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 vitro cell model and 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: WFDC2, 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.
In our former study, we had observed the expression of apoptosis and metastasis-related gene expression in WFDC2 knockdown cells. 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.\textsuperscript{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.\textsuperscript{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. Hoskins et al had reported that SLPI secretion upregulates MMP-9 transcription and secretion in ovarian cancer cells.\textsuperscript{19} Choi also showed that SLPI is associated with MMP-2 and MMP-9 to promote migration and invasion in cancer cells.\textsuperscript{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.

\section*{Methods}

\subsection*{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\textsubscript{2} at 37°C. The PI3K/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).

\subsection*{RNA interference and overexpression transfection}

WFDC2 (NM_001039348) cDNA was inserted into pcDNA3.1 vector to construct the recombination plasmid 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'-GCTCTCTGCCCCAATGATAAGG-3') and the invalid RNAi sequence was (5'-GTTCTCCGAGCTGTCACGT-3').\textsuperscript{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.

\subsection*{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.

\subsection*{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.

\subsection*{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\textsuperscript{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% CO₂. 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 in 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. 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 SPSS13.0 (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. MMPs, especially MMP-2 and MMP-9, can degrade the ECM to regulate cell migration and invasion. 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, 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 WFDC2 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
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, 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, OVCAR8 were chosen for WFDC2 overexpression experiments. Thus, the pCDNA3.1/WFDC2 plasmid was constructed and transfected into OVCAR8 cells. Compared with the control cells, the expression of WFDC2 was dramatically upregulated in OVCAR8/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 OVCAR8/WFDC2 and the control cells and the invasion assay was carried out by transwell polycarbonate plates.

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**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 or 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×magnification); Correlation analysis between WFDC2 and MMP-2 level score in ovarian cancer tissues.

**Abbreviations:** MMP, matrix metalloproteinase; sh, small hairpin.
Remarkably, overexpression of \textit{WFDC2} increased invasion of OVACAR8 cells, while inhibition of MMP-2 rescued the effect of \textit{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 \textit{WFDC2}.

Then, we used siMMP-2 to determine the role of MMP2 in tumor cell metastasis and EMT induced by \textit{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 \textit{WFDC2} may act as an important role in ovarian cancer metastasis and EMT in association with MMP-2.

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

To further illustrate the molecular mechanism of \textit{WFDC2} on cell migration and EMT, we verified whether the P13K/AKT and MAPK/ERK signaling promotes metastasis and EMT mediated by \textit{WFDC2}. As
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 P13K/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 P13K/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
work, we had illustrated the potential clinical relevance of \textit{WFCD2} to ovarian cancer progression, and the results show that increased expression of \textit{WFCD2} correlated with the malignant and peritoneal metastasis of serous ovarian cancer. By means of the shRNA method, we found that knockdown \textit{WFDC2} can inhibit ovarian cancer metastasis and transplant both in vivo and in vitro. Moore et al also described \textit{WFDC2} as a...
promoter of tumor growth. 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. And the MMPs had been considered as the necessary proteases in EMT progression and also an inducer of EMT. 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. 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 increased invasive ability induced by WFDC2 overexpression could be restored 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. In our study, we had observed that the knockdown of WFDC2 suppressed the phosphorylation of AKT and the expression of smad3. 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.

References


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## Supplementary materials

### Table S1 Distribution by tumor characteristics for ovarian cancer patients

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