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ORIGINAL RESEARCH

WFDC2 contributes to epithelial–mesenchymal transition (EMT) by activating AKT signaling pathway and regulating MMP-2 expression

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Objective: To understand the role of *WFDC2* in metastasis of ovarian cancer.
Methods: By knockdown or overexpression of *WFDC2*, we demonstrated the role of *WFDC2* in epithelial–mesenchymal transition (EMT).
Results: We demonstrated that stable knockdown of *WFDC2* suppressed EMT along with the upregulation of E-cadherin and the downregulation of Vimentin. In addition, *WFDC2* knockdown decreases matrix metalloproteinase-2 (MMP-2) expression in in vitro cell model and in in vivo nude mice xenografts. The correlation of *WFDC2* and MMP-2 expression in the clinical sample confirmed that *WFDC2* was tightly correlated with the development of tumor. More importantly, the EMT phenotype and cell invasion induced by *WFDC2* overexpressing can be reversed by the siMMP-2 and P13K/AKT signaling inhibitor.
Conclusion: *WFDC2* contributed to ovarian cancer metastasis and EMT as a positive regulator by activating AKT signaling pathway and inducing MMP-2 expression.
Keywords: WFCD2, ovarian cancer, metastasis, cell migration and invasion, epithelial-mesenchymal transition

Background

The expanding knowledge of cancer biology has led to improved understanding of the molecular mechanisms of ovarian cancer and advancement of targeted therapeutics. *WFDC2* (WAP four-disulfide core domain protein 2), which encodes human epididymis secretory protein 4 (HE4), had been followed with interest as a new serine protease inhibitor belonging to the WAP family. While WAP-type proteins had been identified to be closely related to tumor metastasis by more and more evidence, especially SLPI and P13 (encode secretory leukocyte protease inhibitor[SLPI] and Elafin, respectively).[1–4] Our former work had shown that *WFDC2* is a survival factor for ovarian cancer and its increased expression is associated with malignant and metastasis advantages both in vitro and in vivo.[1,5,21] Otherwise, its physiological and pathological mechanisms in tumorigenesis and metastasis have not been elucidated.

Epithelial–mesenchymal transition (EMT) is a critical process in the metastatic cascade, which is characterized by a fundamental change in cellular morphology and phenotype with increased ability to migrate.[6,7] In recent years, several studies have reported the role of EMT in cancer malignancy.[6,7] and it has been established as a key regulator in many types of cancers including ovarian cancer.[8] However, the mechanisms of EMT regulation in ovarian cancer are still unclear.

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In our former study, we had observed the expression of apoptosis and metastasis-related gene expression in *WFDC2* knockdown cells.[5] To be interesting, *WFDC2* knockdown decreased the expression of matrix metalloproteinase-2 (MMP-2) expression in ovarian cancer cells. As all known, tumor metastasis occurs secondary to tumor cell adhesion, migration, and proteolytic degradation of the extracellular matrix (ECM). MMPs, especially MMP-2 and MMP-9, are prognostic for metastatic potential and outcome in ovarian cancer.[11–13] MMPs also are essential factors of selectively modulating the tumor microenvironment to promote tumor cell metastasis and is considered as an inducer of EMT.[14–17] Bouchad et al reported that *WFDC2* is co-expressed with other WAP structure genes (SLPI and Elafin) on chromosome 20q12-13.1 locus.[18] Hoskins et al had reported that SLPI secretion upregulates MMP-9 transcription and secretion in ovarian cancer cells.[19] Choi also showed that SLPI is associated with MMP-2 and MMP-9 to promote migration and invasion in cancer cells.[20] In view of the above information, we speculated that *WFDC2* may play some role in the ovarian cancer microenvironment rebuilding and promote cell EMT and invasive behavior by regulating MMP-2 expression.

In the present study, we would identify whether *WFDC2* overexpression induced ovarian cancer cell lines to undergo EMT and promoted cell migration and invasion through regulating MMP-2 expression, making *WFDC2* a potential target for gene therapy.

Methods

Cell line and reagents

Human ovarian cancer cell lines SKOV3, HO8910, and OVACAR8 were obtained from Shanghai Institute for Biological Sciences, Chinese Academy of Sciences. All cells were cultured in RPMI-1640 supplemented with 10% FBS and antibiotics (Gibco BRL, Rockville, MD, USA) in an atmosphere of 5% CO_2 at 37°C. The P13K/Akt inhibitor LY294002 (CST, MA, USA) was purchased commercially. Transwell system was purchased from CostarCorning (Corning, NY, USA); puromycin and trizol reagent from Invitrogen (Life Technologies, Carlsbad, CA, USA); and cell culture media (antibiotic, serum, and glutamine) from GIBCO (Life Technologies, Carlsbad, CA, USA). All other molecular reagents and solvents were purchased from SIGMA Corp (St. Louis, MO, USA).

RNA interference and overexpression transfection

WFDC2 (NM_001039348) cDNA was inserted into PcDNA3.1 vetor to construct the recombination plasimid to achieve the overexpression WFDC2 in ovarian cancer cells, while knockdown of *WFDC2* was achieved with cloning small hairpin RNAs (shRNAs) used self-inactivating lentivirus vector containing a CMV-driven GFP reporter (Genepharma Co. Ltd, Shanghai, China). The target sequence for *WFDC2* was (5′-GCTCTCTGCCCAATGATAAGG-3′) and the invalid RNAi sequence was (5′-GTTCTCCGAACGTGTCACGT-3′).[5] Small RNAs (siRNAs) against MMP-2 was also constructed by Shanghai Genepharma Co. Ltd. The results of western blotting and real-time quantitative RT-PCR further confirmed the transfection efficiency.

RNA extraction and real-time RT-PCR

Total RNA was isolated following the manufacturer's instructions (PrimeScript 1st Strand cDNA Synthesis Kit, TAKARA). The β-actin was used to evaluate the efficiency and variability of the reverse transcription step. cDNA samples (0.1 μg) were amplified under conditions recommended by the manufacturer SYBR Green PCR Master Mix (TAKARA): (a) preincubation at 95°C for 1 min; (b) 40 PCR cycles of 95°C for 10 s, and 55°C for 30 s, 70 °C for 30 s.

Western blot

Protein lysates were fractionated on SDS-polyacrylamide gels and transferred to polyvinylidene fluoride (PVDF) membrane and blocked with 5% skimmed milk in Tris-buffered saline with Tween 20. The primary antibodies *WFDC2*, MMP-2, E-cadherin, Vimentin, AKT, ERK, p-AKT, p-ERK, snail, slug, and smad3 were purchased from Cell Signaling Co. Ltd (CST, MA, USA). All antibodies (dilution 1:1,000) were incubated on shaking bed overnight at 4°C, respectively. Secondary antibody (dilution 1:1,000) was incubated at room temperature for 30 mins. Developed films were digitized by scanning, and the densitometric quantification of protein bands was performed with GAPDH as an internal control.

Cell invasion assay

Transwell polycarbonate plates with 6.5 mm diameter tissue culture inserts containing a membrane with 8 μm pores were used for migration and invasion assay (Corning, NY, USA). The cells were cultured in serum-free DMEM medium overnight before the initiation of the experiments. 1×10^5 cells were

seeded into each insert which was precoated with (for the invasion assay) Matrigel (Corning, NY, USA). The DMEM medium with 10% FBS was added to each outer well. The plates were then assembled and incubated for 18 hrs at 37°C, 5% CO_2. After an 18 hr incubation, the plates were rinsed once in PBS, fixed in 70% alcohol for 10 mins, and rinsed with 0.5% crystal violet. The number of cells was counted, and images were obtained under a microscope (100× magnification).

Immunocytochemistry (ICC)

Cells were seeded into 6-well plate with coverslips inside. After appropriate culture, the coverslips were rinsed once in PBS with tween, then fixed in 70% alcohol for 10 mins. The coverslips were incubated with E-cadherin and Vimentin antibodies with dilution rate 1:50 at 4°C for 2 hrs followed by washing with 1× PBS pH 7.4. After washing, the cells were incubated with the secondary antibody. After three washing with 1× PBS, cover slides were analyzed with fluorescence microscope at 200× and 400× magnification.

Immunohistochemistry (IHC)

Immunohistochemistry analysis was performed as previously described.[11] Anti-*WFDC2* antibody and anti-MMP-2 antibody were applied to the slides at a dilution of 1:50 in blocking buffer overnight at 4°C. The slides were then washed and stained by the avidin-biotin method. The slides were lightly counterstained with hematoxylin. The intensity was scored as negative (0), weak (1), medium (2), and strong (3), and the proportion of staining was scored as 1 (≤10%), 2 (11–50%), 3 (51–75%), and 4 (>75%). An overall expression score was calculated by multiplying the scores for intensity and proportion, ranging from 0 to 12.

Statistical analysis

The results were expressed as the mean ± SE. The statistical software (SPSS Inc., Chicago, IL, USA) was used in data processing and analyzing the significance with the one-way ANOVA or unpaired *t*-test. *P*-value <0.05 was considered statistically significant.

Results

WFDC2 knockdown suppresses the expression of MMP-2

Previous data have shown that *WFDC2* knockdown significantly attenuates migration and invasiveness of ovarian cancer cells.[5,21] MMPs, especially MMP-2 and MMP-9, can degrade the ECM to regulate cell migration and invasion.[16–21] In these studies, we detected the expression of MMPs by real-time RT-PCR. The results indicated that the expression of MMP-2 was downregulated by *WFDC2* knockdown (Figure 1A). Then by the means of Western blot and IHC, we have shown that knockdown of *WFDC2* inhibits MMP-2 expression both in vitro and in vivo. MMP-2 was obvious downregulated by *WFDC2* knockdown both in cell model and xenograft of ovarian cancer cells (Figure 1B and C). To further confirm the correlation with *WFDC2* and MMP-2, we also examined the expression of *WFDC2* and MMP-2 in clinical samples with IHC and did the correlation assay. As shown in Figure 1D, a positive correlation between MMP-2 and *WFDC2* expression was observed in 100 ovarian cancer patients (tumor characteristics for ovarian cancer patient see in Table S1 or reference).

WFDC2 promotes cell metastasis by upregulating EMT

Knockdown of *WFDC2* in HO8910 and SKOV3 cells inhibits cell migration and invasion,[5] but the mechanism remains to be studied. In the current study, we further aimed to investigate the effects of *WFDC2* on EMT, which is important in the initiation and promotion of cell migratory and invasive properties. First, the expression of EMT markers was analyzed in *WFDC2* knockdown ovarian cancer cells and the control, respectively. As shown in Figure 2A, knockdown of *WFDC2* increased the expression of E-cadherin, whereas the expression of Vimentin was downregulated. The important regulator of EMT, Slug, and Snail was also decreased by *WFCD2* knockdown. Herein, Smad3, a protein involved in TGF-β and activin-mediated growth modulation, were also decreased by *WFCD2* knockdown (Figure 2B). These data indicated that *WFCD2* is involved in EMT regulation. Moreover, immunofluorescence microscopy confirmed increased levels of E-cadherin and decreased levels of Vimentin in *WFCD2* knockdown HO8910 cells compared with control cells (Figure 2C). The further immunohistochemical analysis of xenograft tumor sections revealed an acquisition of the epithelial nature of the tumor as evidenced by increased E-cadherin expression and decreased levels of Vimentin following *WFCD2* knockdown in HO8910 cells (Figure 2D). The above results indicate a significant correlation between *WFCD2* and the expression of the biochemical markers E-cadherin and Vimentin. Taken

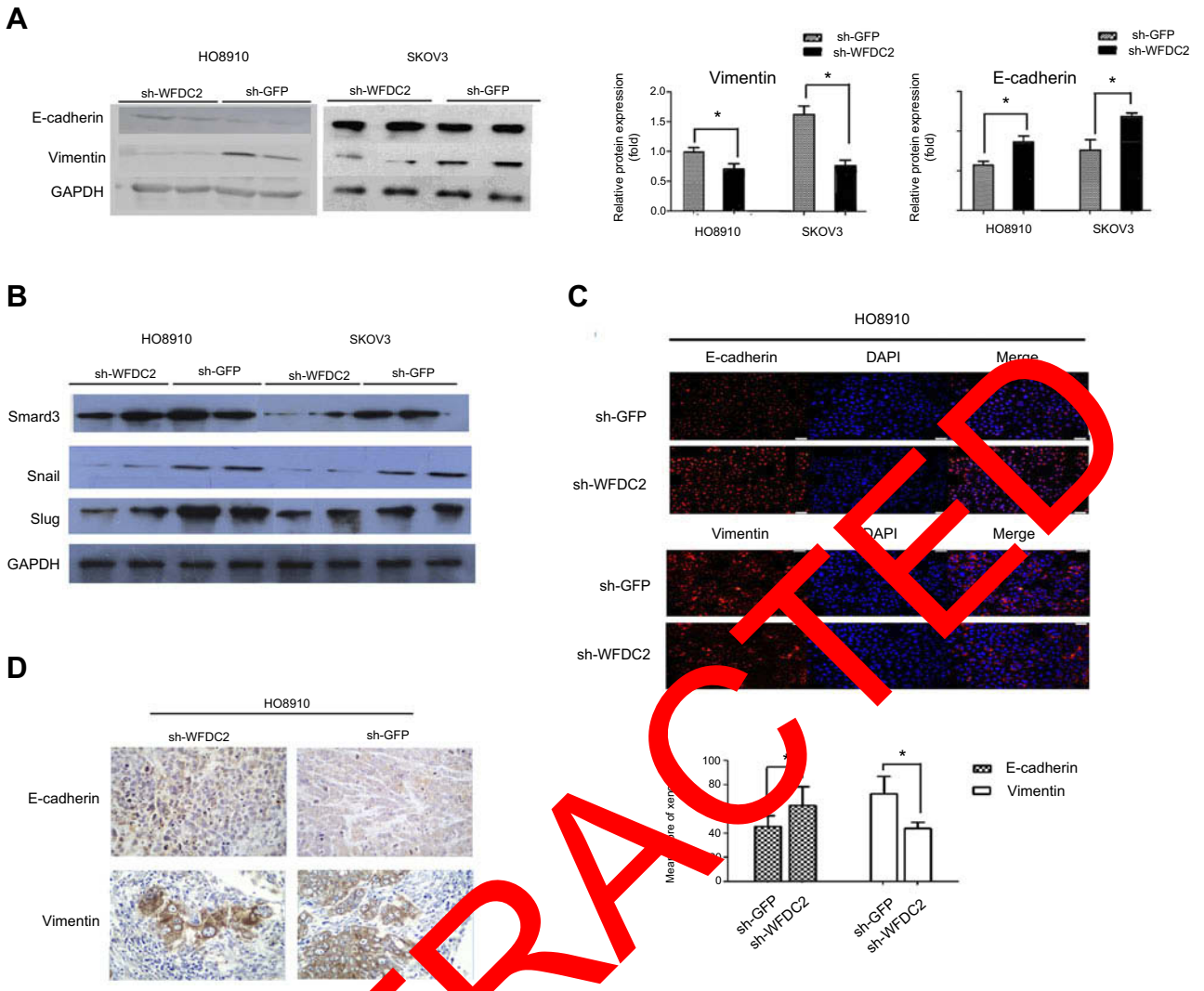

Figure 1 *WFDC2* knockdown suppressed the expression of MMP-2. (**A**) Real-time RT-PCR analysis of MMP-2 in SK-OV-3 and HO8910 cells expressing sh-GFP or sh-*WFDC2*, **P*<0.05 compared to sh-GFP group; (**B**) Western blot analysis of MMP-2 in SK-OV-3 and HO8910 cells expressing sh-GFP or sh-*WFDC2*; **P*<0.05 compared to sh-GFP group; (**C**) MMP-2 immunohistochemistry in sh-GFP and sh-*WFDC2* xenografts; normalized E-cadherin and Vimentin protein levels in sh-GFP or sh-*WFDC2* xenografts. **P*<0.05 compared to sh-GFP group; (**D**) Representative images of MMP-2 and *WFDC2* expression in normal ovarian tissue and primary ovarian cancer tissues are shown (200×magnificaiton); Correlation analysis between *WFDC2* and MMP-2 level score in ovarian cancer tissues.

Abbreviations: MMP, matrix metalloproteinase; sh, small hairpin.

together, these results indicate that WFCD2 is crucial to maintain epithelial characteristics for ovarian cancer and might play some role in EMT.

WFDC2 promotes invasion of ovarian cancer cells in an MMP-2-dependent manner

As cell metastasis had been suppressed by *WFDC2* knockdown in serous ovarian cancer cells, then we examined whether cellular motility could be promoted by overexpression of *WFDC2*. According to the endogenous basal level of *WFDC2* in different ovarian cancer cells, OVACAR8 were chosen for *WFDC2* overexpression experiments.[5] Thus, the PcDNA3.1/*WFDC2* plasmid was constructed and transfected into OVACAR8 cells. Compared with the control cells, the expression of *WFDC2* was dramatically upregulated in OVACAR8/*WFDC2* cells, which was assessed by Western blotting (Figure 3A). To declare whether MMP-2 truly participates in the metastasis induced by *WFDC2*, siMMP-2 (a siRNA especially against MMP-2) was used to treat the OVACAR8/*WFDC2* and the control cells and the invasion assay was carried out by transwell polycarbonate plates.

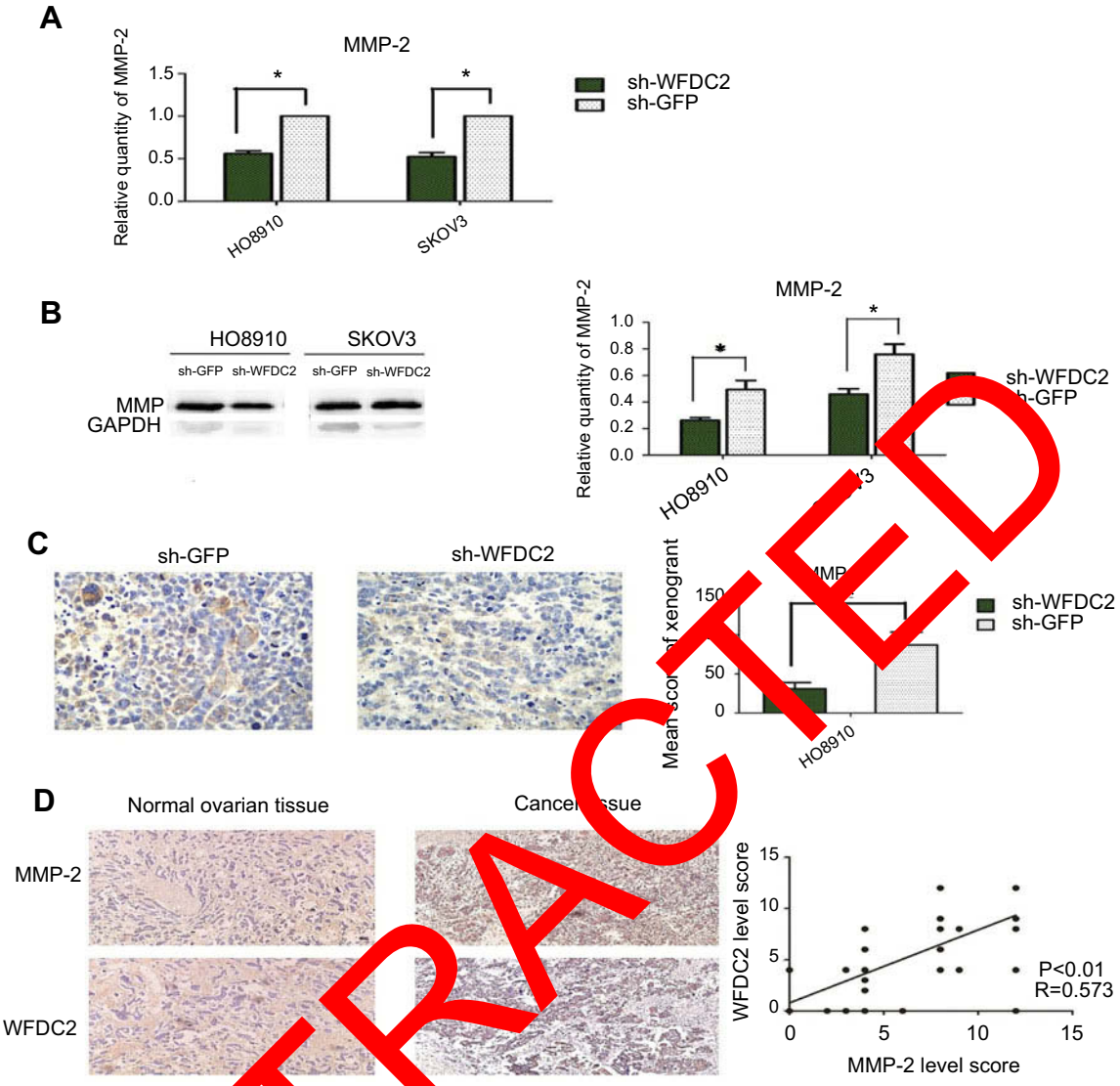

Figure 2 *WFDC2* regulates EMT in ovarian canc (**A**) Western blot analysis of EMT markers (E-cadherin and Vimentin) in SK-OV-3, and HO8910 cells expressing negative control or shRNA, *P< 5 compared to sh FP group. (**B**) Western blot analysis of EMT regulators (smad3, Snail, and Slug) in SK-OV-3, and HO8910 cells expressing sh-GFP or sh-WFD . (**C**) E-cadherin and Vi tin immunofluorescence in sh-GFP or sh-*WFDC2* cells. (**D**) E-cadherin and Vimentin immunohistochemistry in sh-GFP or sh-*WFDC2* xeno afts; norm ed E-cadherin and Vimentin protein levels in sh-GFP or sh-*WFDC2* xenografts. *P<0.05 compared to sh-GFP group in HO8910. **Abbreviations:** sh, smal irpin; E , epithelial–mesenchymal transition.

Remar bly, o erexpression of *WFDC2* increased invasion OVACAR8 cells, while inhibition of MMP-2 rescued the ect of *WFDC2* overexpression on cellular invasion (Figure 3B–C). The results suggest that MMP-2, one of the most important EMT promotors, may be a direct downstream target of *WFDC2*.

Then, we used siMMP-2 to determine the role of MMP2 in tumor cell metastasis and EMT induced by *WFDC2*. As shown in Figure 3C, the expression level of E-cadherin was also increased in siMMP-2-treated cells compared with in control cells, whereas the expression of Vimentin was decreased (Figure 3C). These data suggest that the *WFDC2* may act as an important role in ovarian cancer metastasis and EMT in association with MMP-2.

WFDC2 promotes cell migration and EMT by regulating the AKT pathway in human ovarian carcinoma cells

To further illustrate the molecular mechanism of *WFDC2* on cell migration and EMT, we verified whether the P13K/AKT and MAPK/ERK signaling promotes metastasis and EMT mediated by *WFDC2*. As

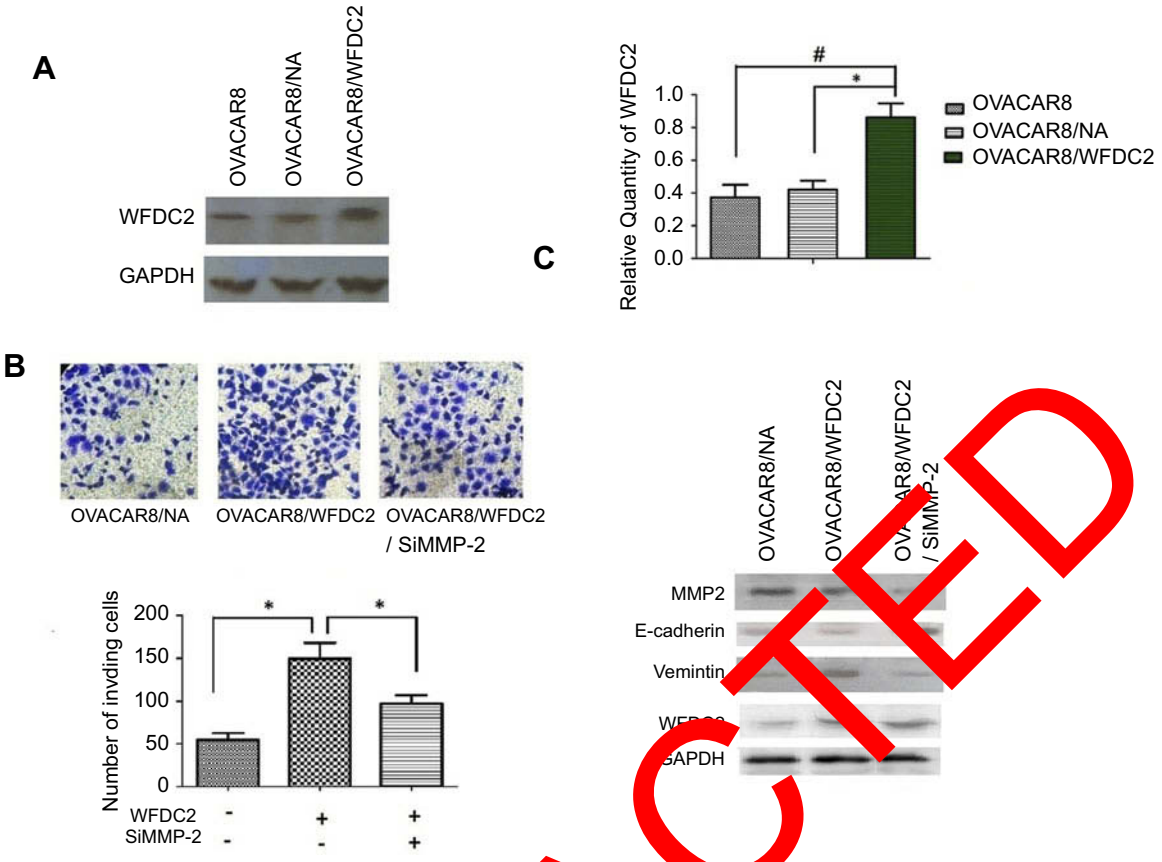

Figure 3 *WFDC2* promote cell invasion in a MMP-2-dependent manner. (**A**) Overexpression of *WFDC2* detected by Western blotting in OVACAR8 cell lines. **P*<0.05 compared to OVACAR8/NA, #*P*<0.05 compared to OVACAR8. (**B**) Invasion of OVACAR8 was inhibited by siMMP-2 by transwell invasion assay with Matrigel (**P*<0.05, compared to OVACAR8 cells with *WFDC2* expression). Histogram plots are representative of the number of cells that invaded through the transwell membranes. (**C**) Detection of MMP-2, Vimentin, and E-cadherin in OVACAR8 cells treated with siMMP-2.

Abbreviations: MMP, matrix metalloproteinase; sh, small hairpin.

shown in Figure 4A, *WFDC2* knockdown had obviously inhibition effect on the AKT phosphorylation (Figure 4A) but not on ERK (data not shown). And overexpression of *WFDC2* promotes AKT phosphorylation as expected (Figure 4B).

We then used the PI3K/AKT signaling inhibitor LY294002 to inhibit the phosphorylation of AKT in OVACAR8/*WFDC2* cells and performed an invasion assay to assess the impact of the *WFDC2* on cellular invasion through PI3K/Akt signaling. As shown in Figure 4C, LY294002 attenuated the cellular invasion of OVACAR8/*WFDC2* cells. By treatment by LY294002 (20 μmol/L), we had observed the inhibition of phosphorylation of AKT and the expression of MMP-2. We also observed the expression level of E-cadherin was also increased in LY294002-treated cells, while Vimentin was decreased compared with control cells (Figure 4D)

All these data suggest the relationship between *WFDC2*, MMP-2, and AKT signaling pathway. Our study shows that *WFDC2* promotes an EMT through the activation of the AKT signaling, leading to the enhanced invasion, migration, and metastasis of serous ovarian cancer cells. Since knockdown of MMP-2 and inhibition of the AKT signaling could inhibit the motility of cells with *WFDC2* overexpression, we propose that AKT and MMP-2 are downstream targets of *WFDC2*, *WFDC2* might activate the AKT signaling to exert MMP-2 and the promoter of EMT and facilitate important functions on ovarian cancer metastasis and (Figure 4E).

Discussion

As the highest lethal gynecological tumors, pathogenesis mechanism of ovarian cancer is not very clear as that in lung cancer, liver cancer and breast cancer. *WFDC2* is known to be highly expressed in ovarian cancer cells and is considered to be a biomarker of ovarian cancer, but its role in the development of ovarian cancer is not yet declared.[1–4] In our previous

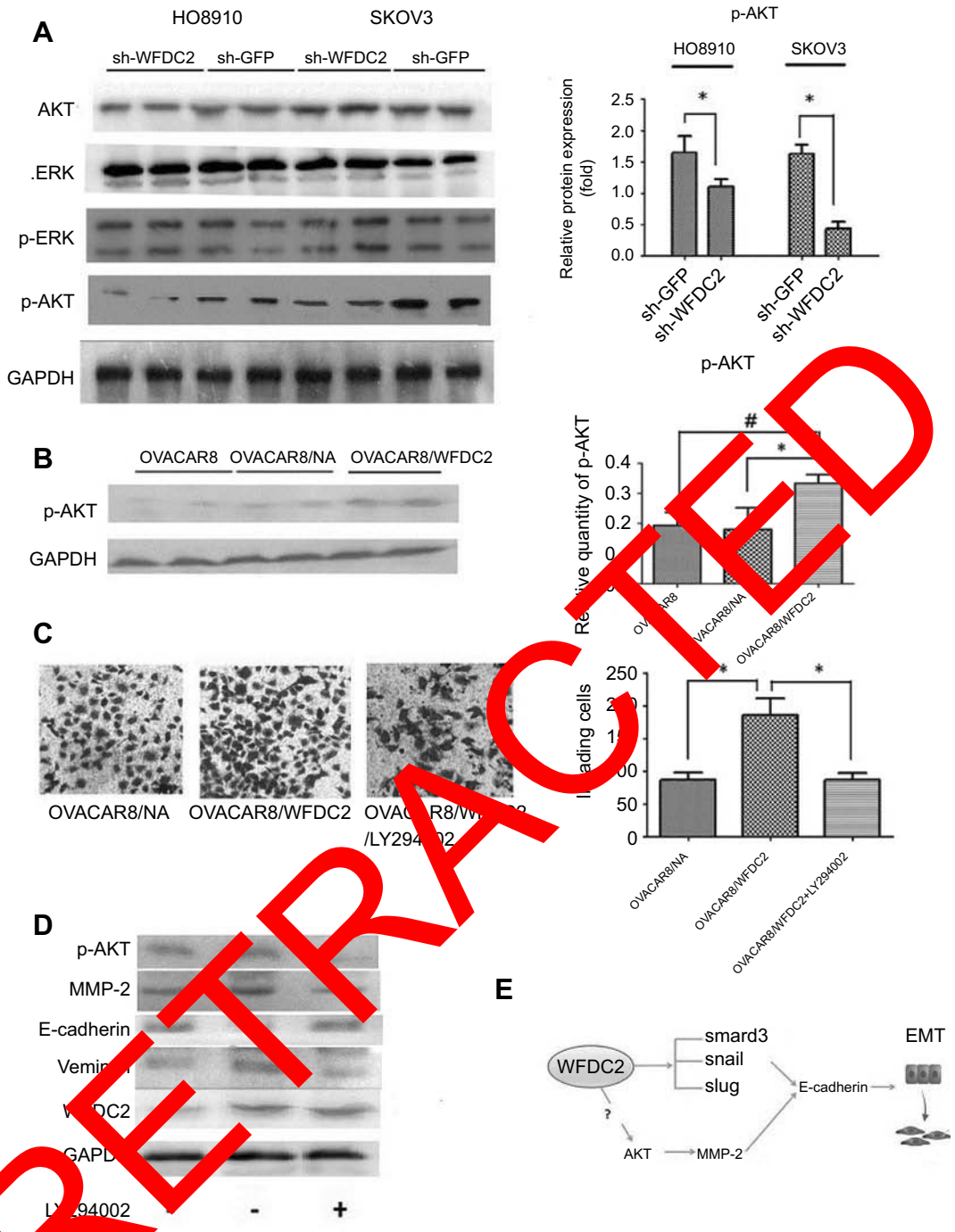

Figure 4 *WFDC2* promotes cell migration and EMT by regulating the AKT pathway in human ovarian carcinoma cells. Knockdown of *WFDC2* suppressed PI3K/AKT signaling but not MAPK/ERK signaling (**P*<0.05 compared to sh-GFP group). (**B**) Overexpression of *WFDC2* activated PI3K/AKT signaling (**P*<0.05 compared to OVACAR8/NA; #*P*<0.05 compared to OVACAR8). (**C**) A transwell assay of OVACAR8/*WFDC2* cells in the presence or absence of LY294002 (0.1 μM); photographs were obtained after 18 hrs of incubation. Data are presented as the mean ± sd (**P*<0.05). (**D**) Detection of p-AKT, MMP-2, Vimentin, and E-cadherin in cells treated with LY294002 (20 μmol/L). (**E**) A schematic diagram of how *WFDC2* promotes cellular metastasis through the AKT-mediated signaling.

Abbreviations: MMP, matrix metalloproteinase; sh, small hairpin; EMT, epithelial–mesenchymal transition.

work, we had illustrated the potential clinical relevance of *WFCD2* to ovarian cancer progression, and the results show that increased expression of *WFCD2* correlated with the malignant and peritoneal metastasis of serous ovarian cancer. By means of the shRNA method, we found that knockdown *WFDC2* can inhibit ovarian cancer metastasis and transplant both in vivo and in vitro. Moore et al also described *WFDC2* as a

promoter of tumor growth.[1,2] To be interesting, we had observed that the ability of ovarian cancer cells to penetrate ECM was greatly reduced with *WFDC2* knockdown in transwell assay. It is well known that MMPs are most important hydrolytic enzymes for the degradation of the ECM.[7,15,17,22] And the MMPs had been considered as the necessary proteases in EMT progression and also an inducer of EMT.[7,17,23,24] We also found a positive correlation of MMP-2 and *WFDC2* not only in cell models, but also in nude mice xenograft and clinical specimens. So we hope to discuss the relationship between *WFDC2* and MMP-2 expression in promoting EMT in this research experience.

First, we analyzed the relationship between *WFDC2* expression and EMT. EMT is a dynamic process, which is characterized by a fundamental change in increasing ability to migrate, as well as the loss of epithelial markers and the acquisition of mesenchymal markers.[8,10,25,26] In this study, we showed *WFDC2* knockdown caused E-cadherin upregulation and Vimentin downregulation, which indicated that *WFDC2* knockdown changed the phenotype of tumor cells and inhibited the EMT progression of tumor cells. Next, a *WFDC2* overexpression cell model had been constructed to explore whether *WFDC2* promotes the generation of EMT by regulating the expression of MMP-2. These results showed that the increasing invasive ability induced by *WFDC2* overexpression could be rested by treating with siRNA against MMP-2. At the same time, the EMT phenotype induced by *WFDC2* overexpression could be also reversed by the siMMP-2. This phenomenon suggests MMP-2 was likely to be a downstream target of *WFDC2*, and *WFDC2* promotes the metastasis and EMT of ovarian cancer cells by regulating the expression of MMP-2.

To declare how *WFDC2* regulated MMP-2 expression, we further analyzed the molecular mechanism of *WFDC2* in cell metastasis. MMP-2, an important protease in EMT progression, could be regulated by activating multiple signal pathways including Smads pathway, ERK-MAPK pathway, and PI3K-AKT pathway.[27–29] In our study, we had observed that the knockdown of *WFDC2* suppressed the phosphorylation of AKT and the expression of smard3. To be interesting, LY294002, a PI3K-AKT pathway inhibitor, reverses MMP-2 expression and cell invasion induced by *WFDC2* overexpression. These results indicate that *WFDC2*-induced activation of PI3K-AKT signaling explains its effect on MMP-2 expression and cell motility, while exactly how *WFDC2* activates the PI3K-AKT pathway needs to be further revealed.

Conclusion

In summary, our study shows that knockdown of *WFDC2* inhibited the EMT progression through activating the PI3K-AKT signaling, leading to the downregulation of MMP-2 and declined the invasion and metastasis of ovarian cancer cells, while overexpression of *WFDC2* entirely reverses these effects. Since knockdown of MMP-2 and inhibition of the PI3K-AKT signaling inhibited the invasion ability of ovarian cancer cells with *WFDC2* overexpression, we propose that both AKT and MMP-2 are downstream targets of *WFDC2* (Figure 4E). Further study is needed to illustrate the specific role of *WFDC2* in the microenvironment rebuilding of ovarian cancer. *WFDC2* overexpression may be not only a biomarker but also a therapeutic target to block the metastasis and recurrence of serous ovarian cancer.

Abbreviation list

WFDC2, WAP four-disulfide core domain 2; EMT, Epithelial–Mesenchymal Transition; IHC, immunohistochemistry; qPCR, quantitative real-time PCR; NC, negative control; MMP2, matrix metalloproteinase 2; AKT, protein kinase B;ERK, extracellular regulated protein kinases,; ECM, extracellular matrix.

Consent for publication

All authors declare that no conflict of interest exists in the submission of this manuscript, and that the manuscript has been approved by all authors for publication.

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Disclosure

The authors report no conflicts of interest in this work.

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Supplementary materials

Table S1 Distribution by tumor characteristics for ovarian cancer patients

Variable	Number of patients (%)	
	n	%
Total		
Age (years)		
≤50	38	38
>50	62	62
FIGO stage		
Stage I	26	28.57
Stage II	21	23.08
Stage III	31	34.07
Stage IV	12	13.19
Grade (epithelial, n=91)		
G1	29	31.87
G2	46	50.55
G3	16	17.58
Histological type		
Serous cystadenocarcinoma	46	50.55
Mucinous cystadenocarcinoma	22	24.18
Endometrioid tumor	14	15.38
Clear cell cacinoma	9	9.89
Transcoelomic metastasis		
No	65	71.42
Yes	26	[illegible]
Lymph node metastasis		
No	74	81.31
Yes	17	[illegible]8.69