

Forkhead box N1 inhibits the progression of non-small cell lung cancer and serves as a tumor suppressor

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Abstract. Forkhead box N1 (FOXN1) belongs to the FOX family of transcription factors, which comprises a diverse group of winged-helix proteins. FOXN1 is a ubiquitously expressed member that has been implicated in the embryo development, metabolism, aging and cancer. However, little is known regarding the role of FOXN1 in non-small cell lung cancer (NSCLC). The aim of the study was to investigate the function of FOXN1 in NSCLC and examine the relevant mechanism. In the present study, using reverse transcription-quantitative polymerase chain reaction, western blotting, transwell assay, MTT assay, luciferase report assay, it was identified that knockdown of FOXN1 increased the proliferation of A549 and H1299 cells, while overexpression of FOXN1 evidently suppressed the cell growth. A Transwell assay was used to determine the relative cell invasion ability, and it was observed that the invading cells were markedly decreased in the FOXN1 overexpression groups; by contrast, reduced expression of FOXN1 demonstrated the potential to promote cell invasion. Furthermore, lower expression of FOXN1 was observed in NSCLC tissues and cell lines as compared with the adjacent non-tumor tissues or human bronchial epithelial cells, respectively. A higher level of FOXN1 was associated with a better prognosis of NSCLC patients. Quantitative chromatin immunoprecipitation analysis and luciferase reporter gene assays revealed that enhancer of zeste homolog 2 (EZH2) and β -catenin were two target genes of FOXN1. Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and western blot analysis indicated that FOXN1 suppressed the expression levels of these target genes at the transcriptional level. In conclusion, the present study demonstrated that FOXN1 served major roles in NSCLC proliferation and invasion by directly repressing EZH2 and

β -catenin, which suggested that FOXN1 may function as a tumor suppressor in NSCLC.

Introduction

Lung cancer is one of the most common types of cancer and the leading cause of cancer-associated mortality worldwide (1,2). Non-small cell lung cancer (NSCLC) is the main type of lung cancer and accounts for nearly 84% of cases. Despite decades of intensive efforts in the cancer research field, the prognosis of patients with NSCLC remains poor, with a 5-year overall survival rate of ~10% (3). Thus, further investigation of novel biomarkers and therapeutic targets for NSCLC is urgently required (4).

The family of forkhead box (FOX) transcription factors, which includes the FOXP subfamily, serves important roles in oncogenesis. Notably, downregulation of FOXP4 markedly reduced the NSCLC cell growth and invasion (5), while FOXP2 worked as a potential novel tumor metastasis suppressor in breast cancer (6). In NSCLC, higher expression of FOXN1 has been associated with improved survival times in patients (7). Using tissue arrays and immunohistochemical analysis, FOXP2 has been observed to function as a novel candidate tumor suppressor gene in different cancer types. However, to our knowledge, the function of FOXN1 in cancer remains unknown. In the present study, the aim was to evaluate the role of FOXN1 in NSCLC.

EZH2 is overexpressed and associated with worse overall survival rate in NSCLC patients (8). Nuclear β -catenin could serve as an important prognosticator for patients with NSCLC (9).

Materials and methods

Cell lines and reagents. Normal human bronchial epithelial (HBE) cells, A549, H1299, SPC-A1, H460, H520 and SK-MES-1 lung cancer cell lines were purchased from the American Type Culture Collection (Manassas, VA, USA). The cells were cultured at 37°C in a humidified atmosphere with 5% CO₂ in RPMI-1640 medium (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Hyclone; Thermo Fisher Scientific, Inc.) and 1% penicillin/streptomycin (Invitrogen; Thermo Fisher Scientific, Inc.). Mouse anti-human FOXN1 polyclonal

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antibody (cat no. sc-271256; 1:500), rabbit anti-human enhancer of zeste homolog 2 (EZH2) polyclonal antibody (cat no. sc-25383; 1:1,000), mouse anti-human β -actin monoclonal antibody (cat. no. SC47778; 1:2,000) horseradish peroxidase-labeled secondary antibodies including goat anti-mouse IgG-horseradish peroxidase (cat no. sc-2005; 1:3,000) and anti-rabbit IgG-horseradish peroxidase (cat no. sc-2004; 1:3,000) were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA).

Transfection for FOXN1 downregulation. Two different FOXN1-short hairpin RNA (shRNA) ShFOXN1#1 TRCN0000013197 (CCGGCCTCCTGCTATGGGCAGACATCTCGAGATGTCTGCCCATAGCAGGAGGTTTTT) ShFOXN1#2 TRCN0000013193 (CCGGGCTCACACTCATCCACACTTACTCGAGTAAGTGTGGATGAGTGTGAGCTTTTT) ShEZH2 TRCN0000040074 (CCGGGCTAGGTTAATTGGGACCAAACCTCGAGTTTGGTCCCAATTAACCTAGCTTTTTTG) Sh β -catenin TRCN0000314991 (CCGGTTGTTATCAGAGGACTAAATACTCGAGTATTTAGTCCTCTGATAACAATTTTTTG) or non-targeting negative control shRNA was designed and synthesized by Sigma-Aldrich (Merck KGaA; Darmstadt, Germany).

Transfection for FOXN1 overexpression. pcDNA3.1-FOXN1 was cloned by GenePharm Co., Ltd. (Shanghai, China). pcDNA3.1 was used the control. Cultured cells were subjected to transfection using Lipofectamine[®] 3000 (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. A total of 20 μ M relative shRNA or 5 μ g relative plasmid were used for transfection. Following incubation at 37°C for 48-72 h, cells were collected and lysed to verify the expression of target genes or proteins by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and western blot analysis, respectively.

Patients. A total of 60 NSCLC patients diagnosed and treated at the People's Hospital of Guizhou Province (Guiyang, China) were enrolled into the present study. Written informed consent was obtained from each patient, and the current study was approved by the Ethics Committee of the People's Hospital of Guizhou Province. Tumor tissues and the adjacent normal tissues were attained during surgery between February 2010 and December 2014. Tissue samples were frozen and stored at -80°C for RT-PCR. Histological and pathological diagnoses were performed by the pathology department. The tissues were embedded in paraffin and cut into 2 μ m sections. Slides were incubated in three washes of xylene for 5 min each, followed by two washes of 100% ethanol for 10 min, 95% ethanol for 10 min and ddH₂O for 5 min. Then the samples were blocked in 10% normal goat serum in PBS at room temperature for 30 min and incubated at 4°C overnight in primary antibody solution of anti-FOXN1 (1:100) The EnVision Detection System kit (Dako; Agilent Technologies, Inc., Santa Clara, CA, USA) was used to visualize the 3,3'-diaminobenzidine chromogen (room temperature for 20 min). Samples were counterstained with hematoxylin (0.2%) at room temperature for 5 min (Zhongshan Golden Bridge Biotechnology Company) according to the Staging Lung Cancer's Revised

International System (10). Neutral gum was used to cover the slides and they were dried at room temperature. Staining was visualized under an Olympus optical microscope (Olympus Corporation, Tokyo, Japan) at x20 magnification for analysis.

Western blot analysis. Lung cancer cells were harvested and lysed using 0.5% NP-40 cell lysis buffer (Sigma-Aldrich; Merck KGaA) for 20 min on ice, and transparent protein lysates were obtained following centrifugation at 12,000 x g for 10 min at 4°C. The Bradford assay reagent (Thermo Fisher Scientific, Inc.) was then used to determine the protein concentration in the lysates. Next, 30 μ g protein from each group was separated by 10% SDS-PAGE and then transferred onto polyvinylidene difluoride membranes. Subsequently, 5% skim milk was used to block nonspecific binding in the samples at room temperature for 1 h, followed by addition of the primary antibodies against FOXN1, EZH2 and β -actin for overnight incubation at 4°C. β -actin served as the internal control. The membranes were then washed with Tris-buffered saline/Tween 20 for 5 min and five times, incubated with respective secondary antibodies goat anti-mouse IgG-horseradish peroxidase (1:3,000) and anti-rabbit IgG-horseradish peroxidase (1:3,000) at room temperature for 1 h and visualized using an enhanced chemiluminescence-based methods (western blotting detection system; Thermo Fisher Scientific, Inc.).

RNA extraction and RT-qPCR. Total RNA was extracted from the tissues or cells using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. The RNA concentration was assessed using a nanophotometer UV/VIS spectrophotometer (Implen GmbH, München, Germany). A total of 2 μ g RNA was converted into cDNA using Revert Aid[™] First Strand cDNA Synthesis kit (Fermentas; Thermo Fisher Scientific, Inc.). The qPCR reaction was subsequently performed on a 7500 Real-Time Detection System (Applied Biosystems; Thermo Fisher Scientific, Inc.) according to the following conditions: Initial step, 95°C for 3 min; second step, 95°C for 10 sec, 60°C for 30 sec and 72°C for 10 sec for a total of 38 cycles. The obtained data were analyzed using the 2^{- $\Delta\Delta C_q$} method, (11) with GAPDH used as an internal control. The primers used were as follows: GAPDH, forward 5'-GAGAAGTATGACAACAGCCTC-3' and reverse 5'-ATGGACTGTGGTCATGAGTC-3'; FOXN1, forward 5'-GCTCCTCACACTATCAGTACC-3' and reverse 5'-AAGATGAGGATGCTGTAGGA-3'.

Invasion assay. Transwell migration chambers (BD Biosciences, Franklin Lakes, NJ, USA) were used to measure the invasive capacity of cells. Briefly, 100 μ l Matrigel (BD Biosciences) was coated onto 8- μ m filter chambers and plated on 24-well plates. A total of 2x10⁴ cells were resuspended using 100 μ l Dulbecco's modified Eagle's medium (DMEM; Invitrogen; Thermo Fisher Scientific, Inc.) basal medium without FBS and seeded into the upper chamber, while 500 μ l DMEM with 10% FBS was added into the lower chamber. Following incubation at 37°C in a 5% CO₂ atmosphere for 12 h, the medium was removed and the cells were washed twice with cool PBS to remove cells from the upper chamber. Subsequent to fixing with 4% paraformaldehyde for 15 min, the invading cells in the lower chamber were stained with 1% crystal violet for

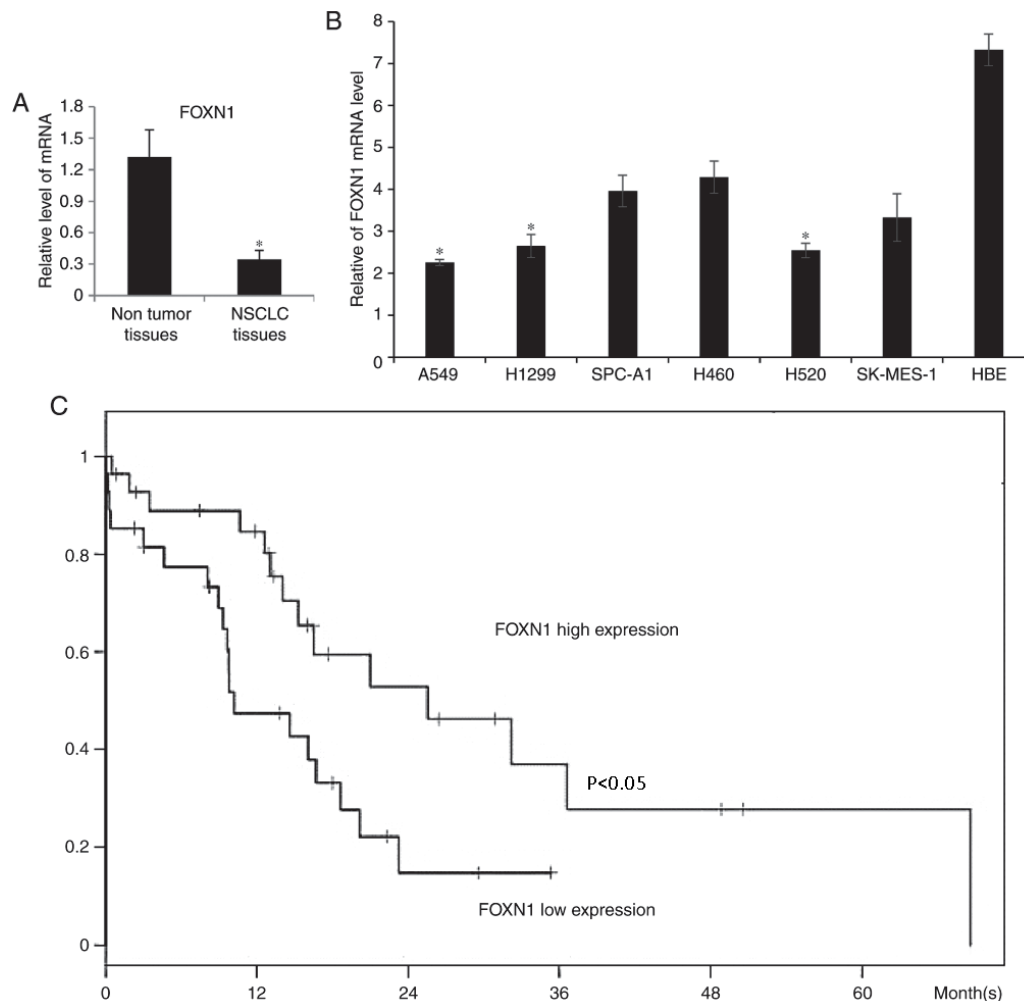


Figure 1. Increased expression of FOXN1 is associated with improved prognosis of NSCLC patients. (A) NSCLC tissues and paired noncancerous lung tissues (n=60), as well as (B) NSCLC cell lines (A549, H1299, SPC-A1, H460, H520 and SK-MES-1) and normal HBE cells, were examined by reverse transcription-quantitative polymerase chain reaction to measure the FOXN1 mRNA levels. *P<0.05 vs. HBE cells. (C) Kaplan-Meier curves were constructed to measure the patient survival rate, and Cox log-rank test was used to measure the prognostic significance. The x-axis represents the survival months, while the y-axis represents the survival probability. (P<0.05). FOXN1, forkhead box N1; NSCLC, non-small cell lung cancer; HBE, human bronchial epithelial cells.

15 min. The number of cells was counted under an upright light microscope in 10 different fields of view (Leica DM4B; Leica Microsystems, Shanghai, China). At least three independent experiments were performed.

MTT assay. Cell proliferation was determined using an MTT proliferation kit (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China). The transfected cells were subjected to centrifugation at 800 x g for 5 min at 4°C, followed by resuspension with complete medium. Next, the cells were seeded into a 96-well plate at a density of 2×10^4 cells per well. Following incubation for 24, 48 and 72 h, MTT reagent was added and an MTT assay was performed following the manufacturer's protocol. Each group was examined in three separate wells.

Quantitative chromatin immunoprecipitation (qChIP) analysis. The qChIP assay was performed using the Chip-IT Express kit (Active Motif, Carlsbad, CA, USA), according to the manufacturer's protocol. qPCR was performed with 5 μ l of the immunoprecipitated target DNA, 1 μ l primers

and 9 μ l mixture (1 μ l enzyme, 2 μ l dNTP and 6 μ l SYBR green solution buffer; all included in the ChIP-IT kit). The primers used were as follows: EZH2, forward, 5'-GAGGCA TGAGAATCGCTTGA-3', and reverse, 5'-GCCGGACCC GTTACTACTTT-3'; β -catenin, forward, 5'-CAGTTGGCA TTACCACTTAT-3', and reverse, 5'-TTCACATCATGGAGGT AGGAT-3'. The PCR amplification was performed at 95°C for 5 min, followed by 35 cycles of 95°C for 20 sec, 55°C for 20 sec and 72°C for 30 sec. The $2^{-\Delta\Delta C_q}$ method of quantification was used, with GAPDH used as an internal control, as per the RT-qPCR section.

Dual-luciferase reporter assay. A dual-luciferase reporter gene system (Promega Corporation, Madison, WI, USA) was used to verify the direct target genes for FOXN1, according to the manufacturer's protocol. Briefly, the full length of EZH2 promoter was synthesized by clonal expansion and cloned into the pmirGLO luciferase gene vector (Promega Corporation). FOXN1 and control plasmids were co-transfected into the cells along with the luciferase reporter vectors using Lipofectamine 2000 (Invitrogen; Thermo Fisher

Table I. Clinicopathological variables in 60 non-small cell lung cancer patients.

Variables	Patient no. (n=60)	FOXN1 protein expression		P-value
		Low (n=37)	High (n=23)	
Age (years)				0.887
<50	32	20	12	
≥50	28	17	11	
Gender				0.210
Male	33	18	15	
Female	27	19	8	
Tumor size				0.017
Small (≤3 cm)	30	14	16	
Large (≥3 cm)	30	23	7	
Metastasis				0.002
Present	36	28	8	
Absent	24	9	15	
TNM stage				<0.001
I + II	28	10	18	
III + IV	32	27	5	
Differentiation				0.580
Well/moderate	34	22	12	
Poor	26	15	11	

Scientific, Inc.). After 36 h at 37°C, the expression of *Renilla* (Takara Biotechnology Co., Ltd., Dalian, China) was used as an internal reference to adjust the number of cells and the transfection efficiency differences.

Statistical analysis. All experiments were performed at least three times. The data were analyzed using SPSS version 17.0 (SPSS, Inc., Chicago, IL, USA). Data was expressed as the mean ± standard deviation of three independent experiments. Differences between the experimental and control groups were compared using Student's t-test to compare differences between two groups. A two-way analysis of variance was performed in cases where more than two groups were compared. The post hoc test employed was Tukey's range test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

FOXN1 is downregulated in NSCLC tissues and cells. In order to understand the role of FOXN1 in NSCLC, 60 pairs of tissue samples were collected from NSCLC patients, and the mRNA expression of FOXN1 was determined by RT-qPCR. As illustrated in Fig. 1A, the expression of FOXN1 was significantly downregulated in tumor tissues when compared with the paired noncancerous tissues ($P < 0.05$). Furthermore, FOXN1 mRNA expression was detected in six NSCLC cell lines, namely A549, H1299, SPC-A1, H460, H520 and SK-MES-1, and normal human bronchial epithelial (HBE) cells. It was observed that the mRNA level of FOXN1 was lower in the NSCLC cell lines, with a significant effect observed for A549,

H1299 and H520 cells, as compared with the HBE cells ($P < 0.05$; Fig. 1B). A549 and H1299 cells were used for subsequent experiments.

Higher expression of FOXN1 is associated with better prognosis in NSCLC patients. To further determine the function of FOXN1 during NSCLC development and progression, the association of FOXN1 expression with the NSCLC patient clinicopathological characteristics, including the gender, age, tumor size, differentiation, metastasis and TNM staging, was analyzed. As shown in Table I, a higher expression of FOXN1 was associated with smaller tumor size, absence of metastasis and lower TNM stage ($P < 0.05$), which indicated an improved prognosis. Furthermore, Kaplan-Meier curves were constructed to analyze the NSCLC patient survival rate. As shown in Fig. 1C, a higher FOXN1 expression was correlated with a better overall survival rate ($P < 0.05$).

FOXN1 suppresses NSCLC cell proliferation. In order to determine the role of FOXN1 in NSCLC cells, the effect of FOXN1 overexpression on A549 and H1299 cell proliferation was investigated. Initially, two specific shRNAs targeted against FOXN1 (namely shFOXN1-1 and shFOXN1-2) were used to reduce the expression of FOXN1 in NSCLC cells. In addition, stable A549 or H1299 cell lines with overexpression of FOXN1 were established by plasmid transfection. The cells transfected with pcDNA3.1 plasmid was named as vector group, while the cells transfected with FOXN1 plasmid was named as FOXN1. The successful knockdown (Fig. 2A) and overexpression (Fig. 2B) of FOXN1 in the cells was confirmed by RT-qPCR. Subsequently, an MTT assay

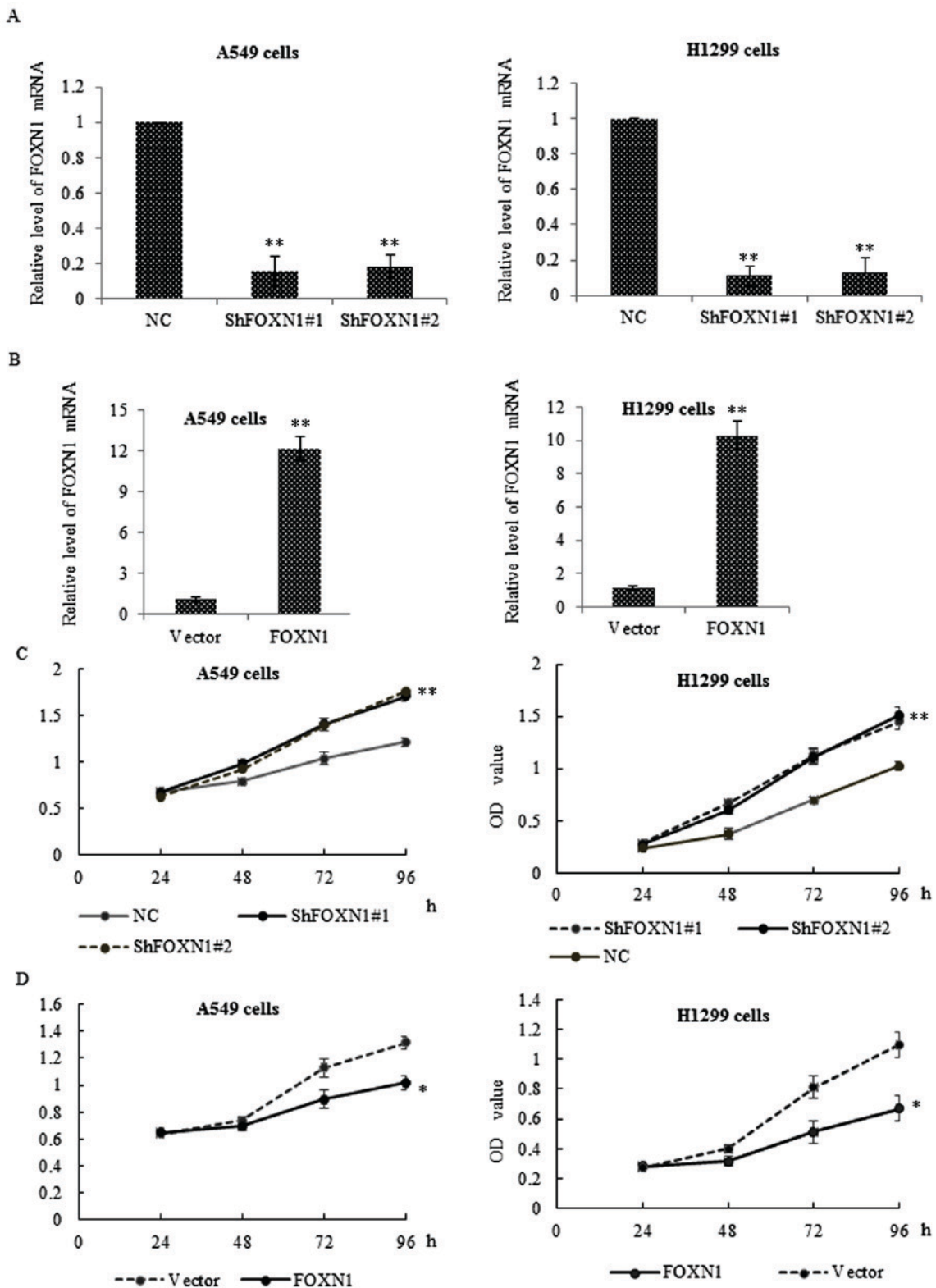


Figure 2. FOXN1 suppresses non-small cell lung cancer cell proliferation. Reverse transcription-quantitative polymerase chain reaction was performed to measure the FOXN1 mRNA expression in A549 or H1299 cells transfected with (A) shRNA targeting FOXN1 or NC shRNA, and (B) a lentivirus expressing FOXN1 or with a vector. The relative FOXN1 mRNA level was normalized against GAPDH expression. Furthermore, an MTT assay was conducted to determine the proliferation of A549 or H1299 cells transfected with (C) shRNA targeting FOXN1 or NC shRNA, and (D) a lentivirus expressing FOXN1 or vector. The results were detected at 24, 48 and 72 h. Values are expressed as the mean \pm standard deviation of three independent measurements. * $P < 0.05$ and ** $P < 0.01$, vs. corresponding control group. FOXN1, forkhead box N1; shRNA, short hairpin RNA; NC, negative control shRNA; OD, optical density.

was performed, which revealed that the cell growth was significantly promoted in cells transfected with shFOXN1 for expression knockdown as compared with the control cells

(Fig. 2C). By contrast, ectopic overexpression of FOXN1 in the A549 and H1299 cells markedly suppressed the cell growth (Fig. 2D).

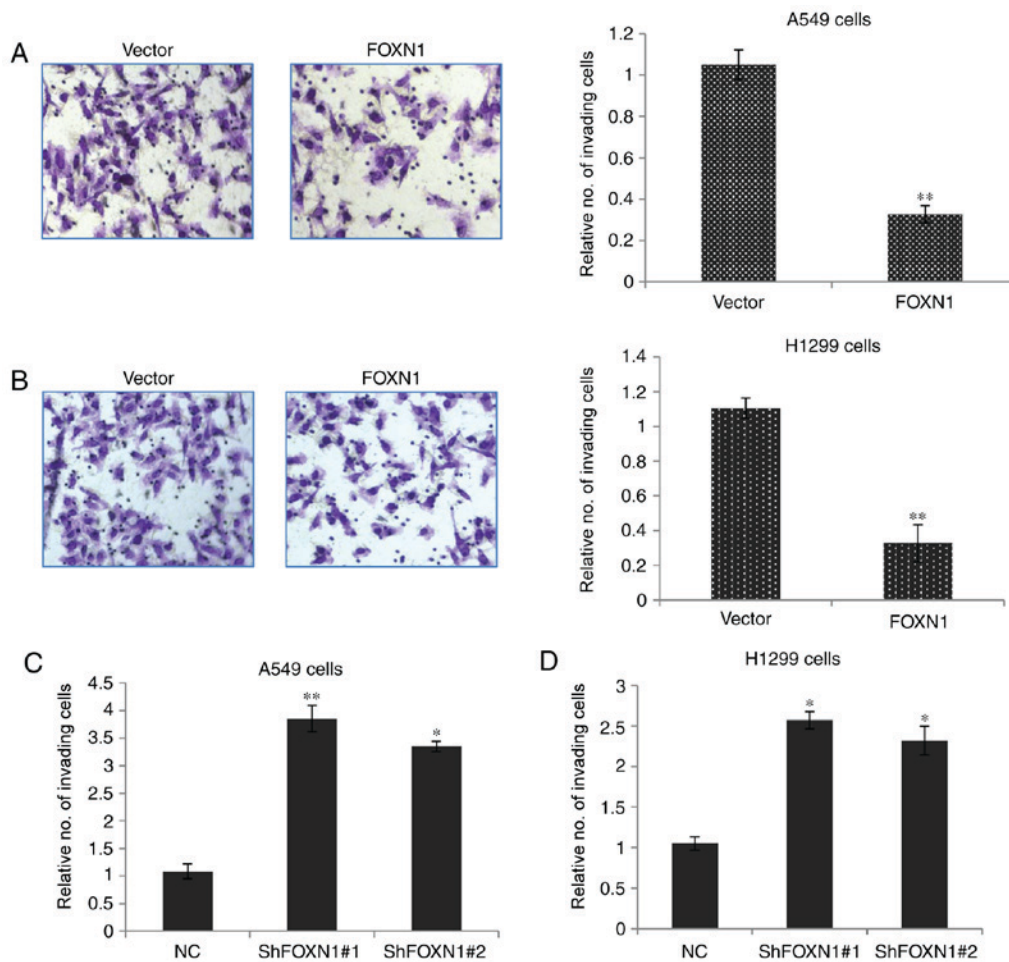


Figure 3. FOXN1 inhibits non-small cell lung cancer cell invasion, as determined by a Transwell assay. (A) A549 or (B) H1299 cells were transfected for 48 h with lentivirus expressing FOXN1 to induce FOXN1 overexpression or a control vector. Images are of cells stained with 1% crystal violet staining, 20x magnification. (C) A549 or (D) H1299 cells were transfected for 24 h with shRNA targeting FOXN1 to inhibit FOXN1 expression or NC shRNA. The number of invading cells was expressed as the mean \pm standard deviation of three independent experiments. * $P < 0.05$ and ** $P < 0.01$, vs. corresponding control group. FOXN1, forkhead box N1; shRNA, short hairpin RNA; NC, negative control shRNA.

FOXN1 inhibits NSCLC cell invasion. To examine whether FOXN1 exerts an effect on the A549 and H1299 cell invasive ability, a Transwell assay was performed. In this assay, the number of cells that migrated from the upper to the lower side of the membrane in the chambers was calculated. As shown in Fig. 3A and B, the number of invading cells was markedly decreased in the FOXN1 overexpression plasmid-transfected cells as compared with the control group. By contrast, a reduced expression of FOXN1 demonstrated the potential to promote cell invasion in A549 (Fig. 3C) and H1299 cells (Fig. 3D), respectively. These results suggested that the overexpression of FOXN1 clearly suppressed the invasion ability of A549 and H1299 cells.

EZH2 and β -catenin are novel target genes of FOXN1. In order to understand the underlying mechanisms of FOXN1 in suppressing NSCLC, a quantitative chromatin immunoprecipitation (ChIP) assay was performed in A549 and H1299 cells, with normal IgG used as a negative control. Different genes involved in proliferation and invasion were selected for investigation, including E-cadherin, N-cadherin, r-catenin, β -catenin, caspase-3, caspase-7, hypoxia-inducible factor 1 α (HIF1A), EZH2, Snail and Twist1. As shown in

Fig. 4A, in A549 cells, the bindings of FOXN1 to E-cadherin and β -catenin promoters were significantly higher when compared with that of the normal IgG. Similar binding enrichment was also detected in H1299 cells (Fig. 4B). FOXN1 is known to typically function as a transcription repressor factor (12); thus, to further support this observation, a dual-luciferase reporter assay was conducted in A549 and H1299 cells. Cells co-transfected with shFOXN1 along with EZH2 or β -catenin promoter-driven luciferase reporters exhibited a moderately enhanced luciferase activity, as compared with cells transfected with *Renilla* and pcDNA3.1 plasmids (Fig. 4C). By contrast, in the FOXN1 overexpression group, reduced luciferase activity was detected (Fig. 4D).

Inhibition of the EZH2/ β -catenin cascade is involved in the suppressive effect of FOXN1 on NSCLC. Consistent with the results of the dual-luciferase reporter assay, in A549 and H1299 cells subjected to FOXN1 knockdown, significantly increased mRNA levels of EZH2 and β -catenin were confirmed by RT-qPCR assay. By contrast, ectopic overexpression of FOXN1 in A549 and H1299 cells markedly suppressed the EZH2 and β -catenin mRNA levels (Fig. 5A). Furthermore, as

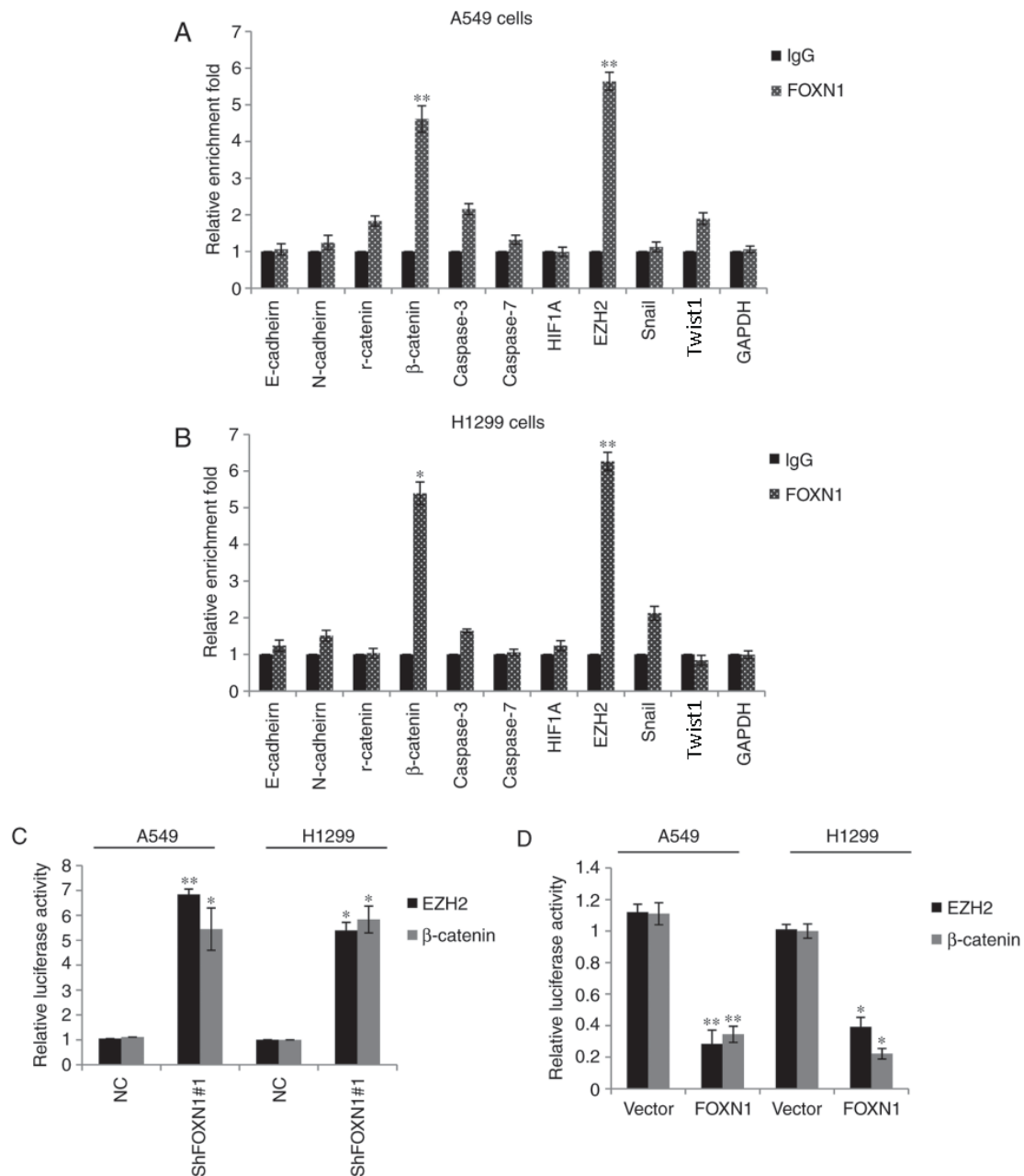


Figure 4. EZH2 and β -catenin are novel target genes of FOXN1. Quantitative chromatin immunoprecipitation experiment was performed in (A) A549 or (B) H1299 cells using an antibody against FOXN1 or normal IgG (negative control). The binding of FOXN1 on EZH2 and β -catenin promoters were measured. A549 or H1299 cells were transfected with EZH2 or β -catenin promoter luciferase constructs along with (C) FOXN1 silencing molecules or NC shRNA, or (D) FOXN1 overexpression construct or pcDNA3.1 plasmids. The luciferase activities were measured and normalized to those of *Renilla*. Values are represented as the mean \pm standard deviation of three independent experiments. * $P < 0.05$ and ** $P < 0.01$, vs. corresponding control group. FOXN1, forkhead box N1; shRNA, short hairpin RNA; NC, negative control shRNA; EZH2, enhancer of zeste homolog 2.

shown in Fig. 5B, shFOXN1 transfection increased the protein levels of EZH2 and β -catenin, while overexpression of FOXN1 decreased the protein levels of EZH2 and β -catenin.

To establish further insight into the mechanism, the study subsequently examined whether EZH2 and β -catenin were involved in the suppressive effect of FOXN1 on NSCLC cell proliferation and invasion. An MTT assay was performed, and revealed that the additional knockdown of EZH2 or β -catenin by shRNA transfection was observed to partially rescue the effect of FOXN1 knockdown on the proliferation potential of A549 and H1299 cells, as compared with the cells with FOXN1 knockdown alone (Fig. 5C). In the Transwell assay,

A549 or H1299 cells co-transfected with shFOXN1 + shEZH2 or shFOXN1 + sh β -catenin demonstrated partial alleviation of the effect of FOXN1 knockdown on the NSCLC cell invasive potential (Fig. 5D).

Discussion

Cell proliferation and metastasis are two important malignant characteristics in NSCLC, which are accompanied by the dynamic changes in the gene expression (13). The results of the present study demonstrated that FOXN1 expression regulated the NSCLC cell growth and invasion.

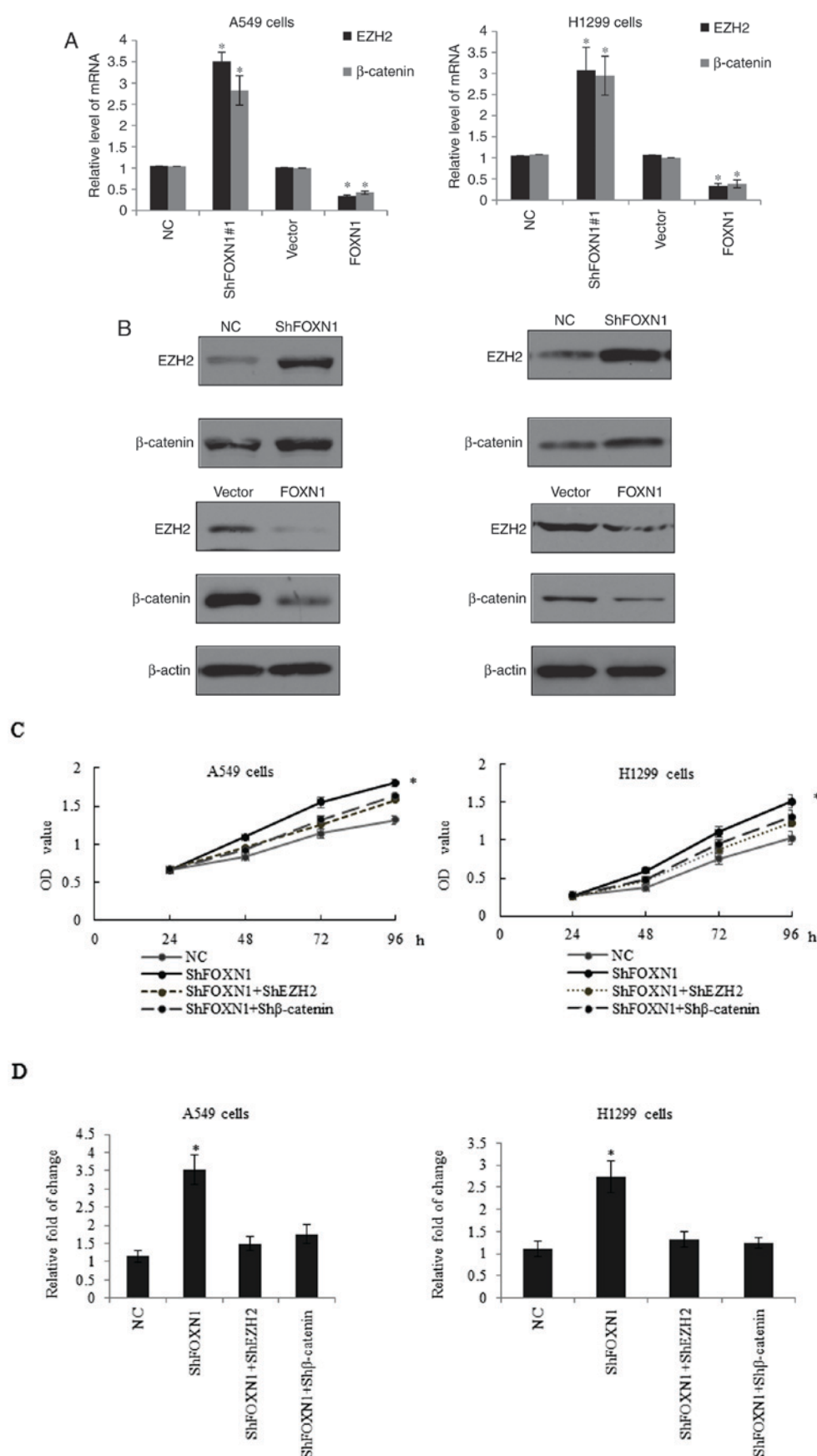


Figure 5. Inhibition of the EZH2/β-catenin cascade is involved in the suppressive effect of FOXN1 on NSCLC. A549 or H1299 cells were transfected with NC shRNA, shRNA targeting FOXN1, FOXN1 overexpression construct or a control vector. (A) EZH2 and β-catenin mRNA levels were measured by reverse transcription-quantitative polymerase chain reaction, while (B) the relative protein levels were detected by western blot analysis. (C) MTT and (D) Transwell assays were performed in A549 cells or H1299 cells transfected with NC, shFOXN1, shFOXN1 + shEZH2, or shFOXN1 + shβ-catenin. Data are represented as the mean ± standard deviation of three independent experiments. * $P < 0.05$ vs. corresponding control group. FOXN1, forkhead box N1; shRNA, short hairpin RNA; NC, negative control shRNA; EZH2, enhancer of zeste homolog 2; OD, optical density.

Overexpression of FOXN1 could inhibit the NSCLC progression. In the NSCLC patients, higher expression of FOXN1 is associated with better prognosis. To our knowledge, this is the first report about the function of FOXN1 in NSCLC.

The current study further revealed that FOXN1 served major roles in NSCLC invasion by directly targeting EZH2 and β -catenin therefore suppressing EZH2 and β -catenin expression. By epigenetically silencing tumor suppressor genes, EZH2 has been reported to promote carcinogenesis, and overexpression of EZH2 was correlated with NSCLC cell migration (14). Furthermore, increased expression of EZH2 has been correlated with lymph node metastasis, tumor size and TNM stage in NSCLC (15). It has also been reported that the expression of EZH2 was significantly higher in NSCLC brain metastases (16). A previous study reported that inhibition of EZH2 sensitized BRG1 and epidermal growth factor receptor mutant lung tumors to topoisomerase II inhibitors, while knockdown of EZH2 reversed the cisplatin-resistance of NSCLC and gastric cancer cells (17). The regulation of EZH2 has been revealed by numerous studies in recent years; for instance, microRNA-138 inhibited tumor growth through repression of EZH2 in NSCLC and osteosarcoma cells (18,19). Furthermore, the long non-coding RNA MALAT1 enhanced the oncogenic activities of EZH2 in castration-resistant prostate cancer (20). These aforementioned findings regarding EZH2 were consistent with the observations of the current study. Furthermore, the present results reported that FOXN1 may be a novel regulator of EZH2 in NSCLC. The inhibition of EZH2 by FOXN1 is of importance in the NSCLC suppression.

It has previously been reported that overexpression of β -catenin was associated with poor overall survival rates in patients with Stage IA-IIA squamous cell lung cancer irrespective of adjuvant chemotherapy (21). By activating the β -catenin signaling pathway, DEAD-box helicase 5 promoted the proliferation and tumorigenesis of NSCLC (22). In addition, through promoting β -catenin degradation, Shisa3 was associated with prolonged survival times in lung cancer (23). The majority of these studies focused on the negative regulation of β -catenin; however, transcriptional repression may also be another important mechanism for β -catenin inhibition (24,25). The present study reported that FOXN1 was a negative regulator of β -catenin, thus contributing toward NSCLC repression. Notably, it was observed that FOXN1 served major roles in NSCLC proliferation and invasion by directly targeting EZH2 and β -catenin, which suggested that FOXN1 may be a multifunctional regulator in NSCLC.

In conclusion, the present study revealed that FOXN1 inhibited the NSCLC progression and served as a tumor suppressor. EZH2 and β -catenin may be potential targets for the development of anti-proliferation and anti-invasion in the treatment of NSCLC. However, further investigation is required in order to determine whether the current results may be translated into the development of valuable NSCLC prognosis biomarkers.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

XJ conceived and designed the work. WW analyzed the data provided technical assistance for the transwell assay. XX and YJ analyzed the data. XJ wrote the paper. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All human tissues are collected under Institutional Review Committee (IRB) and Health Insurance Portability and Accountability Act (HIPAA) approved protocols.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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