Cellular Physiology and Biochemistry

Cell Physiol Biochem 2017;43:339-352
DOI: 10.1159/000480414
Published online: August 31, 2017
Accepted: July 17, 2017

Original Paper

Long Noncoding RNA FAL1 Promotes Cell Proliferation, Invasion and Epithelial-Mesenchymal Transition Through the PTEN/AKT Signaling Axis in Non-Small Cell Lung Cancer

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Key Words
Lncrna • FAL1 • Lung cancer • EMT • PTEN/AKT • Metastasis

Abstract
Background/Aims: Recently, long non-coding RNAs (lncRNAs) have been found to have many biological effects in different cancer stages. Several studies have revealed that focally amplified lncRNA on chromosome 1 (FAL1) regulates cancer progression via p21. However, the expression and mechanism of FAL1 in non-small cell lung cancer (NSCLC) still remain unclear. ***Methods:*** We detected the FAL1 level in NSCLC tissues and in established cell lines using quantitative real-time PCR and evaluated the clinical significance. FAL1 was silenced or overexpressed using siRNA or lentivirus to study whether FAL1 affected cell proliferation, invasion and migration. Xenograft growth and pulmonary metastasis were observed using nude mouse models. The mechanisms were explored with western blotting and immunohistochemistry. ***Results:*** FAL1 was significantly overexpressed in NSCLC compared with adjacent normal tissues, and a high level of FAL1 correlated with poor histological grade, increased lymph node metastasis and advanced TNM stage. Loss- and gain-of-function experiments *in vitro* verified that knockdown of FAL1 inhibited cell proliferation, invasion, migration and EMT via the PTEN/AKT pathway. Furthermore, an *in vivo* assay confirmed that overexpression of FAL1 facilitated tumor growth and metastasis. ***Conclusion:*** FAL1 may promote tumorigenesis and progression of NSCLC through the PTEN/AKT axis, which could lead to lncRNA-related diagnostics and therapeutics in NSCLC.

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Introduction

Among all new cancer cases in 2012 worldwide, lung cancer accounted for 13% of them, and lung cancer mortality has remained the highest of all cancer types during the past several decades [1]. Non-small lung cancer (NSCLC) and small cell lung cancer (SCLC) account for 85% and 15%, respectively, of lung cancer cases [2]. Though diagnostic and therapeutic technologies have advanced rapidly [3, 4], the total five-year survival rate of NSCLC is still as low as 15% [5]. Therefore, it is of critical importance to further explore the mechanism of the development and metastasis of NSCLC to provide more evidence and targets for biological therapies.

Long non-coding RNAs are a subtype of non-coding RNAs. They are longer than 200 nucleotides and were previously regarded as 'transcription noise' that did not have any function [6, 7]. However, due to incisive studies, today, lncRNAs are known to participate in a large range of cellular processes, including proliferation, cell cycle progression, invasion and migration via chromatin modification, transcriptional modification, post-transcriptional modification or competing for microRNAs [8-10]. For example, lncRNA-LOC572558 inhibited bladder cancer cell proliferation and tumor growth by regulating the AKT-MDM2-p53 signaling axis [11]. Additionally, Linc00974 interacting with KRT19 promoted proliferation and metastasis by acting as a sponge of miR-642 in hepatocellular carcinoma [12]. Moreover, lncARSR was transmitted by exosomes and promoted sunitinib resistance in renal cancer [13]. Functional studies have confirmed that many lncRNAs are involved in the development and progression of NSCLC [14], including HOTAIR, H19, CCAT2, and AGAP1-AS1, acting as cancer-promoting genes, and MEG3, LET, GAS5-AS1, and TUG1, behaving as tumor suppressors [15-22].

Focally amplified lncRNA on chromosome 1 (FAL1), a recently verified 566nt-long lncRNA located at 1q21.2, has been confirmed to be upregulated and to promote development in several types of tumors, particularly ovarian cancer [23]. Ectopic expression of FAL1 caused by somatic copy-number alterations (SCNAs) functions as an oncogenic element in ovarian cancer in association with polycomb complex protein BMI1 and p21 [24]. Another study showed that in thyroid cancer FAL1 can regulate the transcription factor E2F to promote aggressive behavior in papillary thyroid cancer [25]. However, little is known about the detailed function and mechanism of FAL1 in NSCLC.

In this study, we first analyzed the expression level of FAL1 in NSCLC tissues compared with paired adjacent tissues and in NSCLC-derived cell lines and elucidated the relationship between FAL1 expression and clinicopathological features. Moreover, with down- and up-regulation of FAL1 in specific cell lines, biological function studies *in vitro* and *in vivo* were performed to detect the contributions of FAL1 to NSCLC development and progression. Then, several investigations of epithelial-mesenchymal transition (EMT) and the expression of PTEN/AKT markers were conducted to profile the mechanism of FAL1. Overall, in this study, we reveal the role that FAL1 plays in NSCLC and provide a potential target for further development of biological diagnostics and therapeutics.

Materials and Methods

Patients and tumor tissues

All of the 100 paired tumor and adjacent tissue samples were collected from patients undergoing open or thoracoscopic surgery from 2011 to 2012 at the Department of Thoracic Surgery, Jiangsu Province People's Hospital. None of the patients received local or systemic chemotherapeutic treatment before the surgery. The samples were conserved in nitrogen before further studies after being removed. We recorded the clinical characteristics of the patients, including TNM staging based on the AJCC staging system (the 7th edition). Written informed consent from all patients and the approval of the Research Ethics Committee of Nanjing Medical University were received before our study.

RNA extraction and qRT-PCR analysis

Total RNA from tissues and cells was extracted using TRIzol reagent (Invitrogen, CA, USA) according to the manufacturer's protocol. The details of qRT-PCR analysis and the primers used are provided (for all online suppl. material, see www.karger.com/doi/10.1159/000480414) in the Supplementary Materials and Methods.

Cell lines and culture conditions

All cell lines (16HBE, A549, SPCA1, H358, H1299, H1975, PC-9 and SK-MES-1) were bought from Shanghai Institutes for Biological Science, China. The eight cell lines were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (Gibco, NY, USA), 100U/mL penicillin and 100μg/mL streptomycin (Gibco, NY, USA) at 37°C in humidified air containing 5% CO_2.

siRNA and plasmid transfection

For knockdown, H1299 cells were cultured in six-well plates. When they reached 70% density after 24 h, cells were transfected with siRNA targeting specific genes or negative control siRNA (siRNA-NC) using Lipofectamine 2000 (Invitrogen, USA). The specific siRNA sequences are provided (see supplementary material) in the Supplementary Materials and Methods.

For overexpression, the full-length FAL1 sequence was cloned into the lentiviral vector pLenti-EF1a-EGFP-F2A-Puro-CMV (Obio, Shanghai, China). After amplification, lentiviral-FAL1 (LV-FAL1) or empty vector (LV-vector) as a control was transfected into SPCA1 cells using polybrene (Obio, shanghai, China) according to the manufacturer's instructions. Then, we selected cells with puromycin and identified the transfection efficiency using qRT-PCR.

Cell proliferation assay

A Cell Counting Kit-8 (CCK8) assay (Dojindo, Tokyo, Japan) was used to monitor cell proliferation, and further details can be found (see supplementary material) in the Supplementary Materials and Methods.

Colony formation assay

Single cell suspensions in 2 ml of 10% FBS RPMI-1640 containing approximately 400 cells were prepared after treatment with specific siRNA, lentivirus or the control, and the cells were seeded in six-well plates. After 15 days of culture at 37°C with 5% CO_2, the cells were fixed in methanol for 30 minutes and stained with crystal violet for 20 minutes. Colonies containing more than 50 cells were counted and compared. The experiment was repeated three times independently for each group.

Wound healing assay

Cells were seeded into six-well plates and cultured to a density of 90%. Then, a line wound was made by scraping a 200μl tip across the confluent cell layer. After being washed with PBS, the cells were cultured in serum-free RPMI-1640 medium. Wound closure was visualized in five random fields using a light microscope (DFC500, Wetzlar, Germany) after 48 h of culture.

Cell invasion and migration assay

Transwell and migration assays were performed with 8-μm-pore chamber inserts (Millipore, MA, USA). Experiments were repeated in triplicate independently. Details of the procedure are described (see supplementary material) in the Supplementary Materials and Methods.

Flow cytometric analysis

In the cell apoptosis assay, transfected cells were collected into a flow tube and washed with pre-chilled PBS three times. After staining with an Annexin V-APC and 7-AAD Double Staining Kit (Keygene, Nanjing, China) according to the manufacturer's recommendations, the cells were analyzed using flow cytometry (FACScan, BD Biosciences, USA). In the cell cycle assay, cells were suspended in 70% cold ethanol overnight after harvest. Then, cells stained with propidium iodide (PI) (Vazyme, Nanjing, China) for 30 minutes were analyzed with a FACScan flow cytometer. The proportion of cells in different cycle phases were calculated and compared.

Xenograft tumor model and in vivo metastasis analysis

A total of 5×10^5 SPCA1 cells transfected with LV-FAL1 or LV-vector were injected into the flanks of female BALB/c-nude mice (5-6 weeks old). Tumor growth was observed each week, and the size of the tumors was calculated as length × width^2 × 0.5(mm^3). The mice were sacrificed after six weeks, and the weight of the xenografts was determined. For the metastasis model, the mice were injected with 1×10^6 SPCA1 cells transfected with the EGFP-expressing LV-FAL1 or LV-vector suspended in 100 μL of PBS. After 6 weeks, the mice were sacrificed, and the lungs were visualized by fluorescence using a 470-nm light source (Lightools Research, Encinitas, CA, USA) to analyze lung metastasis. The number of metastatic tumor nodules was determined, and the lungs were collected for further study. All the animal experiments were performed under the supervision of the Institutional Animal Care and Use Committee of Nanjing Medical University.

Immunohistochemistry and H&E staining.

Immunohistochemistry (IHC) was used to detect the protein level in xenografts, and H&E staining was used to visualize metastatic nodules in the lungs of mice. More information is provided (see supplementary material) in the Supplementary Materials and Methods.

Western-blot analysis

Total protein was extracted from cells using RIPA reagent (Beyotime, Jiangsu, China) containing 100μg/ml PMSF (Beyotime, Jiangsu, China) and 2μg/ml aprotinin (Biosharp, Hefei, China) and quantified using a bicinchoninic acid quantification assay (Pierce Biotechnology, Rockford, IL, USA). The detailed western blotting protocol and the antibodies used are provided (see supplementary material) in the Supplementary Materials and Methods.

Statistical analysis

All the statistical analyses were performed with GraphPad software 5.0 and SPSS 19.0. Student's t-test, one-way ANOVA, and Spearman's test were used to analyze the P value. $P<0.05$ was considered to indicate a statistically significant difference.

Results

FAL1 was upregulated in human NSCLC tissues and cell lines.

To investigate the relationship between FAL1 and NSCLC, we identified the expression of FAL1 in 100 pairs of NSCLC and adjacent normal tissues and in NSCLC-derived cell lines using quantitative real-time PCR. The level of FAL1 was significantly upregulated in 78% (78 of 100) of tumor tissues, suggesting that FAL1 is involved in NSCLC tumorigenesis (Fig. 1A). All seven NSCLC cell lines had higher FAL1 expression than the normal 16HBE cell line, and of these, H1299 cells had the highest FAL1 expression, while SPCA1 cells had the lowest FAL1 level (Fig. 1B). Furthermore, we divided the 100 patients into high and low expression groups based on the median FAL1 level to explore correlations between FAL1 expression and clinicopathological features (Fig. 1C). As shown in Table 1, the expression of FAL1 was significantly correlated with histological grade, lymph node metastasis, tumor size and TNM stage, while no differences were found with gender, age, smoking status, or histological type, indicating that FAL1 may act as an oncogene in NSCLC.

To study the effect of FAL1 *in vitro*, we selected H1299 cells for RNA interference and SPCA1 cells for FAL1 overexpression experiments. Three siRNAs for FAL1 (siRNA-FAL1-1, siRNA-FAL1-2, siRNA-FAL1-3) were transfected into H1299 cells, and qRT-PCR was used to confirm the knockdown efficiency 48 h later. To avoid off-target effects caused by a single siRNA sequence, we double-transfected cells with siRNA-FAL1-2 and siRNA-FAL1-3 according to a study by Lv et al [10].. Compared with negative controls, FAL1 expression in H1299 cells was knocked down by 92% using siRNA-FAL1-2+3 (Fig. 1D). Therefore, we chose the co-transfection group as the siRNA-FAL1 group for further experiments. Meanwhile, lentivir-

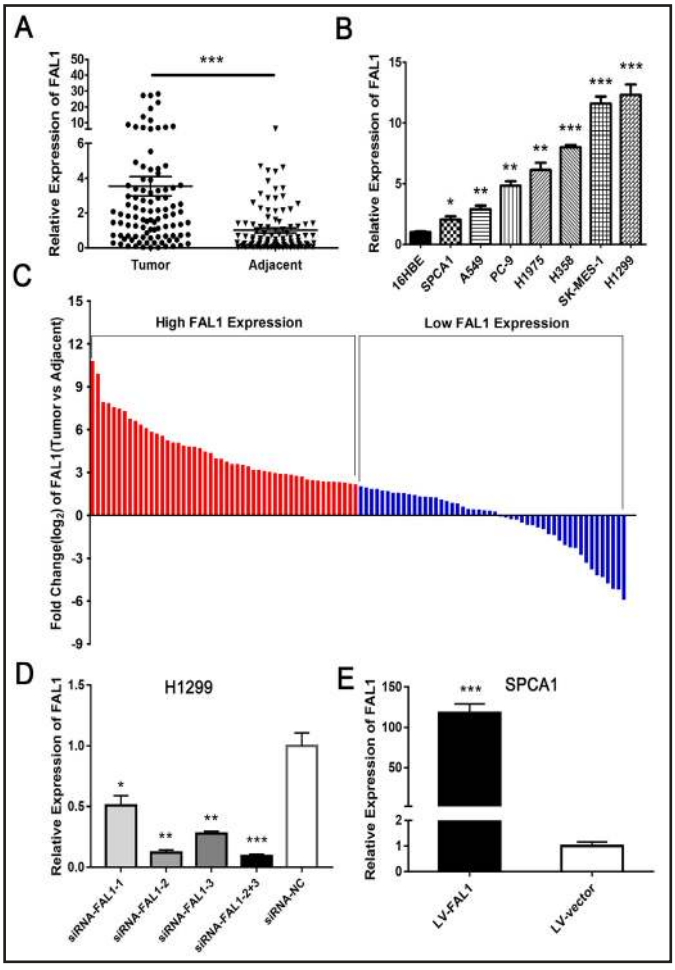

Fig. 1. FAL1 was overexpressed in NSCLC tissues and cell lines. (A) The FAL1 expression level in 100 NSCLC tissues and paired adjacent normal tissues was detected by qRT-PCR. (B) The FAL1 expression level in normal lung 16HBE epithelial cells and seven NSCLC cell lines; β-actin was used as an internal control. (C) The fold change (log2 change) in the FAL1 level between 100 pairs of NSCLC and adjacent normal tissues was measured, and samples were divided into a high FAL1 expression group (50 pairs) and a low FAL1 expression group (50 pairs) based on the median FAL1 level. (D) H1299 cells were transfected with siRNA-FAL1, and the FAL1 expression level was detected by qRT-PCR. (E) SPCA1 cells were transfected with LV-FAL1 or LV-control, and FAL1 expression was examined with qRT-PCR using β-actin as an internal control. The data are shown as the mean ± SD (*P<0.05; **P<0.01; ***P<0.001).

Table 1. Correlation between FAL1 expression and clinicopathological characteristics of NSCLC patients (n=100), [a]The median expression level of FAL1 was used as the cutoff and *Indicates p value<0.05

Characteristics	All Patients	FAL1 low expression (<Median[a])	FAL1 high expression (≥Median[a])	p Chi-squared test p-value
No.	100	50	50	
Age(years)				
<60	68	31	37	0.284
≥60	32	19	13	
Gender				
Male	84	40	44	0.413
Female	16	10	6	
Smoke				
Yes	77	37	40	0.632
No	23	13	10	
Histological grade				
Moderately	61	37	24	0.014*
Poorly	39	13	26	
Histological subtype				
LSC	45	22	23	1.0
LAC	55	28	27	
Lypho metastasis				
Yes	59	21	38	0.001*
No	41	29	12	
Tumor size(cm)				
≤5	53	34	19	0.005*
>5	47	16	31	
TNM stage				
Ⅰa+Ib	19	14	5	0.0008*
Ⅱa+IIb	48	28	20	
IIIa	33	8	25	

FAL1 (LV-FAL1) was used to overexpress FAL1 in SPCA1 cells. After selecting cells using an appropriate concentration of puromycin, the FAL1 level was 110-fold higher than that in the empty vector (LV-vector) group (Fig. 1E).

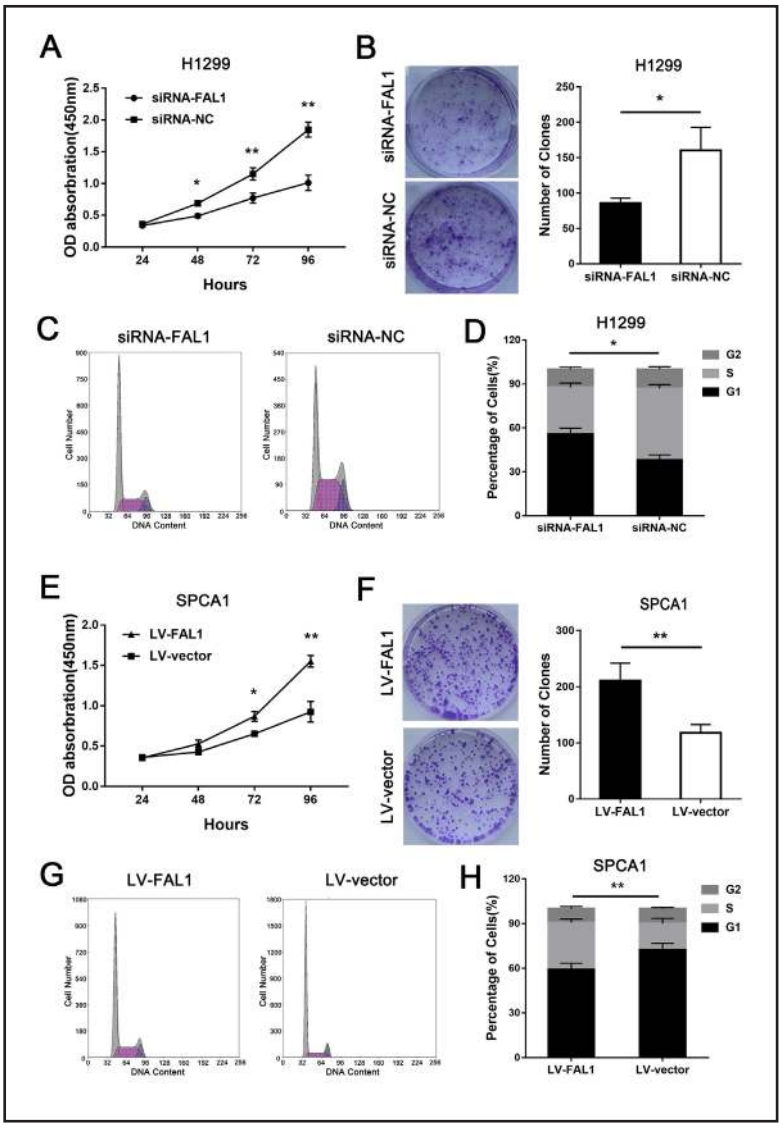

Fig. 2. FAL1 promoted the proliferation and cell cycle of NSCLC cell lines. (A, B, E, F) CCK8 (A, E) and colony formation assays (B, F) were used to detect the viability and proliferation ability of siRNA-FAL1-transfected H1299 cells and LV-FAL1-treated SPCA1 cells compared with control group cells. (C, G) Cell cycle distribution of FAL1 knockdown H1299 cells(C) and FAL1-overexpressing SPCA1 cells (G). (D, H) The percentage of H1299 (D) and SPCA1 (H) cells in different cell cycle phases was measured. The data are shown as the mean ± SD. (*P<0.05; **P<0.01).

FAL1 affected proliferation and cell cycle distribution of NSCLC cells

Since we surmised that FAL1 could promote NSCLC progression, we performed cell functional experiments with transduced cell lines. CCK8 analysis revealed that downregulation of FAL1 significantly repressed the proliferation of H1299 cells (Fig. 2A), while upregulation of FAL1 improved the growth of SPCA1 cells (Fig. 2E). Additionally, colony formation assays showed that the clonogenic ability of H1299 cells was decreased after siRNA-FAL1 interference (Fig. 2B); however, FAL1-overexpressing SPCA1 cells formed more colonies than the control group (Fig. 2F).

To determine whether FAL1 has an effect on cell cycle and apoptosis, we examined the loss- and gain-of-FAL1 cell lines using flow cytometry. As shown in Fig.2, H1299 cells treated with siRNA-FAL1 were arrested in G0/G1 phase compared with the siRNA-NC group (Fig. 2C, D), and overexpression of FAL1 decreased the G0/G1 arrest in SPCA1 cells (Fig. 2G, H). Additionally, we observed the apoptotic rate of FAL1 knockdown H1299 cells and FAL1 upregulated SPCA1 cells but found no differences (data not shown).

Taken together, these results revealed that FAL1 promoted proliferation ability and decreased G1/G0 arrest in NSCLC cells.

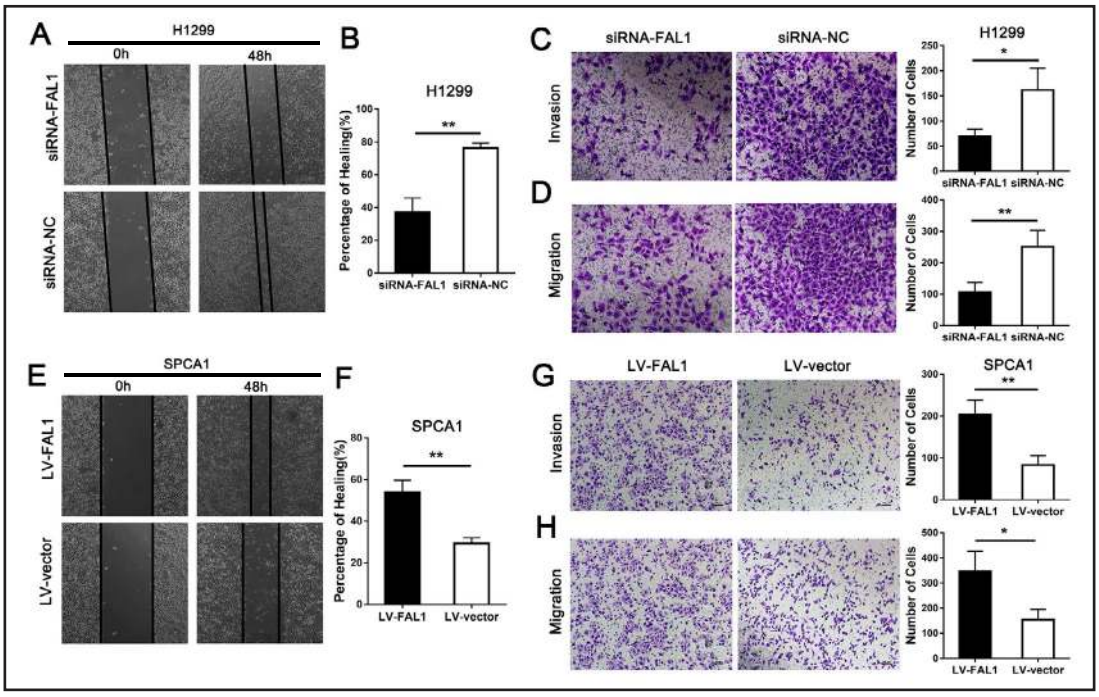

Fig. 3. FAL1 affected NSCLC metastasis *in vitro*. (A, B, E, F) A wound-healing assay was performed in treated H1299 (A, B) and SPCA1 (E, F) cells and compared with the respective control group. (C, G) Transwell invasion and Migration assays were conducted with FAL1 knockdown H1299 cells (C) and FAL1 overexpression SPCA1 cells (G). (D, H) Migration assays of treated H1299 (D) and SPCA1 (H) cells are shown. Six different fields of view were chosen randomly, and images were acquired at 100×magnification. The data are shown as the mean ± SD (*P<0.05; **P<0.01).

FAL1 influenced NSCLC cell invasion and migration

Invasion and migration play important roles in tumor progression and lead to an unfavorable prognosis in NSCLC. To examine the effect of FAL1 on invasion and migration, we performed wound-healing, transwell and Matrigel assays with treated cell lines. Fig. 3 shows that siRNA-FAL1 obviously impaired the wound-healing ability of H1299 cells (Fig. 3A, B), while overexpression of FAL1 in A549 cells led to the opposite effect (Fig. 3E, F). Moreover, the transwell assay produced the same results as the wound-healing assay with regard to changes in cell migration ability (Fig. 3D, H). Then, the Matrigel assay showed that downregulation of FAL1 markedly reduced the number of invasive H1299 cells (Fig. 3C). In contrast, upregulation of FAL1 increased the invasion activity of SPCA1 cells (Fig. 3G). These results indicated that FAL1 played a significant role in facilitating the invasion and migration of NSCLC cells.

FAL1 promoted EMT in NSCLC cells

Because EMT is one of the most well-known processes associated with tumor metastasis [26], we probed the EMT markers ZO-1, E-cadherin, N-cadherin and Vimentin with western blotting. The expression of ZO-1 and E-cadherin was clearly increased, while N-cadherin and Vimentin levels decreased in H1299 cells treated with siRNA-FAL1 relative to siRNA-NC, and the opposite expression results were observed in FAL1-overexpressing SPCA1 cells compared with empty vector-treated cells (Fig. 4A, and see supplementary material, S1A, S1B). These data indicated that FAL1 may influence NSCLC metastasis via EMT.

Regulation of FAL1 affects the expression of the PTEN/AKT signaling axis via BMI1

To further explore the detailed molecular mechanisms of FAL1 and since Hu et al. demonstrated that FAL1 could stabilize BMI1 and promote the function of polycomb

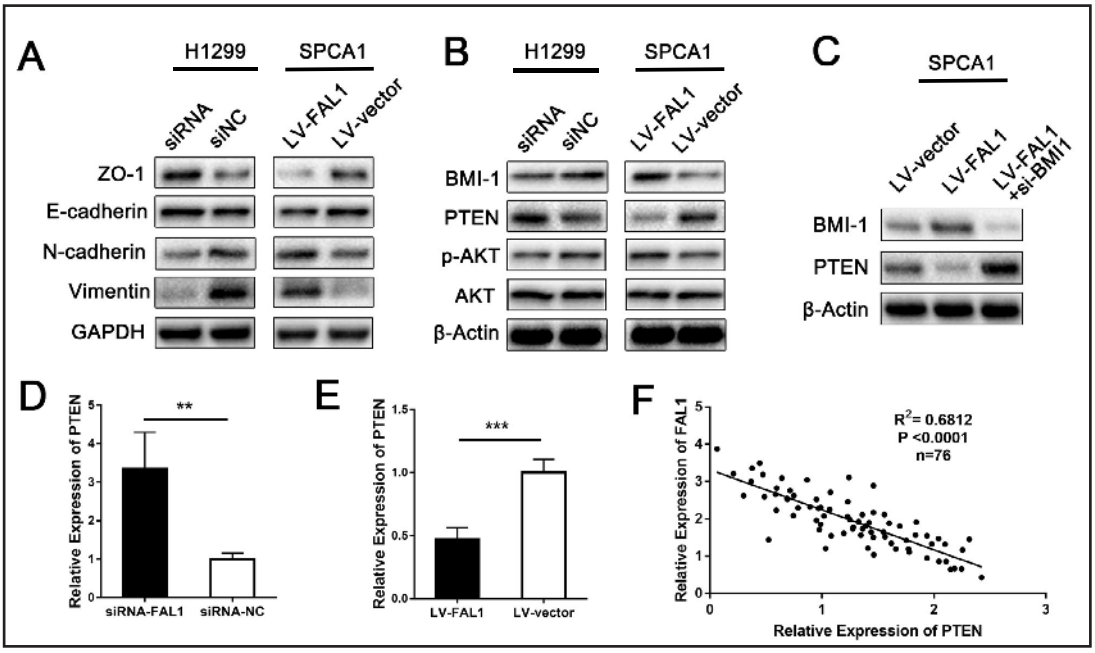

Fig. 4. FAL1 promoted EMT and affected the PTEN/Akt pathway. (A) Western-blot showing the protein level of EMT markers in different treatment cells. GAPDH was used as an internal control. (B) The protein level of PTEN, Akt, p-Akt and BMI1 in FAL1 knockdown H1299 and FAL1 overexpression SPCA1 cells measured by western-blot. β-Actin was used as an internal control. (C) The protein expression of PTEN and BMI1 in siRNA-BMI1 and LV-FAL1 co-treated SPCA1 cells compared with the control group. β-Actin was used as an internal control. (D, E) Following siRNA-FAL1 or LV-FAL1 transfection, the PTEN mRNA level in H1299 (D) and SPCA1 (E) cells was detected using qRT-PCR. (F) Correlation between FAL1 and PTEN expression in 76 NSCLC tissues. Here, siRNA indicates siRNA-FAL1. The data are shown as the mean ± SD (*P<0.05; **P<0.01; ***P<0.001).

repressive complex 1 (PRC1) in ovarian cancer cells [24], we examined the expression of BMI1 in established cells with western blotting and found that BMI1 levels decreased in siRNA-FAL1-treated H1299 cells but increased in FAL1-overexpressing SPCA1 cells (Fig. 4B, and see supplementary material, S1C, S1D). This indicated that FAL1 could act as a protective factor of PRC1 by stabilizing BMI1 in NSCLC, similar to the findings in ovarian cancer cells. Because we found that FAL1 could influence EMT in NSCLC cells, we next sought to explore the relationship between BMI1 and EMT, and found that, according to the report of Song et al. [27]. PTEN is associated with both BMI1 and EMT. PTEN is a cancer suppressor that participates in many activities in several types of tumors, including NSCLC [28]. Therefore, we speculated that FAL1 repressed the expression of PTEN, promoting cell proliferation and stimulating EMT through the PTEN/AKT pathway. To verify our assumption, we detected the mRNA level of PTEN using qRT-PCR in 76 tumor tissues and in established cell lines. PTEN mRNA was highly expressed in FAL1 knockdown H1299 cells, while lower expression was observed in FAL1–overexpressing SPCA1 cells compared with the respective control groups (Fig. 4D, E). More importantly, the level of PTEN was negatively correlated with FAL1 in the 76 NSCLC tissues, with an R^2 value 0.6812 (Fig. 4F). Moreover, the protein expression level of PTEN and the downstream AKT activity in experimental cells was confirmed by western blotting. The decrease in the PTEN level induced by siRNA-FAL1 enhanced AKT phosphorylation, as manifested by upregulation of the p-AKT level without changes in the total AKT level in H1299 cells (Fig. 4B, and see supplementary material, S1C, S1D). Furthermore, we constructed siRNA-BMI1 to downregulate BMI1 in FAL1-overexpressing SPCA1 cells and found that siRNA-BMI1 antagonized the inhibitory effect of FAL1 on PTEN

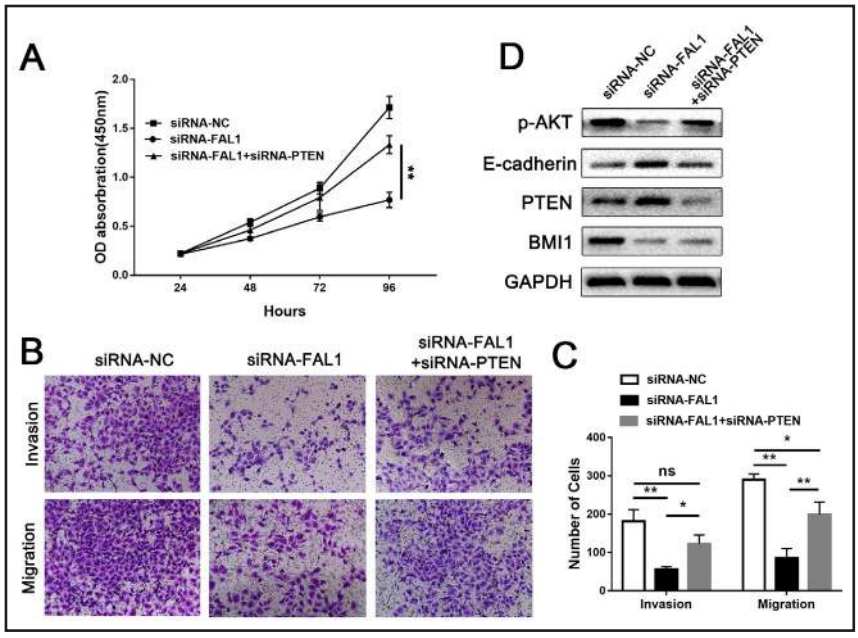

Fig. 5. Knockdown of PTEN reversed the effect of FAL1. (A) A CCK8 assay was used to observe the proliferation ability of H1299 cells after co-transfection with siRNA-FAL1 and siRNA-PTEN. (B, C) Transwell invasion and migration assays were performed to show the effect on metastasis after co-transfection with siRNA-FAL1 and siRNA-PTEN. (D) The protein expression level of E-cadherin, PTEN, p-Akt and BMI1 was detected by western-blot. All images were acquired at 100× magnification. The data are shown as the mean ± SD (*P<0.05; **P<0.01; ns: non-significant).

expression, which confirmed that BMI1 functions as a regulatory factor between FAL1 and PTEN (Fig. 4C, and see supplementary material, S1E).

To further confirm our assumption, we knocked down PTEN expression in FAL1-downregulated H1299 cells using siRNA-PTEN. A CCK8 assay confirmed that the decreased PTEN level reduced the effect of FAL1 interference on cell proliferation (Fig. 5A). In addition, cell invasion and migration ability were markedly rescued by siRNA-PTEN (Fig. 5B, C). Using western blotting, we found that the level of E-cadherin remarkably increased and phosphorylation of Akt was significantly restored after PTEN interference in FAL1 knockdown H1299 cells in contrast to the single siRNA-FAL1 treatment group (Fig. 5D, and see supplementary material, S1F).

Taken together, these results suggested that FAL1 promoted proliferation, invasion, migration and EMT in NSCLC cells *in vitro* by downregulating the PTEN/AKT signaling axis via BMI1.

Overexpression of FAL1 promoted tumorigenesis and metastasis in vivo

To verify whether FAL1 could promote tumorigenesis *in vivo*, SPCA1 cells were stably transfected with LV-FAL1 or LV-vector encoding EGFP, and then, transfected cells were injected into mice. Tumor growth was observed for 6 weeks. Xenografts derived from the overexpression FAL1 group were larger and heavier than those in the control group (Fig. 6A-C). Using qRT-PCR, we confirmed the FAL1 level in xenograft tumors (Fig. 6D). In addition, the proliferation protein marker Ki67 was expressed at significantly higher levels, while PTEN expression was markedly decreased in the LV-FAL1 group compared with the control group (Fig. 6E, F) based on IHC analysis. We next injected LV-FAL1- or LV-vector-transfected SPCA1 cells into the tail vein of mice to probe metastasis *in vivo* (Fig. 6G). After 6 weeks, the data revealed that the number of metastatic tumor nodules in the lungs of the LV-FAL1 group was significantly higher than that in the LV-vector group (Fig. 6H). HE staining was used to visualize the metastatic tumor nodules (Fig. 6I). The *in vivo* probe supported the *in vitro* findings and demonstrated that FAL1 can also increase the oncogenic activity of NSCLC cells *in vivo*.

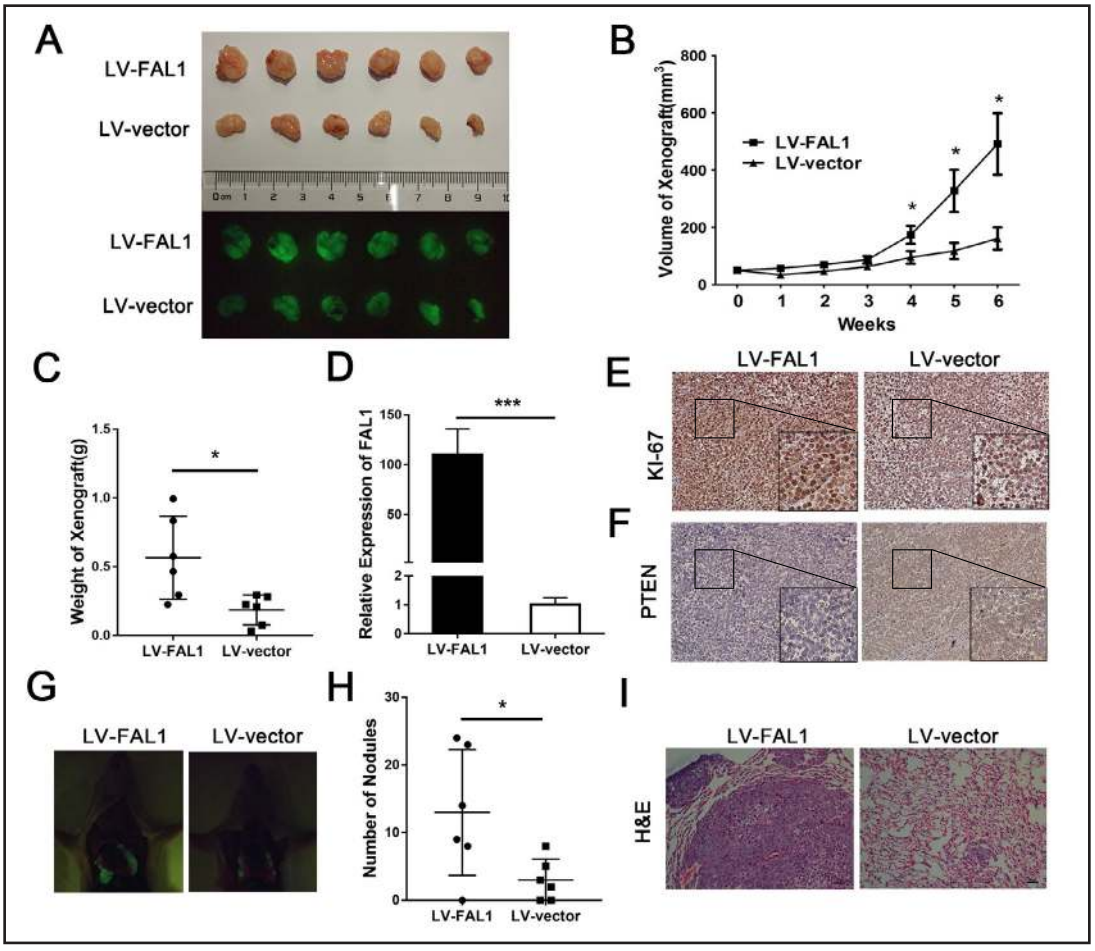

Fig. 6. FAL1 promoted growth and metastasis of NSCLC cells *in vivo*. (A) Tumors from mice injected with LV-FAL1- or LV-vector-transfected SPCA1 cells. The lower image shows the EGFP fluorescence. (B) The tumor volume curve of LV-FAL1- or LV-vector-treated mice is presented. (C) The weight of tumors was measured and analyzed. (D) The relative expression of FAL1 in xenografts was detected. (E) The proliferation ability was examined with Ki67 IHC (100×). (F) IHC of PTEN in xenografts of LV-FAL1-treated SPCA1 cells compared with control xenografts (100×). (G) Examples of lung metastatic nodules visualized with fluorescence. (H) The number of metastatic nodules was determined. (I) HE stained lung sections with metastatic tumor nodules (100×). The data are shown as the mean ± SD (*P<0.05; **P<0.01; ***P<0.001).

Discussion

Ectopic expression of lncRNAs has been found to influence tumor incidence and growth, including NSCLC [29]. Recent studies have shown that FAL1 participated in the tumorigenesis of some carcinomas and promoted cell proliferation in ovarian cancer [23, 24]. However, whether FAL1 functions in NSCLC tumorigenicity, especially invasion and migration, and what mechanisms are involved have not been reported. This study is the first to identify FAL1 as a tumor promoter that (a) is overexpressed in NSCLC tissues and cells; (b) affects proliferation, invasion and migration in NSCLC cells; (c) modulates the PTEN/AKT signaling axis; and (d) promotes tumor development and metastasis *in vivo*.

Lung cancer, especially NSCLC, has the highest fatality and incidence rate of cancers throughout the world and has an unoptimistic prognosis due to its rapid progression and lack of early stage symptoms [5]. To detect NSCLC and determine its prognosis, many biomarkers, including lncRNAs, have been developed [29-32]. For example, MALAT-1 promoted the proliferation and metastasis of NSCLC and acted as an independent positive marker for

poor prognosis [33]. LncRNA-UCA1, through targeting miR-193a-3p, exerted an oncogenic function in NSCLC [34]. Here, we demonstrated that FAL1 was significantly overexpressed in NSCLC tissues relative to paired adjacent tissues. Moreover, high expression of FAL1 was associated with poor histological grade, increased lymph node metastasis, larger tumor size, and a more severe TNM stage compared with the low expression group, according to expression groups determined by the median FAL1 expression level. Alterations in the FAL1 level were caused by SCNA in ovarian cancer according to a study of Hu et al. and stabilized BMI1 to promote ovarian cancer cell proliferation. We next verified that FAL1 knockdown H1299 cells exhibited less proliferation, invasion and migration activity and increased G0/G1 arrest than negative control cells. At the same time, upregulation of FAL1 with LV-FAL1 obviously accelerated SPCA1 cell growth, metastasis and G1/S transition. Thus, for the first time, we confirmed that FAL1 could accelerate the invasion and migration activity of NSCLC cells.

As an important mechanism of tumor metastasis, EMT has been observed in many different types of cancers, including NSCLC [35-37]. EMT is a conserved cellular process in which epithelial cells temporarily lose their cell polarity and develop characteristics of interstitial cells, which can invade and migrate through the body. EMT can be observed by detecting the epithelial markers E-cadherin and ZO-1 and mesenchymal markers, such as Vimentin and N-cadherin [38]. We detected the expression of EMT markers in experimental cells and confirmed that FAL1 could facilitate EMT. Next, to explore the regulatory factor between FAL1 and EMT, we measured the expression level of BMI1, which was reported to have a direct binding relationship with FAL1 in ovarian cancer cells [24]. Therefore, we next speculated that FAL1 could influence tumor development and progression through PRC1 downstream molecules, which have an effect on EMT. Among these, we found that PTEN, which functions as a tumor suppressor, participated in regulation of cell cycle, proliferation, apoptosis, and cell adhesion during the progression of different cancers and was reported to be involved in regulation of EMT pathways in human nasopharyngeal epithelial cells [27, 39]. PTEN is involved in numerous cancers via phosphorylation of the downstream protein AKT [40]. After a series of experiments, we demonstrated that PTEN was reduced in tumor tissues compared with paired adjacent tissues. However, in FAL1 knockdown cell lines, the expression of PTEN improved, and cells overexpressing FAL1 had a lower PTEN level compared with cells in the empty vector group. McCarroll has reported that loss of PTEN/AKT activity can promote tumorigenesis in NSCLC, which explains the changes in proliferation and cell cycle between the treated and control cells [41]. Additionally, as a downstream molecule of many regulatory factors, such as miRNA-10a, TOPK/PBK, PUMA and others, PTEN/AKT has been shown to influence the drug resistance and prognosis of NSCLC [37, 42-44]. Furthermore, we reduced the expression of PTEN in FAL1 knockdown cells and found that the effect of FAL1 siRNA interference was partially rescued, which demonstrated our assumption of the presence of a FAL1-PTEN/AKT-EMT regulation pathway. Thus, we speculated that FAL1 could reduce the activity of PTEN/AKT through BMI1 and then promote NSCLC cell proliferation and facilitate EMT to accelerate metastasis. However, the specific mechanism between PTEN and EMT in NSCLC remains unclear, and according to some reports, Snail and NF-κB may be a bridge between PTEN and EMT in several epithelial cell types [27, 45]. In addition, our study using PTEN knockdown only rescued only a portion of the influence caused by FAL1 downregulation, indicating that there may be more molecular factors involved downstream of FAL1. Certainly, the regulatory mechanism between FAL1 and NSCLC could be a network, and although stronger evidence is needed to prove the conclusion, our study partially revealed that FAL1 plays an important carcinogenic role in development, progression and EMT of NSCLC, which is mediated by PTEN via BMI1.

Finally, two *in vivo* models were also employed to support our conclusion. Overexpression of FAL1 expedited the growth of xenografts in a tumor formation model in nude mice and increased the number of lung metastatic nodules in a metastatic model.

In conclusion, our findings suggest that FAL1 may further explain the pathogenesis of NSCLC and contribute to the development of lncRNA-related diagnostics and therapeutics. Of course, deeper studies are essential to clarify the regulation of FAL1 via other mechanisms based on regulatory biobehaviors in NSCLC.

Acknowledgements

The present study was supported by the National Natural Science Foundation (grant number 81572263) and the science and technology project of Jiangsu Provincial Health Department (grant number H200821).

Disclosure Statement

No conflicts of interest are associated with the submission of this manuscript.

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