A Role for the *WWOX* Gene in Prostate Cancer

Haiyan R. Qin,[1] Dimitrios Iliopoulos,[1] Shuho Semba,[1] Muller Fabbri,[1] Teresa Druck,[1] Stefano Volinia,[1] Carlo M. Croce,[1] Carl D. Morrison,[2] Russell D. Klein,[3] and Kay Huebner[1]

[1]Department of Molecular Virology, Immunology, and Medical Genetics, Comprehensive Cancer Center and [2]Department of Pathology and [3]Department of Hematology and Oncology, The Ohio State University, Columbus, Ohio

Abstract

Expression of the *WWOX* gene, encompassing the common chromosome fragile site FRA16D, is altered in a large fraction of cancers of various types, including prostate cancer. We have examined expression and biological functions of WWOX in prostate cancer. WWOX mRNA and protein expression were significantly reduced in prostate cancer-derived cells (LNCaP, DU145, and PC-3) compared with noncancer prostate cells (PWR-1E), and WWOX expression was reduced in 84% of prostate cancers, as assessed by immunohistochemical staining. Down-modulation of WWOX expression in the prostate cancer-derived cells is due to DNA hypermethylation in the WWOX regulatory region. Treatment with 5-aza-2′-deoxycytidine (AZA), a DNA methyltransferase inhibitor, and trichostatin A, a histone deacetylase inhibitor, led to increased WWOX mRNA and protein expression in prostate cancer-derived cells, most strikingly in DU145 cells. Transfection-mediated WWOX overexpression in DU145 cells suppressed colony growth ($P = 0.0012$), and WWOX overexpression by infection with Ad-WWOX virus induced apoptosis through a caspase-dependent mechanism and suppressed cell growth. Lastly, ectopic expression of WWOX by Ad-WWOX infection suppressed tumorigenicity of xenografts in nude mice, and intratumoral AZA treatment halted tumor growth. The data are consistent with a role for WWOX as a prostate cancer tumor suppressor and suggest that WWOX signal pathways should be further investigated in normal and cancerous prostate cells and tissues. (Cancer Res 2006; 66(13): 6477-81)

Introduction

WWOX (WW domain containing oxidoreductase) is a tumor suppressor gene spanning a genomic region of ∼1 Mb located at chromosome 16q23.3-24.1, a region with a high incidence of loss of heterozygosity (LOH) in breast, prostate, and other cancers (1–3). WWOX protein contains two WW domains in the NH_2-terminal region and a short chain dehydrogenase/reductase (SDR) domain in the central region of the protein; WW domains are involved in protein-protein interactions (4). The WWOX SDR domain has amino acid sequence homology to steroid oxidoreductases, and the WWOX expression level in normal tissues is highest in hormonally regulated tissues, such as testis, prostate, and ovary, suggesting that WWOX may play an important role in hormone-regulated cancers.

Bednarek et al. first reported that ectopic expression of WWOX suppressed breast cancer growth *in vitro* and *in vivo* (5). Consistent with this, we have observed that the expression of WWOX is reduced or lost in breast cancer tissues (6, 7), and Fabbri et al. (8) have shown that Ad-WWOX virus infection of lung cancer cells suppresses xenograft tumorigenicity. The suggestion that the *WWOX* gene is a target of LOH in prostate cancers and may have a function in hormone-responsive tissues led us to hypothesize that WWOX may function as a tumor suppressor in prostate cancer.

Prostate cancer is the most prevalent noncutaneous cancer and the second leading cause of cancer death in males in the United States. It is a heterogeneous disease, varying from clinically indolent to a rapidly fatal systemic malignancy. It develops to advanced stage in ∼25% of patients (9). Androgen ablation remains the primary treatment for advanced prostate cancer, but hormone-refractory prostate cancer recurs invariably within 1 to 2 years (10). At this stage, no therapy has been shown to substantially prolong survival (11). Improved understanding of the molecular mechanisms of prostate cancer development may provide a basis for development of effective treatment strategies.

Extensive research into prostate cancer to date has suggested that numerous molecular abnormalities are involved in prostate cancer development, including chromosomal loss or gain, amplification of oncogenes, and mutation or epigenetic silencing of tumor suppressor genes. In this study, we have investigated the expression of WWOX in prostate cancer cells and tissues and the effect of WWOX restoration on prostate cancer cell growth *in vitro* and *in vivo*.

Materials and Methods

Cell culture. LNCaP, DU145, PWR-1E, and PC-3 cell lines were obtained from the American Type Culture Collection (Manassas, VA). LNCaP, DU145, and PC-3 cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum. PWR-1E cells were maintained in keratinocyte-SFM (Invitrogen Corp., Carlsbad, CA).

***In vitro* adenoviral transduction.** The Ad-WWOX vector was prepared, amplified, and titrated based on manufacturer's instructions (Qbiogene, Carlsbad, CA) and as described previously (8). An Ad-GFP vector served as negative control (Qbiogene). Cells were transduced with Ad-WWOX at appropriate multiplicities of infection (MOI), and transduction efficiency was assessed by visualization of green fluorescent protein–expressing cells using fluorescence microscopy.

Real-time reverse transcription-PCR analysis. Total RNA was isolated using the RNeasy Mini kit (Qiagen, Chatsworth, CA). RNA (2 μg) was reversed transcribed using SuperScript II (Invitrogen). Real-time PCR was done using SYBR Green PCR Master Mix (Bio-Rad, Hercules, CA) under conditions recommended by the manufacturer, using an iCycler iQ Multicolor Real-time PCR Detection System (Bio-Rad). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as an internal reference control;

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

Requests for reprints: Haiyan R. Qin, Department of Molecular Virology, Immunology, and Medical Genetics, Comprehensive Cancer Center, The Ohio State University, Columbus, OH 43210. Phone: 614-688-3317; E-mail: qin.17@osu.edu.

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primer sequences for WWOX and GAPDH were as described (12, 13). WWOX relative expression was determined by the temperature threshold cycle method, and threshold cycle was automatically estimated by the iCycler.

Immunoblot and immunohistochemistry. Immunoblot and immunohistochemistry analyses were carried out as described (14). The following rabbit polyclonal primary antisera and dilutions were used for immunoblotting: anti-WWOX, 1:20,000 (6); anti-caspase-3 (H-277), 1:500 (Santa Cruz Biotechnology, Santa Cruz, CA); anti-caspase-8 (H-134), 1:500 (Santa Cruz Biotechnology); anti-caspase-9 (H-83), 1:500 (Santa Cruz Biotechnology); and anti-Bcl2 (N-19), 1:500 (Santa Cruz Biotechnology). Rabbit polyclonal anti-WWOX (1:5,000) was used for immunohistochemistry.

Prostate tissue microarray. Formalin-fixed and paraffin-embedded tissue blocks from 44 primary prostate cancer patient samples were used in this study. The prostate tissue microarray was constructed by Department of Pathology, The Ohio State University, (Columbus, OH) using a manual tissue arrayer (Beecher Instruments, Sliver Springs, MD). Core samples were retrieved from the selected section in each donor paraffin block and inserted into a receiver paraffin block. The tissue cores are 1.5 mm in diameter, and the arrayed tissue was cut at 4 μm and placed on positively charged slides. Each parallel section was stained with H&E and reviewed to ensure quality control. Immunohistochemical staining was done on the arrayed tissue as described previously (15). WWOX antiserum was used at 1:5,000 dilution. The slides were scored by a pathologist (S.S.) as high (++/+++) or low (−/+) WWOX immunoreactivity.

Analysis of WWOX methylation status. Genomic DNA extraction, bisulfite treatment, and methylation-specific PCR (MSP) were done using primer sequences for methylated and unmethylated WWOX promoter and exon 1 DNA as reported previously (16). The prostate cancer-derived cells were treated with trichostatin A (TSA; 1 μmol/L) for 24 hours, 5-aza-2′-deoxycytidine (AZA; 5 μmol/L) for 120 hours, or AZA for 120 hours followed by TSA for an additional 24 hours. Then, cells were harvested, and RNA and protein were extracted for real-time reverse transcription-PCR (RT-PCR) and immunoblot analysis, respectively.

Cell transfection and colony assay. DU145 cells (7.5×10^5) in 100-mm plates in triplicate were transfected with 6 μg pcDNA3 vector or pcDNA3-WWOX construct using Fugene 6 (Roche Applied Science, Mannheim, Germany). Twelve hours after transfection, cells were cultured in G418-selective (500 μg/mL) medium for 14 days. Cells were then fixed with methanol and stained with Giemsa (Sigma, St. Louis, MO), and visible colonies ≥0.5 mm were counted.

Cell growth and flow cytometric analysis. To determine the optimal MOI for infection, 1×10^5 cells per 60-mm plate, in triplicate, were infected with increasing MOI and counted 7 days after infection using the ViCell counter (Beckman Coulter, Fullerton, CA). For cell growth analysis, 0.5×10^5 cells per well in six-well plates were infected with Ad-WWOX or Ad-GFP at 100 MOI and counted at day 0, 1, 3, 5, and 7 using the Vicell counter. Flow cytometry was done as described (14).

Xenograft analysis. DU145 cells were infected with Ad-GFP or Ad-WWOX at 100 MOI. At 48 hours after infection, cells were collected, and

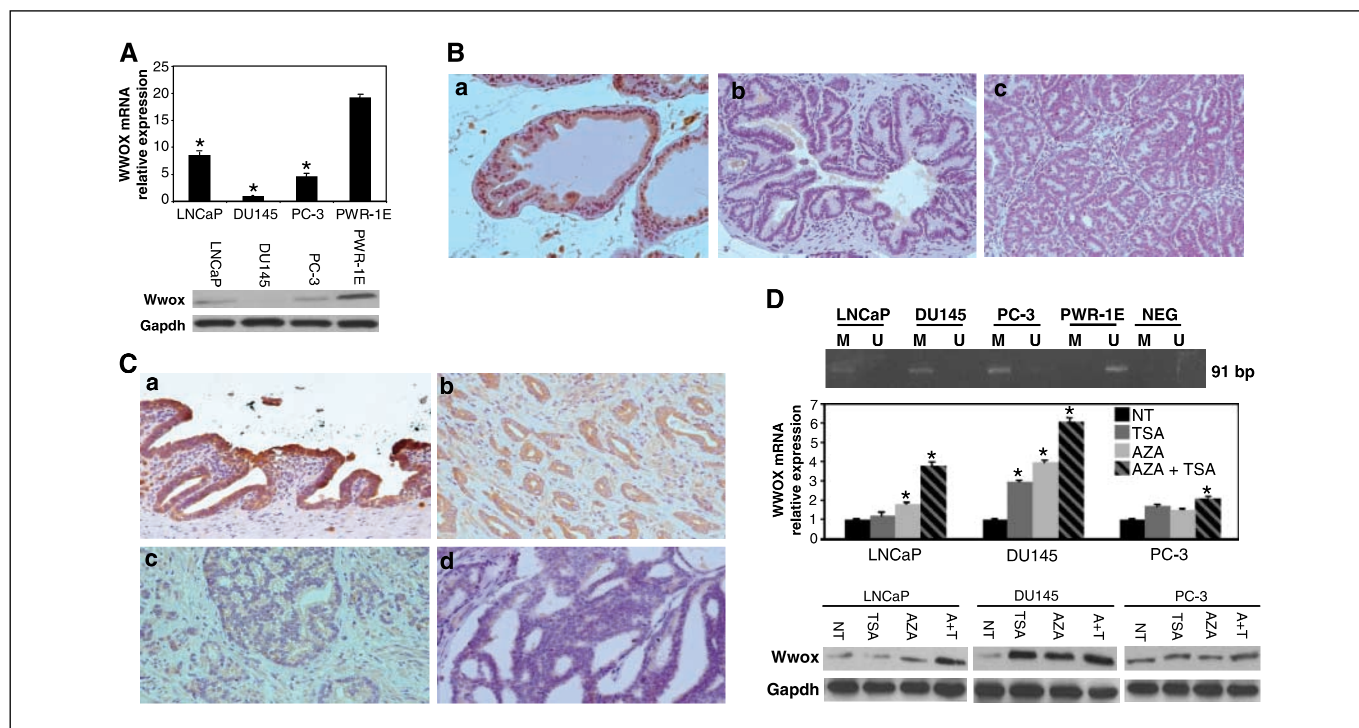

Figure 1. WWOX expression is reduced in prostate cancer. *A, top,* WWOX mRNA relative expression was determined by real-time RT-PCR analysis. GAPDH cDNA served as internal control, and WWOX expression in DU145 cells was set as 1. *Columns,* mean of triplicate samples from two independent experiments; *bars,* SD. *, $P < 0.01$ as determined by one-way ANOVA. *Bottom,* WWOX protein expression was determined by Western blot analysis. GAPDH served as loading control. *B,* WWOX is down-regulated in mouse prostate TRAMP tissues as assessed by immunohistochemical analysis. *a,* high WWOX expression in low-grade PIN and adjacent normal epithelia (magnification, ×200); *b,* low WWOX expression in well-differentiated adenocarcinoma (noninvasive; magnification, ×200); *c,* negative WWOX expression in moderately differentiated adenocarcinoma (invasive; magnification, ×200). *C,* tissue microarray and immunohistochemical analysis of WWOX expression in human prostate cancer tissues. *a,* strong WWOX cytoplasmic expression in adjacent normal prostate epithelium (magnification, ×200); *b,* high WWOX expression; *c,* low WWOX expression; *d,* negative WWOX expression in invasive adenocarcinoma (magnification, ×200). *D, top,* WWOX regulatory region hypermethylation in prostate cancer cells LNCaP, DU145, and PC-3 but not in noncancer PWR-1E cells as determined by MSP analysis. Amplification using WWOX regulatory region primers specific for methylated (*M*) or unmethylated (*U*) DNA sequences. *Middle,* WWOX mRNA expression is increased after treatment with AZA and TSA as determined by real-time RT-PCR analysis. GAPDH cDNA served as internal control, and WWOX expression in nontreated cells was set as 1. WWOX was highly reexpressed after combined AZA and TSA treatment, especially in DU145 cells. *Columns,* mean of triplicate samples from two independent experiments; *bars,* SD. *, $P < 0.01$ as determined by one-way ANOVA. *Bottom,* WWOX protein expression after treatment with AZA and TSA as determined by Western blot. GAPDH served as loading control. Final WWOX protein levels in LNCaP and DU145 were increased after AZA and TSA treatments.

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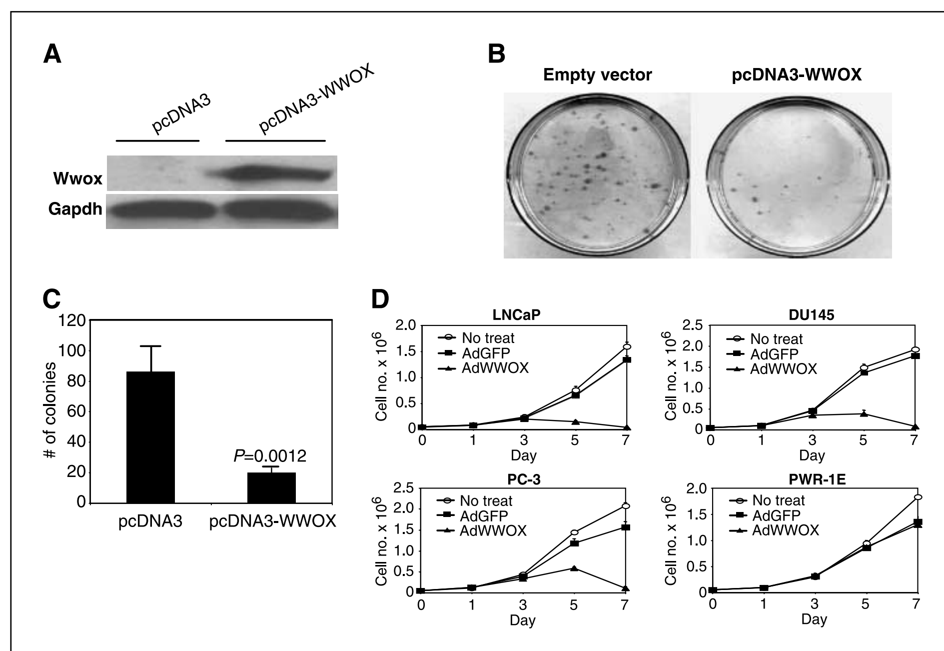

Figure 2. Effect of exogenous WWOX expression on prostate cancer-derived cell growth. *A,* Western blot analysis of WWOX expression in pcDNA3- and pcDNA3-WWOX-transfected DU145 cells 48 hours after transfection, with GAPDH as loading control. *B,* representative plates for colony formation assay in pcDNA3- and pcDNA3-WWOX-transfected DU145 cells. *C,* quantification of colonies per plate from 10-cm plates, in triplicate, for pcDNA3- and pcDNA3-WWOX-transfected DU145 cells. *Columns,* mean of triplicate samples; *bars,* SD. *P* (Student's *t* test) is the significant difference in number of colonies of pcDNA3-WWOX-transfected cells versus empty vector control. *D,* growth of prostate cancer and noncancer cells with no infection, Ad-GFP infection, and Ad-WWOX infection at 100 MOI. WWOX reexpression suppressed the growth of prostate cancer-derived cells (LNCaP, DU145, and PC-3) but not the noncancer prostate cells (PWR-1E). *Points,* mean of triplicate samples; *bars,* SD.

7×10^6 cells were injected into the flanks of 5-week-old female nude mice [National Cancer Institute (NCI), Frederick, MD], five mice per group. Ten control mice were injected with the same number of uninfected DU145 cells. Animals were monitored daily, and tumor sizes were measured every 5 days starting at day 10. When mean tumor volumes for the 10 control mice reached ~ 100 mm^3 (day 15), AZA treatment of five control mice was initiated. AZA (5.0 mg/kg body weight) in 100 μl PBS was given intratumorally on days 15 and 16 and days 22 and 23. At end point (day 35), animals were sacrificed, tumors were weighed, and tumor volumes were calculated using the formula V = (the shortest diameter)^2 × (the longest diameter).

Statistical analysis. The differences in values between experimental groups were analyzed using Student's *t* test and one-way ANOVA, where appropriate. Statistical significance was set at $P < 0.05$.

Results and Discussion

WWOX mRNA and protein expression are reduced in prostate cancer cells and tissues. Alterations of tumor suppressor genes through deletion or epigenetic modification, leading to reduced expression, have been implicated in prostate cancer development (17). Thus, we examined the expression level of WWOX in prostate cancer-derived cells and found that both mRNA and protein expression were significantly reduced in human prostate cancer cells (Fig. 1*A*). Prostate-derived noncancer cells, PWR-1E, exhibit 2.2-fold, 4.1-fold, and 19.2-fold higher WWOX expression than in LNCaP, PC-3, and DU145 cells, respectively, as determined by real-time RT-PCR. Protein expression was barely detectable in DU145 cells and was reduced in LNCaP and PC-3 cells compared with PWR-1E cells, which express abundant WWOX.

WWOX protein expression in mouse prostate transgenic adenocarcinoma mouse prostate (TRAMP) tissues was evaluated by immunohistochemistry. In the TRAMP model, mouse prostate carcinogenesis is driven by prostate-specific expression of SV40 early genes, and exposure to chemical carcinogens is not needed for tumor induction (18). Although WWOX was abundantly expressed in normal- to low-grade prostatic intraepithelial neoplasia (PIN) prostate tissue (Fig. 1*B, a*), WWOX expression was notably reduced in well-differentiated (noninvasive; Fig. 1*B, b*) and moderately-differentiated (invasive) prostate adenocarcinoma (Fig. 1*B, c*).

WWOX protein expression was assessed in 44 primary human prostate cancers on a tissue microarray by immunohistochemical analysis. Representative images are shown in Fig. 1*C*. Similar to mouse prostate tissues, WWOX was highly expressed in adjacent normal human prostate tissues (Fig. 1*C, a*). Of the 44 tumor cases, WWOX expression was high only in 7 (16%) but low to negative in 37 (84%) cases (data not shown).

WWOX regulatory region is hypermethylated in prostate cancer cells. Although point mutations in WWOX are rare (2), LOH at 16q23 is frequent in several cancer types, and homozygous deletions within the *WWOX* gene have been reported in breast, prostate, liver, esophageal, and lung cancer-derived cells (1–3). In addition, WWOX expression is down-regulated through hypermethylation in breast and lung cancers (16). The lack of point mutations and dramatically reduced expression of WWOX in prostate cancer led us to hypothesize that epigenetic silencing might contribute to the down-regulation in prostate cancer. The genomic region of WWOX from −183 to +177, spanning the promoter and exon 1, contains the highest percentage of CpG dinucleotides ($\sim$70%; ref. 16). We characterized the methylation status within this regulatory region by MSP and found that the *WWOX* gene was methylated in the three prostate cancer-derived cells but not in the noncancer PWR-1E cells (Fig. 1*D*). In addition, treatment with TSA, AZA, or the combination of the two drugs led to significant increases of WWOX mRNA expression in the prostate cancer-derived cells, with the most striking increase in DU145 cells followed by LNCaP and PC-3 (Fig. 1*D*). Correspondingly, WWOX protein expression was elevated following the treatment in DU145 and LNCaP, although the increase in WWOX protein in PC-3 was not as striking, suggesting that other mechanism(s) besides methylation contributes to WWOX down-regulation in this cell line. The finding that WWOX expression was restored after inhibition of methylation *in vitro* provides a rationale for consideration of epigenetic therapies for analysis of *in vivo* efficacy.

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Ectopic expression of WWOX suppressed prostate cancer cell growth. Because endogenous WWOX expression is highly reduced in prostate cancer, we sought to overexpress exogenous WWOX and to characterize the effect of WWOX restoration in prostate cancer-derived cells. We first transfected DU145 cells with a pcDNA3-WWOX expression construct. Forty-eight hours following transfection, reexpression of WWOX was confirmed by Western blot (Fig. 2*A*), and a clonogenicity assay was done. Significantly fewer and smaller colonies were formed in pcDNA3-WWOX-transfected cells compared with the vector control (P = 0.0012; Fig. 2*B* and *C*).

To confirm the role of WWOX in suppressing prostate cancer cell growth, we infected prostate cancer and noncancer cells with Ad-WWOX. Because initial experiments suggested that 100 MOI was sufficient for growth inhibition in prostate cancer-derived cells (Supplementary Fig. S1), cell growth analysis was done after infection with Ad-GFP and Ad-WWOX at 100 MOI. Ad-WWOX, but not Ad-GFP infection, suppressed the growth of the three prostate cancer-derived cell lines but had no apparent effect on noncancer PWR-1E cell growth (Fig. 2*D*). Growth suppression occurred earliest in LNCaP cells followed by DU145 and PC-3 cells.

Ad-WWOX transduction induced apoptosis in prostate cancer-derived cells. Four days after infection with Ad-GFP and Ad-WWOX at 100 MOI, cells were collected for flow cytometry analysis to assess the sub-G_1 population serving as an indicator of cell death. No sub-G_1 peak was detected in noninfected control or Ad-GFP-infected cells (Fig. 3*A*). In contrast, Ad-WWOX infection led to detection of 45.40%, 21.56%, and 25.80% of cells in sub-G_1 in LNCaP, DU145, and PC-3 cells, respectively, whereas only 2.71% of cells were in sub-G_1 in PWR-1E cells. At 6 days after infection, >60% of LNCaP, DU145, and PC-3 cells were in sub-G_1 fraction compared with ~10% in PWR-1E (data not shown). To examine the pathway(s) for Ad-WWOX-mediated cell death, cells were collected 4 days after infection, and immunoblot analysis was done for detection of apoptosis-related proteins. As shown in Fig. 3*B*, Ad-WWOX infection resulted in reduced expression of antiapoptotic protein Bcl2 and led to activation of caspase-3 and caspase-9, as indicated by decreased expression of procaspase-3 and procaspase-9 in the three prostate cancer cell lines but not in PWR-1E cells, suggesting that the mitochondria-mediated caspase-dependent pathway is involved in Ad-WWOX-induced apoptosis in the prostate cancer-derived cells. In accordance with our findings, Chang et al. (19) showed that WWOX significantly down-regulated apoptosis inhibitors Bcl2 and Bcl-xL in mouse L929 fibrosarcoma cells, and Fabbri et al. found that Ad-WWOX infection led to caspase-dependent apoptosis in lung cancer-derived cells (8). We did not detect an effect of Ad-WWOX infection on procaspase-8 level (data not shown), suggesting that the death receptor–mediated apoptotic pathway is not activated. Ad-WWOX-induced growth suppression occurred earlier in LNCaP cells, and more LNCaP cells underwent apoptosis compared with DU145 and PC-3 cells, possibly due to the effects of functional p53 and Rb in this cell line.

Ad-WWOX suppressed tumorigenicity in nude mice. Because Ad-WWOX transduction inhibited prostate cancer cell growth and induced apoptosis *in vitro*, we further explored the effect of Ad-WWOX on the growth of prostate cancer-derived cells *in vivo*. Because LNCaP forms tumors slowly in nude mice (20) and because WWOX mRNA and protein expression were restored most dramatically in DU145 cells after AZA treatment *in vitro*, the DU145 cell line was selected for this study. DU145 tumors grew

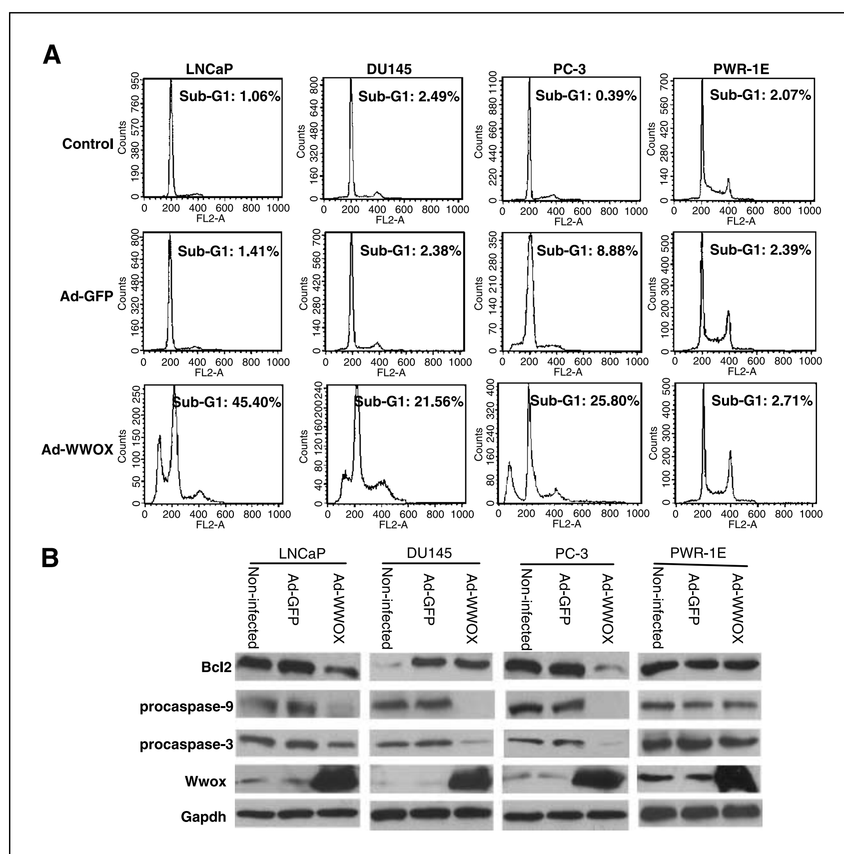

Figure 3. Exogenous WWOX expression induced apoptosis in prostate cancer cells. *A,* cells (5×10^5) were seeded in 100-mm dishes and infected with Ad-GFP or Ad-WWOX at 100 MOI. Cells were harvested 4 days after infection, fixed with 70% cold ethanol, treated with RNase A, and stained with propidium iodide. The samples were analyzed by a FACSCalibur cytometer (BD Biosciences, San Jose, CA). Ad-WWOX infection resulted in significant increase in the sub-G_1 populations in prostate cancer cells (LNCaP, DU145, and PC-3) compared with no infection and Ad-GFP infection. Noncancer PWR-1E cells were not affected. No difference was observed in the sub-G_1 population of Ad-GFP-infected cells compared with uninfected control. *B,* at 4 days after Ad-WWOX infection, the expression level of antiapoptotic protein Bcl2 was reduced in prostate cancer cells but not PWR-1E cells. Caspase-9 and caspase-3 were activated following Ad-WWOX infection, as indicated by reduced expression of procaspase-9 and procaspase-3, compared with uninfected and Ad-GFP-infected controls.

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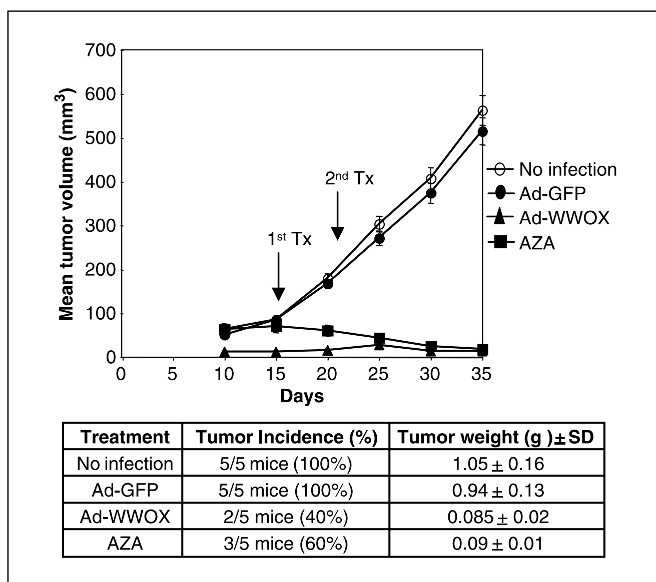

Treatment	Tumor Incidence (%)	Tumor weight (g) ± SD
No infection	5/5 mice (100%)	1.05 ± 0.16
Ad-GFP	5/5 mice (100%)	0.94 ± 0.13
Ad-WWOX	2/5 mice (40%)	0.085 ± 0.02
AZA	3/5 mice (60%)	0.09 ± 0.01

Figure 4. Effect of WWOX restoration on xenograft growth. Ad-WWOX transduction suppressed tumorigenicity of xenografts in nude mice: growth of tumors in mice inoculated with DU145 cells (uninfected, Ad-GFP, and Ad-WWOX-infected) over a 35-day observation period. AZA intratumoral administration restrained tumor growth: AZA (5 mg/kg body weight) treatment was at days 15 and 16 (*1st Tx*) and days 22 and 23 (*2nd Tx*). *Points,* mean tumor volumes; *bars,* SD. Tumor growth was completely halted in mice inoculated with cells infected with Ad-WWOX, whereas Ad-GFP did not result in growth suppression. Tumor growth was also repressed after intratumoral injection of AZA. Tumor incidence and tumor weight in each group are detailed in the table.

progressively in mice inoculated with noninfected control cells, as well as Ad-GFP-infected cells, during the observation period of 35 days (Fig. 4). At end point, little difference was observed in growth of tumors derived from Ad-GFP-infected cells relative to noninfected controls. In contrast, in mice inoculated with Ad-WWOX-infected cells, tumor growth was completely suppressed in three of five mice, and the tumors in the two remaining mice did not actively grow, remaining unchanged throughout the observation period. Furthermore, four intratumoral injections of AZA (5 mg/kg body weight) on days 15, 16, 22, and 23 into small tumors (~100 mm^3) of five control mice suppressed tumor growth. Tumors became undetectable in two of five mice, and the average tumor size in the three remaining mice was reduced to <15 mm^3 at the end of the experiment.

In this first study of the biological effects of WWOX expression in prostate cancer cells, the results suggest that silencing of the WWOX signal pathway is highly relevant to prostate cancer growth and apoptosis and that delineation of this signal pathway will not only help to clarify the role of a significant signal mediator in prostate cancer but may lead to identification of therapeutic targets in addition to WWOX restoration.

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