FOXH1 Promotes Tumor Growth and Metastasis via Regulation of Wnt/β-catenin Signaling Pathway in Lung Cancer

Jun Zhang (ciwei870110@126.com)

Department of Morphological Experiment Center, Medical College of Yanbian University, Yanji City (133000), Jilin Province, China. https://orcid.org/0000-0001-7111-0205

Xian Zhang

Yanbian University Hospital

Shasha Yang

Yanbian University

Yanqiu Bao

Yanbian University Hospital

Dongyuan Xu

Yanbian University

Lan Liu

Yanbian University Hospital

Primary research

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Abstract

Background: Forkhead box protein H1 (FOXH1) is upregulated in a variety of cancer types but its expression patterns and specific functions in lung cancer are unclear at present. The main objective of the present study was to establish whether FOXH1 plays a role in regulation of lung cancer progression.

Methods: The TCGA and Kaplan-Meier plotter dataset was analyzed for possible association between FOXH1 expressions in lung cancer tissues and patient prognosis. A549 and PC9 cells were transfected with shRNA targeting FOXH1 mRNA. The Cell Counting Kit-8 (CCK8), plate clone formation, soft agar, wound healing, transwell invasion and flow cytometry assay were performed to detect the cell proliferation, migration and invasion in lung cancer cells. Tumorigenicity was examined in mouse model system. Western blot analysis was used to detect FOXH1, Matrix metalloproteinase 2 (MMP2), Vimentin, N-cadherin, E-cadherin, Snail, Slug, p-GSK-3β, β-catenin and Cyclin D1. The β-catenin activity was measured by luciferase reporter system assay.

Results: FOXH1 expression in the lung cancer was determined using the TCGA and Kaplan-Meier plotter databases. Higher expression of FOXH1 was observed in tumor tissue relative to normal tissue, which was associated with reduced overall survival. FOXH1 knockdown resulted in significant inhibition of the proliferation, the cell cycle, the migration as well as the invasion of lung cancer cells. Then, we confirmed that FOXH1 knockdown can significantly slow down tumor growth and tumor cell proliferation in vivo using the mouse model. It also significantly reduced the migration and invasion capabilities of lung cancer cells. Using Western blot analyses, we found that FOXH1 depletion inhibited the epithelial-mesenchymal transition of lung cancer cells through downregulation of the mesenchymal cell markers Snail, Slug, MMP2, N-cadherin, and Vimentin, and upregulation of the epithelial marker E-cadherin. Moreover, FOXH1 knockdown significantly downregulated β-catenin and its downstream targets, such as p-GSK-3β and cyclin D1, and also led to direct suppression of β-catenin activity, as determined by the luciferase reporter system assay.

Conclusion: In conclusion, FOXH1 promotes proliferation, migration, and invasion of lung cancer cells via regulation of Wnt/β-catenin signaling. FOXH1 is a prognostic marker and a potential therapeutic target for lung cancer treatment.

Background

Lung cancer is the second most common cancer type worldwide in both men and women. It poses a considerable threat to human health and life and has been the leading cause of cancer death by far, making up about 25% of all cancer-related deaths[1, 2]. Compared to other malignant tumor types, lung cancer has higher incidence and mortality rates, which is attributed to its poorer early diagnosis[3]. In advanced lung cancer, the acquisitive ability of the cancer cells to move and invade nearby tissues is associated with their high metastatic potential. Numerous regulatory mechanisms are involved in tumor metastasis, such as epithelial-mesenchymal transition (EMT), an important physiological process that allows epithelial cells to obtain invasive and motile activity of the mesenchymal cells[4]. The Wnt/β-

catenin signaling pathway regulates cell morphogenesis, gene transcription, differentiation, and proliferation [5, 6]. Slug, Vimentin, MMPs, and other downstream target genes of Wnt/β-catenin are key regulators of EMT [7]. In the early stages of metastasis, cancer cells undergo an EMT-like process controlled by active Wnt/β-catenin signaling[8]. Therefore, elucidation of the mechanisms underlying tumor invasion and metastasis may be the key to improving survival rates of patients with lung cancer.

Forkhead box protein H1 (FOXH1), also designated Fast1, is a member of the FOX family, an evolutionarily conserved transcription factor family [9]. FOXH1 has been studied in several types of cancer cells [10–12], but the function of FOXH1 implicated in cancer progression was still poorly understood. but the function of FOXH1 implicated in cancer progression is still poorly understood. FOXH1 is also reported to be linked with the EMT process in Xenopus tropicalis. EMT can remarkably regulate malignant biological expression of tumor-associated molecules [13–15]. However, there is no data on the expression patterns and potential influence of FOXH1 in lung cancer reported to date. Therefore, we assessed these in the present study, as well as tested the hypothesis that FOXH1 could promote the progression, migration, and invasion of lung cancer by activating Wnt/β-catenin signaling.

Materials And Methods

Antibodies and reagents

Primary antibodies against FOXH1 (Fast1/2), MMP2, Vimentin, N-cadherin, Snail, E-cadherin, Slug, β-actin, β-catenin, p-GSK-3β and cyclinD1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), HRP-conjugated anti-mouse, anti-goat, anti-rabbit secondary antibodies (Zhongshan golden bridge biotechnology, BJ, China), Giemsa stain (Solarbio, BJ, China), Soft agar (Becton Dickinson and Company, Bedford, MA, USA), CCK-8 reagent (BestBio, Shanghai, China) were used in this study.

Cell culture

The human lung cancer cell lines, i.e. PC9, A549, H1299 (NCI-H1299, carcinoma, non-small cell lung cancer), H460 (NCI-H460, carcinoma, large cell lung cancer) and H1975 (NCI-H1975, adenocarcinoma, non-small cell lung cancer), together with human bronchial epithelial cell line (16HBE) were provided by the American Type Culture Collection (ATCC; Manassas, VA, USA). All cell lines were maintained and cultured in Dulbecco's modified Eagle's medium (DMEM Gibco, NY, USA) supplemented with 10% Penicillin-Streptomycin Liquid (Solarbio, BJ, China), 10% fetal bovine serum (FBS, Gibco, NY, USA) in an incubator containing 5% CO^2 maintained at 37°C.

Gene coexpression with FOXH1 in TCGA dataset

The Cancer Genome Atlas (TCGA) is a large-scale cancer genomics program that has molecularly characterized 33 primary cancer types including 3 subtypes of lung cancer. The Oncomine database is a database based on a gene chips and a integrated platform of data mining. In this data, the conditions of screening and mining data can be set up according to own requirements. In this study, the screening conditions were set as: (1) “Cancer Type: Lung cancer”; (2) “Gene: FOXH1”; (3) Dataset Name: TCGA Lung

2; (4) “Analysis Type: Cancer vs Normal Analysis”; (5) Threshold setting conditions (P value < 0.05). As a result, the gene expression profiles including “Lung Adenocarcinoma”, “Papillary Lung Adenocarcinomar” and “Squamous Cell Lung Carcinoma” were selected from the Oncomine database using the above filters. A total of 1016 specimens including 397 normal lung tissues and 619 lung cancer tissues were available in this study (Data reporter ID: 08-145671225; RefSeq Genes: UCSC refGene, July 2009, hg18, NCBI 36.1, March 2006).

Western blotting analysis

For preparing entire cell lysates, cells underwent lysing treatment on ice in RIPA lysis buffer that contained 150 mM NaCl, 50 mM Tris-HCL [pH 7.4], sodium deoxycholate (0.25%), NP40 (1%), 1 mM EDTA, sodium dodecyl sulfate [SDS, 0.1%], mM PMSF. 10 μg/ml aprotinin and 10 μg/ml leupeptin. SDS-PAGE was used to resolve 20 μg of the protein of the cell or the tissue lysate, followed by a transfer to a nitrocellulose membrane (Millipore, Billerica. MA. USA). Tris-buffered saline solution that contained 0.05% Tween 20 were used for blocking the resultant for 1 h at room temperature. The relevant antibodies were added to probe the blots overnight at 4°C. Followed by washing, the blots were probed again with the species-specific secondary antibody coupled to the horseradish peroxidase. Dilutions of all primary and secondary antibodies are listed in Additional file 1 Table. An ECL prime Western blotting kit (Amersham Pharmacia Biotech, Buckinghamshire, UK) were used to assess the immunoreactivity, following the instruction of manufacturer.

Lentiviral vectors and transduction expressed by short hairpin RNA (shRNA)

Lentivirus vectors which encode a shRNA targeting FOXH1 (target sequence: CCGGAGTGAGGGCTTCAGCATCAAGCTCGAGCTTGATGCTGAAGCCCTCACTTTTTTTG; Product Type: SHCLND-NM_003923; TRC number: TRCN0000431226) or shRNA non-targeting control (target sequence: CCGGCAACAAGATGAAGAGCACCAACTCGAGTTGGTGCTCTTCATCTTGTTGTTTT; Product Type: SHC202V) were used to transduce A549 and PC9 cells following the instruction of manufacturer (Sigma Chemical Co). Briefly, $3x10^5$ cells were cultured overnight in a 6-well plate, followed by a transduction with lentiviral particles at 1 multiplicity of infection (MOI) with 8 μg/ml polybrene. The transduction efficiency of each vector was confirmed via the western blotting analysis.

CCK8 assay

The proliferation of cell was measured using the Cell Counting Kit 8 (CCK-8) assay. Cells receiving various treatment or transfection ($1 \cdot 10^3$ cells/well) were planted in the 96-well plates. Then 10 μl CCK-8 solution was used to mix cells in every well respectively after being incubated for 0, 24, 48, 72, 96 and 120 h. Another 4 h of incubation helped to obtain the absorbance values of 450 nm wavelength.

Colony formation assay

Cell culture medium was used to adjust the concentration of the cells to $2 \cdot 10^3$ cells/ml. Then 2 ml cells were plated in each of the 6-well plates, followed by 14 days of incubation. The medium was changed,

and the cell state was observed every 2 days. At the end of incubation, Phosphate-buffered saline (PBS) was applied to wash the cells twice, and 4% paraformaldehyde was used to fix the cells for 15 minutes at 4℃. Followed by washing in PBS twice, the cells were stained with Giemsa solution (2 ml/well) for 30 min, and cell clones were counted.

Soft agar colony formation assay

Cells were suspended in the growth medium that contained 0.35% agar and $1.5 \cdot 10^5$ cells were plated in 6-cm plates (3 ml/well) on the top of a layer of growth medium that contained 0.7% agar (5 ml/well). The agar was added with 500 µl of growth medium coupled with FBS (10%). The cells were incubated for two weeks at 37℃, then the formation of viable colonies was observed using an optical microscope.

Flow Cytometry

After the cultured cells were trypsinized and washed, 75% ethanol were added to suspend the cells and keep at 4℃ overnight to fix the cells. The fixed cells underwent 30 min of incubation with the RNase reagent at 37℃, followed by 30 min of staining with the propidium iodide (PI; 1:100, Propidium iodide cycle Detection kit II, BD Bioscience, USA). The stained cells were acquired using FACS Caliber (BD Company, USA) and analyzed with the standard software. The data were presented as percentages of cells distributed at cell cycle in G1 phase, S phase, and G2 phase, respectively. At least three parallel tests were conducted.

Animal experiments

The present experiment employed female BALB/c nude mice were purchased from Charles River Laboratories (Charles river, Beijing, China), aged at four weeks and maintained under specific pathogen free (SPF) condition in the animal care facility. The mice' health is monitored twice a day during feeding and no adv. adverse events were observed the environmental conditions were 21 ± 2 ℃ and 55%±10% humidity. Each mouse was placed in a 300×210×130mm size cage, they drank water freely and were provided with enough food. All the mice were anesthetized with isoflurane that controlled by a small animal anesthesia machine. The transduction of A549 cells relied on a lentivirus vector that encoded an shRNA targeting FOXH1. After cell harvest, we resuspended $3 \cdot 10^6$ cells in 60 µl DMEM coupled with 20 µl Matrigel (Becton Dickinson and Company, Bedford, MA, USA), followed by orthotopical injection into mouse' right shoulder. Tumor diameter was recorded using a vernier caliper every 2 days. Tumor volume was calculated follows formula: tumor volume (mm^3) = (length×$width^2$)/2. Tumor nodule collection was performed after the euthanization of mouse on day 36. According to the AVMA Guidelines for the Euthanasia of Animals, all the mice were euthanized with an intraperitoneal injection of a three-fold dose of barbiturates. After that we removed tumors immediately and measured the length, width and weight of the tumors. No mice died accidentally during feeding. All surgical procedures and experimental protocols were approved by the Animal Care and Use Committee of Yanbian University Faculty of Medicine. The procedures were performed according to the recommendations of the Yanbian University Institutional Animal Care Use Committee (IACUC).

Transwell assay

The transfected A549 and PC9 cells were resuspended in 100 μl DMEM free of serum and plated in upper chamber of transwell device, with $5 \cdot 10^5$ cells/well. 600 μl complete medium were added into lower chamber as the chemical attractant. After 48 h of incubation in the incubator at 37°C, the cells in upper chamber were removed, and 4% paraformaldehyde were used to fix the cells that invaded the lower chamber, followed by washing in PBS twice and staining with Giemsa solution.

Wound Healing assay

The transfected A549 and PC9 cells were firstly seeded into a 6-well plate at $3 \cdot 10^5$ cells/well. After the cells reached 80% confluence, the tip of a 200 μl pipette was used to scratch the monolayers, and the culture was continued in the FBS-free medium. Subsequently, the wound healing occurred under an inverted microscope and photographed at 0 and 64 h using a digital camera (Olympus, Japan). ImageJ was used to analyze the results of the scratch experiment and calculate the scratch area.

Luciferase reporter gene assay

Luciferase Assay System (Promega, Madison, WI, USA) was used to measure the transcriptional activity assays following the instruction of manufacturer. TOP flash/FOP flash reporter plasmids were used to assess the transcriptional activity dependent on TCF/LEF in Transfected sh-FOXH1 A549 cells and PC9 cells. A Dual-Luciferase Reporter (DLR) Assay Kit (Promega) was applied to measure the luciferase activity following the manufacturer's instruction. A luminometer (Lumat LB9507, Berthold, Bad Wildbad, Germany) was employed to gauge the Firefly and Renilla luciferase activity for the convenience of normalization.

Immunohistochemistry (IHC)

Formalin-fixed, paraffin-embedded tissue sections were cut to 4 μm thickness. After rehydration, the slides were pretreated and deparaffinized through microwave epitope retrieval process (750 W for 15 min in 10 mM citrate buffer, pH 6.0) before application of the primary antibody. The primary antibody (FOXH1 1:100, Ki-67 1:100) was detected using a secondary biotinylated antibody and development by DAB substrate (DAKO, Glostrup, Denmark). Dilutions of primary antibodies are listed in Additional file 1 Table. Staining showed absolute specificity to the nucleus without discernible off-target signal. [16]. Counter-staining was completed with Meyer's hematoxylin.

Analysis

SPSS 17.0 software (IBM, USA) and SigmaPlot 14.0 software (Systat Software, USA) were applied to the statistical analyses. All the experiments were carried out repeatedly for three times, with data represented as the mean ± SD. Student's t-test was used to compare continuous quantities in different groups, and one-way analysis of variance (ANOVA) was carried out for comparisons among ≥ 2 groups. Kaplan Mier methods and logrank test were used to analyze survival data. The p value < 0.05 was considered with statistical significance.

Results

FOXH1 expression is upregulated in lung cancer

The Cancer Genome Atlas (TCGA) is a large-scale cancer genomics program that has molecularly characterized 33 primary cancer types including 3 subtypes of lung cancer. In order to test whether FOXH1 is differentially expressed in lung cancer samples, the normalized gene expression data from TCGA were extracted, and FOXH1 gene expression levels in lung cancer samples were compared with normal tissues using t-tests. The results showed that FOXH1 expression levels are significantly higher in lung adenocarcinoma (p<0.05), papillary lung carcinoma(p<0.05) and squamous cell lung carcinoma (p<0.05) compare to normal samples. The mean fold change of FOXH1 expression in lung adenocarcinoma, papillary lung adenocarcinoma and squamous cell lung carcinoma were 0.168, 0.258, and 0.225, respectively (Fig 1a). Next, to determine whether FOXH1 is a prognostic marker for lung cancer, we included lung adenocarcinoma samples with patient's cancer progression and survival data available in TCGA dataset. The lung cancer patients were stratified into FOXH1 high and FOXH1 low groups using the median of FOXH1 gene expression levels in lung cancer samples. Both progression free survival and overall survival were assessed by Kaplan-Meier plots and logrank tests (Fig 1b). Patients whose samples have low FOXH1 expression level have significantly longer progression free survival and overall survival. Poorer prognosis observed for lung adenocarcinoma patients with higher FOXH1 expression in tumor implies FOXH1 is a prognosis marker and can be a potential treatment target for lung cancer.

The immunohistochemistry staining was applied to determined FOXH1 protein expression levels in the paraffin-embedded lung cancer tissues. Representatively, immunohistochemical detection of FOXH1 in the lung cancer and normal lung tissues according to different samples of lung cancer revealed that the immunoreactivity of FOXH1 was barely expressed in surrounding non-tumor and normal lung tissue compared with the tumor. FOXH1 protein expression was highly detected in the nuclei of tumors (Fig 1c). To establish the functional role of FOXH1 in lung cancer, its expression levels were examined in various lung cancer cell lines and the 16HBE cell line derived from normal lung epithelial cells. As shown in Fig 1d, the expression of FOXH1 in all the lung cancer cell lines was higher than that in 16HBE normal cell line. While A549, PC-9 and H1299 cells had significantly higher FOXH1 expression, the FOXH1 expression in H460 and H1975 cells were only marginally higher. Since A549 and PC9 cell lines simulate lung cancer tissue in terms of the FOXH1 expression level, they were selected for the further experiments.

Knockdown of FOXH1 suppresses growth of lung cancer cells

To test the hypothesis that FOXH1 is an oncogene whose elevated expression in lung cancer cells drives the tumor growth and proliferation, effects of FOXH1 knockdown on cellular growth and cell cycles of lung cancer cells were examined in subsequent experiments. The two lung cancer cell lines, PC9 and A549, were transfected with either a sh-RNA specifically targeting FOXH1 (sh-FOXH1) or a negative control sh-RNA (sh-NC), then a Western blot assay was applied. As shown in Fig 2a, FOXH1 was effectively knocked down in both sh-FOXH1 transfected lung cancer cell lines and its expression level reduced >95% 72 hours after transfection compared to cells transfected with negative control sh-RNA (sh-NC). And the FOXH1 expression level stayed low in the following 14 days. CCK8 assays revealed that the

proliferation of lung cancer cells was suppressed by FOXH1 silencing while the negative control cells proliferated at the same speed as the un-transfected cells (Fig 2b). We also examined the FOXH1 depleted cells in the cell colony formation assays and the soft agar colony formation assays that are extensively applied for evaluating the cellular proliferation *in vitro*. The colony forming capability possessed by A549 and PC9 cells was markedly decreased upon FOXH1 depletion (Fig 2c) and the soft agar colony formation assay showed a significant decrease in colony counts when the A549 and PC9 cells were depleted of FOXH1 (Fig 2d). These results indicated that knockdown of FOXH1suppresses growth of lung cancer cells.

FOXH1 down-regulation inhibits lung cancer cell proliferation *in vivo*

To confirm the oncogenic effect of FOXH1 *in vivo*, a xenograft tumor-bearing model was established by inoculating sh-NC or sh-FOXH1 transfected A549 cells into the nude mice. Thirty-six days after inoculation, the xenografted tumors were harvested and the tumor sizes were measured. As shown in Fig 3a, the tumors induced by sh-FOXH1 transfected A549 cells were significantly smaller than those induced by sh-NC transfected A549 cells in xenografted mice (Fig 3a). Tumors induced by the sh-FOXH1 transfected cells grew significantly slower compared with those in the control mice (Fig 3b). IHC analysis of tumor tissue verified that FOXH1 was barely expressed in the tumor induced by sh-FOXH1transfected cells (Fig 3c). Additionally, we also examined the expression of Ki-67 antigen, a cellular proliferation marker in mouse tumors. As shown in Fig 3c, the proportion of the Ki-67 positive cells was significantly decreased in the tumor induced by FOXH1 depleted A549 cells. Collectively, these results suggested that FOXH1 depletion efficiently suppresses tumor growth *in vivo*.

Knockdown of FOXH1 suppresses lung cancer cell invasion and migration

Since cancer cell invasion and migration are essential for cancer advancement and malignancy, we investigated how FOXH1 expression affects the mobility of lung cancer cells.

Effects of FOXH1 on cell proliferation and migration were investigated through wound healing assay. FOXH1-suppressed A549 and PC9 cells showed a marked decrease proliferative and migration ability compared to the sh-NC control (Fig 4a). Cell cycle progression was further assessed via flow cytometry. Knockdown of FOXH1 led to an increase in G1 phase from 64.5% to 74.88% (A549 cells) or 55.85% to 66.18% (p <0.05) (PC9 cells), and decrease in G2 phase in A549 and PC9 cells from 10.52% to 6.40% or 23.30% to 13.55% (p <0.05), and decrease in S phase in A549 and PC9 cells from 24.89% to 18.73% or 20.85% to 20.28% (Fig 4b) indicating the FOXH1 knockdown inhibits cell cycle progression. In the transwell invasion assay, suppression of FOXH1 led to cell invasion decrease from 108 to 16 (p <0.001) in A549 cells and from 162 to 19 (p <0.001) in PC9 cells, relative to corresponding control groups (Fig 4c). These results clearly suggest that knockdown of FOXH1 inhibits migration, proliferation and invasion of lung cancer cells.

Knockdown of FOXH1 decreased EMT marker alterations in PC9 and A549 cells

As cellular invasion and migration are the main phenotypes of epithelial-mesenchymal transition (EMT), a process that involves in tumor metastatic expansion or cancer advancement, we further assessed whether FOXH1 knockdown affects EMT-specific molecules in lung cancer cells using the A549 and PC9 cell lines that stably express sh-FOXH1 and sh-NC.

Western blot analysis results of A549 and PC9 cells with FOXH1 knockdown are shown in Fig 5. The mesenchymal markers Matrix metalloproteinase-2 (MMP2), Vimentin, N-cadherin, Snail and Slug were significantly down-regulated, whereas the epithelial markers E-cadherin was notably upregulated by FOXH1 depletion in both lung cancer cell lines (Figs 5a and b). No changes were found in the negative control cell lines. Our findings indicate that FOXH1 can regulate the metastatic potential of lung cancer cells via activation of EMT.

Knockdown of FOXH1 suppresses β-Catenin signaling in lung cancer

Wnt/β-catenin signaling pathway is essential in regulating multiple processes, including cell proliferation, migration, and invasion in various cancer types, it may also play an important role in EMT. To further explore and clarify the molecular mechanisms underlying FOXH1-mediated tumor promotion and malignancy in lung cancer, effects of FOXH1 on Wnt/β-catenin signaling pathway were assessed. First, we evaluated expression of β-catenin signaling molecules β-catenin, cyclin D1 and p-GSK-3β via western blot analysis.

As shown in Fig 6a, the protein levels of β-catenin and its two downstream targets, cyclin D1 and p-GSK-3β, were significantly decreased in A549 and PC9 cells transfected with sh-FOXH1. Next, we used a Dual-Luciferase Reporter (DLR) assay for further determination of changes in β-catenin activity induced by FOXH1. TCF/LEF-dependent transcriptional activity of β-catenin in A549 and PC9 cells was measured after transfection with TOP/FOP flash reporter plasmids. Compare to the sh-NC control group, sh-FOXH1 induced a significant decrease in TOP luciferase reporter activity (Fig 6b), thus a decreased Wnt/β-catenin activity. Based on the results above, we conclude FOXH1 exerts its activity upstream of the β-catenin, and consequently, its silencing inhibits activation of Wnt/β-catenin signaling in lung cancer.

Discussion

We observed the upregulation of FOXH1 in lung cancer tissues using the Cancer Genome Atlas (TCGA) datasets. We additionally confirmed that FOXH1 levels in lung cancer cells increases. Moreover, we demonstrated that FOXH1 knockdown blocks the Wnt/β-catenin pathway in lung cancer cells, thereby resulting in the inhibition of proliferation, migration, and invasion of these cells. Mechanistically, FOXH1 knockdown directly suppresses β-catenin activity, which mediates oncogenic functions by regulating the expression of EMT-associated Slug, Vimentin, E-cadherin, and MMPs.

The human FOXH1 gene is recently reported to be over-expressed in the majority of tissues and some cancer cell lines [10, 17–21].However, the potential relationship between FOXH1 expression and lung cancerogenesis, as well as its implications, has not yet been investigated. Analyses of data from the

comprehensive public cancer database TCGA revealed significantly higher expression of FOXH1 in lung cancer tissues compared to normal tissues (Fig. 1a). In addition, higher FOXH1 expression is associated with reduced overall survival in lung cancer patients (Fig. 1b), supporting our hypothesis that FOXH1 regulates lung cancer progression. FOXH1 expression is thus identified as an important prognostic factor for poor survival in lung cancer.

The series of events promoting tumor cell invasion and migration may have significance in the prognosis of cancer patients [22]. EMT drives metastasis during progression of multiple cancer types, including lung cancer [23, 24]. EMT is stimulated under conditions where epithelial cells lose connexins, like E-cadherin, and acquire mesenchymal marker proteins, like N-cadherin and Vimentin [15]. Identification of EMT status may thus aid in clarifying the mechanisms underlying lung cancer metastasis. An association of FOXH1 with EMT has been previously reported [11, 12]. In our experiments, FOXH1 knockdown inhibited cell proliferation, altered cell cycle, and suppressed cell invasion and migration. Moreover, our xenograft mouse model demonstrated inhibited proliferation of FOXH1-depleted A549 cells *in vivo*. We also showed that FOXH1 knockdown led to significant suppression of mesenchymal cell marker expression (Slug, Snail, MMP2, N-cadherin, and Vimentin) and stimulation of epithelial cell marker expression, further supporting the correlation of FOXH1 and EMT progression.

The Wnt/β-catenin signaling pathway regulates cell morphogenesis, gene transcription, differentiation, and proliferation [5, 6]. Slug, Vimentin, MMPs, and other downstream target genes in the Wnt/β-catenin pathway are key regulators of EMT[7]. In the early stages of metastasis, cancer cells undergo an EMT-like process that is controlled by Wnt/β-catenin signaling activation [8]. Wnt/β-catenin is suggested to be involved in the regulation of different tumor types through modulatory effects on downstream targets, such as c-myc, cyclin D1, and GSK-3β[25–27]. In our experiments, cyclin D1 β-catenin and GSK-3β levels decreased in the FOXH1 knockdown cells. To further verify the relationship between FOXH1 and β-catenin, we subsequently explored the signaling pathway involving these factors using the TOP/FOP luciferase reporter system. FOXH1 knockdown induced significant reduction of TOP/FOP luciferase activity, confirming that the Wnt/β-catenin pathway is activated downstream by FOXH1 in lung cancer cells.

Conclusions

In conclusion, FOXH1 activates Wnt/β-catenin signaling, which facilitates the EMT process in lung cancer cells. Suppressing FOXH1 expression can effectively inhibit lung cancer cell growth, supporting potential the clinical utility of FOXH1 as a novel therapeutic target specific to lung cancer, which should be explored in further studies.

Abbreviations

CCK-8: Cell Counting Kit-8; DAB: Diaminobenzidine; DLR: Dual-Luciferase Reporter; DMEM: Dulbecco's modified Eagle's medium; EMT: epithelial-mesenchymal transition; FBS: Fetal bovine serum; FOXH1:

Forkhead box protein H1; IHC: Immunohistochemistry; MMP2: Matrix metalloproteinase; NC: negative control; PBS: Phosphate-buffered saline; shRNA: short hairpin RNA; TCGA: The Cancer Genome Atlas.

Declarations

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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 upon reasonable request.

Ethics approval and consent to participate

All surgical procedures and experimental protocols were approved by the Animal Care and Use Committee of Yanbian University Faculty of Medicine. The procedures were performed according to the recommendations of the Yanbian University Institutional Animal Care Use Committee (IACUC).

Competing interests

The authors declare that they have no competing interests.

Consent for publication

Not applicable.

Authors' contributions

Jun Zhang: Conceptualization, Methodology, Writing - Original Draft.

Xian Zhang: Validation, Data Curation.

Shasha Yang: Investigation.

Yanqiu Bao: Software, Formal analysis.

Dongyuan Xu: Resources, Conceptualization, Supervision, Project administration,

Funding acquisition.

Lan Liu: Conceptualization, Supervision, Funding acquisition.

All authors read and approved the final manuscript

Authors' information

Jun Zhang[1,3,#] , Xian Zhang[2,#], Shasha Yang[1], Yanqiu Bao[2], Dongyuan Xu[1*], Lan Liu[2*]

Jun Zhang and Xian Zhang contributed equally to this work.

Corresponding author: Correspondence to Dongyuan Xu and Lan Liu.

Author details

1.Department of Morphological Experiment Center, Medical College of Yanbian University, Yanji City (133000), Jilin Province, China.

Jun Zhang, Shasha Yang, Dongyuan Xu

2.Department of Pathology, Affiliated hospital of Yanbian University, Yanji City (133000), Jilin Province, China.

Xian Zhang, Yanqiu Bao, Lan Liu

3.Department of Histology and Embryology, Jilin Medical University, Jilin City (132013), Jilin Province, China.

Jun Zhang

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Figures

Fig.1

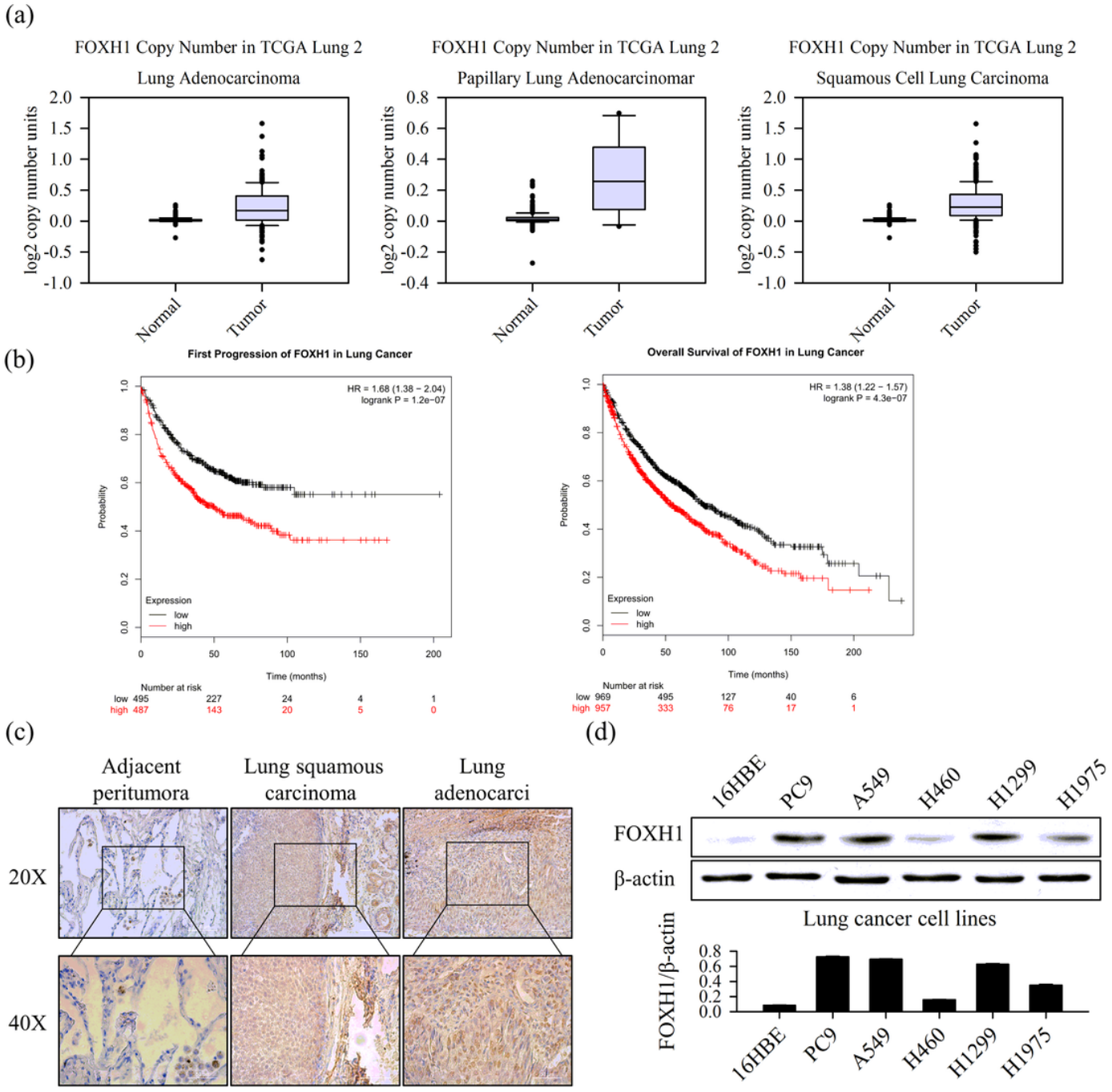

Figure 1

The expression of FOXH1 in lung cancer tissues and cell lines. (a)The lung cancer tumor exhibited an obviously higher FOXH1 expression than normal group in the TCGA database. (b) Higher expression of FOXH1 in lung cancer causes lower survival time of lung cancer. (c) IHC staining for FOXH1 in lung cancer tissues, negative expression of FOXH1 in para-carcinoma tissues, strong expression of FOXH1 in tumor tissues was mainly located in the nucleus. (d) Western blot analysis of FOXH1 expression in the lung cancer cell lines (PC9, A549, H460, H1299 and H1975) and human bronchial epithelial cells (16HBE).

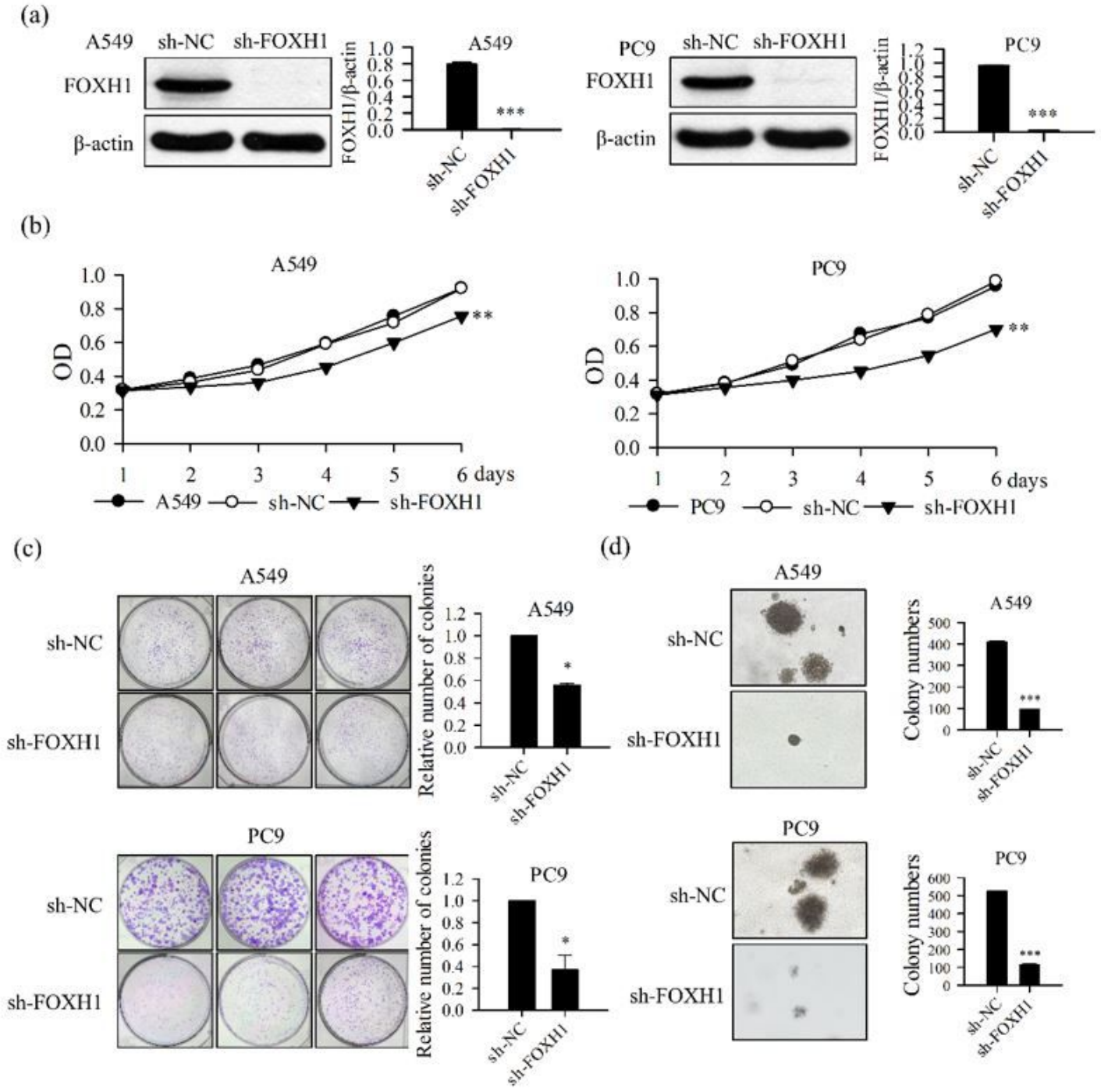

Figure 2

Knockdown of FOXH1 suppresses proliferation of the lung cancer cells. (a) A549 and PC9 cells transfected with the sh-FOXH1 plasmid and the sh-NC (sh-Nontarget Control) plasmid, FOXH1 expression was examined by western blot (***, p□0.001, vs. sh-NC). (b) CCK8 assay applied to determine the cell viability in sh-FOXH1 transfected A549 and PC9 cells (**, p□0.01 vs. sh-NC). (c) Cell colony formation assay was used to determine colony forming capability in sh-FOXH1 transfected A549 and PC9 cells. The relative number of colonies is performed with histogram. (*, p□0.05 vs. sh-NC) (d) Cellular proliferation in sh-FOXH1 transfected A549 and PC9 cells were evaluated via the soft agar colony formation assay. The colonies were counted (***, p□0.001 vs. sh-NC).

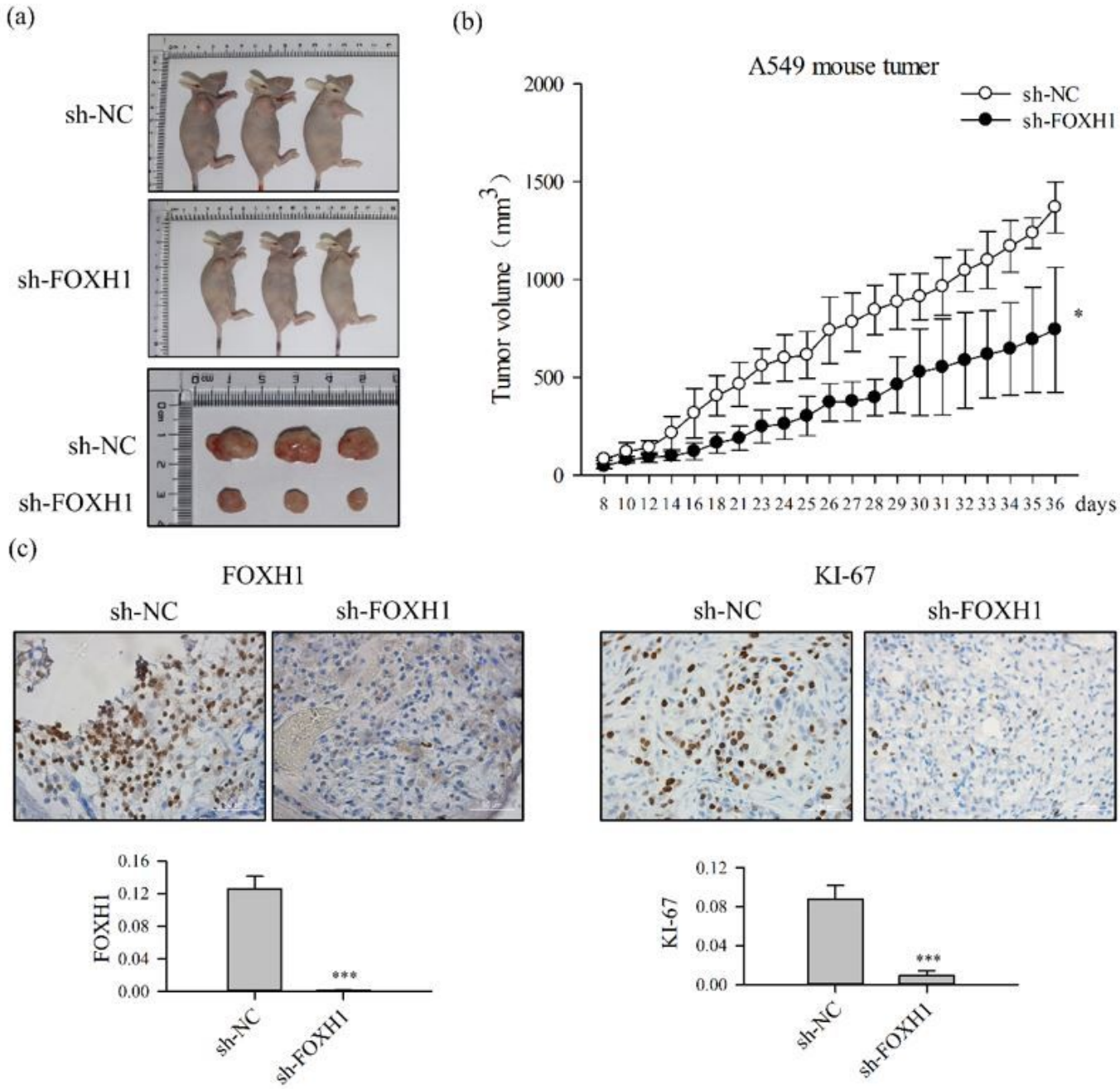

Figure 3

FOXH1 down-regulation inhibits tumorigenicity in mouse model. (a) Growth of the tumor masses in A549 cells which transduced with sh-NC or sh-FOXH1 were implanted into the shoulders of nude mice. (b) Tumor growth curve in nude mice. Tumor volume measurement was performed by digital caliper after tumor cells were injected subcutaneously into the shoulders of nude mice for 8 days. When measurement was terminated, the animals were sacrificed on day 36, and tumor tissues were collected. FOXH1 knockdown cell group grew slower than the control group and formed tumor masses (*, p□0.05 vs. sh-NC). (c) Immunohistochemistry assay of FOXH1 and KI-67 expression from tumor xenografts in each group of nude mice. (***, p□0.001 vs. sh-NC).

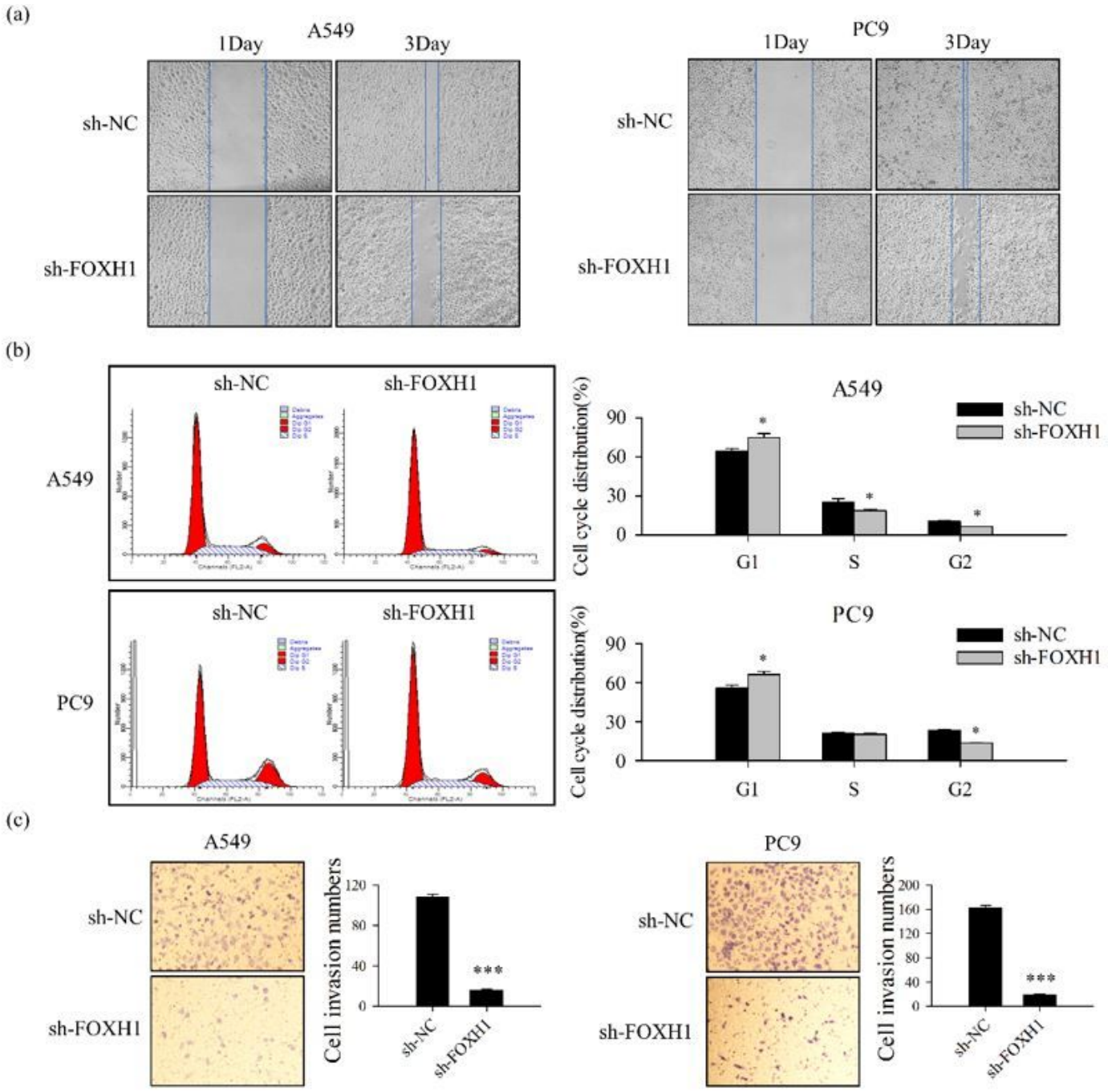

Figure 4

The effects of sh-FOXH1 on cell invasion, proliferation and migration of lung cancer cells. (a)Wound healing assay was performed to determine the migration ability of sh-FOXH1 transfected A549 and PC9 cells, the wounded area was examined by phase-contrast microscopy at 1day and 3 days. (b) Cell cycle analyses in the sh-FOXH1 transfected A549 and PC9 cells by flow cytometry. (*, p□0.05 vs. sh-NC) (c) Transwell invasion assays was performed to determine cell invasion in sh-FOXH1 transfected A549 and PC9 cells. The invasion cells number were counted (***, p□0.001 vs. sh-NC).

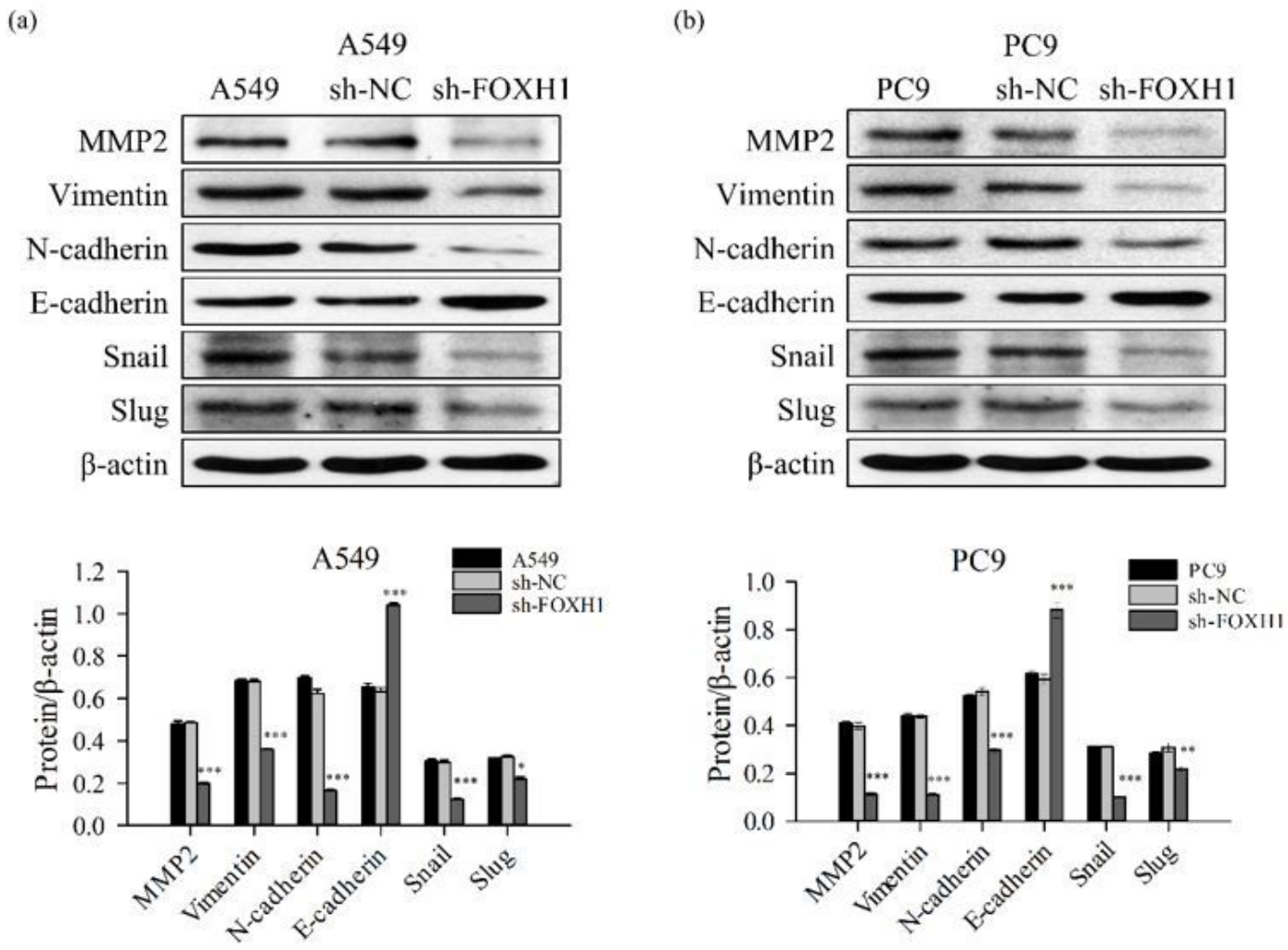

Figure 5

Knockdown of FOXH1 decreased EMT marker alterations in A549 and PC9 cells. (a)Western blot of EMT-related molecular markers, include MMP2, Vimentin, N-cadherin, E-cadherin, Snail and Slug in the sh-NC or sh-FOXH1 transfected A549 cells. β-actin is served as the internal control (*, p□0.05, ***, p□0.001 vs. sh-NC). (b)Western blot of EMT-related molecular markers, include MMP2, Vimentin, N-cadherin, E-cadherin, Snail and Slug in the sh-NC or sh-FOXH1 transfected PC9 cells. β-actin is served as the internal control (**, p□0.01, ***, p□0.001 vs. sh-NC).

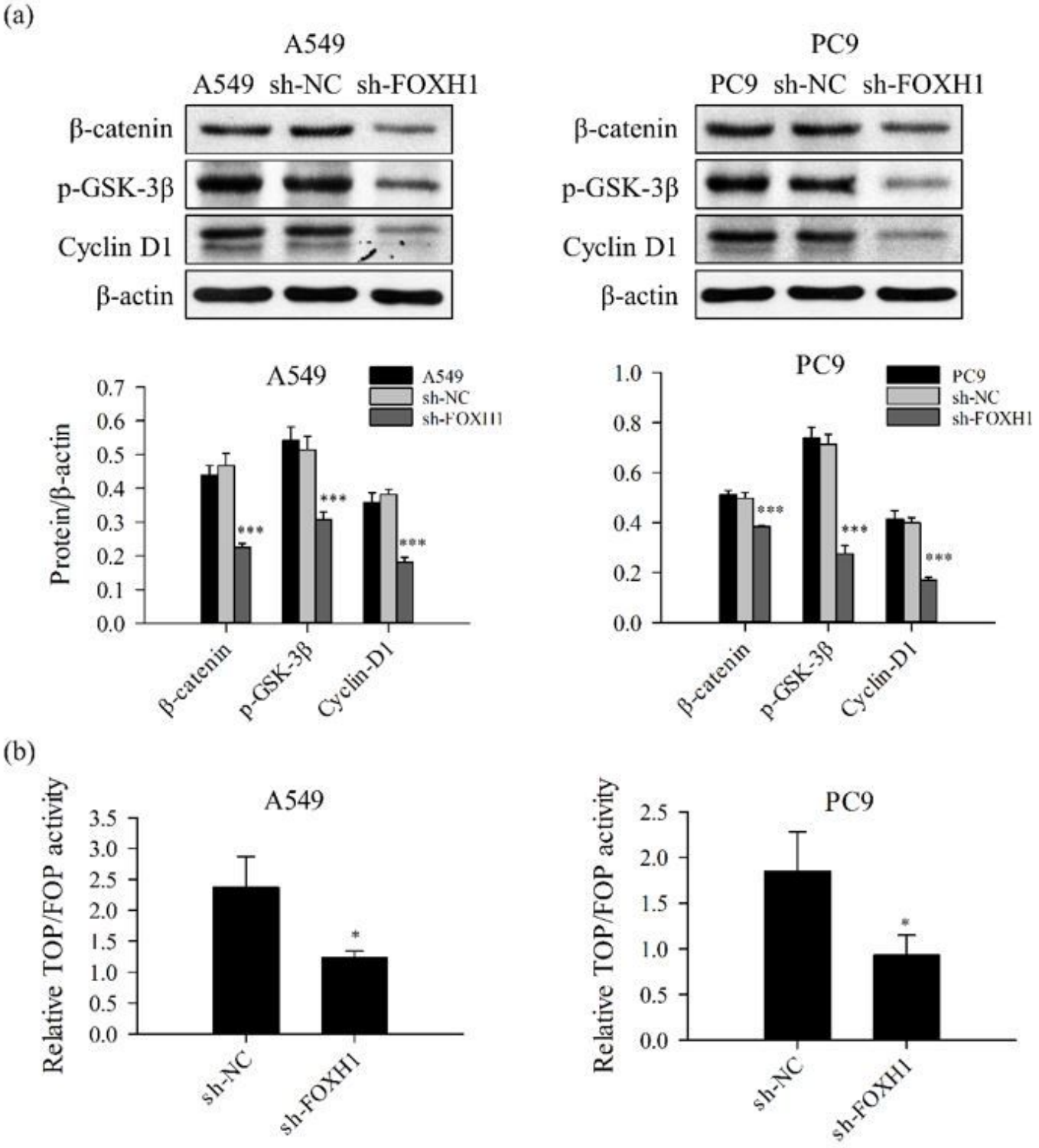

Figure 6

Knockdown of FOXH1 suppresses β-Catenin signaling in lung cancer. (a)Western blotting showing that FOXH1 knockdown decreased the expression of β-catenin, p-GSK-3β and cyclin D1 in A549 and PC9 cells. β-actin served as the internal control (***, p□0.001 vs. sh-NC). (b)TCF/LEF-dependent transcriptional activity of β-catenin in A549 and PC9 cells transfected with TOP/FOP flash reporter plasmids. Assays of relative luciferase activity in cells were performed (*, p□0.05 vs. sh-NC).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Additionalfile1Table.xlsx