Role of the *WWOX* gene, encompassing fragile region *FRA16D*, in suppression of pancreatic carcinoma cells

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The *WW-domain-containing oxidoreductase (WWOX)* gene spans the common chromosomal fragile site *FRA16D* (16q23.2) and is believed to be a tumor suppressor in various human malignancies. We have previously shown frequent down-modulation of Wwox expression in pancreatic carcinoma (PC); however, biological function of Wwox in pancreatic duct carcinogenesis remains unknown. In PANC-1 (Wwox-negative) PC-derived cells, restoration of recombinant *WWOX* gene expression with adenoviral gene delivery (Ad-*WWOX*) effectively increased the number of cells with subG$_1$ DNA contents in a multiplicity of infection-dependent manners: Ad-*WWOX* infection up-regulated caspase-3 activity and reduced procaspase-3 and procaspase-8 levels. We also confirmed that restoration of *WWOX* gene suppressed cell growth *in vitro* and tumorigenicity *in vivo*. In addition, transduction of wild-type *WWOX*-expressing vector inhibited PANC-1 colony formation; however, substitution of Y33 of Wwox with arginine did not lead to inhibition of colony formation, suggesting the biological significance of the WW1 domain of Wwox for its tumor-suppressing activity. In PC tissue samples, abundant cytoplasmic Wwox expression was detected in the normal pancreatic duct epithelium, whereas Wwox expression was frequently reduced not only in a large fraction of PC but also in precancerous lesions in accord with the pancreatic intraepithelial neoplasia (PanIN) grade, which was closely correlated with patients' poorer outcome. Interestingly, the existence of Wwox expression was associated with elevated mothers against decapentaplegic homolog 4 (Smad4) protein levels *in vitro* and *in vivo*. These findings suggest that down-modulation of Wwox expression is an early event and may be associated with the down-regulation of Smad4 protein levels during pancreatic duct carcinogenesis. (*Cancer Sci* 2008; 99: 1370–1376)

Pancreatic carcinoma (PC) is among the most aggressive and lethal human diseases, with a very poor prognosis; even with complete surgical resection and adjuvant chemotherapy, the 5-year survival rate is less than 20%.[1] Infiltrating carcinomas of the exocrine pancreas arise from histologically identifiable intraductal precursors that undergo a series of architectural and cytologic changes. These intraductal lesions of the pancreas are also known as pancreatic intraepithelial neoplasias (PanIN), and they progress from flat to papillary without atypia to papillary with atypia to carcinoma *in situ*.[2,3] Multiple genetic and epigenetic alterations have been documented in PC: numerous alterations in *KRAS*,[4] *HER-2*,[5] *CDKN2A*,[4,6] *BRCA2*,[7] *TP53*,[8] and *SMAD4*,[9] have been described in a variety of PanIN using both genetic and immunohistochemical analyses, and some *in situ* lesions eventually progress to infiltrating carcinoma.[3] But much remains unknown about development and progression of pancreatic duct lesions.

Common chromosome fragile sites in the human genome are particularly susceptible to damage by environmental carcinogens. Common fragile sites have been observed at or near structural chromosome defects recognized in various cancers, and such instability contributes to neoplasia by virtue of altered expression of the associated genes. For instance, homozygous deletion and chromosomal breakage at *FRA3B* (3p14.2) have been shown to result in a loss of *fragile histidine triad (FHIT)* gene expression in many human cancers.[10,11] Restoration of the *FHIT* gene in cancer cells effectively induces caspase-dependent apoptosis with a suppression of the Akt–survivin pathway,[12,13] confirming the role of Fhit as a tumor suppressor. Similarly, the *WW-domain-containing oxidoreductase (WWOX)* gene encompasses *FRA16D* (16q23.2). Frequent loss of heterozygosity (LOH) at the *WWOX* locus, hypermethylation of the *WWOX* promoter and resultant loss of Wwox expression has been reported in breast,[14] esophageal,[15] and other human malignancies.[16–18]

Wwox protein contains two WW domains (WW1 and WW2) at the N-terminus and a central short-chain dehydrogenase/ reductase (SDR) domain that has amino acid sequence homology with steroid oxidoreductases.[19–21] Recent studies have revealed the tumor suppressor effects of Wwox in human cancers: restoration of the *WWOX* gene by adenoviral gene delivery was found to suppress tumor growth and increase the number of cells with subG$_1$ DNA content.[21,22] Interestingly, the WW1-domain-specific proline-rich ligand, the PPXY motif, has been identified as critical for Wwox function as a regulator of the subcellular localization of p73,[23] and AP-2 γ transcription factors.[24] In particular, the substitution of Y33 with arginine diminished Wwox interaction with v-erb-a erythroblastic leukemia viral oncogene homolog 4 (ErB4),[25] and Jun,[26] suggesting that Y33 within the WW1 domain, a physiological phosphorylation target for Src tyrosine kinase, is critical for Wwox-mediated protein–protein interactions. Furthermore, osteosarcomas in juvenile *Wwox*$^{-/-}$ mice and lung papillary carcinoma in adult *Wwox*$^{+/-}$ mice occurred spontaneously,[27] and treatment with ethylnitrosourea and *N*-nitrosomethylbenzylamine effectively induced lung tumors/ lymphomas,[27] and forestomach tumors in *Wwox*$^{+/-}$ mice,[28] respectively, confirming the Wwox tumor-suppressor effects *in vivo*.

We have previously demonstrated the possible function of Wwox in PC: hypermethylation-mediated down-regulation of Wwox expression levels was frequently detected in PC-derived cells and PC tissue samples. In addition, transfection of *WWOX* inhibited colony formation of PC cell lines by triggering apoptosis.[29] In this study, we further investigated the effect of Wwox in the regulation of cell growth and apoptosis in PC-derived cells using adenoviral gene delivery. Moreover, we investigated the expression of Wwox in PC tissue samples and adjacent PanIN to find out the role of Wwox in pancreatic duct carcinogenesis.

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Materials and Methods

Cell culture and tissue samples. Human PC-derived cell line PANC-1 (Wwox-negative,[29] American Type Culture Collection, Manassas, VA, USA) was routinely maintained in Roswell Park Memorial Institute media (RPMI)-1640 supplemented with 10% fetal bovine serum. QBI-HEK 293CymR cells (Qbiogene, Irvine, CA, USA) were used for generation, amplification and titration of recombinant adenoviruses.[12,13] In total, 32 formalin-fixed and paraffin-embedded sporadic PC specimens surgically removed at Kobe University Hospital (Kobe, Japan) were employed. Informed consent was obtained from all patients and the study was approved by the Kobe University Institutional Review Board. None of these cases had received adjuvant chemotherapy or radiotherapy before surgery. Histological examination was performed according to the General Rules for the Study of Pancreatic Cancer,[30] along with tumor–lymph nodes–metasteses (TNM) classification.[31] Non-neoplastic ducts including normal pancreatic ducts and PanIN[32] adjacent to PC were included in the analyses.

Immunoblot. Samples were extracted in cell lysis buffer containing 50 mM TRIS-HCl (pH 7.4), 125 mM NaCl, 0.1% Triton X-100, 5 mM ethylenediamine tetraacetic acid (EDTA), 1% (v/v) protease inhibitor cocktail (Sigma, St. Louis, MO, USA) and 1% (v/v) phosphatase inhibitor cocktail (Sigma). Total protein (20 μg) was denatured and subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis followed by electrotransfer to Hybond-C membrane (GE Healthcare, Piscataway, NJ, USA). Membranes were probed with the following antibodies: anti-Wwox,[21] anti-procaspase-3 (Cell Signaling, Beverly, MA, USA), anti-procaspase-8 (Cell Signaling), anti-Smad4 (Cell Signaling), anti-Smad2/3 (Cell Signaling), anti-green fluorescent protein (GFP; MBL, Nagoya, Japan) and anti-β-actin (Sigma). After probing with appropriate secondary antibodies, signals were detected by chemiluminescence substrate.

Gene transduction. Adenoviruses carrying human recombinant wild-type *WWOX* (Ad-*WWOX*) and *GFP* (Ad-*GFP*) were prepared, amplified, and titrated as described elsewhere.[21] Ad-*GFP* virus was used as a non-specific control for gene transfer. PANC-1 cells were incubated with adenoviral aliquots at a desired multiplicity of infection (MOI) for 4 h before addition of culture medium (>25 × volume of virus inoculum). Transduction efficiency was assessed by visualization of GFP-expressing cells using fluorescence microscopy. Human *WWOX* complementary DNA (cDNA) was subcloned into the mammalian expression vector pRcCMV (Invitrogen, Carlsbad, CA, USA) to generate p*WWOX* vector. Also, site-directed mutagenesis was performed using p*WWOX*-Y33R, as well as the SDR domain-defective mutant *WWOX*-expressing vectors pΔ*WWOX* and pΔ*WWOX*-Y33R. Each plasmid was transfected into PANC-1 cells using Lipofectamine (Invitrogen).

Flow cytometric analysis and caspase-3 activity assay. To analyze cellular DNA content, PANC-1 cells were collected and fixed in 70% methanol, treated with RNase A and stained with propidium iodide. The analysis was performed with a fluorescence-activated cell sorting (FACS) Calibur cytometer (BD Biosciences, San Jose, CA, USA). The activity of caspase-3 was evaluated by the caspase-3 colorimetric substrate/inhibitor (Sigma). Briefly, PANC-1 cells (5×10^6 cells/dish) infected with Ad-*WWOX* or Ad-*GFP* (MOI 0–50) were suspended in cell lysis buffer (50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid [HEPES], 5 mM 3,3-cholamidopropyl-dimethylammonio-1-propanesulfonate [CHAPS] and 1 mM dithiothreitol [DTT]). In total, 100 μL of assay buffer (20 mM HEPES, 0.1% CHAPS, 5 mM DTT and 2 mM EDTA) and 10 μL of acetyl-Asp-Glu-Val-Asp p-nitroanilide (Ac-DEVD-pNA) substrate were added to triplicate culture wells in 96-microwell plates. N-Ac-Asp-Glu-Val-Asp-Asp-CHO (Ac-DEVD-CHO), an aldehyde caspase-3-specific inhibitor, was also used to test the specificity of caspase-3. The plates were then incubated at 37°C for 2 h and caspase-3 activity in the lysate was determined by absorbance at 405 nm on a microwell plate reader.

Cell growth test, colony formation assay and tumorigenicity tests. For the cell growth test, PANC-1 cells were seeded in a 100-mm plate at a density of 1×10^5 cells and infected with Ad-*WWOX* and Ad-*GFP* (MOI 25). Viable cells were counted. For colony formation assay, PANC-1 cells (1.0×10^4) in 100-mm plates in triplicate were transfected with 6 μg wild-type and mutant *WWOX*-expressing vectors using Lipofectamine (Invitrogen). Forty-eight hours after transfection, cells were fixed with methanol and stained with Giemsa, and visible colonies (>0.5 mm in diameter) were counted. For tumorigenicity test, viable PANC-1 cell numbers were counted 24 h after infection by Ad-WWOX or Ad-GFP (MOI 25). Cells were then collected, and 1×10^6 cells were injected into the flanks of 10-week-old female nude mice (Japan Clea, Tokyo, Japan), five mice per group. Five control mice were injected with the same number of uninfected PANC-1 cells. Animals were monitored daily and subcutaneous (s.c.) tumor sizes were measured twice a week. At endpoint (7 weeks), animals were sacrificed, tumors were weighed and tumor volumes were calculated using the formula V = (the shortest diameter)^2 × (the longest diameter).

Immunohistochemistry. A modified version of the immunoglobulin enzyme bridge technique with labeled streptavidin biotin (LSAB) kit (Dako, Glostrup, Denmark) was used.[33] Briefly, deparaffinized and rehydrated sections were autoclaved in a citrate buffer. After blocking of endogenous peroxidase activity and non-specific reactions, the primary antibodies against Wwox,[21] and Smad4 (Cell Signaling) were applied to sections and were subsequently incubated with biotinylated monkey antirabbit immunoglobulin G (IgG). Streptavidin conjugated to horseradish peroxidase was used to immerse with 3,3-diaminobenzidine tetrahydrochloride. Immunohistochmical results were evaluated by two pathologists (S.N. and S.S.) who were blinded to clinical information. Immunoreactivities of Wwox and Smad4 were graded according to the number of stained cells and the staining intensity in individual cells as follows: (–), almost no positive cells; (+), 5–50% of tumor cells showed weak immunoreactivity; (++), >50% of tumor cells showed weak immunoreactivity or tumor cells showed intense immunoreactivity. Similarly, Smad4 immunoreactivity was graded as follows: (–), negative; (+), positive.

Statistical analysis. Statistical analysis was conducted using χ^2 test to evaluate the relationship between Wwox immunoreactivity and clincopathologic characters. Student's two-sided *t*-test was used to compare values of test and control samples. Survival curves were drawn according to the Kaplan–Meier method and differences between the curves were analyzed by applying the log-rank test. *P*-values less than 0.05 were considered statistically significant.

Results

Restoration of *WWOX* induces caspase-dependent apoptosis and suppresses tumor growth *in vitro* and *in vivo*. The tumor-suppressing and apoptosis-inducing effects of Wwox were investigated in order to characterize the functions of Ad-*WWOX* in PC cells. In PANC-1 cells, transduction of Ad-*WWOX*, but not that of Ad-*GFP*, induced apoptosis; 48 h after infection, Ad-*WWOX* led to a MOI-dependent elevation in the number of cells with subG$_1$ DNA contents (Fig. 1a,b). Although restoration of the *WWOX* gene with Ad-*WWOX* increased the number of cells with subG$_1$ DNA contents in PK-1 (Wwox-negative) cells, Ad-*WWOX* infection was less effective in PK-9 (Wwox-positive) cells (data not shown). Activation of caspase-3 was also MOI-dependent, which was confirmed by detection of reduced levels of procaspase-3

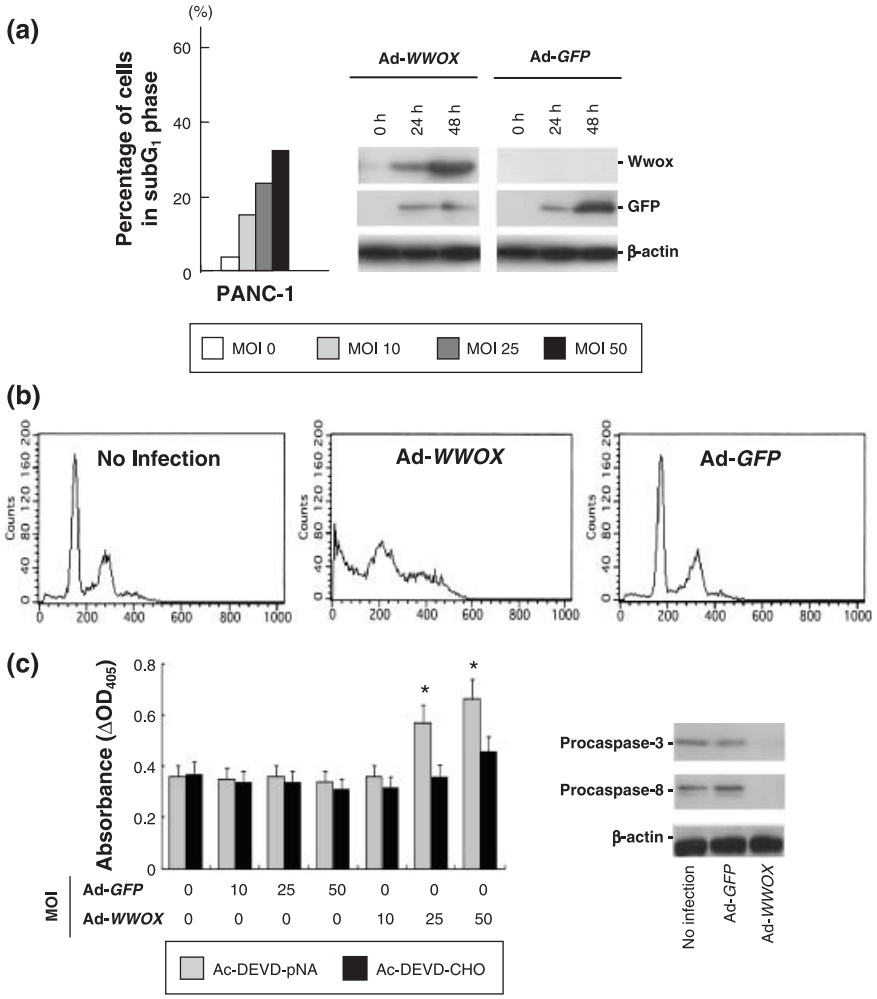

Fig. 1. Restoration of WW-domain-containing oxidoreductase (Wwox) expression induces caspase-dependent apoptosis in PANC-1 cells. (a) Percentage of cells in subG$_1$ DNA content for adenoviral gene delivery (Ad)-*WWOX* infection. Ad-*antigreen fluorescent protein* (*GFP*) did not increase the fraction of subG$_1$ DNA content more than 5% (data not shown). Recombinant Wwox protein was detected by immunoblot. (b) Representative results of flow cytometric analysis. Cells were collected 48 h after Ad-*WWOX* or Ad-*GFP* infection (multiplicity of infection [MOI] 25). (c) Increased activity of caspase-3 by infection of Ad-*WWOX*. Decrease of procaspase-3 and procaspase-8 determined by immunoblot are also shown. *$P < 0.05$.

and procaspase-8 expression (Fig. 1c). We also evaluated the growth-suppressing effect of Wwox and found that Ad-*WWOX* effectively suppressed cell growth *in vitro* (Fig. 2a) and tumorigenicity *in vivo* (Fig. 2b).

Since the physiological phosphorylation site Y33 of Wwox is essential for Wwox-mediated tumor suppression,[23–26] the biological significance of Y33 in the WW1 domain was confirmed, as compared with the significance of the C-terminus SDR domain (Fig. 3a,b). Interestingly, the Y33 mutant Wwox did not lead to inhibition of colony formation; thus, the Y33 mutant Wwox was much less effective than the SDR-deleted mutant in terms of suppressing colony formation (Fig. 3c,d).

Reduction of Wwox expression during pancreatic duct carcinogenesis. The expression levels of Wwox in non-neoplastic pancreatic duct and PC tissue samples were determined by immunohistochemistry. Abundant cytoplasmic Wwox expression was detected in the normal pancreatic duct epithelium, whereas Wwox expression was reduced in precancerous lesions in accord with the PanIN grade (Fig. 4a). In PC, absent (–) or low (+) Wwox expression was frequently detected (Fig. 4b), with a significant correlation with frequent incidence of lymph node metastasis ($P = 0.026$; Table 1). We also performed microsatellite analysis,[34] and methylation-specific polymerase chain reaction (MSP),[17] to investigate the genetic and epigenetic backgrounds of these Wwox-negative PCs. The frequencies of LOH at D16S3029 within the *WWOX* locus and hypermethylation of the *WWOX* promoter CpG (cytosine and guanine separated by a phosphate) island were 22% and 20%, respectively; however, hypermethylation of the *WWOX* exon 1 CpG island was detected in 83% of Wwox-negative PC (data not shown).[17] Among 32 patients with PC who underwent curative surgery and who received follow-up care at Kobe University Hospital, a significant difference in survival rates was detected depending on Wwox level: the absent (–) and low (+) Wwox expression was significantly associated with shorter survival times, as compared with tumors expressing high (++) levels of Wwox expression ($P = 0.024$; Fig. 4c).

Wwox restores Smad4 levels in PC cells. Inactivating mutations in components of the transforming growth factor beta (TGF-β)–Smad signaling pathway results in resistance to the antiproliferative effects of TGF-β in PC cells.[35–37] To gain a better understanding of the role of Wwox on the TGF-β–Smad signaling pathway, we investigated the association between Wwox and Smad4 expression. Loss of Smad4 expression tended to associate with low Wwox levels (Fig. 5a,b). In PANC-1 cells, restoration of the Wwox expression elevated Smad4 protein levels 48 h after p*WWOX* transfection (Fig. 5c). No alteration was found at the messenger RNA (mRNA) levels of Smad4 expression (data not shown).

Discussion

Carcinoma cell lines and primary tumors exhibit hemizygous or homozygous deletion with endpoints within fragile regions of the human genome, especially within the most active common fragile site, *FRA3B*, which is encompassed by the *FHIT* gene.[11] As has been observed with the *FHIT* gene and

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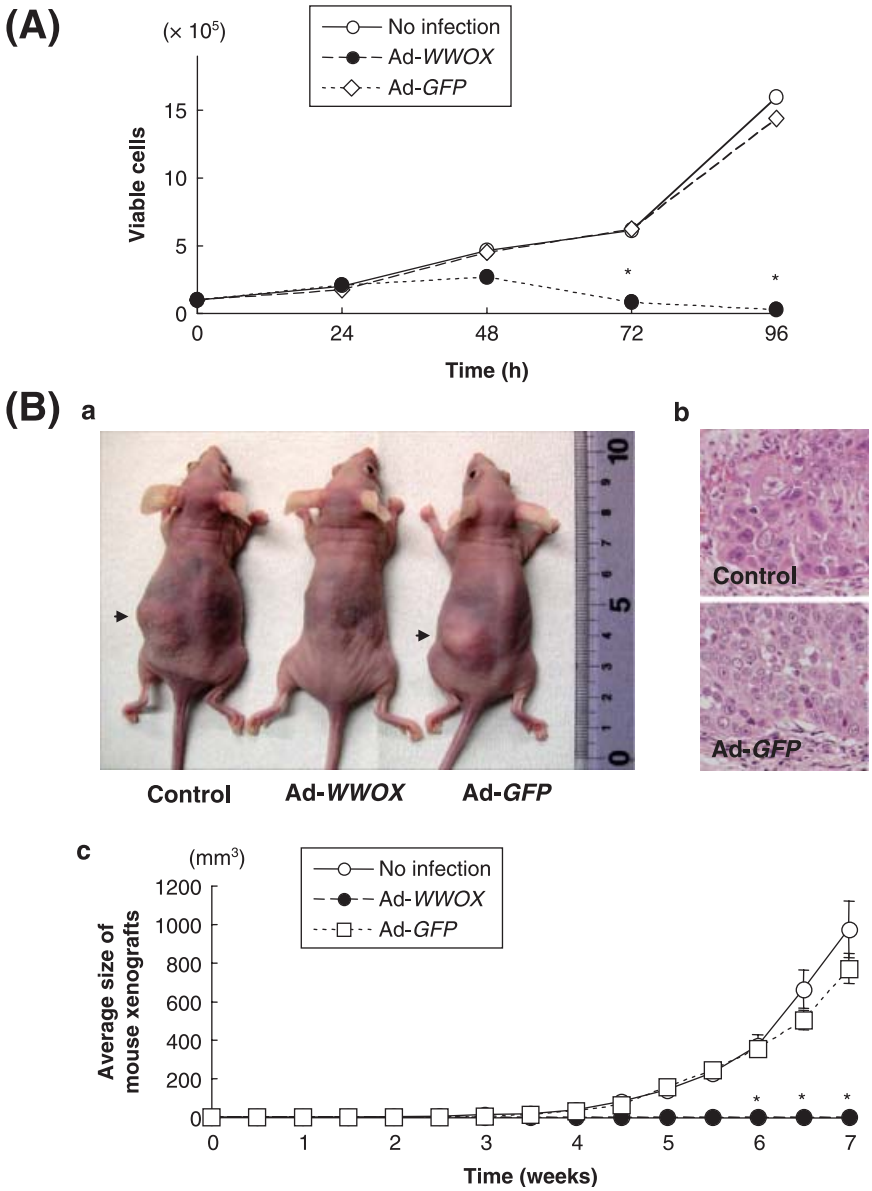

Fig. 2. Growth suppression by restoration of the *WW-domain-containing oxidoreductase* (*WWOX*) gene in PANC-1 cells *in vitro* and *in vivo*. (A) Cell growth test after transduction of the *WWOX* gene with adenoviral (Ad)-*WWOX* (multiplicity of infection [MOI] 25). (B) Tumorigenicity of Ad-*WWOX* infected PANC-1 cells. (a) Representative mouse subcutaneous (s.c.) tumors at 7 weeks. (b) Histological examination of the s.c. tumors (hematoxylin & eosin (HE). staining). (c) Restoration of Wwox expression inhibited s.c. tumor formation. $*P < 0.05$.

other fragile-site-related genes, deletion at a certain locus and loss of protein expression has been shown to promote cell transformation and immortalization in various human malignancies; this is likewise the case with the *WWOX* (*FRA16D*) locus and Wwox expression.[38–40] In this study, decreased Wwox expression has been detected in PC cells and tissues, as compared with that of normal pancreatic tissue. Since the *WWOX* gene is encompassed by *FRA16D*, and loss of Wwox expression was frequently detected in many human malignancies, we hypothesized that a reduction in Wwox expression may be closely associated with a deletion at this locus; LOH at D16S3029 was, however, infrequent.

Conversely, hypermethylation at *WWOX* regulatory sites was frequently detected, suggesting that epigenetic regulation of *WWOX* transcripts, particularly by hypermethylation at the exon 1 CpG island, may play an important role in pancreatic tumorigenesis.[29] According to previous studies, hypermethylation at both the promoter and exon 1 CpG islands of the *WWOX* gene is a relatively common feature in lung and breast cancers,[41,42] whereas hypermethylation at either of these sites does not appear to be associated with bladder cancer.[17] In heterochromatin, most candidate CpG sites expand from the promoter region to the exon 1 region and the methylated CpG sites are bound by methyl-cytosine-binding protein complexes, which exclude transcription factor complexes.[43] We assessed the importance of the exon 1 CpG island in the regulation of *WWOX* expression in PC and confirmed that demethylation at the *WWOX* regulatory sites resulted in restoration of Wwox protein expression in MIA PaCa-2 cells (from undifferentiated human pancreatic carcinoma cell line; supplementary Fig. S1). Recently, post-transcriptional regulation of Wwox protein levels has been documented: phosphorylation of Wwox at Y287 by Ack1, an activated Cdc42-associated kinase, promotes Wwox degradation, consequently stimulating prostate tumorigenesis.[44] Further investigation will be needed to clarify the mechanism by which Wwox expression levels may be down-regulated.

Molecular genetic analyses have provided a convincing line of evidence that pancreatic duct lesions are the precursors to infiltrating PC. Almost all of the genetic alterations that have been identified in infiltrating PC have also been detected in these ductal lesions and, remarkably, the prevalence of these genetic alterations increases as the degree of cytological and

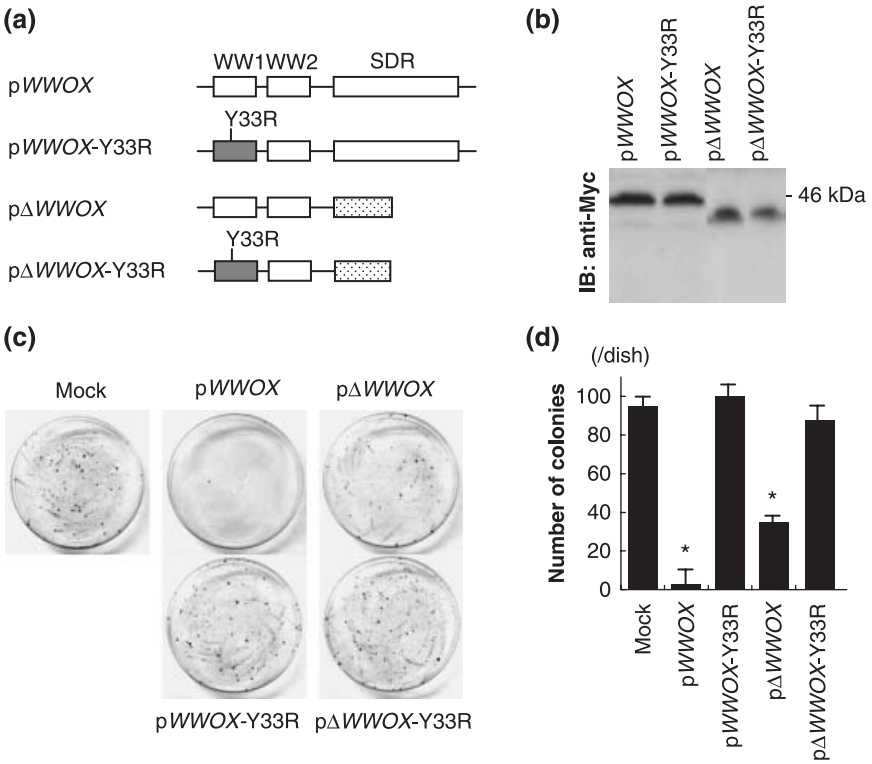

Fig. 3. Biological significance of the WW1 domain in PANC-1 cells. (a) A scheme of the wild-type and mutant WW-domain-containing oxidoreductase (*WWOX*)-expressing vector constructs. Substitution of Y33 with arginine was conducted to generate Y33R mutants, and the short-chain dehydrogenase /reductase (SDR)-domain deletion mutants were also designed. (b) Detection of recombinant Wwox protein by immunoblot. The N-termini of these proteins were tagged with Myc. (c) Representative plates for colony formation assay. (d) Quantification of colonies per plate from 100-mm plates, for pcDNA3 (mock), p*WWOX*, p*WWOX*-Y33R, pΔ*WWOX* and pΔ*WWOX*- Y33R-transfected PANC-1 cells. **P* < 0.05.

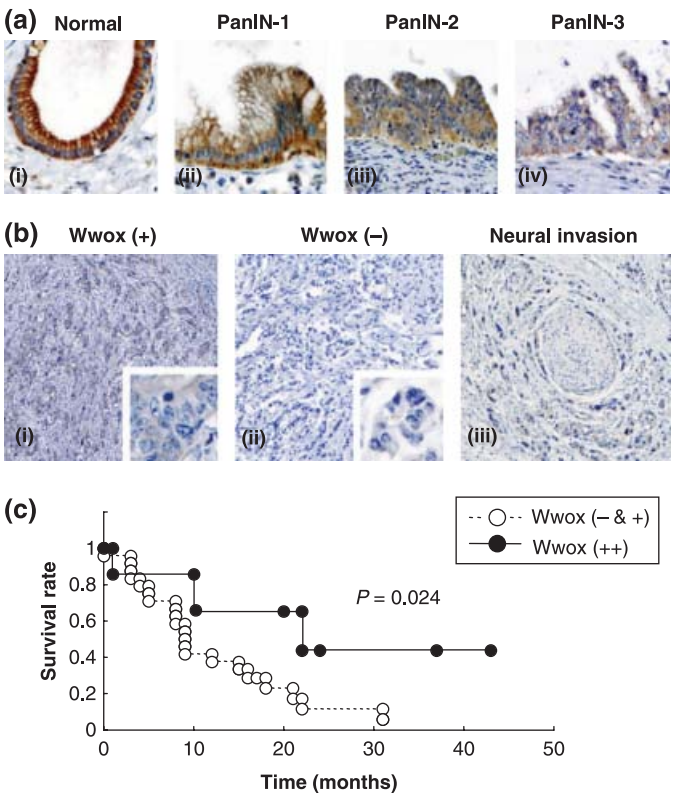

Fig. 4. WW-domain-containing oxidoreductase (Wwox) expression in pancreatic carcinoma (PC) tissue samples. (a) Wwox expression in-non-neoplastic pancreatic duct epithelia (i) and pancreatic intraepithelial neoplasia (PanIN) (ii, PanIN-1; iii, PanIN-2; and iv, PanIN-3). (b) Wwox expression in PC. Representative tumors with weak (i) and negative Wwox expression (ii & iii) are shown. (c) Kaplan–Meier curves of the overall survival of PC patients whose cancer samples are positive (++), and negative or weak (– & +) for Wwox expression.

Table 1. Results of WW-domain-containing oxidoreductase (Wwox) expression in pancreatic carcinoma (PC): association with clinicopathological findings

	Wwox expression†			*P*-value*
	Total *n* (%)	(– & +) *n* (%)	(++) *n* (%)	
Total	32 (100%)	25 (79%)	7 (21%)	
Histology‡				
Moderately diff. type	29 (91%)	23 (72%)	6 (19%)	0.887
Poorly diff. type	3 (9%)	2 (6%)	1 (3%)	
Tumor size‡				
TS1 + TS2	14 (44%)	10 (31%)	4 (13%)	0.351
TS3 + TS4	18 (56%)	15 (47%)	3 (9%)	
Type of tumor growth‡				
INFβ	27 (84%)	21 (66%)	6 (18%)	0.704
INFγ	5 (16%)	4 (13%)	1 (3%)	
Lymphatic vessels infiltration				
Negative	3 (9%)	2 (6%)	1 (3%)	0.881
Positive	29 (91%)	23 (72%)	6 (19%)	
Venous vessels infiltration				
Negative	5 (16%)	2 (7%)	3 (9%)	0.057
Positive	27 (84%)	23 (71%)	4 (13%)	
Lymph node metastasis				
Negative	7 (22%)	3 (9%)	4 (13%)	0.026
Positive	25 (78%)	22 (69%)	3 (9%)	
Clinicopathological stage‡				
I + II + III	10 (31%)	7 (22%)	3 (9%)	0.885
IV	22 (69%)	18 (56%)	4 (13%)	

**P*-values less than 0.05 were considered to be statistically significant.
†Evaluation of Wwox expression was described in the text.
‡Histology, tumor size, type of tumor growth and clinicopathological stage were classified according to the General Rules for the Study of Pancreatic Cancer,[30] along with TNM classification.[31]

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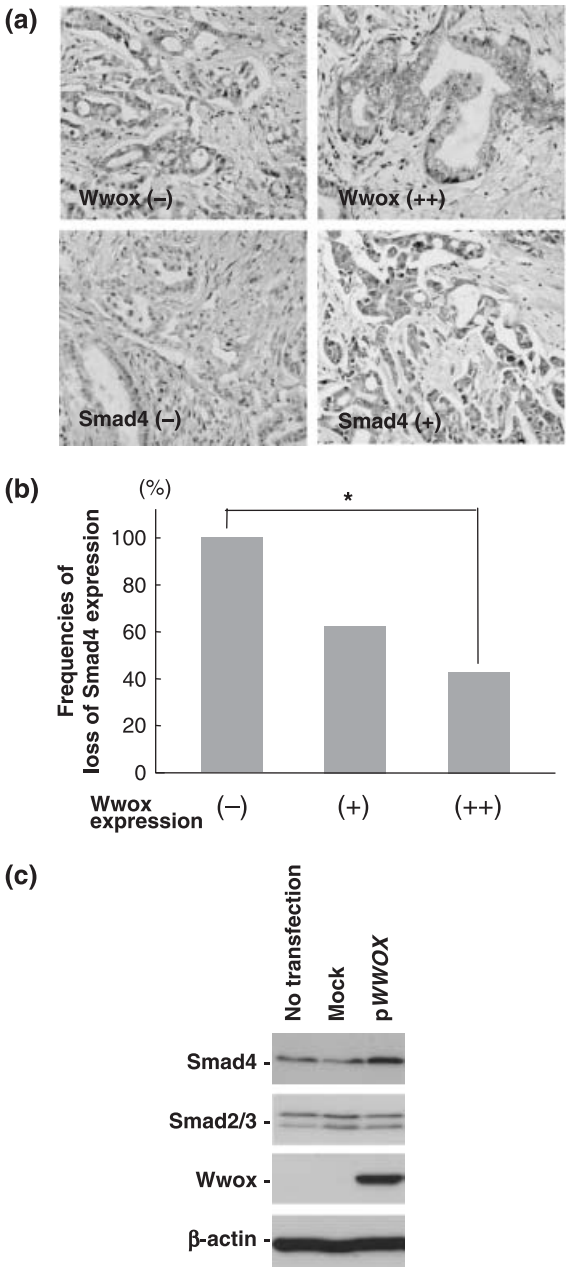

Fig. 5. Association between WW-domain-containing oxidoreductase (Wwox) and mothers against decapentaplegic homolog 4 (Smad4) expression in pancreatic carcinoma (PC) cells. (a) Representative immunohistochemistry results for Wwox and Smad4. (b) Frequencies of loss of Smad4 expression in PC samples. (c) Wwox modulated Smad4 expression at the protein level in PANC-1 cells. p*WWOX* was transfected into PANC-1 cells and the cells were collected 48 after transfection.

architectural atypia in the duct lesions increases.[32] Interestingly, in PanIN, reduced Wwox expression was detected in accord with the progression of morphological features. This finding suggests that suppression of Wwox expression may accelerate the accumulation of genetic changes in oncogenic (*KRAS* and *HER-2*) and tumor suppressor proteins (*CDKN2A*, *TP53* and *SMAD4*), consequently leading to the transformation of pancreatic duct lesions. Also, loss of Wwox expression was found to be correlated with the grade of PC malignancy, as determined by the incidence of lymph node metastasis and poorer outcome. These findings suggest the significance of reduced Wwox expression during pancreatic duct carcinogenesis and progression of PC.

Here we noted that restoration of *WWOX* expression induced caspase-dependent apoptosis and suppressed cell growth *in vitro* and *in vivo*. The *WWOX* gene encodes a 46-kDa protein that contains two WW domains (WW1 and WW2), which are involved in protein–protein interactions, and an SDR domain, which may be involved in sex-steroid metabolism.[45] Ludes-Meyers *et al*.[46] identified the specific proline-rich ligand for Wwox as PPXY motif and demonstrated the N-terminal WW1 domain is responsible for this interaction. Various transcription factors have been identified as Wwox-interacting proteins: p73, the p53 family protein, at ^{482}PPPPY^{488} motif,[23] Ap-2 γ at ^{56}PPPYFPPPY^{64} motif,[24] and Jun proto-oncogene, at ^{67}PPVY^{70} motif physically interact with Wwox,[26] respectively, inhibiting nuclear transport and resulting in down-regulation of their transcriptional activity. In this study, p*WWOX* transfection increased Smad4 expression at the protein level, but not at the mRNA level, suggesting the Wwox-mediated post-transcriptional regulation of Smad4 levels. The *SMAD4* gene (also referred to *DPC4*, for deleted in pancreatic carcinoma locus 4) shares characteristics with typical tumor suppressor genes,[47] about half of PC contain either homozygous deletions of the *SMAD4* locus or inactivating mutations in one allele associated with LOH, and a resultant loss of Smad4 expression is frequently detected in PC.[9,47] Indeed, in the context of TGF-β signaling, Smad4 plays a central role in the nuclear transport of Smad2 and Smad3 proteins; Smad4 forms complexes with Smad2 and Smad3 after activation of TGF-β receptor, which results in tumor suppression by the up-regulation of the cyclin-dependent kinase inhibitors p15 and p21, as well as the up-regulation of other cell-cycle and cell-death regulators.[48] Therefore, Wwox may exhibit such tumor suppressor effects through TGF-β–Smad signaling by increasing Smad4 levels. Smad4 can be proteasomally degraded after polyubiquitination by Smurf1 and Smurf2 E3 ligase complexes, which possess the WW domain.[49,50] Although these Smurfs cannot bind to Smad4 directly, the PPXY motif within Smad2 and Smad7 enable the formation of complexes with Smad4 and Smurfs, which subsequently leads to Smad4 degradation. Further investigation will be necessary to elucidate the mechanism by which Smad4 levels can be increased by restoration of the *WWOX* gene.

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Supplemental material

The following supplementary material is available for this article:

Fig. S1. Supplemental data 1. Hypermethylation-mediated reduction of WW-domain-containing oxidoreductase (Wwox) expression in MIA PaCa-2 cells. Human pancreatic carcinoma (PC)-derived cell line MIA PaCa-2 (American Type Culture Collection, Manassas, VA, USA) was routinely maintained in Roswell Park Memorial Institute media (RPMI)-1640 supplemented with 10% fetal bovine serum. (A) The effect of 5-aza-2′-deoxycytidine (5-aza-dC; Sigma, St. Louis, MO, USA), trichostatin A (TSA; Sigma), and a combination of the two drugs on the *WWOX* methylation status in MIA PaCa-2 cells. For methylation-specific polymerase chain reaction (MSP), genomic DNA was treated with sodium bisulfite (Qiagen, Hilden, Germany) and was analyzed by MSP using primer sets within CpG (cytosine and guanine separated by a phosphate) sites in the *WWOX* promoter and exon 1.[12] Polymerase chain reaction (PCR) samples were resolved by electrophoresis on a 2% agarose gel. For treatment with 5-aza-dC and TSA, cells were seeded in a 100-mm plate at a density of 1×106 cells. After 24 h, cells were treated with 5-aza-dC (5 μM) and/or TSA (1 μM). Genomic DNA and cell lysate were isolated 5 days after addition of 5-aza-dC and/or TSA treatments.[22] M, methylated; U, unmethylated. (B) Restoration of Wwox protein by treatment with 5-aza-dC and TSA in MIA PaCa-2 cells. Relative intensity of these bands are visualized.

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