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ORIGINAL ARTICLE

WWOX inhibits the invasion of lung cancer cells by downregulating RUNX2

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The WW domain-containing oxidoreductase (WWOX) is a tumor suppressor that is lost or decreased in most human tumors. The role of WWOX in human lung carcinoma invasion is still not clear. This study aimed to elucidate the potential role of WWOX in lung cancer cell invasion. WWOX mRNA levels in human lung cancers and lung cancer cell lines were assayed by quantitative real-time PCR. WWOX in lung cancer cell lines was manipulated by transfection of expression vector or small interfering RNA. Cell migration and invasion were assessed by wound healing and/or transwell migration and invasion assays. The protein levels of WWOX, E-cadherin and RUNX2 were analyzed by western blot or immunofluorescence. WWOX expression is inversely correlated to invasiveness of lung cancer. WWOX overexpression in highly invasive H1299 cells reduced cell motility and invasiveness, and inhibited the expression of RUNX2 and its target gene matrix metalloproteinase-9 (MMP-9). Silencing WWOX in less invasive NL9980 cells resulted in opposite effects. Overexpressing RUNX2 in H1299 or silencing RUNX2 in NL9980 cells reversed the effects of WWOX. These results suggested that WWOX inhibited the invasive phenotype of lung cancer through downregulating the expression of RUNX2.

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INTRODUCTION

Lung cancer is one of the most common cancers and the leading cause of tumor-related deaths worldwide.[1] Whereas traditional cancer treatments, for example, surgery, radiotherapy and chemotherapy, have not been desirable in controlling lung cancer progression and increasing survival rates, recent advances in molecular diagnosis and immunotherapy have improved the outcome of lung cancers[2] despite the heterogeneity and complexity of histopathologic classification and clinical behavior of lung cancers.[3]

WWOX (WW domain-containing oxidoreductase) gene is a tumor suppressor that is originally identified from a region located at chromosome region 16q23.3-24.1 encompassing the common fragile site FRA16D that is frequently aberrant in breast cancers.[4] The *WWOX* gene encodes a 46 kDa protein that is composed of two WW domains, a nuclear localization motif and a C-terminal short-chain alcohol dehydrogenase/reductase domain.[4,5] WWOX is a tumor suppressor that is altered in many malignancies.[6] WWOX promoter hypermethylation is detected in 3/4 of lung cancers[7] and its expression is lost or strongly diminished in a majority of non-small-cell lung cancers.[8] Loss of the whole or part of WWOX gene predisposes individuals to lung cancer in the Chinese population.[9] Overexpression of WWOX could prevent lung cancer growth *in vitro* and *in vivo*.[10,11] Although WWOX has been shown to inhibit the invasion and metastasis of many cancers[12–14] and it does so in osteosarcoma by inhibiting RUNX2 expression,[14,15] it is still not known whether WWOX also plays a role in lung cancer cell invasion. This study aims to investigate the role of and the mechanism governing WWOX in lung cancer cell invasion.

MATERIALS AND METHODS

Tumor tissues samples

The protocols were reviewed and approved by institutional review committee of Jiangnan University (Wuxi, China). An informed consent form was signed by every participant. A total of 30 lung cancer samples were obtained from surgical resection of primary lung carcinomas with 14 cases of stages I, 9 cases of stage II and 7 cases of III according to the TNM (tumor–node–metastasis) classification. In addition, 15 normal lung tissues matching the tumor samples were also collected.

Cell culture

Human lung cancer cell lines NL9980, A549, NCI-H1299 and HBE were obtained from American Type Culture Collection (Manassas, VA, USA). NL9980 and A549 were cultured in Dulbecco's modified Eagle's medium (Gibco, Shanghai, China) containing 10% fetal bovine serum (fetal bovine serum) (Gibco), and NCI-H1299 and HBE in RPMI-1640 (Gibco) containing 10% fetal bovine serum at 37 °C in a humidified incubator with 5% CO_2.

Transfection of overexpression plasmids

The pcDNA3.0-wwox and pcDNA3.0-runx2 mammalian expression vectors were purchased from GenePharma (Suzhou, China). H1299 cells were transiently transfected with pcDNA3.0 empty vector, pcDNA3.0-wwox and pcDNA3.0-wwox/pcDNA3.0-runx2 using Lipofectamine 2000 (Invitrogen, Shanghai, China) according to the manufacturer's instructions. At 6 h after transfection, the medium containing transfection reagents was replaced with fresh medium. The transfected H1299 cells were harvested for subsequent experiments 48 h after transfection.

Transfection of siRNA

Small interfering RNA (siRNA) duplexes targeting human WWOX and RUNX2 as well as nontargeting control siRNA were purchased from GenePharma. The RNA sequences were 5′-GGACGGCUGGGUUUACUACTT-3′ and

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5′-GUAGUAAACCCAGCCGUCCTT-3′ for WWOX and 5′-GGUCCUAU GACCAGUCUUATT-3′ and 5′-UAAGACUGGUCAUAGGACCTT-3′ for RUNX2. NL9980 cells were transfected with these siRNA duplexes using Lipofectamine 2000 according to the manufacturer's instructions.

Quantitative real-time PCR analysis

Quantitative real-time PCR was essentially performed as previously described.[16] Total RNA was extracted from normal and tumor lung tissues with the RNAprep Pure Tissue Kit (Tiangen, Beijing, China) and from lung cancer cells with the RNAprep Pure Cell/Bacteria Kit (Tiangen) according to the manufacturer's protocols. The complementary DNA was synthesized with the PrimeScript RT reagent kit (Takara, Beijing, China) according to the manufacturer's instructions. Real-time PCR was performed using SYBR Green PCR Master Mix (Roche, Indianapolis, IN, USA) on a StepOne (Applied Biosystems, Foster City, CA, USA). The PCR conditions were 95 °C for 3 min followed by 40 cycles of 95 °C for 15 s, 58 °C for 15 s and 68 °C for 30 s. The primers were synthesized by Sangon (Shanghai, China) and their sequences were 5′-CGTCGAACGCAGTGCATCCT-3′ and 5′-CATCCCTCCCAG ACCCTCCA-3′ for WWOX (NM_016373); 5′-GTCATCCAGTTTGGTGTCGC-3′ and 5′-GGACCACAACTCGTCATCGT-3′ for MMP-9 (NM_004994); 5′-GCTTCA TTCGCCTCACAAACAA-3′ and 5′-GGTCTCGGTGGCTGGTAGTG-3′ for RUNX2 (NM_001024630); and 5′-GAAATCCCATCACCATCTTCCAGG-3′ and 5′-GAGC CCCAGCCTTCTCCATG-3′ for GAPDH (NM_002046). The relative gene expression levels were calculated by $2^{-\Delta\Delta Ct}$ method and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) served as the internal control.

Immunofluorescence

Subconfluent monolayers of H1299 and NL9980 cells were seeded onto coverslips in 24-well plates. Cells were fixed with whole-cell fixative for 10 min on ice, washed with phosphate-buffered saline and incubated in permeabilizing solution (0.25% Triton X-100 in phosphate-buffered saline) for 10 min on ice. Then, the coverslips were incubated with 1:50 diluted antibodies against WWOX (Cell Signaling Technology, Beverly, MA, USA), E-cadherin (Abcam, Cambridge, MA, USA) or GAPDH (Abcam) overnight at 4 °C. The slides were washed and next incubated with Alexa Fluor 488-Affinipure Goat anti-mouse or Alexa Fluor 594-Affinipure anti-rabbit IgG (Jackson ImmunoReseach, West Grove, PA, USA) at room temperature for 30 min in the dark. Nuclei were stained with DAPI (4',6-diamidino-2-phenylindole).

Western blotting

Western blot was performed according to previously described method with some modifications.[17] Briefly, cells were lysed in ice-cold RIPA buffer (150 mM NaCl, 50 mM Tris pH 7.4, 1 mM EDTA, 0.1% Triton X-100) supplemented with a protease inhibitor tablet. Proteins were separated on 8% SDS–polyacrylamide gel electrophoresis gels and transferred onto nitrocellulose membranes. The membranes were incubated with a rabbit polyclonal antibody to WWOX, RUNX2 (Abcam) or GAPDH at 4 °C overnight, washed and incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG antibody (Jackson ImmunoReseach) at room temperature for 30 min before the specific protein bands were visualized using Western Detection kit (Beyotime, Shanghai, China). Protein bands were scanned and analyzed using ImageJ (NIH, Bethesda, MD, USA). The optical density of specified protein was expressed by the ratio between target protein and loading control (GAPDH).

Scratch-wound healing assay

A published protocol[18] was used for scratch-wound healing assay with some modification. Cells were cultured in serum-free medium overnight and wounded by scratching with sterile 200 μl pipette tips. The cell migration was expressed as the percentage of reduction of gap distance after 12 h.

Transwell invasion and migration assays

Cell invasion and migration assays with Transwell systems were basically following previously described methods.[19] Briefly, 24-well inserts with (cell invasion assay) or without (cell migration assay) 10 mg ml^{-1} Matrigel (Sigma, St Louise, MO, USA) coating were used for cell invasion or migration assays. The 5×10^4 cells were resuspended in medium containing 1% fetal bovine serum and plated into the upper chambers of Transwell plates (Millipore, Beijing, China) and the lower chambers were filled with media containing 10% fetal bovine serum. The cells were cultured at 37 °C for 24 h. The cells on the upper side were wiped off with cotton swabs and the filters were then fixed in methanol and stained with 1% crystal violet. The cells on the lower surface were counted from 5 different random fields at ×200 magnification. The relative cell migration (or invasion) rate was calculated against the number of migrating (or invading) cells of control that was set as 100%.

Statistical analysis

All *in vitro* experiments were performed independently at least three times. Data were expressed as mean ± s.d. Differences between groups were assessed with *T*-tests using Microsoft Excel. A *P*-value of < 0.05 was considered statistically significant.

RESULTS

Reduction or loss of WWOX expression was correlated with lung cancer invasion *in vivo* and *in vitro*

The WWOX mRNA level was greatly depressed in tumor tissues (0.68 ± 0.12) compared with that of normal tissues (11.26 ± 3.68) ($P < 0.0001$) (Figure 1a). Moreover, the mRNA level of WWOX was lower in TNM stage II/III tumors (0.46 ± 0.12) than in stage I (1.91 ± 0.26) ($P < 0.01$) (Figure 1b).

The WWOX mRNA levels of lung cancer cell lines H1299, A549 and NL9980 were significantly lower than that of normal human bronchial epithelial cell line (HBE) (Figure 1c). The mRNA levels of the three examined lung cancer cell lines were in the order of H1299 < A549 < NL9980 (Figure 1c). Meanwhile, H1299 cells showed much stronger invasiveness than NL9980 cell in transwell invasion assays (Figure 1d).

WWOX inhibits lung cancer cell migration and invasion

We next examined the roles of WWOX on the invasiveness of lung cancer cells by overexpressing WWOX in H1299 cells (Figures 2a and c) and silencing WWOX in NL9980 cells with siRNA (Figures 2b and c). WWOX-overexpressing H1299 (H1299-wwox) cells showed a more cohesive and epithelioid phenotype than H1299-mock (Figure 2d), whereas WWOX-silenced NL9980 (NL9980-siwwox) cells showed lamellipodium-type extension phenotype compared with NL9980-siNC cells (Figure 2d). Meanwhile, ectopically expressed WWOX increased the level of cell adhesion molecule E-cadherin in H1299 cells (Figure 2e, left panel) and silencing WWOX decreased E-cadherin level in NL9980 cells (Figure 2e, right panel).

The migration ability of H1299 cells was significantly inhibited (40–65%) by WWOX overexpression in would healing (Figure 3a, left panel) and transwell migration (Figure 3b, left panel) assays. On the other hand, silencing WWOX promoted the migration of NL9980 cells (Figures 3a and b, right panels). Similarly, overexpressing WWOX inhibited the invading ability of H1299 cells (Figure 3c, left panel), whereas silencing WWOX increased the number of invading NL9980 cells (Figure 3c, right panel).

WWOX inhibits lung cancer cell invasion by downregulating RUNX2

The protein levels of RUNX2 were negatively regulated by WWOX in lung cancer cells (Figures 4a–c). Overexpressing WWOX in H1299 cells reduced RUNX2 level (Figures 4a and b), whereas targeting WWOX with siRNA resulted in an increase of RUNX2 protein in NL9980 cells (Figures 4a and c). Consequently, the expression of matrix metalloproteinase-9 (MMP-9), a downstream target gene of RUNX2, was significantly lower in H1299-wwox cells than in H1299-NC cells (Figure 4d), whereas it was substantially higher in NL9980-siwwox cells than in NL9980-siNC cells (Figure 4e). Moreover, RUNX2 overexpression (Figures 5a and b) significantly enhanced cell invasion of H1299-wwox cells (Figures 5d and e), whereas RUNX2 silencing (Figures 5b and c)

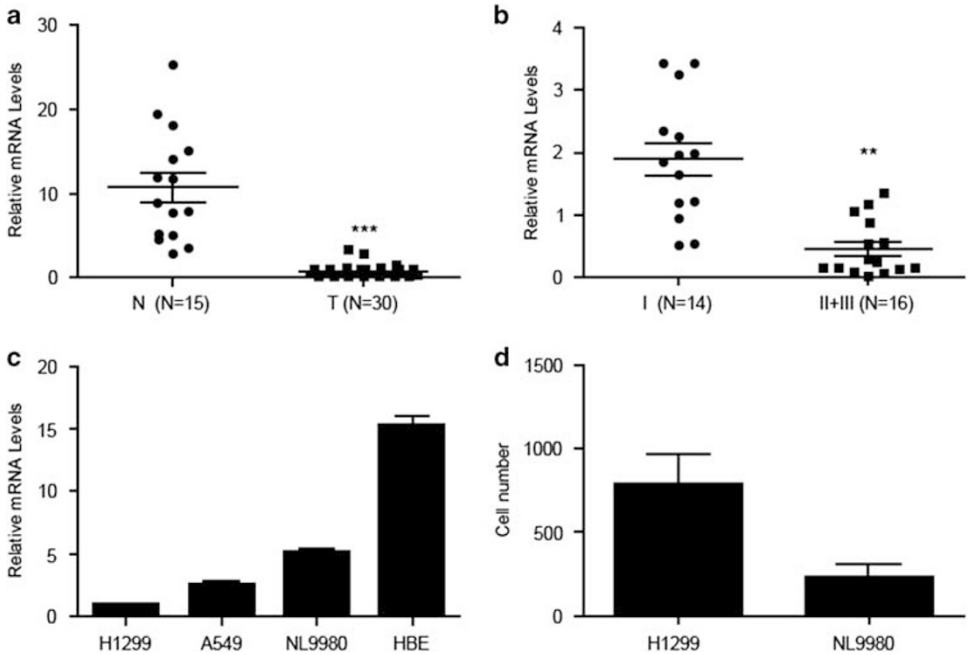

Figure 1. Loss of WWOX (WW domain-containing oxidoreductase) expression is associated with tumor invasion in lung cancers and lung cancer cell lines. (**a**) Analysis of WWOX mRNA levels in normal lung tissues (N) and lung carcinoma tumors (T) by quantitative real-time PCR. (**b**) Comparison of WWOX mRNA levels in lung carcinomas with different tumor–node–metastasis classifications. (**c**) The level of WWOX expression in H1299, A549, NL9980 and HBE cell lines was evaluated by real-time PCR. (**d**) The invading abilities of the lung cancer cell lines were assessed in a transwell invasion assay. $^{**}P < 0.01$; $^{***}P < 0.001$.

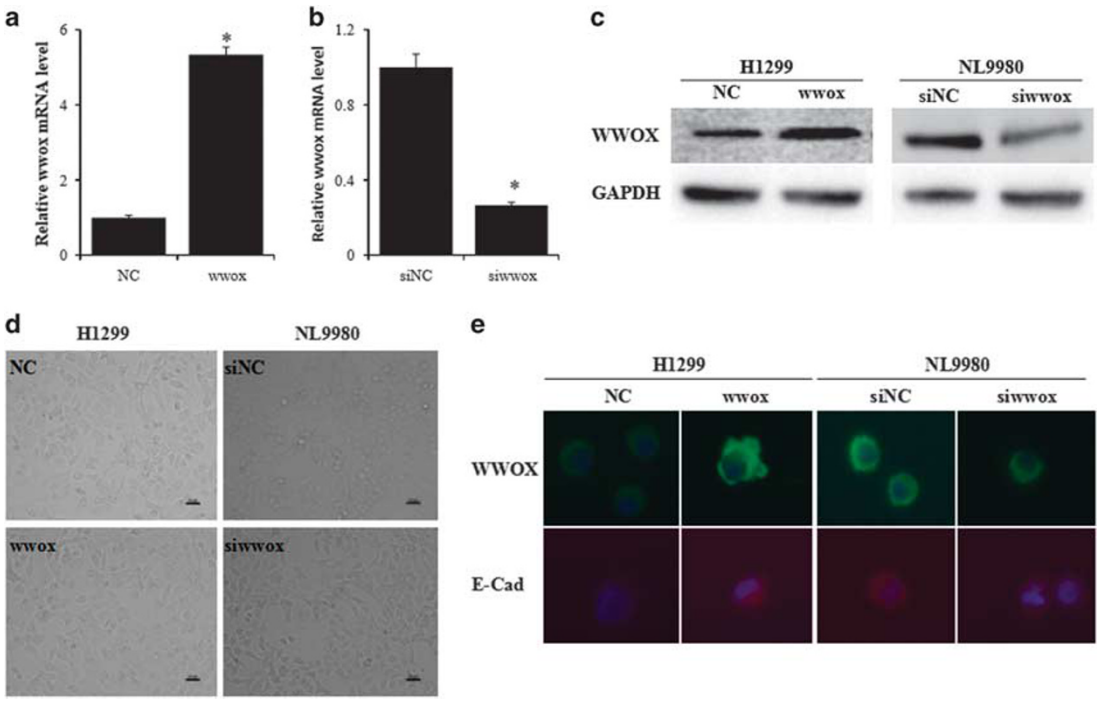

Figure 2. WWOX (WW domain-containing oxidoreductase) regulates cell–cell adhesion in lung cancer cells. WWOX mRNA levels in H1299 after transfecting overexpression vector (**a**) and NL9980 after small interfering RNA (siRNA transfection) (**b**) were assessed by quantitative real-time PCR (qPCR). (**c**) WWOX protein levels in H1299 transfected with empty pcDNA vector or pcDNA-wwox (left panel) and NL9980 transfected with control siRNA or siRNA targeting WWOX (right panel). (**d**) The morphology of H1299 cells transfected with empty vector (H1299-NC) or WWOX expression vector (H1299-wwox) (left panel) and NL9980 cells transfected with negative control siRNA (NL9980-siNC) or siRNA targeting WWOX (NL9980-siwwox) (right panel). Bar, 20 μm. (**e**) The expression of WWOX and E-cadherin in H1299-NC, H1299-wwox, NL9980-siNC and NL9980-siwwox was evaluated by immunofluorescence (magnification × 400). $^{*}P < 0.05$.

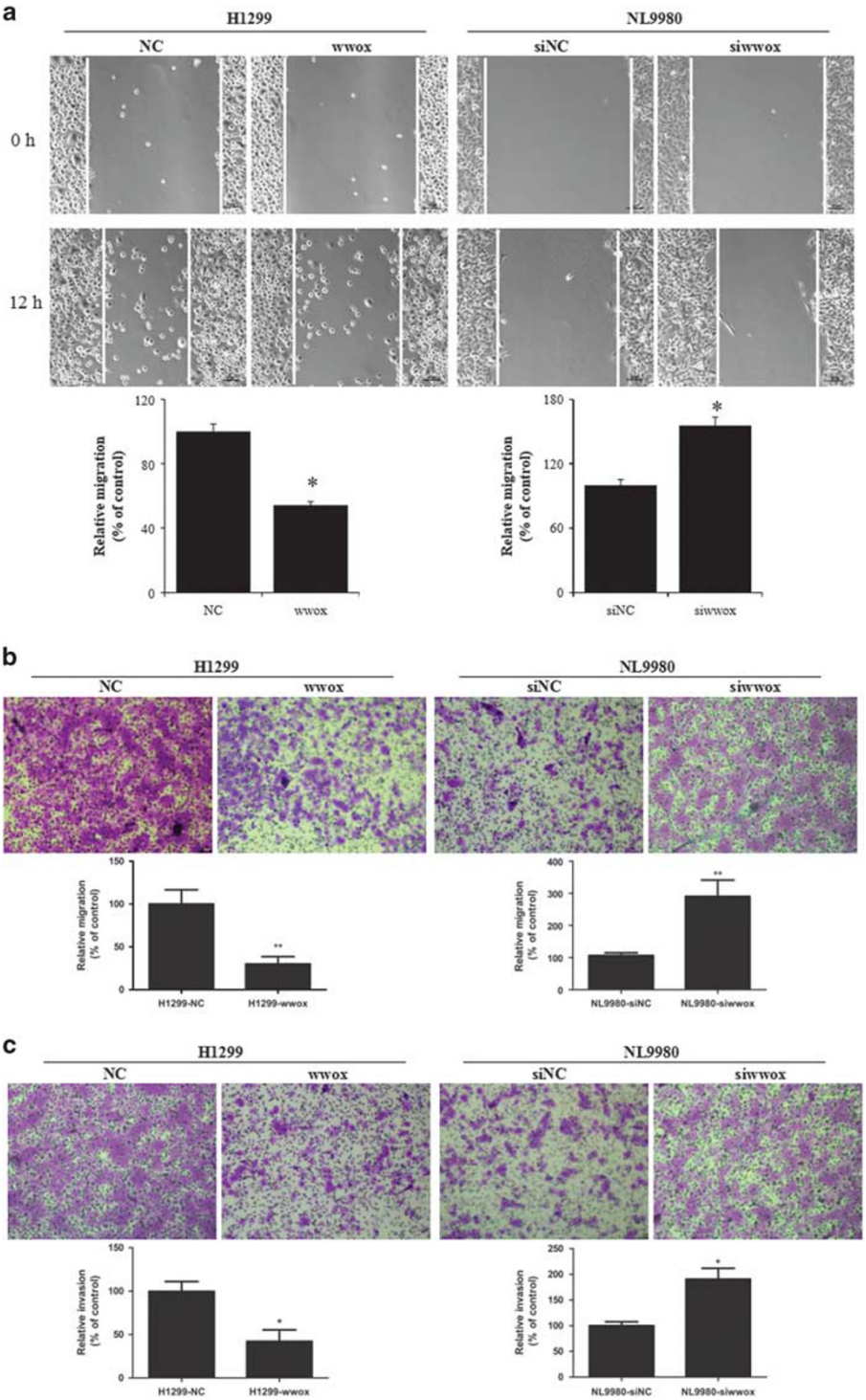

Figure 3. Effects of WWOX (WW domain-containing oxidoreductase) on lung cancer cell migration and invasion. The migration ability of H1299 (left panels) and NL9980 (right panels) cells was assessed by scratch-would healing assay (**a**) and transwell migration assay (**b**). The invasiveness of H1299 (left panel) and NL9980 (right panel) cells was evaluated by transwell invasion assay (**c**). Bar, 100 μm. $^{*}P < 0.05$; $^{**}P < 0.01$.

blocked cell invasiveness induced by WWOX silencing in NL9980 cells (Figures 5f and g).

DISCUSSION

The loss or significant reduction of tumor-repressing WWOX in human lung cancers was prevalent,[7,8] and the lower WWOX level was associated with more aggressive tumors and poorer prognosis.[8] WWOX was also shown to be absent or reduced in many other cancers including epithelial ovarian cancer,[20] pancreatic cancer,[21] head and neck squamous cell carcinoma[22] and breast cancer.[23] The loss/reduction of WWOX expression might be rooted from deletion mutation,[9,20] hypermethylation and destabilizing mutations.[7] The preservation of intratumoral

WWOX expression was associated with better clinical outcomes in non-small-cell lung cancer patients after resection surgeries.[24] These data demonstrated that the loss or reduction of WWOX function might be a critical step during the tumorigenesis and the gain of invasiveness of lung cancer and many other cancers.

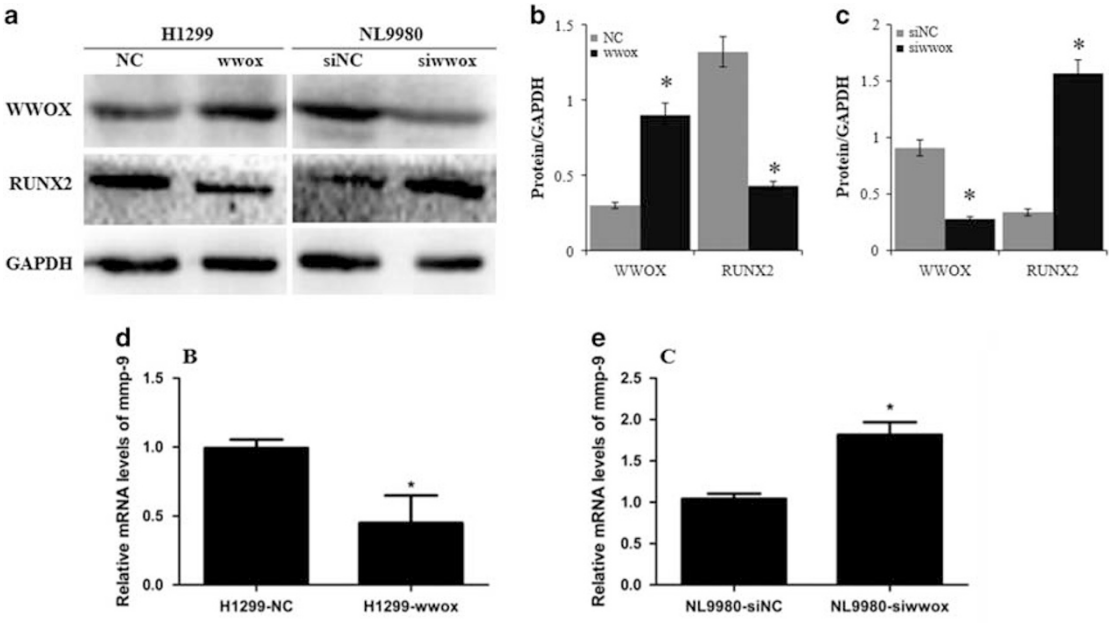

Figure 4. WWOX (WW domain-containing oxidoreductase) inhibited RUNX2 expression in lung cancer cells. (**a**) WWOX and RUNX2 protein levels in H1299 (left panel) and NL9980 (right panel) cells were analyzed by western blot. Quantitative analysis of WWOX and RUNX2 protein levels of H1299 (**b**) and NL9980 (**c**) cells were expressed as ratio of either WWOX or RUNX2 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Relative mRNA levels of matrix metalloproteinase-9 (MMP-9) of H1299 (**d**) and NL9980 (**e**) were determined by quantitative real-time PCR. $*P < 0.05$.

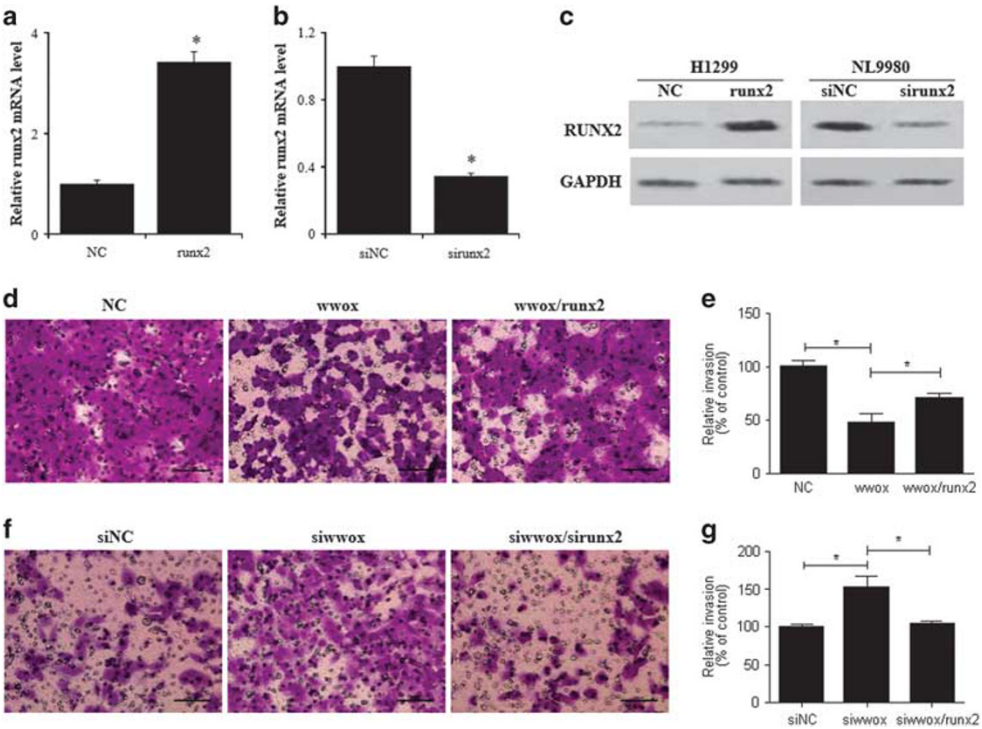

Figure 5. RUNX2 abrogated WWOX (WW domain-containing oxidoreductase) inhibition of lung cancer cell invasiveness. RUNX2 mRNA levels in H1299 after transfecting overexpression vector (**a**) and NL9980 after small interfering RNA (siRNA) transfection (**b**) were assessed by quantitative real-time PCR (qPCR). (**c**) RUNX2 protein levels in H1299 transfected with empty pcDNA vector or pcDNA-wwox (left panel) and NL9980 transfected with control siRNA or siRNA targeting RUNX2 (right panel). Transwell invasion assay showed that overexpressed WWOX inhibited lung cancer cell invasion that was reversed by RUNX2 overexpression in H1299 cell (**d**, **e**). The invasiveness of NL9980 was enhanced by WWOX silencing, and the increase was abolished by siRNA targeting RUNX2 (**f**, **g**). Bar, 100 μm. $*P < 0.05$.

WWOX protein was shown to interact with SMAD3 to disrupt its distribution that resulted in reduced occupancy of SMAD3 on target gene promoters and the inhibition of the activation of transforming growth factor-β target genes involved in tumorigenesis and metastasis.[25] WWOX could also be involved in ATM (ataxia telangiectasia-mutated)-dependent activation of ATR (ataxia telangiectasia and Rad3-related protein) checkpoint proteins upon single-stranded breaks that improved genetic stability.[26] In endometrial adenocarcinoma, WWOX regulated the expression of genes involved in epithelial-to-mesenchymal transition, metalloproteinases and cadherin and integrin signaling pathways to inhibit cancer invasion and recurrences.[27] With the enhancement of HDAC3, WWOX interacted with the BCL9-2, inhibited BCL9-2 activity and interfered with β-catenin–TCF1 interaction in breast cancer.[28] These results demonstrated that WWOX functioned as a tumor suppressor through regulating a host of different downstream targets. The current study demonstrated that RUNX2 was inhibited by WWOX in lung cancer cells to reduce the invasiveness of lung cancer.

RUNX2 has been shown to promote cancer metastasis.[14,28–30] RUNX2 promoted breast cancer cells migrating to and colonizing in bones through upregulating integrin α5.[30] By transcriptionally activating CXCR4 expression, RNNX2 promoted gastric cancer cell migration and invasion *in vitro* and enhanced gastric cancer invasion and metastasis *in vivo*.[29] Overexpressing WWOX in osteosarcoma cells strongly inhibited cancer metastasis through inhibiting the expression of RUNX2 and RUNX2 target genes[14] that was in accordance with the current observation that WWOX inhibited RUNX2 and consequently reduced MMP-9 expression in lung cancer cells.

In conclusion, WWOX expression was strongly inhibited in human lung cancers and lung cancer cell lines. Reintroducing WWOX into lung cancer cells inhibited their invasive phenotype through downregulating RUNX2 and its target genes including MMP-9 expression. These results demonstrated that WWOX–RUNX2 axis could serve as prognostic biomarkers and therapeutic targets of lung cancers.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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