WWOX Gene Expression Abolishes Ovarian Cancer Tumorigenicity *In vivo* and Decreases Attachment to Fibronectin via Integrin α_3

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Abstract

The WW domain–containing oxidoreductase (*WWOX*) gene is located at FRA16D, a common fragile site involved in human cancer. Targeted deletion of *Wwox* in mice causes increased spontaneous tumor incidence, confirming that *WWOX* is a bona fide tumor suppressor gene. We show that stable transfection of *WWOX* into human PEO1 ovarian cancer cells, containing homozygous *WWOX* deletion, abolishes *in vivo* tumorigenicity, but this does not correlate with alteration of *in vitro* growth. Rather, *WWOX* restoration in PEO1, or *WWOX* overexpression in SKOV3 ovarian cancer cells, results in reduced attachment and migration on fibronectin, an extracellular matrix component linked to peritoneal metastasis. Conversely, siRNA-mediated knockdown of endogenous *WWOX* in A2780 ovarian cancer cells increases adhesion to fibronectin. In addition, whereas there is no *WWOX*-dependent difference in cell death in adherent cells, *WWOX*-transfected cells in suspension culture display a proapoptotic phenotype. We further show that *WWOX* expression reduces membranous integrin α_3 protein but not integrin α_3 mRNA levels, and that adhesion of PEO1 cells to fibronectin is predominantly mediated through integrin α_3. We therefore propose that *WWOX* acts as an ovarian tumor suppressor by modulating the interaction between tumor cells and the extracellular matrix and by inducing apoptosis in detached cells. Consistent with this, the suppression of PEO1 tumorigenicity by *WWOX* can be partially overcome by implanting these tumor cells in Matrigel. These data suggest a possible role for the loss of *WWOX* in the peritoneal dissemination of human ovarian cancer cells. [Cancer Res 2009;69(11):4835–42]

Introduction

The *WWOX* gene extends over >1 Mb and spans a common fragile site that has been implicated in human tumorigenesis (1). *WWOX* encodes a 46-kDa protein containing two WW domains and an oxidoreductase domain (2, 3). *WWOX* is homozygously deleted in a number of human cancers (4). In addition, decreased or aberrant *WWOX* expression has been shown in multiple tumor types (4–9). Bednarek and colleagues (10) reported that ectopic *WWOX* expression suppressed the *in vivo* tumorigenicity of breast cancer cells in nude mice and their clonogenicity in soft agar, but did not alter *in vitro* cell proliferation. Similarly, adenoviral transfection of *WWOX* into lung and prostate cancer cell lines deficient in *WWOX* expression resulted in decreased tumorigenicity in nude mice (11, 12). Two independent mouse knockout models for *WWOX* showed increased spontaneous tumor incidence, confirming that *WWOX* is a bona fide tumor suppressor. Targeted deletion of *Wwox* resulted in spontaneous development of osteosarcomas in juvenile $Wwox^{-/-}$ mice and lung papillary carcinoma in adult $Wwox^{+/-}$ mice (13). The same study observed an increased rate of ethylnitrosourea-induced lung tumors and lymphomas in $Wwox^{+/-}$ mice compared with wild-type littermates. The same group showed that *N*-nitrosomethylbenzylamine induced forestomach tumors in 96% of $Wwox^{+/-}$ mice compared with 29% of $Wwox^{+/+}$ mice (14), with 27% of the $Wwox^{+/-}$ mice exhibiting invasive squamous cell carcinomas compared with none of the $Wwox^{+/+}$ mice. Independently, Ludes-Meyers and colleagues (15) created *Wwox* hypomorphic mice using a gene trap vector. These mice developed B-cell lymphomas at increased incidence, further suggesting a role for *Wwox* in multiple tumor types.

Induction of apoptosis has been proposed as the possible mechanism of *WWOX* tumor suppression. Transfection of Ad-*WWOX* virus in lung and prostate tumors induced apoptosis via a caspase-dependent mechanism (11, 12). The same group have shown that WWOX can directly bind p73 protein via its WW domains, resulting in enhanced p73-mediated apoptosis (16). Similarly, Chang and colleagues (17) reported that WWOX can bind the p53 tumor suppressor protein and enhance the apoptotic response to tumor necrosis factor. However, suppression of tumorigenicity in breast cancer was not associated with increased apoptosis (10), suggesting that other mechanisms and contexts of *WWOX* tumor suppression exist.

We previously identified that *WWOX* is disrupted by homozygous deletion (4) and shows frequent and significantly decreased expression (7) in ovarian cancer. In addition, in a panel of 444 human ovarian cancers, Nunez and colleagues (18) showed that significant loss of WWOX protein occurred in 30% of serous ovarian tumors, and was even more common in the two most aggressive ovarian cancer histotypes (mucinous and clear cell). Loss of *WWOX* expression in ovarian tumorigenesis may result from epigenetic promoter methylation, which has been shown in breast and lung cancer (19), and was detected by methylation microarray analysis in ovarian tumors (20). Loss of WWOX expression in ovarian cancer associated with advanced stage disease and decreased overall survival, further supporting an ovarian tumor suppressor role of *WWOX* (18).

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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Here we report that transfection of *WWOX* into ovarian cancer cells lacking *WWOX* expression abolishes their *in vivo* tumorigenicity in nude mice, promotes apoptosis in suspension culture *in vitro*, but does not alter their *in vitro* growth rate. Furthermore, *WWOX* reduces membranous integrin α_3 activity and decreases tumor cell adhesion to fibronectin. We propose that *WWOX* suppresses the tumorigenicity and regulates the peritoneal dissemination of ovarian cancer cells by modulating the interaction of tumor cells with the extracellular matrix (ECM) and promoting apoptosis in detached cells.

Materials and Methods

Tumor cell lines. The PEO1 ovarian cancer cell line (21) is homozygously deleted for *WWOX* exons 4 to 8 (4) and lacks WWOX protein expression. Clonality was achieved by transfection with a hygromycin resistance vector and then positively selecting for growth. The *WWOX* open reading frame was cloned into the pEF6/V5-His-TOPO cloning vector (Invitrogen) and transfected using Effectene (Qiagen) to produce several independent clonal lines as previously described (7). A2780 and SKOV3 human ovarian cancer cell lines (22, 23) were obtained from the European Collection of Cell Cultures.

Cells were maintained in RPMI containing 10% FCS, 100 units/mL penicillin, 100 μg/mL streptomycin (and 50 μg/mL hygromycin in PEO1 cells) and cultured at 37°C, 5% CO_2.

Tumorigenicity. The procurement, husbandry, and experiments on nude mice conformed to the United Kingdom Co-ordinating Committee on Cancer Research Guidelines for the Welfare of Animals in Experimental Neoplasia. Cells (5×10^6) in 100 μL serum-free medium [or 50 μL serum-free medium and 50 μL Matrigel (BD Biosciences) for Matrigel experiments] were injected s.c. into each flank of five nude mice for each cell line.

Growth curves. Log-phase cells (1×10^5 per well) were seeded into six-well plates and incubated at 37°C, 5% CO_2 and then harvested and counted at 0, 2, 5, 7, 9, and 11 d.

Soft-agar proliferation assays. One percent Seaplaque agarose (2 mL/well; BioWhittaker) was added to six-well plates and incubated overnight (4°C). Five thousand cells in 3 mL of 0.4% Seaplaque agarose were added to each well. After 24 h, 1 mL of medium was added. Colonies were counted by direct microscopy after 28 d.

Clonogenicity assays. Log-phase cells were syringed and counted using a hemocytometer. Two hundred cells (multiplicity <1.05) in 4 mL of medium were seeded into 10-mL Petri dishes and incubated for 21 d. Cells were fixed in 2 mL of 2:1 acetone/methanol and stained with hematoxylin, and then colonies were counted.

Transwell migration assays. Transwell cell culture inserts (Costar) were prepared as per manufacturer's instructions. Each transwell was coated with fibronectin or bovine serum albumin (BSA; control), blocked with 0.1% BSA, and then washed with PBS. Cells (5×10^4) in 100 μL serum-free medium were aliquoted into the upper compartment of each transwell and incubated for 84 h.

Transient transfections. Cells (5×10^5) were transfected with 1 to 1.5 μg of WWOX-expressing plasmid or empty vector, in a 1:3 ratio with Lipofectamine 2000 (Invitrogen), in 500 μL of RPMI. This was replaced after 4 to 6 h with medium containing 10% FCS, and cells were replated 1:3 after 12 h. Cells were used 48 h later, unless specified otherwise.

RNAi transfections. Cells (2.5×10^5 per well) were incubated in a six-well plate for 2 d. One hundred microliters of 2 μmol/L siRNA Smartpool for *WWOX* or nontargeting oligonucleotide 1 (Perbio) were mixed with 100 μL of OptiMem medium for 5 min. Separately, 5 μL of Dharmafect transfection reagent 1 (Perbio) were incubated with 195 μL of OptiMem medium for 5 min, then combined with relevant siRNA solution for 20 min. Reagents were mixed with 2.6 mL of RPMI containing 10% FCS and added. Mock-treated cells received transfection reagent but no siRNA, and untreated cells received only fresh medium. Attachment assays were done 96 h after transfection.

Attachment assays. Fibronectin (BD Biosciences; 100 μL of 10 μg/mL) was incubated in multiple wells of a 96-well plate overnight (4°C) before washing twice with PBS and blocking with 200 μL of 0.1% BSA in PBS. Cells (5×10^4) in 200 μL serum-free medium were then incubated for 15 to 120 min. After two further washes, adherent cells were quantified by sulforhodamine B (SRB) assay. For integrin blocking experiments, 7×10^5 cells were resuspended in 100 μL of a 1:50 dilution of either anti–α_2 integrin (P1E6), anti–α_3 integrin (P1B5), or antihuman IgG (HP6017) antibodies (Chemicon). After 1-h incubation, cells were washed thrice and plated for attachment to fibronectin as above.

Integrin expression profiling. Adhesion of cells to anti-integrin antibodies was quantified using the Alpha/Beta Integrin-Mediated Cell Adhesion Array Combo Kit (Chemicon) according to the manufacturer's instructions. Cells (2×10^5) in 100 μL PBS were added per well and incubated for 1.5 h. The wells were washed and stained, and binding was quantified by measuring absorbance (570 nm).

Fluorescence-activated cell sorting staining of integrin. Cells were washed twice in PBS and fixed with 2% paraformaldehyde (PolySciences) at room temperature for 30 min. After washing, cells were blocked with 500 μL of 1% BSA (Sigma). Cells (1×10^5) were stained with 100 μL of 1:100 dilution of mouse anti–α_3 (P1B5) or anti–$\alpha_5\beta_1$ (JBS5) integrin antibodies (Chemicon) in 1% BSA in PBS, followed by 100 μL of 1:500 dilution of goat anti-mouse FITC-conjugated antibody (Sigma) in 1% BSA in PBS. After three washes and fixation in 2% paraformaldehyde, cells were permeabilized with 500 μL of BD Perm/Wash (BD Biosciences) and stained with 100 μL of 1:200 rabbit anti-human WWOX antibody (10) in BD Perm/Wash, followed by 100 μL of 1:20 dilution of goat anti-rabbit APC-conjugated antibody (R&D Systems). Cells were counted on a FACSCanto flow cytometer (BD Biosciences), and data analyzed with FACSDiva version 6.0 software.

Measurement of apoptosis. Apoptosis was assessed using the TACS Annexin V-FITC Apoptosis Detection Kit (R&D Systems) according to the manufacturer's instructions. Cells (1×10^5) were incubated (± 10 μmol/L cisplatin) for 48 h. Cells were incubated with Annexin V Incubation Reagent containing propidium iodide for 15 min before reading by flow cytometry (FACSCalibur, Becton Dickinson). Apoptosis was further assessed based on the cell morphology of cytocentrifuged cells fixed in methanol and stained with REASTAIN Quick-Diff Kit (REAGENA) using oil immersion microscopy. At least 500 cells were counted per slide. In nonadherent experiments, 2.5×10^5 cells were plated on poly(2-hydroxyethyl methacrylate)–coated six-well plates to prevent adhesion (24). Cells were incubated for 20 h at 37°C before cytocentrifugation, and apoptosis was determined as above.

Results

***WWOX* suppresses *in vivo* tumorigenicity but not *in vitro* proliferation.** PEO1 cells were transfected with a *WWOX*-expressing plasmid or empty vector as previously described (7). PEO1 parental cells and vector-transfected control cells express no full-length *WWOX* mRNA or protein (due to a homozygous deletion), but *WWOX*-transfected clones express variable levels of *WWOX* as assessed by quantitative reverse transcription-PCR and immunoblotting (Supplementary Fig. S1). When grown s.c. in nude mice, PEO1 parental cells and vector-transfected controls formed large tumors, but there was no growth of four independent *WWOX*-transfected clones (Fig. 1*A*). This shows that reconstitution of *WWOX* expression in *WWOX*-null PEO1 ovarian cancer cells results in abolition of tumorigenicity in nude mice. The finding that the rate of growth of the vector-transfected controls was less than that of the parental PEO1 cells may be secondary to clonal heterogeneity.

Although *WWOX* abolished tumorigenicity of PEO1 cells *in vivo*, it had no effect on their *in vitro* growth when plated on plastic (Fig. 1*B*) or in soft agar (Fig. 1*C*). Thus, although *WWOX* has a tumor suppressor role *in vivo*, it does not alter cell proliferation *in vitro*..

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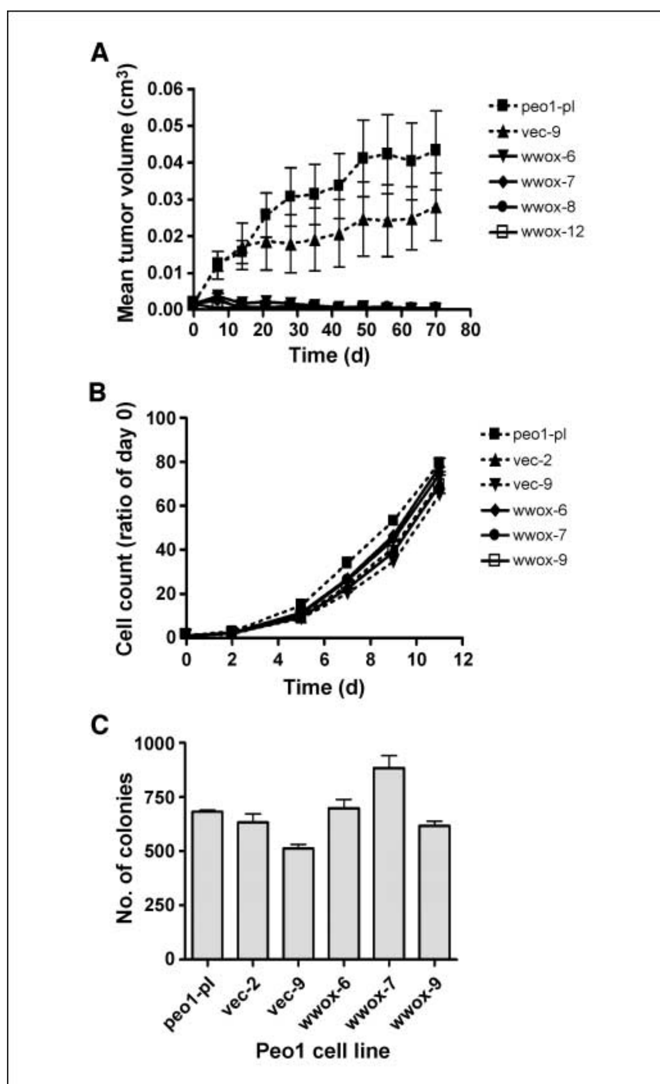

Figure 1. WWOX reconstitution abolishes PEO1 tumor growth *in vivo* but not *in vitro*. *A,* s.c. tumor growth of parent (*peo1-pl*), vector-transfected (*vec-9*), and WWOX-transfected (*wwox-6, wwox-7, wwox-8, wwox-12*) cells. *Points,* mean tumor size ($n = 10$); *bars,* SD. *B,* growth rate *in vitro*. Vec-2 is a vector-transfected line and wwox-9 is a WWOX-transfected cell line. Log-phase cells (1×10^5) were seeded in six-well trays; cells were harvested and Coulter counted. *Points,* mean of triplicate experiments; *bars,* SE. *C,* soft-agar clonogenicity. Five thousand log-phase cells were incubated, and colony numbers counted after 28 d. *Columns,* mean of duplicate experiments; *bars,* SD.

***WWOX* expression does not increase the rate of apoptosis in adherent ovarian cancer cells.** Several previous studies reported that *WWOX* overexpression resulted in increased apoptosis. To determine whether reconstituted *WWOX* expression in PEO1 cells induced apoptosis, compared with the parent line and vector-transfected controls, we examined Annexin V positivity by fluorescence-activated cell sorting (FACS) after 48 hours in culture. There was no significant difference in Annexin V staining between the parent line, vector controls, and *WWOX*-transfected clones (Fig. 2*A*). We next examined Annexin V staining in these cell lines following 48 hours of exposure to cisplatin (10 μmol/L), a chemotherapeutic drug used to treat ovarian cancer and known to induce cell death in these cells, but again found no evidence of increased apoptosis associated with *WWOX* expression (Fig. 2*A*). We confirmed these results by comparing DNA condensation in these cells, with and without exposure to cisplatin, and by examining the proportion of sub-G_1 cells by FACS, and again showed no effect associated with *WWOX* expression (data not shown). We also performed clonogenicity assays on the *WWOX*- and vector-transfected PEO1 cells, and showed no *WWOX*-associated difference in colony-forming ability in the absence or presence of cisplatin (Fig. 2*B*).

As additional confirmation, apoptosis was examined in another ovarian cancer cell line. SKOV3 ovarian cancer cells (low levels of endogenous *WWOX*; Supplementary Fig. S2) were transiently transfected with a *WWOX*-expressing plasmid, and again this resulted in no alteration in apoptosis, as assessed by Annexin V assay (Supplementary Fig. S3).

Assessment of the effect of *WWOX* on proliferation and apoptosis *in vivo*. Because *WWOX* reconstitution abolished tumorigenicity in nude mice but had no effect on proliferation or apoptosis of adherent cells *in vitro*, an assessment of the effect of *WWOX* on proliferation and apoptosis *in vivo* was done. *WWOX*-transfected and control cells were again injected s.c. into nude mice, but this time the experiment was terminated after 14 days (before the *WWOX*-expressing tumors disappeared). Immunohistochemistry for Ki67 was done as a surrogate for proliferation and terminal deoxyribonucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) assay for apoptosis. More than 65% of tumor cells in all xenografts were Ki67 positive regardless of *WWOX* expression (Supplementary Fig. S4*A* and *B*). Although there was some variation, there was greater heterogeneity within tumors than between different tumors. Thus, the effect on tumor regression *in vivo* did not seem to be attributable to altered proliferation. TUNEL positivity was <3%, but again there was considerable

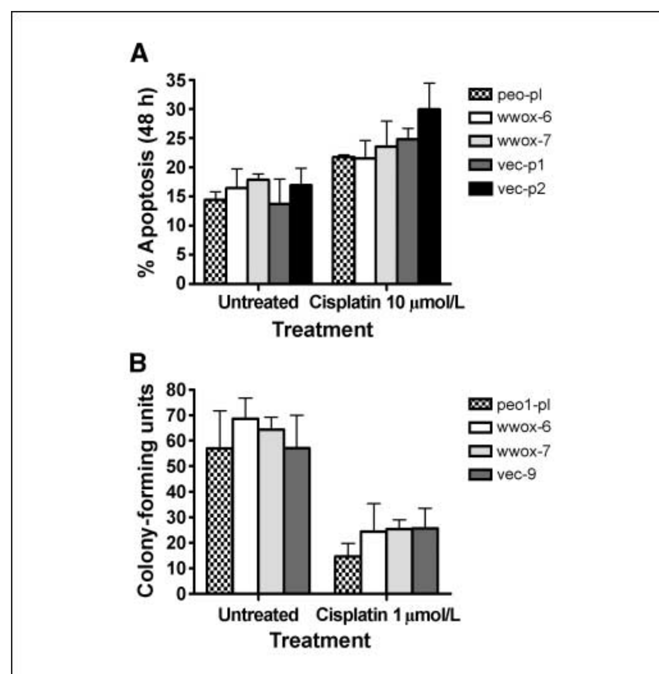

Figure 2. Effect of WWOX reconstitution on apoptosis in PEO1 cells. *A,* Annexin V assay. Cells (1×10^5) were incubated for 48 h (±10 μmol/L cisplatin). Apoptosis was assessed by flow cytometry using recombinant human Annexin V-FITC. *Columns,* mean of triplicate experiments; *bars,* SE. *B,* colony-forming efficiency. Two hundred cells were seeded (±1 μmol/L cisplatin) onto gridded Petri dishes for 21 d before counting. *Columns,* mean of triplicate experiments; *bars,* SE.

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heterogeneity within individual tumors (data not shown). More apoptotic cells were identified using morphologic criteria than by TUNEL assay, but there were no significant differences between groups (Supplementary Fig. S4*C* and *D*). In all tumors, there were areas of more pronounced cell death associated with inflammation and fibrosis, but at the time point examined, no significant differences were noted. Differential apoptosis or proliferation may, however, have been identified if further time points had been explored in this xenograft model.

***WWOX* reduces tumor cell adhesion to fibronectin.** To understand how *WWOX* might suppress *in vivo* tumorigenicity of ovarian cancer cells, we next examined the interaction of tumor cells with the ECM. *WWOX*-transfected PEO1 cells adhered more slowly to fibronectin as compared with vector-transfected controls and showed significantly reduced adhesion after 2 hours (Fig. 3*A*; $P < 0.005$). Similarly, decreased adhesion to fibronectin was observed after 15 minutes in SKOV3 ovarian cancer cells overexpressing *WWOX* following transient transfection, as compared with vector-transfected controls (Fig. 3*B*; $P < 0.05$). We also used specific siRNA oligos to knock down *WWOX* in the A2780 ovarian cancer cell line (which expresses moderate levels of the gene). A2780 cells adhered more rapidly to fibronectin than did PEO1 cells, but again the cells expressing *WWOX* (those treated with nontargeting siRNA or no siRNA) adhered more slowly as compared with those that had *WWOX* knockdown (*WWOX* siRNA treated), and showed a significant difference in attachment after 30 minutes (Fig. 3*C*; $P < 0.05$). Thus, *WWOX* is able to modulate tumor cell binding to fibronectin in several ovarian cancer cell lines (PEO1, SKOV3, and A2780).

In addition to its involvement in cellular adhesion, fibronectin is also involved in cellular migration. Integrins bind ECM ligands such as fibronectin and cluster at the cell surface, resulting in recruitment of adaptor and signaling proteins required for cellular migration (25). Therefore, the effect of *WWOX* on migration was assessed in transwell migration assays. *WWOX*-transfected PEO1 cells showed a decreased capacity to migrate toward fibronectin compared with the parental PEO1 cells or vector-transfected controls (Fig. 3*D*). However, *WWOX* expression had no effect on Matrigel invasion of PEO1 cells (data not shown).

Tumorigenicity of *WWOX*-expressing PEO1 cells in nude mice when injected along with Matrigel. In view of our data showing abolished *in vivo* tumorigenicity and reduced *in vitro* cell

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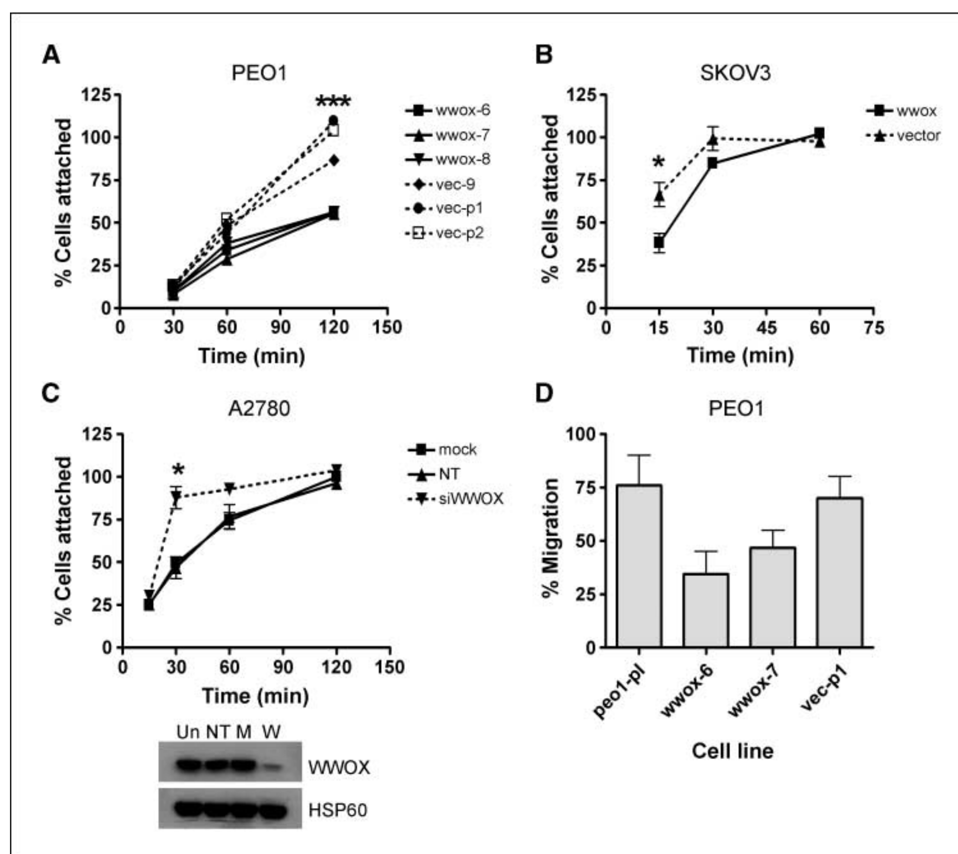

Figure 3. Effect of WWOX expression on cell adhesion to fibronectin and on migration. Cell adhesion measured by SRB staining. *A,* PEO1 (no endogenous WWOX). *Solid lines,* stable WWOX transfectants. *Dashed lines,* vector transfectants. *Points,* mean of triplicate experiments; *bars,* SE. ***, $P < 0.005$, WWOX-transfected compared with vector-transfected cells. *B,* SKOV3 (low endogenous WWOX). *Solid line,* transient WWOX transfection. *Dashed line,* vector transfection. *Points,* mean of triplicate experiments; *bars,* SE. *, $P < 0.05$, WWOX-transfected compared with vector-transfected control. *C,* A2780 (moderate endogenous WWOX). *Dashed line,* siRNA knockdown of WWOX (*siWWOX*). *Solid lines,* mock transfected and nontargeting siRNA (*NT*) transfected. *Points,* mean of triplicate experiments; *bars,* SE. *, $P < 0.05$, WWOX siRNA–transfected compared with mock or nontargeting controls. A representative immunoblot is shown, confirming WWOX knockdown following siRNA treatment. Protein was extracted at time of adhesion assay (96 h posttransfection). Blot was probed for WWOX and HSP60. *Un,* untreated; *NT,* nontargeting siRNA treated; *M,* mock treated; *W,* WWOX siRNA treated. *D,* migration of PEO1 parent, WWOX-transfected, and vector-transfected cells toward fibronectin. Migration was expressed as the ratio of cells on the undersurface to that on the upper surface of the transwell [measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide], and normalized for migration toward BSA in the control wells. *Columns,* means of five experiments; *bars,* SE.

adhesion to ECM components, with no evidence of apoptosis in *WWOX*-transfected ovarian cancer cells, we hypothesized that loss of adhesion-mediated signaling decreased the ability of the cancer cells to survive *in vivo*. In an attempt to enhance adhesion-mediated signaling in the *WWOX*-expressing cells, the s.c. tumorigenicity experiments were repeated with *WWOX*- and vector-transfected PEO1 cells embedded in Matrigel, an artificial ECM commonly used for *in vivo* tumorigenicity experiments to increase the grafting success. This was in an attempt to create a privileged environment rich in ECM components and determine whether this increased the survival of the *WWOX*-expressing cells. In the previous tumorigenicity experiment (without Matrigel), *WWOX* expression abolished tumor growth. In the presence of Matrigel, however, all cells survived with similar growth rates until about 56 days when the *WWOX*-expressing cells started to die (Fig. 4*A*). By 84 days, there were no significant tumor masses remaining in the mice injected with the *WWOX*-expressing cells. In contrast, the parental PEO1 cells and vector-transfected controls (none of which express *WWOX*) continued to grow throughout this time. These findings suggest the possibility that the excess of matrix proteins in Matrigel could compensate for the reduced adhesion of the *WWOX*-expressing cells, and support their growth until such time as the resorption of Matrigel led to tumor mass regression.

***WWOX* affects the survival of PEO1 cells when grown in suspension.** Considering the results above where we show decreased cellular adhesion to fibronectin in *WWOX*-expressing ovarian cancer cells, together with enhanced *in vivo* survival of these cells when xenografted in the presence of Matrigel (compared with when they are xenografted in the absence of Matrigel), we repeated the apoptosis assay using poly(2-hydroxyethyl methacrylate)–coated plates to prevent cellular adhesion. This showed that whereas there is no difference in the rates of apoptosis (between *WWOX*-expressing and *WWOX* nonexpressing cells) when the cells are allowed to adhere, the rate of cell death in nonadherent culture is approximately three times higher in the *WWOX*-transfected PEO1 cells (Fig. 4*B*). Therefore, as well as causing a reduction in migration toward and attachment to fibronectin, *WWOX* reconstitution in PEO1 cells resulted in a decreased ability to survive in suspension.

***WWOX* modulates tumor cell adhesion by regulating integrin α_3 activity.** The altered interaction with fibronectin in *WWOX*-expressing ovarian cancer cells suggests an effect on integrin function. To investigate this, integrin binding assays were done using a commercial kit (Chemicon). These showed that α_2 and α_3 were the main functioning α integrins in PEO1 cells (Fig. 5*A*), with very little binding to α_1, α_4, α_5, or α_V detected. In addition, the level of membranous α_2 and α_3 integrins in the *WWOX*-transfected PEO1 cells was significantly decreased compared with vector-transfected controls ($P < 0.05$). The *WWOX*-mediated decrease in membranous integrin α_3 expression was also shown by FACS in transiently transfected PEO1 cells (Fig. 5*B*; $P < 0.05$), although no change in integrin α_3 mRNA level was detected (Supplementary Fig. S5).

We also confirmed these findings by FACS in transiently transfected SKOV3 cells. Again, WWOX transfection resulted in significantly decreased membranous integrin α_3 compared with vector controls (Fig. 5*C*; $P < 0.05$).

Although integrin α_3 is known to bind several ligands, including fibronectin, the major integrin for fibronectin binding is integrin $\alpha_5\beta_1$. PEO1 expresses very little integrin $\alpha_5\beta_1$ (see above), and this is unaltered by *WWOX* transfection (data not shown). In contrast, SKOV3 cells express high levels of integrin $\alpha_5\beta_1$, and this is significantly reduced following *WWOX* transfection (Fig. 5*C*; $P < 0.05$). This suggests a link between *WWOX* expression and the level of integrins that interact with fibronectin.

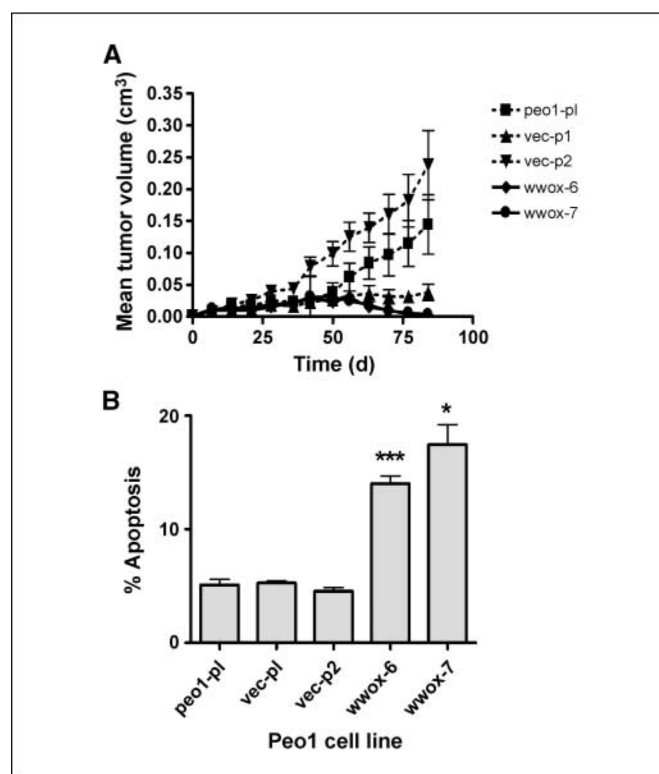

Figure 4. Effect of environment on survival of WWOX-expressing PEO1 cells. *A,* s.c. tumor growth of parent, vector-transfected, and WWOX-transfected cells injected in the presence of Matrigel. *Points,* mean tumor sizes ($n = 10$); *bars,* SD. *B,* apoptosis of cells cultured in six-well poly(2-hydroxyethyl methacrylate)–coated plates, assessed microscopically based on the cell morphology of cytocentrifuged cells. More than 500 cells were counted per slide. *Columns,* mean of triplicate experiments; *bars,* SE. *, $P < 0.05$; ***, $P < 0.005$.

Because integrin $\alpha_5\beta_1$ levels are low in PEO1 cells, we wanted to confirm whether fibronectin binding of these cells was actually mediated by either integrin α_2 and/or α_3, whose expression, as we have shown, is altered by *WWOX*. We repeated the adhesion assays, preincubating the cells with integrin α_2 or α_3 function-blocking antibodies. These studies showed that blocking integrin α_3 greatly reduced binding to fibronectin of all PEO1 clonal lines, compared with cells preincubated with a nonspecific IgG antibody (Fig. 6*A*; $P < 0.05$), confirming that fibronectin binding in these cells is mediated through α_3. In contrast, preincubation with α_2 blocking antibody had very little effect on fibronectin binding in these cells (Fig. 6*B*). The above data therefore suggest that *WWOX* reduces membranous α_3 integrin (and $\alpha_5\beta_1$ integrin in SKOV3 cells), which results in decreased tumor cell adhesion to fibronectin.

Discussion

Our data show an important role for *WWOX* in regulating ovarian cancer development and progression. We showed abolition of tumorigenicity following transfection of *WWOX* in human PEO1 ovarian cancer cells, further supporting a tumor suppressor role for *WWOX* in human ovarian cancer. Transfection of *WWOX* in these cells did not cause decreased cell growth *in vitro*, but was associated with altered membranous integrin levels and decreased adhesion and migration on fibronectin. We postulate that *WWOX*-

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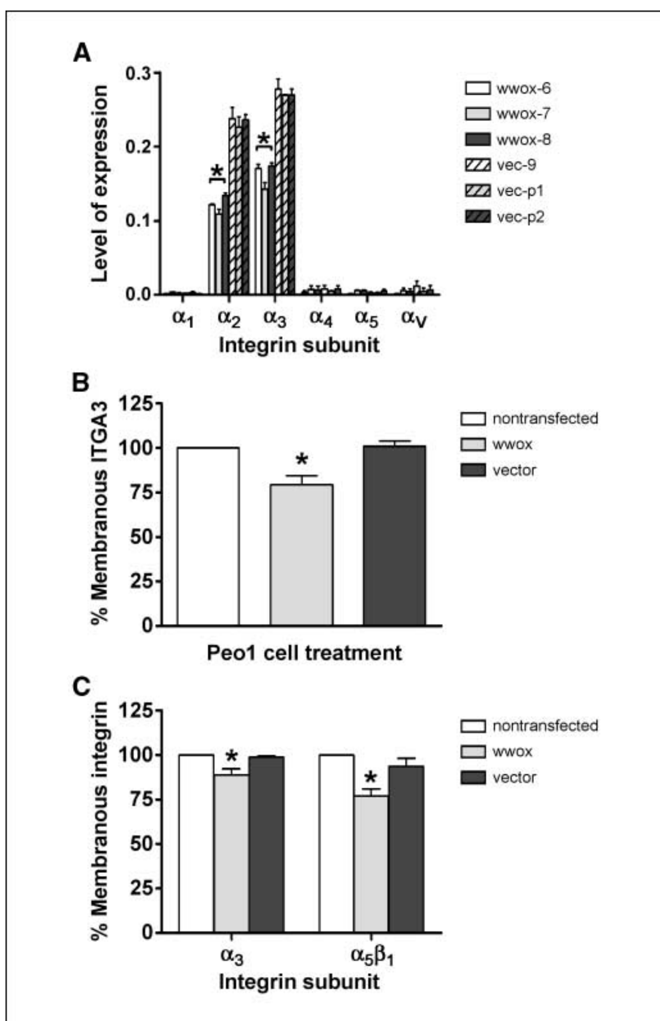

Figure 5. Effect of WWOX expression on integrin activity. *A,* stable WWOX transfection into PEO1 cells reduces expression of both α_2 and α_3 integrins compared with vector-transfected controls. Attached cells were measured by crystal violet staining. *Columns,* mean of duplicate experiments; *bars,* SD. *, $P < 0.05$, WWOX-transfected compared with vector-transfected controls. *B,* FACS staining of membranous integrin α_3 levels in transiently WWOX-transfected PEO1 cells. *Columns,* mean of triplicate experiments; *bars,* SE. * $P < 0.05$, WWOX-transfected compared with vector-transfected control. *C,* FACS staining of membranous integrin α_3 and $\alpha_5\beta_1$ levels in transiently WWOX-transfected SKOV3 cells. *Columns,* mean of six experiments; *bars,* SE. *, $P < 0.05$, WWOX-transfected compared with vector-transfected control.

mediated inhibition of adhesion results in decreased *in vivo* tumorigenicity. This hypothesis is supported by the fact that *WWOX*-transfected PEO1 ovarian cancer cells were able to grow and survive for around 56 days when injected into the flanks of nude mice in the presence of Matrigel, before the tumor started to regress, whereas the same tumor without Matrigel did not grow.

Interestingly, *WWOX* transfection into ovarian cancer cells did not induce apoptosis, as determined by Annexin V staining, sub-G_1 fraction, or nuclear morphology in normal (adherent) *in vitro* cell culture. This is consistent with the findings of Bednarek and colleagues (10) in breast cancer, but differs from those of Fabbri and colleagues (11) and Qin and colleagues (12) in lung and prostate cancer cells, respectively, which showed an increase in the sub-G_1 cell population, activation of caspase-3, or cleavage of poly(ADP-ribose) polymerase-1 following transient transfection with adenoviral *WWOX* constructs in cells deficient for endogenous *WWOX*. This difference may result from disparity in transfection techniques and resultant dose effects (stable transfection of plasmid or retrovirus vectors versus transient transfections of adenoviral vectors), although all these studies show similar *WWOX*-mediated suppression of *in vivo* tumorigenicity. However, our data show that in culture conditions designed to prevent cell adhesion, *WWOX*-transfected cells showed greatly increased apoptosis. *WWOX*-induced reduction in integrin expression may account for this increase in apoptosis because integrins such as α_3 are known to affect both cell-cell contact and survival in other cell types (26, 27). No difference in apoptosis was observed in tumors *in vivo* at the time point examined, however.

Ovarian cancer classically spreads by locoregional peritoneal dissemination. Many of the steps in locoregional peritoneal dissemination involve the integrin family of cell adhesion molecules. Integrin $\alpha_5\beta_1$ is important in the formation of multicellular spheroids—clumps of tumor cells that traverse the abdominal cavity (28)—and in the adhesion and migration of ovarian cancer cells to ECM and mesothelial cells (29–31). Integrin $\alpha_2\beta_1$ can promote disaggregation of multicellular spheroids and attachment to ECM (32), whereas $\alpha_3\beta_1$ regulates invasion and migration of ovarian cancer cells on ECM (33), and both $\alpha_3\beta_1$ and

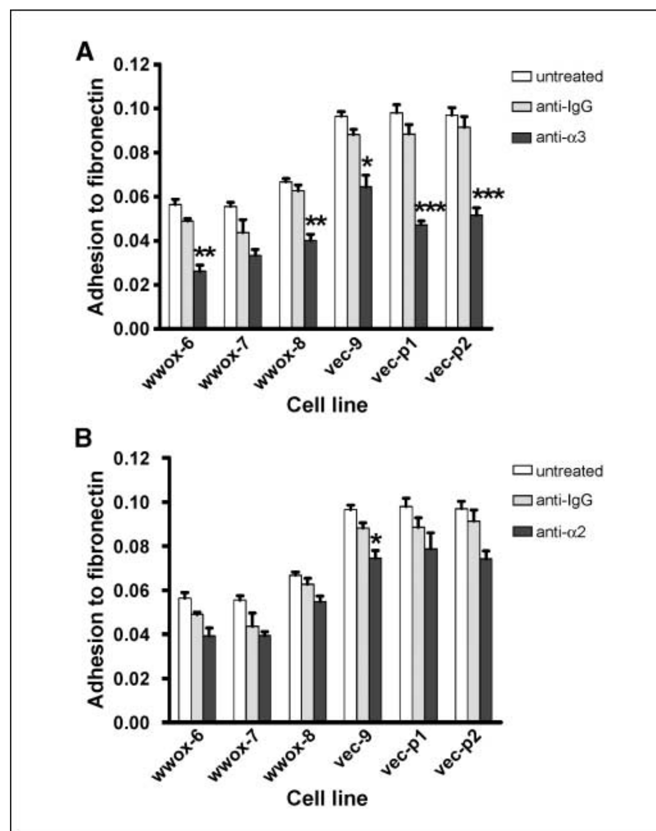

Figure 6. Effect of integrin function-blocking antibodies on binding of PEO1 cells to fibronectin. *A,* preincubation with anti-α_3 antibody or control IgG antibody before adhesion assay. Attached cells were quantified by SRB assay. *Columns,* mean of triplicate experiments; *bars,* SE. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.005$, cells blocked with anti-α_3 antibody compared with anti-IgG antibody–treated controls. *B,* preincubation with anti-α_2 antibody or control IgG antibody before adhesion assay. Attached cells were quantified by SRB assay. *Columns,* mean of triplicate experiments; *bars,* SE. *, $P < 0.05$, cells blocked with anti–α_2 integrin antibody compared with anti-IgG antibody–treated controls.

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$\alpha_2\beta_1$ are associated with locoregional peritoneal dissemination in gastric cancer (34, 35), likely suggesting a similar role in ovarian cancer. Furthermore, increased expression of fibronectin, a major ligand of several integrin family members including $\alpha_5\beta_1$ and $\alpha_3\beta_1$, has been reported in disseminated peritoneal tumor compared with primary ovarian tumors (36). Tumor cell integrins are not only important for peritoneal attachment but they also subsequently mediate vital intracellular signaling survival pathways for cell proliferation (33, 37) and suppression of apoptosis (38, 39) and can increase chemoresistance in tumor cells (40, 41). Our data suggest that loss of *WWOX* during ovarian cancer development would result in increased levels of membranous integrins and increased adhesion and migration of tumor cells on ECM, as well as suppression of apoptosis in detached tumor cells. We thus speculate that this would enhance locoregional peritoneal tumor spread. Consistent with this hypothesis, loss of WWOX protein expression has previously been correlated with advanced stage disease and poorer survival in ovarian cancer patients (42).

There are several mechanisms by which WWOX may modulate integrin function. WWOX has been reported as a Golgi-associated protein (10) and may therefore affect integrin protein maturation and exocytosis, as was described recently for presenilin proteins (43). Alternatively, several studies have shown that WWOX can bind and inhibit various transcription factors (16, 44, 45). We have shown that WWOX does not directly affect the mRNA levels of α_3 integrin, but it may still modulate the transcription of another factor that can regulate integrin function. A third possible mechanism is that WWOX may play a role at the cell periphery in modulating "inside-out" signaling of integrin activation. Supporting this are reports of two WWOX-binding proteins, ACK1 and Ezrin, with links to cell adhesion and migration. The nonreceptor tyrosine kinase ACK1 functions as an oncogene in several cancer types (46). It is regulated by integrin signaling and promotes cell motility via Cdc42 (47), and it has recently been shown to phosphorylate WWOX protein and target it for proteasomal degradation (48). Ezrin is a cytoplasmic protein linking transmembrane signaling to cytoskeletal reorganization and can bind WWOX to the cytoskeleton during differentiation of gastric parietal cells (49). Ezrin has also been shown to decrease cell-ECM adhesion, motility, and invasion in colorectal cancer cells (50).

We have shown a novel role for *WWOX* in decreasing integrin activity and the adhesion of tumor cells to fibronectin. *WWOX* was further found to induce apoptosis in detached cells. These data suggest an important role for loss of the *WWOX* tumor suppressor in promoting the spread of ovarian cancer. Attachment of tumor cells to ECM enhances intracellular survival signaling that promotes proliferation and chemoresistance (40), and this signaling would be expected to be increased on loss of *WWOX*. Identifying these intracellular pathways should therefore identify novel therapeutic targets for improved treatment of disseminated ovarian tumors.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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