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Original Paper

FOXD1 Promotes Cell Growth and Metastasis by Activation of Vimentin in NSCLC

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Key Words
NSCLC • FOXD1 • Vimentin • Cell migration • Cell invasion

Abstract:
Background/Aims: Forkhead box D1 (FOXD1) has a well-established role in early embryonic development and organogenesis and functions as an oncogene in several cancers. However, the clinical significance and biological roles of FOXD1 in non-small cell lung cancer (NSCLC) remain largely unknown. ***Methods:*** A total of 264 primary NSCLC tissue samples were collected. The expression levels of FOXD1 in these samples were examined by immunohistochemical staining. The expression of FOXD1 was knocked down by lentiviral shRNA. The relative expression of FOXD1 was determined by qRT-PCR, Western blotting and immunofluorescence image. The functional roles of FOXD1 in NSCLC were demonstrated cell viability CCK-8 assay, colony formation, cell invasion and migration assays, and cell apoptosis assay *in vitro*. *In vivo* mouse xenograft and metastasis models were used to assess tumorigenicity and metastatic ability. The Chi-square test was used to assess the correlation between FOXD1 expression and the clinicopathological characteristics. Survival curves were estimated by Kaplan-Meier method and compared using the log-rank test. The Cox proportional hazards model was used for univariate and multivariate analyses. ***Results:*** We determined that higher levels of FOXD1 were present in NSCLC tissues, especially in metastatic NSCLC tissues. FOXD1 was also higher in all NSCLC cells compared with normal human bronchial epithelial cells. A higher expression level of FOXD1 was associated with malignant behavior and poor prognosis in NSCLC patients. Knockdown of FOXD1 significantly inhibited proliferation, migration, and invasion *in vitro* and tumor growth and metastasis *in vivo*, and it increased the apoptosis rates of NSCLC cells. Mechanistic analyses revealed that FOXD1 expressed its oncogenic characteristics through activating Vimentin in NSCLC. Multivariate Cox regression analysis indicated that FOXD1 was an independent prognostic factor both for overall survival (OS) and disease-free survival (DFS)

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in NSCLC patients. ***Conclusion:*** Our results indicated that FOXD1 might be involved in the development and progression of NSCLC as an oncogene, and thereby might be a potential therapeutic target for NSCLC patients.

Introduction

Lung cancer is the leading cause of cancer deaths worldwide [1] and has become the second leading cause of death in China, with an increased incidence of 465% over the past 30 years [1, 2]. Non-small cell lung cancer (NSCLC) accounts for over 80% of all lung cancer cases, and approximately 70% of NSCLC patients are diagnosed with advanced disease [1, 3]. Although surgical resection and adjuvant therapy have improved in recent years, the 5-year survival rate of NSCLC remains poor due to the lack of obvious symptoms for early diagnosis, recurrence and metastasis [3, 4]. Hence, a better understanding of the molecular mechanisms of NSCLC development and progression will facilitate the development of new targets for therapy.

FOXD1, a member of the forkhead family, is primarily expressed in human embryonic tissues, where it regulates organogenesis [5]. Furthermore, FOXD1 was found to be a mediator and indicator of cell reprogramming by mediating self-renewal and differentiation [6, 7]. Increasing evidence has indicated that cell reprogramming is associated with tumor initiation and progress [8]. For example, FOXD1 was found to be increased in prostate cancer tissues and associated with lymph node metastasis [9]. Another study has shown that FOXD1 promoted cell proliferation and chemotherapy resistance by inducing the G1 to S phase transition in breast cancer [10]. FOXD1 was reported to be significantly upregulated in glioma samples, and further investigations suggested that FOXD1 directly regulated ALDH1A3 transcription to activate clonogenic and tumorigenic potential of mesenchymal stem-like cells of glioma [8]. These findings strongly indicated that FOXD1 enrichment in tumor cells might affect biological functions, including cell proliferation, apoptosis, invasion, and metastasis. Interestingly, FOXD1 was also reported to be overexpressed in human NSCLC, and patients with high FOXD1 expression survived for a significantly shorter time than those with low FOXD1 expression [11]. However, the exact roles and molecular mechanisms of FOXD1 contributing to NSCLC progress have remained largely unknown.

In our present study, we first confirmed that the expression of FOXD1 was significantly increased in NSCLC tissues. Both *in vitro* and *in vivo* assays showed that short hairpin RNA (shRNA)-mediated FOXD1 knockdown inhibited NSCLC cell proliferation, invasion, and metastasis. Mechanistic studies showed that FOXD1 promoted NSCLC cell invasive potential by upregulating the expression of Vimentin. Our results provide an important mechanism of FOXD1 regulation of cell proliferation and invasion during NSCLC progression.

Materials and Methods

Patients and samples

A total of 264 primary NSCLC cases who underwent surgical resection of NSCLC confirmed by pathological evaluation were collected at the Department of Respiration of Shanghai 10th People's Hospital affiliated with Tongji University in Shanghai, China. Paired NSCLC tissue and adjacent normal lung tissue samples were obtained from these 264 patients between Jan 2007 and Dec 2010. None of the patients had received adjuvant chemotherapy, radiation or any other treatment before resection, and all patients were followed-up. All tumor tissues were classified according to the 2010 lung cancer staging system of the AJCC [12]. Written informed consent was obtained from all patients. The use of these tissue specimens was approved by the ethics committee of Shanghai 10th People's Hospital and conducted in adherence with the Declaration of Helsinki protocols. Specimens were frozen in liquid nitrogen and stored at −80 °C before use.

Immunohistochemical analysis

Immunohistochemical (IHC) staining was performed using the Dako Envision system (Dako, Carpinteria, CA, USA) according to the manufacturer's instructions. In short, paraffin-embedded tissues were cut into 3-5 μm sections for dewaxing and rehydration. The sections were microwaved in 0.1 M citrate buffer (pH=6.0) and incubated in 3% H_2O_2 at room temperature for 30 min followed by incubation with 10% goat serum at room temperature for 30 min. Primary antibody (anti-FOXD1 rabbit polyclonal antibody, 1:40, Proteintech, Chicago, IL, USA; mouse anti-vimentin, 1:400, Zymed Laboratories, USA) was added and the sections were incubated overnight at 4 °C. Then, biotin-labeled secondary antibody (CW Bio，Beijing, China) was added and the sections were incubated at room temperature for 30 min. Next, the sections were immersed in freshly prepared 3, 3'-diaminobenzidine (DAB) for color development for 5 min. The sections were then thoroughly rinsed with water and stained with hematoxylin. Five high-power fields (400×) were randomly selected for each specimen slide. When the cellular membrane and/or cytoplasm were stained brown, the cells were positive.

Cell culture

Five NSCLC cell lines, A549, 95-C, 95-D, NCI-H460, and NCI-H1299, and a normal human bronchial epithelial cell line (16HBE) were obtained from the Institute of Biochemistry and Cell Biology of the Chinese Academy of Sciences (Shanghai, China). The cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) (Invitrogen, Carlsbad, USA), 100 μg/ml streptomycin and 100 units/ml penicillin (HyClone, Logan, USA) in a humidified atmosphere with 5% CO_2 at 37 °C.

Cell transfection and lentivirus production

The lentiviral shRNA constructs for FOXD1 knockdown were purchased from Sigma-Aldrich (St. Louis, MO, USA), the sequences of the FOXD1 shRNA was 5'-CCGGTTGTTAATAACGCTATGTTAGCTCGAGCTAACATAGCGTTATTAACAATTTTTG-3', while the sequences of specificity control for the shRNA was 5'-CCGGAATGCCTACGTTAAGCTATACCTCGAGGTATAGCTTAACGTAGGCATTTTTTTG-3'. The lentivirus of shFOXD1 was generated by co-transfecting HEK293T with the pPACKH1 Lentivector Packaging KIT (Sigma-Aldrich). To generate the stable cell line, cells were transfected with lentiviruses for 24 h and replaced with complete culture medium. The Vimentin-siRNA (siVimentin) and negative control siRNA were all purchased from Santa-Cruz Biotechnology (Dallas, TX, USA). Approximately 1×10^5 cells were plated in 6-well dishes before transfection and grown without antibiotics. Cells were transfected using Lipofectamine® RNAiMAX reagent (Invitrogen) according to the protocol. Cells were analyzed by Western blot analysis 24-48 h after transfection.

RNA extraction and quantitative real-time PCR

Total RNA was extracted from cells using TRIzol reagent (Invitrogen) according to the manufacturer's protocol. Reverse transcription was performed with the Superscript first-strand synthesis system (Invitrogen, Carlsbad, USA) according to the manufacturer's instructions. The following primers used for real-time PCR were as follows: FOXD1 forward, 5'-AAGAACCCGCTGGTGAAG-3' and reverse, 5'-GTCCAGTAGTTGCCCTTGC-3'; and GAPDH forward, 5'-ACAACTTTGGTATCGTGGAAGG-3' and reverse, 5'-GCC ATCACGCCACAGTTTC-3'. The relative expression of FOXD1 mRNA was normalized to the expression level of GAPDH mRNA. qRT-PCR was performed on an ABI 7900HT instrument (Applied Biosystems, USA). All of the determinations were performed in triplicate and all experiments were carried out at least three independent times.

Cell viability, colony formation and apoptosis assays

Cell viability was measured using the Cell Counting Kit 8 (CCK8, Dojindo, Japan) according to the manufacturer's instructions. In brief, cells were seeded in 96-well plates at a density of 1×10^3 cells/well in 100 μl of medium. Then, 100 μl of CCK-8 was added to the culture at 1, 2, 3 and 4 days. The optical density (OD) was measured at 450 nm in a spectrophotometer (Spectra Max Plus, Molecular Devices, Sunnyvale, USA).

Cells were collected in the logarithmic phase of growth and seeded into 6-well plates at a density of 800 cells/ well for 14 days. The medium was refreshed with medium containing 10% FBS once every 3 days. The cells were then fixed with 4% paraformaldehyde for 30 min and stained with crystal violet (Shanghai Jingtian Biotech Co., Ltd., JT-00518) for 20 min. Representative photographs were captured, and colonies with more than 40 cells were counted.

Apoptotic cells were examined using an Annexin V-PE Apoptosis Detection Kit (Millipore, USA). The samples were harvested and then stained with 5 μl of Annexin V-PE and 5 μl of PI in the dark for 15 min. Cellular apoptosis was detected by flow cytometry and the results were analyzed using the Cell-QuestTM Pro software (BD Biosciences, MA, USA).

Migration and invasion assays

Cells (3×10^5/well) were suspended in 100 μl of serum-free DMEM and added to the upper chamber of an insert (8 μm pore size, Millipore, USA) coated with or without Matrigel (BD) for the transwell migration and invasion assay. Then, 600 μl DMEM with 10% FBS was added to the lower chamber. Cells were fixed and stained with 0.05% crystal violet after 36 h of incubation at 37 °C for the migration and invasion assays. The stained cells were counted in 6 random fields of each chamber under a microscope (×100 magnification) and photographed using an IX71 inverted microscope (Olympus, Tokyo, Japan) at 200× magnification.

Immunofluorescence

For immunofluorescence microscopy, the cells treated with shFOXD1 or SCR were cultured on cover slips and fixed with 4% (vol/vol) paraformaldehyde (Sigma-Aldrich) for 15 min and then permeabilized in 0.1% Triton X-100 (Sigma-Aldrich). The cells were washed with a 2% glycine solution and blocked with PBS containing 2% FBS and 0.5% saponin for 30 min at 37°C The primary antibody against vimentin (1:100, CST, Danvers, MA, USA) was added and incubated overnight at 4°C, followed by incubation with Alexa Flour 488-conjugated secondary antibodies (1:1000, Sigma, St. Louis, MO, USA) for 1 h. DAPI (Sigma, St. Louis, MO, USA) was used to counterstain DNA. Immunofluorescence images for Vimentin were viewed with confocal laser-scanning microscopy (Fluoview FV1000, Olympus, Japan).

Western blot analysis

The cultured cells were harvested and lysed with RIPA lysis buffer (Promega) for 30 min at 4 °C. A total of 60 μg of total protein was loaded into 10% SDS-PAGE and transferred electrophoretically onto a PVDF membrane (Millipore, Germany). The membranes were blocked with 1% bovine serum albumin (BSA) in TBST buffer (Tris Buffer Saline containing 0.1% Tween-20) for 1 h at room temperature and subsequently incubated with primary antibodies against FOXD1 (Proteintech) or E-cadherin, N-cadherin, Vimentin (Santa Cruz Biotechnology Inc. Dallas, TX, USA) or MMP-9, MMP-2, GAPDH (Cell Signaling Technology, USA) overnight at 4°C. After extensive washing with TBST buffer, the blots were then probed with horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. Protein bands were detected by the enhanced chemiluminescence reagent ECL (Millipore, MA, USA) and the intensity of the bands was analyzed using ImageJ software (National Institutes of Health, USA).

In vivo tumor formation and metastasis assays in nude mice xenograft model

Six-week-old male nude BALB/c mice (4-6 weeks old; body weight, 18–22 g) were purchased from the Shanghai Laboratory Animal Center of the Chinese Academy of Sciences (Shanghai, China). Nude mice were injected subcutaneously into the left flanks with NCI-H460 cells (1×10^7) infected with lentiviral constructs carrying either the shFOXD1 vector or the control vector. Two perpendicular diameters (a: the largest; b: the smallest) of the tumor were measured with a caliper for 5 weeks monitoring. Tumor volume (V) was calculated using the following formula: $V = a\times b^2\times0.5$. Tumor weights were also measured. All the mice were euthanized 5 weeks later. Mouse xenograft tumor tissues were harvested and tissue sections were then subjected to IHC staining with antibodies against FOXD1 and ki-67 (Sigma-Aldrich). Mouse lungs were fixed with 10% neutral-buffered formalin, embedded in paraffin, sectioned at 4 μm and stained with H&E. The number of pulmonary metastatic tumor nodules was counted under a light microscope at magnifications of ×100 and ×200.

Statistical analysis

The data were assessed using SPSS 18.0 software (IBM, SPSS, Chicago, IL, USA). All of the experiments were performed in triplicate, and Student's t-test was used to analyze the significance of the different levels between two groups. The Chi-square test was used to assess the correlation between FOXD1 expression and the clinicopathological characteristics. Survival curves were estimated by the Kaplan-Meier method and compared using the log-rank test. The Cox proportional hazards model was used for univariate and multivariate analyses. The data are expressed as the mean ± standard deviation (SD) and differences of $P<0.05$ were considered to be statistically significant.

Results

FOXD1 is frequently upregulated in human NSCLC

The mRNA expression level of FOXD1 was determined in 264 pairs of NSCLC tissue and paired adjacent non-tumor tissue by qRT-PCR. As shown in Fig. 1A, FOXD1 was significantly increased in NSCLC tumor tissue compared with the paired non-tumor tissue. Furthermore, the mRNA level of FOXD1 was significantly higher in NSCLC patients with metastasis than in patients without metastasis (Fig. 1B). FOXD1 protein expression was confirmed by IHC staining. Our results showed that non-tumor lung tissues were stained slightly positive for FOXD1, with positive staining of FOXD1 in NSCLC tissue, especially in NSCLC tissues with metastasis (Fig. 1C). Finally, protein expression levels of FOXD1 were measured in five NSCLC cell lines and 16HBE cell by Western blotting. Expression levels of FOXD1 were elevated in all NSCLC cell lines compared to 16HBE cells, especially in NCI-H460 and NCI-H1299 cells, which have the highest endogenous FOXD1 levels (Fig. 1D). These results suggested that FOXD1 might act as an oncogene in the development of NSCLC.

Knockdown of FOXD1 inhibits the proliferation of NSCLC

Since FOXD1 was upregulated in NSCLC tissues, lentiviral-mediated FOXD1 knockdown was used to assess the effect of FOXD1 on cell proliferation. First, shRNA#1, #2, and #3 stable silencing FOXD1 were established in NCI-H460 and NCI-H1299 cell lines. Western blotting showed that FOXD1 was obviously knocked down in both shRNA#1 cell lines (Fig. 2A). Then, shRNA#1 (named shFOXD1) was used in the following experiments. Cell viability was significantly reduced in both stable FOXD1-knockdown NCI-H460 and NCI-H1299 cell lines compared with SCR by the CCK-8 assay (Fig. 2B). Consistent with the results of CCK-8, the results of the colony formation assays indicated that the stable FOXD1-knockdown NSCLC cells exhibited a weakened capacity for colony formation compared with SCR (Fig. 2C). Next, flow cytometric analysis was performed to further evaluate the effects of FOXD1 on the proliferation of NSCLC cells by altering cell apoptosis. Silencing FOXD1 significantly increased the percentage of apoptosis rates compared with SCR (Fig. 2D). Collectively, our data from the FOXD1-knockdown cells indicated that FOXD1 promoted NSCLC cell proliferation by affecting cell apoptosis.

FOXD1 silencing impairs NSCLC cell migration and invasion

Uncontrolled cell proliferation and metastasis are the main characteristics of tumor aggressive behaviors [13-15]. It is necessary to evaluate the effects of FOXD1 on the migratory and invasive capacity of NSCLC cells. Transwell migration and Matrigel invasion assays showed that deletion of FOXD1 significantly decreased the number of migrated and invaded NCI-H460 and NCI-H1299 cells (Fig. 3A). Since our data suggest the potential involvement of FOXD1 in the metastatic capacity of the cancer phenotype, we explored whether FOXD1 regulates gene expression of markers of metastasis. Interestingly, FOXD1 silencing resulted in an obvious decrease in the protein expression of Vimentin, N-cadherin, MMP-2 and MMP-9, while endogenous FOXD1 knockdown caused an increase in E-cadherin. These data might explain the underlying mechanisms of FOXD1-induced cell invasion.

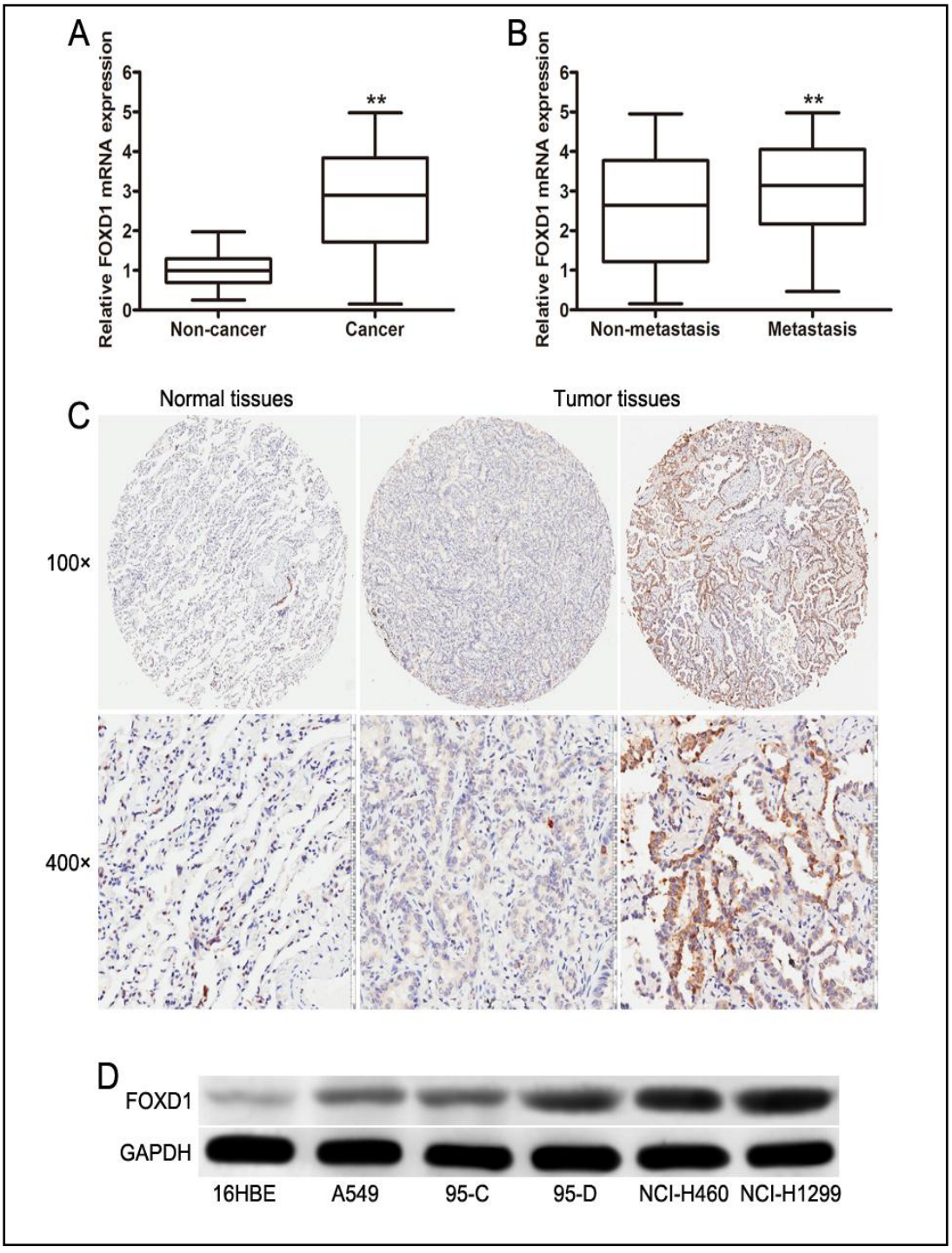

Fig. 1. Expression of FOXD1 is elevated in NSCLC tissues and cells. (A) qRT-PCR was performed to detect the FOXD1 mRNA expression in 264 pairs of NSCLC tissues and corresponding noncancerous lung tissues. (B) The mRNA level of FOXD1 was measured in primary NSCLC cancer tissues of 115 patients without metastasis and 149 patients with metastasis. (C) FOXD1 protein expression in normal lung tissues and NSCLC was detected by immunohistochemistry. Left, normal lung tissue; middle, weakly positive staining of FOXD1 in NSCLC tissue; right, strongly positive staining of FOXD1 in NSCLC tissue. (D) Western blots of FOXD1 expression in the 16HBE cell line and the A549, 95-C, 95-D, NCI-H460, and NCI-H1299 NSCLC cell lines. ** P<0.01.

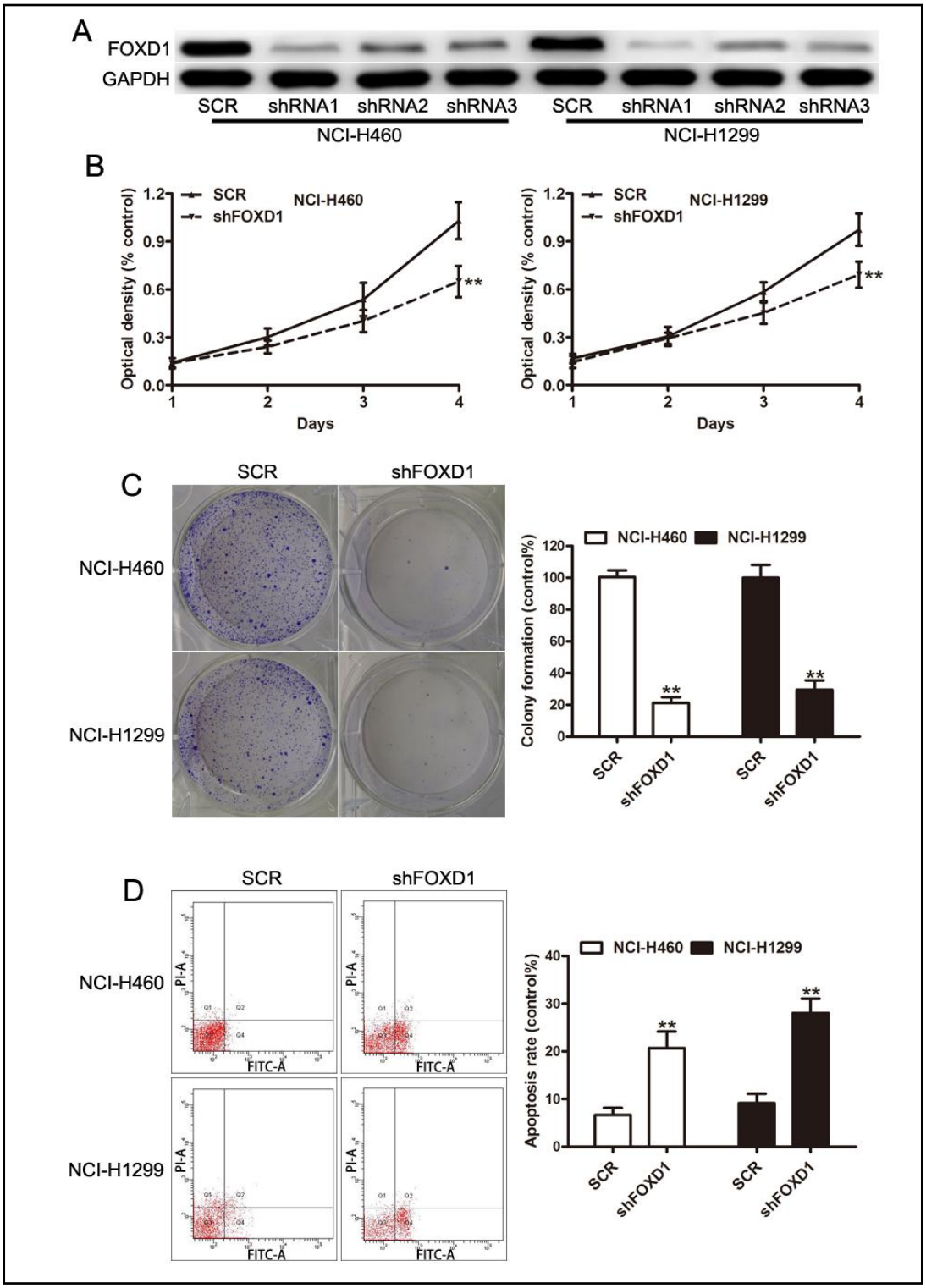

Fig. 2. Down-regulation of FOXD1 inhibits NSCLC proliferation. (A) The expression of FOXD1 in NCI-H460 and NCI-H1299 cells is interfered by lentiviral-mediated FOXD1 shRNA. Western blotting was performed to confirm the knockdown efficiency. GAPDH was used as the loading control for Western blotting. The (B) CCK-8 and (C) the clone formation assay detected cell proliferation after transducing with FOXD1 shRNA. (D) Cell apoptosis analysis revealed that FOXD1 has influenced the apoptosis of NCI-H460 and NCI-H1299 cells. **P<0.01.

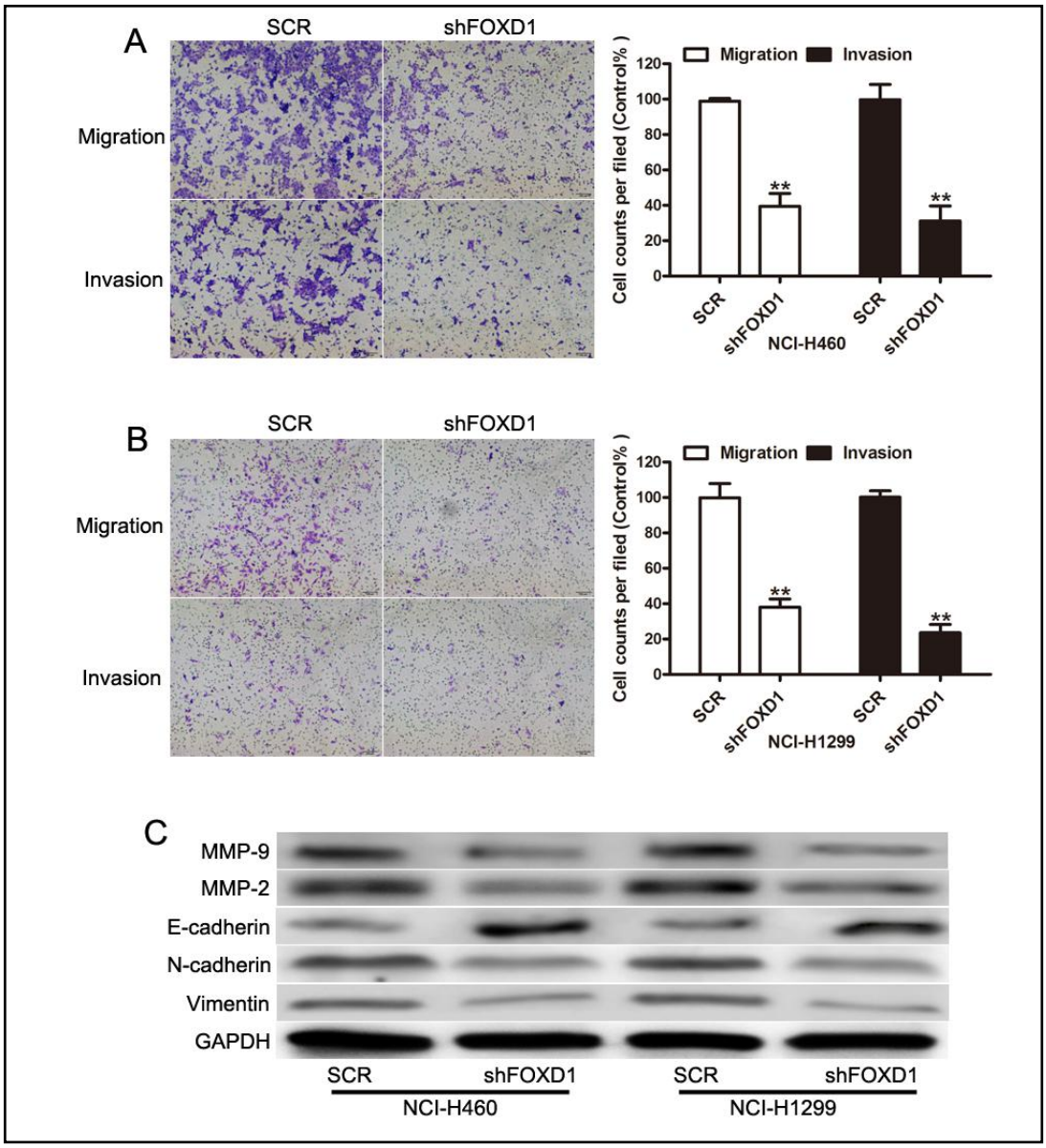

Fig. 3. FOXD1 knockdown decreases NSCLC cell migration and invasion. Representative images and quantification of migration and invasion of NCI-H460 (A) and NCI-H1299 (B) cells after transducing with FOXD1 shRNA. (C) The protein expression of Vimentin, N-cadherin, E-cadherin, MMP-2 and MMP-9 in NCI-H460 and NCI-H1299 cells transduced with FOXD1 shRNA or SCR. ** P<0.01.

FOXD1 depletion in NSCLC cells results in impaired tumor growth and metastasis in vivo

Given that NSCLC cells infected with shFOXD1 exhibited reduced proliferative capacity, migratory and invasive capacity *in vitro*, we further confirmed the effects of FOXD1 on the proliferative and metastatic capacity of NSCLC cells *in vivo*. NCI-H460 cells infected with shFOXD1 or SCR were injected subcutaneously into the left flanks of nude mice to produce a xenograft model. Gross specimens dissected from the mice pictorially demonstrate this difference in volume (Fig. 4A). Both tumor volume and weight were measured to analyze the tumor growth. The nude mice implanted with NCI-H460 cells treated with lentiviral shFOXD1 vector showed significantly smaller (P<0.01) (Fig. 4B) and lighter (P<0.01) (Fig. 4C) implantation tumors than nude mice that had been implanted with NCI-H46 cells treated with the control vector. Ki-67 is an excellent marker to determine the growth of a tumor cell, and

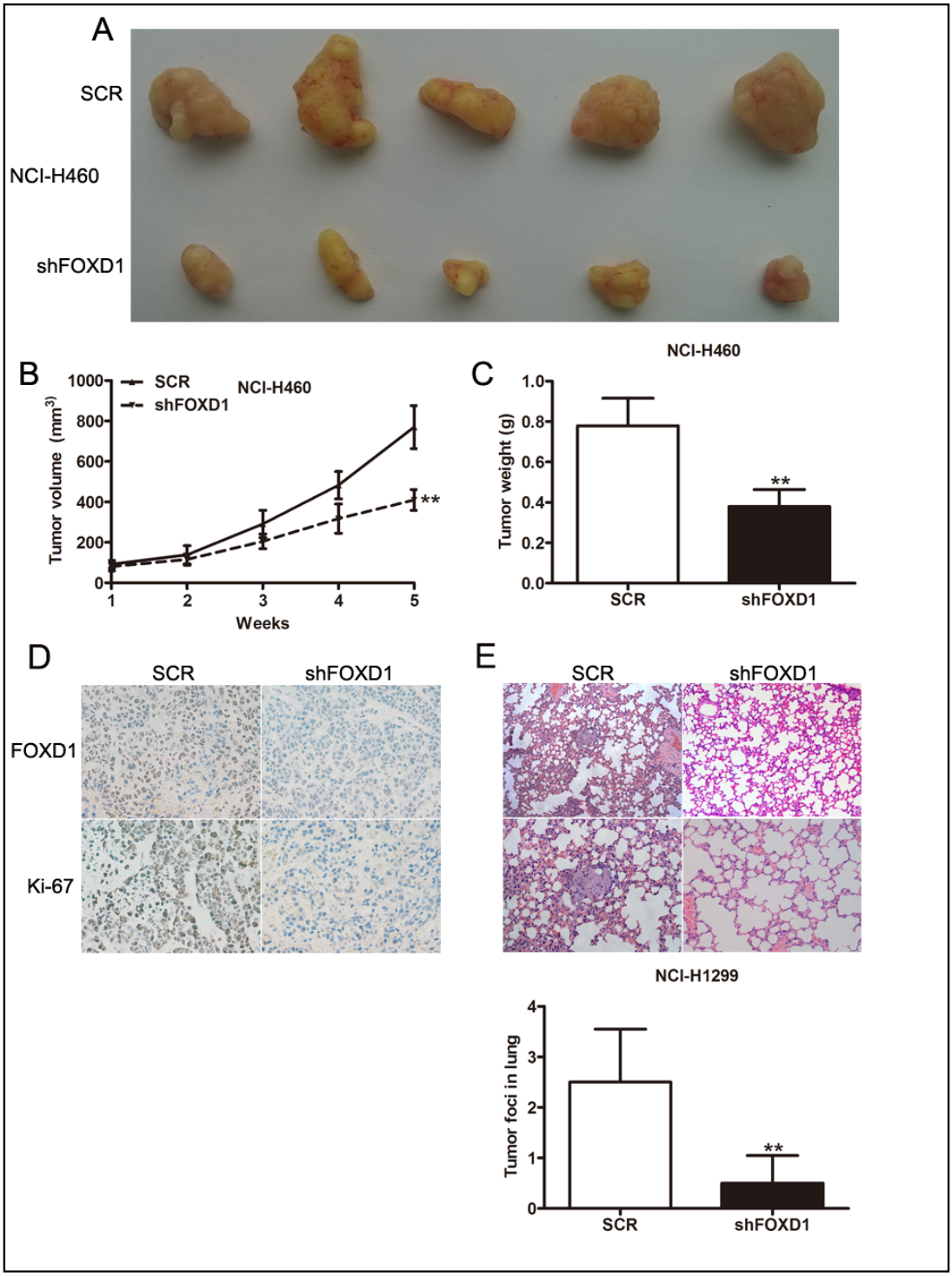

Fig. 4. Knockdown of FOXD1 represses tumor growth and metastasis in vivo. (A) Representative images of tumors formed by NCI-H460 isolated from nude mice. (B) Tumor cell growth course in nude mice. (C) Tumor weight in nude mice. (D) Representative images of FOXD1 and Ki-67 staining in tumors isolated from nude mice. (E) Representative H&E stained images of metastatic nodes from mouse lungs. **P<0.01.

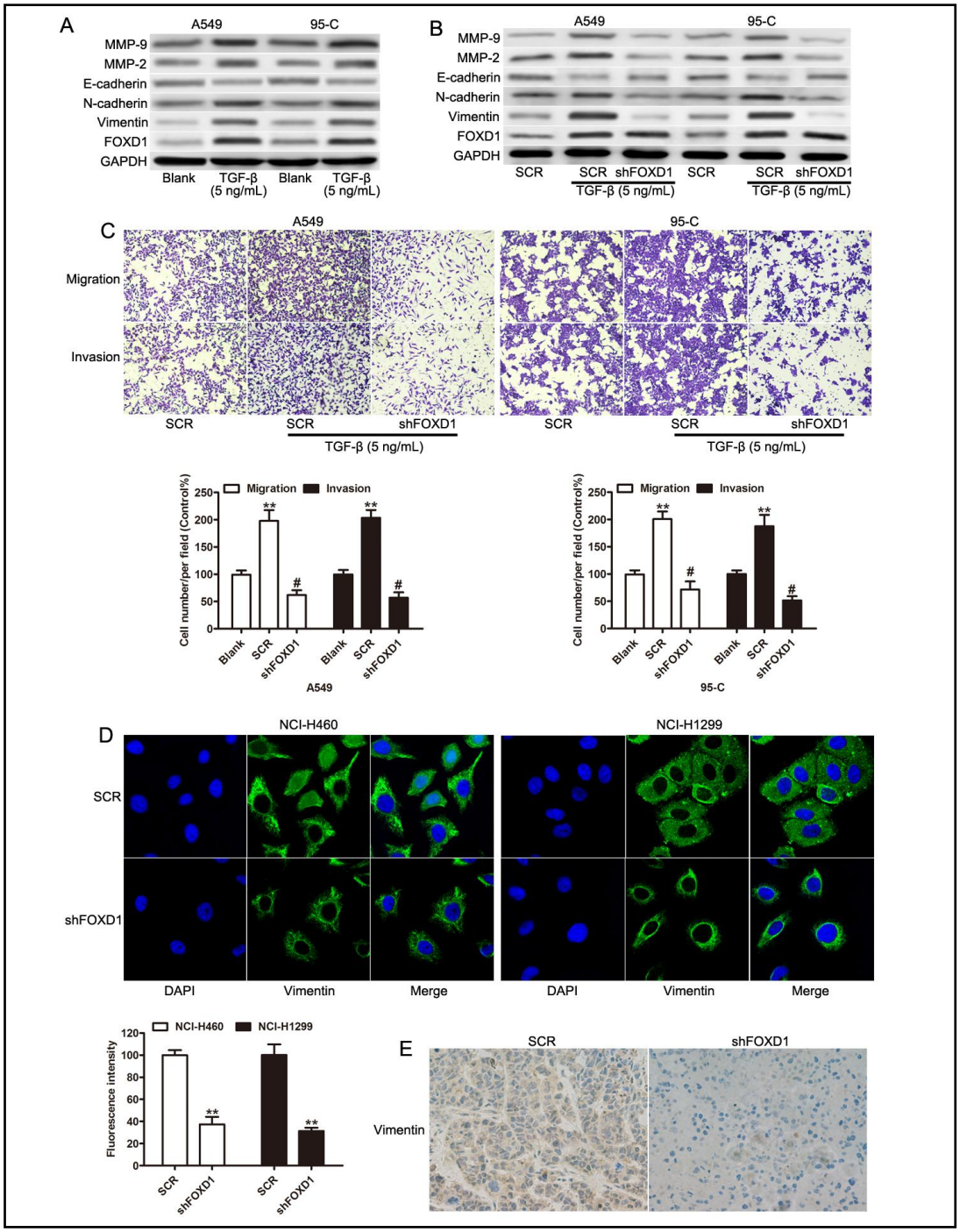

Fig. 5. FOXD1 promotes NSCLC cell migration and invasion through Vimentin. (A) N-cadherin, E-cadherin, MMP-2, MMP-9, FOXD1 and Vimentin expression was increased during TGF-β1 (48 h) induced cell migration and invasion in A549 and 95-C cells. (B) The protein expression of FOXD1, Vimentin, N-cadherin, E-cadherin, MMP-2 and MMP-9 in A549 and 95-C cells transfected with negative control (NC) or Vimentin siRNA after TGF-β1 stimulation. (C) Representative images and quantification of migration and invasion of A549 and 95-C cells transfected with negative control (NC) or Vimentin siRNA after TGF-β1 stimulation. (D) Immunofluorescence analysis of Vimentin in NCI-H460 and NCI-H1299 cells transduced with FOXD1 shRNA or SCR. Green signals indicate that Vimentin and nuclei were counterstained with DAPI. (E) IHC staining showed the expression of Vimentin in tumor xenografts isolated from nude mice. ** P<0.01.

our results show that knockdown of FOXD1 expression caused a significant decrease in the number of Ki-67 positive cells in FOXD1 silencing xenograft tumors compared with SCR (Fig. 4D). Histologic sections of the lungs were obtained from mice that had been implanted with tumor cells and H&E stained. The number of metastatic nodules was counted under a microscope. The nude mice implanted with NCI-H460 cells infected with shFOXD1 had a significantly lower number of pulmonary metastases than nude mice that had been implanted with NCI-H46 cells treated with the control vector (P<0.01) (Fig. 4E). Overall, these results suggested that FOXD1 depletion can inhibit tumor aggressiveness by reducing cell proliferation and metastasis both *in vitro* and *in vivo*.

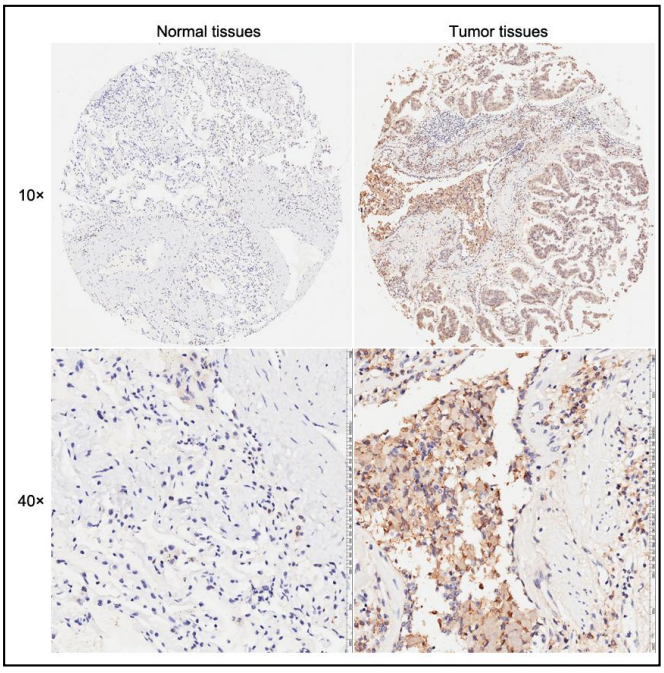

Fig. 6. Representative photomicrographs of the immunohistochemical expression of Vimentin in adjacent normal tissues and NSCLC tumors.

FOXD1 is essential for TGF-β-induced Vimentin expression, cell migration and invasion

Previous studies reported that TGF-β functions as a major inducer of cancer progression [16, 17]. Interestingly, a significant increase in FOXD1 in A549 and 95-C cells was observed after treatment with 5 ng/ml TGF-β (Fig. 5A). Vimentin is a widely considered as a marker of the epithelial mesenchymal transition (EMT), which can be induced by TGF-β in several cancers [18, 19]. Consisted with these results, Vimentin was also increased after TGF-β treatment, and TGF-β also induced higher expression of Vimentin, N-cadherin, MMP-2 and MMP-9 and lower expression of E-cadherin (Fig. 5A). Next, we examined whether FOXD1 regulates the expression of the mesenchymal gene vimentin upon TGF-β stimulation. As we expected, FOXD1 silencing decreases TGF-β-induced expression of Vimentin, N-cadherin, MMP-2 and MMP-9, while increasing the expression of E-cadherin (Fig. 5B). In addition, an *in vitro* transwell assay showed that knockdown of FOXD1 decreased NSCLC cell migration and invasion induced by TGF-β (Fig. 5C), thus indicating that FOXD1 is involved in TGF-β-induced migration and invasion. Moreover, knockdown of FOXD1 decreased the intensity (green) of Vimentin in NCI-H460 and NCI-H1299 cells (Fig. 5D). Immunohistochemical analysis of tumor sections indicates low Vimentin expression from FOXD1 silenced tumors compared with SCR tumors (Fig. 5E). These data suggest that FOXD1 promotes NSCLC progress associated with induced vimentin and increased invasive ability.

Combination of FOXD1 and Vimentin exhibits improved prognostic accuracy for NSCLC

We analyzed the relationship between FOXD1 and Vimentin expression and patient survival. The immunohistochemical expression of Vimentin in NSCLC tumors was more positive than the adjacent normal tissues (Fig. 6). Samples were classified into two groups according to the mean value of FOXD1 expression. As shown in Table 1, high FOXD1 expression was significantly associated with the tumor-node-metastasis (TNM) stage of tumor progression. Our results also showed that higher protein levels of Vimentin were significantly associated with tumor histology grade, TNM stage and tumor progression (Table 1). The prognostic value of FOXD1 and Vimentin was analyzed by comparing the OS and DFS

Table 1. Associations between FOXD1/Vimentin expression level and clinicopathologic features of NSCLC patients (n =264)

Variables	FOXD1 Expression, n		P	Vimentin Expression, n		P
	Low n=132	High n=132		Low n=132	High n=132	
Sex						
Female	13	15	0.689	10	18	0.110
Male	119	117		122	114	
Age,y						
≤60	66	68	0.806	72	62	0.218
>60	66	64		60	70	
Histology						
Squamous cell carcinoma	100	90	0.357	96	94	0.921
Adenocarcinoma	19	27		23	23	
Others	13	15		13	15	
Smoking						
Yes	106	113	0.252	108	100	0.228
No	26	19		24	32	
Histology grade						
Well	26	24	0.338	31	19	0.046
Moderate	97	92		93	96	
Poor and Unknown	9	16		8	17	
TNM stage						
I- II	67	48	0.018	69	46	0.004
III-IV	65	84		63	86	
Tumor progression						
Metastasis	85	64	0.009	93	56	<0.001
No metastasis	47	68		39	76	

Table 2. Univariate Analyses of Factors Associated with Overall Survival and Disease Free Survival. Abbreviation: 95% CI, 95% confidence interval; AFP, alpha-fetoprotein; NS, not significant (Cox proportional hazards regression model)

Parameter	Overall Survival		Disease Free Survival	
Variable	Hazard ratio (95% CI)	P Value	Hazard ratio (95% CI)	P Value
Sex (male vs. female)	0.883(0.568-1.375)	NS	0.910(0.591-1.402)	NS
Age, y (>60 vs. ≤60)	1.099(0.837-1.441)	NS	1.100(0.845-1.433)	NS
Smoking (yes vs. no)	1.146(0.819-1.603)	NS	1.149(0.830-1.591)	NS
Histology (Adenocarcimoma vs Squamous cell carcinoma)	1.089 (0.890-1.333)	NS	1.024(0.833-1.259)	NS
Histology grade (Poor vs. well)	1.386(1.054-1.823)	0.020	1.380(1.053-1.809)	0.020
TNM stage (III/IV vs. I/II)	1.838 (1.387-2.435)	<0.001	1.921(1.461-2.524)	<0.001
Tumor progression (Metastasis vs. No metastasis)	1.879(1.428-2.471)	<0.001	2.028(1.552-2.650)	<0.001
FOXD1 (high vs. low)	2.044(1.550-2.696)	<0.001	1.954(1.495-2.554)	<0.001
Vimentin (high vs. low)	2.276(1.720-3.013)	<0.001	2.530(1.922-3.331)	<0.001

Table 3. Multivariate Analyses of Factors Associated with Overall Survival and Disease Free Survival. Abbreviation: 95% CI, 95% confidence interval; NS, not significant (Cox proportional hazards regression model)

Variable	Overall Survival		Disease Free Survival	
	Hazard ratio (95% CI)	P Value	Hazard ratio (95% CI)	P Value
Histology grade (Poor vs. well)	0.989(0.723-1.352)	NS	0.899(0.661-1.222)	NS
TNM stage (III/IV vs. I/II)	1.426(0.919-2.211)	NS	1.383(0.908-2.106)	NS
Tumor progression (Metastasis vs. No metastasis)	1.087(0.689-1.715)	NS	1.225(0.789-1.901)	NS
FOXD1 (high vs. low)	2.017(1.525-2.668)	<0.001	1.947(1.484-2.556)	<0.001
Vimentin (high vs. low)	1.920(1.306-2.822)	<0.001	2.338(1.750-3.124)	<0.001
Combination of FOXD1 and Vimentin Overall		<0.001		<0.001
High FOXD1/High Vimentin vs. low FOXD1/low Vimentin	4.547(2.912-7.102)	<0.001	4.696(3.049-7.234)	<0.001

of patients with high and low FOXD1 or Vimentin expression (n=132 each group). Kaplan-Meier analysis showed that patients with low FOXD1 expression had a significantly higher postoperative 5-year OS and a significantly higher postoperative DFS than patients with high FOXD1 expression (Fig. 7A and 7B) (P<0.01). When patients were divided according to the level of Vimentin expression, those with low Vimentin expression had a significantly higher 5-year OS and significantly higher 5-year DFS than those with high Vimentin expression (Fig. 7C and 7D) (P<0.01). Univariate analysis showed that FOXD1, Vimentin, histology grade, TNM stage and tumor progression were significantly associated with OS and DFS in NSCLC patients (Table 2). However, sex, age, smoking, and histology did not significantly associate with OS and DFS (Table 2). In addition, multivariate analysis showed that FOXD1 and Vimentin were independent prognostic indicators for both OS and DFS (Table 3). In the combined analysis of FOXD1 and Vimentin expression, patients with low FOXD1 and low Vimentin expression had the highest 5-year OS and the lowest DFS, and the opposite pattern was observed in patients with high FOXD1 and Vimentin expression (Fig. 7E and 7F). Taken

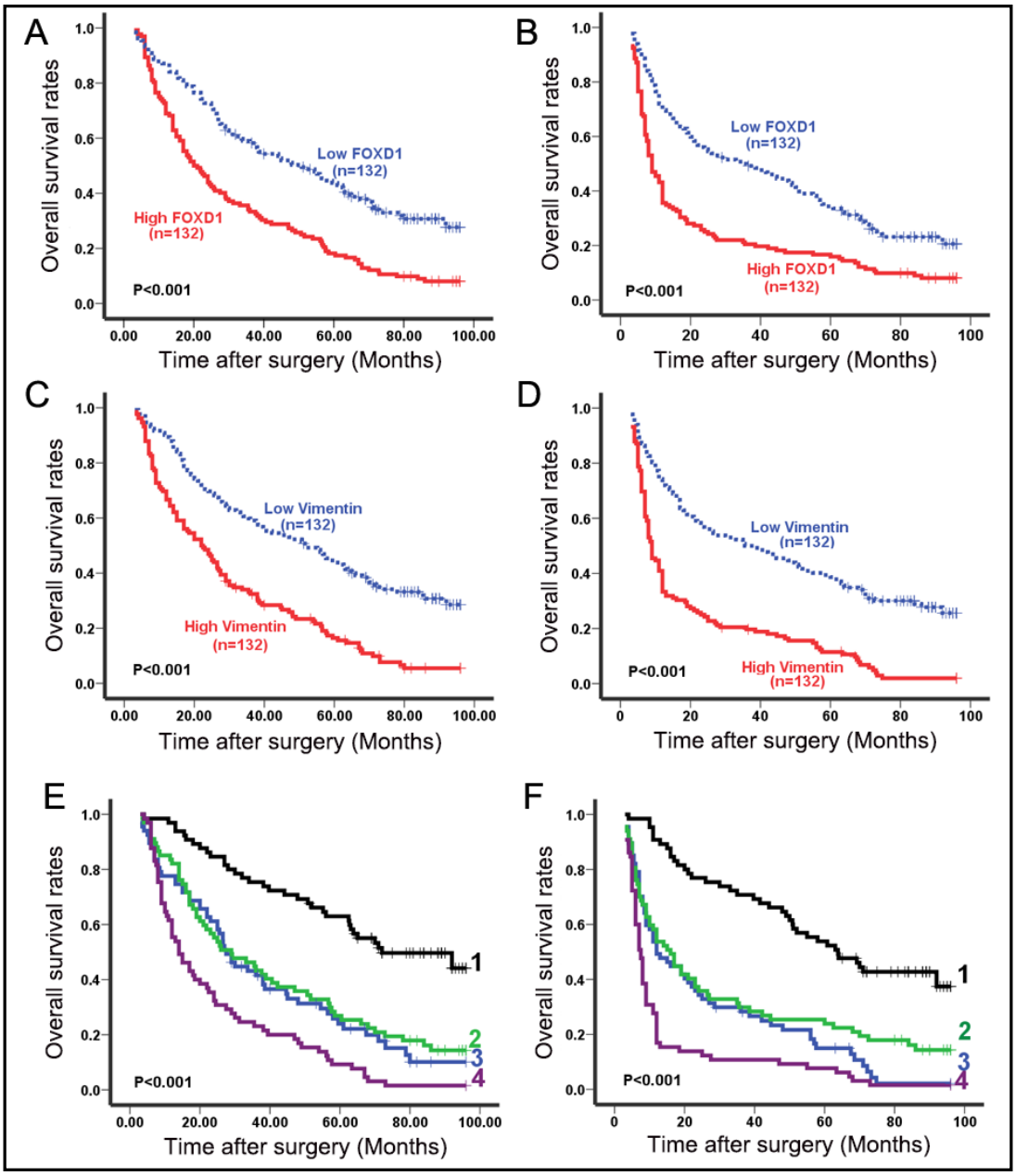

Fig. 7. Prognostic significance of FOXD1 and Vimentin expression in NSCLC patients. (A and B) Kaplan-Meier analysis of the correlation between FOXD1 expression and overall survival (A) and disease-free survival (B) in NSCLC patients. (C and D) Kaplan-Meier analysis of the correlation between Vimentin expression and OS (C) and DFS (D) in NSCLC patients. (E and F) Patients with low FOXD1 and low Vimentin had the longest OS (E) and lowest possibility of DFS (F) according to the cut-off values of FOXD1 and Vimentin, which were defined as the median of the cohort. 1: low FOXD1 and low Vimentin; 2: high FOXD1 and low Vimentin; 3: low FOXD1 and high Vimentin; 4: high FOXD1 and high Vimentin.

together, these results indicate that FOXD1 and Vimentin expression levels are positively correlated with poor OS and a greater recurrence rate in NSCLC patients, suggesting that FOXD1 and Vimentin are prognostic indicators in NSCLC.

Discussion

The FOX family of genes, characterized by a common 100 amino acid winged-helix DNA-binding domain, regulates a wide spectrum of biological processes, including cell growth, survival, longevity, immunity, metabolism, differentiation, development and migration [20]. Thus, the dysregulation of FOX genes is also observed in a variety of cancerspage5. The FOXD1 gene is located on chromosome 5q12 and is involved in a wide array of biological processes, including proliferation, invasion and tumorigenesis [5, 7, 8]. A previous study used microarray datasets from 90 lung cancer specimens to analyze the levels of FOXD1 mRNA and found that the aberrant FOXD1 mRNA was correlated with NSCLC patient survival. They also reported that FOXD1-knockdown led to suppression of cell proliferation and thought that the expression status of FOXD1 is a novel prognostic factor of NSCLC [11].

In this study, we have demonstrated that levels of FOXD1 were increased in clinical NSCLC tissues compared to adjacent normal lung tissues, especially in metastatic NSCLC tissues. FOXD1 was also increased in NSCLC cell lines, including A549, 95-C, 95-D, NCI-H460, and NCI-H1299, compared with normal human bronchial epithelial cells. These results are consistent with a previous study that showed that FOXD1 expression was elevated in NSCLC tissues and NSCLC cell lines, including H358, H520, and H522 [11]. Furthermore, we revealed that a higher expression level of FOXD1 was associated with malignant behavior and poorer prognosis of NSCLC patients. It is worth mentioning that FOXD1 is after all a transcription factor so nuclear localization would be expected, but FOXD1 was mainly in cytoplasm, which was previously reported [8]. However, we don't found that high levels of FOXD1 expression were significantly associated with these clinicopathological findings, including the presence of squamous cell carcinoma, male gender and history of heavy smoking.

To determine the biology function of FOXD1 in NSCLC, gain-and loss-of-function were used. Knockdown of FOXD1 significantly inhibited proliferation, migration, and invasion *in vitro* and tumor growth and metastasis *in vivo*, and increased the apoptosis rates of NSCLC cells. The results were consistent with other studies, which showed significant growth inhibition in several cancer cell types [7, 10, 21]. In breast cancer, it was suggested that FOXD1 induces the G1/S transition and promotes the cell cycle progression by down-regulation of p27 expression [10]. In glioma, reduced expression of FOXD1 was associated with the inhibition of glioma cell proliferation, cell survival and migration, implying that FOXD1 is an oncogene in glioma [21]. It was found that knockout of FOXD1 inhibits downstream transcriptional Dax1, which is a component of the autoregulatory network for maintaining pluripotency, and the fate mapping analyses further reveal that >95% of induced pluripotent stem cell (iPSC) colonies are derived from FOXD1-positive cells. Thus, FOXD1 is a mediator and indicator of successful progression of reprogramming [7]. It was found that the gene encoding the small leucine-rich proteoglycan decorin is repressed by FOXD1 in cortical interstitial cells, partially rescuing the failure of progenitor cell differentiation [5].

Previous studies have shown that overexpression of vimentin may predict the progression and unfavorable survival of NSCLC [22-24]. Vimentin is essential to the progression and prognosis of cancer through the EMT, a critical event in the invasion, progression and metastasis of epithelial cancers [25, 26]. In the present study, we found that FOXD1 expression was related to increased expression of Vimentin. Interestingly, high levels of FOXD1 expression were significantly associated with the high level of Vimentin. When the FOXD1 was knocked out, Vimentin expression was down-regulated. The mechanistic analyses revealed that FOXD1 performed its oncogenic characteristics through activating Vimentin in NSCLC.

A meta-analysis of observational studies with 4118 NSCLC cases predicted the prognostic values of Vimentin expression in NSCLC and revealed that Vimentin is a helpful potential biomarker in NSCLC [27], but there is no information regarding the relationship between FOXD1 and Vimentin expression in lung cancer. FOXD1 expression was identified as a prognostic factor in the previous univariate analysis and multivariate Cox analysis [11]. In the present study, the prognosis of patients was also investigated. Patients with high FOXD1

expression survived for a significantly shorter time than those with low FOXD1 expression. Multivariate Cox regression analysis indicated that FOXD1 was an independent prognostic factor for OS and DFS in NSCLC patients. In addition, overexpression of vimentin may also predict the progression and an unfavorable survival of NSCLC. In the combined analysis of FOXD1 and Vimentin expression, high FOXD1 and Vimentin expression were associated with the worst prognosis. To the best of our knowledge, this is the first study to investigate the relationship between FOXD1 and Vimentin in the prognosis of NSCLC patients.

Conclusion

In summary, we showed that knockdown of FOXD1 could inhibit cell proliferation and invasion *in vitro* and *in vivo* and that high expression of FOXD1 is associated with worse prognosis of NSCLC. Our findings suggest that FOXD1 acts as an oncogenic factor in NSCLC partly by activating Vimentin. Hence, as an independent prognostic factor, FOXD1 could be an innovative treatment target for NSCLC.

Abbreviations

FOXD1 (Forkhead box D1); NSCLC (non-small cell lung cancer); OS (overall survival); DFS (disease-free survival); ShRNA (short hairpin RNA); IHC (Immunohistochemical); DAB (diaminobenzidine); FBS (fetal bovine serum); CCK8 (Cell Counting Kit 8); OD (optical density); SD (standard deviation); EMT (epithelial mesenchymal transition); TNM (tumor-node-metastasis); iPSC (induced pluripotent stem cell.).

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Disclosure Statement

The authors declare that they have no competing interests.

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