

# Wilms Tumor Gene Protein 1 Is Associated With Ovarian Cancer Metastasis and Modulates Cell Invasion

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Supported by a 2005–2006 Penny Severns Breast, Cervical and Ovarian Cancer Fund grant from the Illinois Department of Public Health (to M.V.B.); a 2006 Ovarian Cancer Research Foundation Program of Excellence award (to M.V.B.); and National Cancer Institute Research Grant R01 CA86984 (to M.S.S.)

We thank Dr. Peter Penzes for critical reading of the article. We also thank Dr. Marianne Kim (Dr. Jonathan Licht Laboratory) for helpful discussions and for providing us with *in vitro*-transcribed WT1 protein and HEK293 cell lysate for the positive detection of WT1 on Western blot analysis.

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Received August 1, 2007; revision received October 16, 2007; accepted October 26, 2007.

**BACKGROUND.** Although metastatic disease is the primary cause of death from epithelial ovarian carcinoma, to the authors' knowledge the cellular mechanisms that regulate intraperitoneal metastasis are largely unknown. Metastasizing ovarian carcinoma cells encounter a collagen-rich microenvironment because the submesothelial matrix is comprised mainly of interstitial collagens Types I and III.

**METHODS.** Immunohistochemistry using primary and metastatic ovarian carcinoma samples was employed to detect expression of Wilms tumor gene protein 1 (WT1). Three-dimensional (3D) collagen culture, real-time reverse transcriptase-polymerase chain reaction, and immunofluorescent staining were used to evaluate changes in WT1 RNA and protein expression in response to 3D collagen culture. Boyden chamber invasion assay, scratch-wound motility assay, and Western blot analysis were used to establish the function of WT1 in ovarian carcinoma cells.

**RESULTS.** To model intraperitoneal invasion *in vitro*, ovarian cancer cells were cultured in a 3D collagen microenvironment. 3D collagen culture resulted in robust induction of WT1 at the mRNA and protein levels. WT1 expression was prevalent in primary ovarian tumors and was retained in paired peritoneal metastases. Functional studies supported a role for WT1 in intraperitoneal invasion, because siRNA knockdown of WT1 expression reduced the ability of ovarian cancer cells to invade 3D collagen gels.

**CONCLUSIONS.** The data from the current study identify a novel regulatory mechanism for the control of WT1 expression and provide evidence for a functional role of WT1 protein in the control of cellular invasive activity. *Cancer* 2008;112:1632–41. © 2008 American Cancer Society.

**KEYWORDS:** ovarian carcinoma, Wilms tumor gene protein 1 (WT1), 3-dimensional collagen, tumor microenvironment, invasion, migration, metastasis.

Epithelial ovarian carcinoma is the leading cause of death from gynecologic malignancy.<sup>1</sup> The lack of reliable biomarkers for widespread early detection combined with relatively nonspecific symptoms lead to the diagnosis of many new patients with advanced ovarian carcinoma (stage III/IV) with poor prognosis. Because the majority of patients with late-stage disease die of metastatic disease, a more detailed understanding of factors that regulate metastasis is warranted. Recent studies have supported a role for the tumor microenvironment (comprised of the primary tumor, neighboring cells, stromal elements, and secretory products) in influencing malignant transformation and tumor progression.<sup>2–4</sup> However, to our knowledge, the role of microenvironmental factors in the development and progression of ovarian carcinoma is not well understood.

Epithelial ovarian tumors are believed to originate from the normal ovarian epithelium that covers the ovarian surface. Malignant cells are shed into the peritoneal cavity and may adhere to the mesothelial surface of intraperitoneal organs, initiate mesothelial cell retraction, and invade the submesothelial matrix to anchor secondary lesions. Microenvironmental factors, particularly Type I collagen, may play a major role in ovarian tumor metastasis because both the submesothelial matrix and the peritoneal fluid are rich in interstitial collagens.<sup>5,6</sup> Aggregation of collagen-binding integrins induces expression of the transcription factor designated early growth response 1 (EGR1)<sup>7</sup> and subsequent induction of membrane type 1-matrix metalloproteinase (MT1-MMP) activity that potentiates collagen invasion<sup>8,9</sup> and thereby likely impacts the anchoring of metastatic lesions.

Wilms tumor gene protein 1 (WT1) is a zinc-finger protein that binds to a DNA sequence element common to the EGR family of transcriptional activators.<sup>10,11</sup> Expression of WT1 is positively associated with epithelial ovarian cancer,<sup>12–20</sup> although to our knowledge its function in ovarian tumor progression and metastasis have not been explored to date. In the current study, a panel of primary ovarian tumors and a subset of primary tumors with matched peritoneal metastases were evaluated for WT1 expression. The role of the interstitial collagen microenvironment in the regulation of WT1 expression was examined and the functional contribution of WT1 protein to cellular invasion and migration was evaluated using siRNA knockdown of WT1 protein. Results demonstrated positive WT1 staining in a majority of primary ovarian tumors and in all metastatic lesions of serous and endometrioid histotype. WT1 expression was found to be strongly induced by culture on 3-dimensional (3D) collagen gels, a model system proposed to mimic submesothelial anchoring. Down-regulation of WT1 protein using siRNA resulted in decreased invasion into 3D collagen gels and retarded cell motility in wound healing assay, without altering the expression of the collagenolytic proteinase MT1-MMP. Together, these data identify a novel regulatory mechanism for the control of WT1 expression and provide evidence of a functional role of WT1 protein in the control of cellular invasive activity via modulation of cell migration.

## MATERIALS AND METHODS

### Materials

Monoclonal anti-Wilms tumor gene protein 1 (WT1) (clone 1E9) was purchased from Chemicon International (Temecula, Calif). Polyclonal antihuman MT1-

MMP (hinge region) antibodies, monoclonal anti- $\beta$ -tubulin (clone TUB2.1) and antimouse immunoglobulin G horseradish peroxidase-conjugated antibodies, human collagen Type I, and bacterial collagenase were obtained from Sigma Chemical Company (St. Louis, Mo). Goat antimouse-Alexa488 antibody was purchased from Invitrogen Corporation (Carlsbad, Calif). VECTASHIELD mounting medium with 4',6-diamidino-2-phenylindole (DAPI) was obtained from Vector Laboratories (Burlingame, Calif).

### Immunohistochemistry

Immunohistochemical analysis was performed retrospectively on tumor tissue microarrays prepared by the Pathology Core Facility of the Robert H. Lurie Comprehensive Cancer Center at Northwestern University assembled from tissue originally taken for routine diagnostic purposes as well as surgeries for the resection of metastatic ovarian carcinoma obtained under a protocol approved by the Institutional Review Board. The microarray tissue specimens included 114 primary human ovarian carcinomas (56 serous, 37 endometrioid, 6 mucinous, and 15 clear cell). An array of paired primary and metastatic ovarian carcinoma tissues was also prepared, and included 17 primary and 17 metastatic ovarian carcinoma tissues obtained from each individual patient during the same surgical procedure (15 serous ovarian carcinomas and 2 endometrioid ovarian carcinomas). All cores measured 1 mm in greatest dimension. Samples were cut 3- $\mu$ m to 4- $\mu$ m thick and deparaffinized. Antigen retrieval was accomplished by heat induction at 99°C for approximately 45 minutes. Immunohistochemical staining with antibodies to WT1 (Chemicon) at 1:200 dilution was performed according to standard procedures. Analysis of tissue sections was performed by light microscopy by a pathologist (B.P.A.) without prior knowledge of the clinical variables. Scoring of WT1 was assigned according to the intensity of the staining using a 4-tiered scale and graded as 0 (negative; with <25% cells positive), 1+ (weak; 25–50% positive cells), 2+ (moderate; 51–75% positive cells), or 3+ (strong; >75% positive cells). Membranous and/or cytoplasmic staining was not considered positive. Statistical analyses were performed by the Biostatistics Core Facility of the Robert H. Lurie Comprehensive Cancer Center.

### Cell Culture

The ovarian carcinoma cell lines DOV13 and OVCA433 were provided by Dr. R. Bast, Jr (University of Texas M.D. Anderson Cancer Center, Houston, Tex) and maintained in complete media (Gibco Invi-

trogen, Carlsbad, Calif) supplemented with 10% fetal bovine serum (Gibco Invitrogen) at 37°C in 5% carbon dioxide as previously described.<sup>21</sup>

### 3D Collagen Type I Cell Culture Model

To mimic the initial stages of submesothelial matrix invasion by ovarian cancer cells, cells in suspension were plated on top of a 3D collagen Type I (3DCI) gel, cultured for various periods of time, and collected in the presence of collagenase as described previously.<sup>7</sup> Control cells were plated on thin-layer collagen Type I (2-dimensional collagen Type I [2DCI]) and prepared by coating tissue culture plates by passive adsorption with a dilute (10 µg/mL) solution of rat tail collagen Type I overnight.

### mRNA Extraction and cDNA Synthesis

Total mRNA was purified from 1 to 2 × 10<sup>6</sup> cells using Aurum Total RNA Mini Kit (Bio-Rad Laboratories, Hercules, Calif) according to the manufacturer's instructions. cDNA was synthesized from 10 µg of total RNA using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories). mRNA purification and cDNA synthesis experiments were repeated 4 times.

### Quantitative Real-time Polymerase Chain Reaction

Real-time polymerase chain reaction (PCR) was performed with the ABI Prizm (Applied Biosystems, Foster City, Calif) according to the manufacturer's instructions. SYBR green was used for quantitative PCR as a double-stranded DNA-specific fluorophore. The PCR was conducted by initial denaturation for 10 minutes at 95°C followed by 40 cycles of 94°C for 15 seconds and 60°C for 30 seconds using the iTaq SYBR Green Supermix (Bio-Rad Laboratories). To determine the specificity of the PCR primers, melting curves were collected by heating the products at 95°C, then cooling down to 65°C, and then slowly melting at 0.5°/second up to 95°C. Primers for WT1 mRNA detection were constructed in accordance with the requirements (sense: 5'TGCCTCGA-GAGCCAGC 3'; antisense: 5'GTGTGACCGTAGCTGG 3') and, as a control, previously published primers were synthesized.<sup>22</sup> Both sets of primers yielded similar results. The efficiency of the amplification was determined using the standard curves method. Relative quantification of gene expression between experimental (3DCI) and control (2DCI) samples was measured by normalization against endogenous RPL-19 using the  $\Delta C_T$  method.<sup>23</sup> Before using RPL-19 as a control, it has been established that its expression correlated well with the total RNA concentration and did not change with the time and treatment

used in our studies. Fold changes were quantified as  $2^{-(\Delta C_{T\text{sample}} - \Delta C_{T\text{control}})}$ , as described previously.<sup>23</sup>

### Western Blot Analysis

Cells incubated under various conditions were collected as described previously<sup>7</sup> and lysed with buffer containing 50 mM of Tris, 150 mM of NaCl, 1 mM of CaCl<sub>2</sub>, 1 mM of MgCl<sub>2</sub>, 1% NP40, and proteinase inhibitor cocktail (Roche, Basel, Switzerland). Cell lysates (20 µg) were electrophoresed on 9% sodium dodecyl sulfate-polyacrylamide gels under reducing conditions,<sup>24</sup> electroblotted to a polyvinylidene difluoride (PVDF) membrane,<sup>25</sup> and blocked with 5% skim milk in Tris-buffered saline with 0.1% Tween 20 (TBST) (25 mmol/L of Tris [pH 7.5], 150 mmol/L of NaCl, and 0.1% Tween 20) for 1 hour at room temperature (20°C). Membranes were incubated for 1 to 2 hours at room temperature with antibodies derived against proteins of interest. The antibodies were used at the following dilutions: 1:1000 for antihuman MT1-MMP polyclonal antibody in 3% bovine serum in TBST, and 1:1000 for anti-β-tubulin monoclonal antibody in 5% skim milk in TBST. Immunoreactive bands were observed with an anti-(rabbit-IgG)-peroxidase or anti-(mouse-IgG)-peroxidase (1:1000 in 5% skim milk in TBST) and enhanced chemiluminescence using LAS3000 (Fujifilm, Tokyo, Japan) and LAS3000 ImageReader software. Band intensities were determined using LAS3000 ImageGauge software according to the manufacturer's instructions.

### Immunocytochemistry

Cells were cultured on glass slides coated with a thin layer of collagen Type I or covered with 3DCI gels as described earlier. Conditioned media were then removed and cells were fixed onto the glass slide, followed by permeabilization for 1 hour with 0.1% Triton X-100 in phosphate-buffered saline (PBS) containing 10% goat serum. Permeabilized cells were incubated for 24 hours at 4°C with a 1:200 dilution of monoclonal anti-WT1 antibody in PBS containing 2% goat serum. DAPI staining used to observe DNA. Mouse antihuman WT1 antibody was used at a 1:500 dilution, and secondary goat antimouse-Alexa488 antibody at a 1:500 dilution was used to observe WT1 expression in cells. Images were taken with an Axiovert Zeiss fluorescent microscope (Zeiss Inc, Thornwood, NY).

### Transient Transfections

Transient transfections were performed using the lipofection method with Lipofectamine (Invitrogen, Carlsbad, Calif.) as a vehicle. WT1 and control siRNA (Santa Cruz Biotechnology, Santa Cruz, Calif) were

transiently transfected into DOV13 and OVCA433 cells according to the manufacturer's instructions. Down-regulation of WT1 mRNA expression in cells transfected with WT1 siRNA was verified by real-time reverse transcriptase (RT)-PCR in comparison with the cells transfected with control siRNA, and amounted to 2.5-fold and 6.5-fold for DOV13 and OVCA433, respectively. WT1 was efficiently silenced on the protein level in both DOV13 and OVCA433 cell lines as well.

### Cellular Invasion Assay

Invasion assays were performed using Transwell chambers (0.8  $\mu$ m; Becton-Dickinson Immunocytometry Systems, Braintree, Mass) as described previously.<sup>9</sup> In brief, Transwell inserts were coated on the bottom with a thin layer of human collagen (Sigma Chemical Company) as a chemoattractant for 1 hour at 37°C. The inner well of the filters contained 20  $\mu$ g of human collagen that was allowed to air-dry overnight. Cells were grown to confluency and starved overnight in serum-free, insulin-free media to stop proliferation. Cells (70,000) were plated in the collagen-coated inserts and allowed to invade the collagen gel for 20 hours at 37°C in serum-free conditions. Filters were collected and the cells adhering to the lower surface were fixed and stained using the Diff-Quik staining kit (Dade Behring, Deerfield, Ill) according to the manufacturer's instructions. Cells in several random fields were counted and averaged. Invading cells were expressed as a percentage of the total cells added to the invasion chamber.

### Wound Healing Assay

DOV13 and OVCA433 cells were cultured in standard conditions, as described above, until 70% to 80% confluency after the transient transfections with either WT1 siRNA, control siRNA, or PBS as indicated in earlier. Scratch wounds were introduced into the confluent monolayers using a pipette tip and wound closure was monitored over time and photographed using a Zeiss Axiovert microscope at  $\times$ 8.8 magnification on the objective lens. The distance between the wound edges was measured using Zeiss Axiovert software in 10 random locations to calculate relative wound closure relative to the initial wound.

## RESULTS

### Analysis of WT1 Expression in Primary and Metastatic Ovarian Carcinomas

Previous studies have demonstrated WT1 expression in epithelial ovarian carcinoma, with serous ovarian carcinomas reported as variably (54–100%) positive

for WT1, whereas the mucinous, clear cell, and endometrioid histotypes were predominantly negative.<sup>12–20</sup> Generally, correlations with tumor stage or grade were not reported. In the current study, a total of 114 primary ovarian carcinomas were evaluated for WT1 expression, including 56 serous, 37 endometrioid, 6 mucinous, and 13 clear cell carcinomas (Table 1). Overall, 56% of all tested specimens were positive for WT1, characterized by diffuse staining, including 77% of serous and 38% of endometrioid ovarian carcinomas (Figs. 1A and 1B). Positive staining was observed predominantly in stage III and IV tumors (Table 1). One case of mucinous ovarian carcinoma (total number,  $n = 6$ ) (Fig. 1C) and 2 cases clear cell ovarian carcinoma (total number,  $n = 15$ ) (Fig. 1D) were positive for WT1 and displayed focal staining.

To determine whether elevated WT1 expression is observed in metastatic lesions, 17 cases of paired primary and metastatic tumor tissue were evaluated. All the metastases were positive for WT1, with strong staining (2+ to 3+) observed in 88% of cases (Table 2). In 8 cases (47%), WT1 expression levels were higher in the metastasis compared with the matched primary tumor (Table 2) (Fig. 2B). The majority of primary tumors exhibiting very strong (3+) staining maintained very strong expression in the metastatic lesion (Table 2). It is interesting to note that 1 of the specimens had a primary tumor that was negative for WT1, but a paired sample of metastatic tissue was positive (Table 2, Case 16). Only 2 cases exhibited weaker WT1 expression in the metastatic lesion compared with the primary tumor (Table 2). Overall, 88% of cases demonstrated WT1 expression levels in the metastasis that were greater than or equal to those in the corresponding primary lesion.

### Expression of WT1 is Up-regulated by 3D Collagen

Because WT1 expression was correlated with advanced stage disease and was prevalent in extra-ovarian metastases, additional studies were performed to evaluate potential mechanisms by which the expression of WT1 may be regulated. We have previously demonstrated that ovarian carcinoma cells adhere preferentially to interstitial collagen Type I,<sup>9,21</sup> a major component of the submesothelial matrix into which ovarian cancer cells locally invade and anchor secondary lesions.<sup>7–9,21,26</sup> Culturing ovarian carcinoma cells in 3DCI engages the  $\alpha$ 3 $\beta$ 1-integrin-signaling pathway<sup>9,27</sup> and, through up-regulation of EGR1,<sup>7</sup> causes overexpression of MT1-MMP, which contributes to collagen invasion.<sup>8,9</sup> To evaluate the effect of 3DCI culture on WT1 expression, 2 representative serous ovarian carcinoma cell lines (DOV13 and OVCA433) were cultured on 3DCI compared

**TABLE 1**  
**Distribution of WT1 Staining According to Stage in Ovarian Tumors of Various Histotypes**

Stage	Staining intensity*	Serous	Endometrioid	Mucinous	Clear cell
IV	Strong positive (3+)	2	No samples	No samples	No samples
	Positive (2+)	3			
	Weakly positive (1+)	3			
	Negative	3			
	Total tumors	11			
	Total positive	8 (73%)			
III	Strong positive (3+)	21	7	0	0
	Positive (2+)	6	3	1	2
	Weakly positive (1+)	7	0	0	0
	Negative	5	7	1	5
	Total tumors	39	17	2	7
	Total positive	34 (87%)	10 (59%)	1 (50%)	2 (29%)
II	Strong positive (3+)	0	0	No samples	0
	Positive (2+)	0	2		0
	Weakly positive (1+)	2	0		0
	Negative	2	7		3
	Total tumors	4	9		3
	Total positive	2 (50%)	2 (22%)		0 (0%)
I	Strong positive (3+)	0	1	0	0
	Positive (2+)	1	0	0	0
	Weakly positive (1+)	0	1	0	0
	Negative	1	9	4	3
	Total tumors	2	11	4	3
	Total positive	1 (50%)	2 (18%)	0 (0%)	0 (0%)
Overall total	Total tumor	56	37	6	13
	Total positive	45 (82%)	14 (38%)	1 (17%)	2 (15%)

WT1 indicates Wilms' tumor gene protein 1.

\* WT1 staining intensity was designated as negative, 1+ (weakly positive), 2+ (positive), and 3+ (strong positive).

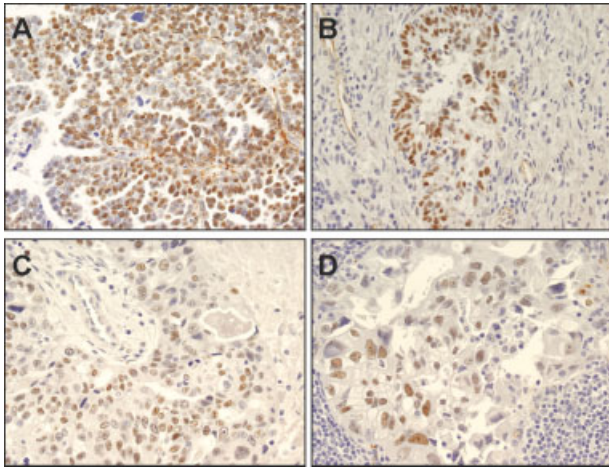
with thin-layer collagen Type I (2DCI) and changes in WT1 mRNA expression were quantified using real-time RT-PCR. WT1 mRNA was found to be increased in cells cultured on 3DCI (28-fold for OVCA433 and 12-fold for DOV13), with maximum induction observed at 30 minutes of 3DCI culture (Fig. 3A). The increase in WT1 mRNA was followed by increased WT1 protein expression in 3DCI gels after 5 hours of culture, as demonstrated by immunocytochemistry and Western blot analysis (Figs. 3B-3E). Together these data demonstrate that 3DCI is a potent inducer of WT1 expression in serous ovarian carcinoma cells.

### WT1 Modulates Collagen Invasion Through Modulation of Cell Migration

Culturing ovarian carcinoma cells in 3DCI provides an in vitro model for the microenvironmental regulation of cellular behavior, because metastasizing cells locally invade the submesothelial collagen Type I-rich matrix to anchor secondary lesions. To determine whether the WT1 protein may play a role in collagen invasion, the WT1 gene was silenced in the DOV13 and OVCA433 cell lines using siRNA and the

ability of cells to invade 3DCI gels was evaluated. Cells with silenced WT1 invaded collagen approximately 30% to 40% less efficiently than wild-type cells or cells transfected with control siRNA (Figs. 4A and 4B), indicating that WT1 expression modulates collagen invasion.

Invasion of collagen Type I by ovarian cancer cells is accomplished through the collagenolytic activity of the MT1-MMP.<sup>7-9,27</sup> Culturing ovarian cancer cells in 3DCI up-regulates the EGR1 gene, resulting in transcriptional activation of the MT1-MMP promoter.<sup>7</sup> Because WT1 is also an EGR family protein that binds to the same consensus-binding sequence,<sup>10,11</sup> the effect of WT1 on MT1-MMP expression was evaluated. To examine the potential role of WT1 as a regulator of MT1-MMP, DOV13 cells were transfected with WT1 siRNA, control siRNA, and PBS. Transfected cells were cultured on thin-layer and 3DCI for 24 hours followed by analysis of MT1-MMP expression by Western blot analysis (Fig. 4C). WT1 silencing was not found to lead to down-regulation of MT1-MMP on 3DCI, indicating that the enhanced collagen invasion observed earlier does not result



**FIGURE 1.** Immunohistochemical analysis of Wilms tumor gene protein 1 (WT1) expression in primary human epithelial ovarian tumors. Tumors were stained with an antibody to WT1 (at 1:200 dilution). (A and B) Representative WT1-positive cases of (A) serous and (B) endometrioid ovarian tumors. (C and D) WT1-positive examples of (C) mucinous and (D) clear cell ovarian carcinomas.

from WT1-induced changes in MT1-MMP expression.

It has been demonstrated that a 17AA(-)KTS(-) isoform of WT1 was able to induce morphologic changes and promote cell migration.<sup>28</sup> Although a detailed WT1 isoform analysis was not performed in the course of the current study, several WT1 bands were observed by Western blot analysis (Fig. 3D). To evaluate further the role of WT1 in promoting ovarian cancer cell migration, cells were transfected with WT1 siRNA, control siRNA, and PBS and analyzed using a scratch-wound motility assay (Fig. 4D and 4E). Both DOV13 and OVCA433 cell lines that were transfected with WT1 siRNA demonstrated slower migration compared with the control. These data suggest that modulation of collagen invasion (Figs. 4A and 4B) by WT1 may be manifested mechanistically via altered cell motility.

**DISCUSSION**

WT1 was initially identified as one of the key players in the development of the childhood kidney neoplasm Wilms tumor.<sup>10,22,29</sup> Since its discovery, a growing number of studies have demonstrated the involvement of WT1 in cancers of other origins.<sup>30-32</sup> WT1 functions as a transcriptional regulator, but may function as either an activator or repressor depending on cellular context.<sup>30</sup> Similarly, WT1 has been reported to function as both a tumor suppressor and an oncogene in tumors of different origin.

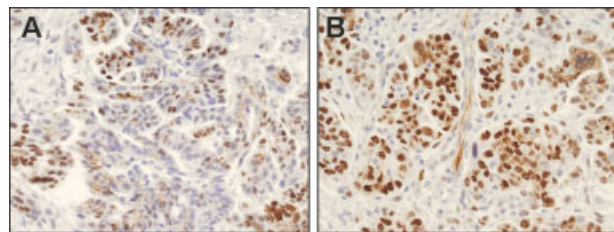
**TABLE 2**  
Immunohistochemical Analysis of Primary and Metastatic Ovarian Cancer Tumors

Case No.*	Specimen type	WT1†
1	Primary	2+, f
	Metastasis	2+, d
2	Primary	3+, d
	Metastasis	3+, d
3	Primary	2+, d
	Metastasis	3+, d
4	Primary	2+, f
	Metastasis	3+, d
5	Primary	2+, d
	Metastasis	3+, d
6	Primary	3+, d
	Metastasis	3+, d
7	Primary	2+, d
	Metastasis	3+, d
8	Primary	3+, d
	Metastasis	3+, d
9	Primary	2+, d
	Metastasis	2+, f
10	Primary	1+, f
	Metastasis	2+, d
11	Primary	2+, d
	Metastasis	3+, d
12	Primary	3+, d
	Metastasis	3+, d
13	Primary	2+, d
	Metastasis	1+, d
14	Primary	2+, f
	Metastasis	2+, d
15	Primary	2+, d
	Metastasis	1+, d
16, endometrioid	Primary	Negative
	Metastasis	2+, d
17, endometrioid	Primary	1+, d
	Metastasis	2+, d

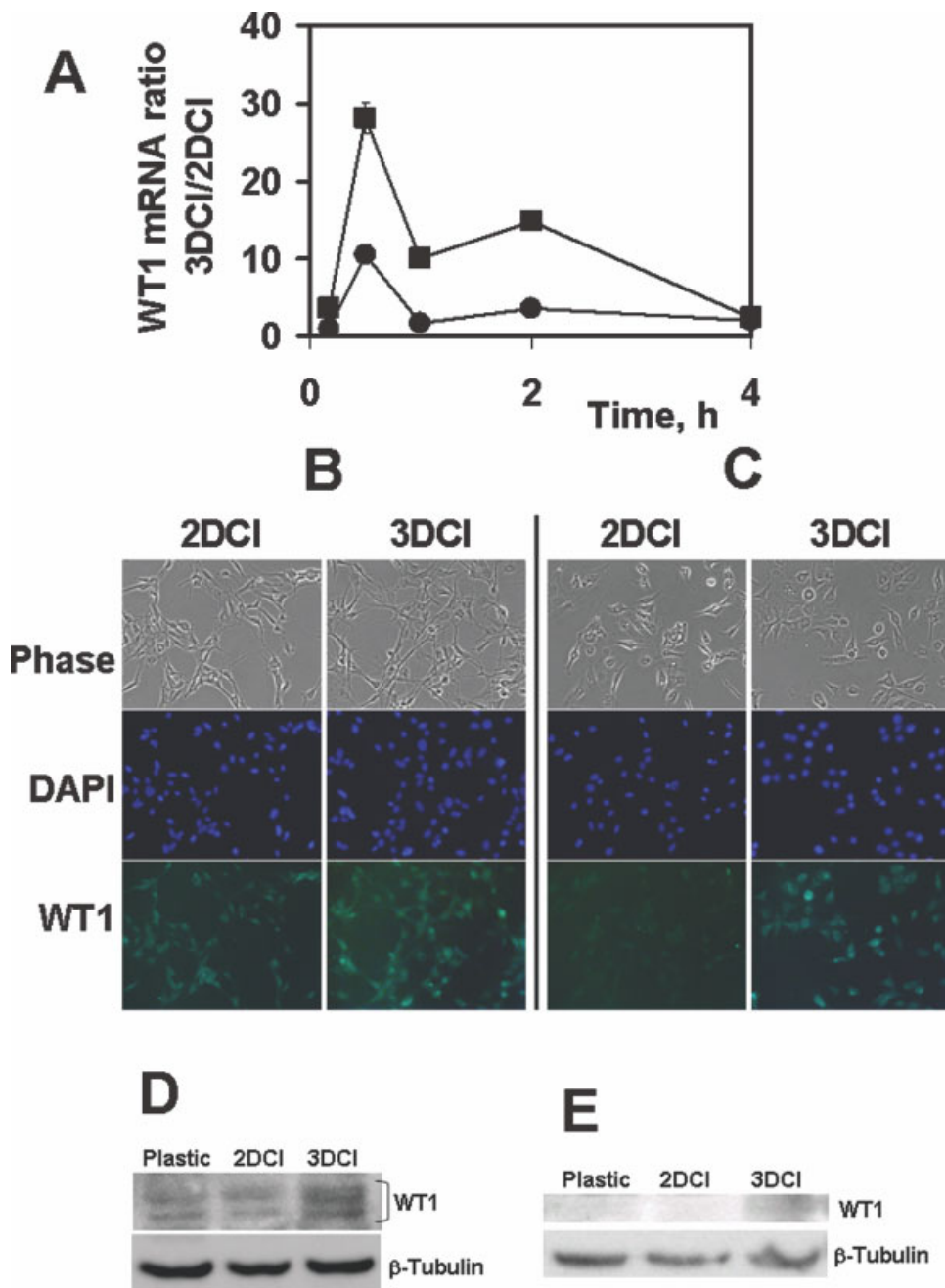
WT1 indicates Wilms' tumor gene protein 1; f, focal; d, diffuse.

\* Fifteen tumors were classified as serous histotype (Samples 1-15) and 2 tumors were classified as endometrioid histotype (Samples 16 and 17, as indicated).

† WT1 staining intensity was described as negative, 1+ (weakly positive), 2+ (positive), and 3+ (strong positive).



**FIGURE 2.** Immunohistochemical analysis of Wilms tumor gene protein 1 (WT1) expression in paired primary and metastatic human epithelial ovarian tumors. (A and B) Paired (A) primary tumor and (B) the corresponding metastatic lesion were stained with antibody directed against WT1 (at 1:200 dilution).

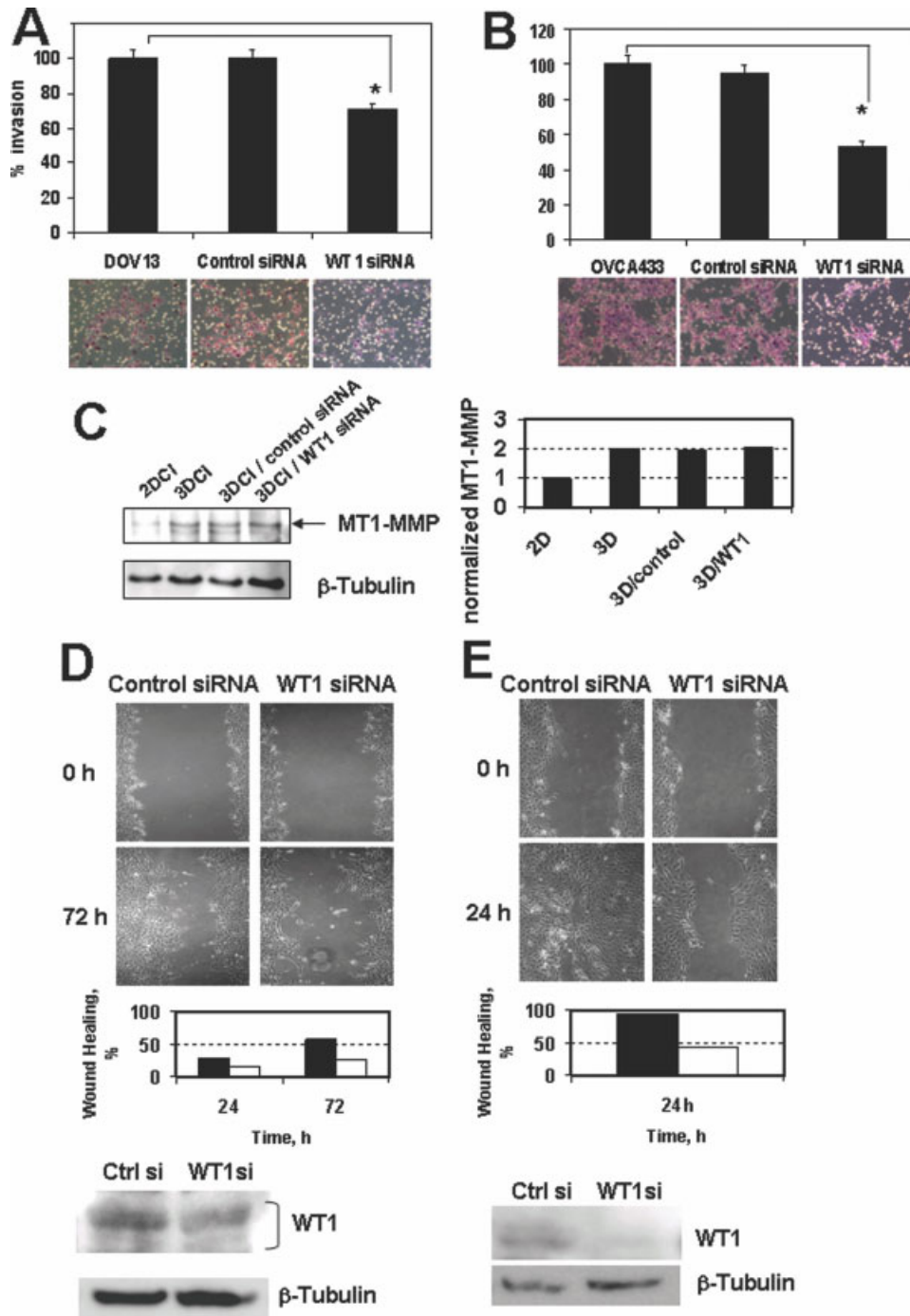


**FIGURE 3.** Three-dimensional (3D) collagen culture increases expression of Wilms tumor gene protein 1 (WT1). (A) Cells were cultured on thin-layer collagen Type I coating (2DCI) or 3D collagen Type I (3DCI) gels for the indicated time periods. Total mRNA was purified, cDNA was synthesized, and quantitative real-time reverse transcriptase-polymerase chain reaction was performed. Results are plotted as the expression ratio, indicating the fold change of mRNA expression in cells cultured on 3DCI gel compared with 2DCI. Closed circles (●) and closed squares (■) represent data obtained for the DOV13 and OVCA433 cell lines, respectively. Data are shown as the mean  $\pm$  standard deviation, and are an average of 3 independent experiments. Immunofluorescent images of (B) DOV13 and (C) OVCA433 were cultured for 5 hours on 2DCI and 3DCI. The upper panels show bright field images of the cells, the middle panels show nuclear staining with 4', 6-diamidino-2-phenylindole (DAPI), and the bottom panels represent WT1-specific staining in cells. Images were taken at  $\times 32$  magnification using an Axiovert Zeiss fluorescent microscope. Western blot analysis of WT1 expression in (D) DOV13 cells and (E) OVCA433 cells cultured on tissue culture-treated plastic, 2DCI, and 3DCI.

For example, WT1 functions as a tumor suppressor in the formation of Wilms tumors, whereas high WT1 expression is reported to be correlated with poor prognosis for patients with acute myeloid leukemia, astrocytic tumor, and breast cancer.<sup>33-35</sup> Poor outcome for clear cell ovarian adenocarcinoma patients is correlated with epigenetic silencing of WT1<sup>36</sup>; however, expression of WT1 in women with serous epithelial ovarian carcinoma is indicative of an unfavorable prognosis.<sup>37</sup> It has been speculated that WT1 is involved in the maintenance of a mesenchymal-

epithelial balance during development<sup>30</sup>; thus, it is interesting to speculate that aberrant regulation of WT1 expression in ovarian tumors may function to promote the mesenchymal-epithelial transition observed in early ovarian tumorigenesis.<sup>38-40</sup>

It has been suggested that WT1 expression is tissue specific and is controlled by additional regulatory elements.<sup>41</sup> For example, HER-2/*neu* and Pea3 were previously identified as increasing WT1 expression,<sup>42,43</sup> whereas WT1 expression was reportedly down-regulated by the Sry transgene.<sup>44</sup> Exogenous



**FIGURE 4.** Wilms tumor gene protein 1 (WT1) gene silencing retards collagen invasion through migration. (A and B) Boyden chamber 3-dimensional (3D) collagen invasion assay. (A) DOV13 and (B) OVCA433 ovarian cancer cell lines were transfected with siRNA for WT1 or control siRNA, as indicated, and invasion of 3D collagen gels was evaluated in a modified Boyden chamber assay. Histograms show the percentage of invading cells, with invasion of wild-type cells designated as 100%. Data represent an average of 3 independent experiments. \*indicates  $P < .05$ . Representative bright field images of cells invading collagen Type I in Boyden chambers are shown below the histograms. (C) WT1 does not affect membrane type 1-matrix metalloproteinase (MT1-MMP) expression on 3D collagen. Ovarian cancer cells (DOV13) were transfected with WT1 siRNA, control siRNA, and phosphate-buffered saline (PBS) and cultured on thin-layer collagen Type I coating (2DC1) or 3D collagen Type I (3DC1) gel for 24 hours. Cell lysates were examined for MT1-MMP expression using Western blot analysis. The histogram to the right demonstrates a quantitative analysis of MT1-MMP expression compared with the loading control,  $\beta$ -tubulin, and is an average of 2 independent experiments. The standard deviation was  $\pm 10\%$ . (D and E) Scratch-wound motility assay. (D) DOV13 cell monolayers were wounded and observed over the indicated time periods, 24 hours and 72 hours. (E) OVCA433 cell monolayers were wounded and observed after 24 hours. Note that the cell motility of both cell lines that were transfected with PBS was the same as that for cells transfected with the control siRNA, and therefore the former are not shown in the panel. Photographs of the wounds are representative of 2 independent experiments that were performed and quantified. Histograms illustrate the percentage of cell motility in the wound healing assay with the standard deviation of  $\pm 10\%$ . Black and open bars represent the percentage of wound healing in cell monolayers transfected with control siRNA and WT1 siRNA, respectively. Images from Western blot analysis show down-regulation of WT1 protein using WT1-specific siRNA.

factors, such as the addition of curcumin to leukemic cells<sup>45</sup> and hypoxic culture conditions (through HIF1)<sup>46</sup> can also down-regulate WT1. Recent studies have linked WT1 with  $\beta$ -catenin signaling,<sup>47–51</sup> suggesting a potential mechanism for the activation of Tcf/Lef-responsive target genes. WT1 was shown to play various roles, including promotion of cell adhesion by transcriptional activation of the  $\alpha$ 4 integrin<sup>52</sup> and cell migration and invasion via modulation of the cytoskeleton.<sup>28</sup> The current data support a model wherein metastasizing ovarian tumor cells encounter intraperitoneal collagen, both in the submesothelial matrix<sup>5</sup> and in ascites as a component of the ovarian tumor fibroproliferative response,<sup>6</sup> resulting in integrin-mediated up-regulation of WT1 expression. However, although to our knowledge prospective target genes of WT1 in this model are unknown, functional studies support WT1 as an active participant in invasion through enhanced cell motility because silencing of WT1 expression significantly impacts both motility and the ability to invade 3D collagen-rich matrices.

The data from the current study are in agreement with previous studies demonstrating a high level of WT1 expression in serous ovarian carcinomas, with more restricted expression noted in other histotypes.<sup>12–20</sup> Furthermore, the current study results indicate that WT1 expression is most prevalent in advanced stage tumors (stage III/stage IV) as well as in 100% of metastatic lesions, suggesting that WT1 expression is enhanced during progression to metastasis.<sup>53</sup> Several investigations have evaluated WT1 as a target for therapeutic intervention in various types of malignant tumors and a WT1 peptide vaccine reportedly induced regression of leukemia, as well as lung and breast cancers.<sup>54</sup> Data from the current study and others<sup>53</sup> support WT1 as a potential novel therapeutic target for the control of serous ovarian carcinoma metastasis.

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