

The role of WWOX tumor suppressor gene in the regulation of EMT process via regulation of CDH1-ZEB1-VIM expression in endometrial cancer

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Abstract. This study defines the role of WWOX in the regulation of epithelial to mesenchymal transition. A group of 164 endometrial adenocarcinoma patients was studied as well as an ECC1 well-differentiated steroid-responsive endometrial cell line, which was transduced with WWOX cDNA by a retroviral system. The relationship between WWOX gene and EMT marker (*CDH1*, *VIM*, *ZEB1*, *SNAIL*) expression on mRNA (RT-qPCR) and protein levels (western blotting) was evaluated. The EMT processes were also analysed *in vitro* by adhesion of cells to extracellular matrix proteins, migration through a basement membrane, anchorage-independent growth and MMP activity assay. DNA microarrays (HumanOneArray™) were used to determine WWOX-dependent pathways in an ECC1 cell line. A positive correlation was observed between WWOX and *ZEB1*, and a negative correlation between *CDH1* and *VIM*. WWOX expression was found to inversely correlate with the risk of recurrence of tumors in patients. However, in the WWOX-expressing ECC1 cell line, WWOX expression was found to be inversely related with *VIM* and positively with *CDH1*. The ECC1/WWOX cell line variant demonstrated increased migratory capacity, with increased expression of metalloproteinases MMP2/MMP9. However, these cells were not able to form colonies in suspension and revealed decreased adhesion to fibronectin and fibrinogen. Microarray analysis demonstrated that WWOX has an impact on the variety of

cellular pathways including the cadherin and integrin signaling pathways. Our results suggest that the WWOX gene plays a role in the regulation of EMT processes in endometrial cancer by controlling the expression of proteins associated with cell motility, thus influencing tissue remodeling, with the suppression of mesenchymal markers.

Introduction

Endometrial cancer is one of the most common malignant tumors of the reproductive tract among women in developed countries. In Poland in 2010, over five thousand women were diagnosed with endometrial cancer of whom over a thousand died (<http://epid.coi.waw.pl/krn/>). The average age at diagnosis is 65, and mortality rate increases with the age of patient (1,2). Many potential risk factors have been characterized, such as obesity, menopausal status, unopposed estrogens, low parity and nullparity and history of cancer in family (1-3) however, the mechanisms by which they work are not fully understood.

Moreover, conditions such as diabetes mellitus and polycystic ovarian syndrome increase the risk of endometrial cancer development (4). Most endometrial cancers are sporadic, and can be divided into two general groups based on histopathological features and molecular profiles: endometrioid endometrial cancers (EECs) and non-endometrioid endometrial cancers (NEECs) (1-3). Type I, or endometrioid endometrial cancers (EEC), are more common and characterized by estrogen-dependence, microsatellite instability, loss of heterozygosity of the *PTEN* tumor suppressor gene and genetic alterations in the RAS and WNT pathways. Type II, non-endometrioid endometrial cancers (NEECs), are characterized by estrogen-independence with an abnormal accumulation of p53 protein, inactivation of the *p16* gene, overexpression of the *HER2/neu* gene and decreased expression of the *CDH1* gene (5). However, it is important to note that the molecular pathways of the two cancer types are not fully understood.

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The epithelial-mesenchymal transition (EMT) is a complex process which results in transformation of epithelial to mesenchymal cells. Cells of the epithelium are characterized by polarity and non-motility, while mesenchymal cells are loosely organized and able to migrate (6-8). These features are a consequence of changes in gene expression profile i.e., increases in expression of mesenchymal markers such as vimentin and N-cadherin, and downregulation of epithelial markers such as E-cadherin and cytokeratins, which act as thigh and adherence junction proteins (9,10). Although EMT is associated with embryogenesis and organ development under physiological conditions, it has also been associated with cancer progression and metastasis (10-12). A number of transcription factors, i.e., SNAI1, SNAI2, TWIST and ZEB1/2, which are known as the E-cadherin repressors, are the key regulators triggering EMT progression (9,11,13). Changes in EMT marker expression are important in endometrial cancer, as cancer cells invade the myometrium, and this invasion is considered one of the most important prognostic factors for types I and II EC (14).

The WW domain-containing oxidoreductase gene (*WWOX*), also known as *FOR* or *WOX1*, is a tumor suppressor gene which spans more than one million base pairs mapped at locus 16q23.3-q24 in the common chromosomal fragile site FRA16D. The *WWOX* gene encodes a chain of 414 amino acids constituting a 46-kDa protein containing two WW domains in the NH₂-terminal region with a short-chain dehydrogenase/reductase domain, which suggests that it may interact with the receptors regulating steroid hormones. The first WW domain interacts with many partners such as ERBB4 (15,16), transcription factor AP2γ (17,18), YAP (15), Jun (19), HIF1α (20) and others (21). A high level of *WWOX* expression has been observed in hormone-dependent tissues such as those of the ovary, mammary gland or testis, which together with the enzymatic specificity of the SDR domain, indicates its role in the metabolism of steroid hormones (22,23). Loss of *WWOX* expression has been observed during the development of various types of cancer. Moreover, numerous studies have demonstrated correlations between the decrease of *WWOX* expression and the presence of adverse prognostic factors, including shorter survival and a higher degree of malignancy in various types of carcinoma, especially such hormone-dependent ones as those of the breast (5,16,24), prostate (25), ovary (26) and others (27-31). The suppressive character of the *WWOX* gene has been confirmed in many *in vitro* studies, where *WWOX* overexpression resulted in inhibition of cell proliferation, increased apoptosis and inhibition of anchorage-independent growth (32-34). Moreover, mice with a knocked out *WWOX* gene resulted in a greater incidence of numerous disorders such as bone metabolic defects, improper glandular development and disruption of steroidogenesis (23). However, the experiments performed in our laboratory on variety of tumor types, indicated that *WWOX* functioning in the cell appears to be more complex and is probably not only limited to well-known tumor suppressor properties.

We redirected our interest into *WWOX* regulation of cell adhesion and motility properties, as Gourley *et al* reported that overexpression of *WWOX* in an ovarian cancer cell line resulted in decreased adhesion to fibronectin (35), and *WWOX* ectopic overexpression in breast cancer and colon cancer cell

lines has also been observed to result in increased potential of invasion with the modulation of cell adhesive properties (36,37). Moreover, in our previous study on endometrial cancer, we observed that *WWOX* negatively correlated with *CDH1* expression, a well known epithelial marker downregulated during EMT (38). The aim of the study, as a continuation of previous observations, was to determine its role in the EMT process using both an *in vitro* model and endometrial tumor samples.

The overexpression of *WWOX* in an ECC1 well-differentiated steroid-responsive endometrial cell line was induced via stable retroviral transduction. The changes in global gene expression profile after *WWOX* induction were assessed by microarray and the biological effects connected with EMT process were assessed by migration through a basement membrane, anchorage-independent growth, adhesion to ECM proteins and MMPs activity. RT-qPCR analysis of chosen EMT markers was also performed on 164 tumor samples.

Materials and methods

Tumor samples. The study included 164 endometrioid endometrial carcinomas (EECs, type I) obtained from the Department of Gynecological Oncology, Medical University of Lodz. The samples were classified according to FIGO classification system and also classified into risk of recurrence category according to Pecorelli (39). The mean age was 62 years (median 61; range 31-83 years). Tumor samples were stored at -80°C in RNA Later buffer (Ambion®; Thermo Fisher Scientific, Waltham, MA, USA).

The study was performed after receiving the consent of the patients, and was approved by the the Ethics Committee of the Medical University of Lodz (RNN/208/11/KE).

Cell lines and reagents. The ECC-1 cell line was obtained from the American Type Culture Collection (ATCC). This endometrial cancer cell line is hormone-responsive, as well as estrogen (α and β), androgen and progesterone receptor-positive (40). Cells were cultured in advanced RPMI-1640 medium supplemented with heat-inactivated fetal bovine serum (Invitrogen Life Technologies, Carlsbad, CA, USA), L-glutamine (Invitrogen Life Technologies), HEPES buffer (Invitrogen Life Technologies) and antibiotics (penicillin 50 U/ml; streptomycin 50 μg/ml; neomycin 100 μg/ml) (Invitrogen Life Technologies). The cells were incubated at 37°C in a humidified incubator with 5% CO₂.

Transduction. The process was performed with retroviral vector pLNCX2, either with or without *WWOX* cDNA. To increase the effectiveness of transduction, the infectious medium was supplemented with 8 μg/ml polybrene (Sigma-Aldrich, MO, USA). After 24 h, the medium was removed and replaced with advanced RPMI-1640 (Invitrogen Life Technologies) with standard supplements. For selection, the cells were cultured in the presence of 400 mg/ml G418 (Sigma-Aldrich) for 14 days. The stable transductants were analyzed by RT-qPCR and western blot assay.

RT-qPCR. Total RNA from cells and tumor tissues was isolated using TRIzol reagent (Invitrogen Life Technologies),

and 10 µg of RNA was reverse-transcribed with ImProm RT-II reverse transcriptase (Promega Corp., Madison, WI, USA).

RT-qPCR was performed with LightCycler 480 II (Roche Diagnostics GmbH, Mannheim, Germany). PCR products were detected with SYBR Green I and qPCR Core kit for SYBR Green I (Eurogentec, Southampton, UK). Reactions were performed in duplicate. The relative expression of *WWOX*, *CDH1*, and *VIM* were analysed, as were two *CDH1* repressors: *ZEB1* and *SNAIL*. The relative expression of all investigated genes was normalized to three reference genes: *RPLPO*, *RPS17* and *H3F3A*. All information regarding primer sequences, PCR reaction conditions and the length of received products is available upon request. The relative expression level of all investigated genes was calculated using the Roche algorithm (41) with the Universal Human Reference RNA (Stratagene, La Jolla, CA, USA) as a calibrator.

Microarray assay. The microarrays were performed using Human OneArray Whole Genome Microarray v 5.1 (Phalanx Biotech, USA) in four replicates (dual labelling protocol). A ULS™ Labeling kit (Kreatech Diagnostics, The Netherlands) was used to alternately/cross label single-stranded cDNA of two variants (ECC1/vector, ECC1/*WWOX*) with Cy3 and Cy5 as well as Universal Human Reference RNA (Stratagene).

The slide was prepared for hybridization and pre-hybridization according to the manufacturer's protocol. The hybridization process was performed in a 2X SSPE humidity chamber at 42°C for 16-18 h. The following buffers were used for post-hybridization washes: 1X SSPE/0.03% SDS (2 min, 42°C), 1X SSPE (2 min, RT), 0.1X SSPE (rinsed several times, RT) (Phalanx Biotech).

ProScanArray (Perkin-Elmer, USA) was used for scanning and ScanArray Express for preliminary normalization. Lowess and statistical analysis were performed with TM4 software suite provided by The Institute for Genomic Research (<http://www.tm4.org/>). After modified t-test analysis, the fold change was calculated and the Pantherdb online ontology application (www.pantherdb.org) was used to classify the received genes according to ontological terms. The results were regarded as significant at $p < 0.05$.

Protein extraction and western blotting. The endometrial cancer cells were lysed in RIPA protein extraction buffer supplemented with proteases, phosphatase inhibitor cocktail and PMSF (Sigma-Aldrich). The protein concentration was measured using the Bradford method (Bio-Rad Laboratories, CA, USA), run on 10% SDS-PAGE gel electrophoresis and transferred to a PVDF membrane (Sigma-Aldrich). The membranes were blocked in a buffer of 5% non-fat milk in TBST for 1 h at room temperature and then incubated for 19 h at 4°C with primary antibodies to goat anti-human *WWOX*, 1:100; mouse anti-E-cadherin, 1:100; mouse anti-vimentin, 1:100 (sc:20529, sc:8426, sc:51721, Santa Cruz Biotechnology Inc., Dallas, TX, USA). Following incubation, the membranes were washed three times with TBST and incubated with secondary antibodies conjugated with alkaline phosphatase (Sigma-Aldrich) for 1 h. The membranes were washed three times in TBST and developed using Novex® AP Chromogenic Substrate (Invitrogen Life Technologies). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) at a dilution of 1:1,000

was used as the reference protein (sc-59540, Santa Cruz Biotechnology Inc.). All biological tests were performed in a minimum of three replicates.

Adhesion assay. Cell adhesion was evaluated using Cell Biolabs CytoSelect™ Cell Adhesion assay kit (Cell BioLabs, San Diego, CA, USA) according to the manufacturer's instructions. This test provides a rapid and quantitative method for accessing the cell adhesion potential. The ECC1 cells were seeded at a density of 1.5×10^5 cells in a total volume of 150 µl serum-free media to each well. The cells were incubated for 1 h at 37°C. The absorbance was measured at 560 nm with a BioTek plate reader (BioTek US, USA).

Cell invasion assay. The cell invasiveness potential was determined using a Colorimetric CytoSelect™ Cell Invasion Assay Kit Cell (Cell BioLabs) according to the manufacturer's protocol. The cell suspension was incubated at a density of 3×10^5 /well in serum-free media for 48 h. The absorbance was measured at 560 nm on a BioTek reading plate (BioTek US). Each variant was tested in quadruplicate.

Zymography. ECC1 cells were seeded in 6-well plates at a density of 1.5×10^6 /well. Twenty-four hours before collection, the medium was replaced with serum-free RPMI medium. Qubit® Protein assay kits (Invitrogen Life Technologies) were used to quantify protein content. Samples containing 15 µg of protein were separated in 10% SDS-page gel containing 2 mg/ml gelatin. After electrophoresis, the gel was washed twice for 30 min in 2.5% Triton X-100 wash buffer and then incubated at 37°C for 18 h in a solution containing 50 mM Tris (pH 7.5) (Sigma-Aldrich), 10 mM CaCl_2 (Sigma-Aldrich) and 200 mM NaCl (Sigma-Aldrich). Following this, the gel was stained with 0.125% Coomassie brilliant blue R-250 (Sigma-Aldrich) and de-stained with solution (30% methanol, 10% acetic acid, 60% distilled water) (Sigma-Aldrich). A protein marker was used to measure the molecular weights of the analysed enzymes. Densitometric analysis with ImageJ Analysis 1.34s software (Wayne Rasband, National Institutes of Health, USA, <http://rsb.info.nih.gov/ij/>) was used to quantify MMP activities.

Soft agar. A soft agar assay was performed to analyze the differences in anchorage-independent growth between ECC1/*WWOX* and ECC1/vec. Six-well plates were coated with 2 ml 0.9% low melting agarose (AppliChem, MO, USA) in RPMI-1640 with 10% FBS. Both variants of ECC1 cells were suspended in 0.3% low melting agarose at a concentration of 10^4 cells per well. The cells were incubated at 37°C in a humidified incubator for 14 days. The plates were stained with 1 ml of 0.005% crystal violet (Sigma-Aldrich) in water and colonies were counted using ImageJ Analysis 1.34 s software (Wayne Rasband, National Institutes of Health, USA, <http://rsb.info.nih.gov/ij/>).

Statistical analysis. The correlation of expression was performed using the non-parametric Spearman's rank correlation test. The Mann-Whitney t-test was used to determine the differences between the levels of expression of the investigated genes and clinical factors in patients with endometrial cancer.

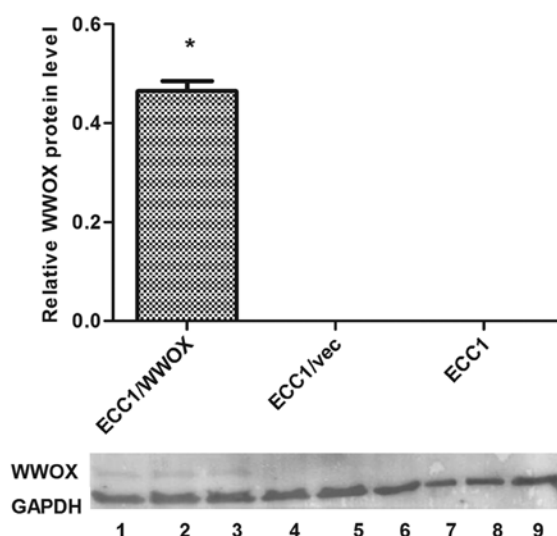


Figure 1. Confirmation of ECC1 cell line transfection on the protein level. The values are mean \pm SD. * $p < 0.05$. Lanes 1-3, ECC1/WWOX; lanes 4-6, ECC1/vec; lanes 7-9, ECC1.

The Aspin-Welsch t-test was used to determine the statistical significance of the difference observed between cell variants in all biological tests.

Results

Confirmation of WWOX gene transduction in ECC1. The transduction resulted in the increase of WWOX expression both on the mRNA and protein levels. A >180-fold change of WWOX gene expression was observed on the mRNA level: the mean relative expression values being 0.019 and 3.34 for ECC1/vector and ECC1/WWOX respectively. The change on the protein level is shown (Fig. 1).

Microarray analysis. Our microarray analysis revealed more than 800 genes with changed expression as the result of WWOX ectopic overexpression ($p < 0.05$). The identified genes belong to several signalling pathways associated with regulation of cell differentiation, apoptosis and cellular communication/tissue architecture (Table I). The predominant ones being the Wnt signalling pathway (13 genes), integrin signalling pathway (10 genes), inflammation mediated by chemokine and cytokine signalling pathway (6 genes), gonadotropin-releasing hormone receptor pathway (11 genes), angiogenesis (6 genes), TGF- β signalling pathways (8 genes), apoptosis (5 genes), and the cadherin signaling pathway (6 genes). The ontology classification (by cellular pathways) by WWOX overexpression is included in Table II. The two main EMT markers found in the cadherin signalling pathway were vimentin and E-cadherin.

A 1.6-fold increase in expression of the *CDH1* gene and a 1.9-fold decrease in expression of *VIM* mRNA were observed in the ECC1 cell line overexpressing WWOX. Microarray data were submitted to the GEO Database (reference series: GSE61041).

Microarray validation. The observed change in expression of *CDH1* and *VIM* was confirmed with RT-qPCR: a 3.5-fold

Table I. Ontological analysis of genes in biological process regulated by WWOX gene in ECC1 cell line.

Biological process	No. of genes
Metabolic process	368
Primary metabolic process	299
Unclassified	266
Cellular process	262
Cell communication	147
Biological regulation	138
Nucleobase-containing compound metabolic process	138
Protein metabolic process	136
Localization	124
Transport	120
Developmental process	117
RNA metabolic process	102
Regulation of biological process	87
Transcription, DNA-dependent	82
Transcription from RNA polymerase II promoter	80
Multicellular organismal process	70
Single-multicellular organism process	70
Regulation of nucleobase-containing compound metabolic process	69
System development	69
Cell cycle	68
Response to stimulus	65
Immune system process	62
Regulation of transcription from RNA polymerase II promoter	62
Cellular protein modification process	61
Protein transport	60
Intracellular protein transport	60
System process	60
Regulation of catalytic activity	51
Regulation of molecular function	51
Neurological system process	48
Cellular component organization or biogenesis	46
Cellular component organization	43
Mesoderm development	43
Nervous system development	42
Ectoderm development	42
Cell adhesion	41
Biological adhesion	41
Cell-cell signaling	41
Anatomical structure morphogenesis	28
Cell-cell adhesion	27
Cellular component morphogenesis	26
Death	26
Cell death	26
Apoptotic process	26
Mitosis	26

Table II. The number of genes in pathways regulated by *WWOX* gene in ECC1 cell line.

Pathway	No. of genes
Unclassified	667
Wnt signalling pathway	13
Gonadotropin-releasing hormone receptor pathway	11
Integrin signalling pathway	10
TGF- β signalling pathway	8
Alzheimer disease-presenilin pathway	7
Angiogenesis	6
Inflammation mediated by chemokine and cytokine signalling pathway	6
Huntington disease	6
Cadherin signalling pathway	6
Apoptosis signalling pathway	5
Heterotrimeric G-protein signalling pathway-Gq α and Go α mediated pathway	5

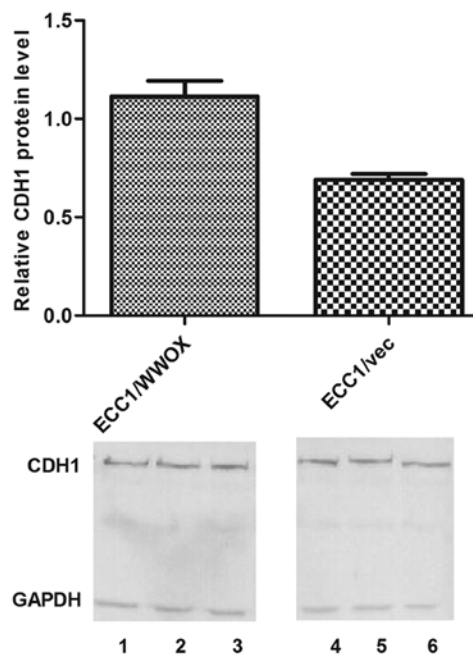


Figure 2. Western blot analysis of the level of CDH1 protein. The values are mean \pm SD. Lanes 1-3, ECC1/WWOX; lanes 4-6, ECC1/vec.

increase of *CDH1* (mean relative expression 0.48 vs 1.67 for ECC1/vector and ECC1/WWOX respectively); 2-fold decrease for *VIM* (0.00035 vs 0.00019 for ECC1/vector and ECC1/WWOX respectively). Similar changes were also noted on protein level (Figs. 2 and 3).

The influence of WWOX on invasiveness, adhesion potential and cell growth in suspension. Overexpression of the *WWOX* gene resulted in a 1.3-fold increase in migration in comparison to the ECC1/vector ($p < 0.05$) (Fig. 4) and greater induction of metalloproteinase activity i.e., MMP-2 and -9 (Fig. 5). These

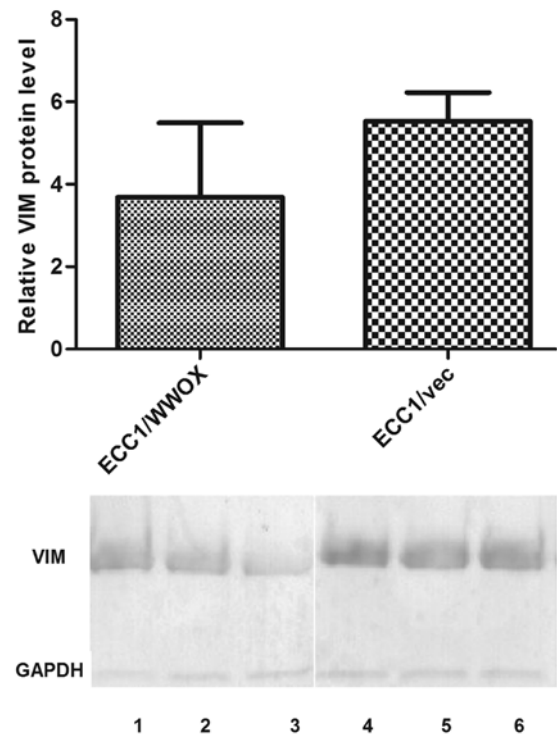


Figure 3. Western blot analysis of the level of VIM protein. The values are mean \pm SD. Lanes 1-3, ECC1/WWOX; lanes 4-6, ECC1/vec.

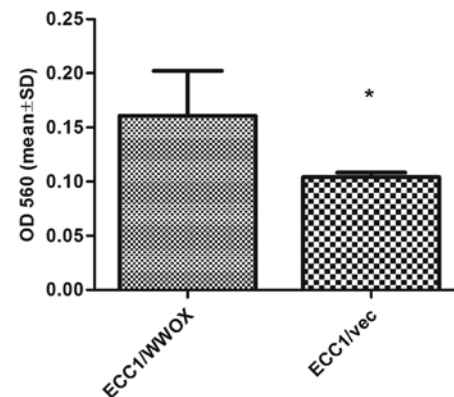


Figure 4. The ability to cross a membrane coated with a basement membrane matrix of ECC1/WWOX. The values are mean \pm SD. * $p < 0.05$.

results suggest that increased expression of *WWOX* influences the motility and invasive potential of the cells. However, the soft agar assay, which measures the ability of cells to grow in suspension, in a similar way to cancer cells being able to survive after detachment, revealed a reduced colony number for the ECC1/WWOX cell line variant (Fig. 6, $p < 0.05$).

The invasion and growth in soft agar assay results indicate that even though ECC1/WWOX cells demonstrated a higher basic membrane transition ability, they were unable to survive after detachment. The cells overexpressing *WWOX* demonstrated a significantly lower ability to adhere to fibrinogen (1.8-fold change; $p < 0.05$) (Fig. 7).

Expression analysis in tumor samples. In the tumor samples, the EMT markers were also analyzed in relation to *WWOX*.

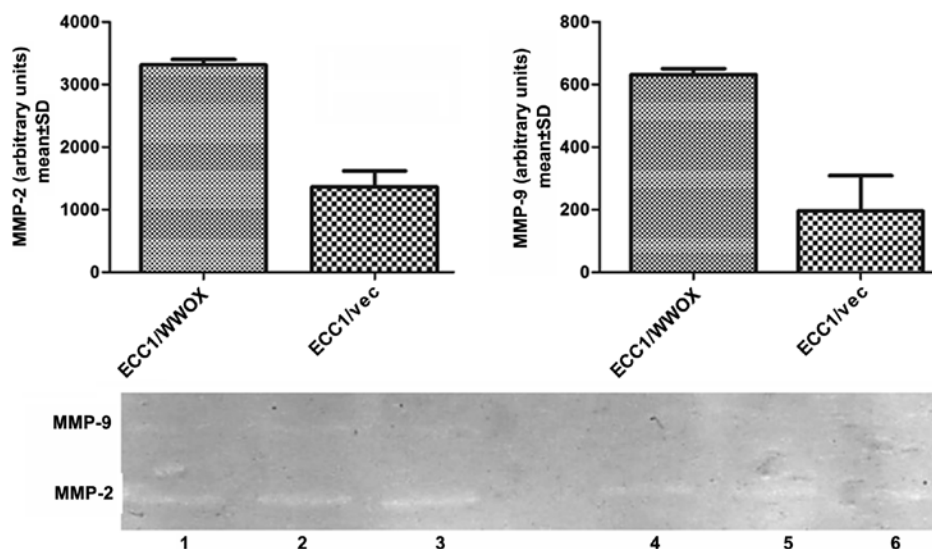


Figure 5. MMP-2 and MMP-9 activities analyzed by gelatin zymography of ECC1/WWOX. The values are mean \pm SD. Lanes 1-3, ECC1/WWOX; lanes 4-6, ECC1/vec.

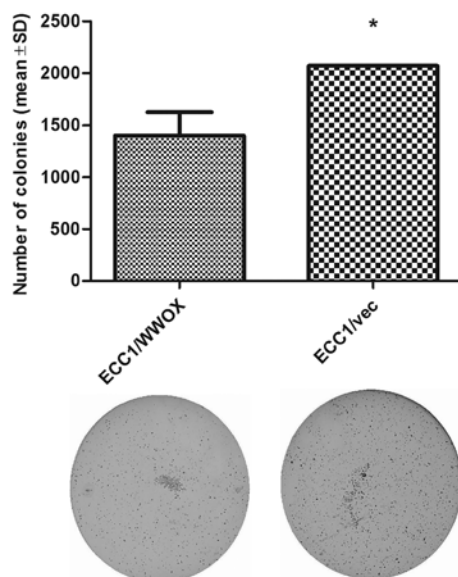


Figure 6. Colony formation assays of ECC1/WWOX. The colonies were counted using ImageJ software (mean \pm SD). * p <0.05.

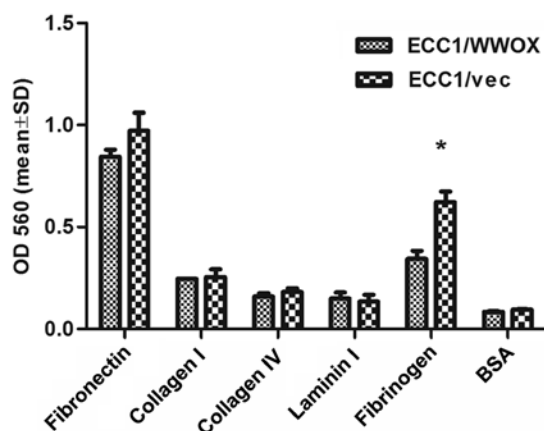


Figure 7. Adhesion to ECM proteins of ECC1/WWOX and ECC1/vec control cells. The values are mean \pm SD. * p <0.05, BSA, bovine serum albumin.

Table III. The correlation analysis between the levels of expression of *WWOX* and other EMT tumor-related genes in endometrial carcinoma patients.

Gene	Spearman's rank correlation	P-value
<i>WWOX/VIM</i>	-0.268	0.0005
<i>WWOX/ZEB1</i>	0.260	0.0004
<i>WWOX/CDH1</i>	-0.512	0.0001
<i>WWOX/SNAI1</i>	0.029	0.7114 (NS)

ZEB1 and *SNAI1* were included in the EMT marker gene panel. A positive correlation was observed between *WWOX* and *ZEB1* ($R_s=0.26$; $p=0.0004$), and a negative correlation between *WWOX* and *VIM* ($R_s=-0.268$, $p=0.0005$). A negative correlation is known to exist between *WWOX* and *CDH1* (38) and this was confirmed by the increased number of samples. The correlation with *SNAI1* was statistically insignificant ($R_s=0.029$; $p>0.05$). The remaining correlations are presented in Table III. Similar to other tumor types, the expression of *WWOX* in endometrial cancer inversely correlated with the risk of cancer recurrence, i.e., tumors with low risk demonstrated higher *WWOX* expression than those with medium and high risk. A statistically significant difference was found between the low ($n=38$, median expression 2.33) and medium ($n=83$, median expression 1.12) risks of disease recurrence ($p=0.023$). More detailed information of all correlations with clinical factors is presented in Table IV.

Discussion

The process of epithelial to mesenchymal transition and its regulation seems to be important in endometrial carcinogenesis and disease progression. A review of the literature indicates that this is the first report to investigate the influence

Table IV. The correlation analysis between *WWOX*, *ZEB1*, *VIM*, *CDH1* genes and clinical factors in endometrial carcinoma patients.

Clinical factor	n	Median of <i>WWOX</i> mRNA level (range)	P-value	Median of <i>ZEB1</i> mRNA level (range)	P-value	Median of <i>VIM</i> mRNA level (range)	P-value	Median of <i>CDH1</i> mRNA level (range)	P-value
Risk of recurrence									
Low	38	2.33 (1.24-3.40)	0.012 (low/medium)	0.35 (0.18-0.76)	0.06 (low/medium)	0.06 (0.02-0.17)	0.44 (low/medium)	0.34 (0.05-0.69)	0.023 (low/medium)
Medium	83	1.12 (0.75-1.43)	0.19 (medium/high)	0.15 (0.10-0.23)	0.13 (high-medium)	0.05 (0.01-0.10)	0.68 (medium/high)	0.69 (0.51-0.94)	0.15 (medium/high)
High	25	1.24 (0.86-2.77)	0.44 (low/high)	0.25 (0.11-0.51)	0.58 (low/high)	0.04 (0.01-0.12)	0.23 (low/high)	0.34 (0.10-0.95)	0.69 (low/high)
NS	18								
Grade									
1	48	1.86 (1.05-2.57)	0.05 (1 vs 2)	0.15 (0.08-0.45)	0.42 (1 vs 2)	0.03 (0.01-0.08)	0.06 (1 vs 2)	0.26 (0.07-0.70)	0.048 (1 vs 2)
2	79	1.03 (0.75-1.37)	0.14 (2 vs 3)	0.30 (0.18-0.47)	0.06 (2 vs 3)	0.09 (0.04-0.14)	0.14 (2 vs 3)	0.79 (0.53-1.03)	0.1 (2 vs 3)
3	30	1.59 (0.83-4.21)	0.86 (1 vs 3)	0.13 (0.09-0.21)	0.44 (1 vs 3)	0.05 (0.01-0.09)	0.83 (1 vs 3)	0.39 (0.16-0.77)	0.86 (1 vs 3)
NS	7								
FIGO stage									
I	98	1.30 (0.90-1.72)	0.87 (I vs II)	0.19 (0.12-0.34)	0.70 (I vs II)	0.06 (0.03-0.11)	0.48 (I vs II)	0.64 (0.47-0.79)	0.35 (I vs II)
II	25	1.36 (0.64-2.86)	0.44 (II vs III)	0.19 (0.09-0.43)	0.38 (II vs III)	0.03 (0.03-0.19)	0.96 (II vs III)	0.94 (0.4-1.28)	0.11 (II vs III)
III	26	1.34 (0.86-4.27)	0.43 (I vs III)	0.23 (0.11-0.51)	0.60 (I vs III)	0.05 (0.01-0.12)	0.34 (I vs III)	0.29 (0.10-0.95)	0.33 (I vs III)
NS	15								
Myometrium invasion									
<1/2	83	1.61 (1.24-2.12)	0.12	0.20 (0.12-0.34)	0.96	0.07 (0.04-0.11)	0.18	0.53 (0.32-0.70)	0.30
>1/2	69	0.97 (0.81-1.36)		0.19 (0.12-0.31)		0.04 (0.01-0.08)		0.65 (0.24-1.15)	
Without	4	0.72		0.18		0.13		0.33	
NS	8								
Lymph node metastasis									
Negative	125	1.34 (0.97-1.66)	0.42	0.17 (0.11-0.27)	0.13	0.05 (0.02-0.08)		0.65 (0.49-0.83)	0.17
Positive	24	1.34 (0.86-2.65)		0.27 (0.11-0.63)		0.05 (0.01-0.13)		0.29 (0.10-0.64)	
NS	15								

NS, not specified. Bold indicates statistically significant correlation.

of the *WWOX* gene on EMT in endometrial tumor samples and in an endometrial cancer cell line model.

The present study is a continuation of previous observations that *WWOX* inversely correlates with *CDH1* expression, which suggests that alterations in the expression of this tumor suppressor gene probably influence the process of cell adhesion (38).

WWOX overexpression was induced in a well-differentiated endometrial cancer cell line and its effect on EMT progression was analysed on microarrays and by multiple biological assays. The microarray analysis revealed significant changes ($p < 0.05$) in the expression of two main EMT markers, oppositely regulated in relation to *WWOX* expression level i.e., *CDH1* was upregulated and *VIM* was downregulated. This result was confirmed on both the protein and mRNA levels.

This observed upregulation of *CDH1* in the cancer cell line stands in contrast to our previous report, which revealed a negative correlation between *CDH1* and *WWOX*. However, in the present study, the larger, tumor sample population also demonstrated a negative correlation. This discrepancy between the cell line model and the tumor samples in the present study may be explained by comparing the complexity of tumor architecture between the ECM matrix and the cell line in a 2D *in vitro* culture. The well-differentiated nature of the cell line within the experiment may also have an impact on *WWOX* functioning, as shown in previous studies on colon cancer cell lines (36). However, the relationship between *CDH1* and *WWOX* implies that the *WWOX* gene has an influence on the regulation of the adhesion process. The loss of E-cadherin expression is a hallmark of EMT, and its reduced transcription in endometrial cancer was found to correlate with the depth of myometrial invasion (14,42).

In the increased number of tumor samples used in the present study, loss of *CDH1* expression tended to be observed in grade 3 tumors and those with the highest risk of recurrence ($p > 0.05$). However, interestingly, grade 2 tumors and those with medium risk of recurrence demonstrated significantly higher *CDH1* expression on the mRNA level in comparison to grade 1 tumors and those with a low risk of recurrence.

WWOX mRNA expression was found to have an inverse association with risk of recurrence and tumor grade. Tumors with low risk of recurrence and grade 1 demonstrated significantly higher *WWOX* expression, which confirms that loss of *WWOX* is connected with tumor progression, as seen in other tumor types (30,43,44). The inverse correlation with *WWOX* and *CDH1* seen in tumor samples may also suggest *WWOX* regulatory role in the process of differentiation, since reduced *CDH1* protein level is associated with diminished differentiation (42).

Even though the overexpression of *WWOX* resulted in the increase of *CDH1* expression in the *in vitro* model, the result of the invasion assay revealed that the ECC1/*WWOX* cell line variant had increased invasion capacity and migratory potential, as overexpression of *WWOX* resulted in increased expression of metalloproteinases MMP2/MMP9. This observation is consistent with previous findings in breast and colon cancer cell lines ectopically overexpressing *WWOX* protein (36,37), but also implies that the interaction between *WWOX* and *CDH1* may be modulated by other EMT-related genes. Hence, *WWOX* expression was compared with *ZEB1* and

SNAIL1 expression on the mRNA level. Of those two genes, a positive correlation was only noticed between *WWOX* and *ZEB1*.

The observed positive correlation between *WWOX* and *ZEB1* may further confirm the idea that *WWOX* may play a important role in EMT induction in terms of cell remodelling, with suppressed acquisition of the mesenchymal phenotype; this negative correlation was previously noted with vimentin, a mesenchymal marker, both in an *in vitro* model and in tumor samples.

In a normal human uterus, *ZEB1* expression is regulated by steroid hormones. It is higher in both stroma and the myometrium in the secretory phase than in the proliferative phase. While the *ZEB1* gene is silenced in the normal endometrial epithelium (45), it is expressed in the tumor-associated stroma of endometrial cancers, as well as in epithelial-derived cancer cells, with its level correlating with disease staging (45,46). Thus, activation of *ZEB1* expression in epithelial cancer cells causes EMT, through repression of *CDH1* and induction of vimentin (46). In the present study, especially in tumor samples, it seems that the negative correlation between *WWOX* and *CDH1* may be a result of *ZEB1* interplay in the functional axis of *WWOX*-*CDH1*, and is not present in the stable micro-environment of cell culture.

However, *ZEB1*-regulated acquisition of mesenchymal phenotype was found to be incompleting in both the *in vitro* model and tumor samples. High *WWOX* expression resulted in decreased *VIM* expression. This negative correlation was confirmed on the protein level in the *in vitro* ECC1 cultures and has recently also been observed for ovarian cancer (47). The possibility that mesenchymal progression was incomplete as a result of *VIM* downregulation is confirmed by the observation that the *WWOX*-transduced cells were not able to form colonies in suspension, suggesting that they have lost some of their aggressiveness. This observation is consistent with previous findings on ovarian and cervical cancer cell lines (32,33) and confirms the tumor suppression properties of *WWOX*. Moreover, our findings also support those of Gourley *et al* that *WWOX* may be involved in tissue architecture organization through modulation of the adhesion potential of cells to different ECM proteins (35). Significantly reduced adhesion to fibrinogen and tendency to fibrinonection was demonstrated by ECC1 cells with high ectopic expression of *WWOX*.

The received tendency for decreased adhesion to fibronectin is consistent with previous findings in ovarian cancer (35), but this is the first study to reveal reduced adhesion to fibrinogen after *WWOX* overexpression. Importantly, it has been hypothesized that fibrinogen is able to facilitate metastasis by enhancing adhesion, thus promoting the survival of tumor cells circulating in blood vessels. Elevated fibrinogen expression was associated with metastasis in lung, colon, ovarian and breast cancers (48,49). Moreover, in breast cancer, fibrinogen-mediated migration was observed only in such malignant tumor cell lines as MDA-MB-231 and MCF-7, whereas this ability was decreased in the non-malignant MCF-10A cell line (49). Hence, our observations on the endometrial cancer cell line seems to confirm that *WWOX* overexpression mediates the loss of aggressiveness and that this gene is involved in the regulation of adhesion potential.

In conclusion, this study investigated the role of the *WWOX* gene in the process of EMT in endometrial cancer. On both tumor and cell line models, statistically significant correlations were noted with *CDH1* and vimentin, which are the main markers of EMT. Our observations indicate that *WWOX* may be involved in the initiation of EMT, leading to changes in cell adhesion and motility. They also suggest that *WWOX* plays a suppressive role in the process of mesenchymal phenotype acquisition, resulting in reduction of cell features associated with aggressiveness. However, further research in this direction is required.

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References

- Amant F, Moerman P, Neven P, Timmerman D, Van LE and Vergote I: Endometrial cancer. *Lancet* 366: 491-505, 2005.
- Leslie KK, Thiel KW, Goodheart MJ, De Geest K, Jia Y and Yang S: Endometrial cancer. *Obstet Gynecol Clin North Am* 39: 255-268, 2012.
- Merritt MA and Cramer DW: Molecular pathogenesis of endometrial and ovarian cancer. *Cancer Biomark* 9: 287-305, 2010.
- Troisi R, Potischman N, Hoover RN, Siiteri P and Brinton LA: Insulin and endometrial cancer. *Am J Epidemiol* 146: 476-482, 1997.
- Bednarek AK, Laffin KJ, Daniel RL, Liao Q, Hawkins KA and Aldaz CM: *WWOX*, a novel WW domain-containing protein mapping to human chromosome 16q23.3-24.1, a region frequently affected in breast cancer. *Cancer Res* 60: 2140-2145, 2000.
- Thiery JP, Acloque H, Huang RY and Nieto MA: Epithelial-mesenchymal transitions in development and disease. *Cell* 139: 871-890, 2009.
- Garg M: Epithelial-mesenchymal transition - activating transcription factors - multifunctional regulators in cancer. *World J Stem Cells* 5: 188-195, 2013.
- Kalluri R and Weinberg RA: The basics of epithelial-mesenchymal transition. *J Clin Invest* 119: 1420-1428, 2009.
- Tanaka Y, Terai Y, Kawaguchi H, Fujiwara S, Yoo S, Tsunetoh S, Takai M, Kanemura M, Tanabe A and Ohmichi M: Prognostic impact of EMT (epithelial-mesenchymal-transition)-related protein expression in endometrial cancer. *Cancer Biol Ther* 14: 13-19, 2013.
- Huber MA, Kraut N and Beug H: Molecular requirements for epithelial-mesenchymal transition during tumor progression. *Curr Opin Cell Biol* 17: 548-558, 2005.
- Thiery JP: Epithelial-mesenchymal transitions in development and pathologies. *Curr Opin Cell Biol* 15: 740-746, 2003.
- Grünert S, Jechlinger M and Beug H: Diverse cellular and molecular mechanisms contribute to epithelial plasticity and metastasis. *Nat Rev Mol Cell Biol* 4: 657-665, 2003.
- Montserrat N, Mozos A, Llobet D, Dolcet X, Pons C, de Herreros AG, Matias-Guiu X and Prat J: Epithelial to mesenchymal transition in early stage endometrioid endometrial carcinoma. *Hum Pathol* 43: 632-643, 2012.
- Abal M, Llauroadó M, Doll A, Monge M, Colas E, González M, Rigau M, Alazzouzi H, Demajo S, Castellví J, *et al*: Molecular determinants of invasion in endometrial cancer. *Clin Transl Oncol* 9: 272-277, 2007.
- Aqeilan RI, Donati V, Palamarchuk A, Trapasso F, Kaou M, Pekarsky Y, Sudol M and Croce CM: WW domain-containing proteins, *WWOX* and *YAP*, compete for interaction with *ErbB-4* and modulate its transcriptional function. *Cancer Res* 65: 6764-6772, 2005.
- Aqeilan RI, Donati V, Gaudio E, Nicoloso MS, Sundvall M, Korhonen A, Lundin J, Isola J, Sudol M, Joensuu H, *et al*: Association of *Wwox* with *ErbB4* in breast cancer. *Cancer Res* 67: 9330-9336, 2007.
- Aqeilan RI, Palamarchuk A, Weigel RJ, Herrero JJ, Pekarsky Y and Croce CM: Physical and functional interactions between the *Wwox* tumor suppressor protein and the AP-2gamma transcription factor. *Cancer Res* 64: 8256-8261, 2004.
- Guler G, Huebner K, Himmethoglu C, Jimenez RE, Costinean S, Volinia S, Pilarski RT, Hayran M and Shapiro CL: Fragile histidine triad protein, WW domain-containing oxidoreductase protein *Wwox*, and activator protein 2gamma expression levels correlate with basal phenotype in breast cancer. *Cancer* 115: 899-908, 2009.
- Gaudio E, Palamarchuk A, Palumbo T, Trapasso F, Pekarsky Y, Croce CM and Aqeilan RI: Physical association with *WWOX* suppresses c-Jun transcriptional activity. *Cancer Res* 66: 11585-11589, 2006.
- Abu-Remaileh M and Aqeilan RI: Tumor suppressor *WWOX* regulates glucose metabolism via *HIF1α* modulation. *Cell Death Differ* 21: 1805-1814, 2014.
- Abu-Odeh M, Bar-Mag T, Huang H, Kim T, Salah Z, Abdeen SK, Sudol M, Reichmann D, Sidhu S, Kim PM, *et al*: Characterizing WW domain interactions of tumor suppressor *WWOX* reveals its association with multiprotein networks. *J Biol Chem* 289: 8865-8880, 2014.
- Safuda-Gorgul A, Seta K, Nowakowska M and Bednarek AK: *WWOX* oxidoreductase - substrate and enzymatic characterization. *Z Naturforsch C* 66: 73-82, 2011.
- Aqeilan RI, Hagan JP, de Bruin A, Rawahneh M, Salah Z, Gaudio E, Siddiqui H, Volinia S, Alder H, Lian JB, *et al*: Targeted ablation of the WW domain-containing oxidoreductase tumor suppressor leads to impaired steroidogenesis. *Endocrinology* 150: 1530-1535, 2009.
- Aqeilan RI and Croce CM: *WWOX* in biological control and tumorigenesis. *J Cell Physiol* 212: 307-310, 2007.
- Qin HR, Iliopoulos D, Semba S, Fabbri M, Druck T, Volinia S, Croce CM, Morrison CD, Klein RD and Huebner K: A role for the *WWOX* gene in prostate cancer. *Cancer Res* 66: 6477-6481, 2006.
- Nunez MI, Rosen DG, Ludes-Meyers JH, Abba MC, Kil H, Page R, Klein-Szanto AJ, Godwin AK, Liu J, Mills GB, *et al*: *WWOX* protein expression varies among ovarian carcinoma histotypes and correlates with less favorable outcome. *BMC Cancer* 5: 64, 2005.
- Kosla K, Pluciennik E, Kurzyk A, Jesionek-Kupnicka D, Kordek R, Potemski P and Bednarek AK: Molecular analysis of *WWOX* expression correlation with proliferation and apoptosis in glioblastoma multiforme. *J Neurooncol* 101: 207-213, 2011.
- Kuroki T, Yendamuri S, Trapasso F, Matsuyama A, Aqeilan RI, Alder H, Rattan S, Cesari R, Noll ML, Williams NN, *et al*: The tumor suppressor gene *WWOX* at FRA16D is involved in pancreatic carcinogenesis. *Clin Cancer Res* 10: 2459-2465, 2004.
- Pluciennik E, Nowakowska M, Wujcicka WI, Sitkiewicz A, Kazanowska B, Zielińska E and Bednarek AK: Genetic alterations of *WWOX* in Wilms' tumor are involved in its carcinogenesis. *Oncol Rep* 28: 1417-1422, 2012.
- Żelazowski MJ, Pluciennik E, Pasz-Walczak G, Potemski P, Kordek R and Bednarek AK: *WWOX* expression in colorectal cancer - a real-time quantitative RT-PCR study. *Tumor Biol* 32: 551-560, 2011.
- Li YP, Wu CC, Chen WT, Huang YC and Chai CY: The expression and significance of *WWOX* and β -catenin in hepatocellular carcinoma. *APMIS* 121: 120-126, 2013.
- Xiong Z, Hu S and Wang Z: Cloning of *WWOX* gene and its growth-inhibiting effects on ovarian cancer cells. *J Huazhong Univ Sci Technolog Med Sci* 30: 365-369, 2010.
- Qu J, Lu W, Li B, Lu C and Wan X: *WWOX* induces apoptosis and inhibits proliferation in cervical cancer and cell lines. *Int J Mol Med* 31: 1139-1147, 2013.
- Bednarek AK, Keck-Waggoner CL, Daniel RL, Laffin KJ, Bergsagel PL, Kiguchi K, Brenner AJ and Aldaz CM: *WWOX*, the FRA16D gene, behaves as a suppressor of tumor growth. *Cancer Res* 61: 8068-8073, 2001.
- Gourley C, Paige AJ, Taylor KJ, Ward C, Kuske B, Zhang J, Sun M, Janczar S, Harrison DJ, Muir M, *et al*: *WWOX* gene expression abolishes ovarian cancer tumorigenicity in vivo and decreases attachment to fibronectin via integrin $\alpha 3$. *Cancer Res* 69: 4835-4842, 2009.
- Nowakowska M, Pospiech K, Lewandowska U, Piastowska-Ciesielska AW and Bednarek AK: Diverse effect of *WWOX* overexpression in HT29 and SW480 colon cancer cell lines. *Tumor Biol* 35: 9291-9301, 2014.

37. Lewandowska U, Zelazowski M, Seta K, Byczewska M, Pluciennik E and Bednarek AK: WWOX, the tumor suppressor gene affected in multiple cancers. *J Physiol Pharmacol* 60 (Suppl 1): 47-56, 2009.
38. Płuciennik E, Kośła K, Wójcik-Krowiranda K, Bieńkiewicz A and Bednarek AK: The WWOX tumor suppressor gene in endometrial adenocarcinoma. *Int J Mol Med* 32: 1458-1464, 2013.
39. Pecorelli S: Revised FIGO staging for carcinoma of the vulva, cervix, and endometrium. *Int J Gynaecol Obstet* 105: 103-104, 2009.
40. Mo B, Vendrov AE, Palomino WA, DuPont BR, Apparao KB and Lessey BA: ECC-1 cells: A well-differentiated steroid-responsive endometrial cell line with characteristics of luminal epithelium. *Biol Reprod* 75: 387-394, 2006.
41. Pfaffl MW, Horgan GW and Dempfle L: Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. *Nucleic Acids Res* 30: e36, 2002.
42. Sakuragi N, Nishiya M, Ikeda K, Ohkouch T, Furth EE, Hareyama H, Satoh C and Fujimoto S: Decreased E-cadherin expression in endometrial carcinoma is associated with tumor dedifferentiation and deep myometrial invasion. *Gynecol Oncol* 53: 183-189, 1994.
43. Płuciennik E, Kusińska R, Potemski P, Kubiak R, Kordek R and Bednarek AK: WWOX - the FRA16D cancer gene: Expression correlation with breast cancer progression and prognosis. *Eur J Surg Oncol* 32: 153-157, 2006.
44. Wang X, Chao L, Ma G, Chen L, Zang Y and Sun J: The prognostic significance of WWOX expression in patients with breast cancer and its association with the basal-like phenotype. *J Cancer Res Clin Oncol* 137: 271-278, 2011.
45. Spoelstra NS, Manning NG, Higashi Y, Darling D, Singh M, Shroyer KR, Broaddus RR, Horwitz KB and Richer JK: The transcription factor ZEB1 is aberrantly expressed in aggressive uterine cancers. *Cancer Res* 66: 3893-3902, 2006.
46. Singh M, Spoelstra NS, Jean A, Howe E, Torkko KC, Clark HR, Darling DS, Shroyer KR, Horwitz KB, Broaddus RR, *et al*: ZEB1 expression in type I vs type II endometrial cancers: A marker of aggressive disease. *Mod Pathol* 21: 912-923, 2008.
47. Yan H and Sun Y: Evaluation of the mechanism of epithelial-mesenchymal transition in human ovarian cancer stem cells transfected with a WW domain-containing oxidoreductase gene. *Oncol Lett* 8: 426-430, 2014.
48. Palumbo JS, Kombrinck KW, Drew AF, Grimes TS, Kiser JH, Degen JL and Bugge TH: Fibrinogen is an important determinant of the metastatic potential of circulating tumor cells. *Blood* 96: 3302-3309, 2000.
49. Sahni A, Arévalo MT, Sahni SK and Simpson-Haidaris PJ: The VE-cadherin binding domain of fibrinogen induces endothelial barrier permeability and enhances transendothelial migration of malignant breast epithelial cells. *Int J Cancer* 125: 577-584, 2009.