Inhibition of Breast Cancer Cell Growth *In vitro* and *in vivo*: Effect of Restoration of Wwox Expression

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**Abstract**

**Purpose:** The WWOX gene is down-regulated in breast cancer and loss of Wwox expression correlates with important clinical features of breast cancer. Thus, we have examined the effect of restoration of Wwox expression in breast cancer-derived cells.

**Experimental Design:** Wwox protein expression was restored by the following: (a) infection with a recombinant adenovirus carrying WWOX cDNA (Ad-WWOX) or (b) treatment with the DNA methyltransferase inhibitor, 5-aza-2'-deoxycytidine, to activate the endogenous WWOX gene, in breast cancer-derived cells *in vitro* and *in vivo*.

**Results:** Restoration of Wwox expression led to suppression of growth of Wwox-deficient breast cancer-derived cells, through activation of the intrinsic caspase pathway, but did not affect growth of Wwox-sufficient MCF7 cells. Intratumoral Wwox restoration, through Ad-WWOX infection or endogenous Wwox reactivation by 5-aza-2'-deoxycytidine injection, suppressed tumor growth in nude mice by inducing apoptosis. Alteration of global methylation levels was not observed.

**Conclusions:** The results confirm that overexpression of exogenous Wwox inhibits breast cancer cell growth *in vitro* and *in vivo* and, perhaps more importantly, shows that restoration of endogenous Wwox expression, and likely other proteins, by treatment with a *de novo* methyltransferase inhibitor, also inhibits breast cancer cell growth and reverses breast cancer xenograft growth.

Breast cancer is the leading cause of death for American women between ages 50 and 60 years. Despite advances in chemotherapy, radiotherapy, and adjuvant hormonal therapy, one third of patients with breast cancer relapse and die of disease (1). New therapeutic strategies are needed to improve this outcome.

The WWOX gene spans >0.5 Mb at 16q23.3-24.1, a chromosome region involved in loss of heterozygosity in various types of cancer (2). Esophageal squamous cell carcinoma, non–small cell lung cancer, and breast cancer showed a high loss of heterozygosity rate, low mutation rate, and expression of aberrant transcripts of the WWOX gene (see ref. 3 for review). Wwox protein contains two WW domains in the NH2-terminal region and a short chain dehydrogenase/reductase domain. WW domains are involved in protein-protein interactions with Ap2γ, Ap2α, ErbB4, p73 proteins (4–6), and possibly p53 (7). The short chain dehydrogenase/reductase domain has homology to steroid oxidoreductases and Wwox levels are high in normal hormonally regulated tissues, such as prostate, ovary, and breast.

Wwox expression is reduced in 63% of invasive breast carcinomas and is correlated with estrogen receptor α levels (8, 9). Expression of many tumor suppressor genes is down-regulated in cancer by epigenetic mechanisms, and CpG-rich islands of gene promoter regions are frequently methylated in cancers (see ref. 10 for review). A role for epigenetic mechanisms of WWOX transcriptional regulation was suggested for hemopoietic and pancreatic neoplasms (11, 12), and WWOX promoter methylation status was associated with lack of expression. Wwox is also inactivated in breast and lung cancers by regulatory region DNA methylation; promoter methylation was also detected in tissue adjacent to breast cancer, and methylation in WWOX exon 1 distinguished breast cancer DNA from DNA of adjacent and normal tissue (13).

Restoration of expression of tumor suppressors, such as p53 and Fhit, has been shown to block cancer cell growth and induce apoptosis in cancer cells and tumors (14–18). Likewise, Wwox restoration in lung cancer cells *in vitro* and in xenografts suppressed growth (19), and restoration of Wwox expression in lung cancer cells, by expression of an inducible WWOX plasmid, also suppressed tumor growth (20). To investigate tumor suppression in breast cancer, we have now examined the *in vitro* and *in vivo* effects of Wwox protein expression in breast cancer cells with high (MCF7), low (BT-474), or no (MDA-MB-231 and HCC1937) endogenous Wwox expression, by Ad-WWOX infection or by endogenous Wwox reactivation.
through methytransferase inhibition. Both methods of Wwox restoration to the Wwox-deficient breast cancer cells were associated with effective induction of apoptosis in vitro and suppressed breast cancer xenograft growth in vivo, without effecting the Wwox-sufficient MCF7 cells or causing persistent changes in global methylation levels.

Materials and Methods

Cell culture
Breast cancer-derived cell lines were obtained from the American Type Culture Collection (Manassas, VA). MCF7 and BT-474 cells were cultured in DMEM supplemented with 10% fetal bovine serum; MDA-MB-231 and HCC1937 cells were maintained in RPMI 1640.

In vitro adenoviral transduction
The WWOX adenovirus, also carrying the green fluorescent protein (GFP) gene, was constructed using the Adenovator-CMV5GaO-IRESGFP transfer vector (Qiogene, Carlsbad, CA) as described (8, 19). Ad-GFP virus served as control (Qiogene). Cells were transduced with Ad-WWOX or Ad-GFP at appropriate multiplicity of infection (MOI), and transduction efficiency was assessed by visualization of GFP-expressing cells.

Real-time PCR analysis
Real-time PCR was done using SYBR Green PCR Master Mix (Bio-Rad, Hercules, CA) and gyceraldehyde-3-phosphate dehydrogenase served as an internal reference control. Primer sequences for WWOX and gyceraldehyde-3-phosphate dehydrogenase were as described (13).

Immunoblot analysis
Protein extraction and immunoblot analysis were done as described (5).

Cell proliferation and death assays
Cell counts. Cells (10^5) were plated in six-well plates, infected at MOIs of 10, 25, 50, 100, and 200, and counted in a Beckman Coulter (Fullerton, CA) Counter using the Vi-CELL viability program (analyzing 100 images). Cell growth was calculated as a percentage

Coulter (Fullerton, CA) Counter using the Vi-CELL viability program at MOIs of 10, 25, 50, 100, and 200, and counted in a Beckman

Cultivation, stained with propidium iodide, and analyzed by flow cytometry by EPICS-XL scanner (Beckman-Coulter).

CCK-8 cell viability assay. CCK-8 is a sensitive nonradioactive colorimetric assay for determining cell growth (Dojindo Molecular Technologies, Gaithersburg, MD). The inhibition factor was calculated as fold difference relative to untreated counterpart cells; 96-well plates were used and each cell line was plated in 12 wells.

Bisulfite modification and methylation-specific PCR
Cellular DNA was bisulfite modified as described (13), purified using Wizard DNA purification resin (Promega Corp., Madison, WI), denatured in NaOH, ethanol precipitated, resuspended in 20 μL water, and stored at –20°C. Modified DNA (2 μL) was amplified using primers described previously (13). Specific primers covering CpG sites were designed to distinguish, by direct sequencing, between methylated and unmethylated sequences of bisulfite-modified DNA.

Inhibition of de novo methyltransferase and histone deacetylation in vitro
DNA demethylation and histone deacetylation were effected in vitro by treating BT-474 and MDA-MB-231 cells with 5-aza-2′-deoxycytidine (DAC) and trichostatin A. Maximal Wwox reexpression was observed with the following regimen, which was used in subsequent experiments:

1 x 10^6 cells were plated in a 75-cm^2 flask and grown as described above; every 24 h for 5 days, medium was replaced with fresh medium containing 5 μmol/L DAC. After the 5th day, medium was replaced with medium containing 1 mmol/L trichostatin A, incubation was continued for 24 h, and cells were collected.

WWOX down-regulation by RNA interference
WWOX small interfering RNA (siRNA) and scrambled control siRNA were purchased from Ambion, Inc. (Austin, TX). MDA-MB-231 cells (2 x 10^5) were cultured in a six-well plate, transfected with 50 nmol/L WWOX siRNA, and treated with 5 μmol/L DAC. Cell growth was assessed by Vi-CELL counter.

In vivo studies
Ad-WWOX viral therapy. Animal studies were done under an approved protocol according to institutional guidelines. (a) In vitro treatment: BT-474 and MDA-MB-231 cells were infected with Ad-GFP or Ad-WWOX at MOI of 100; 48 h after infection, 10^5 cells were injected s.c. into the flanks of 6-week-old female nude mice (National Cancer Institute, Frederick, MD), five mice per group; five control mice were injected with 10^5 uninfected cells. Animals were monitored daily, and tumor sizes were measured every 5 days starting at day 14. At the end point (day 24), animals were sacrificed, tumors were weighed, and volumes were calculated using the formula V = (the shortest diameter)² x (the longest diameter). (b) In vivo treatment: 5 mice were injected with the 10^5 uninfected BT-474 or MDA-MB-231 cells. Animals were monitored daily, and tumor sizes were measured every 5 days starting at day 14. When mean tumor volumes reached 80 mm^3 (day 14), five mice were intratumorally injected with Ad-GFP, five mice were injected with Ad-WWOX, and five mice were untreated (control group). Tumor growth was monitored, and volumes were calculated.

Epigenetic therapy. (a) In vitro treatment: five control mice received s.c. flank injections of 10^5 uninfected MDA-MB-231 cells; another five mice received s.c. flank injections of 10^5 cells that had been treated, in vitro, with 5 days of DAC. (b) In vivo treatment: 10 mice received s.c. flank injections of 7 x 10^6 MDA-MB-231 cells. Before mean tumor volumes reached 80 mm^3 (day 17), treatment was initiated with 2.0 mg/kg DAC. Treatments were given on days 15 and 16, days 21 and 22, and days 28 and 29. Five (intratumoral PBS) mice received intratumoral injections of 100 μL PBS and five (intratumoral DAC) received intratumoral injections of 2.0 mg/kg DAC.

Ex vivo studies
Immunoblot analysis. Protein lysates from tumors of MDA-MB-231 untreated, Ad-GFP-, Ad-WWOX-, and DAC-treated mice were evaluated by immunoblot analysis using Wwox, caspase-9, and caspase-3 antisera.

Assessment of global methylation levels. DNA was extracted from untreated and in vivo DAC-treated MDA-MB-231 tumors (day 27) and bisulfite treated. Methylation status of Alu-repetitive elements was determined by PCR amplification [5’-GATCTTTTTATTAATAATAATGACAAAATTAGT-3’ (forward primer) and 5’-GATCCCAACTAATAATGACAAAAATTAGT-3’ (reverse primer)] of Alu sequences as described (21). The PCR product was digested with Mbol, which cleaves elements that were originally methylated. The digested PCR product was separated by gel electrophoresis and stained with ethidium bromide. The upper band represents unmethylated Alu elements. Wwox promoter methylation status was assessed as described previously (13).

Statistics
The results of all in vitro and in vivo assays were expressed as mean ± SDs. Student’s two-sided t test was used to compare the values of the test and control samples. P < 0.05 was considered significant.

Results

Growth of Ad-WWOX-infected breast cancer cells in vitro. To study the effects of restoration of Wwox expression in breast
cancer cells, MCF7, BT-474, MDA-MB-231, and HCC1937 cells were infected with Ad-WWOX, MOI of 100, for 6 days. MCF7 cells express high endogenous WWOX mRNA and protein levels, whereas BT-474 cells express low endogenous Wwox, and MDA-MB-231 and HCC1937 cells are Wwox negative (Fig. 1A). Cell counts showed significant inhibition of cell growth in Ad-WWOX-infected cells, BT-474, MDA-MB-231, HCC1937, but not in Wwox-positive MCF7 cells, compared with Ad-GFP-infected cells (Fig. 1B-E). Ad-WWOX inhibited cell growth 6-fold in BT-474 and MDA-MB-231 cells and 5-fold in HCC1937 cells (Fig. 1F). MCF7 cell growth was not inhibited by Ad-WWOX, suggesting that endogenous WWOX sufficient cells are not affected by Wwox overexpression.

In addition, all the cell lines were infected with Ad-GFP and Ad-WWOX at increasing MOIs (0, 25, 50, 100, 200, and 500; Supplementary Fig. S1). MCF7 cell growth was affected only at MOI of 500, probably due to cytotoxicity (Supplementary Fig. S1A). BT-474 cell growth was ~30% inhibited by Ad-WWOX MOI of 25 but not by Ad-GFP infection (Supplementary Fig. S1B), suggesting that Wwox expression, and not adenoviral transduction, was responsible for cell growth inhibition. MDA-MB-231 and HCC1937 cells were ~70% and ~60% growth inhibited, respectively, by Ad-WWOX MOI of 25 infection (Supplementary Fig. S1C and D).

**Cell cycle kinetics of Ad-WWOX-infected cells.** Cell cycle alterations induced by Wwox overexpression were assessed after Ad-WWOX infection. MCF7, BT-474, MDA-MB-231, and HCC1937 cells were infected with increasing MOIs and fractions of transduced cells were monitored by confocal microscopy and flow cytometry. To exclude virus-specific effects, we compared Ad-GFP- to Ad-WWOX-infected cells at

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**Fig. 1.** Effect of Wwox expression on breast cancer cell growth in vitro. A, Wwox mRNA and protein expression is reduced in breast cancer cells. Top, Wwox mRNA relative expression was determined by real-time PCR analysis. Glyceraldehyde-3-phosphate dehydrogenase (Gapdh) served as control. Columns, mean of triplicate samples from two independent experiments; bars, SD. P < 0.001, determined by Student’s t test analysis. Bottom, Wwox protein expression was determined by immunoblot analysis. Wwox protein level is high in MCF7 cells, highly reduced in BT-474, and not detected in MDA-MB-231 and HCC1937 cells. B to E, growth of breast cancer cells after Ad-GFP or Ad-WWOX infection (MOI of 100). WWOX restoration suppressed BT-474, MDA-MB-231, and HCC1937 cell growth starting from day 4 after transduction. F, CCK-8 cell viability test. Ad-WWOX infection inhibited BT-474, MDA-MB-231, and HCC1937 cell growth.
the same MOIs. An apoptotic (sub-G1) population was observed after Ad-WWOX infection in BT-474, MDA-MB-231, and HCC1937 cells but not in endogenous Wwox-positive MCF7 cells. Ad-GFP infection did not modify cell cycle profiles. At 144 h after Ad-WWOX infection (MOI of 100), 39.7% of BT-474 cells, 53.1% of MDA-MB-231 cells, and 71.4% of HCC1937 cells were in the sub-G1 fraction; only 4.5% of MCF7 cells were in the sub-G1 fraction (Fig. 2A). Wwox induction of cell death was MOI and time dependent (data not shown).

**Analysis of apoptotic pathways in Wwox-reexpressing cells.** To investigate mechanism of Ad-WWOX-induced apoptosis, we assessed expression of proteins involved in the apoptotic cascade, including Bcl-2, Bax, caspase-3, caspase-8, and caspase-9. In Ad-WWOX-infected cells, with restored Wwox expression, the level of Bax was increased and Bcl-2 decreased. Procaspase-9 and procaspase-3 levels were decreased in Ad-WWOX-infected MDA-MB-231 cells, but the procaspase-8 level was not significantly affected; caspases were not activated in uninfected and Ad-GFP-infected cells (Fig. 2B). MCF7 cells were unaffected by exogenous Wwox expression and modulation of apoptotic proteins was not detected (data not shown).

**Epigenetic regulation of WWOX in breast cancer cells.** To determine if WWOX is epigenetically regulated in breast cancer cell lines, we did methylation-specific PCR in the WWOX regulatory region (promoter and exon 1). WWOX was unmethylated and highly expressed in MCF7 cells. In the WWOX-negative cells, MDA-MB-231 and HCC1937, the WWOX regulatory region was methylated in both regions only in MDA-MB-231 cells (data not shown).

Treatment with 5 μmol/L DAC for 5 days increased the endogenous Wwox level 6-fold in MDA-MB-231 cells, whereas combination treatment did not further increased significantly the expression (Fig. 3A). Wwox expression was not affected significantly by epigenetic treatments in BT-474 and HCC1937 cells.

To determine if the antiproliferative effect of demethylation was associated with Wwox reexpression, MDA-MB-231 cells were transfected with WWOX siRNA and treated with 5 μmol/L DAC. Treatment with 50 nmol/L WWOX siRNA down-regulated the DAC-induced, Wwox expression dramatically (Fig. 3B) and these cells showed an increase in the proliferation rate (P < 0.001) 96 h after DAC treatment (Fig. 3C). Thus, although it is likely that DAC treatment restores expression of other genes in these cells, restoration of Wwox expression is important for the DAC-induced antiproliferative effect.

**Tumorigenicity of Ad-WWOX-infected breast cancer cells.** Nude mice were inoculated with 10^7 BT-474, HCC1937, and MDA-MB-231 cells infected in vitro, MOI of 100, with Ad-GFP or Ad-WWOX, and cultured for 24 h. Uninfected cells served as tumorigenic controls. At 42 days after injection, tumor growth was completely suppressed in mice inoculated with Ad-WWOX-infected BT-474 and MDA-MB-231 cells (Fig. 4). Ad-GFP-infected cells showed tumor growth similar to uninfected cells.

To determine if intratumoral infection might also suppress tumor growth, 10 nude mice were inoculated with BT-474 or MDA-MB-231 cells and when tumors reached ~80 mm^3, five mice bearing tumors of the two cell types were inoculated with Ad-GFP or Ad-WWOX at days 15 and 16 (first cycle) and days 27 and 28 (second cycle). On day 35, the BT-474 tumors injected with Ad-WWOX had regressed to an average of 27.6 mm^3 and further regressed to an average of 16.1 mm^3 by day 42, with palpable tumors not detected in three of five mice. Similarly, MDA-MB-231 tumors regressed to an average of 31.8 mm^3 by day 35 and 18.8 mm^3 by day 42, with complete regression in one mouse.

**Tumor suppression by endogenous WWOX reactivation.** Untreated and in vitro DAC-treated MDA-MB-231 cells were injected s.c. into the flanks of nude mice. We tested the cell viability before injections and we found that 85% to 91% of the cells were viable. By day 15, mice injected with the in vitro DAC-treated cells developed very small tumor tumors (average, 29 mm^3; Fig. 5A). In contrast, all five mice from the control group, which had received untreated MDA-MB-231 cells and intratumoral injections of PBS, developed tumors (average, 500 mm^3) by day 35. A second group of five mice injected with untreated MDA-MB-231 cells were treated in vivo with DAC as follows: when tumors reached an average size of 80 mm^3, DAC (2.0 mg/kg) was injected intratumorally at days 15 and 16 (first cycle), days 21 and 22 (second cycle), and days 28 and 29 (third cycle). This protocol led to complete regression of tumors in three of five mice by day 42 and nearly complete
regression in two of five, with small residual tumors (12 and 24 mm³; Fig. 5A).

To assess Wwox expression ex vivo, proteins were extracted from tissue fragments of MDA-MB-231 tumors at day 42 and immunoblotted. Wwox expression was detected in the in vivo DAC-treated residual tumor tissue but not in the PBS-treated (Fig. 5B). Wwox was found unmethylated in its promoter region by methylation-specific PCR in the DAC-treated tumors. In addition, evidence of apoptotic activity was observed in the in vivo DAC-treated remnant tumor tissue, evidenced by reduced levels of procaspase-3 (Fig. 5B).

To determine if global methylation levels were affected by in vivo DAC treatment, the methylation status of Alu-repetitive elements was assessed by PCR and restriction digestion to quantify DNA methylation. The global methylation levels in PBS-treated (29-34%) and DAC-treated (28-32%) tumor tissues were similar (Fig. 5C), indicating that Alu sequences were not affected by in vivo DAC treatment or had recovered unmethylated status after the final DAC dose.

Discussion

Genes at fragile sites are frequently inactivated early in the neoplastic process, especially after exposure to environmental carcinogens (22, 23). We have been interested in studying the effects of loss of fragile gene expression in the development...
of cancer and therapeutic effects of their restoration. Previous studies showed that the fragile WWOX gene is inactivated in a significant fraction of breast cancers (8, 9), mainly due to DNA hypermethylation (13). DNA hypermethylation can be reversed by DNA methyltransferase inhibitors, such as DAC (24) or zebularine (25), agents that have therapeutic potential for cancers. Thus, we determined if restoration of Wwox overexpression in breast cancer cells suppresses cancer cell growth and induces apoptosis in vitro by induction of apoptosis in the Wwox-deficient cancer cells, whereas Ad-GFP did not modify cell cycle profiles, confirming a Wwox-specific effect. To examine apoptotic pathway(s) induced by Wwox overexpression, immunoblot analyses were done to detect activation of proapoptotic and antiapoptotic proteins. Ad-WWOX infection resulted in reduced levels of antiapoptotic protein Bcl-2, and Bax levels were increased and led to activation of the initiator caspase-9 and effector caspase-3, as indicated by decreased expression of procaspase-9 and procaspase-3. Procaspase-8 was not activated, suggesting that the intrinsic (mitochondrial)-mediated caspase-dependent pathway is involved in the Ad-WWOX-induced apoptosis in breast cancer cells, as was shown after Ad-WWOX infection of lung and prostate cancer-derived cells (19, 26).

Because Ad-WWOX transduction inhibited breast cancer cell growth and induced apoptosis in vitro, we explored the effect of Ad-WWOX on the growth of breast cancer cells in vivo. We observed suppression of in vivo tumorigenicity of breast cancer cells by Ad-WWOX transduction in BT-474 and MDA-MB-231 cells and Ad-WWOX infection by intratumoral injections was very effective in suppressing tumor growth. The results lend support to proposals that Wwox loss has an important role in breast cancer development and Wwox restoration has therapeutic potential. The finding that the WWOX regulatory region was methylated in MDA-MB-231 cells, together with our report that WWOX is frequently hypermethylated in breast cancers (13), suggested that Wwox might be restored in breast cancer cells by DAC treatment. Additionally, previous studies showed that DAC treatment induces apoptosis in MDA-MB-231 cells in vitro (24) and we have shown that WWOX is one of the genes reactivated in vitro after DAC treatment. Thus, we treated MDA-MB-231 xenografts with DAC when tumors reached \( \sim 80 \) mm\(^3\) in volume and observed suppression of tumor growth in nude mice. Previous clinical studies have shown that the use of demethylating agents, such as decitabine, in patients was highly cytotoxic and nonspecific (27–29). Recently Issa et al. (30) showed that decitabine is effective, specific, and noncytotoxic when used in doses 30-fold lower than the maximum tolerated dose. To increase specificity of demethylating agents and decrease global effects, it will be necessary to design protocols that effectively induce endogenous tumor suppressors while minimizing effects on global gene expression and patient toxicity. Our approach showed that a DNA methyltransferase inhibitor can be effective in low, multiple doses that act as targeted therapy rather than a cytotoxic agent (31). The low doses of DAC that we used, compared with higher doses reported previously (32, 33), restored Wwox expression and induction of apoptosis without lasting alteration of global methylation levels. DAC treatment of MDA-MB-231 tumors likely induced reexpression of numerous proteins in addition to Wwox (34–37), but the decrease in effect on cell growth observed in the WWOX siRNA experiment strongly suggests that Wwox restoration plays an important role in the growth-suppressive effect of DAC in breast cancer cells.

In conclusion, overexpression of exogenous Wwox or restoration of endogenous Wwox protein expression in breast cancer cells suppresses cancer cell growth and induces apoptosis in vitro and suppression of xenograft tumor growth, suggesting that reactivation of the Wwox signal pathway in breast cancers has therapeutic potential.
References


Clin Cancer Res 2007;13(1) January 1, 2007 274 www.aacrjournals.org

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