cells. Our data indicated *WT1* may also enhance OC cell migration and invasion through activating ERK1/2 signaling. Previous ChIP-DNA selection and ligation (DSL) experiment identified a cohort of genes whose promoters were targeted by *WT1* and showed these *WT1*-bound targets were enriched in

cell apoptosis, Wnt signaling pathway, MAPK signaling, cell migration, cell proliferation and cell adhesion [47]. Possibly, *WT1* may activate ERK1/2 signaling by regulation of transcription factors, such as *sprouty1* [48,49]. Further studies aimed at the identification of additional *WT1* co-factors, such as those that regulate the ERK1/2 signaling, will help explain the full range of *WT1* activity in OC metastasis.

In summary, the present study explored the link between *WT1* and EMT and their potential roles in OC metastasis. We provided here that *WT1* promoted migration, invasion and EMT in OC cells. Mechanistically, we showed that *WT1* acted as an oncogene in the progression of OC via negative modulation of E-cadherin by directly binding to its promoter and promoting EMT process through indirect downregulation of E-cadherin via activating ERK1/2 pathway. Our studies may help establish *WT1* as a good target for therapeutic intervention in advanced ovarian cancer treatment.

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## Disclosure statement

The authors declare no conflict of interest.

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