

WWOX, the FRA16D Gene, Behaves as a Suppressor of Tumor Growth¹

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Abstract

We recently reported the cloning of *WWOX*, a gene that maps to the common fragile site FRA16D region in chromosome 16q23.3-24.1. It was observed that the genomic area spanned by *WWOX* is affected by chromosomal translocations and homozygous deletions. Furthermore, the high incidence of allelic loss in breast, ovarian, prostate, and other cancers affecting this region suggests that *WWOX* is a candidate tumor suppressor gene. Expression of *WWOX* is highly variable in breast cancer cell lines, with some cases showing low or undetectable levels of expression. In this report, we demonstrate that ectopic *WWOX* expression strongly inhibits anchorage-independent growth in soft agar of breast cancer cell lines MDA-MB-435 and T47D. Additionally, we observed that *WWOX* induces a dramatic inhibition of tumorigenicity of MDA-MB-435 breast cancer cells when tested *in vivo*. We also detected the common occurrence of aberrant *WWOX* transcripts with deletions of exons 5–8 or 6–8 in various carcinoma cell lines, multiple myeloma cell lines, and primary breast tumors. These aberrant mRNA forms were not detected in normal tissues. Interestingly, we further observed that proteins encoded by such aberrant transcripts display an abnormal nuclear localization in contrast to the wild-type *WWOX* protein that localizes to the Golgi system. Our data indicate that *WWOX* behaves as a potent suppressor of tumor growth and suggest that abnormalities affecting this gene at the genomic and transcriptional level may be of relevance in carcinogenesis.

Introduction

We recently cloned and described *WWOX*, a gene spanning a genomic region of more than 1 million nucleotide bp located in chromosome 16q23.3-24.1, an area also recognized as the common fragile site FRA16D (1). Common fragile sites are areas of chromosome breakage, which can be induced by exposure to inhibitors of DNA replication such as aphidicolin. FRA16D and FRA3B were found to be the most frequently expressed of the more than 80 described common chromosomal fragile sites (2). It has been suggested that DNA instability at common fragile sites is associated with cancer and that such fragility may affect the function of genes encoded in those chromosome regions. Recently, a detailed physical map of FRA16D was described. Based on this map and our positional cloning data, we can conclude that *WWOX* is the gene that occupies most of this region of chromosome 16 (3).

Allelic losses and chromosomal fragility affecting the chromosome 16 region spanned by *WWOX* were described in breast, prostate, ovarian, and other cancers (4–9). In studies from our laboratory, we previously observed that chromosome 16q (6) and, in particular, the area occupied by *WWOX* were already affected by a high incidence of loss of heterozygosity at preinvasive stages of breast cancer develop-

ment (7). Furthermore, we determined that the area spanned by *WWOX* is affected by hemizygous loss in most breast cancer cell lines, and one case of homozygous deletion was also described (1). Other investigators detected homozygous losses in the same region, and more recently, some of these deletions were confirmed to be affecting *WWOX* exons in ovarian and other cancer cell lines.³ We and others also observed that previously identified chromosomal translocation break points t(14;16)(q32;q23), found in several multiple myeloma cell lines, are located within the *WWOX* gene as well (1, 3). After our cloning of *WWOX*, other investigators cloned the same gene, which they named *FOR*, and reported the existence of alternative mRNA 3'-end-spliced variants (10).

WWOX is a 414-amino acid protein and contains two WW domains in the NH₂ terminus and a short chain dehydrogenase domain in the central portion of the protein. WW motifs are known to be involved in protein-protein interactions (11). Based on the high expression of *WWOX* in hormonally regulated tissues (testis, prostate, and ovary) and its amino acid sequence homology to specific oxidoreductases, we postulated that *WWOX* may be an enzyme involved in steroid metabolism (1). Recently, the mouse homologue was cloned, and it was reported to associate with p53 and to potentially play a role in apoptosis (12).

In this report, we provide evidence for the first time that *WWOX* behaves as a suppressor of tumor growth when ectopically expressed in breast cancer cell lines. We also report the detection of aberrantly spliced *WWOX* mRNA forms with deletion of exons 5–8 or 6–8 in cancer cell lines and primary breast tumors. These aberrant mRNA forms were not detected in normal tissues. Interestingly, we also determined that proteins translated from such abnormal transcripts have a different cellular localization than wild-type *WWOX* protein.

Materials and Methods

Cell Lines and Normal and Tumor Tissues. Cell lines used in these experiments were derived from our collection or obtained from American Type Culture Collection. Normal and breast tumor samples were obtained from the Cooperative Human Tissue Network. RNA was isolated using Trizol reagent (Life Technologies, Inc.) according to the manufacturer's protocol. Isolated RNAs were treated with DNase I (Promega) before cDNA synthesis, which was performed using Superscript II Reverse Transcriptase (Life Technologies, Inc.) according to the manufacturer's protocol. For the methylation study, MDA-MB-435 cells were treated in culture with 5-aza-2'-deoxycytidine (3.5 μg/ml) for 48 h.

Real-time and Nested RT-PCR.⁴ Real-time RT-PCRs were performed with primers for the wild-type *WWOX* transcript designed to span intron 8 (forward primer 5'-TCGCAGCTGGTGGGTGAC-3' located on exon 8 and reverse primer 5'-AGCTCCCTGTTGCATGGACTT-3' located on exon 9).

Real-time RT-PCR was performed using a Perkin-Elmer Biosystems Gene Amp 7700 Sequence Detection System. All reaction components were pur-

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³A. J. Paige, K. J. Taylor, C. Taylor, S. G. Hillier, S. Farrington, D. Scott, D. J. Porteous, J. F. Smyth, H. Gabra, and J. E. Watson, *WWOX*: a candidate tumor suppressor gene involved in multiple tumor types, submitted for publication.

⁴The abbreviations used are: RT-PCR, reverse transcription-PCR; BFA, brefeldin A; GFP, green fluorescence protein.

chased from PE Biosystems. Detection of double-stranded PCR products was performed with SYBR Green I. All reactions were performed in triplicate. Relative levels of expression were normalized using the β 2-microglobulin gene (5'-TGAGTGCTGTCTCCATGTTTGA-3' and 5'-TCTGCTCCCCAC-CTCTAAGTTG-3'; Ref. 13) as an internal reference control.

Nested RT-PCR to determine the existence of aberrant transcripts of *WWOX* was performed using primers spanning the whole open reading frame (forward primer, 5'-AGGCAGTGCGCAGGCGTGAGC-3'; reverse primer, 5'-CAGC-CCTGGCACTTGGCTGAGG-3'; nested primer set; forward primer 5'-AG-CAGGCGTGAGCGGTCGG-3' and reverse primer 5'-TGCCTGAGGGGA-CACACACAGG-3').

RT-PCR screening for aberrantly spliced *WWOX* Δ 6-8 mRNA was performed using forward primer 5'-GAGTTCCTGAGCGAGTGGACCG-3' and reverse primer 5'-ACGGTGGTGGCAGCTCCCTGTTGCCATTCTTC-3', which is positioned on a novel exon-exon junction between exons 5 and 9 and does not amplify the wild-type transcript. These RT-PCR reactions were performed using the Expand High Fidelity PCR System (Roche).

Bisulfite DNA Sequencing. Cell line DNA was isolated using standard methods, and bisulfite sequencing was performed as described previously (14). Primers for amplification of modified DNA were as follows: (a) for the sense strand, 5'-TAGTTTTTATTATTATTAGTTTTTATTATT-3' and 5'-AATAC-TACATCCTAAACAACAA-3'; (b) for nested PCR; 5'-AGTTTTTATTATTAT-GAGTTTTTATTAAAT-3' and 5'-CCRCRCAATACTACATCCTA-3'; (c) for the antisense strand, 5'-GGGATGAGGTYGTTTTGTTT-3' and 5'-TCATA-AATCTCTATTAAACAACAA-3'; and (d) for nested PCR, 5'-GYGTAGTGT-TGTATTTGAAT-3' and 5'-TCACAATCTCTATTATATATTTTAACTA-3'. For sequencing, we used nested primers 5'-TCCTCCCCRCRCAAATAAC-3' and 5'-TTATTATTATGAGTTTTTATTAAATAATAG-3' for the sense strand and 5'-ACCTAAACTACATTTCCCATATCC-3' and 5'-TAGTGTGTATT-TGAATAGTAG-3' for the antisense strand.

Vector Construction and Stable Transfection. *WWOX* wild-type cDNA was cloned into the pHOOK3 vector (Invitrogen) or the pLNCX2 retroviral vector (Clontech). Transfections were performed using a standard calcium phosphate precipitation method with 10 μ g of DNA from the vector or *WWOX* cDNA clone.

For retroviral transduction, *WWOX* cloned into pLNCX2 vector was transduced into the PT67 packaging cell line (Clontech). Positive transfectants were selected for 1 week with G418 (200 μ g/ml). Viruses were produced according to the manufacturer's protocol. Target cells were grown to 30% confluence and infected with viruses (approximately 10^6 colony-forming units/ml) mixed with cell-specific culture medium with Polybrene as vehicle (8 μ g/ml). After 24 h, medium was replaced, and stable transfectants were selected with 200 μ g/ml G418 for 2 weeks.

Soft Agar Growth Assay. The assay was performed using 5×10^3 MDA-MB-435 cells in 2 ml of medium (DMEM + 10% FBS) supplemented with 0.34% agarose (FMC) and layered on a 3-ml base of 0.9% agarose with medium. For T47D cells, we used 5×10^4 cells and DMEM + 10% FBS + 10 μ g/ml insulin medium. Experiments were performed in triplicate in 35-mm, 6-well plates. After 4 weeks of growth, cells were stained by incubation with nitroblue tetrazolium, cultures were photographed, and colonies with a diameter > 200 μ m were counted and measured using NIH Image analysis software.

In Vivo Tumorigenicity Assay. We used female BALB/c athymic nude mice (National Cancer Institute). Eight-week-old animals (5 animals/construct) received bilateral injection into the thoracic mammary fat pad area of 1×10^6 cells/side MDA-MB-435/vector or MDA-MB-435/*WWOX*. Tumor growth was monitored for a period of 7 weeks. Tumor diameter was determined by caliper measurements once a week, and tumor volume was calculated using the formula $\pi/6 \times \text{larger diameter} \times (\text{smaller diameter})^2$. At the end of the experiment, tumors were dissected, and the individual tumor wet weight was determined.

GFP-WWOX Fusion and Cellular Localization. GFP-WWOX NH₂-terminal fusion proteins were constructed using pEGFP-N Vector Systems (Clontech). MCF-10F cells (1×10^7) were electroporated in 400 μ l of complete medium with 20 μ g of the GFP-WWOX construct DNA. Cells were plated into 4-well coverslip chambers and incubated for 24 h before confocal microscopy analysis. The Golgi system was visualized with anti-Golgi 58 K monoclonal mouse antihuman antibody (Sigma Chemical Co.) and Cy5-labeled antimouse IgG donkey secondary antibody (Jackson ImmunoResearch

Laboratories). BFA (Sigma Chemical Co.) treatment was performed 72 h after electroporation (5 μ g/ml) for 45 min, followed by a 35-min recovery in BFA-free media. Mitochondria were visualized using MitoTracker Red CMXRos (Molecular Probes).

Results

Analysis of *WWOX* Expression in Cancer Cell Lines and Breast Tumors

Using Northern blot analysis, we previously observed that breast cancer cell lines express highly variable levels of *WWOX* mRNA and that some of these cell lines displayed very low or undetectable levels of *WWOX* mRNA expression (1). Therefore, to perform a comparative analysis of *WWOX* levels of expression, we performed a quantitative analysis using a real-time RT-PCR approach. The results with regard to the relative expression of *WWOX* are summarized in Fig. 1.

Some breast cancer cell lines showed very low or almost undetectable levels of *WWOX* expression as determined by this very sensitive assay, especially MDA-MB-435, MDA-MB-231, BT-549, and T47D. Interestingly, by surveying the literature, we observed that several of these same breast cancer cell lines were described as highly tumorigenic, as determined by their ability to form tumors in nude mice (15, 16). In contrast, MCF-7 and BT-20, which showed higher levels of *WWOX* expression, have a much lower tumorigenic potential (16).

Promoter Methylation Study

Very often, silencing of expression of a tumor suppressor gene is the result of methylation of cytosine residues in CpG pairs within the gene's promoter region. Because we found that *WWOX* is variably expressed in breast cancer cells and that some cell lines show very low or undetectable levels of expression, we performed bisulfite genomic sequencing of the *WWOX* promoter region in several of the breast cancer cell lines. This analysis was performed on cells that showed the lowest levels of *WWOX* mRNA (BT-549, MDA-MB-231, and MDA-MB-435) and on cells with a high level of *WWOX* transcription (MCF-7 and SKBr-3). We sequenced a CpG-rich area surrounding the translation start codon (from -630 to +280) including a putative TATA box (-301 to -292). In summary, we did not find any evidence of CpG methylation in this region. We also did not observe any significant increase in the level of *WWOX* expression after treatment of MDA-MB-435 cells with 5-aza-2'-deoxycytidine, an inhibitor of CpG methylation (Fig. 1).

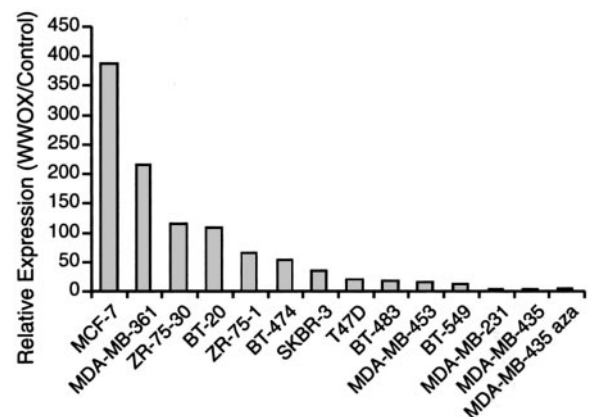
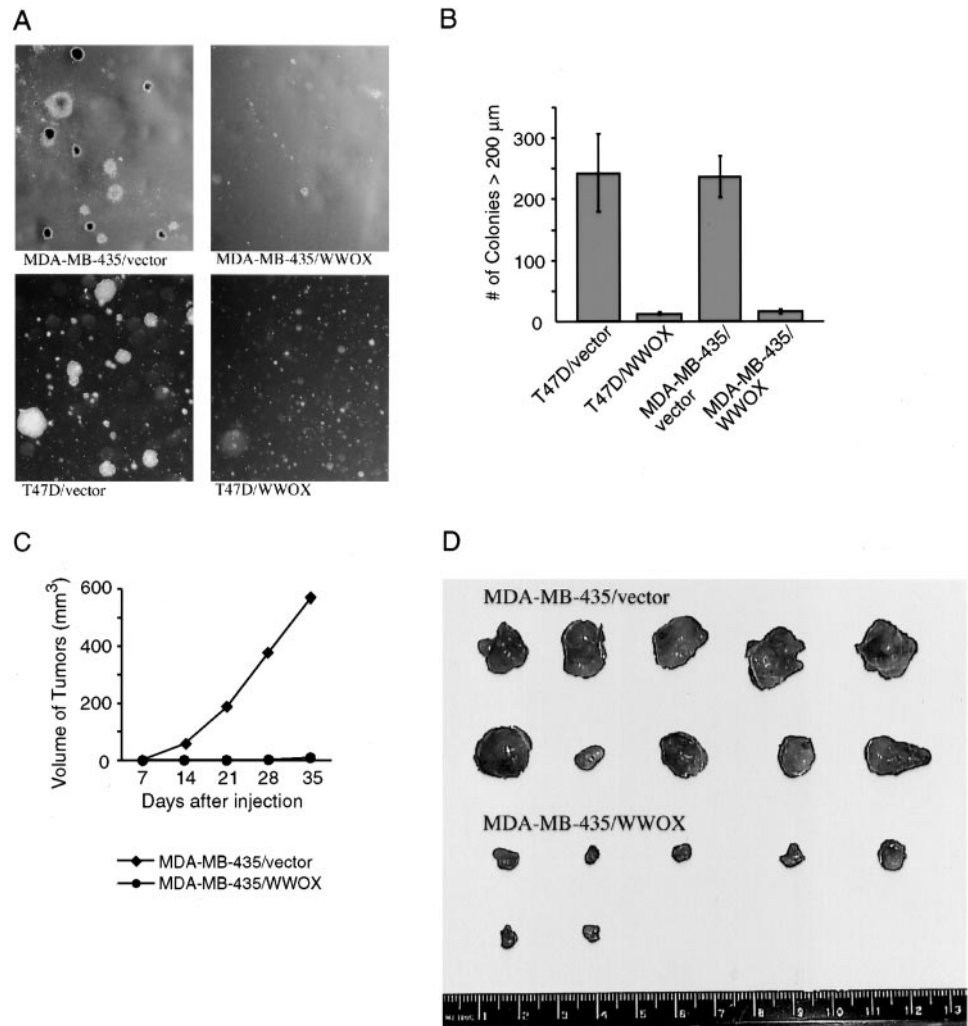


Fig. 1. *WWOX* mRNA expression in breast cancer cell lines as determined by real-time RT-PCR analysis. Levels of *WWOX* expression are represented as compared with those of the control gene β 2-microglobulin.

Fig. 2. Soft agar growth assay (A and B). Ability of *WWOX* to suppress anchorage-independent growth of MDA-MB-435 and T47D breast cancer cell lines transfected with expression vector carrying *WWOX* cDNA or with control vector only. A, low-magnification photographs of representative colonies formed by 30 days after plating by MDA-MB-435 or T47D cells ectopically expressing *WWOX* (right panels) or by cells transfected with vector only (left panels). B, comparative analysis of the number of colonies larger than 200 μm in diameter. Colonies were stained by incubation with nitroblue tetrazolium, complete culture fields were photographed, and colony counting and measurements were performed using NIH Image analysis software. C and D, suppression of tumorigenesis of MDA-MB-435 breast cancer cells in nude mice by ectopically increased *WWOX* expression. Five animals per condition (*i.e.*, cells transfected with vector alone or vector with *WWOX*) received bilateral injection into the thoracic mammary fat pads of 1×10^6 cells/site as described in "Materials and Methods." Tumor growth was monitored for 7 weeks, and caliper measurements were conducted once a week. C, comparative analysis on the rate of tumor growth from cells transfected with vector alone or vector with *WWOX*. Tumor volumes were calculated as determined in "Materials and Methods." Note the clear difference in tumor latency and rate of tumor growth. D, the top two rows display the tumors formed by MDA-MB-435/vector cells in nude mice at the end of the observation period. A total of 10 tumors developed in 10 injection sites. The bottom two rows display the tumors formed by MDA-MB-435 *WWOX*-transfected cells at the end of the same observation period (7 tumors developed; 10 injection sites). Note the dramatic difference in size between both groups ($P = 0.00001$).



Phenotype Analysis of *WWOX* Transfectants

In Vitro Studies. It has been demonstrated previously that restoring or increasing the expression of a tumor suppressor gene in cancer cell lines negatively affects anchorage-independent growth and/or tumorigenic potential. To determine whether increased *WWOX* expression may change the phenotype of cancer cell lines expressing low levels of *WWOX* mRNA, we used recombinant retroviruses carrying the cloned *WWOX* cDNA to transduce breast cancer cell lines MDA-MB-435 and T47D. Expression of the ectopic *WWOX* cDNA in transduced cells was confirmed using real-time RT-PCR (data not shown). We first compared the ability to grow in monolayer cultures of *WWOX* versus empty vector breast cancer line transfectants, and we found no detectable differences (data not shown). However, dramatic differences were observed when we compared their ability to grow in soft agar. We found that increased *WWOX* expression strongly inhibits growth in soft agar of MDA-MB-435 cells (Fig. 2A). The average number of colonies formed by MDA-MB-435/vector cells was 236 ± 33 , whereas MDA-MB-435/*WWOX* cells formed only 17 ± 3 colonies ($P = 0.006$). Similarly, T47D cells transfected with *WWOX* showed a dramatic decrease in their ability to grow in soft agar (14 ± 2 colonies compared with 243 ± 64 colonies formed by T47D/vector cells; $P = 0.02$; Fig. 2B). In summary, breast cancer cells that are ectopically expressing *WWOX* formed fewer and much smaller colonies than did control cells transfected with vector only.

In Vivo Studies. Because growth in soft agar does not necessarily reflect the ability to form tumors, we performed intramammary fat pad

injections in nude mice of the MDA-MB-435/*WWOX* and MDA-MB-435/vector cells to compare their tumorigenicity. Tumor growth was monitored for 7 weeks, and tumor size was measured once a week as described in "Materials and Methods." Tumors generated by the MDA-MB-435/vector cells were already palpable by 7 days after injection and grew rapidly in size from that point on. On the other hand, for the MDA-MB-435/*WWOX*-injected animals, the first very small tumors were detected by 25 days postinjection, but only in some of the mice. Fig. 2C illustrates a dramatic difference in kinetics of tumor growth as determined by caliper size measurements between both groups of animals. At termination of the experiment, MDA-MB-435/vector cells formed tumors displaying an average weight of 821 ± 295 mg, with tumors formed at all of the injection sites (10 of 10 sites), whereas MDA-MB-435/*WWOX* cells formed much smaller tumors with an average wet weight of 51 ± 22 mg, with tumors formed at 7 of 10 sites (Fig. 2D). Such a remarkable difference in the tumor growth rate and size ($P = 0.00001$) strongly supports our conclusion that *WWOX* behaves as a suppressor of tumor growth.

Analysis of Aberrantly Spliced *WWOX* mRNAs

Northern blot analyses from breast cancer, ovarian cancer, and multiple myeloma cell lines showed the presence of transcripts of smaller size in some of the cancer cell lines analyzed, which could represent abnormal *WWOX* mRNAs (Fig. 3A). Therefore, to further investigate the presence of such putative aberrant transcripts and

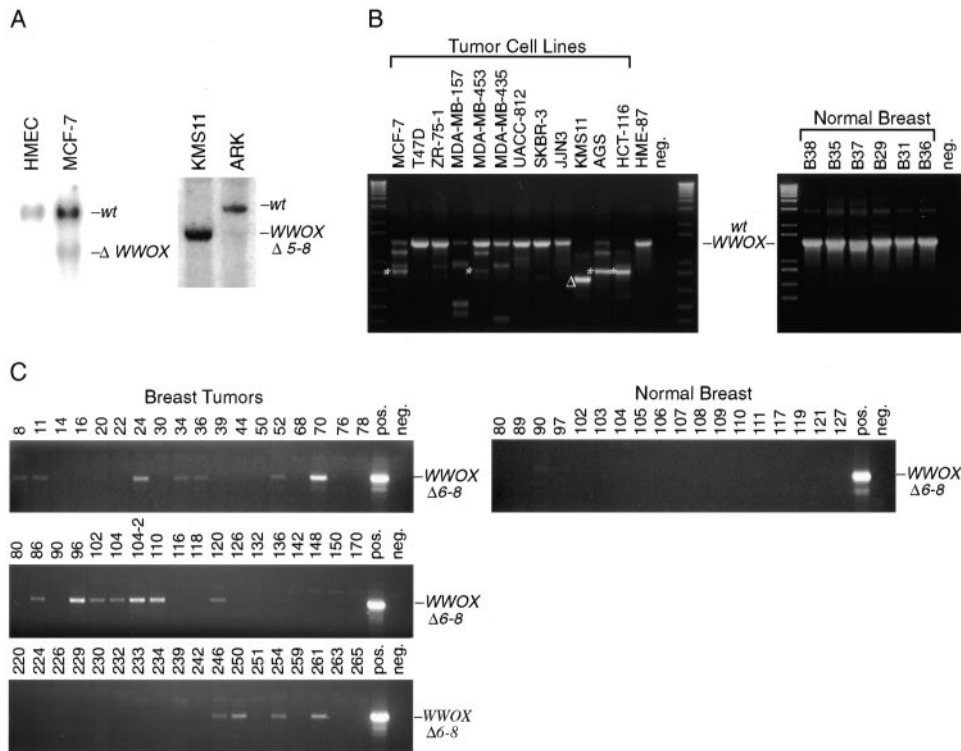


Fig. 3. A, Northern blot analyses demonstrating the detection of aberrant *WFOX* transcripts in breast cancer cell line MCF-7 (left panel) and in multiple myeloma line KMS11 (right panel). The KMS11 transcript is that of the *WFOX* Δ 5-8 form (sequence reported to GenBank under accession number AF395124). Human normal mammary epithelial cells and ARK (multiple myeloma cell line) cells show the normal size wild-type *WFOX* transcript. B, RT-PCR analysis of full-length *WFOX* cDNA and detection of aberrant transcripts in cancer cell lines and human breast tumors. RT-PCR analysis of *WFOX* transcripts in various cancer cell lines (left panel) and normal human mammary gland organoids (right panel). MCF-7, T47D, ZR-75-1, MDA-MB-157, MDA-MB-453, MDA-MB-435, UACC-812, and SKBr-3 are breast cancer cell lines; JN3 and KMS11 are multiple myeloma cell lines; AGS is a gastric adenocarcinoma cell line; HCT-116 is a colon adenocarcinoma cell line; HME-87 is a normal breast epithelial cell line; and B38, B35, B29, B31, and B36 are normal mammary organoid samples. The whole open reading frame (exon 1-exon 9) 1422-bp *WFOX* wild-type fragment was amplified using a nested PCR approach. Aberrant transcripts labeled with an asterisk represent the *WFOX* Δ 6-8 versions, and those labeled with Δ identify the *WFOX* Δ 5-8 cDNA form. Note the detection of the *WFOX* Δ 6-8 form in cancer cell lines MCF-7, MDA-MB-453, AGS, and HCT-116 and the detection of the *WFOX* Δ 5-8 form in myeloma cell line KMS11. C, RT-PCR analysis of the *WFOX* Δ 6-8 transcript in human breast cancer samples. Amplification was made specific for this product by using a 3' primer spanning the exon 5-9 junction ("Materials and Methods"). Left panels, breast cancer samples; right panel, normal breast adjacent tissue samples; pos., positive control (plasmid containing *WFOX* Δ 6-8 cDNA); neg., negative control.

characterize them, we used a full-length mRNA nested RT-PCR approach.

Confirming the Northern analysis observations, several cancer cell lines showed smaller size amplification products (Fig. 3B) in addition to the normal PCR product (1422 bp). All such RT-PCR products were isolated and sequenced. Sequencing of the shorter transcripts allowed us to conclude that they represent abnormally spliced versions of *WFOX*. Interestingly, we detected the presence of transcripts displaying deletions of exons 6-8 (*WFOX* Δ 6-8) in MDA-MB-453, MCF-7, HCT-116 (colon adenocarcinoma), and AGS (gastric adenocarcinoma) cancer cell lines (Fig. 3B). We also observed a deletion of exons 5-8 as the highly and only expressed transcript obtained from KMS11, a multiple myeloma cell line. This deletion found in KMS11 also results in a frameshift. The first 136 of the 311 amino acids in the new protein are conserved, but the following 175 amino acids are different than those in the wild-type protein. The cDNA sequence of such aberrant transcripts has been reported to GenBank with accession numbers AF395123 and AF395124, respectively. Other aberrant transcripts displaying no wild-type translation start codon were found in breast cancer cell lines MDA-MB-157 and MDA-MB-435. In contrast, RT-PCR analysis of several normal breast tissues showed only the wild-type length of *WFOX* mRNA (Fig. 3B).

Due to the finding of *WFOX* Δ 6-8 aberrant transcripts in breast cancer lines, we designed a specific primer set to detect this form. To provide specificity, the 3' RT-PCR primer was designed to span the abnormal exon 5-9 junction. Using this primer set, we performed the screening of cDNAs obtained from a panel of 53 fresh breast cancer

samples in search of the abnormal *WFOX* Δ 6-8 product. We detected the *WFOX* Δ 6-8 aberrant transcript in 17 of the 53 specimens, which represents 32% of the examined tumors (Fig. 3C). In contrast, we did not detect the *WFOX* Δ 6-8 transcripts in any of the 18 adjacent, normal breast tissue samples tested (Fig. 3C).

Normal and Abnormal *WFOX* Cellular Localization

To determine the normal cellular localization of *WFOX* and compare proteins resulting from the aberrant transcripts detected in cancer cells, we used *WFOX* proteins fused to the GFP. Confocal microscopy analysis of normal breast MCF-10F cells transiently transfected with GFP-*WFOX* showed that *WFOX* is a cytoplasmic protein localized in distinct perinuclear particles. Dual-color detection of GFP-*WFOX* and a mitochondria-specific staining allowed us to conclude that *WFOX* does not localize in mitochondria (Fig. 4A). In a second experiment, we determined that GFP-*WFOX* is localized within the Golgi complex due to its colocalization with antibody anti-Golgi 58 K protein, which is specific for an epitope located on the Golgi membrane (Fig. 4B). To confirm this finding, we exposed GFP-*WFOX*-transfected cells to BFA. BFA is known to cause disassembly of the Golgi complex and redistribution of its contents to the cytoplasm. After BFA treatment, we observed that GFP-*WFOX* was diffusely distributed in the cytoplasm, thus confirming our original localization. After removing BFA from the culture media and allowing a short time for recovery, GFP-*WFOX* was again found to be localized within the recovered Golgi apparatus (Fig. 4C). Our con-

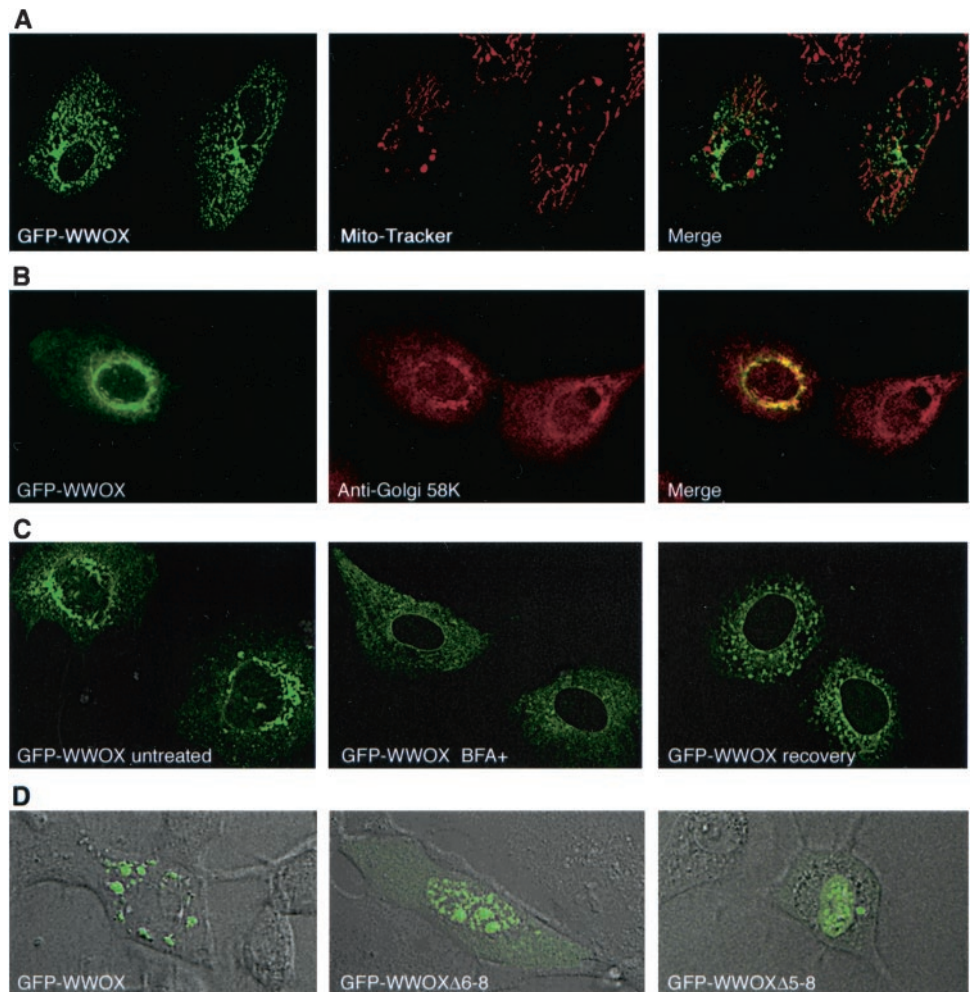


Fig. 4. Wild-type WWOX is localized in the Golgi complex, but aberrant versions of the protein are localized in the cell nucleus. *A*, left panel, GFP-WWOX-transfected MCF-10F cells; middle panel, mitochondria stained with MitoTracker Red CMXRos; right panel, merged image. Note that there is no colocalization of GFP-WWOX and mitochondria staining in the MCF-10F cells. *B*, left panel, GFP-WWOX; middle panel, anti-Golgi 58 K staining; right panel, merged image demonstrates the colocalization of GFP-WWOX with the Golgi-specific staining. *C*, exposure of GFP-WWOX-transfected cells to BFA. Left panel, untreated MCF-10F cells; middle panel, cells after a 45-min incubation with BFA (note that the GFP-WWOX cellular localization was affected by the BFA exposure); right panel, cells after a 30-min recovery from BFA treatment. *D*, combined Nomarski imaging and GFP fusion protein detection. Left panel, WWOX wild-type protein; middle and right panels, aberrant proteins as indicated. Note the distinct nuclear localization of the aberrant proteins, in contrast with the wild-type WWOX cytoplasmic localization.

firmed localization of WWOX to the Golgi system is in disagreement with previous studies with the mouse homologue protein, which was reported to localize in mitochondria (12). The reason for this discrepancy is unclear at this point.

Interestingly, in sharp contrast to the wild-type WWOX protein localization within the Golgi complex, we found that GFP-WWOX Δ 6-8 and the GFP-WWOX Δ 5-8 forms localize to the cell nucleus (Fig. 4D). These WWOX abnormal proteins display a specific pattern in the nucleus, which may be associated with distinct nuclear particles and/or putative transcription sites.

Discussion

Among the characteristic hallmarks of tumor suppressor genes is the inactivation of both alleles. Tumor suppressor alleles are usually silenced by point mutations, complete or partial deletions, or promoter methylation. More recently, haploinsufficiency has been postulated as an additional possible mechanism of tumor suppressor inactivation (17). In this report, we provide evidence demonstrating that WWOX behaves as a tumor suppressor gene when ectopically expressed in breast cancer cells with low or undetectable levels of endogenous expression. We demonstrated that WWOX expression induces a strong suppression of anchorage-independent growth of breast cancer cell lines T47D and MDA-MB-435. More importantly, tumorigenicity of the MDA-MB-435 breast cancer line was dramatically reduced by ectopically expressed WWOX. In previous studies, we have determined that the MDA-MB-435 cell line was hemizygous for WWOX (1). In these studies, we further concluded that the undetectable

WWOX expression in this and other cell lines does not appear to result from a promoter methylation phenomenon of the remaining allele. Therefore, at least in these cases, the lack of expression is likely the result of transcriptional regulation events.

It was intriguing to observe that in several cancer cell lines and in more than 30% of primary breast tumors, we found the expression of WWOX aberrant mRNA transcripts displaying deletions of exons encoding major portions of the enzymatic WWOX domain (WWOX Δ 5-8 and WWOX Δ 6-8). Furthermore, we found no evidence of such aberrant transcripts in any normal tested breast tissues obtained from either normal mastectomy specimens or normal samples adjacent to tumors.

Interestingly, the aberrant proteins resulting from the WWOX Δ 5-8 and WWOX Δ 6-8 transcripts were found to localize in the cell nucleus, in contrast to the normal Golgi complex localization of the wild-type protein. The resultant disruption in the catalytic function of the oxidoreductase domain consequential to the deletion of exons 5 or 6 through 8, plus the shift in normal cellular localization, may dramatically alter the function of WWOX in the cell. Hypothetically, different protein partners could bind to the WW domains of WWOX in the nucleus than in its normal cytoplasmic localization. It is also possible that the WWOX Δ 6-8 and WWOX Δ 5-8 proteins may behave in a dominant negative fashion, competing with wild-type WWOX for its normal partners and eventually transporting them to the cell nucleus.

It is worth mentioning that the findings of abnormalities affecting WWOX transcription as well as the aberrations affecting the genomic region spanned by this gene, *i.e.*, the FRA16D, are very similar to

those found with the *FHIT* gene, which is also a gene spanning a very large genomic area and localizing to another common chromosomal fragile site, FRA3B (18, 19).

Interestingly, it was also shown that *FHIT* behaves as a tumor suppressor gene when ectopically expressed in tumor cells (20). In addition, *FHIT* (+/-) heterozygous mice are highly sensitive to tumorigenicity upon carcinogen exposure (21). It has been suggested, however, that genomic abnormalities affecting *FHIT* could be just a consequence of its location within an unstable genomic region, thus questioning the role of *FHIT* in carcinogenesis (22). On the contrary, we argue that it is precisely the localization of genes such as *WWOX* and *FHIT* within very unstable chromosomal regions (FRA16D and FRA3B) that makes them prime targets to be affected by genomic aberrations during carcinogenesis. Furthermore, the accumulated data indicate that inactivation by diverse mechanisms of these genes appears to be of importance in carcinogenesis.

We speculate that the normal cellular function of *WWOX* may be affected by various different mechanisms. As mentioned, we and others have previously demonstrated a high incidence of loss of heterozygosity affecting *WWOX* in various cancer types (1, 23, 24). In our previous studies, we already observed a high incidence of allele losses at preinvasive stages of breast cancer (7), suggesting that one of the *WWOX* alleles is inactivated very early in breast carcinogenesis. Such single allele loss by itself may be of importance and lead to phenotypic abnormalities as observed in cases of tumor suppression inactivation due to haploinsufficiency; an example of this is the mechanism postulated to operate in the inactivation of the p27^{kip1} gene (17).

In addition, other mechanisms may be operational to further affect the expression of the remaining *WWOX* allele, such as that seen in the case of the MDA-MB-435 breast cancer cell line, e.g., transcriptional down-regulation. Furthermore, other, more classical mechanisms of additional *WWOX* inactivation also appear to be operational in some cases, as was observed by others via homozygous deletions of exonic regions in small cell lung cancer and ovarian cancer cell lines.³ Finally, a mechanism that also needs to be further investigated is the possible dominant negative effect exerted by the aberrant *WWOX* transcripts detected in cancer cell lines and primary tumors. As mentioned, this becomes even more relevant due to the described abnormal cellular localization of the aberrant *WWOX* proteins and their potentially disabled enzymatic function. The sum of the evidence provided indicates that aberrations at both the genomic and transcriptional level affecting *WWOX*, the FRA16D gene, may be of importance in carcinogenesis.

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