

FOXM1 Drives Epithelial-Mesenchymal Transition Program Through E-Cadherin Promoter Biding Ability in Non-Small-Cell Lung Cancer Cells

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Abstract

Background: Forkhead box (FOX) gene family plays a critical role in regulating Epithelial-mesenchymal transition (EMT) program, and in which, FOXM1 can mediate multiple malignant process in many type of tumor cells. However, the modulate functions of FOXM1 on EMT in non-small-cell lung cancer (NSCLC) cells, especially the transcriptional function on E-cadherin coding gene CDH1 remains unclear. This article mainly focuses on FOXM1, exploring its mechanism in regulating EMT of NSCLC cells, and FOXM1 inhibitor thiothrepton's effects in EMT intervention.

Methods: Morphological changes of overexpressed cells were observed by HE staining. The effects of scratch test, Transwell chamber test and Western-blot analysis on cell migration and invasion ability and the expression of EMT-related markers were analyzed. Dual luciferin reporter enzyme assay and nuclear transcription factor immunoprecipitation assay (ChIP, immunofluorescence) revealed the transcriptional regulation of FOXM1 on EMT markers. MTT assay and clone formation assay were used to determine the effect of thiomycin on the viability of NSCLC cells and the ability of cell clone formation.

Results: After overexpression of FOXM1, the cells showed intermediate epithelial-mesenchymal morphology, but not complete mesenchymal morphology, and their migration and invasion abilities were enhanced. The protein expression levels of N-cadherin, Snail1 and Vimentin were increased, while the expression levels of E-cadherin were decreased. On the contrary, knockdown of FOXM1 expression showed the opposite result. The double luciferin reporter enzyme assay showed that FOXM1 inhibited the luciferin reporter vector CDH1-2000-promoter. ChIP results confirmed that FOXM1 could bind endogenous to CDH1 gene promoter. In cells overexpressing FOXM1, knockdown of Snail further promotes FOXM1-mediated CDH1 transcription. MTT results and clone formation experiments showed that thiomycin had inhibitory effect on the proliferation of NSCLC cells. Morphological observation, cell migration assay and Transwell chamber assay showed that thiomycin inhibited TGF- β 1-induced enhanced cell migration and invasion. Western-blot analysis showed that thiomycin down-regulated the expression of FOXM1, N-cadherin, Snail, and Vimentin induced by TGF- β 1, while blocking the expression of E-cadherin induced by TGF- β 1 decreased.

Conclusion: FOXM1 can directly bind to the promoter of E-cadherin encoding gene, and can indirectly inhibit E-cadherin expression by stimulating Snail. Overexpression of FOXM1 can promote EMT progression in NSCLC cells. Therefore, down-regulation of FOXM1 can inhibit this process. In addition, thiothrepton, a FOXM1 inhibitor, blocked proliferation, colony formation, and EMT progression in NSCLC cells.

Background

Lung cancer is one of the most malignant cancer with the highest morbidity and mortality worldwide, and around 85% of which is non-small-cell lung cancer (NSCLC)¹. Owing to extremely complex of NSCLC pathogenesis and absence of effective early screening techniques, most patients diagnosed with NSCLC

have already been in advanced stage with distant metastasis, and the five-year survival rate is extremely low. Despite many promising anti-NSCLC strategies including surgical or TKI drugs, a large number of patients still cannot benefit from them, and their high prices also lay burden on many families².

Substantial evidences show that epithelial-mesenchymal transition (EMT) has been recognized as a hallmark of NSCLC metastasis. EMT is a biological process by which tumor cells undergo a complex transformation from epithelial phenotype to mesenchymal phenotype, rendering NSCLC cells to travel across original sites, cellular matrix and vascular system to generate distant colonization in other organs³. Compellingly, EMT is not only associated with a high rate of metastasis, but also highly relevant to recurrence and even drug resistance after clinical surgery or TKI treatment, as it also endows tumor cells with cancer stem cell properties^{4, 5}. However, the molecular mechanism of EMT has remained elusive.

Recent studies have found that the forkhead box (FOX) family plays an important role in the regulation of EMT. It is worth pondering that FOX genes promote cancer development⁶, while others can function as tumor suppressors^{7, 8}. Among FOX gene family, FOXM1 involves in cell proliferation, mitosis and also plays a critical role in embryonic development⁹. Recent years, FOXM1 has been widely recognized as an oncogene that improves tumor progression¹⁰⁻¹². A large number of clinical samples have been collected and analyzed, and their data implies that FOXM1 can be used as an important risk factor for judging the poor prognosis of tumor patients^{13, 14}. Intriguingly, evidences also support FOXM1 is a pivotal molecule in the process of pulmonary fibrosis¹⁵ and endothelial cells transformation¹⁶ mediated by type II EMT, which plays a important role in TGF- β -induced EMT and promotes transcription of Snail^{15, 16}. The regulation role of FOXM1 in EMT may not single point regulation, but a more complex interaction pattern. According to www.researchgate.net, FOXM1 was found to be highly expressed in NSCLC tissues, and the tendency of FOXM1 to be involved in the proliferation and invasion of NSCLC was confirmed in the Kong FF study¹⁷. Thus, understanding the causal relationship between FOXM1 and EMT can provide new strategies for treating NSCLC metastasis.

EMT-related protein E-cadherin is encoded by CDH1 gene, which plays an important role in tumor invasion and metastasis¹⁸. The absence or low expression of E-cadherin is common in tumor cells, which mediates the decrease of adhesion between cells, the enlargement of tumor cell space, and the easy shedding of cells, giving tumors strong ability of EMT. Therefore, CDH1 can be used as a tumor suppressor gene¹⁹. Literature has shown that E-cadherin may be a target of FOXM1²⁰, and FOXM1 participates in tumor EMT by down-regulating E-cadherin, but the mechanism of action has not been explored.

In this study, we determined that FOXM1 can directly bind to E-cadherin encoding gene CDH1 promoter, and regulate EMT in NSCLC cells via FOXM1-Snail-E-cadherin axis. Further studies showed that altered FOXM1 expression or use FOXM1 inhibitor thiostrepton can suppress EMT process in NSCLC cells. These

findings indicate that FOXM1 acts as an upstream of EMT, and establish the potential therapeutic roles of targeting FOXM1 for treating NSCLC.

Materials And Methods

Cell culture

A549, H1650, H1299, H1975, HCC827 cell lines were purchased from Chinese Academy of Sciences Shanghai Cell Bank. PC9 was a kindly gift from Dr. Sui Yuxia (Fujian Provincial Hospital, China). All cell lines were cultured in RPMI-1640 (Hyclone) supplemented with 10% FBS (Gibco), 100 U/ml penicillin and 100 µg/ml streptomycin (Gibco). Cells were incubated at 37°C with 5% CO₂.

Plasmids preparation, siRNA, shRNA and transfection

The Flag-FOXM1 lenti-virus was purchased from GENECHM, China. Si-Snail was purchased from RaboBio, China. Dual luciferase report vector pEZX-basic and pEZX-CDH1-2000 were purchased from GeneCopoeia. Different truncations of E-cadherin promoters were amplified from pEZX-CDH1-2000 and sub-cloned to pEZX-basic by In-fusion cloning kit (Vazyme, China). The primers used for promoter cloning were as follows: Forward CDH1-1500: 5'-catttctactactagtagcggtTCTCCTGACCTCGTGATCTGCC-3'; Forward CDH1-1000: 5'-catttctactactagtagcggtAAATTAGGCCGCTCGAGGC-3'; Forward CDH1-500: 5'-catttctactactagtagcggtGCCCGACTTGTCTCTCTACAA-3'; all the Reverse primer was CDH1+35: 5'-catggtggcggatcctctagaCAAGCTCACAGGTGCTTTGCA-3'.

Two shRNAs that target FOXM1 were cloned to pLKO.1. After annealing the forward and reverse primers, their ends were phosphorylated with PNK (NEB). The annealed double-strand DNA were ligated to the AgeI and EcoRI double-digested pLKO.1. The forward sh1 primers was: 5'-CCGGCGCTACTTGACATTGGACCAATTCAAGAGATTGGTCCAATGTCAAGTAGCGTTTTTTGGTACC-3', reverse was: 5'-AATTGGTACCAAAAACGCTACTTGACATTGGACCAATCTCTTGAATTGGTCCAATGTCAAGTAGCG-3'; The forward sh2 primers was: 5'-CCGGGCGTATTTCTTAGCTCATTATTCAAGAGATAATGAGCTAAGGAAATACGCTTTTTTTGGTACC-3', reverse was: 5'-AATTGGTACCAAAAAGCGTATTTCTTAGCTCATTATCTCTTGAATAATGAGCTAAGGAAATACGC-3'.

All of the plasmids, siRNAs and shRNAs were transfected using lipofectamine 2000 (Thermo Fisher) and performed according to the manufacturer's instruction.

Western blot analysis

Cells lysates were prepared by RIPA (Solarbio, China) with %1 PMSF (Solarbio, China). Protein samples was separated by 10% SDS-PAGE (Beyotime Biotechnology, China) and transferred onto PVDF membranes. The membranes were blocked in 5% skim milk in TBST at room temperature for 2 h and then incubated with primary antibodies at 4°C overnight. After 3 times washing with TBST, membranes were

incubated with Goat anti-rabbit secondary antibodies (1:4,000 in TBST) at room temperature for 2h. After 3 times washing with TBST, the target proteins were visualized using enhanced chemiluminescence kit (Proteintech) according to the manufacturer's protocol. Antibodies against FOXM1 and GAPDH were purchased from Proteintech. WB-used antibodies against E-cadherin, N-cadherin, Vimentin, Snail, ZEB1 were included EMT-sampler Kit, which was purchased from Cell Signaling Technology.

Immunofluorescence

Cells grown on slides were fixed in 4% paraformaldehyde for 15 min, and then washed 3 times in PBS for 5 min each. The cells were permeabilized by treatment with 0.5% triton for 10 min, and then washed 3 times with PBS for 5 min each. Cells were blocked for 30 min using undiluted goat serum. Next, cells were incubated with primary antibody (0.5 mg /mL BSA dilution, 1: 200) overnight at 4 °C. After rinsed 3 times with PBST, the secondary antibody (0.5 mg / mL BSA dilution, 1: 200) was added and incubated at room temperature for 2h in the dark. After 3 times washed with PBST, nuclei were stained for 10 min, then rinsed with PBS 3 times. Then the slides were observed using fluorescence microscope. Antibodies used in immunofluorescence including FOXM1, Vimentin, E-cadherin, Snail and Cy3-linked anti-rabbit were purchased from Proteintech.

Migration assay

Cells were trypsinized and grown as a confluent monolayer in six-well plates. The next day, cells were scratched using a 10 μ L pipette tip against a ruler. Wash 3 times with PBS to remove the cells that had been scratched. RPMI-1640 complete medium, drugs or reagents were added. Took pictures under an inverted microscope at 0h and 24h and Image J was used to analyze the scratch area.

Invasion assay

The tips used must be placed at -20 °C for more than 2h in advance. Dilute Matrigel gel (Corning) by 1: 8 on ice, and spread 60 μ L of gel per chamber. Set the gel in a 37°C incubator for 1h. The cell density was adjusted to 2 x 10⁵ cells/mL with serum-free medium, and 100 μ L was added into the invasion chamber. Add RPMI-1640 complete medium (containing 10% FBS) to the lower chamber and incubate for 24 h. Discard the small chamber culture medium and wash twice with PBS. Wipe the Matrigel and cells on the upper surface with a cotton swab, fix with 4% paraformaldehyde for 20 minutes, stain with 0.1% crystal violet for 20 minutes, and wash with water for more than 3 times. Five fields of each well were photographed under an inverted microscope and counted.

Dual luciferase report assay

Cell lysates were prepared 48h post transfected with different truncations of E-cadherin promoters in dual-Luciferase Reporter vector, and luciferase activity was measured by Dual-Luciferase Reporter Assay Kit (Promega) according to the manufacturer's instruction. In si-Snail experiments, the cells were transfected with siRNAs 24h prior to luciferase plasmids transfection.

Chromatin Immunoprecipitation (ChIP) Assay

ChIP was performed as described in ChIP Kit (Beyotime Biotechnology, China). In brief, 4 μ g FOXM1 antibody was used to immunoprecipitate DNA, then PCR was performed to amplify E-cadherin promoter. Two primers were used: Forward, 5'-CAACAGCATAGGGAGACATT-3', Reverse, 5'-TGTAGAGCTTCATGGGTAGT-3'.

Proliferation assay

5000 cells were seeded into a 96-well plate with a volume of 200 μ L per well. The outermost periphery of the 96-well plate was filled with 200 μ L of PBS. After 24h incubation at 37 °C, thioestrepton (Yuanye Bio, Shanghai, China) was added to the final concentrations of 0, 1, 2, 5, 10, 20, 30, 50, 75, 100 μ M, 6 bio-replicates for each concentration. After the end of the culture at 24h or 48h, add 20 μ L of MTT solution (Biosharp, 5mg/ml in PBS) to each well. Carefully discard the culture supernatant after 4 h incubation. Add 150 μ L DMSO to each well and shake for 10 minutes to fully melt the crystals. The wavelength of 490nm was selected to measure the results using microplate Reader (BioTec Instruments, Inc).

Colony formation assay

Cells were split to 6 wells plates, 300 cells/well. After 24 hours of incubation, thioestrepton was added to make the final concentrations of 0, 10, 20, and 30 μ M, with triplicates for each concentration. After 48 h culturing in a 37 °C, replaced all wells with RPMI-1640 complete medium. After continuous cultured for 14 days, discarded the supernatant and carefully washed twice with PBS. Add 1% crystal violet (0.5g crystal violet in 5mL 95% ethanol) and stain for 5 minutes, then slowly wash away the staining solution with tap water and dry at room temperature. Colonies were counted using Image J.

Statistical analysis.

Student's t test was used to determine significance between two groups, whereas comparisons between multiple groups were performed using two-way ANOVA. GraphPad Prism 6 was used to perform the statistical analysis. Significance: * $p \leq 0.05$, ** $p \leq 0.01$, *** ≤ 0.001 , **** ≤ 0.0001 .

Results

1 FOXM1 is abnormally highly expressed in NSCLC which indicates a poor prognosis.

The abnormal expression of FOXM1 has been continuously identified from tumor samples in recent years. We first used the GEPIA database²¹ to interrogate the role of FOXM1 in NSCLC patients.

FOXM1 expression was significantly higher in NSCLC tissues than in normal tissues (Fig. 1A) and increased significantly with tumor progression (Fig. 1B). Patients with abnormally high FOXM1 expression suggest a significant reduction in poor overall survival in NSCLC patients (Fig. 1C).

The expression of FOXM1 in NSCLC tissues is significantly higher than that in normal tissues (Fig. 1A), and increased significantly with tumor progression (Fig. 1B). Patients with abnormally high FOXM1 expression suggest a significant reduction in poor overall survival in NSCLC patients (Fig. 1C). Substantial evidences show that FOXM1 is highly relevant to EMT in many kinds of tumors. We then utilized GEO database (<https://www.ncbi.nlm.nih.gov/geo/>), and R software to analyze FOXM1 expression profiling data (GSE2222, FOXM1 knockdown in breast cancer cells²²) to carry out secondary mining to preliminary obtain its overall impact on EMT. Corresponding to previous studies, Gene Set Enrichment Analysis (GSEA) found that the overall gene expression level associated with EMT was down-regulated owing to knocking down FOXM1 in this study (Fig. 1D).

To further explore the abnormal relationship of FOXM1 and EMT in NSCLC, we next detected the expression of FOXM1 and some EMT marker proteins in different NSCLC cells (Fig. 1E). Mesenchymal cells such as H1299 and H1975 have high expression of FOXM1, while displaying a low expression of E-cadherin. Other NSCLC cells, more corresponding to epithelial phenotypes, however, exhibited low expression of FOXM1. To monitor EMT formation, we used TGF- β 1 to trigger EMT in A549. Of note, TGF- β 1 treatment also led to an increasing expression of FOXM1 as well as other EMT phenomenon appeared (Fig. 1F). These data indicate FOXM1 has some correlation with EMT, implying FOXM1 is of great significance in the early course of EMT and recurrence and metastasis in NSCLC.

2 FOXM1 inhibits E-cadherin expression by binding to CDH1 promoter

To study the underlying regulation mechanism of FOXM1 in EMT, we utilized Cistrome (<http://cistrome.org/db/#/>) ChIP-Seq database²³ to explore the potential binding targets of FOXM1. In the ChIP-seq experimental data from Sanders et al²⁴, FOXM1 in MCF7 cells has a certain binding activity to the CDH1 promoter²⁵, encoding E-cadherin, which was among top putative binding targets (Fig. 2A and 2B). Therefore, we asked whether FOXM1 can bind to CDH1 promoter in NSCLC cells. We constructed FOXM1 stable expression in A549 cell and tested the binding ability of FOXM1 to CDH1 promoter by dual luciferase reporter assay and ChIP. Different truncation versions of CDH1 promoter (pEZXC-DH1-2000, pEZXC-DH1-1500, pEZXC-DH1-1000, pEZXC-DH1-500) were cloned to dual luciferase reporter vector (pEZXC-basic), and were transfected into FOXM1-stable cells (Fig. 2C). As expected, the result shows that the luciferase activity ratio of pEZXC-basic, empty vector, is very low. The ratio are significantly higher in pEZXC-DH1-1500, pEZXC-DH1-1000, pEZXC-DH1-500 transfection groups. However the figure for pEZXC-DH1-2000 transfection reduced (Fig. 2C), which indicates FOXM1 may have an inhibitory effect on the 1500-2000bp, prior to starting ATG in the E-cadherin promoter. Consistent with luciferase reporter assay, ChIP further validated that FOXM1 can directly bind to CDH1 promoter (Fig. 2D). These data suggest that CDH1 promoter is a target binding site of FOXM1.

3 FOXM1 also regulate E-cadherin expression in the upstream of Snail

FOXM1 is essential in the progression of type II EMT, which was identified by inducing the expression of Snail^{15, 16}. Snail is also an upstream transcription factor for EMT. In this experiment, we knocked down

Snail in FOXM1 overexpressing cells, and transfected pEZX-CDH1-2000 recombinant vector to examine Firefly/Renilla luciferase activity. This Ratio was significantly enhanced in si-Snail group (Fig. 2E), which suggest Snail functions between FOXM1 and E-cadherin. Next, we used cellular immunofluorescence to visualize this upstream and downstream relationship. The fluorescence of E-cadherin restored (Fig. 2F), while the fluorescence of FOXM1 was not affected after Snail knockdown in both control and overexpressed FOXM1 cell (Fig. 2G). Thus, our dual luciferase report and immunofluorescence data demonstrate that FOXM1 also regulates EMT through FOXM1-Snail-E-cadherin axis.

4 FOXM1 enhances the expression of EMT markers and promotes migration and invasion ability of NSCLC cells.

EMT-producing cells can exhibit morphological changes, and we already found CDH1 is a direct binding target of FOXM1. To gain a more comprehensive understanding about FOXM1 and EMT, we used FOXM1 stable over-expressed A549 cells as a model. In this study, Control and Mock group showed epithelial morphology, while the FOXM1 overexpression group tended to be spindle-shaped morphology that similar to mesenchymal cells (Fig. 3A). Accordingly, WB results showed that mesenchymal proteins such as Vimentin, N-cadherin, and Snail were up-regulated, and E-cadherin expression was down-regulated in FOXM1 over-expressed cell (Fig. 3B), indicating that overexpression of FOXM1 can affect EMT signals. Given these results, we sought to measure whether knockdown FOXM1 can result in decreased expression of EMT markers. Mesenchymal like H1975 cells has highly expressed FOXM1, which was selected as a knockdown model to reverse the EMT process. The two shFOXM1 constructs were transfected to H1975 cells, and the results showed that down-regulated FOXM1 decreased EMT markers including N-cadherin, Vimentin, and Snail, whereas increased E-cadherin expression (Fig. 3C).

In order to confirm altered expression of FOXM1 can disturb the mobility of NSCLC cells, we determined the migration and invasion ability in these cell models. Indeed, over-expressed FOXM1 in A549 cells significantly promoted cell migration and invasion (Fig. 3D-G). By contrast, knockdown of FOXM1 in H1975 cells dramatically attenuated the migration and invasion in vitro (Fig. 3H-K). Taken together, these data suggest FOXM1 is critical for EMT initiation, and highly expressed FOXM1 is responsible for EMT-induced invasiveness in NSCLC cells.

5 FOXM1 inhibitor thiostrepton reverse TGF- β 1-induced EMT properties in NSCLC cells

To further demonstrate that FOXM1 is a druggable target in suppressing EMT. We employed thiostrepton, an effective FOXM1 inhibitor, to prevent EMT in a pharmacological way. We decided to use A549 and PC9 cells to generate TGF- β 1-induced EMT models, because they were originally epithelial-like. We first used MTT assay and colony formation assay to evaluate 24, 48h survival of thiostrepton-treated A549 and PC9 cells. MTT data displayed a dramatically decrease in the cell growth both in time and dose-dependent manner (Fig. 4A). Consistent with MTT, thiostrepton significantly inhibited colony formation of A549 and PC9 cells (Fig. 4B). Intriguingly, thiostrepton also reverse the TGF- β 1-induced EMT-like phenotype, as fusiform cells were gradually returned to the rounded shape (Fig. 4C). By WB, we observed that thiostrepton inhibited the expression of FOXM1 in A549 and PC9 cells induced by TGF- β 1 (Fig. 4D

and 4E). Meanwhile, the mesenchymal phenotype-related proteins N-cadherin and Snail were down-regulated, whereas the expression of E-cadherin was restored (Fig. 4D and 4E). But ZEB1 was not significantly affected. As EMT biomarkers also influence migration and invasion, we measured these ability under the treatment of thiostrepton. As expected, thiostrepton drastically repressed the migration and invasion both in A549 and PC9 cells (Fig. 5A-5H), and this was not due to anti-proliferation of thiostrepton because it didn't kill too much cells from 10–30 μ M during first 24h (Fig. 4A). Together, these results provide an evidence that thiostrepton has a great impact on NSCLC cell and EMT progression property.

Discussion

Tumor metastasis is a very complicated and delicate process, which is produced by co-regulation of exogenous factors from tumor milieu and internal multiple molecules. However, tumor cells often undergo EMT at the beginning of metastasis. The biological process of EMT is to reduce cell adhesion, as well as acquire a mesenchymal phenotype. E-cadherin plays a vital role in maintaining the adhesion and morphology of cells, and its down-regulation is related to the specific transcription characteristics of tumor cells^{26–28}. FOX genes family have been identified to be involved in the development of EMT, and the mechanism of FOXM1 mediates EMT in NSCLC remained elusive. This work focused on the mechanism of FOXM1 in mediating EMT in NSCLC, particularly the transcriptional regulation of E-cadherin promoter. We also observed some evidences for down-regulating EMT after targeting FOXM1, providing new insight into the prevention and treatment of NSCLC metastasis.

In this study, we confirmed that FOXM1 is up-regulated in NSCLC and relevant to EMT process. We observed that cells with high expression of FOXM1 had low E-cadherin expression, and other cells with low expression of FOXM1 had the opposite features. Next, we proposed and confirmed the transcriptional regulation effect of FOXM1 on the promoter of E-cadherin encoding gene CDH1. In the luciferase report assay, FOXM1 has positive regulation for pEZXC-DH1-1500, pEZXC-DH1-1000, and pEZXC-DH1-500 recombinant promoter reporter vectors. The binding effect is consistent with previous work²⁹, although raised a question about why FOXM1 facilitate E-cadherin. However, FOXM1 generated a negative regulatory effect on the pEZXC-DH1-2000 recombination promoter reporter vector. In addition, earlier studies have shown that FOXM1 can promote Snail expression result in a type II EMT^{15, 16}. Therefore, we further hypothesized that Snail expression promoted by FOXM1 can achieve the second step inhibition of E-cadherin. Interestingly, knockdown of Snail increased the relative fluorescence activity of pEZXC-DH1-2000 in FOXM1-overexpressed cell, and knockdown of Snail did not impair FOXM1 expression, indicating that FOXM1 promotes EMT through FOXM1-Snail-E-cadherin axis (Fig. 5I).

In order to further elucidate the effect of FOXM1 in regulating EMT, we examined EMT markers and cell migration and invasion ability by targeting FOXM1. Overexpression of FOXM1 in A549 cell promoted the EMT process, presented by the up-regulation of mesenchymal markers and enhanced the invasion and metastasis of the cells. Conversely, H1975 cells with high FOXM1 expression level were knocked down, the EMT features and invasion and metastasis were suppressed. Intriguingly, the reversal effect of

FOX M1 inhibitor thio strepton also can suppress cell migration and invasion, and prevent the EMT process in TGF- β 1 induced cells. These findings are in line with previous studies, and verified that FOX M1 can promote EMT.

There has many limitations in this single network between FOX M1 and EMT, and it is not possible to fully understand the nature of the EMT process. In NSCLC cells, FOX M1 may be able to recruit other functional molecules to achieve overall regulation of EMT. For example, FOX M1 can bind to PDGF-A and promote its expression in malignant glioma cells to maintain its tumor stem cell characteristics. FOX M1 can also recruit β -cantenin/TCF4 to jointly promote STAT3 expression and interfere with FOX M1 can sensitize glioblastoma to temozolomide³⁰. The malignant loop of p65/FOX M1/ β -cantenin plays an important role in tumor progression³¹. Niclosamide can effectively interfere with this cycle by inhibiting p65 and FOX M1 from entering the nucleus and their transcriptional function, thereby preventing the self-renewal ability of chronic myeloid leukemia stem cells³¹. These works also emphasize the inhibitory effect of FOX M1 on EMT intervention. Combined with previous studies, our observations suggest that the potential anti-tumor effect of thio strepton can be further explored, and a more scientific theoretical basis and anti-tumor auxiliary strategy can be taken into consideration.

In summary, FOX M1 plays an important role in facilitating EMT through directly regulate E-cadherin or FOX M1-Snail-E-cadherin axis. Our work deciphered an important methods to interfere with the EMT process in NSCLC cells by targeting FOX M1.

Declarations

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Declared none

Authors' contributions

This study was designed by JSR and LW. XLL and ZSJ did the experiments. YZS and YXL analyzed and interpreted the data. JSR and LW conceptualized and supervised the project. XLL, and ZSJ were major contributors in writing the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

Not applicable.

Ethics approval and consent to participate

Not applicable.

Consent for publication

We confirm that written inform consent has been taken from the patients for this study.

Availability of data and materials

Relevant data can be obtained by emailing us

Conflict of interest

All the authors are agreement with the content of the manuscript and declare that this manuscript is original, has not been submitted to or under consideration by another publication, has not been previously published in any language or form.

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Figures

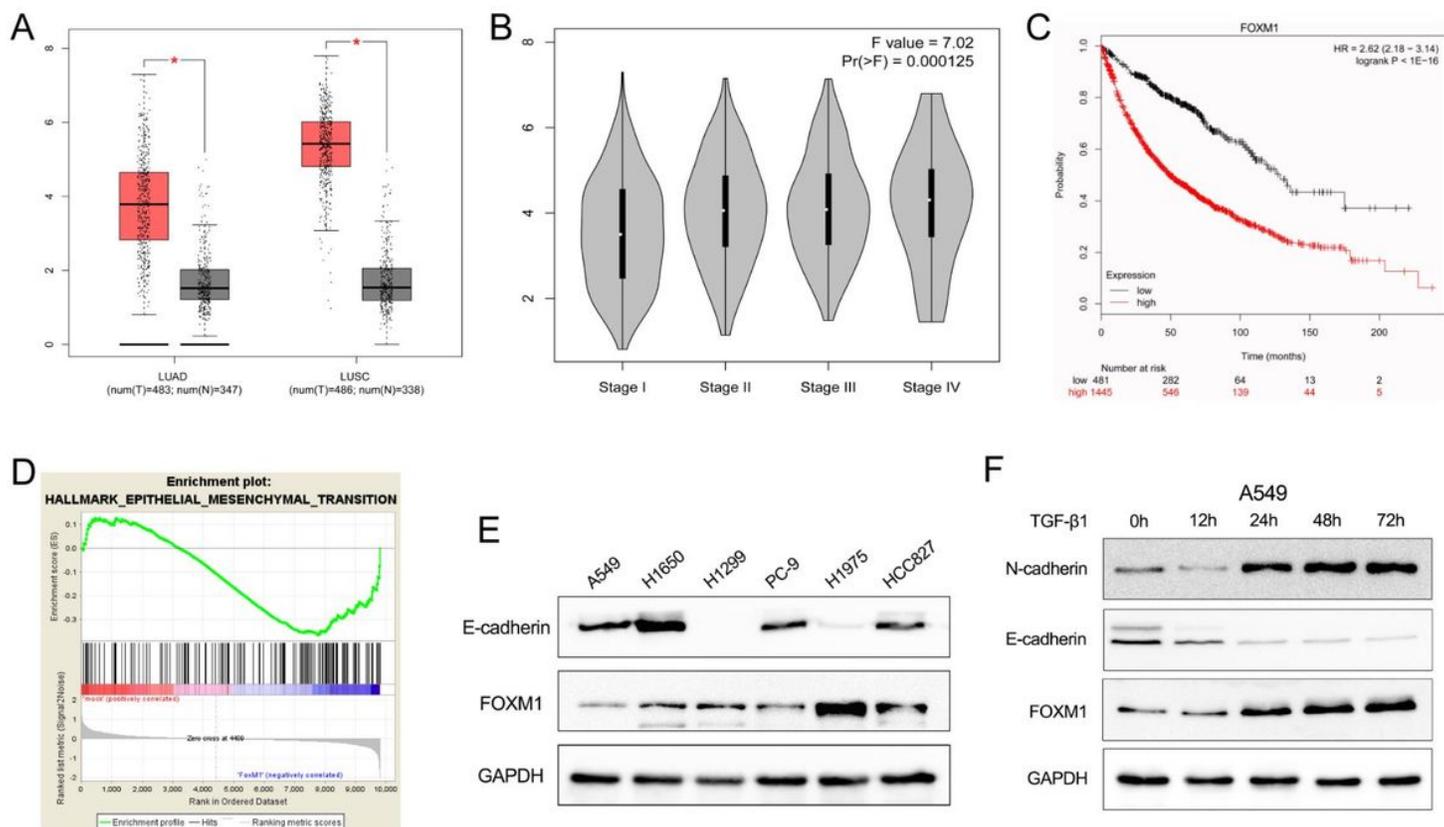


Figure 1

FOXM1 is up-regulated in NSCLC and relevant to EMT. (A) FOXM1 is significantly higher in LUAD and LUSC (red) when compared with their corresponding non-tumor tissue (grey). (B) FOXM1 is gradually up-regulated with the tumor deteriorate degree. (C) FOXM1 positive expressed NSCLC patients (red)

displayed a worse survival rate than FOXM1 negative expressed NSCLC patients (black). (D) The effect of FOXM1 knockdown on overall EMT signaling in GSE2222 profiling analyzed by GSEA. Control cells versus FOXM1 knockdown cells are presented by red part and blue part respectively. (E) Expression of FOXM1, E-cadherin, Vimentin and FOXM1 were analyzed by western blot in different NSCLC cell lines. (F) FOXM1 was found to be induced during treatment of TGF- β 1, and E-cadherin, N-cadherin were detected to prove the EMT initiation function of TGF- β 1. LUAD, lung adenocarcinoma; LUSC, lung squamous cell carcinoma.

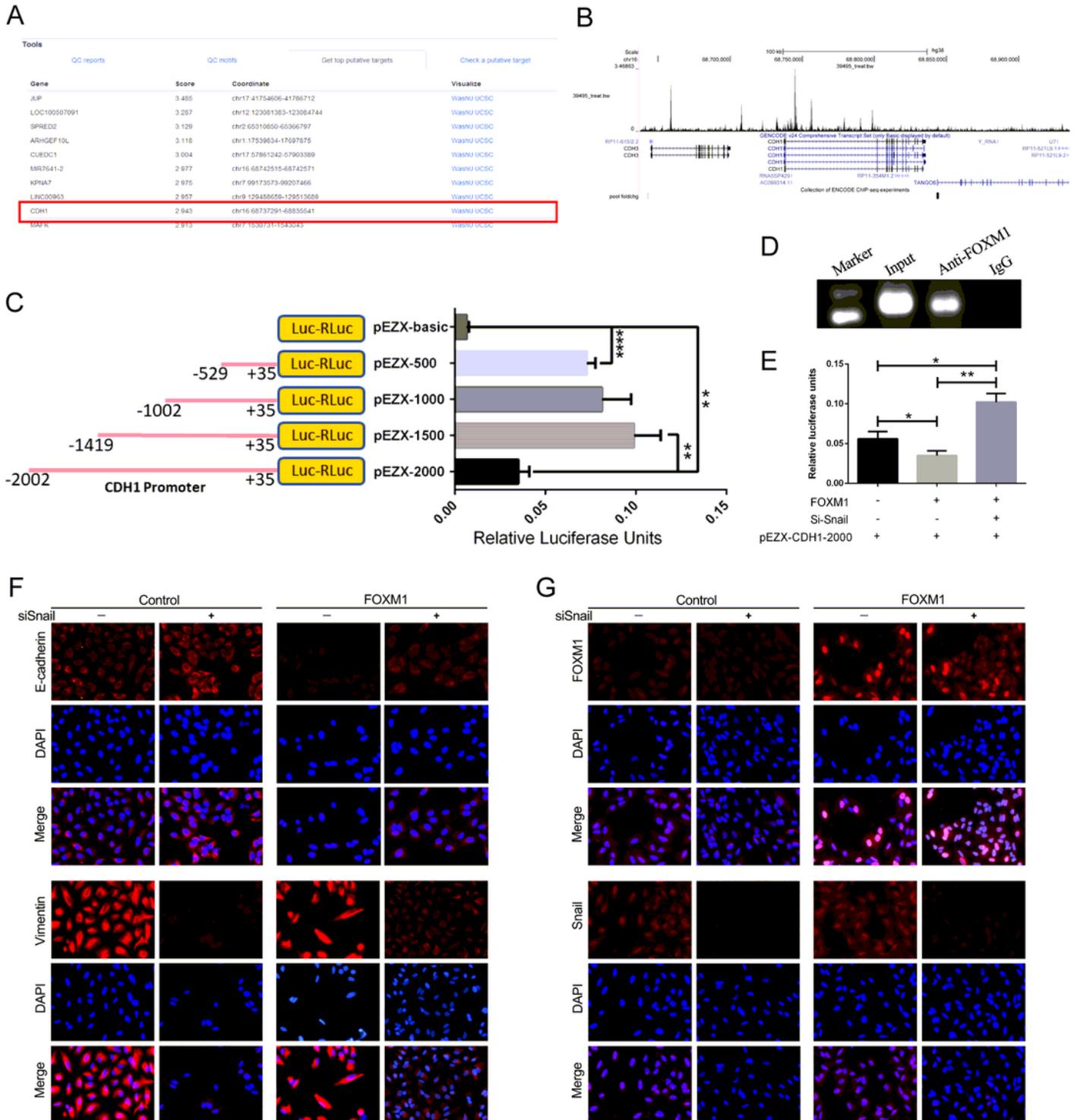


Figure 2

FOXM1 regulate E-cadherin expression through directly binding to its promoter or FOXM1-Snail-E-cadherin axis. (A) E-cadherin encoding gene CDH1 is among the top putative binding targets of FOXM1, which is highlighted by red frame. (B) Visualized ChIP-seq data reflect binding ability of FOXM1 to CDH1 in genome. (C) Dual luciferase report assay. Left panel: schematic diagram showing different length of CDH1 promoter constructs in dual luciferase reporter vector. Right panel: different corresponding CDH1 promoter constructs were transfected to FOXM1 overexpressed cell. After 48 h, luciferase activities were determined and normalized. (D) The binding of FOXM1 to CDH1 amplified by PCR and detected in gel electrophoresis in ChIP assay. (E) Dual luciferase report assay measured the effect of si-Snail on full length of CDH1 promoter in control A549 cell and FOXM1 overexpressed A549 cell. (F) and (G) IF visualized the effect of Snail knockdown on E-cadherin, Vimentin (F), FOXM1 and Snail (G) in control A549 cell and FOXM1 overexpressed A549 cell. ChIP, chromatin immunoprecipitation.

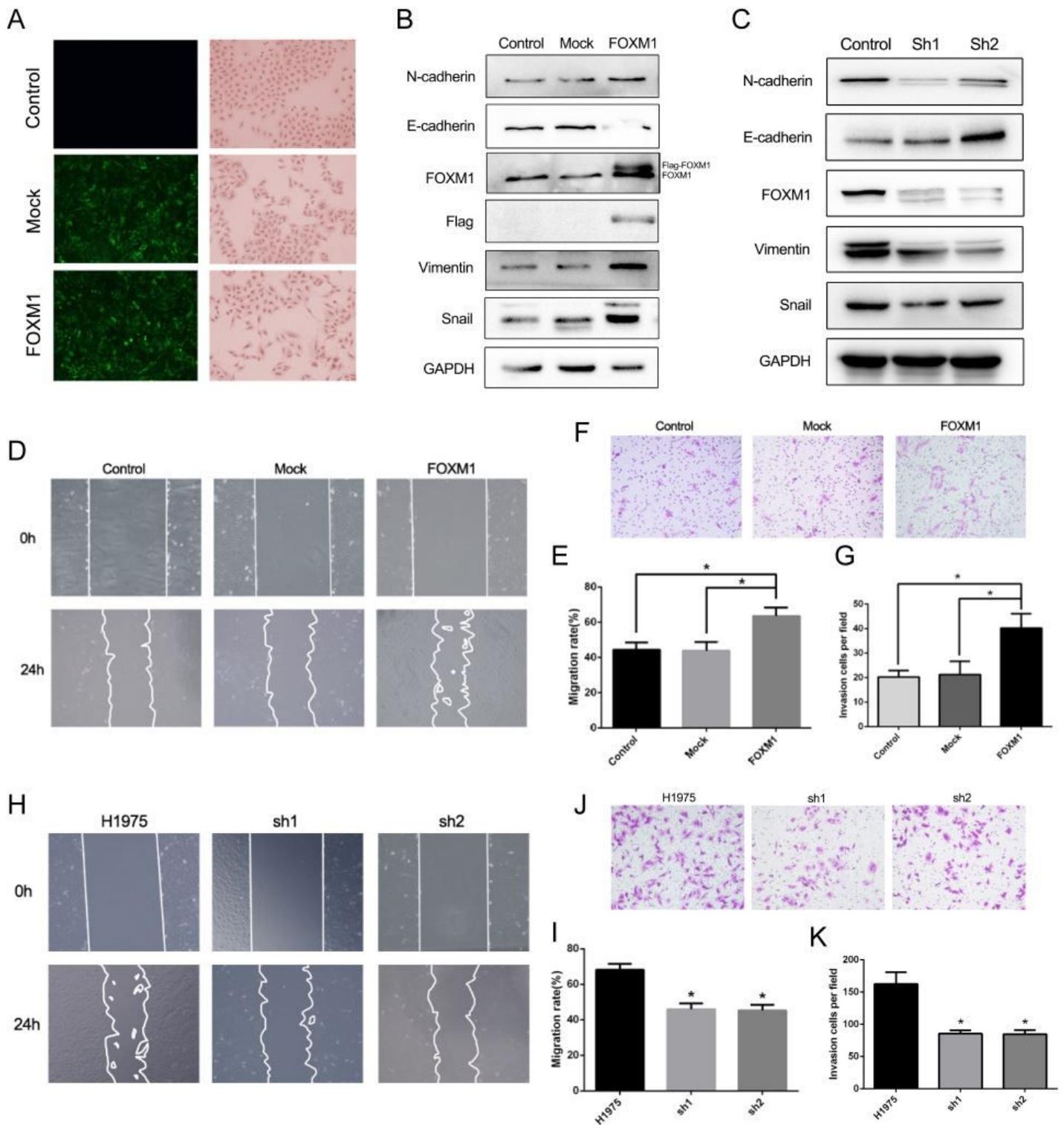


Figure 3

FOXM1 regulate the expression of EMT-related proteins and migration and invasion abilities in A549 and H1975 cells. (A) FOXM1 and non-FOXM1 lenti-virus containing GFP was transduced to A549 cells to make stable expressed cells. Right panels show that only FOXM1 lenti-virus transduce generate morphology change. (B) Expression of N-cadherin, E-cadherin, FOXM1, Vementin and Snail were analyzed by western blot in A549 cell, and A549 cell with stable empty vector (Mock) or FOXM1 overexpression. (C)

Expression of N-cadherin, E-cadherin, FOXM1, Vimentin and Snail were analyzed by western blot in H1975 cell, and A549 cell with FOXM1 knockdown by sh1 and sh2. (D-G) Migration ability and invasiveness of A549 cell, and A549 cell with stable empty vector (Mock) or FOXM1 overexpression were evaluated by scratch assay and matrigel invasion assay. Migration data (E) and invading cells (G) were collected and counted in three independent experiments. (H-K) Knockdown FOXM1 suppress H1975 cell migration (H) and invasion (J). Migration data (E) and invading cells (G) were collected and counted in three independent experiments.

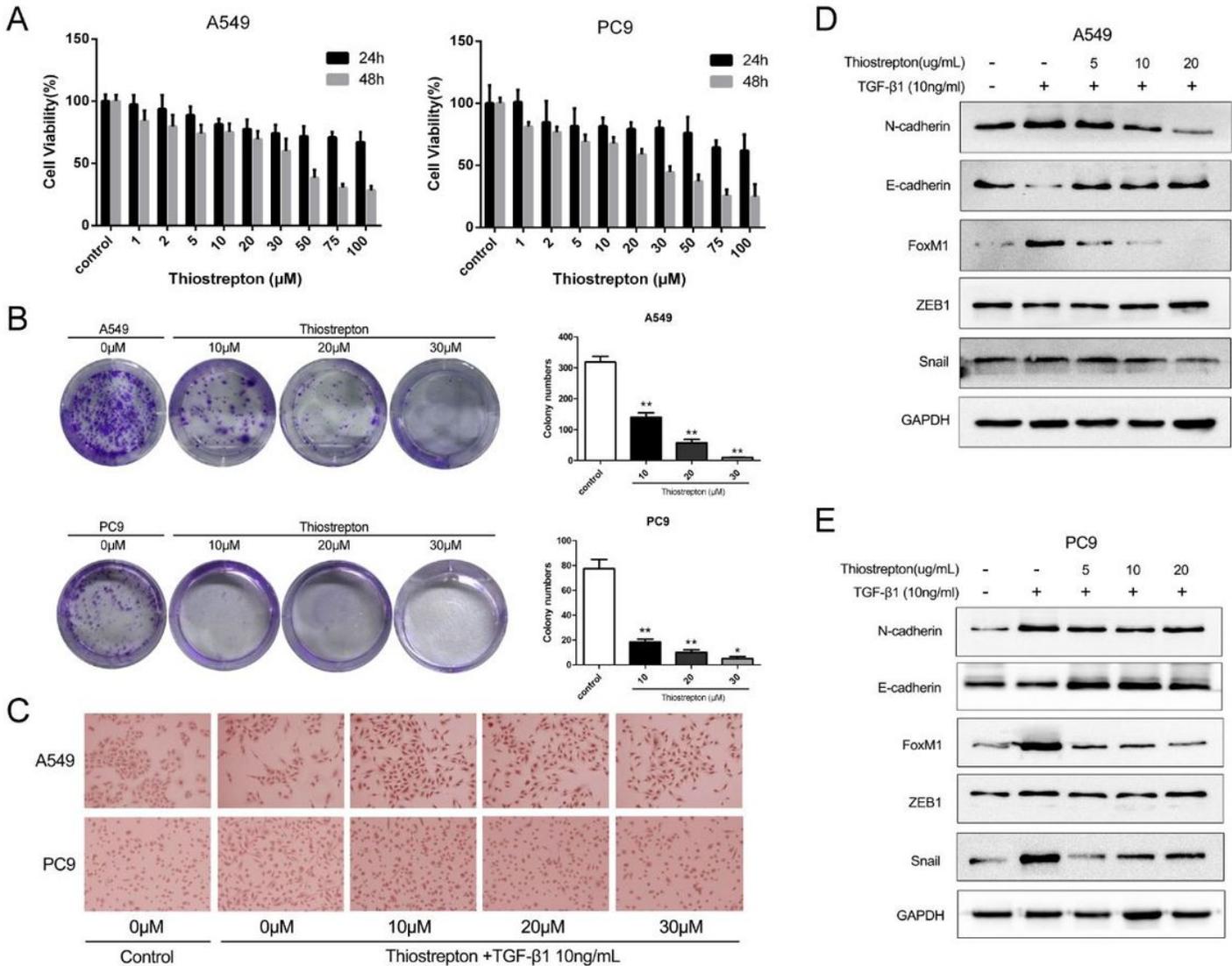


Figure 4

Thiostrepton prevent A549 cell and PC9 cell proliferation and colony formation, and reverse TGF- β 1 induced EMT. (A) Cell proliferation dose-inhibition curves for A549 and PC9 treated with thiostrepton for 24h and 48h. (B) Cells were incubated for 14d after being treated by 0, 10, 20, and 30 μM thiostrepton. Left panels: A549 (up) and PC9 (down) colonies obtained in representative wells are shown. Right panels: the counting number of colonies of A549 (up) and PC9 (down) are shown in bar charts. (C) A549 and PC9 cells were incubated with TGF- β 1 or in combination with 0, 10, 20, and 30 μM thiostrepton. TGF- β 1

triggers spindle cell shape formation compared to control cells, but this was gradually reversed by the presence of thioestrepton. (D) Expression of N-cadherin, E-cadherin, FOXM1, ZEB1 and Snail in A549 and PC9 cells undergo TGF- β 1 treatment treated with or without thioestrepton were evaluated by western blot.

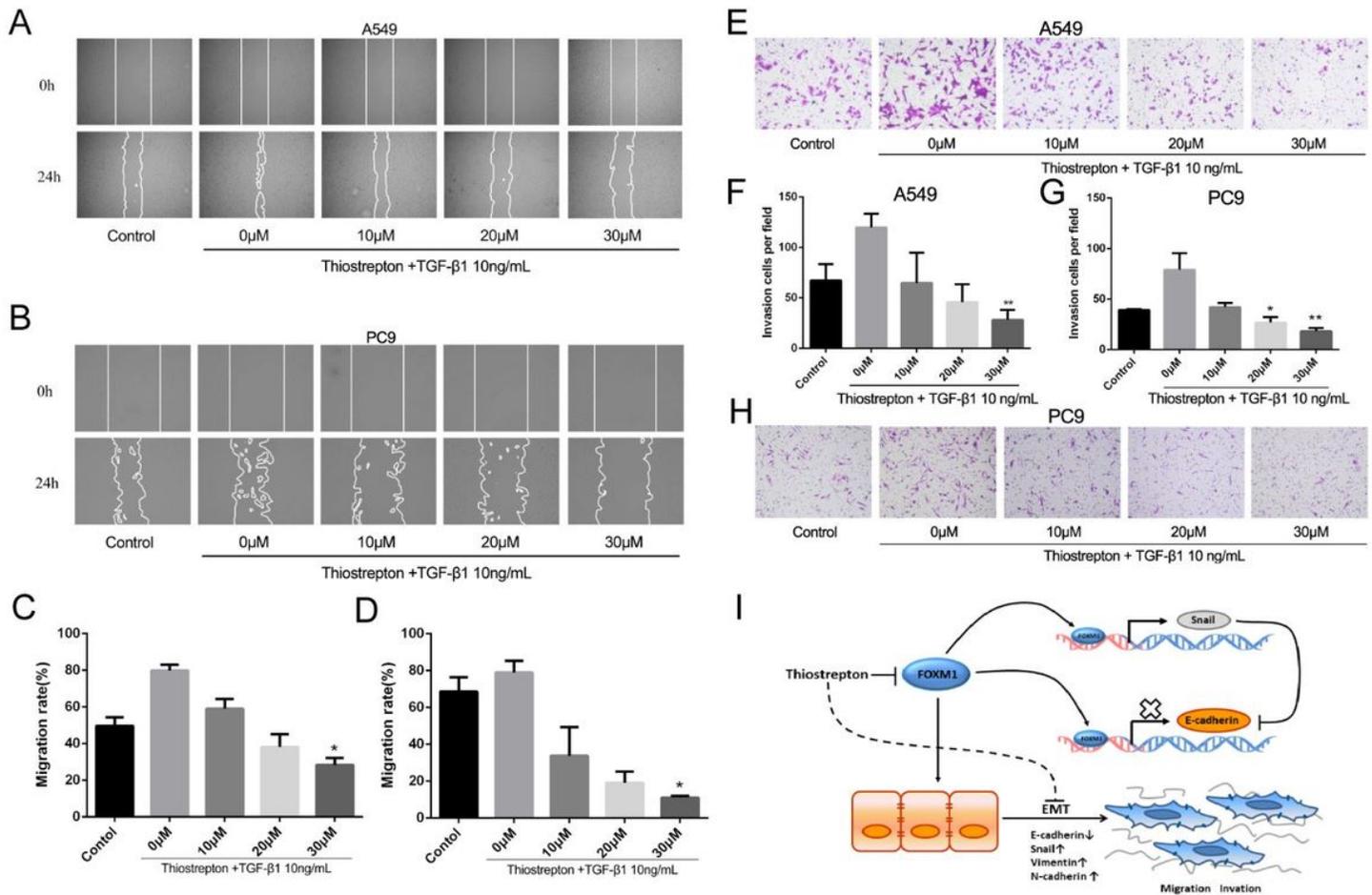


Figure 5

Inhibition of FOXM1 by thioestrepton attenuates TGF- β 1 induced migration and invasion ability. (A-D) migration ability and (E-H) invasiveness of A549 and PC9 incubated with TGF- β 1 in the presence with or without thioestrepton are presented. The percentage of migration rate of A549 and PC9 are described in (C) and (D) respectively, whereas (F) and (G) show invasion cell number per field respectively. (I) The mechanism of EMT in NSCLC cells based on FOXM1 manipulation.