Cancer Research

Frequent Attenuation of the WWOX Tumor Suppressor in Osteosarcoma Is Associated with Increased Tumorigenicity and Aberrant RUNX2 Expression

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Abstract

The WW domain-containing oxidoreductase (WWOX) is a tumor suppressor that is deleted or attenuated in most human tumors. *Wwox*-deficient mice develop osteosarcoma (OS), an aggressive bone tumor with poor prognosis that often metastasizes to lung. On the basis of these observations, we examined the status of WWOX in human OS specimens and cell lines. In human OS clinical samples, WWOX expression was absent or reduced in 58% of tumors examined ($P < 0.0001$). Compared with the primary tumors, WWOX levels frequently increased in tumors resected following chemotherapy. In contrast, tumor metastases to lung often exhibited reduced WWOX levels relative to the primary tumor. In human OS cell lines having reduced WWOX expression, ectopic expression of WWOX inhibited proliferation and attenuated invasion *in vitro*, and suppressed tumorigenicity in nude mice. Expression of WWOX was associated with reduced RUNX2 expression in OS cell lines, whereas RUNX2 levels were elevated in femurs of *Wwox*-deficient mice. Furthermore, WWOX reconstitution in HOS cells was associated with downregulation of RUNX2 levels and RUNX2 target genes, consistent with the ability of WWOX to suppress RUNX2 transactivation activity. In clinical samples, RUNX2 was expressed in the majority of primary tumors and undetectable in most tumors resected following chemotherapy, whereas most metastases were RUNX2 positive. Our results deepen the evidence of a tumor suppressor role for WWOX in OS, furthering its prognostic and therapeutic significance in this disease. *Cancer Res; 70(13); 5577–86. ©2010 AACR.*

Introduction

The WW domain-containing oxidoreductase (*WWOX*) gene encodes a 46-kDa tumor suppressor that is altered in most human cancers (1, 2). *WWOX* spans the second most active common fragile site, FRA16D, frequently involved in cancer (3, 4). Loss of heterozygosity (LOH), homozygous deletions (HD), hypermethylation, and chromosomal translocations affecting *WWOX* have been reported in many malignancies. *WWOX* inactivation has been associated with more aggressive tumors and with poor prognosis (2, 5, 6). Recently, we have generated a targeted ablation of the *Wwox* gene in mice (7). Analysis of *Wwox*-mutant mice shows a bona fide tumor suppressor function of WWOX. More than 30% of *Wwox*-deficient juvenile mice develop periosteal osteosarcomas (OS; ref. 7). Importantly, molecular characterization of these tumors identifies RUNX2 as a partner of WWOX (8).

RUNX2 is an essential transcription factor for bone formation, and accumulative data have shown that its levels are upregulated in many malignancies, including OS and metastatic breast and prostate cancers (9–14). Our recent findings show that WWOX physically associates with RUNX2 and functionally suppresses its transactivating function in osteoblasts and in neoplastic cells (8). Furthermore, analysis of *Runx2* mRNA expression in femurs of *Wwox*-deficient mice revealed its significant upregulation (8), suggesting that WWOX may regulate RUNX2 levels. Interestingly, osteoblasts isolated from *Wwox*-deficient mice exhibit an autonomous defect in differentiation (8).

OS is the most common primary malignant bone tumor in childhood and adolescence (15). This highly aggressive tumor

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usually involves long bones and frequently metastasizes to the lungs. Little is known of the etiology of OS and lesser still of the various interactions that occur between host and tumor cells to regulate the growth and progression of OS *in vivo*. OS is genetically heterogeneous, marked by multiple random chromosomal aberrations with only a few recurring deletions and amplifications (16, 17). Recently, it has been shown that mice with osteoblast-restricted loss of p53 and Rb uniformly develop OS (18). Molecular analyses of OS have also revealed alterations in transforming growth factor-β/bone morphogenetic protein, Wnt/β-catenin, and cell cycle–related pathways (10, 19–21). RUNX2 levels are increased in human OS (10, 12–14); however, the cause of this elevation is not known. Molecular markers predictive of OS tumor progression, metastasis, and response to chemotherapy are lacking, preventing development of more biologically tailored therapies for high-risk patients.

Here, we report immunohistochemical studies of WWOX and RUNX2 expression in a series of human OS samples recovered as biopsies from untreated primary tumors, bulk resections following chemotherapy, and excised metastases. We also describe the effect of overexpressing WWOX in human and in murine OS cell lines on the proliferation, migration, and invasion *in vitro* of the cell lines and on their xenograft growth in nude mice. Our findings suggest that reduction of WWOX expression is a common event in human OS and that its expression could serve as a prognostic and therapeutic indicator for assessing OS progression.

Materials and Methods

Cell lines and cell culture

OS-derived cell lines were obtained from the American Type Culture Collection; human MG-63 cell line was a gift from Prof. Zvi Bar Shavit (Hebrew University), and MLOY4 cells were received from Prof. Linda Bonewald (University of Kansas). Cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum.

In vitro viral transduction

The *WWOX* adenovirus transduction has been previously described (22). Ad-*GFP* virus served as a control. Cells were transduced with Ad-*WWOX* or Ad-*GFP* at appropriate multiplicities of infection (MOI), and transduction efficiency was assessed by visualization of green fluorescent protein (GFP)–expressing cells. To generate a lentiviral *WWOX* vector, human *WWOX* cDNA was cloned in lentiviral vector containing neomycin as the selection marker (gift from Dr. Ittai Ben-Porath, Hebrew University) using the Gateway cloning system (Invitrogen).

In vivo studies

Animal studies were done under an approved protocol according to institutional guidelines. KHOS, HOS, and K7M2 OS cells were infected with Ad-*WWOX* or Ad-*GFP* at a MOI of 100. Twenty-four hours after infection, 10^7 cells were injected s.c. into the flanks of 6-week-old female nude mice (five mice per group; Charles River Laboratories). Five control mice were injected with 10^7 uninfected cells. Animals were monitored daily, and tumor sizes were measured every 5 days. At the end point (day 28), animals were sacrificed, tumors were weighed, and tumor volumes were calculated as previously described (22).

Immunohistochemistry

Human OS tissue sections were obtained following Institutional Review Board approval. For each tumor, immunohistochemistry was performed on one representative formalin-fixed, paraffin-embedded section chosen by review of the H&E-stained slides. Polyclonal anti-WWOX antibody (1:5,000 dilution; ref. 23) or monoclonal anti-RUNX2 antibody (1:100 dilution; ref. 12) was used. Immunohistochemical staining and scoring of WWOX and RUNX2 staining were determined by at least two pathologists. For WWOX, tumors were grouped into three categories (strong, reduced, and absent) based on the intensity of cytoplasmic staining in all tumor cells present on the slides. The "strong" category was assigned when the intensity was equivalent to that found in osteoblasts and chondrocytes from healthy tissues. The "reduced" category was used for tumors with clearly diminished intensity, and the "absent" category for tumors with no WWOX immunostaining. When present, osteoblasts in reactive bone surrounding tumors and chondrocytes in bronchial cartilage served as internal positive controls. Nuclear RUNX2 staining was scored as either positive or negative. Tumors with only scattered RUNX2-positive cells (<2% of all tumor cells) were considered negative.

Statistical analysis

Results of *in vitro* and *in vivo* experiments were expressed as mean ± SD or SE. Fisher's exact test, Student's *t* test, and 95% confidence interval (CI) based on exact binomial CI were used to compare values of test and control samples. $P < 0.05$ indicated significant difference.

Detailed Materials and Methods are provided as Supplementary Data.

Results

High incidence of OS in *Wwox*-deficient mice

We previously reported that ~30% of *Wwox*-deficient mice developed spontaneous OS (7). This incidence was based on sections through paraffin-embedded bone. Because only few serial slides contain tumor, we developed a screening approach using a more sensitive detection method by micro–computed tomography (μCT) imaging of intact limbs (Supplementary Fig. S1A). Irregular protrusions on the endosteal or periosteal sides of the cortex seen on μCT were then subjected to histologic assessment (Supplementary Fig. S1B). Using this approach, 100% of *Wwox*-deficient mice had developed OS by 18 days of age and 68% of mice had bilateral involvement (Supplementary Table S1). These new findings strengthen the hypothesis that *WWOX* deficiency can contribute to human OS.

Table 1.

A. WWOX immunostaining in human OS

Specimen	Total	Altered*	%	95% CI†	*P*‡
Normal bone	12	0	0	00.00–26.46	—
OS					
Biopsies	34	20	59	40.70–75.35	0.0004
Resections	34	20	59	40.70–75.35	0.0004
Metastases	15	8	53	26.59–78.73	0.003
Total OS	83	48	58	46.49–68.60	<0.0001

B. RUNX2 immunostaining in human OS

Specimen	Total	Positive		Negative		95% CI
		No.	%	No.	%	
Biopsies	20	12	60	8	40	36.05–80.88
Resections	25	4	16	21	84	5.45–41.91
Metastases	11	8	73	3	27	39.03–93.98
Total OS	56	24	43	32	57	56.60–88.54

*Altered represents reduced and absent WWOX immunostaining.
†95% CIs are based on exact binomial CI.
‡*P* values are based on Fisher's exact test versus the normal bone samples.

Frequent alteration of WWOX expression in human OS

To assess the clinical significance of WWOX protein expression in human OS, 83 OS samples from 51 patients (Supplementary Table S2) were analyzed by immunohistochemistry and compared with 12 normal bone and cartilage tissues (Table 1A). These included 34 pretreatment biopsies, 34 posttreatment resections, and 15 posttreatment metastasis resections. Representative examples of OS with normal, reduced, and absent WWOX immunoreactivity are shown in Fig. 1A. WWOX expression was uniformly strong in the cytoplasm of the 12 normal bone and cartilage specimens (Supplementary Fig. S2; Fig. 1A). In OS specimens, strong WWOX expression was detected in 42% (35 of 83), whereas 58% (48 of 83) exhibited absent or reduced (grouped as Altered) WWOX immunoreactivity ($P < 0.0001$; Table 1A). These data indicate that WWOX expression is significantly altered in OS tumors compared with normal bone.

WWOX expression following chemotherapy

After biopsy diagnosis, OS is treated with a chemotherapeutic induction regimen of cisplatin, doxorubicin, and high-dose methotrexate before surgical resection (24). To determine the effect of chemotherapy on WWOX expression, the immunohistochemical expression of WWOX was compared in pretreatment biopsies and posttreatment resection specimens from 16 OS patients. WWOX expression increased in 44% (7 of 16) of the paired samples, 5 of which were initially WWOX negative (95% CI, 19.75–70.12; Fig. 1B; Supplementary Table S3). Another 44% (7 of 16) of the paired samples showed no change in WWOX; however, all expressed WWOX in the pretreatment biopsies. WWOX decreased in postchemotherapy specimens in 22% (2 of 16) of the paired samples. These results suggest that chemotherapy, which induces tumor cell normalization, is accompanied by restoration of WWOX expression. Interestingly, all samples where WWOX increased had a poor response to chemotherapy (<90% necrosis), whereas 67% of the remaining paired samples had a favorable response (Supplementary Table S4).

WWOX expression in metastatic lesions

Because OS frequently metastasizes, we determined whether WWOX expression in metastases was different than in primary OS by comparing immunohistochemical expression in paired samples from nine patients who had a pretreatment OS biopsy and a posttreatment metastasectomy. Nearly half (four of nine) of paired specimens showed reduced or absent WWOX expression in the metastasis compared with the primary tumor (95% CI, 13.70–78.80; Fig. 1C; Supplementary Table S5). WWOX levels were unchanged in three of the paired samples (two already had reduced WWOX levels in the primary tumor), whereas two metastases exhibited higher WWOX expression compared with the primary tumor. These results suggest that decreased WWOX levels are associated with a more aggressive tumor cell at the metastatic site.

WWOX expression in OS cell lines

To determine the expression of WWOX in OS cells, we examined mRNA levels and protein expression in five human and three mouse OS cell lines. Four human OS cell lines (MG-63, KHOS, SAOS2, and HOS) exhibited significantly lower

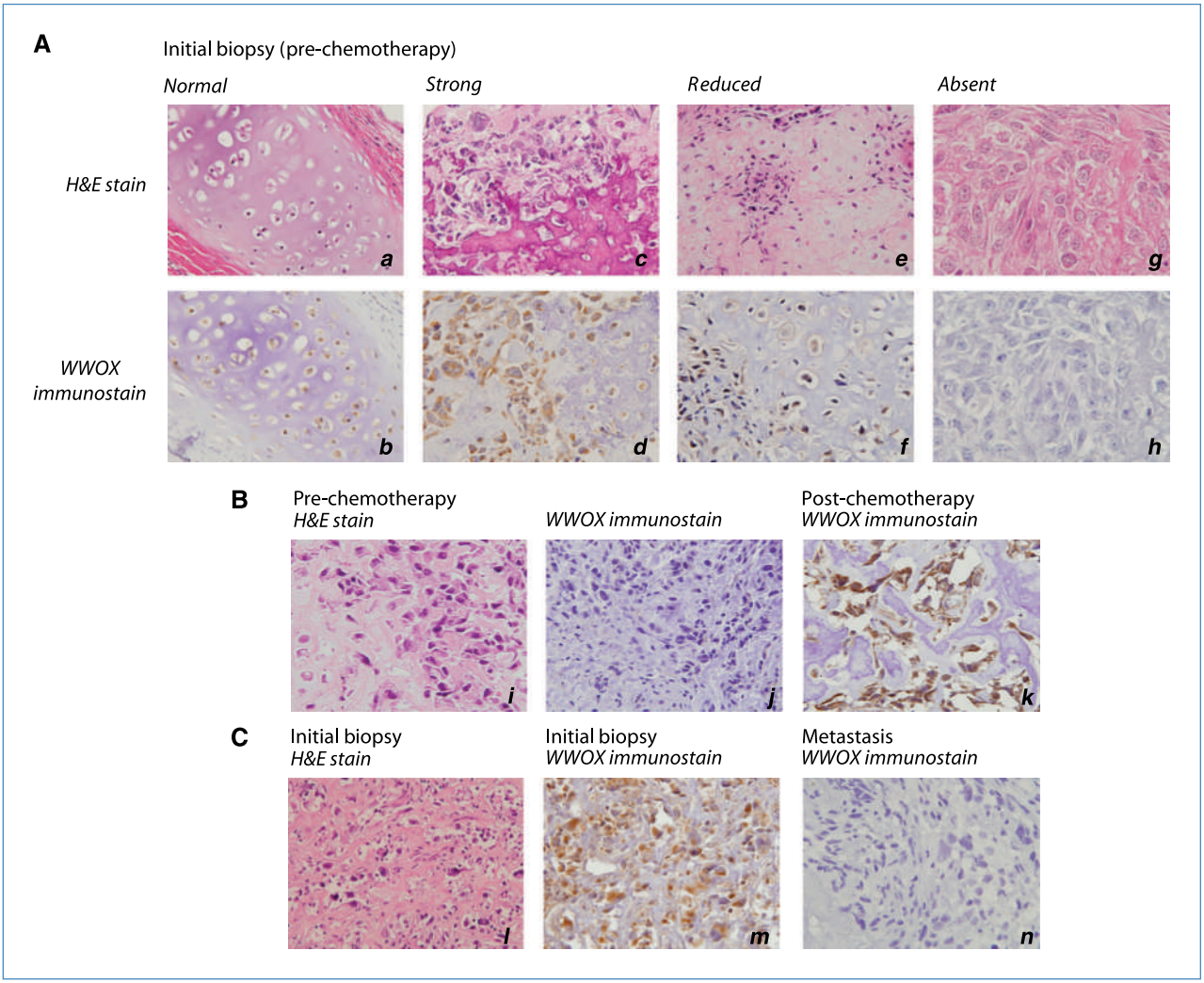

Figure 1. Altered WWOX expression in human OS cases. A, patterns of WWOX immunohistochemical expression in human OS. a and b, H&E and WWOX immunostaining showed uniformly strong WWOX expression in peribronchial cartilage and normal bone (see Supplementary Fig. S1). Magnification, ×400. c and d, H&E and OS sections showing strong WWOX expression. Magnification, ×600. e and f, H&E and OS sections with reduced WWOX expression. Magnification, ×400. WWOX is reduced in both the chondroid and more primitive cells of the tumor. g and h, H&E and OS sections with absent WWOX expression. Magnification, ×1,000. B, immunohistochemical staining of WWOX shows its absence in the pretreatment biopsy (i and j; magnification, ×600) and appearance of WWOX in a posttreatment resection (k; magnification, ×1,000) from the same patient. C, immunohistochemical staining of WWOX shows its presence in the biopsy section (l and m; magnification, ×600) and its absence in a metastasis resection (n; magnification, ×1,000) from the same patient. In each panel, one representative picture is shown from each group.

WWOX expression than in hBM, *WWOX*- positive MCF-7 breast cells, and murine preosteoblastic MC3T3 cells (Fig. 2A). *WWOX* mRNA expression in these human OS cells was comparable with MDA-MB-231 cells, a breast cancer cell line that expresses very low levels of *WWOX* (25). By contrast, *Wwox* mRNA expression in the K7M2 mouse OS cell line was comparable with mouse bone marrow, MC3T3, and MLOY4 normal osteocytes (Fig. 2B). Protein expression was barely detected in the four human cell lines compared with MCF-7 cells, which express abundant WWOX (Fig. 2C). The K7M2 mouse OS cells exhibited comparable WWOX protein levels with the mouse preosteoblastic MC3T3 cells (Fig. 2C). These results indicate that WWOX expression is altered in most human OS cells.

Effects of WWOX overexpression on OS proliferation, apoptosis, and tumorigenicity

Because four of five human OS cell lines exhibited low endogenous WWOX expression, we determined whether overexpressing WWOX would alter their growth. We overexpressed WWOX in three OS cell lines using Ad-*WWOX* (22) or Ad-*GFP* at a MOI of 100. Immunoblot analysis 72 hours after infection confirmed WWOX overexpression in all Ad-*WWOX*–infected cells (Fig. 3A). Next, we measured cell proliferation by the 2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide inner salt (XTT) assay. Two OS cell lines (KHOS and HOS) that have very low endogenous WWOX levels exhibited reduced proliferation following Ad-*WWOX* infection compared with Ad-*GFP* infection

(Fig. 3B). In contrast, K7M2 cells, which endogenously express higher levels of WWOX, had no change in proliferation following WWOX overexpression (Fig. 3B).

Previous studies have shown that ectopic WWOX expression promotes apoptosis (22). We therefore used propidium iodide and flow cytometry to assess whether WWOX overexpression induces apoptosis in the OS cell lines. A sub-G_1 population was present in Ad-*WWOX* infected in KHOS and HOS cells but not in K7M2 cells (Supplementary Fig. S3). At 72 hours after Ad-*WWOX* infection (MOI, 100), but not after Ad-*GFP* infection, the sub-G_1 fraction was increased ~6-fold in KHOS cells and ~3-fold in HOS cells.

We next measured tumorigenicity of OS cells infected with Ad-*WWOX* or Ad-*GFP* by inoculating nude mice with 1×10^7 cells. At 28 days after injection, three of five and two of five mice inoculated with Ad-*WWOX*–infected HOS cells and KHOS cells, respectively, displayed no tumors, whereas all Ad-*GFP*–infected mice had tumors (data not shown). The average volume of tumors at day 28 after inoculation with Ad-*WWOX*–infected cells showed significant suppression of tumor growth in KHOS and HOS cells, but not in WWOX-positive K7M2 cells, compared with tumors from Ad-*GFP* treatment (Fig. 3C).

WWOX expression affects OS progression

Because OS cells often metastasize, we used lentiviral vectors to generate stable clones of HOS and KHOS cells expressing *WWOX* or empty vector (EV) and then measured their proliferation and cell migration characteristics. HOS-*WWOX* and KHOS-*WWOX* stable clones had increased WWOX expression compared with HOS-EV and KHOS-EV clones (Fig. 4A; Supplementary Fig. S5A). We performed XTT, colony formation, and soft agar assays and found that HOS-*WWOX* and KHOS-*WWOX* cells had reduced proliferation, survival, and anchorage-independent cell growth compared with HOS-EV and KHOS-EV clones and their respective parental cell lines (Fig. 4B; Supplementary Figs. S4 and S5).

HOS-*WWOX* cells had low cell motility compared with HOS-EV and HOS cells in a wound-healing assay (Fig. 4C). In a Matrigel invasion assay, HOS-EV clones behaved similar to HOS cells, whereas HOS-*WWOX* cells exhibited reduced invasiveness (Fig. 4D). Similar results were observed when KHOS-*WWOX* stable clones were compared with KHOS-EV clones (Supplementary Fig. S5).

Loss of WWOX expression is associated with elevated RUNX2 levels in several OS cell lines

We reported recently that WWOX suppresses the transactivation function of RUNX2 (8); therefore, we set out to determine whether WWOX tumor suppressor function in OS is associated with elevated levels of RUNX2. First, we examined RUNX2 in femurs of *Wwox*-deficient mice and found elevated immunohistochemical levels of RUNX2 compared with wild-type littermate control mice (Supplementary Fig. S6). Next, by

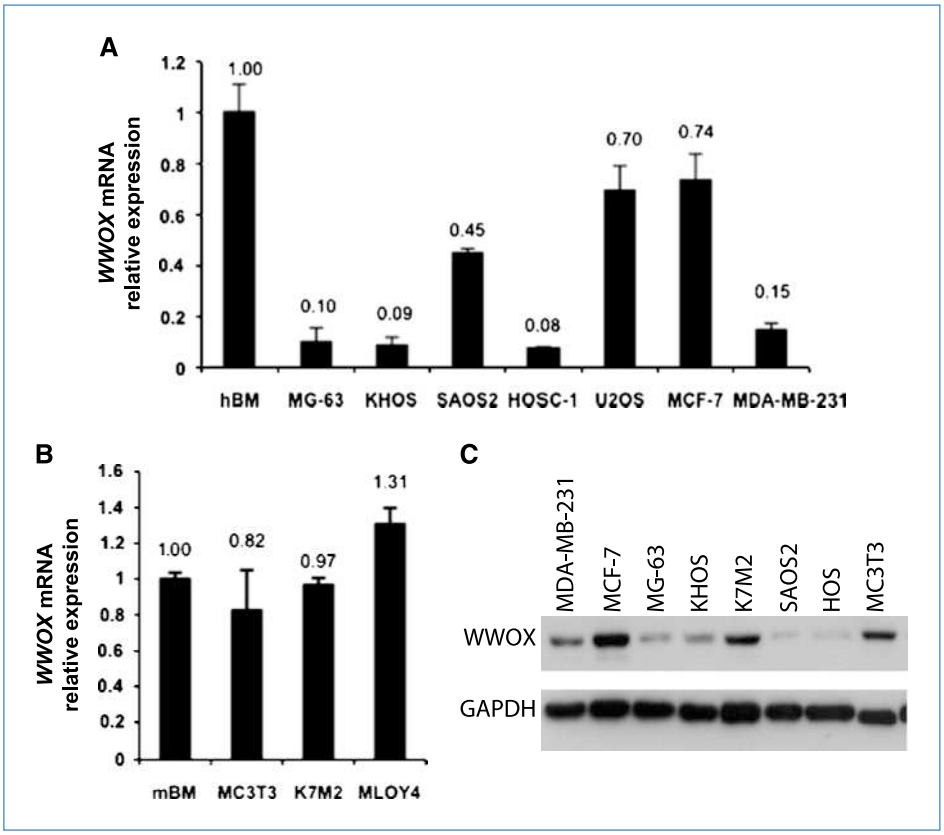

Figure 2. WWOX expression is reduced in OS cell lines. A and B, *WWOX* mRNA relative expression in human (A) and mouse (B) OS cell lines was determined by real-time PCR analysis. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) cDNA served as internal control, and *WWOX* expression in bone marrow was set as 1. Columns, mean of triplicate samples from two independent experiments; bars, SD. C, WWOX protein expression was determined by Western blot analysis. GAPDH served as loading control. MCF-7 (*WWOX*-positive) and MDA-MB-231 (*WWOX*-reduced) breast cancer cells served as positive controls for *WWOX* mRNA levels and WWOX protein expression.

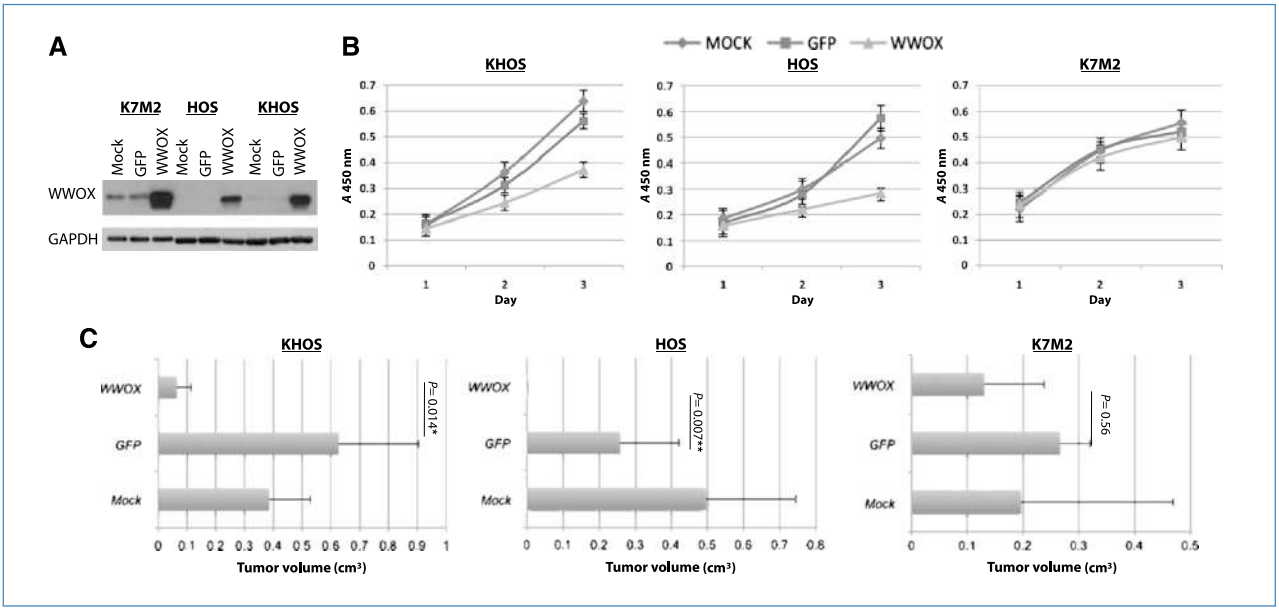

Figure 3. Ectopic expression of WWOX in human OS cells suppresses growth and tumorigenicity. A, Western blot analysis of WWOX expression in OS cells with no infection, infection with Ad-*GFP*, or infection with Ad-*WWOX* at a MOI of 100. Lysates were collected 72 h after infection. B, proliferation rate of the three different OS cells with no infection, infection with Ad-*GFP*, or infection with Ad-*WWOX* at a MOI of 100. C, tumor volume of untreated, Ad-*GFP*–infected, and Ad-*WWOX*–infected KHOS, HOS, and K7M2 OS cancer cells.

Western blot analysis in cell lines, we also found an inverse correlation between levels of WWOX and RUNX2 in several lines (Fig. 5A). The breast cancer cell line MCF-7 has high levels of WWOX and absent RUNX2, whereas WWOX levels are low and RUNX2 is expressed in the highly metastatic cell line MDA-MB-231. Similarly, murine cell lines MC3T3 and K7M2 express WWOX and have reduced RUNX2 expression, whereas human OS cell lines HOS, SAOS2, and KHOS have reduced WWOX expression and elevated RUNX2 expression. Interestingly, the inverse correlation between WWOX and RUNX2 was not observed in two less differentiated OS cell lines, MG-63 and U2OS. Importantly, overexpression of WWOX in the lentivirus-transduced HOS-*WWOX* cells caused a significant reduction in *RUNX2* mRNA and protein as assessed by quantitative reverse transcription-PCR (qRT-PCR) and Western blot (Fig. 5B and C). Similarly, downstream target genes of RUNX2, matrix metalloproteinase-9 (*MMP9*), type I collagen (*COL1A1*), and, to a lesser extent, osteocalcin (*OC*), showed marked downregulation in HOS-*WWOX* cells compared with HOS-EV cells (Fig. 5B). These data suggest that WWOX expression may contribute to OS formation, at least in part, through regulating RUNX2 expression.

Aberrant RUNX2 expression in human OS

Because the majority of human OS examined express elevated levels of RUNX2 (10, 14), and our findings showed that WWOX expression is attenuated in OS, we tested whether WWOX and RUNX2 expression are inversely correlated in human OS specimens. Immunohistochemical analyses of 56 of our OS cases (Supplementary Table S2) revealed that 60% (12 of 20) of pretreatment biopsies were positive for RUNX2 (Table 1B). Only 16% (4 of 25) of posttreatment resections were positive for RUNX2, indicating that the majority of resected samples had lost this highly metastatic marker. Paired pretreatment biopsy and posttreatment resections were available for 12 OS patients. Eight of the biopsies were RUNX2 positive and all (100%) became RUNX2 negative after treatment, whereas one in four of the biopsies that were RUNX2 negative became RUNX2 positive after treatment (Supplementary Tables S4 and S6), suggesting a poor outcome. A similar comparison was performed using paired pretreatment biopsies and resected metastases from seven OS patients. Interestingly, among the seven patients for whom pretreatment biopsies and metastasectomy specimens were available, all pretreatment biopsies were RUNX2 positive and five remained RUNX2 positive in the metastases (Supplementary Table S7), consistent with poor prognosis.

Although WWOX and RUNX2 results suggested an inverse association in human OS, this was not evident when paired comparisons were performed on the 56 available cases (Supplementary Table S8). In pretreatment biopsies, 78% (seven of nine) of WWOX strong tumors were also RUNX2 positive. In WWOX reduced or absent biopsies, 55% (6 of 11) were also RUNX2 negative. Evaluation of both WWOX and RUNX2 levels following chemotherapy revealed that, of the 6 of 12 patients with increased WWOX following chemotherapy, 3 (50%) had a concomitant decrease in RUNX2. Two others were RUNX2 negative in both biopsy and resection such that 83% (five of six) of posttreatment specimens with increased WWOX were RUNX2 negative (Supplementary Table S4). Two of the three metastases with decreased WWOX also had decreased RUNX2 despite findings that most of the metastases are RUNX2 positive (Supplementary Table S9).

Discussion

We find that WWOX expression is absent or reduced in 58% of OS patient specimens ($P < 0.0001$) and in the majority of human OS cell lines. In some OS patients, chemotherapy restored WWOX levels and the metastases had reduced levels. The observed alterations in WWOX expression were accompanied, at least in part, by aberrant RUNX2 expression, a factor known to be expressed in OS (10, 14). Our data also revealed that WWOX expression suppresses tumorigenicity both *in vitro* and *in vivo* in nude mice and that ectopic WWOX expression diminishes tumor progression of metastatic OS cell lines. These findings provide the first evidence that WWOX expression is clinically and therapeutically significant in OS.

Consistent with other types of human cancer (1, 2, 5), we observed that a significant proportion of OS samples exhibited either markedly reduced (41%) or absent (17%) WWOX expression (Fig. 1; Table 1A). WWOX protein and mRNA levels are significantly reduced in many human OS cell lines compared with control cells (Fig. 2). Previous studies have shown that hypermethylation of the regulatory region of *WWOX* is associated with low or absent protein expression (5); however, we could not confirm this in OS cell lines (data not shown). Alteration of *WWOX* in cancer has also been shown to be associated with LOH, HD, and enhanced protein degradation (5). Tumor suppressor genes at common fragile sites are frequently inactivated early in neoplastic progression (26). It is thus possible that inactivation of *WWOX* could occur during the extensive proliferation necessary for early bone growth, contributing to development of OS in childhood.

Because loss of WWOX results in OS formation in mice (ref. 7; Supplementary Fig. S1), and reduced levels are present in human OS cell lines and tissues, we determined whether ectopic expression of WWOX could reverse malignancy. In restoring WWOX expression in two WWOX-reduced OS cell lines by infection with Ad-*WWOX*, we observed a decreased tumorigenicity *in vitro*, as indicated by reduced proliferation (Fig. 3B) and increased apoptosis (Supplementary Fig. S3), and *in vivo*, as indicated by marked reduction of tumor growth in immunocompromised mice (Fig. 3C). Thus, ectopic expression of WWOX in OS cells can potentially reverse malignant properties despite other cancer-associated genetic alterations that have accumulated in these cell lines. Decreased tumorigenicity was not observed in K7M2 OS cells that express WWOX, suggesting that WWOX overexpression may not affect the cellular phenotype in WWOX-sufficient cells.

We previously showed that WWOX, via its first WW domain, associates with RUNX2 and suppresses its transactivation of the OC promoter (8). Increased RUNX2 levels have been reported in human OS (10, 12–14). Our data show that RUNX2 protein and mRNA levels were also high in femurs of *Wwox*-deficient mice and in some OS cell lines with low endogenous levels of WWOX, suggesting an inverse correlation with WWOX (Supplementary Fig. S6; ref. 8). *RUNX2* autoregulatory mechanisms have been described in osteoblasts (27, 28), and several lines of evidence indicate that RUNX2 expression is regulated by different tumor suppressors and oncoproteins implicated in the pathogenesis of OS, including pRb, p53, and MDM2 (18, 29, 30). Additionally, WW domain-containing proteins such as YAP (31) and the potent activator TAZ (32) interact with the same protein domain as WWOX. Therefore, it is possible that when WWOX is absent,

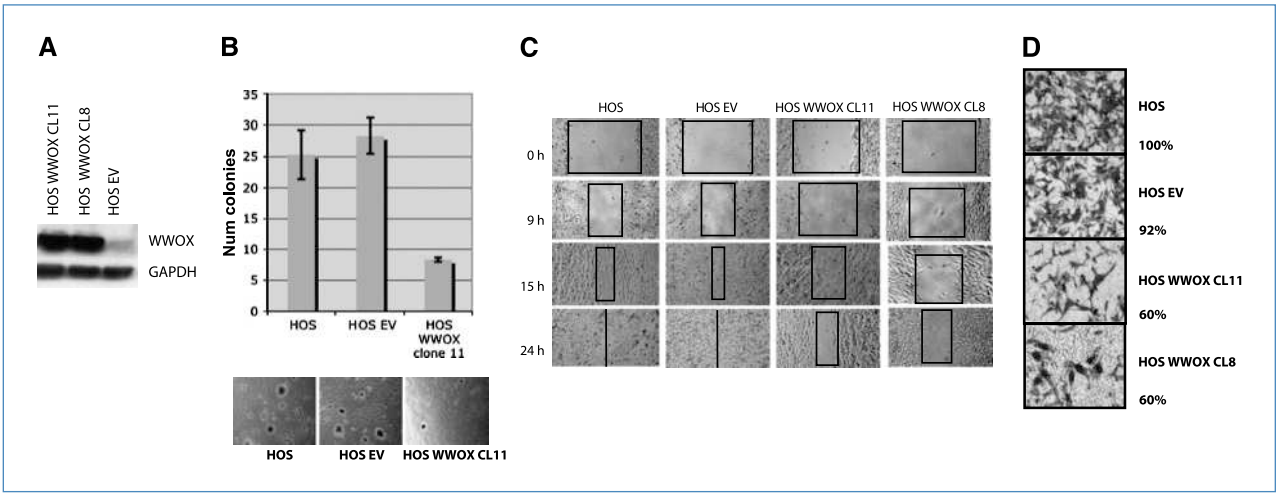

Figure 4. Restoration of WWOX in HOS OS cells suppresses tumor progression. A, establishment of HOS stable clones expressing *WWOX* and EV using lentiviral vector. Western blot analysis of WWOX expression in HOS-WWOX and HOS-EV cells showing restoration of WWOX in HOS clones 11 (CL11) and 8 (CL8). B, soft agar growth assay showing ability of *WWOX* to suppress anchorage-independent growth of HOS-WWOX stable clones (clone 11 is shown as an example) compared with HOS-EV clone. Top, quantification of colonies for HOS, HOS-EV, and HOS-WWOX. Columns, mean of triplicates samples; bars, SE. $P = 0.001$. Bottom, low-magnification photographs of representative colonies formed by 30 d after plating. C, wound-healing assay showing reduced migration of HOS-WWOX stable clones compared with control clones. Wound closures were photographed at indicated time after wounding. The square represents wound. D, Matrigel invasion assay where HOS stable WWOX clone exhibits markedly reduced Matrigel invasion properties ($P = 0.003$).

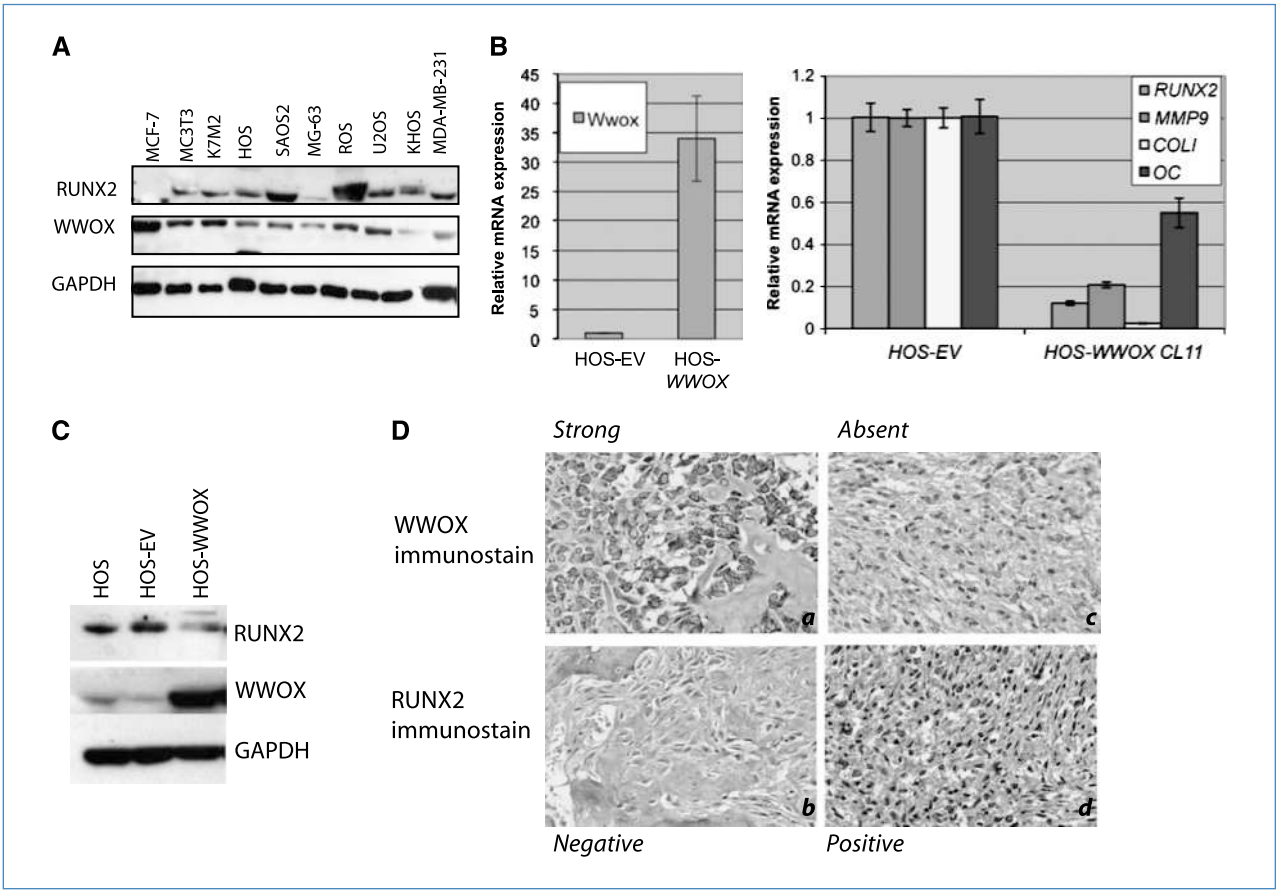

Figure 5. Inverse association of WWOX and RUNX2 expression in OS. A, Western blot analysis of WWOX and RUNX2 expression in OS cell lines. HOS, SAOS2, and KHOS (WWOX-reduced) cells have higher levels of RUNX2 compared with MC3T3 and K7M2 (WWOX-positive) cells. B, real-time PCR analysis of *WWOX, RUNX2*, and RUNX2 downstream targets, *MMP9, OC*, and *COLIA1*, in HOS-WWOX and HOS-EV stable clones. Restoration of WWOX in HOS cells (left) leads to downregulation of RUNX2 levels and downstream target. Columns, mean of triplicates samples; bars, SD. C, Western blot analysis of RUNX2 expression in HOS clones. HOS-WWOX clone exhibits reduced RUNX2 expression compared with HOS-EV and parental cells. D, immunohistochemical staining for WWOX and RUNX2 in human OS. Example of a tumor with normal WWOX expression (*a*) that was RUNX2 negative (*b*). Example of tumors with reduced or absent WWOX (*c*) but positive for RUNX2 (*d*). Magnification, ×600.

reduced, or nonfunctional, other WW domain-containing proteins may regulate RUNX2 transcription, contributing to its increased levels in OS.

In human OS samples, we found that 60% of prechemotherapy OS biopsies were RUNX2 positive (Table 1B). Interestingly, when paired comparisons were performed on 56 human OS samples, an inverse relationship between WWOX and RUNX2 was not evident (Supplementary Table S8). More than half of WWOX altered primary tumors were also RUNX2 negative, and ~80% that were WWOX strong were also RUNX2 positive. The strong inverse relationship found *in vitro* may reflect *ex vivo* selection of WWOX altered cell lines due to their enhanced growth properties, although we did observe an inverse correlation *in vivo* in *Wwox*-deficient mice. It is possible that the immunohistochemistry performed on fixed human tissue samples lacked the sensitivity to detect changes in WWOX and RUNX2 expression seen by qRT-PCR assays and Western blots, performed on cell lines. Alternatively, the relationship between WWOX and RUNX2 may be more complex *in vivo*, in part due to multiple additional aberrations that occur in the tumors. Support for this latter hypothesis is found in the less differentiated cell lines, which showed both WWOX and RUNX2 expression to be either reduced (MG-63) or preserved (U2OS; Fig. 5A). An expanded study will be required to explore this aberrant relationship in human OS.

After tumor staging, the most important predictor of disease-free survival in OS is responsiveness to chemotherapy. Ninety percent or greater tumor necrosis at resection (good response) is associated with a more favorable prognosis. Currently, there are no molecular markers to further stratify the poor responding patients for additional therapy (33–35). We examined WWOX immunoreactivity in paired biopsy and resection specimens and found that in nearly half of postchemotherapy resections, WWOX expression was increased (Supplementary Table S3). None of the resections having increased WWOX expression had high (>90%) rates of tumor necrosis. At present, our sample size is too small to assess

the significance of this observation. However, we speculate that WWOX expression might serve as another predictor of tumors with favorable and less favorable prognoses (Supplementary Table S4).

Metastasis, a hallmark of malignancy (36), is found in up to 20% of patients with OS at initial diagnosis and is associated with a poor prognosis (33, 35). Our analysis revealed that nearly half of metastases had reduced or absent levels of WWOX compared with their primary tumor (Supplementary Table S5). We also showed that stable restoration of *WWOX* in metastatic HOS and KHOS cell lines inhibited anchorage-independent growth and colony formation (Fig. 4), and reduced migration and Matrigel invasion *in vitro* (Fig. 4; Supplementary Fig. S5), suggesting that loss of WWOX expression might be associated with development of metastases.

Intriguingly, the majority of OS cases experienced a loss of RUNX2 following chemotherapy (Supplementary Tables S4 and S6). Nearly all cases where WWOX increased following chemotherapy were also RUNX2 negative, suggesting a normalization of the WWOX-RUNX2 relationship with chemotherapy. Another significant outcome of our study is that the majority of metastatic tumors expressed RUNX2 (Supplementary Tables S7 and S8), consistent with its oncogenic potential (9). Similar to the primary tumors, an inverse relationship was not found between WWOX and RUNX2 in most resections and metastases. It is possible that failure to restore the inverse relationship between WWOX and RUNX2 levels with chemotherapy identifies cases with an inherent predisposition toward metastasis. Larger case series will be necessary to evaluate whether assessing WWOX and RUNX2 expression will be a useful prognostic tools in OS.

In conclusion, WWOX expression is lost or reduced in a large portion of human OS and seems accompanied by an aberrant relationship with RUNX2. Ectopic expression of WWOX in human OS cells suppressed cancer cell growth, diminished migration and invasion *in vitro*, and suppressed xenograft tumor growth. Tumors that increased WWOX levels and lost RUNX2 expression following chemotherapy had poor necrosis responses, suggesting that a subset of poor responders may have improved prognosis due to tumor cell normalization. This normalization was not found in metastases, which largely retained patterns seen in the primary tumors despite chemotherapy. Our findings suggest potential prognostic value in examining WWOX and RUNX2 in OS patients.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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