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ORIGINAL ARTICLE

Inhibition of miR-24 suppresses malignancy of human non-small cell lung cancer cells by targeting *WWOX* in vitro and in vivo

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Keywords
Apoptosis; caspase 3; miR-24; MMP9; *WWOX*.

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Abstract

Background: We investigated the effect of micro-RNA 24 (miR-24) and *WWOX* on non-small cell lung cancer (NSCLC) cell proliferation and migration in vitro and in vivo.

Methods: We performed bioinformatics analysis and 3′ untranslated region luciferase assay to investigate the direct target of miR-24. Proliferation, apoptosis, and transwell invasion assays were employed to evaluate the effect of *WWOX* overexpression with pcDNA3-WWOX and knocking down miR-24 with miR-24 small interfering RNA. Quantitative real-time PCR, Western blot, and immunohistochemistry were also used to investigate miR-24 and c-Kit expression, and apoptosis and invasion-related proteins. Finally, we constructed a tumor xenograft model in nude mice to confirm the effect of miR-24 on NSCLC cell proliferation in vivo.

Results: According to our experimental data, miR-24 inhibition could induce apoptosis by activating caspase 3 and suppress the viability and proliferation of NSCLC cells in vitro and in vivo. MiR-24 downregulation could reduce the invasive ability of NSCLC cells by downregulating MMP9. *WWOX* was identified as a functional target of miR-24. *WWOX* overexpression generated the same effect with antagonizing miR-24, while blocking *WWOX* counteracted the tumor suppressive effect caused by miR-24 inhibition. MiR-24 may function as an oncogene and play an important role in the cell growth and migration of NSCLC.

Conclusions: Our findings enhance understanding of the miR-24 regulatory network and the molecular mechanism that underlies the oncogenesis and development of NSCLC. Suppressing the effect of miR-24 on cancer cells using a miR-24 inhibitor may be an attractive therapeutic strategy against NSCLC.

Introduction

Eighty-percent of all lung cancer cases are of non-small cell histology. Non-small cell lung cancer (NSCLC) is the leading cause of cancer-related death worldwide. Only 17% of all patients diagnosed survive more than five years,[1] highlighting the extreme lethality of lung cancer. Therefore, understanding the molecular mechanism underlying NSCLC pathogenesis is crucial to management of the disease. Fortunately, important advances have been achieved in the treatment of advanced NSCLC with the introduction of new antiblastic and biological agents.

MicroRNAs (miRNAs) comprise a large family of regulatory RNAs that repress the expression of target messenger RNAs (mRNAs) in a sequence specific manner.[2] miRNAs play important roles in diverse biological processes, including development, differentiation, proliferation, and apoptosis.[3–5] Moreover, aberrant miRNA expression patterns have been found in human cancers, including lung cancer.[6–8] MiR-24 is generally upregulated during the differentiation of hematopoietic cell lines[9] in thymic development.[10] Because miR-24 is upregulated in diverse cell types during terminal differentiation, we intended to identify its function and the target genes it regulates in NSCLC.

The *WWOX* gene spans the FRA16D common chromosomal fragile site and encodes a member of the short-chain dehydrogenases/reductases (SDR) protein family. Expression of WWOX-encoded protein induces apoptosis, while defects in this gene are associated with multiple types of cancer. However, the role of *WWOX* in regulating NSCLC cell proliferation and motility has not yet been elucidated.

Apoptosis is a well-orchestrated and programmed cell death that occurs in multicellular organisms. Certain kinds of damage trigger a series of biochemical steps, leading to characteristic cell morphology and death.[11] It seems clear that the tight regulation of apoptotic function through miRNAs is critical to many cellular processes and the development of cancer. However, the relationship between miR-24 and NSCLC cell proliferation and apoptosis is not clear.

In this study, we performed a 3′ untranslated region (UTR) luciferase assay and observed that luciferase activity was increased after co-transfection of the miR-24 inhibitor and *WWOX* 3′UTR vector. MiR-24 binds directly to the 3′-UTR of *WWOX* to suppress gene expression. Inhibition of miR-24 induces apoptosis and suppresses the cell proliferation and migration ability of NCI-H358 and NCI-H1299 human NSCLC cells. Moreover, inhibition of miR-24 also suppresses the tumor growth of mice with severe combined immunodeficiency in a tumor xenograft model. *WWOX* overexpression showed the same effect with antagonizing miR-24. In summary, our findings suggest that miR-24 regulates the viability and migration of NSCLC cells via the direct targeting of *WWOX*. This may eventually provide practical information for the management of NSCLC.

Methods

Patient samples

Fifteen patients (7 men, 8 women; mean age 45.5 years), confirmed by pathological examination with NSCLC at the Sichuan Academy of Medical Sciences and Sichuan Provincial People's Hospital were enrolled in the study. Informed consent was obtained for the use of tissue samples. Three patients were at tumor node metastasis (TNM) stage I, four at stage II, six at stage III, and two at stage IV. TNM staging was performed according to American Joint Committee on Cancer (6th edition) criteria.

MicroRNA (miR) target prediction

We predicted the putative downstream messenger RNA (mRNA) target of miR-24 using the most frequently used prediction algorithms, TargetScan version 7.0 (Whitehead Institute for Biological Research, Cambridge, UK) and PicTar (Rajewsky Lab, New York, NY, USA).

Plasmid and oligonucleotides

MiR-24 inhibitor and *WWOX* small interfering RNA (siRNA) were commercially synthesized with antisense oligonucleotide (OriGene, Beijing, China). The 3′-UTR of the *WWOX* gene carrying the predicted miR-24 binding site was cloned by PCR. We inserted this fragment upstream of the reporter gene in the pGL3-basic/luciferase vector and tested the luciferase activity using the Dual-Luciferase Reporter Assay system (Promega, Madison, MI, USA), following the manufacturer's instructions. To construct a *WWOX* overexpression plasmid, we amplified the full-length human *WWOX* gene (without the 3′-UTR) using a complementary (DNA) clone as a template and inserted it into the pcDNA3 vector. The insertions were verified by DNA sequencing.

Cell culture and transfection

NCI-H358 cells were cultured in RPMI-1640 (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS) and 1000 U/ml penicillin/streptomycin (P/S). NCI-H1299 cells were grown in Dulbecco's modified Eagle medium supplemented with 10% FBS and 50 μg/mL kanamycin. The two human NSCLC cell lines were incubated in a humidified atmosphere at 37°C with 5% CO_2. Transfection was performed using a Lipofectamine 2000 Reagent (Invitrogen, Carlsbad, CA, USA), following the manufacturer's instructions.

RNA isolation and quantitative real-time PCR

RNA was extracted from cells using TRIzol (Invitrogen). In miRNA quantitation, complementary DNA was generated with the stem-loop reverse transcript primer and Moloney murine leukemia virus (M-MLV) reverse transcriptase (Promega) using 1 μg of small RNA as a template. To detect the *WWOX* level, complementary DNA was generated with oligo(dT) primers and M-MLV reverse transcriptase (Promega) using 4 μg of large RNA as a template. PCR amplification was performed using a SYBR Premix Ex *Taq* II (Perfect Real-Time) kit (Takara Bio, Shiga, Japan) and an ABI PRISM 7300 Sequence Detection system (Applied Biosystems, Foster City, CA, USA). U6 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were used as an endogenous control. The primers used were as follows: U6 forward 5′-GCTTCGGCAGCACATATACTAAAAT-3′; reverse 5′-CGCTTCACGAATTTGCGTGTCAT-3′; GAPDH forward 5′-CTCCTCCTGTTCGACAGTCAGC-3′; reverse

5′-CCCAATACGACCAAATCCGTT-3′; WWOX forward 5′-TCCTCAGAGTCCCATCGATTT-3′; reverse 5′-CGGCA GCAGTTGTTGAAGTA-3′.

Western blot

Cells were lysed and the protein was harvested 48 hours after transfection. Immunoblot assays were performed using antibodies against WWOX, MMP-9, and caspase 3, as well as GAPDH. All antibodies were purchased from Beijing Bioss Biotechnology, Inc. (Beijing, China). LabWorks image acquisition and analysis software (UVP, LLC; Analytik Jena AG, Upland, CA, USA) was used to acquire images of bands of interest and to quantify protein intensities.

Proliferation assay

To evaluate the viability of NSCLC cells, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide (MTT) assay was performed. Ten microliters of MTT (0.5%) was added into the culture solution at 24, 48, and 72 hours after transfection. The absorbance at 570 nm was measured using the μQuant universal microplate spectrophotometer (Bio-Tek Instruments, Winooski, VT, USA).

Apoptosis assay

NCI-H358 and NCI-H1299 cells were transfected with synthesized oligonucleotides or control vectors. After an additional incubation of 36 hours, the cells were harvested, stained with 7-Aminoactinomycin D and phycoerythrin-labeled anti-annexin-V antibody, and analyzed by fluorescence-activated cell sorting (FACS).

Transwell invasion assay

Transwell invasion assay was conducted by planting 5×10^4 NCI-H358 cells coated with 40 μl of Matrigel diluted to 4 μg/μL with RPMI-1640 medium or 9×10^4 NCI-H1299 cells with 1 μg/μL of Dulbecco's modified Eagle medium on the upper chamber of each insert (Corning, Cambridge, MA, USA); 800 μL of medium supplemented with 20% FBS was added to the lower chambers. Cells attached to the lower surface were stained for 25 minutes with crystal violet and then photographed for counting.

Tumor xenograft model in nude mice

NCI-H358 cells were suspended in 150 μL of serum-free RPMI 1640 and subcutaneously injected into the flank of five-week-old female nude mice. The mice with severe combined immunodeficiency were randomly divided into two groups of seven. The two groups were treated with miR-24 inhibitor or scrambled oligo through local injection of the xenograft tumor at multiple sites every five days for 25 days. Tumor size was measured every three days after 10 days of injections. The tumor volume was calculated as follows: length × width^2 × 1/2. All experiments were performed according to American Association for the Accreditation of Laboratory Animal Care guidelines for human treatment of animals.

Immunohistochemistry

The sections were pretreated with microwave irradiation, blocked, and incubated using polyclonal rabbit anti-human c-Kit (Beijing Bioss Biotechnology Inc.). Staining intensity was assessed.

Statistical analysis

The results were reported as the mean ± standard deviation of at least three independent experiments. The data was processed using GraphPad Prism 5 software (GraphPad Software, Inc., San Diego, CA, USA) with the two-tailed Student's *t*-test. A P value of < 0.05 was considered significant.

Results

Identification of *WWOX* as a direct target of miR-24

To identify the potential downstream molecules regulated by miR-24, we used TargetScan and PicTar to predict the target genes. *WWOX* was chosen for further study. Base-pairing complementation revealed that the 3′ UTR of *WWOX* had significant complementarity with miR-24 (Fig 1a), and the species conservation information of the interaction of miR-24 and *WWOX* were predicted by TargetScan software (Fig 1b). We then examined the expression of miR-24 and *WWOX* in 15 pairs of human NSCLC tissue by quantitative real-time PCR. Spearman's rank correlation analysis revealed a negative correlation between miR-24 and *WWOX* expression (Fig 1c). To further demonstrate that *WWOX* was directly targeted by miR-24 in NSCLC cells, dual-luciferase reporter assay was performed to observe the interaction between miR-24 and 3′-UTR of *WWOX* mRNA. The miR-24 inhibitor obviously reduced the miR-24 level (Fig 1e) and elevated WWOX luciferase activity (Fig 1d) and protein levels (Fig 1f) in NCI-H358 and NCI-H1299 cells. These results revealed that miR-24

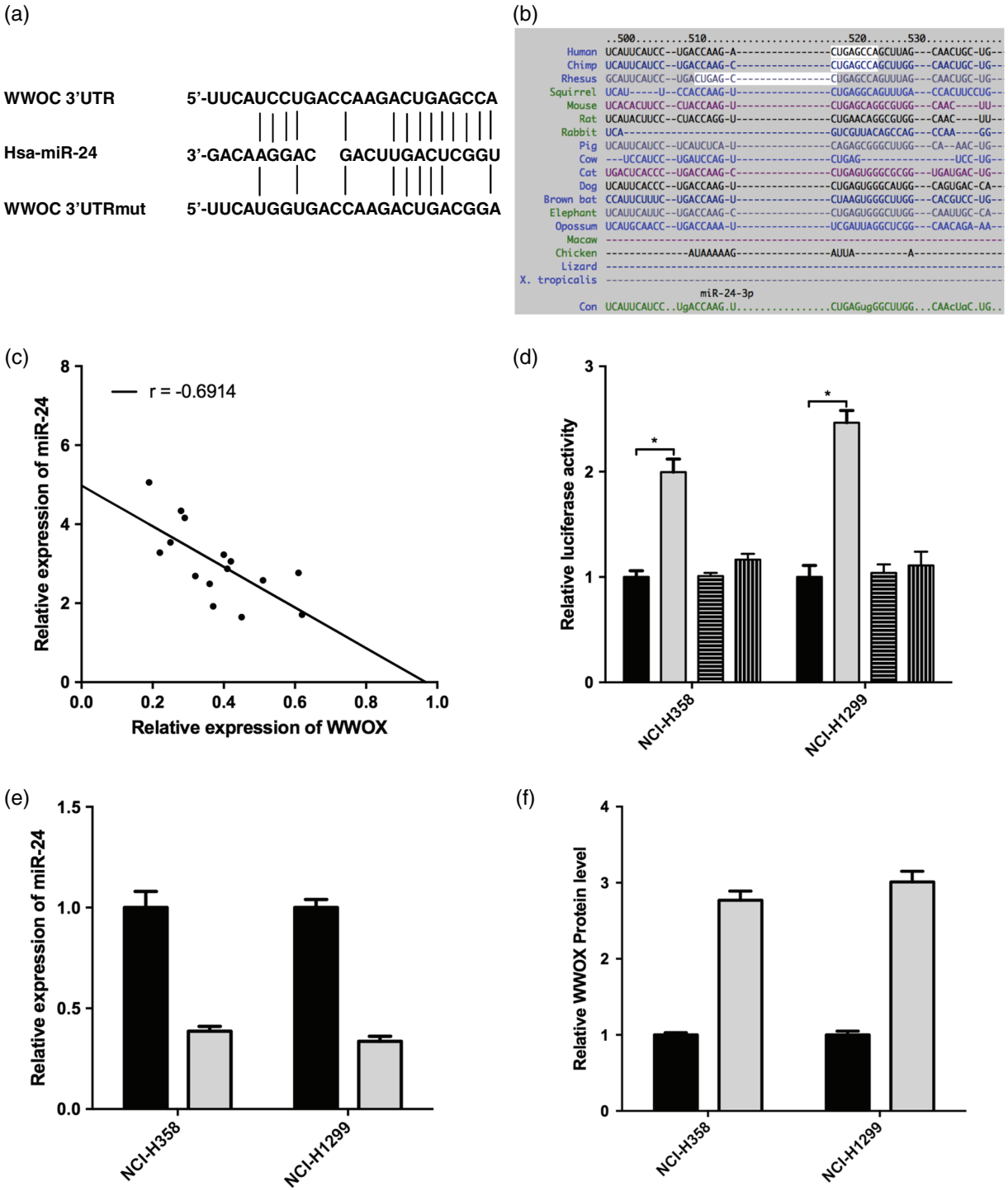

Figure 1 MiR-24 downregulates *WWOX* by binding to its 3′ untranslated region (UTR). (**a**) Predicted miR-24 binding sites in the 3′UTR of *WWOX*. (**b**) Species conservation information of the interaction between miR-24 and *WWOX* was predicted using TargetScan software. (**c**) The correlation between miR-24 and *WWOX* expression. MiR-24 and *WWOX* messenger RNA (mRNA) levels in 15 pairs of human non-small cell lung cancer (NSCLC) tissues were measured by quantitative real-time PCR (qRT-PCR); U6 and β- Actin were used for normalization. (**d**) Luciferase reporter assays in NSCLC cells, following co-transfection with the *WWOX* 3′UTR and miR-24 inhibitor. The data shows fold changes (mean ± standard deviation) of three independent experiments. (■) Ctrl+WWOX-3′UTR, (□) miR-24 inhibitor+WWOX-3′UTR, (▤) Ctrl+WWOX-3′UTRmut, and (▥) miR-24 inhibitor +WWOX-3′UTRmut. (**e**) The miR-24 mRNA level after treatment with the miR-24 inhibitor, by qRT-PCR. (**f**) *WWOX* protein level after treatment with the miR-24 inhibitor, by Western blot. (■) Ctrl and (□) miR-24 inhibitor. *$P < 0.05$.

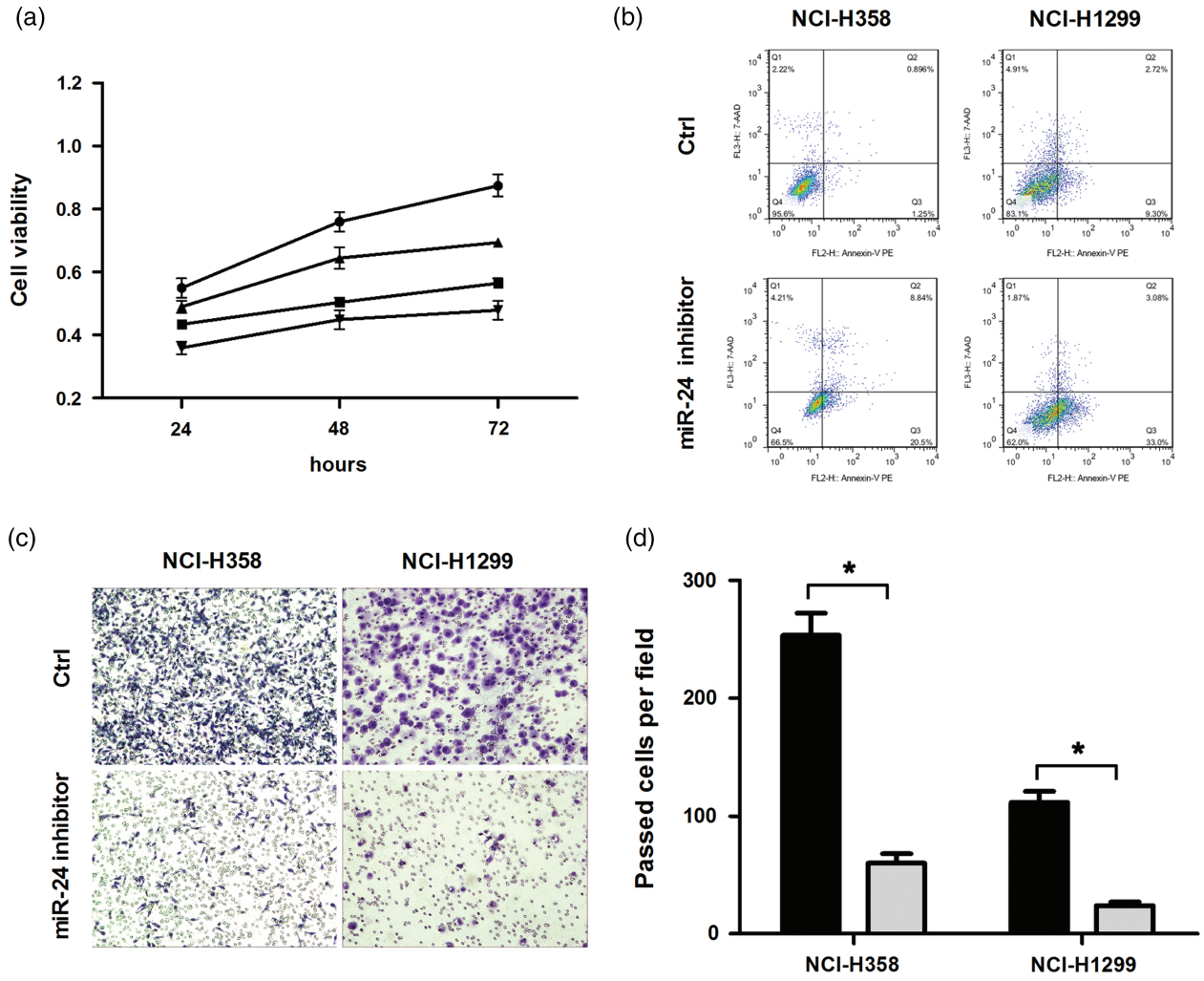

Figure 2 Downregulation of miR-24 induces apoptosis and suppresses the proliferation and invasion of non-small cell lung cancer (NSCLC) cells. (**a**) A cell viability assay. Absorbance at 570 nm was measured at 24, 48, and 72 hours after transfection. (–●–) NCI-H358+Ctrl, (–■–) NCI-H358+miR-24 inhibitor, (–▲–) NCI-H1299+Ctrl, and (–▼–) NCI-H1299+miR-24 inhibitor. (**b**) Apoptosis was measured by fluorescence-activated cell sorting with 7-AAD and phycoerythrin staining. (**c**) Transwell invasion assay of NSCLC cells (a representative image is shown). (**d**) Cells in three random fields of view at 100x magnification were counted and expressed as the average number of cells per field. (■) Ctrl and (□) miR-24 inhibitor. *$P < 0.05$.

binds directly to the 3′ UTR of *WWOX* to suppress gene expression.

Downregulation of miR-24 suppresses the proliferation and invasion of non-small cell lung cancer (NSCLC) cells in vitro

To determine the effects of miR-24 on the malignant behavior of NSCLC cells, miR-24 inhibitor was transfected into NCI-H358 and NCI-H1299 cells. The viability decreased in cells that had low levels of miR-24 compared to control cells, as observed in MTT assay (Fig 2a). To further investigate cell viability suppression caused by the miR-24 inhibitor, we performed FACS to detect the impact of miR-24 on NSCLC cell apoptosis. The miR-24 inhibitor significantly increased apoptosis in NCI-H358 and NCI-H1299 cells compared to the control (Fig 2b). Transwell invasion assay revealed that the passing of DNA in cells transfected with the miR-24 inhibitor decreased compared to the control (Fig 2c,d). The two NSCLC cell lines showed similar results. The data indicated that downregulation of miR-24 suppressed NSCLC cell viability and invasion in vitro.

WWOX could induce apoptosis and suppress invasion of NSCLC cells

To investigate the function of *WWOX* on the phenotype of NSCLC cells, we constructed a *WWOX* overexpression vector. After the efficiency of the vector was confirmed by Western blot (Fig 3a), we transfected it into the two NSCLC cell lines

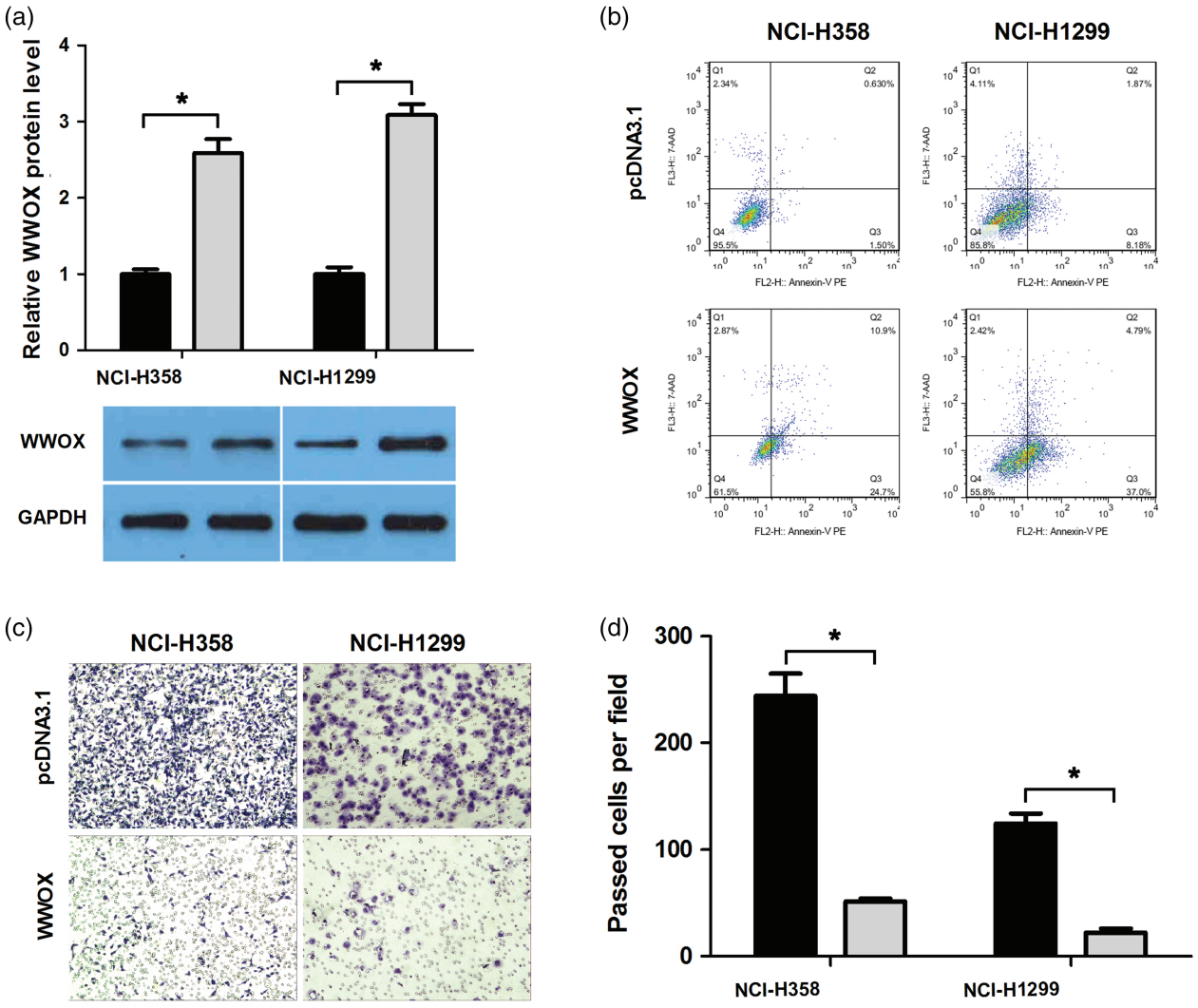

Figure 3 *WWOX* overexpression inhibits the malignant phenotype of non-small cell lung cancer (NSCLC) cells. (**a**) The efficiency of the *WWOX* overexpression plasmid was measured by Western blot. (**b**) Apoptosis assay in NCI-H358 and NCI-H1299 cells by fluorescence-activated cell sorting. (**c**) Transwell invasion assay of NSCLC cells (a representative image is shown). (**d**) Cells in three random fields of view at 100x magnification were counted and represented as the average number of cells per field. **P* < 0.05. (■) pcDNA3.1 and (□) WWOX. GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

and found that apoptosis was increased in the *WWOX* overexpression group compared to the control (Fig 3b). Meanwhile, the number of passed NSCLC cells was decreased by *WWOX* expression, as observed in transwell invasion assay (Fig 3c,d). These results revealed that *WWOX* could induce NSCLC cell apoptosis and inhibit invasion in vitro.

Knockdown of *WWOX* restored the influence of the miR-24 inhibitor on apoptosis and invasion

To confirm that the function of miR-24 is mediated by repression of *WWOX* and not by the targeting of other cellular molecules, a rescue experiment was performed. *WWOX* siRNA and miR-24 inhibitor were co-transfected into NCI-H358 and NCI-H1299 cells. The miR-24 inhibitor group expressed a higher level, while the co-transfection group expressed a lower level of *WWOX* by Western blot (Fig 4a). *WWOX* siRNA counteracted the effect of the miR-24 inhibitor on *WWOX* expression. As is shown in the FACS and transwell invasion assay, *WWOX* siRNA also counteracted the effects of the miR-24 inhibitor on apoptosis (Fig 4b) and cell invasion (Fig 4c,d). These results demonstrated the mechanism of miR-24, at least partially, is to targeting *WWOX* in NSCLC.

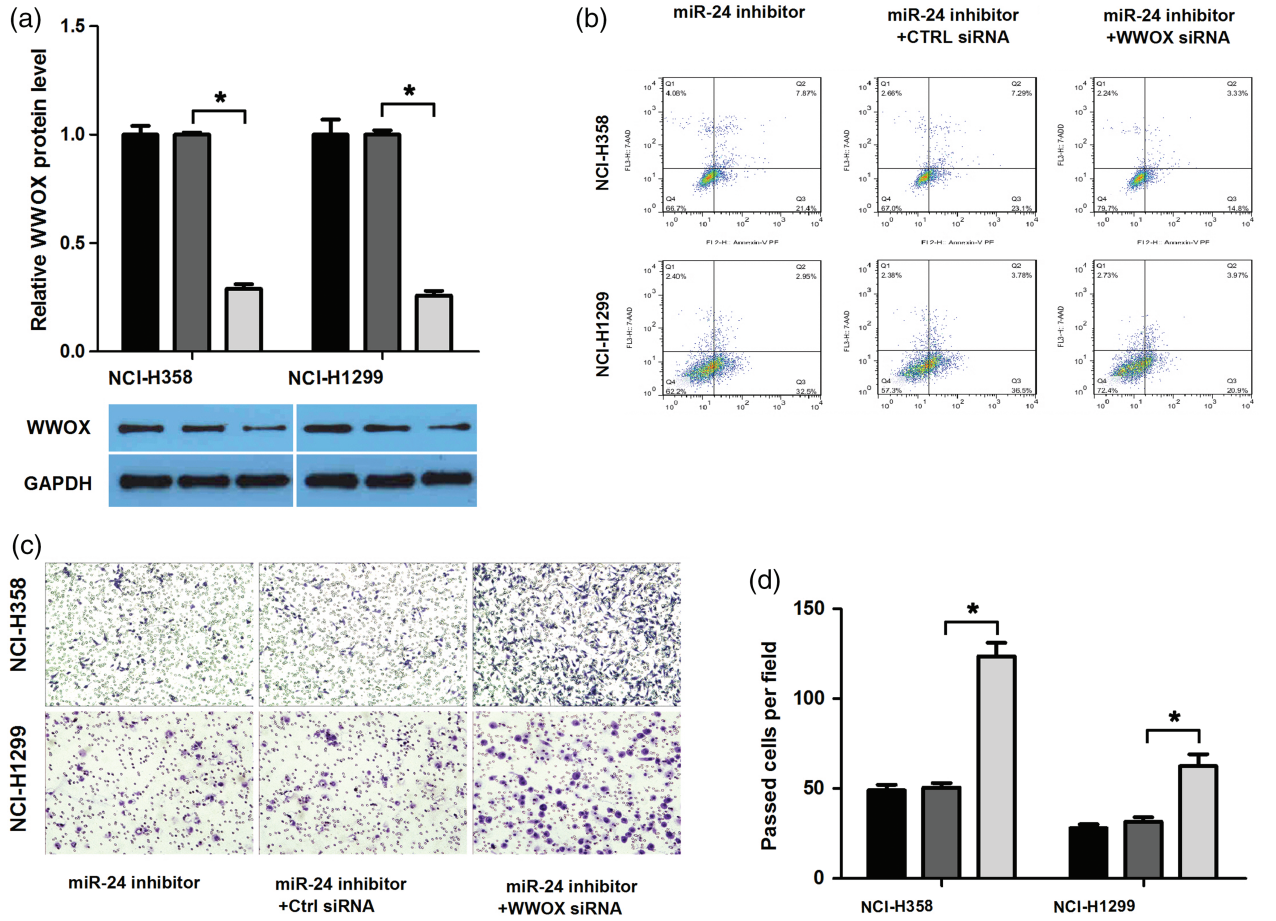

Figure 4 *WWOX* knockdown restored the influence of the miR-24 inhibitor on apoptosis and invasion. (**a**) The miR-24 inhibitor was transfected with or without *WWOX* small interfering RNA (siRNA). The *WWOX* protein level was tested by Western blot in a rescue experiment. (**b**) Apoptosis assay by fluorescence-activated cell sorting. (**c**,**d**) Transwell invasion assay in NCI-H358 and NCI-H1299 cells. Error bars indicate the mean ± standard deviation of three independent experiments, $*P < 0.05$. (■) miR-24 inhibitor, (■) miR-24 inhibitor+Ctrl siRNA, and (□) miR-24 inhibitor+WWOX siRNA.

miR-24 and *WWOX* influence caspase 3 and MMP9 expression in NSCLC cells

In order to explore the mechanisms underlying miR-24 and *WWOX* regulation of apoptosis and invasion of NSCLC cells, we detected the manner of expression of apoptosis and invasion-related proteins (caspase 3 and MMP9). According to Western blot results, the protein level of activated caspase 3 increased when miR-24 expression was inhibited (Fig 5a) or when *WWOX* expression was ectopic (Fig 5b) in NCI-H358 and NCI-H1299 cells. However, MMP9 protein levels were reduced in the same experiment (Fig 5a,b). *WWOX* siRNA counteracted the effect of the miR-24 inhibitor on caspase 3 and MMP9 expression (Fig 5c). These results suggested that miR-24 downregulation induces apoptosis, possibly by activating caspase 3, and suppresses invasion by inhibiting MMP9.

miR-24 downregulation suppresses NSCLC cell proliferation in vivo

To further confirm the effect of miR-24 on NSCLC cell proliferation in vivo, we constructed a tumor xenograft model in nude mice. Mice were treated with miR-24 inhibitor or scrambled oligo through local injection of the xenograft tumor; the tumor nodules are shown in Figure 6a. The tumor volumes were smaller in the miR-24 inhibitor group than in the control (Fig 6b). To verify that the decrease in the tumor volume of xenograft nodules was caused by miR-24 downregulation, we tested the levels of miR-24 and *WWOX* in the xenograft tumors. The miR-24 mRNA levels were lower and the WWOX protein levels higher in the miR-24 inhibitor group than in the control (Fig 6c,d). Immunohistochemical assay showed that c-Kit (a well-known oncogene) expression was lower in the

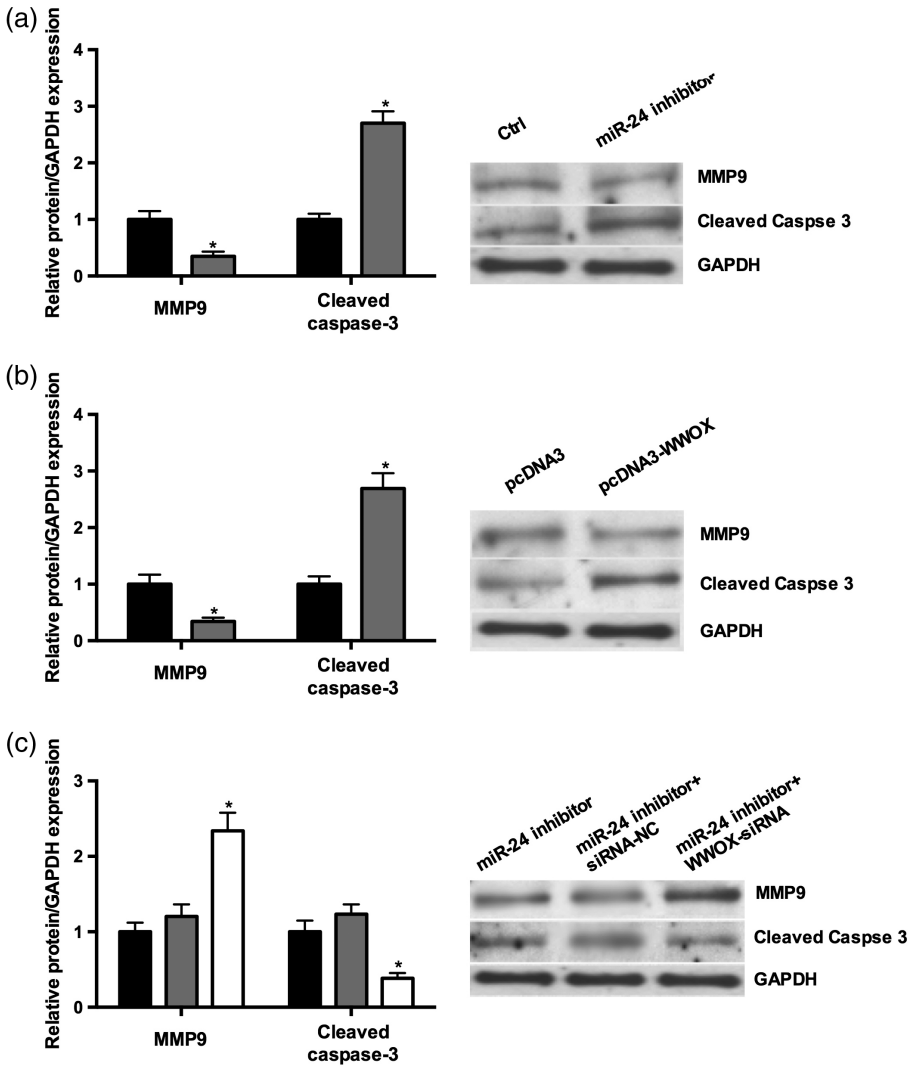

Figure 5 Influence of miR-24 and *WWOX* on caspase 3 and MMP9. (**a**) Regulation of the miR-24 inhibitor on caspase 3 and MMP9 by Western blot. (■) Ctrl and (▩) miR-24 inhibitor. (**b**) The influence of *WWOX* on caspase 3 and MMP9. (■) pcDNA3 and (▩) pcDNA3-WWOX. (**c**) *WWOX* small interfering RNA (siRNA) restored the effect of the miR-24 inhibitor on caspase 3 and MMP9. (■) miR-24 inhibitor, (▩) miR-24 inhibitor+siRNA-NC and (□) miR-24 inhibitor+WWOX-siRNA. Error bars indicate the mean ± standard deviation of three independent experiments, $*P < 0.05$. GAPDH, glyceraldehyde 3-phosphate dehydrogenase; NC, negative control.

miR-24 inhibitor group (Fig 6e), confirming that the miR-24 inhibitor has a tumor suppressive function. The result revealed that downregulation of miR-24 suppresses NSCLC cell proliferation in vivo.

Discussion

Only one third of the patients diagnosed with NSCLC are eligible for surgery[12] and post-surgical survival is limited. Emerging evidence suggests that the deregulation of miRNAs and their target genes constitutes a complex interacting network that controls cancer progression, including NSCLC. For example, miR-130a targets MET and induces TRAIL-sensitivity in NSCLC by downregulating miR-221 and 222,[13] and miR-195 suppresses NSCLC by targeting CHEK1.[14] Other miRNAs related to NSCLC include miR-29b,[15] miR-582-3p,[16] miR-326[17] and miR-205.[18]

We focused on miR-24, which has been reported as an oncogene in many cancers, including gastric,[19] pancreatic,[20] and breast cancers.[21,22] However, the role of miR-24 in NSCLC has not previously been well illustrated. Based on our experimental data, low expression of miR-24 induced apoptosis and inhibited cell proliferation and migration in NSCLC cells by directly targeting *WWOX*, indicating the oncogenic property of miR-24 in NSCLC. Meanwhile, Zhao *et al.* found that a high level of miR-24 promotes cell proliferation by targeting NAIF1 in NSCLC,[23] which is in accordance with our observation. One miRNA may regulate many target genes, and one gene may be targeted by many miRNAs. To determine which downstream targets are also regulated by miR-24 requires further study. Researchers have discovered that miR-24 functions as a tumor suppressor in nasopharyngeal carcinoma and prostate cancer,[24,25] which does not match our observation. One possible explanation is that miRNA expression

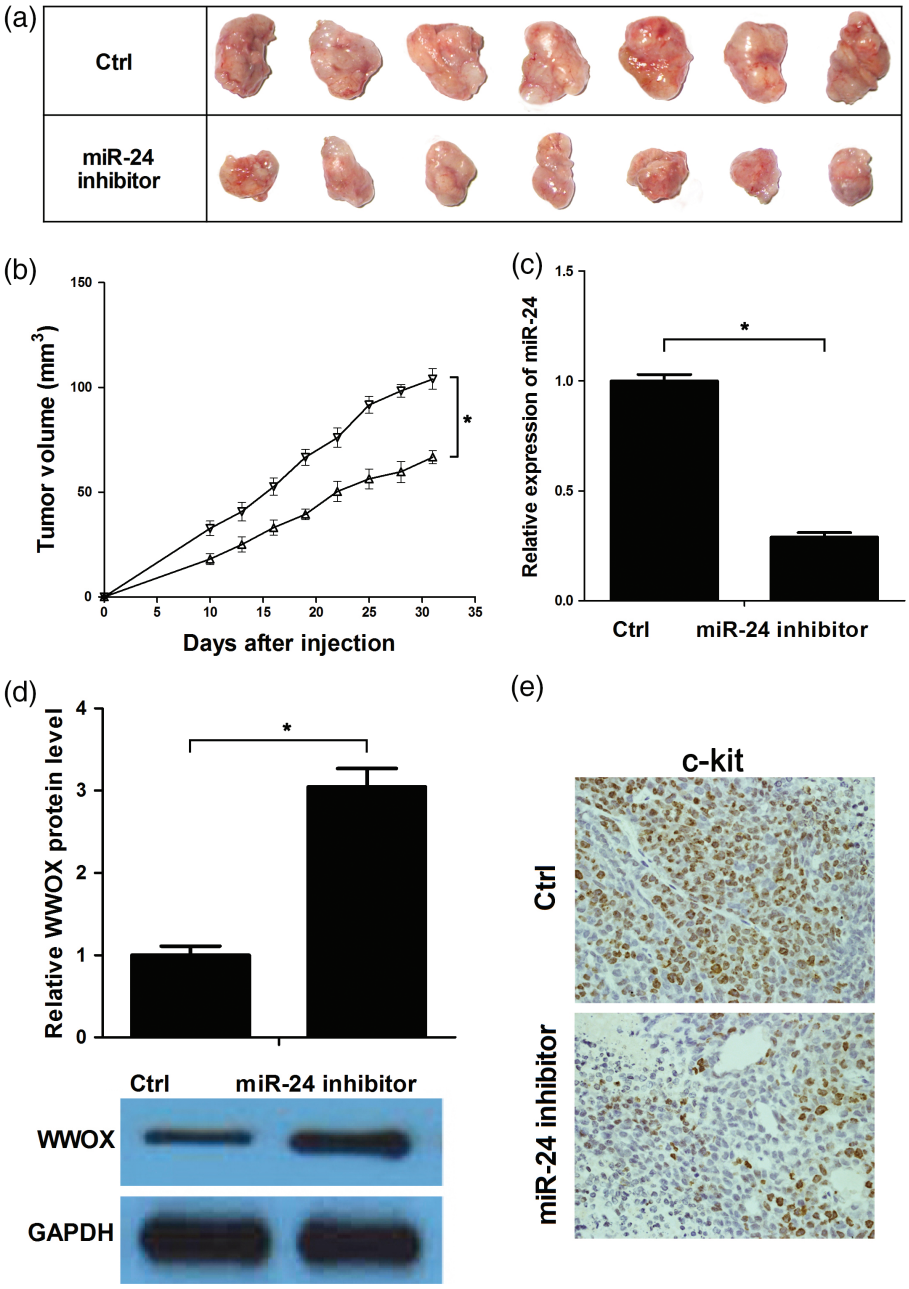

Figure 6 MiR-24 facilitates non-small cell lung cancer (NSCLC) cell growth in a tumor xenograft model. (**a**) An equal number of NCI-H358 cells was injected into two groups of nude mice treated with miR-24 inhibitor or scrambled oligo through local injection of the xenograft tumor every five days, seven days after the injection. The image shows the tumor xenograft. (**b**) Tumor size was measured every three days, after seven days of injections. The tumor volume was calculated as follows: length × width^2 × 1/2. (—▽—) Ctrl and (—△—) miR-24 inhibitor. (**c**) miR-24 and (**d**) *WWOX* expression in the xenograft tumor. (**e**) c-Kit expression in the xenograft tumor by immunohistochemistry. Error bars indicate the mean ± standard deviation of three independent experiments, **P* < 0.05. GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

depends on the cellular context, and the function of miRNA is tumor-type specific.[26]

WWOX, generally known as a tumor suppressor gene,[27,28] is located at a common fragile region, FRA16D,[29] and is reported to be involved in several tumor types, including ovary, breast, and esophagus tumors.[30–32] Sai *et al.* demonstrated that the *WWOX* gene is altered by deletion and/or aberrant expression in a significant number of lung cancers and may contribute to the pathogenesis of NSCLC.[33] Baykara *et al.* reported that hypermethylation of the *WWOX* gene promoter and mutations in this gene may be related to lung carcinogenesis. By contrast, our results showed that miR-24 binds directly to the *WWOX* mRNA to suppress its expression.[34] Thus, *WWOX* may be involved in the oncogenesis and development of NSCLC at both transcriptional and post-transcriptional levels.

Based on our observations, miR-24 inhibitor-mediated apoptosis suppresses NSCLC cell proliferation. To explore the intermediate signaling molecule, we examined the level of caspase 3 expression, a member of the cysteine-aspartic acid protease family. As expected, the protein level of activated caspase 3 increased when miR-24 expression was

inhibited, indicating the important role of caspase 3 in cell apoptosis, caused by the miR-24 inhibitor in NSCLC cells. However, whether other members of caspase family also participate in miR-24-related apoptosis requires further investigation. Proteins of the matrix metalloproteinase (MMP) family are involved in extracellular matrix breakdown in normal physiological processes, such as embryonic development, reproduction, and tissue remodeling, as well as in disease processes, such as arthritis and metastasis. Surprisingly, we discovered that MMP9 is an important intermediator in the miR-24-related migration process of NSCLC cells. The miR-24 inhibitor suppresses NSCLC cell migration by downregulating MMP9.

Collectively, the results of our study provide evidence of the coordination of miR-24 and *WWOX* in the regulation of cell apoptosis, proliferation, and migration during NSCLC development. Inhibition of miR-24 expression may serve as an alternative treatment modality for NSCLC.

Disclosure

No authors report any conflict of interest.

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