

Research Paper

# FOXMI Promotes Lung Adenocarcinoma Invasion and Metastasis by Upregulating SNAIL

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## Abstract

The forkhead box M1 (FOXMI) transcription factor is one of the key genes inducing tumor invasion and metastasis by an unknown mechanism. In this study, we set out to investigate the effects of FOXMI overexpression on metastatic human lung adenocarcinoma and the underlying mechanism. FOXMI expression was analyzed in 78 frozen lung adenocarcinoma tissue samples using an Affymetrix microarray and a 155-paraffin-embedded lung adenocarcinoma tissue microarray with immunohistochemical detection. FOXMI was found to be overexpressed in lung adenocarcinoma, particularly in metastatic patients, compared to non-metastatic patients. Knockdown of FOXMI by a specific siRNA significantly suppressed EMT progression, migration and invasion of lung adenocarcinoma cells *in vitro*, and tumor growth and metastasis *in vivo*, whereas restored expression of FOXMI had the opposite effect. FOXMI binds directly to the SNAIL promoter through two specific binding sites and constitutively transactivates it. Collectively, our findings indicate that FOXMI may play an important role in advancing lung adenocarcinoma progression. Aberrant FOXMI expression directly and constitutively activates SNAIL, thereby promoting lung adenocarcinoma metastasis. Inhibition of FOXMI-SNAIL signaling may present an ideal target for future treatment.

Key words: Lung adenocarcinoma, Invasion, Metastasis, FOXMI, SNAIL

## Introduction

Lung cancer is currently the most frequent cause of major cancer incidence and mortality worldwide (1). Two main histological categories are included: small-cell lung cancer (SCLC) and non-small-cell lung cancer (NSCLC). NSCLC comprises about 80% of all diagnosed lung cancer, and lung adenocarcinoma is the most common subtype of NSCLC (2). Among patients with lung cancer, 70% present with advanced-stage disease when diagnosed, which has al-

ready spread locally or to distant organs (3). While targeted therapy, based on patient-specific molecular aberrations, has recently led to significant responses in lung adenocarcinomas (4, 5), about half of the lung cancer patients do not harbor known driver mutations and cannot be treated with targeted therapy (6). Therefore, novel insights into the molecular mechanisms underlying lung adenocarcinoma pathogenesis as well as regulators and potential targets for treat-

ment are critically needed.

FOXM1, a transcriptional factor of the forkhead box family, is involved in the regulation of organism development and cell proliferation (7, 8). FOXM1 is considered to be a key regulator of both G1-S and G2-M phases of the cell cycle and mitotic spindle integrity (9). Furthermore, FOXM1 is aberrantly overexpressed in the majority of human solid cancers and highly associated with poor clinical prognosis, including glioma, colon cancer, and hepatocellular carcinomas (10-13). Recent studies indicated that aberrant FoxM1 drives the overexpression of some oncogenes and plays an important role in angiogenesis, cell cycle acceleration, metastasis, and drug resistance (14-16). We have previously reported that FOXM1 activates Wnt signaling in glioma stem cells and induces self-renewal (17). In addition, abnormal expression of FOXM1 leads to overexpression of plasminogen activator, urokinase receptor (PLAUR), and this novel FOXM1-PLAUR signaling plays an important role in colon cancer progression and metastasis (18). FOXM1 transcriptionally regulates the expression of the Cav-1 gene and positively transactivates it, promoting pancreatic cancer epithelial-mesenchymal transition (EMT), invasion, and metastasis (19). FOXM1 promotes breast cancer metastasis by increasing nuclear retention of SMAD3 (20). Interestingly, FOXM1 is aberrantly expressed in non-small cell lung carcinomas, suggesting it plays an important role in lung carcinogenesis (21). However, the precise function of FOXM1 and the underlying mechanisms of its activity in lung adenocarcinoma EMT and metastasis remain unclear.

SNAIL is the key regulator of EMT, inducing it by partially repressing epithelial markers and up-regulating mesenchymal markers (22, 23). SNAIL is a member of the Snail family of zinc finger transcription factors (24), aberrantly overexpressed in multiple malignant tumors, including colon cancer, hepatocellular carcinoma, and breast cancer (25-27). The expression of SNAIL is positively associated with a more aggressive phenotype, higher risk for distant metastases and poorer survival rates (28, 29). Furthermore, SNAIL could promote an invasive phenotype in lung carcinoma, whereas the underlying mechanisms are unknown (30). In the present study, we sought to determine the role of FOXM1 in lung adenocarcinoma invasion and metastasis and its molecular mechanism. We identified that FOXM1 directly regulates SNAIL expression in lung adenocarcinoma and sequentially promotes tumor metastasis and invasion. This important FOXM1-SNAIL signaling may be used for future target therapy of lung adenocarcinoma.

## Materials and Methods

### Human sample specimens and immunohistochemistry analysis

The use of human lung adenocarcinoma specimens was approved by the institutional review board of The Shanghai Cancer Center at Fudan University. 78 frozen lung adenocarcinoma tissue samples were used in the gene chip microarray assay, including 21 grade I, 21 grade II and 36 grade III. In the grade I group, 7 patients are metastatic. In the grade II group, 12 patients are metastatic. In the grade III group, 26 patients are metastatic, respectively. 155-paraffin-embedded lung adenocarcinoma tissue samples were used in the tissue microarray assay, including 51 grade I, 41 grade II and 63 grade III. In the grade I group, 15 patients are metastatic. In the grade II group, 23 patients are metastatic. In the grade III group, 46 patients are metastatic, respectively. We stained tissue sections (4  $\mu$ m) from paraffin-embedded lung adenocarcinoma specimens with an antibody against human FOXM1 (Santa Cruz Biotechnology, Dallas, TX, USA) or an anti-human SNAIL antibody (Abgent, San Diego, CA, USA). For negative controls, the primary antibody was replaced with control IgG (Santa Cruz Biotechnology, Dallas, TX, USA). Staining results were scored by two investigators blinded to the clinical data. We quantitatively scored the tissue sections according to percentage of positive cells and staining intensity, as previously described (31).

### Cell lines and culture conditions

The human lung cancer cell lines NCI-H1650, H1299, A549, HCC827, NCI-H358, SPCA1, and H460 were obtained from the China Center for Type Culture Collection. Lung cancer cell lines were cultured in Roswell Park Memorial Institute (RPMI)-1640 medium supplemented with 10% fetal bovine serum (FBS), sodium pyruvate, nonessential amino acids, and L-glutamine (Life Technologies, Carlsbad, CA, USA).

### Transient or stable transfection of lung adenocarcinoma cell lines

To overexpress FOXM1, we transfected NCI-H358 and HCC827 cells with pcDNA3.1-FOXM1 or control vector pcDNA3.1 plasmids (32). Stably transfected cell lines were isolated by selection with G418 (500  $\mu$ g/mL; Life Technologies, Carlsbad, CA, USA). To inhibit FOXM1 expression, we transfected A549 and NCI-H1650 cells with a FOXM1 siRNA oligonucleotide of the sequence CUCUUCUCCUCA-GAUAUAdTdT (17) or with control siRNA. Sh-FOXM1 lentiviruses were generated with oligonucleotides

(5'-ggatccccgctcttccctcagatatattgatccgtatatctgagga gaagagttttccaactcgag-3'). To establish stable cell lines, Sh-FOXM1 lentiviruses was transduced into lung adenocarcinoma cells with polybrene (6 µg/mL; Sigma-Aldrich, St. Louis, MO, USA). Cells were then selected with 10 µg/mL puromycin for 14 days.

### Western blot assay

Standard Western blotting was carried out using whole-cell protein lysates of lung adenocarcinoma cells and primary antibodies against FOXM1 (K-19, Santa Cruz Biotechnology, USA), E-cadherin (EPR699, Abcam, Cambridge, UK), N-cadherin (EPR1791-4, Abcam, Cambridge, UK), vimentin (EPR3776, Abcam, Cambridge, UK), and SNAIL (RB1400, Abgent, USA) and secondary antibodies (anti-rabbit IgG or anti-mouse IgG; Cell Signaling Technology, Danvers, MA, USA). Equal-amount protein sample loading was monitored using an anti-Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody (EPR6256, Abcam, Cambridge, UK).

### Reverse transcription (RT) and real-time PCR

Total RNA was isolated from cells using TRIzol reagent (Life Technologies, USA). First-strand cDNA was synthesized from 1 µg of total RNA using Moloney murine leukemia virus reverse transcriptase (Life Technologies, USA). Real-time PCR analysis of gene expression was carried out using 2 µL of cDNA and the SYBR Green Master Mix (Promega, Madison, WI, USA), as recommended by the manufacturer. GAPDH was used as an internal control. PCR products were run in triplicate for target and internal control genes.

FOXM1 primers 5'-AAGGTTGAGGAGCCTTCGAG-3' (sense) and 5'-ATTCGGTCGTTTCTGCTGCTT-3' (antisense); SNAIL primers 5'-CCTCCCTGTCAGATGAGGAC-3' (sense) and 5'-CCAGGCTGAGGTATTCCTTG-3' (antisense); E-cadherin primers 5'-TGCCCAGAAAATGAAAAGG-3' (sense) and 5'-GTGTATGTGGCAATGCGTTC-3' (antisense); Vimentin primers 5'-GAGAACTT TGCCGTTGAAGC-3' (sense) and 5'-GCTTCCTGTAGGTGGCAATC-3' (antisense); N-cadherin primers 5'-ACAGTGGCCACCTACAAAGG-3' (sense) and 5'-CCGAGATGGGGTTGATAATG-3' (antisense); GAPDH primers 5'-CGGATTTGGTCGTATTGG-3' (sense) and 5'-TCCTG GAAGATGGTGATG-3' (antisense);

### Cell scratch-wound assay

Lung adenocarcinoma cells were grown in 6-well plates until confluence. A cell scratch-wound was generated by scraping with a 10-µL tip. After 12 h, the wounded cells were photographed, and cell migration was assessed by measuring gap sizes in multiple fields.

### Cell migration and invasion assays

Cell migration assays were conducted using a polycarbonate membrane pre-coated with Matrigel (24-well Transwell; Corning, Corning, NY, USA), and cell invasion assays were performed using a modified 24-well Boyden chamber with a membrane that was pre-coated with Matrigel (BD Biosciences, San Jose, CA, USA), respectively. Briefly, 24 hours after transfection of NCI-H1650 cells with FOXM1 siRNA and NCI-H358 cells with pcDNA3.1-FOXM1, or their respective controls, 500 µL of cells ( $1 \times 10^5$ /mL) in serum-free medium were loaded into the upper wells, while medium containing 20% FBS was placed in the lower wells as a chemoattractant. Cells on the bottom surface of filters were fixed, stained with gentian violet, and counted microscopically in five randomly selected fields at 200× magnification.

### Cell immunofluorescence

A549, NCI-H1650, HCC827, and NCI-H358 cells were cultured on Falcon chamber slides (BD Biosciences, USA) until 50–60% confluence before being fixed with 4% paraformaldehyde and permeabilized with 0.3% Triton X-100. Cells were immersed three times with cold phosphate-buffered saline (PBS), incubated with FOXM1 (Santa Cruz Biotechnology, USA), E-cadherin, N-cadherin, vimentin (Abcam, Cambridge, UK), and SNAIL (Abgent, USA) primary antibodies at 4°C overnight, subsequently incubated with corresponding Alexa Fluor-conjugated secondary antibodies (Life Technologies, USA) at room temperature for 1 h, and mounted using ProLong® Gold Antifade Reagent with DAPI (Life Technologies, USA). Microscopic images of cells were obtained using a Leica inverted fluorescence microscope (Leica Microsystems, Wetzlar, Germany) with ProgRes Image Capture Software (JENOPTIK Optical Systems, Jena, Germany) and a Leica Confocal LAS-AF SP5 System (Leica Microsystems, Germany).

### Promoter reporter and dual luciferase assay

A 2.0-kb *SNAIL* promoter was cloned into pGL3-Basic Luciferase Reporter Vectors (Promega, USA). Site-specific mutagenesis of the *SNAIL* promoter was carried out using a QuikChange Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA, USA), according to the manufacturer's instructions. Primers used to generate the mutant vector were as follows: mut1, 5'-TGTGAGGTTTA TGCCAGAGCCACCC-3' (sense) and 5'-GGGTGGCTCTGGCATAAACCTCACA-3' (antisense); mut2, 5'-GTAATTATCTGTGCACTTCGTCTGTC-3' (sense) and 5'-GACAGACGAAGTGCACAGATAATTAC-3' (antisense); mut1and2, 5'-TGTGAGGTTTATGC CAGAGCCACCC-3' (sense) and 5'-GACAGACGAA

GTGCACAGATAATTAC-3' (antisense). The mutation was confirmed by DNA sequencing. *SNAIL* promoter activity was normalized by cotransfection with a  $\beta$ -actin/Renilla luciferase reporter, containing a full-length Renilla luciferase gene (31). Both, firefly and Renilla luciferase activity were quantified using a Dual-Luciferase Reporter Assay System (Promega, USA) 24 h after transfection.

### Chromatin immunoprecipitation (ChIP) assay

Tumor cells ( $5 \times 10^6$ ) were prepared for the chromatin immunoprecipitation (ChIP) assay with the ChIP assay kit (Millipore, Billerica, MA, USA), according to the manufacturer's protocol. The resulting precipitated DNA samples were analyzed using PCR to amplify a potential binding site 1 region of the *SNAIL* promoter with the primers 5'-AGACAGTAGTTCTGCCCTTCAGGT-3' (sense) and 5'-ATGGAGCCGTGTTACAGCCT-3' (antisense), and a potential binding site 2 region of the *SNAIL* promoter with the primers 5'-AGTTGCCACTTCTCCCTCGGCCT-3' (sense) and 5'-GGAACGGTGCTCTTGCT-3' (antisense). PCR products were resolved electrophoretically on a 2% agarose gel and visualized using ethidium bromide staining.

### Animal experiments

All procedures involving mice were conducted in accordance with Fudan University Shanghai Cancer Center Animal Care guidelines. All efforts were made to minimize animal suffering, to reduce the number of animals used, and to utilize possible alternatives to *in vivo* techniques. Tumor cells in the exponential growth phase were harvested by brief exposure to 0.25% trypsin/0.02% EDTA solution (w/v). Cell viability was determined using Trypan blue exclusion, and only single-cell suspensions that were more than 95% viable were used. Groups of five nude mice were injected with tumor cells either subcutaneously ( $1 \times 10^6$  per mouse) or into the tail vein ( $5 \times 10^6$  per mouse). Subcutaneously injected animals were killed 6 weeks later or when they had become moribund, and tumors were removed and weighed. Tail-injected animals were killed 4 weeks after the injection or when they had become moribund, their lungs were removed, and surface metastases were counted. Every surface was examined by two investigators who were unaware of the experimental protocol and scored separately. Tissue was fixed in 10% buffered formalin, immersed in an ascending series of alcohols, and paraffin embedded. 4  $\mu$ m sections were cut and stained with hematoxylin and eosin (H & E).

### Statistical analysis

The significance of the data from patient speci-

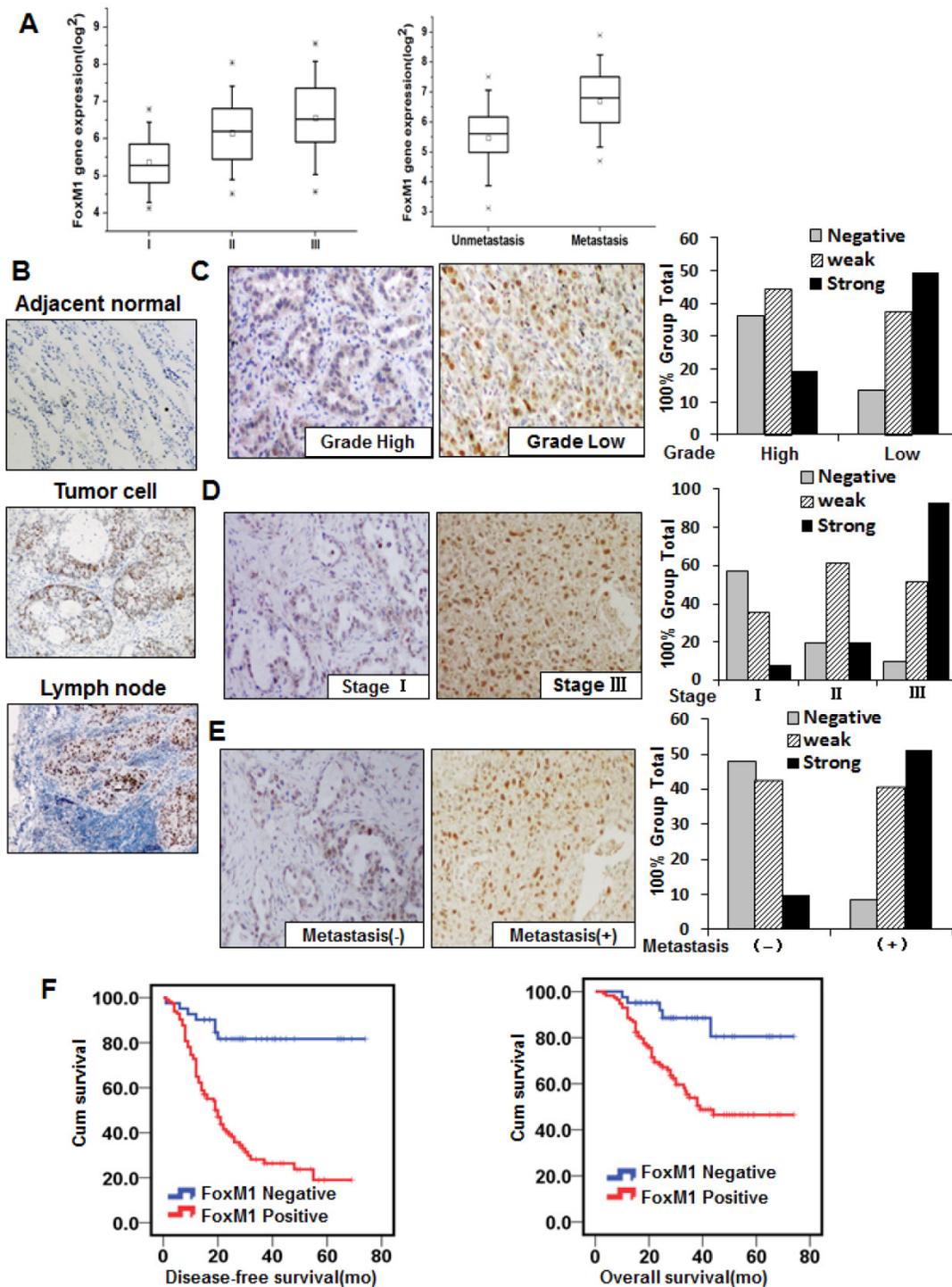
mens was determined by the Pearson correlation coefficient. The 2-tailed  $\chi^2$  test was used to determine the significance of differences between covariates. Survival durations were calculated using the Kaplan–Meier method. The log-rank test was used to compare cumulative survival rates in patient groups. The significance of *in vitro* and *in vivo* data was determined by Student's *t*-test (two-tailed), considering  $P < 0.05$  as significant.

## Results

### FOXM1 expression in human lung adenocarcinoma specimens and its association with lung cancer pathologic features

To screen for novel molecular events that lead to metastasis of lung adenocarcinoma, genome-wide gene expression profiling was conducted on 78 frozen lung adenocarcinoma samples, using the Affymetrix GeneChip<sup>®</sup> Human Genome U133 Plus 2.0 microarray. FOXM1 expression was elevated in the stage II and III groups (I vs. II,  $P < 0.05$ ; I vs. III,  $P < 0.01$ ; **Fig. 1, left panel**), as well as in the metastasis group ( $P < 0.05$ ; **Fig. 1, right panel**). These results were confirmed by semiquantitative RT-PCR experiments using the same 78 lung adenocarcinoma samples (Supplementary Material: **Fig. S1**).

To further evaluate the effect of FOXM1 expression on lung adenocarcinoma progression and metastasis, we investigated the expression of FOXM1 protein with the 155-primary lung adenocarcinoma tissue microarray from stages I to III. We observed FOXM1-positive staining in the nuclei of tumor cells and FOXM1-negative or weak FOXM1-positive staining in adjacent normal lung cells (**Fig. 1, B**). Increasing FOXM1 expression correlated with decreasing levels of tumor differentiation, indicating that FOXM1 expression is significantly upregulated in poorly differentiated tumors (**Fig. 1, C**). In addition, FOXM1 expression was positively correlated with disease stages, particularly in early-stage (stage I) and late-stage (stage III) tumors (**Fig. 1, D**). Moreover, FOXM1 expression in distant metastasis specimens was dramatically higher than in non-metastasis specimens (**Fig. 1, E**). Correlations between FOXM1 expression and clinicopathologic characteristics are summarized in Supplementary Material: **Table S1**, showing that increased FOXM1 expression is correlated with disease stage ( $P = 0.001$ ), pT classification ( $P = 0.001$ ), distant metastasis ( $P = 0.001$ ), vessel invasion ( $P = 0.002$ ), and disease differentiation ( $P = 0.001$ ). These findings indicate that FOXM1 expression plays a critical role in lung adenocarcinoma development and progression and is a valuable biomarker for this disease.



**Figure 1. FOXM1 expression in lung adenocarcinoma specimens and its association with lung adenocarcinoma pathologic features.** A, Genome-wide gene expression profiling was conducted on 78 frozen lung adenocarcinoma samples, using the Affymetrix GeneChip<sup>®</sup> Human Genome U133 Plus 2.0 microarray. FOXM1 expression was elevated in the stage II and III groups (I vs. II,  $P < 0.05$ ; I vs. III,  $P < 0.01$ ; left panel), as well as in the metastasis group ( $P < 0.05$ ; right panel). B, Tissue microarray immunostaining using a specific antibody against FOXM1. Representative images of FOXM1 protein expression in adjacent normal lung tissue, lung adenocarcinoma tissue, and lymph node tissue are shown (200 $\times$ ). Note that the majority of adjacent normal lung tissue cells are negative for FOXM1 expression, whereas lung adenocarcinoma tissue cells and lymph node cells are strongly positive for FOXM1 expression. C, FOXM1 expression is positively correlated with tumor differentiation, and representative images of high and low grade tumors are shown (200 $\times$ ). The numbers of samples of low and high grade were 67 and 88, respectively. D, FOXM1 expression was positively correlated with disease stage, and representative images of stages I and III are shown (200 $\times$ ). The sample numbers of stages I, II, and III were 51, 41, and 63, respectively. E, FOXM1 expression was positively correlated with tumor metastasis, and representative images of tumors from patients with or without distant metastases are shown (200 $\times$ ). The numbers of patients with or without distant metastases were 84 or 71, respectively. F, DFS (left) and OS (right) rates were estimated using the Kaplan–Meier method. Both rates were significantly worse in patients with FOXM1-positive primary tumors than in patients with FOXM1-negative primary tumors ( $P < 0.001$  for DFS,  $P = 0.003$  for OS).

To assess the clinical significance of FOXM1 overexpression in lung adenocarcinoma, we scored

FOXM1 staining with the 155-primary lung adenocarcinoma tissue microarray as previously described

(31). There was a significant difference between the FOXM1-positive and FOXM1-negative groups in the number of patients who developed metastasis from primary lung adenocarcinoma after surgery. Positive FOXM1 expression was associated with a significantly increased risk of distant metastases (HR 5.739; 95% CI, 2.64–12.47;  $P < 0.001$ ; **Fig. 1, left panel**). Moreover, the Overall Survival (OS) was significantly higher in patients with FOXM1-negative tumors than in those with FOXM1-positive tumors (HR 4.06; 95% CI, 1.62–10.20;  $P = 0.003$ ; **Fig. 1, right panel**), indicating that FOXM1 is a negative total survival marker for lung adenocarcinoma patients. These results strongly indicate that FOXM1 expression is directly associated with metastasis and prognosis of patients with lung adenocarcinoma.

### Altered FOXM1 expression affects EMT induction in human lung adenocarcinoma cells

As the overexpression of FOXM1, examined by immunohistochemistry, was associated with lung adenocarcinoma metastasis and ascending clinical stage, we subsequently investigated whether FOXM1 was capable of inducing EMT in human lung adenocarcinoma cells. First, we screened expression levels of FOXM1 in different lung adenocarcinoma cell lines (**Fig. 2**). Then, we chose two cell lines expressing high levels of FOXM1 (A549, NCI-H1650) and two expressing low levels (HCC827, NCI-H358), and carried out Western blot (**Fig. 2, upper panel**), real-time PCR (**Fig. 2, lower panel**) and cell immunofluorescence (**Fig. 2, C**) analyses with these four cell lines. We found that FOXM1 expression correlated with the expression levels of EMT markers: the expression of an epithelial marker (E-cadherin) was higher in the low-level FOXM1-expressing cells (HCC827, NCI-H358) than in the high-level FOXM1-expressing cells (A549, NCI-H1650; **Fig. 2**). In contrast, the expression of mesenchymal markers (vimentin, N-cadherin) was lower in the low-level FOXM1-expressing cells (HCC827, NCI-H358) than in the high-level FOXM1-expressing cells (A549, NCI-H1650; **Fig. 2**).

To determine the effect of altered FOXM1 expression on EMT induction in lung adenocarcinoma cells, we transfected the FOXM1 expression vector pcDNA3.1-FOXM1 (or the control vector) into NCI-H358 cells. We found that elevated expression of FOXM1 in NCI-H358 cells (**Fig. 2, D**) significantly increased vimentin and N-cadherin expression but decreased E-cadherin expression, both on the mRNA (**Fig. 2, lower panel**) and protein level (**Fig. 2, upper panel**). In contrast, FOXM1-specific siRNA led to a concomitant decrease in vimentin and N-cadherin but

increase in E-cadherin expression, both on the mRNA (**Fig. 2, D**; **Fig. 2, E lower panel**) and protein level (**Fig. 2, E upper panel**) in NCI-H1650 cells.

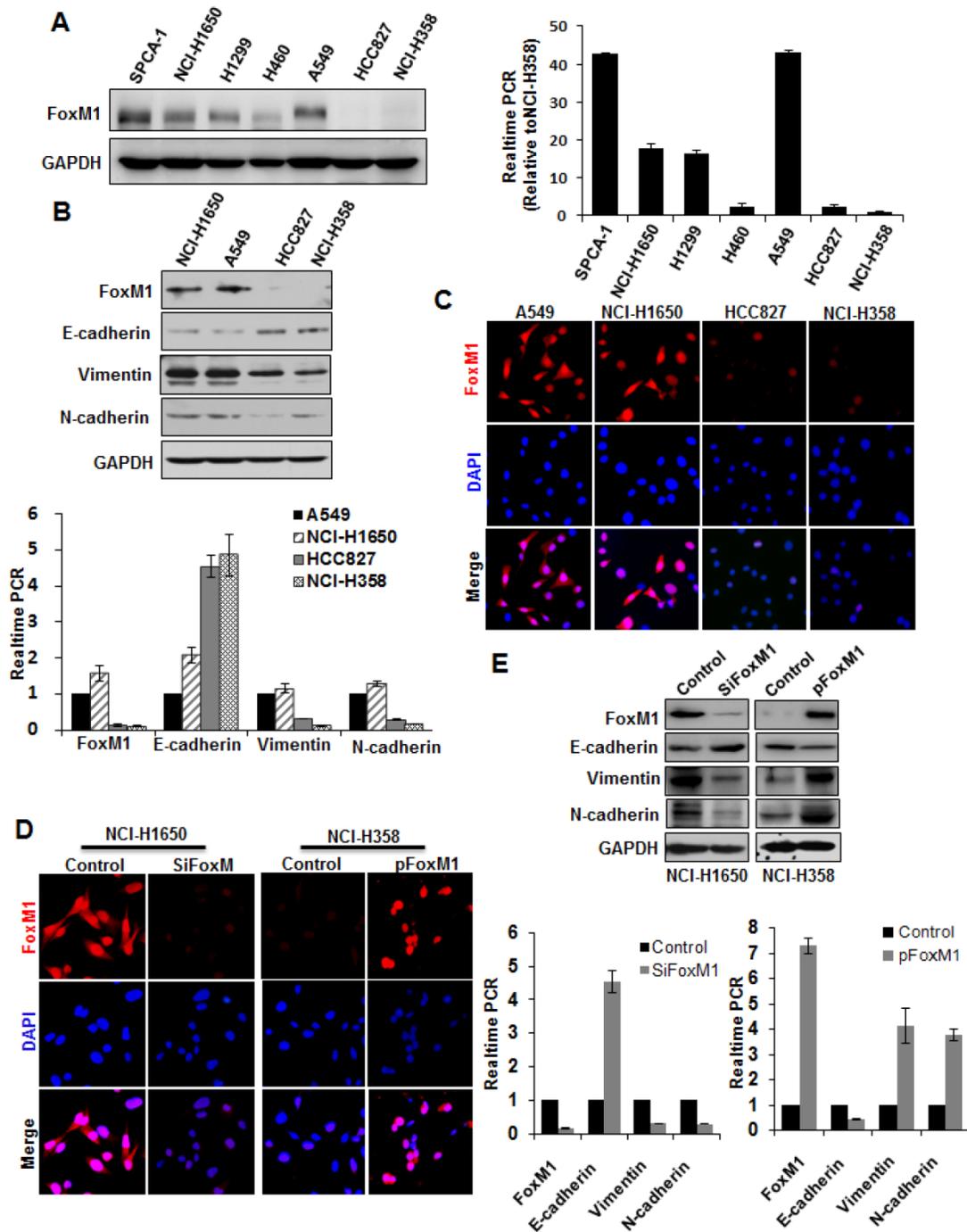
Immunofluorescence staining further revealed that E-cadherin (**Fig. 3, A**) was strongly downregulated in FOXM1 siRNA-transfected NCI-H1650 cells, while levels of N-cadherin, vimentin and fibronectin were dramatically induced in FOXM1 silenced NCI-H1650 cells (**Fig. 3, A**). Ectopic expression of FOXM1 in NCI-H358 cells also decreased the levels of E-cadherin (**Fig. 3, B**) and increased the levels of N-cadherin, vimentin and fibronectin (**Fig. 3, B**). These results indicate that altered FOXM1 expression affects EMT induction in lung adenocarcinoma cells.

### Altered FOXM1 expression affects migration and invasion of human lung adenocarcinoma cells *in vitro*

To determine the effect of altered FOXM1 expression on migration and invasion of lung adenocarcinoma cells, FOXM1-transfected and FOXM1-siRNA-transfected lung adenocarcinoma cells were wounded by scratching and maintained at 37°C for additional 12 h. Down-regulation of FOXM1 attenuated the flattening and spreading of NCI-H1650 cells, whereas ectopic FOXM1 expression strongly promoted the flattening and spreading of NCI-H358 cells (**Fig. 4, A1, B1**). These results were confirmed by migration and invasion assays, revealing that the migratory and invasive ability were attenuated in FOXM1-siRNA-transfected NCI-H1650 cells, whereas the levels of both migration and invasion of FOXM1-transfected NCI-H358 cells were significantly higher than those of control cells (**Fig. 4, A2, B2**).

### Effects of altered FOXM1 expression on tumorigenicity and metastasis of human lung adenocarcinoma cells *in vivo*

To determine whether FOXM1 plays an important role in the tumorigenicity and metastasis of human lung adenocarcinoma cells *in vivo*, we injected FOXM1-shRNA-transfected A549 cells into either the subcutis or the caudal vein of nude mice in groups of five. Consistent with the effect of altered FOXM1 expression on migration and invasion of colon cancer cells *in vitro*, knockdown of FOXM1 significantly inhibited lung tumor growth (**Fig. 5, A, B**) and abrogated lung metastases (**Fig. 5, C, D**) of A549 cells in nude mice, whereas overexpression of FOXM1 significantly promoted tumor growth (**Fig. 5, E, F**) and increased lung metastases (**Fig. 5, G, H**) of NCI-H358 cells. Our data clearly indicate that FOXM1 is oncogenic and promotes tumorigenicity and metastasis of lung adenocarcinoma *in vivo*.



**Figure 2. Correlation between FOXM1 expression and EMT potential of lung adenocarcinoma cells.** A, Western blot (left panel) and real-time PCR (right panel) analysis of FOXM1 protein expression in lung adenocarcinoma cell lines. Quantitative real-time PCR results were standardized in relation to GAPDH and expressed as multiples of NCI-H358 results. B, Western blot (upper panel) and real-time PCR (lower panel) analysis of expression levels of FOXM1, E-cadherin, vimentin, and N-cadherin. Note that high-level FOXM1-expressing cells (NCI-H1650 and A549) showed high-level expression of mesenchymal markers (vimentin, N-cadherin) and low-level expression of epithelial markers (E-cadherin), whereas low-level FOXM1-expressing cells (HCC827 and NCI-H358) showed the opposite. C, Cell immunofluorescence staining for FOXM1 (red) and nuclei (DAPI; blue). Note that NCI-H1650 and A549 cells displayed strong positive FOXM1 staining, whereas HCC827 and NCI-H358 cells displayed weak positive FOXM1 staining. D, Cell immunofluorescence staining for FOXM1 (red) and nuclei (DAPI; blue). NCI-H1650 cells were transfected with control siRNA (“control”) or siRNA against FOXM1 (“siFOXM1”), whereas NCI-H358 cells were transfected with control pcDNA3.1 vector (“control”) or pcDNA3.1-FOXM1 expression vector (“p FOXM1”). E, Western blots (upper panel) and real-time PCR (lower panel) analysis of EMT markers. Total protein lysates or RNA were prepared from NCI-H1650 cells with FOXM1 knockdown or NCI-H358 cells with FOXM1 overexpression (as described in D).

**A close relationship between altered expression of FOXM1 and SNAIL in lung adenocarcinoma**

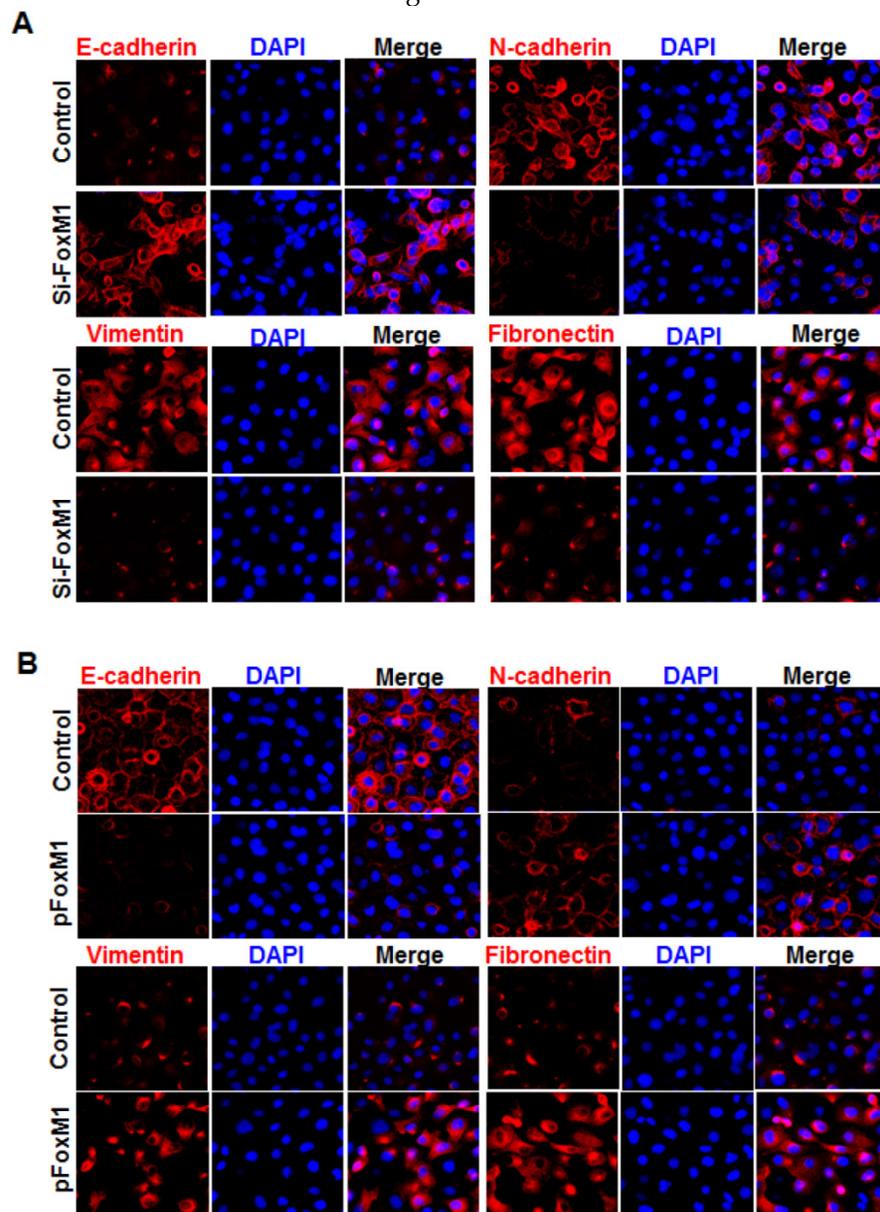
To explore the mechanisms underlying FOXM1 overexpression in lung adenocarcinoma, we analyzed

both FOXM1 and SNAIL expression in tissues and cell lines. By analyzing consecutive primary lung adenocarcinoma sections, we observed that FOXM1 expression was significantly correlated with SNAIL expression. Lung adenocarcinoma, but not adjacent tissues,

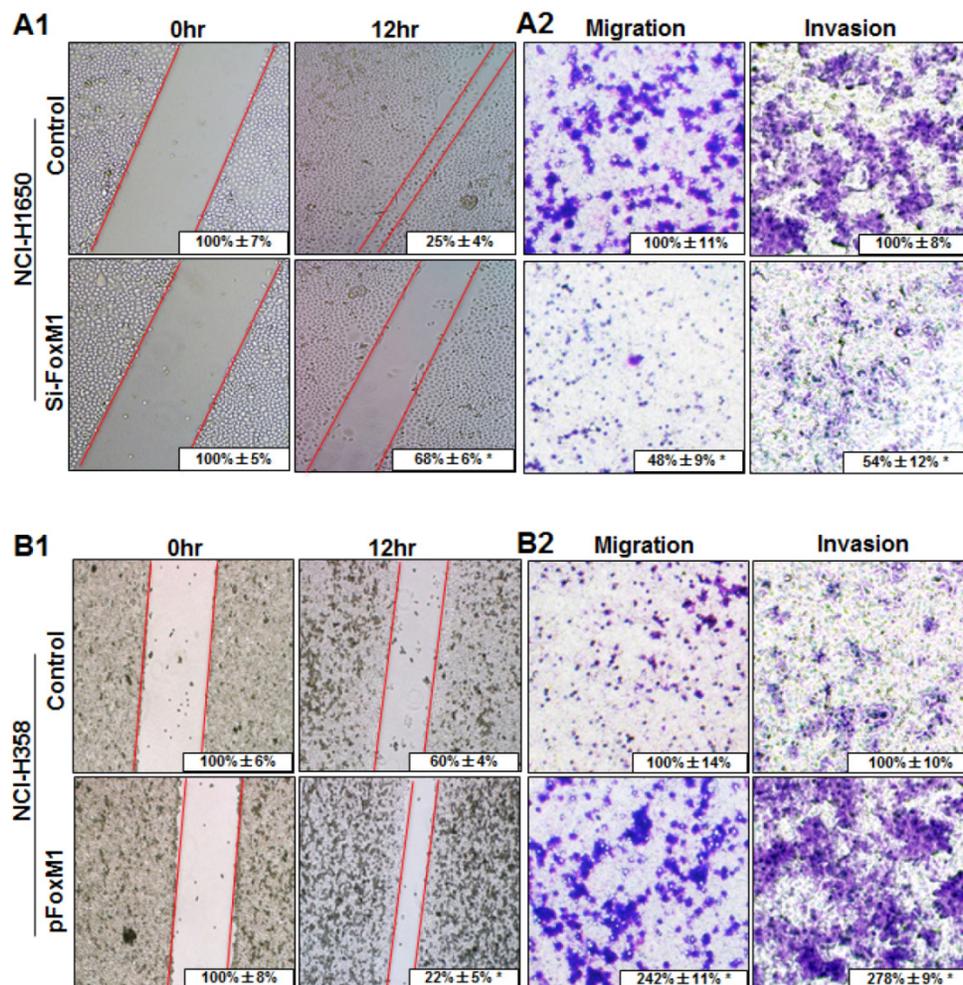
expressed both FOXM1 and SNAIL (Fig. 6, A1), and their direct correlation was found statistically significant ( $r = 0.645$ ;  $P < 0.01$ ; Fig. 6, A2). Consistently, the expression of FOXM1 also correlated with the expression of SNAIL in lung adenocarcinoma cell lines (Fig. 6, B).

To further explore the molecular mechanisms of regulation of SNAIL expression by FOXM1, we first determined the impact of altered FOXM1 expression on SNAIL expression in human lung adenocarcinoma cell lines. We found that knockdown of FOXM1 expression in NCI-H1650 and A549 cells led to significantly decreased SNAIL mRNA and protein expression (Fig. 6, C1, C2). Conversely, increased expression of FOXM1 in NCI-H358 and HCC827 cells led to sig-

nificantly increased SNAIL mRNA and protein levels (Fig. 6, D1, D2). Furthermore, to determine the role of FOXM1 in regulating SNAIL transcription, we generated the *SNAIL* promoter pLuc-SNAIL and cotransfected it together with FOXM1 siRNA into NCI-H1650 and A549 cells, causing knockdown of FOXM1 and suppression of the *SNAIL* promoter in both cell lines (Fig. 6, E1). Conversely, overexpression of FOXM1 after cotransfection of pcDNA3.1-FOXM1 together with pLuc-SNAIL into NCI-H358 and HCC827 cells activated the *SNAIL* promoter (Fig. 6, E2). Collectively, our findings indicate that FOXM1 regulates SNAIL expression, most likely at the transcriptional level.



**Figure 3. Influence of FOXM1 expression on EMT marker alteration of lung adenocarcinoma cells.** Cell immunofluorescence staining for E-cadherin (red), N-cadherin (red), vimentin (red), fibronectin (red), and nuclei (DAPI; blue), respectively, using NCI-H1650 cells with FOXM1 knockdown (A) or NCI-H358 cells with FOXM1 overexpression (B).



**Figure 4.** Influence of FOXM1 expression on lung adenocarcinoma cell migration and invasion. NCI-H1650 (A) and NCI-H358 cells (B) were transfected with FoxM1 siRNA and pcDNA3.1-FoxM1 for 48 h, respectively. For the cell scratch-wound assay, NCI-H1650 (A1) and NCI-H358 cells (B1) were wounded by scratching and maintained at 37°C for additional 12 h. Cells were photographed, and cell migration was assessed by measuring gap sizes (inserted numbers representing percentage area of gap  $\pm$  SD). Migration and invasiveness of NCI-H1650 (A2) and NCI-H358 cells (B2) cells were determined as described in Materials and Methods. Representative migrating or invading tumor cells were photographed. Data represent the mean  $\pm$  SD of triplicates ( $P < 0.01$  in a comparison of the FoxM1 siRNA-treated group or the pcDNA3.1-FoxM1-treated group with control groups).

### Direct transcriptional regulation of SNAIL expression by FOXM1 in lung adenocarcinoma cells

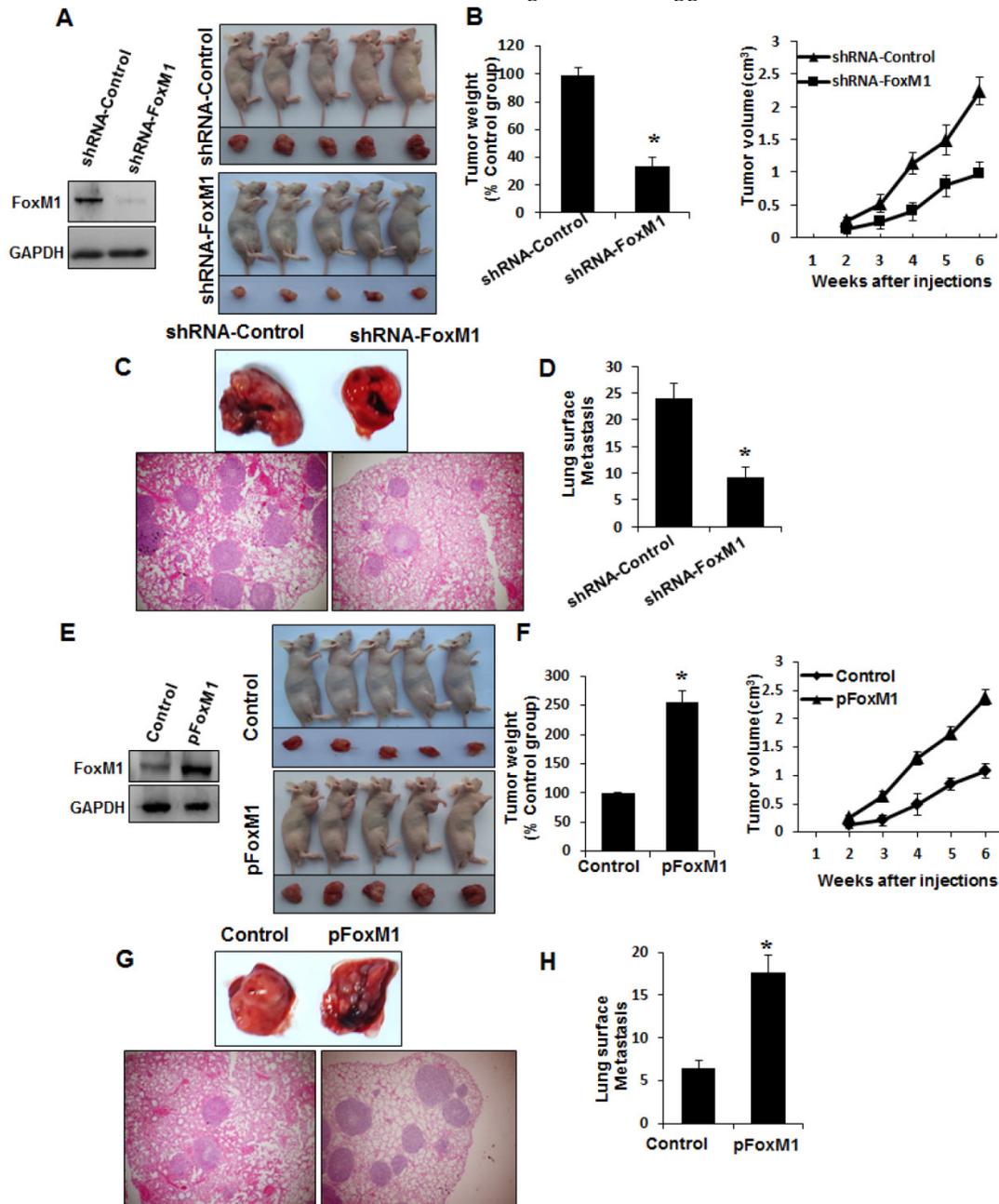
To determine whether SNAIL could be a direct transcriptional target of FOXM1, we scanned approximately 2-kb of the promoter region of *SNAIL* with the FOXM1 DNA-binding consensus sequence (31) and found two FOXM1 putative binding sites (−747 to −735 and −1290 to −1280) (Fig. 6, F). To confirm that FOXM1 binds directly to the human *SNAIL* promoter, we performed ChIP assays using two pairs of PCR primers for sequences in the vicinity of these potential FOXM1 binding sites. Indeed, ChIP assays in NCI-H1650 cells, using antibodies specific to FOXM1, showed that FOXM1 protein binds directly to the endogenous *SNAIL* promoter in both sites (Fig. 6, G1). Moreover, in NCI-H358 cells, normally expressing low FOXM1 levels, there is an increased binding

of FOXM1 protein to the *SNAIL* promoter after transfection with pcDNA3.1-FOXM1 (Fig. 6, G2). These results were further confirmed by promoter mutation assays. Various mutant reporters were generated from the wild-type *SNAIL* promoter construct, including a FOXM1-binding site 1 mutation only (pGL3-SNAIL Mut1), a FOXM1-binding site 2 mutation only (pGL3-SNAIL Mut2), and a mutation of both site 1 and site 2 (pGL3-SNAIL Mut3). We transfected these mutant luciferase reporters into NCI-H1650 or A549 cells and compared their activity with that of the wild-type *SNAIL* promoter (Fig. 6, H1, H2). Disruption of one or both of the FOXM1-binding sites significantly attenuated *SNAIL* promoter activity in NCI-H1650 or A549 cells. In summary, these findings show that FOXM1 binds specifically to FOXM1-binding sites in the *SNAIL* promoter, suggesting that SNAIL is a direct transcriptional target of FOXM1.

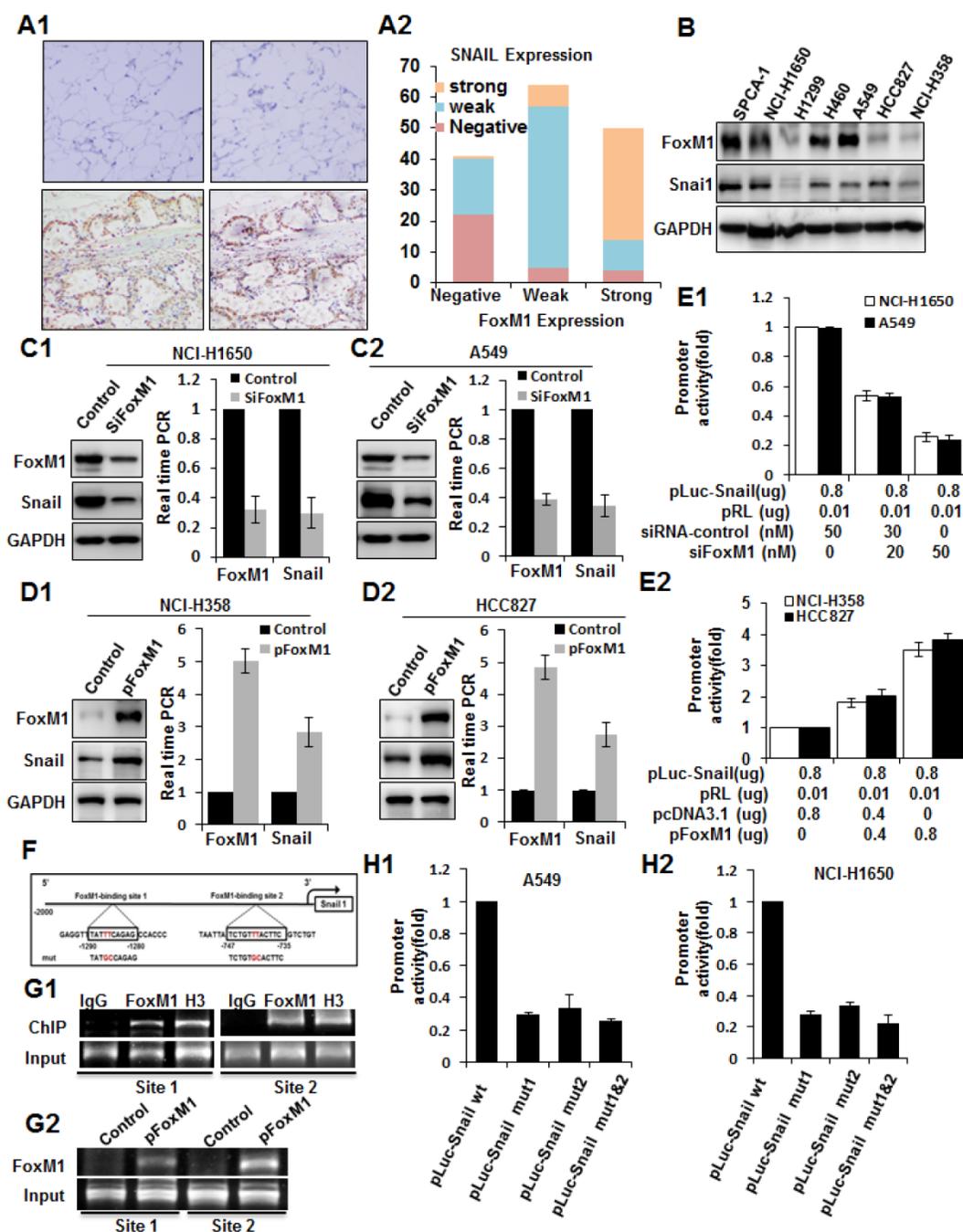
### Discussion

In this study, we determined the critical role of FOXM1 and SNAIL in lung adenocarcinoma pathogenesis and its underlying mechanisms. We observed that elevated FOXM1 expression was directly correlated with lung adenocarcinoma progression and metastasis. FOXM1-positive staining of lung adenocarcinoma cells could be used to identify a greatly increased risk of metastasis in patients after surgery,

which might serve as a valuable prognostic marker. We also found that FOXM1 transcriptionally activates the SNAIL gene, thereby constituting an important signaling pathway that directly affects EMT, invasion, and metastasis of lung adenocarcinoma cells *in vitro* and *in vivo*. Collectively, our clinical and mechanistic evidence strongly suggests that dysregulated FOXM1 expression causes abnormal SNAIL expression and critically contributes to lung adenocarcinoma pathogenesis and aggressive behavior.



**Figure 5. Influence of FOXM1 expression on lung adenocarcinoma growth and metastasis.** NCI-H1650 cells with FoxM1 knockdown (A, B) or NCI-H358 cells with FoxM1 overexpression (C, D) were injected into nude mice (n = 5) either subcutaneously into the right scapular region ( $1 \times 10^6$  per mouse) or into the caudal vein ( $5 \times 10^6$  per mouse). Tumor-bearing mice were sacrificed when they became moribund or after 6 weeks (subcutaneous injection) or after 4 weeks (tail injection). Shown are IB blot of the shFoxM1 knockdown or pFoxM1 overexpress (A, E, left panel), gross tumors (A, E, right panel), gross lung metastatic tumors (C, G, upper panel) and H & E-stained sections of liver (C, G, lower panel); tumor weights (B, F, left panel), tumor volumes (B, F, right panel) and the numbers of lung surface metastases (D and H).  $P < 0.01$  in a comparison of the FoxM1 shRNA-treated group or the pcDNA3.1-FoxM1-treated group with control groups.



**Figure 6. Coexpression of FOXM1 and SNAIL in lung adenocarcinoma and direct binding of FOXM1 to the SNAIL promoter.** A, Immunohistochemical staining (A1) of lung adenocarcinoma specimens using specific anti-FOXM1 and anti-SNAIL antibodies. Shown are representative images of negative (upper 2 panels) and positive (lower 2 panels) FOXM1 and SNAIL staining of lung adenocarcinoma sections (200 x). FOXM1 expression levels were directly correlated with SNAIL expression levels ( $r = 0.645$ ;  $P < 0.01$ ). A2) in primary lung adenocarcinoma, as analyzed using the Pearson test. B, Western blot analysis of FOXM1 and SNAIL protein expression in lung adenocarcinoma cell lines. C, NCI-H1650 (C1) and A549 (C2) cells transfected with FOXM1-siRNA or control siRNA. Western blot (left) and RT-PCR (right) analysis of FOXM1 and SNAIL expression. D, NCI-H358 (D1) and HCC827 (D2) cells transfected with pcDNA3.1-FOXM1 or control pcDNA3.1. Western blot (left) and RT-PCR (right) analysis of FOXM1 and SNAIL expression. E, Effects of FOXM1 expression on SNAIL promoter activity. NCI-H1650 and A549 cells were cotransfected with 0.8  $\mu$ g of the SNAIL promoter-luciferase construct pLuc-Snail and 0–50 nmol/L FOXM1-siRNA or control siRNA (E1), whereas NCI-H358 and HCC827 cells were cotransfected with 0.8 mg pLuc-Cav and 0–0.8  $\mu$ g of pcDNA3.1-FOXM1 or pcDNA3.1 (E2). Promoter activities were determined using the Dual-Luciferase Assay Kit. F, Sequences and positions of putative FOXM1-binding elements in the SNAIL promoter. G, Binding of FOXM1 to the SNAIL promoter *in vivo*. ChIP assays were done with NCI-H1650 (G1) and NCI-H358 (G2) cells treated with pcDNA3.1-FOXM1 or pcDNA3.1. Chromatin fragments of cells (1% of total cell lysates as input) were immunoprecipitated with an anti-FOXM1 antibody or control IgG and subjected to PCR. H, SNAIL promoter activity with or without mutations in the FOXM1 binding sites, as determined by luciferase activity in A549 (H1) and NCI-H1650 (H2) cells.

Previous evidence had indicated that FOXM1 might serve as a novel prognostic biomarker involved in the tumorigenesis and metastasis of several malignancies (33). Some studies had suggested a potential

role of FOXM1 in NSCLC patients, e.g., Yang *et al* (34) demonstrated that overexpression of FOXM1 was related to poor survival in 69 squamous cell carcinoma patients, not including lung adenocarcinoma. Xu

*et al* (35) found that FOXM1 overexpression was significantly associated with several clinicopathological parameters such as TNM staging, tumor stage, lymph node metastasis and poorer prognosis in 175 NSCLC patients. The results of these studies were not exactly equivalent to ours, maybe due to different study populations, varied sample quality or cell type (NSCLC vs. lung adenocarcinoma). In our current study, FOXM1 expression was first investigated in 78 frozen lung adenocarcinoma tissue samples using Affymetrix microarrays, then confirmed in the same 78 frozen lung adenocarcinoma tissue samples using real-time PCR and with a 155-paraffin-embedded lung adenocarcinoma tissue microarray using immunohistochemistry. We found that elevated expression of FOXM1 was significantly associated with poorer tumor differentiation and advanced disease stage, particularly with distant metastasis and metastasis-free survival time. Thus, we present very important clinical evidence, indicating that FOXM1 promotes lung adenocarcinoma metastasis.

Both our *in vivo* and *in vitro* assays supported this notion. Altered FOXM1 expression affected the ability of migration and invasion of lung adenocarcinoma cell lines. Enhanced expression of FOXM1 in lung adenocarcinoma cell lines was associated with much higher tumor growth and metastatic ability, compared to control cells in the mouse model. Our findings are consistent with recent studies, which have indicated a critical role for FOXM1 in tumorigenesis and metastasis of pancreatic, breast and lung cancer cells (19, 20, 36, 37).

Cancer metastasis is a complex process, and its cellular and molecular mechanisms remain to be determined (38, 39). EMT is a critical process providing epithelial-derived tumor cells with the ability of increased migration and invasiveness, contributing to tumor metastasis (40, 41). In the current study, we found that the expression of FOXM1 is much higher in cell lines of mesenchymal phenotype, such as A549, than in cell lines of epithelial phenotype, such as NCI-H358. Elevated FOXM1 expression could contribute to the acquisition of the EMT phenotype by up-regulating mesenchymal cell markers vimentin and N-cadherin and down-regulating the epithelial cell marker E-cadherin, whereas decreased FOXM1 expression could cause the opposite. Our new findings were consistent with recent studies, which have shown a correlation between FOXM1 up-regulation and cancer cell EMT in human pancreatic cancer cells (19) and another correlation between EMT during radiation-induced pulmonary fibrosis and FOXM1 overexpression (42).

Assuming a crucial role of FOXM1 in the metastasis of malignancies, it is necessary to explore the

underlying mechanisms responsible for FOXM1 overexpression in lung adenocarcinoma. Previous studies showed that FOXM1 increases nuclear localization of beta-catenin in glioma stem cells (17) and activates TGF-beta signaling in breast cancer cells (20). In lung adenocarcinoma cell lines, these two important pathways may also be induced by FOXM1, as has been implied in other previous studies (43, 44). The results of our current study indicate that SNAIL, which plays an important role in inducing and promoting EMT in epithelial cells (45), is a novel downstream target of FOXM1. We show that the expression levels of FOXM1 correspond directly to the expression levels of SNAIL, both in human lung adenocarcinoma tissues and lung adenocarcinoma cell lines. By using a well-established FOXM1 overexpression and knock-down system, we found that overexpression of FOXM1 led to increased expression of SNAIL, whereas knockdown of FOXM1 had the opposite effect. Moreover, we noted that FOXM1 could transactivate SNAIL promoter activity, in agreement with recent studies, showing that FOXM1 translationally regulated SNAIL during lung inflammation (42), yet not indicating the exact binding site of FOXM1 in the human SNAIL promoter. Our current study specifically identified two potential FOXM1-binding sites in human SNAIL promoters. Mutations in these sites significantly attenuated FOXM1-mediated transactivation of the human SNAIL promoter, and ChIP assays confirmed the recruitment of FOXM1 to these binding sites in the human SNAIL promoter *in vitro*.

## Conclusions

In summary, this study provides both clinical and molecular evidence supporting the regulatory role of FOXM1 in SNAIL expression and a novel FOXM1-SNAIL signaling pathway in tumorigenesis and metastasis of lung adenocarcinoma. Our study not only indicates a very important molecular mechanism of lung adenocarcinoma progression and metastasis, but it also provides a promising molecular target for designing optional therapeutic modalities to control the progression and metastasis of lung adenocarcinoma.

## Supplementary Material

Table S1 and Fig.S1.

<http://www.ijbs.com/v11p0186s1.pdf>

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### Authors' contributions

All authors participated in the interpretation of the studies, analysis of the data and review of the manuscript. Ping Wei, Xiaoyan Zhou, and Xiang Du participated in the study design and supervision. Ping Wei, Yiqing Wang, and Nu Zhang conducted the experiments. Dawei Li and Lisha Wang conducted *in vitro* experiments. Xiangjie Sun, Chen Shen and Yusi Yang collected the clinical and follow-up data. Ping Wei wrote the manuscript.

### Competing interests

The authors declare that they have no competing interests.

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