

## Editorial Expression of Concern

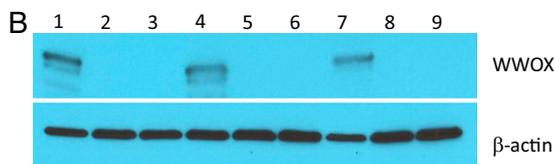
### MEDICAL SCIENCES

PNAS is publishing an Editorial Expression of Concern regarding the following article: “*WWOX* gene restoration prevents lung cancer growth *in vitro* and *in vivo*,” by Muller Fabbri, Dimitrios Iliopoulos, Francesco Trapasso, Rami I. Aqeilan, Amelia Cimmino, Nicola Zanesi, Sai Yendamuri, Shuang-Yin Han, Dino Amadori, Kay Huebner, and Carlo M. Croce, which appeared in issue 43, October 25, 2005, of *Proc Natl Acad Sci USA* (102:15611–15616; first published October 13, 2005; 10.1073/pnas.0505485102).

The editors wish to note that Fig. 1*B*,  $\beta$ -actin panel, appears to have duplicated bands. The authors note that “because this issue was first raised more than 7 years after publication, the original data are not available to confirm whether an error was made in the figure construction.” However, the authors state that any error in figure construction does not affect their scientific conclusions.

The authors have provided an image from a replicate experiment completed in 2014 which, the authors state, “confirms the results shown in Figure 1*B*. This confirmation supports the conclusions in this work.” The image for Fig. 1*B* and its legend appear below.

Inder M. Verma  
*Editor-in-Chief*



**Fig. 1*B*.** Expression of Wwox protein. (*B*) Expression of Wwox after infection with Ad-WWOX (25  $\mu$ g loaded). Lane 1, H1299, Ad-WWOX-infected; lane 2, H1299, Ad-GFP-infected; lane 3, H1299; lane 4, H460, Ad-WWOX-infected; lane 5, H460, Ad-GFP-infected; lane 6, H460; lane 7, A549, Ad-WWOX-infected; lane 8, A549, Ad-GFP-infected; lane 9, A549.

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# WWOX gene restoration prevents lung cancer growth *in vitro* and *in vivo*

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The *WWOX* (WW domain containing oxidoreductase) gene at the common fragile site, FRA16D, is altered in many types of cancer, including lung cancer. We have examined the tumor suppressor function of *WWOX* in preclinical lung cancer models. The *WWOX* gene was expressed in lung cancer cell lines through recombinant adenovirus (Ad) infection (Ad-*WWOX*), and through a drug [ponasterone A, (ponA)]-inducible system. After *WWOX* restoration *in vitro*, endogenous *Wwox* protein-negative cell lines (A549, H460, and H1299) underwent apoptosis through activation of the intrinsic apoptotic caspase cascade in A549 and H460 cells. Ectopic expression of *Wwox* caused dramatic suppression of tumorigenicity of A549, H460, and H1299 cells in nude mice after Ad-*WWOX* infection and after ponA induction of *Wwox* expression in H1299 lung cancer cells. Tumorigenicity and *in vitro* growth of U2020 (*Wwox*-positive) lung cancer cells was unaffected by *Wwox* overexpression. This study confirms that *WWOX* is a tumor suppressor gene and is highly effective in preventing growth of lung cancer xenografts, whether introduced through viral infection or by induction of a silent *WWOX* transgene.

adenovirus | inducible expression | viral gene transduction

Lung cancer is the leading cause of cancer mortality in the United States (1), with an incidence of  $\approx 170,000$  new cases per year in the United States (1), and mortality is very high. Nonsmall cell lung cancer (NSCLC) accounts for  $\approx 80\%$  of lung cancers. Surgery remains the main therapy for NSCLC, but a large fraction of patients cannot undergo curative resection. Despite new drugs and therapeutic regimens, the prognosis for lung cancer patients has not significantly changed in the last 10 years. Recombinant virus gene therapy has been investigated in lung cancer patients; adenovirus (Ad) and retrovirus encoding wild-type p53 have been injected intratumorally in lung cancer clinical trials (2–6). Recombinant Ad injection in lung cancer phase I studies (7) has demonstrated safety and feasibility, and phase I/II clinical trials are currently recruiting patients to evaluate toxicity and efficacy of gene therapy with recombinant Ads.\*\*

Lung cancer is associated with early loss of expression of the *FHIT* (fragile histidine triad) gene (8) at fragile site FRA3B (9). Fragile regions are particularly susceptible to damage on exposure to environmental carcinogens, which are etiological factors in lung cancer. Recently, Yendamuri *et al.* (10) have demonstrated that the *WWOX* (WW domain containing oxidoreductase) gene is also altered in a fraction of nonsmall cell lung cancers. *WWOX* is located at fragile site FRA16D (11) and encodes a 414-aa protein with two WW domains and a short-chain dehydrogenase domain. WW domains are protein–protein interaction domains, and *Wwox* interactors with important signaling roles in normal epithelial cells have been identified. *Wwox* interacts with p73 and can trigger redistribution of nuclear p73 to the cytoplasm, suppressing its transcriptional activity (12). *Wwox* also interacts with Ap2- $\gamma$  transcription factors with roles

in cell proliferation (13). Most recently, *Wwox* has been reported to compete with Yap protein for binding to the intracellular ErbB4 domain, a transcriptional activator (14). Thus, the *Wwox* pathway includes a number of downstream signaling proteins that may also serve as cancer therapeutic targets.

The *WWOX* gene is altered in many types of cancer, including breast, ovary, prostate, bladder, esophagus, and pancreas (15–19). In nonsmall cell lung cancer, transcripts missing *WWOX* exons were detected in 26% of tumors and in five of eight cell lines (10). *WWOX* allele loss occurred in 37% of tumors, and the promoter is hypermethylated in 62.5% of squamous cell lung carcinomas (10, 19). To investigate tumor suppression in lung cancer, we studied *in vitro* and *in vivo* effects of *Wwox* protein expression in *Wwox*-negative (A549, H460, and H1299) and -positive lung cancer cells (U2020) by infection with Ad carrying the *WWOX* gene; H1299 cells were also stably transfected with an inducible *Wwox* expression vector, which allows induction of near physiologic levels of protein. *Wwox* restoration effectively induced apoptosis *in vitro* and suppressed lung cancer tumorigenicity in nude mice, with no effect on lung cancer cells that constitutively express the *Wwox* protein.

## Materials and Methods

**Cell Culture.** *Wwox*-negative A549, H460, and H1299 and *Wwox*-positive U2020 lung cancer cell lines from American Type Culture Collection were maintained in RPMI medium 1640 with 10% FBS. HEK-293 CymR cells from Qbiogene (Carlsbad, CA) were cultured in DMEM with 10% FBS. H1299 cells do not express p53, whereas A549 and H460 express wild-type p53 (20).

**Recombinant Ads and *in Vitro* Transduction.** *WWOX* cDNA from normal human liver RNA (Ambion, Austin, TX) was reverse-transcribed by SuperScript First-Strand Synthesis (Invitrogen). Double-stranded cDNA was prepared by PCR amplification using the following conditions: 95°C for 3 min, 30 cycles at 94°C for 30 sec, 65°C for 60 sec, 72°C for 30 sec, and 72°C for 7 min; *WWOX* forward 5'-GCCAGGTGCCTCCACAGTCAGCC-3' and *WWOX* reverse 5'-TGTGTGTGCCCATCCGCTCT-GAGCTCCAC-3' primers were used. The cDNA was cloned into Adenovator-CMV5(CuO)-IRES-*GFP* transfer vector (Qbiogene) (11). This vector allows transgene expression driven by the cumate-inducible CMV5(CuO) promoter. An internal

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Abbreviations: Ad, adenovirus; ponA, ponasterone A; moi, multiplicity of infection; PARP, poly(ADP-ribose) polymerase.

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**Table 1. Tumor weight (in grams)  $\pm$  SD in nude mice**

Treatment	Wwox <sup>-</sup>			Wwox <sup>+</sup>
	A549	H460	H1299*	U2020
Untreated	0.86 $\pm$ 0.15	0.64 $\pm$ 0.11	1.87 $\pm$ 0.33	0.57 $\pm$ 0.09
Ad-GFP	0.81 $\pm$ 0.16	0.61 $\pm$ 0.15	1.66 $\pm$ 0.28	0.55 $\pm$ 0.05
Ad-Wwox	0.08 $\pm$ 0.03	0.03 $\pm$ 0.04	0.10 $\pm$ 0.26	0.59 $\pm$ 0.03

\*H1299/I<sup>-</sup>, 1.98  $\pm$  0.41; H1299/I<sup>+</sup>, 0.21  $\pm$  0.31.

**Cell Cycle Kinetics of Infected Cells.** Cell cycle alterations induced by Wwox overexpression were assessed after infection at several mois, with Ad-Wwox or Ad-GFP. A sub-G<sub>1</sub> population was observed after Ad-Wwox infection in A549, H460, and H1299 cells that do not express endogenous Wwox but not in endogenous Wwox-positive U2020 cells. Ad-GFP infection did not modify cell cycle profiles. At 96 hr after Ad-Wwox infection (moi = 100), 58% of A549, 94% of H460, and 17% of H1299 cells were in the sub-G<sub>1</sub> fraction; 7% of U2020 cells were in the sub-G<sub>1</sub> fraction (Fig. 2). Wwox induction of cell death was moi- and time-dependent (data not shown).

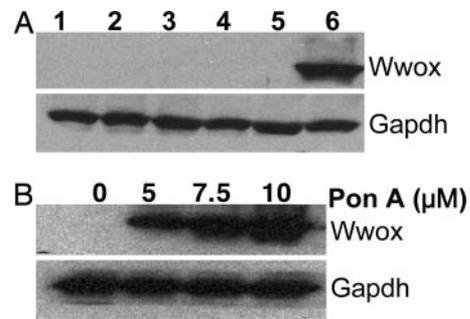
**Apoptotic Pathways in Wwox-Reexpressing Cells.** A549, H460, H1299, and U2020 lung cancer cell lines were infected with increasing mois, and the fraction of transduced cells was monitored by confocal microscopy and cell cycle kinetics analyses. Significant differences were observed in cell growth for Ad-Wwox and Ad-GFP infection, at a range of mois, in lung cancer cell lines (A549, H460, and H1299) lacking endogenous Wwox (Fig. 3A). U2020 cells were unaffected by exogenous Wwox expression.

To study Wwox-induced apoptotic pathways, expression of downstream apoptotic effectors was assessed *in vitro*. At 96 hr after infection, pro-caspase 3 and full-length PARP-1 levels were reduced in Ad-Wwox-infected A549 and H460 cells compared with Ad-GFP control cells. In H1299 cells, a decrease of full-length PARP-1 was observed. Cleavage of precursors was not observed in infected U2020 cells (Fig. 3B).

**Effects of Conditional Wwox Expression in H1299 Cells.** H1299/I clone 7 expressed the Wwox transgene only on induction with ponA (Fig. 4A) and was used in subsequent experiments. Wwox expression increased in a dose-dependent manner after ponA treatment (Fig. 4B) from 24 to 72 hr (Fig. 4C).

Clone 7 H1299/I<sup>-</sup> (uninduced) cells were plated, and, 24 hr later (day 1), Wwox expression was induced by 10  $\mu$ M ponA. Maximum expression was observed at day 4 and significantly affected cell proliferation by day 5 (Fig. 4D), causing reduction in cell numbers and suggesting that Wwox inhibits growth of H1299 cells.

**Tumorigenicity of Ad-Wwox-Infected Lung Cancer Cell Lines.** Nude mice were inoculated with  $5 \times 10^6$  A549, H460, and U2020 cells infected *in vitro* at a moi of 100 with Ad-GFP or Ad-Wwox and cultured for 24 hr. Uninfected cells served as tumorigenic controls. At 28 days after injection, tumor growth was completely suppressed in mice inoculated with Ad-Wwox-infected H460 cells (Fig. 5A). The average tumor weights for controls (Ad-GFP and untreated H460 cells) at day 28 were 0.61  $\pm$  0.15 g and 0.64  $\pm$  0.11 g, respectively. At 28 days, two of five mice inoculated with Ad-Wwox-infected A549 cells showed no tumors, and average tumor weight was 0.08  $\pm$  0.03 g, significantly lower ( $P < 0.001$ ) than tumors of Ad-GFP-infected A549 (0.81  $\pm$  0.16 g) and mock-infected A549 (0.86  $\pm$  0.15 g) cells (Table 1). In mice injected with infected U2020 cells, no tumor growth suppression was observed (Fig. 5A).



**Fig. 6.** *Ex vivo* analysis of H1299/I<sup>-</sup> and H1299/I<sup>+</sup> cells. (A) Protein lysates from H1299 (lane 1), uninduced H1299/I<sup>-</sup> (lanes 2, 3, and 4), and induced H1299/I<sup>+</sup> (lane 5) tumors tested for Wwox expression by immunoblot analysis. Wwox was not expressed in the H1299/I<sup>-</sup> or H1299/I<sup>+</sup> tumors. (B) A portion of the H1299/I<sup>+</sup> tumor was plated and cultured, and cells were treated with ponA. Wwox was reexpressed after 48 hr of treatment with 10  $\mu$ M ponA, indicating the presence of the inducible Wwox plasmid.

**Effect of Induced Wwox Expression on Tumorigenicity.** We next compared tumorigenicity of H1299 cells infected with Ad-Wwox or induced to express Wwox by ponA treatment. Nude mice were inoculated with  $1 \times 10^7$  cells 24 hr after infection with Ad-Wwox or Ad-GFP. Five mice were also injected with  $1 \times 10^7$  uninduced H1299/I<sup>-</sup> (H1299/I<sup>-</sup>) and  $10^7$  H1299/I<sup>+</sup> cells 24 hr after ponA treatment. At 28 days after injection, three of five and four of five mice inoculated with Ad-Wwox-infected H1299 cells and H1299/I<sup>+</sup> cells, respectively, displayed no tumors (Fig. 5B). Average weight of tumors from Ad-Wwox-infected (0.10  $\pm$  0.26 g) and H1299/I<sup>+</sup> (0.21  $\pm$  0.31 g) cells was significantly reduced compared with tumors from Ad-GFP (1.66  $\pm$  0.28 g), H1299/I<sup>-</sup> (1.98  $\pm$  0.41 g), and parental H1299 (1.87  $\pm$  1.33 g) cells (Table 1). Thus, Wwox expression, delivered by viral infection (Ad-Wwox) or by induction of expression of an inactive “endogenous” Wwox gene (H1299/I<sup>+</sup>), was effective in suppressing lung cancer cell growth in nude mice.

**Wwox Expression in H1299/I+ Explanted Tumors.** To assess Wwox expression *ex vivo*, we performed immunoblot analysis of proteins extracted from fragments originating from parental H1299, H1299/I<sup>-</sup>, and H1299/I<sup>+</sup> tumors; Wwox expression was not found in any of the tumors (Fig. 6A). Explanted, cultured fragments from H1299/I<sup>+</sup> tumors were examined for retention of inducible Wwox plasmid by treating with ponA and testing for Wwox expression by immunoblot analysis. The detection of Wwox induction in H1299/I<sup>+</sup> explants revealed that the Wwox plasmid was present and inducible (Fig. 6B), suggesting that the small tumors were derived from inoculated cells that had lost expression of Wwox due to absence of inducer *in vivo*.

## Discussion

Innovative therapeutic strategies are urgently needed for lung cancer treatment. Because genes at common fragile sites are frequently inactivated early in the neoplastic process, especially on exposure to environmental carcinogens, we have been interested in the effect of loss of fragile gene expression in development of cancer and therapeutic effects of their restoration (22). A number of studies have suggested that the fragile Wwox gene is inactivated in a significant fraction of lung cancers (10, 16), particularly by promoter hypermethylation (16). Hypermethylation is reversible, a strategy with promise for cancer therapy. Thus, we have determined whether restoration of Wwox expression in lung cancer cells lacking expression of endogenous Wwox would reverse malignancy despite numerous cancer-associated genetic alterations that have accumulated in lung cancer cell lines. We have restored Wwox expression in four lung cancer cell

lines by infection with Ad-*WWOX* and observed dramatic loss of tumorigenicity of the lung cancer cells that lacked endogenous *Wwox*.

Introduction of the *WWOX* gene in the three *Wwox*-negative cell lines resulted in induction of apoptosis *in vitro*, as shown by the fraction of cells with sub-G<sub>1</sub> DNA content and by suppression of cell growth in culture. The fraction of Ad-*WWOX*-infected H1299 cells with sub-G<sub>1</sub> DNA content was lower than for the other two *WWOX*-negative cell lines, possibly because apoptosis may occur later after restoration of *Wwox* expression in H1299 cells; another possibility is that expression of p53 in A549 and H460 cells had an additive effect with expression of *Wwox* protein, although the tumor suppressive effect was similar in the three lung cancer cell lines. The U2020 lung cancer cells expressing endogenous *Wwox* were not affected by overexpression of *Wwox*, suggesting that normal *Wwox*-expressing lung cells would be unaffected by *Wwox* overexpression after *WWOX* gene therapy. Growth of all three lung cancer cells *in vitro* was adversely affected by overexpression of *Wwox* after virus infection or *ponA* induction, as shown by the downturn in cell number after a few days of *Wwox* overexpression. It will be interesting to examine *Wwox* binding to known interacting proteins at days 2–5 in these *in vitro* overexpression cultures to define the signal events directly downstream of *Wwox* expression after *WWOX* infection or induction.

We observed efficient suppression of *in vivo* tumorigenicity of lung cancer cell lines by Ad-*WWOX* transduction in three *WWOX*-negative lung cancer cell lines and by induction of *Wwox* expression in stably transfected H1299 lung cancer cells. The tumorigenicity of the aggressive H460 cell line was completely suppressed by Ad-*WWOX* treatment at 28 days after injection. A significant reduction in tumor occurrence and size was observed in animals injected with *WWOX*-transduced A549 and H1299 cells. The results suggest that *Wwox* loss may play an important role in the pathogenesis of lung cancer. It is interesting that both

methods of *Wwox* restoration in H1299 cells appeared to result in more dramatic effects *in vivo* than *in vitro*, possibly because the *in vivo* microenvironment somehow activates the *Wwox* apoptotic pathway.

This study demonstrates that *WWOX* induces cell growth inhibition and apoptosis in lung cancer cells. In A549 and H460 cell lines, we observed caspase-dependent induction of apoptosis through the intrinsic pathway. In H1299 cells, we observed cleavage of full-length PARP-1, but procaspase 3, 9, and 8 were not cleaved, possibly because apoptosis occurs later in these cells. *Wwox* and Fhit protein expression is frequently reduced in lung, breast, and bladder cancers in association with promoter hypermethylation (16). Epigenetic alterations can be reversed by specific agents or inhibitors, suggesting such inhibitors as therapeutic agents (23–26). The *ponA*-inducible expression of *Wwox* can be considered a model for the effects of *WWOX* reactivation after silencing by epigenetic mechanisms. The extent of loss of tumorigenicity after restoring inducible *Wwox* expression was comparable to the tumor suppression observed after Ad-*WWOX* expression, both *in vitro* and *in vivo*, suggesting that massive overexpression of *Wwox* is not necessary to effect tumor suppression. This finding suggests that drugs capable of reactivating the epigenetically silenced *WWOX* gene could be effective in treatment of lung cancer.

In conclusion, restoration of *Wwox* protein expression in lung cancer cells is followed by induction of apoptosis *in vitro* and suppression of tumorigenicity *in vivo* and suggests that reactivation of the *Wwox* signal pathway is a potential target for lung cancer prevention and therapy.

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