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Long non-coding RNA FAL1 regulated cell proliferation through Akt pathway via targeting PDK1 in esophageal cancer cells

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Abstract. – OBJECTIVE: Esophageal cancer (EC) is one of the most common cancers in the world. Long non-coding RNA focally amplified lncRNA on chromosome 1 (FAL1) is an oncogene which is frequently and focally amplified in human cancers. However, the role of FAL1 in EC remains unknown. The aim of the present study was to evaluate the effect of FAL1 on EC cell lines and explore the underlying mechanism.

PATIENTS AND METHODS: The expression levels of FAL1 in EC tissues and cell lines were detected by RT-PCR. Then, Eca109 cell, a human esophageal cancer cell line, was transfected with FAL1-overexpressing vector or FAL1-siRNA or empty vector. The cell proliferation was measured by MTT assay. The cell apoptosis was assessed by TUNEL assay. The cell invasion was determined by transwell assay. The interaction effect between FAL1 and 3-phosphoinositide-dependent protein kinase 1 (PDK1) was evaluated by chromatin immunoprecipitation (ChIP) assay.

RESULTS: FAL1 was significantly up-regulated in EC tissues and human EC cell lines including Eca109, KYSE150, Eca9706, Kyse30, and TE-1 cells. Overexpression of FAL1 promoted cell proliferation, invasion ability, and cell cycle, but it inhibited cell apoptosis in EC cell lines. Overexpressed FAL1 activated Akt pathway via interacting with PDK1 in EC cell lines.

CONCLUSIONS: FAL1 regulated cell proliferation through Akt pathway via targeting PDK1 in EC cells. These findings revealed that FAL1 exhibited oncogenic activity in EC, and inhibiting FAL1 might be useful for preventing the progression of EC.

Key Words:

Esophageal cancer, Long non-coding RNA FAL1, Akt pathway, PDK1, Cell proliferation.

Introduction

Esophageal cancer (EC) is one of the most common cancers in the world with high morbidity and mortality[1]. EC has been proved to possess extremely aggressive modality and poor survival rate[2]. There are two main subtypes of EC, esophageal squamous-cell carcinoma and esophageal adenocarcinoma[1]. And squamous-cell carcinoma remains the most common subtype which accounts for approximately 90% of EC cases[1]. EC usually occurs in middle-aged and elderly people, but rarely occurs in young people[1]. Clinical studies proved that gastroesophageal reflux disease (GERD), cigarette smoking, alcohol, and obesity are the main risk factors for EC[1]. Numerous studies[3,4] indicated that identification of certain genes might be beneficial for underlying EC pathogenesis.

Non protein-coding RNA (ncRNA) is a group of RNAs that are not translated into protein[5]. Long non-coding RNA (lncRNA) is one subtype of ncRNA with longer than 200 nucleotides[6]. Although thousands of lncRNAs have been identified, the biological functions of the majority of lncRNAs still remain unknown[7]. Increasing studies[8,9] proved that lncRNAs have the ability to interact with various molecules and then form a variety of complexes including RNA-RNA, RNA-DNA, or RNA-protein complexes through their specific functional domains. And lncRNAs have been reported to function in cell physiology and pathology. Therefore, lncRNAs play an important role in many diseases, such as cancers[9].

Focally amplified lncRNA on chromosome 1 (FAL1) is a kind of lncRNA which is frequently and focally amplified in human cancers acting as

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an oncogene[10,11]. It has been reported[12-14] that FAL1 plays an important role in non-small cell lung cancer, thyroid cancer, and ovarian cancer. However, the role of FAL1 in EC remains unknown.

In the present study, we evaluated the expression level of FAL1 in EC tissues and cell lines, and further investigated the effect of FAL1 on EC cell lines. The results would provide evidence for developing novel therapies of EC.

Materials and Methods

Patients

A total of 73 patients with EC who hospitalized in The Second Affiliated Hospital of Guangxi Medical University during May 1, 2013 to March 20, 2015 were enrolled. Patients were classified into three grades according to the World Health Organization classification criteria: Grade II (n=19), Grade III (n=37), and Grade IV (n=17). And 13 healthy volunteers also participated in the present research. The blood samples from the 73 EC patients and 13 healthy volunteers were collected and stored. In addition, 8 EC tissues and 3 adjacent tissues were collected from the patients with EC who received surgical resection. The informed consent was obtained from the patients and volunteers. The present investigation was approved by the Ethics Committee of The Second Affiliated Hospital of Guangxi Medical University, China.

Cell Culture and Treatments

Human normal esophageal epithelial cell line HEEC, and human esophageal cancer cell lines, Eca109, KYSE150, Eca9706, Kyse30, and TE-1 cells were obtained from Cell Bank of the Chinese Academy of Sciences (Shanghai, China). Human esophageal cancer cell lines, CaES-17 cell, was purchased from the China Center for Type Culture Collection (Wuhan, China). The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Gibco, Carlsbad, CA, USA) supplemented with fetal bovine serum (FBS, Gibco, Carlsbad, CA, USA), 100 U/mL penicillin (Sigma-Aldrich, St. Louis, MO, USA) and 100 μg/mL streptomycin (Sigma-Aldrich, St. Louis, MO, USA) and maintained in a humidified incubator at 37°C with 5% CO_2.

The small interfering RNA (siRNA) targeting FAL1 (FAL1-siRNA) were designed and synthesized by Shanghai GenePharma Co., Ltd. (Shanghai, China). The Eca109 cell lines were transfected with FAL1-overexpressing vector or empty vector or FAL1-siRNA using lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

RT-PCR

The total RNA of the EC tissues, adjacent tissues, and esophageal epithelial cells were extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Then, the RNA was reverse transcribed for cDNA synthesis using a high capacity RNA-to-cDNA Kit (Applied Biosystems, Thermo Fisher Scientific, Inc., Foster City, CA, USA). Subsequently, the mRNA expressions were measured using a SYBR ExScript qRT-PCR kit (TaKaRa, Dalian, China). GAPDH was used as an internal control. The reaction was performed on an ABI 7500 system (Applied Biosystems, Thermo Fisher Scientific, Inc.). The $2^{-\Delta\Delta Cq}$ method was used for the calculation of the relative mRNA expression level. The primers used in the study were listed as follows: FAL1, sense 5'-CTCG GATC CGCG CATC TCCT ACGG CCTC CAGG ACAG AGG-3', and anti-sense 5'-CGAG CGGC CGCA GACA TCCA AGTG TCCT GTGT AATA GGCT G-3'; cyclin D1, sense 5'-GCTG CTCC TGGT GAAC AAGC-3', anti-sense 5'-CACA GAGG GCAA CGAA GGTC-3; MMP7, sense 5'-GTAG AACG CGAC CATG GCCG-3', anti-sense 5'-CGAC AAGG CGTA AGGT GCTG C-3'; p21, sense 5'-ACCG GGTA ATGC GCTC AGTA A-3', anti-sense 5'-GTAG CGTC AAGG GTAA ATAG G-3'; FASL sense 5'-GATT AAGG GCAG GCGA GGCG-3', anti-sense 5'-CTGA AGCA CCAT AACC AGGA C-3'; p27, 5'-GCCA TAGC GTAA GACA GGCT C-3', anti-sense 5'-GACA CCGG GTTA TTGA GGAT-3'; Bim, 5'-TGGA GACC TCGA ATTG TCG-3', anti-sense 5'-TAAG GCCT CGCA CGGT ACA-3'; caspase-3, sense 5'-ACCG ATGT CGAT GCAG CTAA-3', anti-sense 5'-AGGT CCGT TCGT TCCA AAAA-3'; GAPDH, sense 5'-GAGA GACC CTCA CTGC TG-3', anti-sense 5'-GATG GTAC ATGA CAAG GTGC-3'.

MTT Assay

MTT assay was performed to evaluate the cell proliferation of Eca-109 cells at 0, 1, 2, 3, 4 days after transfection. Briefly, Eca-109 cells (10^3 cells/well) with different treatments were seeded into 96-well plates and incubated for 24 h. Then, 20 ml of MTT (5 mg/ml, Sigma-Aldrich, St. Louis, MO, USA) was added to the cells and then cultured for another 4 h. Subsequently, 150 ml of DMSO

(Sigma-Aldrich, St. Louis, MO, USA) was added and incubated for 10 min in the dark to dissolve the formazan crystal. Finally, the absorbance was determined at the wavelength of 570 nm using a microplate reader (Bio-Tek Instruments Inc., Winooski, VT, USA).

TUNEL Assay

After transfection, cells were cultured for 24 h and then fixed with 4% paraformaldehyde at 25°C for 30 min. Then, the cells were incubated in permeabilization solution (0.1% Triton X-100 in 0.1% sodium citrate, Invitrogen, Carlsbad, CA, USA) for 3 min on ice. Then 50 µl of TUNEL reaction mixture (Roche, Basel, Switzerland) was added and incubated for 60 min at 37°C in the dark. After washing for three times, 50 µl of PI (Sigma-Aldrich, St. Louis, MO, USA) was added and incubated for 20 min in the dark. Then, the cells were observed under a fluorescence microscope.

Cell Invasion Detection

In vitro cell invasion ability was detected using transwell assay. The 24-well transwell units with polycarbonate filters (Corning Costar, Corning, NY, USA) were used for the assay. The Eca-109 cells were placed in the upper part of the transwell and incubated for 36 h. Cells in the upper chamber were removed by cotton swab and the invaded cells were located on the underside of the filter. The cells were stained with hematoxylin for 10 min and counted. The numbers of invaded cells were calculated by averaging the total number of cells from three dependent experiments.

Western Blot

The Eca-109 cells were lysed using RIPA buffer (Solarbio, Beijing, China), and the protein concentration was measured using a BCA protein assay kit (Beyotime Biotechnology, Shanghai, China). A total of 20 µg protein from each sample was separated using 10% SDS-PAGE and then transferred onto PVDF membranes. After blocking with 5% skimmed milk at room temperature for 1 h, the membranes were incubated with anti-cyclin D1 (dilution 1:500, Abcam, Cambridge, MA, USA), anti-MMP7 (dilution 1:500, Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-p21 (dilution 1:400, Cell Signaling Technology, Danvers, MA, USA), anti-p-Akt (dilution 1:500, Abcam, Cambridge, MA, USA), anti-Akt (dilution 1:500, Abcam, Cambridge, MA, USA), and anti-β-actin (dilution 1:500, Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 4°C for 8 h. Subsequently, the membranes were incubated with secondary antibody (dilution 1:2000, Abcam, Cambridge, MA, USA) at room temperature for 1 h. Finally, the bands were visualized using an enhanced chemiluminescence (ECL) kit (Thermo Scientific, Waltham, MA, USA).

Luciferase Reporter Assay

The full-length promoters of FOXO1 and p21 were inserted into the pGL3 vector (Promega, Madison, WI, USA). The pGL3-FOXO1 promoter or pGL3-p21 promoter and FAL1-siRNA or FAL1-overexpressing vector or vector was co-transfected into the Eca-109 cells. Then, the cells were harvested and the luciferase activity was detected using a Dual-Luciferase Reporter Assay kit (Promega, Madison, WI, USA) according to the manufacturer's protocol.

Chromatin Immunoprecipitation (ChIP) Assay

The ChIP assay was performed as described in the published methods[15]. Eca-109 cells with different transfections were treated with 1% methanol and incubated at room temperature for 10 min. Then, the cells were lysed in SDS-containing lysis (Solarbio, Beijing, China) buffer and sonicated to obtain the soluble chromatin. The supernatant was collected by centrifugation and incubated overnight with 3-phosphoinositide-dependent protein kinase 1 (PDK1) antibody or control IgG. The protein-DNA complex was then collected by protein A Agarose (Millipore, Corp, Billerica, MA, USA). After elution and reverse cross-link, the purified DNA from the immune complex was subjected to RT-PCR.

Statistical Analysis

The statistical analysis was performed using the GraphPad Prism software (version 4.0, GraphPad Prism Software, CA, USA). The Student's *t*-test was used for statistical comparisons between two groups. Analysis of Variance (ANOVA) with Tukey's post-hoc testing was used for statistical comparisons among more than three groups. $p < 0.05$ was considered statistically significant.

Results

LncRNA FAL1 Was Over-Expressed in EC Tissues and Cell Lines

To evaluate the expression level of FAL1 in EC, RT-PCR was performed to detect the ex-

pression level of FAL1 in 8 EC tissues and 3 adjacent tissues. As shown in Figure 1A, FAL1 was overexpressed in EC tissues. To further investigate the association between FAL1 level and EC grades, the expression level of FAL1 in 73 patients with EC was measured. The results in Figure 1B showed that expression level of FAL1 in patients with Grade II, III, and IV was higher than that in healthy volunteers. Besides, the patients in Grade III and IV exhibited a higher level of FAL1 compared to patients in Grade II. We also found that the expression level of FAL1 was significantly up-regulated in human EC cell lines including Eca109, KYSE150, Eca9706, Kyse30, and TE-1 cells (Figure 1C).

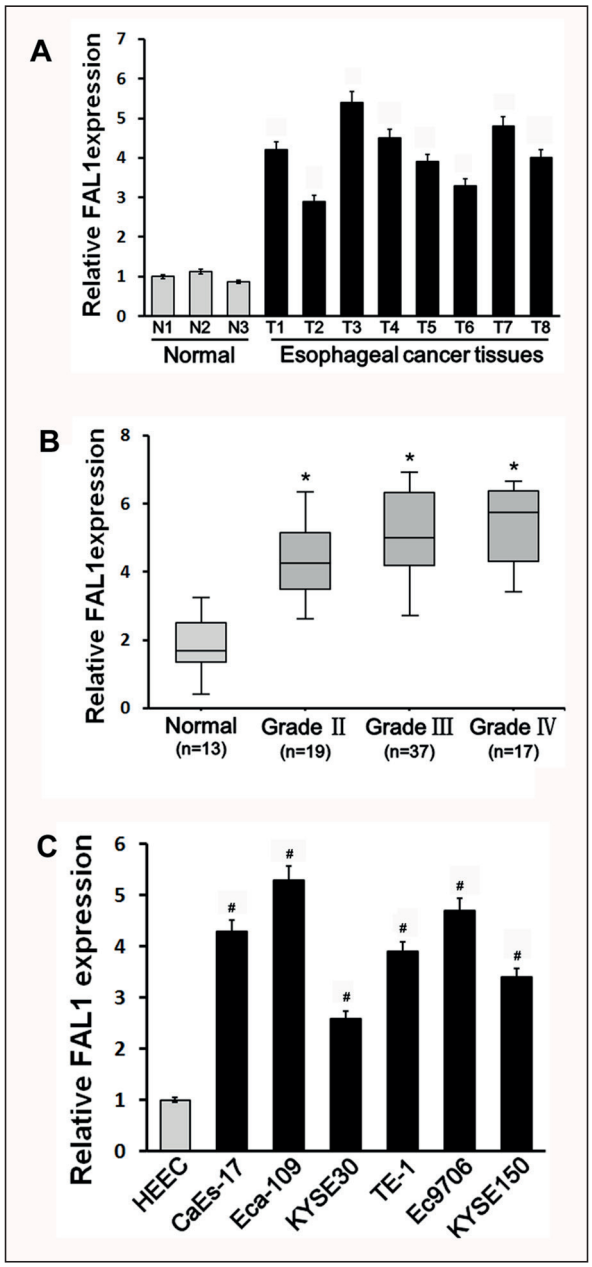

Figure 1. LncRNA FAL1 was over-expressed in EC tissues and cell lines. **A,** RT-PCR was performed to detect the expression level of FAL1 in 8 EC tissues and 3 adjacent normal tissues. **B,** The expression levels of FAL1 in 73 patients with EC were measured by RT-PCR. *$p < 0.05$ *vs.* normal group. **C,** The expression levels of FAL1 in normal esophageal cells HEEC and human EC cell lines including Eca109, KYSE150, Eca9706, Kyse30, and TE-1 cells were detected by RT-PCR. *$p < 0.05$ *vs.* HEEC cells.

Effect of lncRNA FAL1 on Cell Proliferation, Apoptosis, Invasion Ability and Cell Cycle

To investigate the effect of FAL1 on EC cells, FAL1-siRNA or FAL1-overexpressing vector were transfected into Eca109 cells. The transfection efficiency was confirmed by RT-PCR. As shown in Figure 2A, the expression of FAL1 was markedly increased or decreased in the cells transfected with FAL1-overexpressing vector or FAL1-siRNA, respectively. The results of MTT assay indicated that overexpression of FAL1 markedly promoted the cell proliferation of Eca109 cells, while FAL1-siRNA clearly inhibited the cell proliferation (Figure 2B). The cell apoptosis was evaluated by TUNEL assay. The results proved that cell apoptosis was significantly decreased in the cells transfected with FAL1-overexpressing vector, and noticeably increased in the cells transfected with FAL1-siRNA (Figure 2C). Transwell assay was performed to detect the cell invasion ability. The results in Figure 2D revealed that overexpressed FAL1 elevated invasion ability of Eca109 cells and FAL1-siRNA reduced the invasion ability.

The protein and mRNA levels of several cell cycle-related genes including cyclinD1, MMP7, and p21 were measured by Western blot and RT-PCR. As shown in Figures 3A and 3B, the overexpression of FAL1 distinctly induced the expression of cyclinD1 and MMP7 at both protein and mRNA levels. While the expression of cyclinD1 and MMP7 were reduced by FAL1-siRNA. The effects of FAL1 overexpression and FAL1-siRNA on p21 expression were opposite compared to that on cyclinD1 and MMP7. Further investigation indicated that FAL1 would target the promoter of FOXO1 and p21 (Figures 3C and 3D). In addition, the mRNA levels of several genes which are associated with cell cycle and cell apoptosis, including FASL, p27, Bim, and caspase-3, were significantly decreased by FAL1 overexpression.

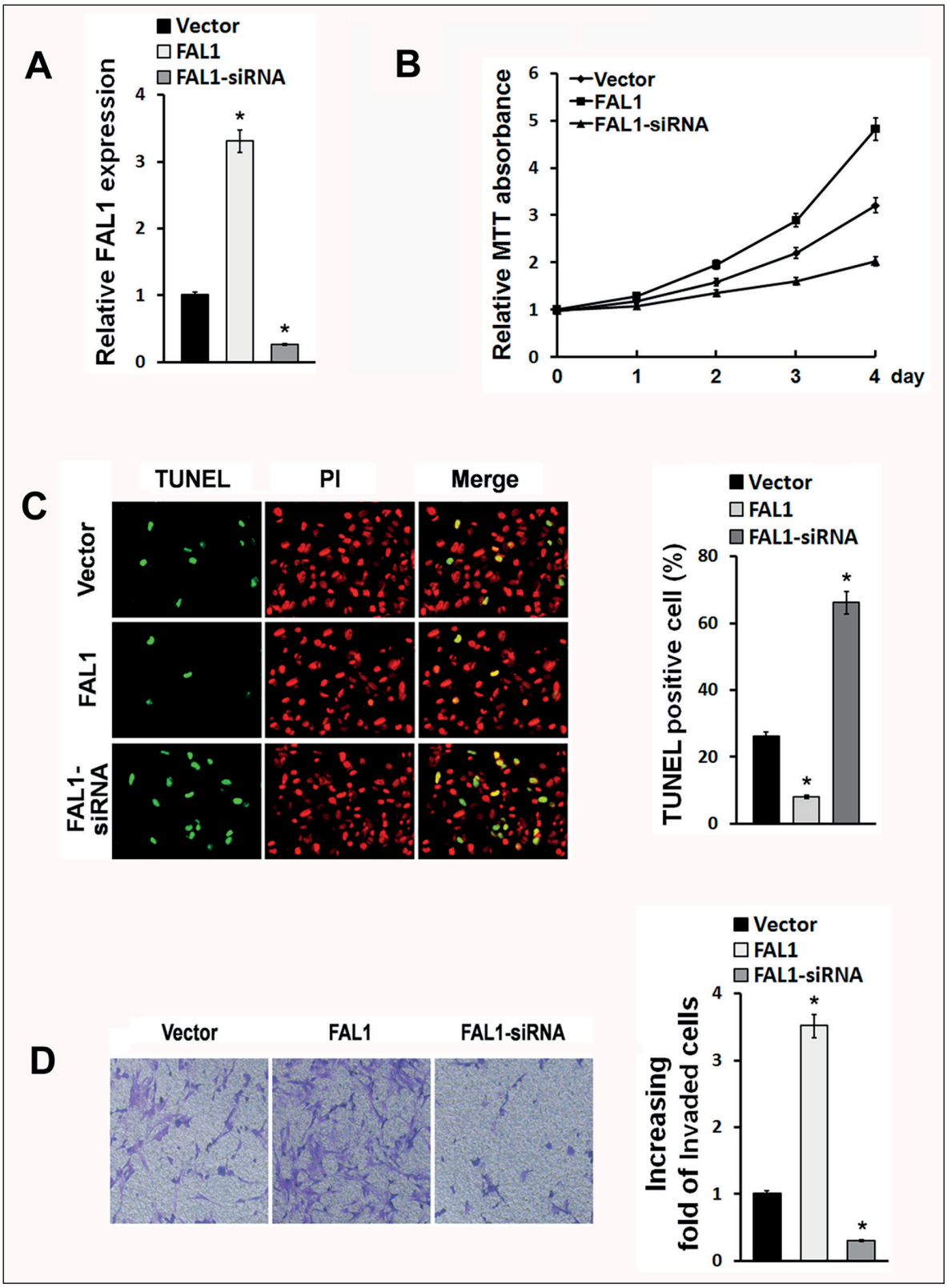

Figure 2. Effect of lncRNA FAL1 on cell proliferation, apoptosis, and invasion. To investigate the effect of FAL1 on EC cells, FAL1-siRNA or FAL1-overexpressing vector or empty vector were transfected into Eca109 cells. **A,** The transfection efficiency was confirmed by RT-PCR. **B,** The cell proliferation was measured by MTT assay. **C,** The cell apoptosis was assessed by TUNEL assay. **D,** The cell invasion was determined by transwell assay. *$p < 0.05$ *vs.* cells transfected with empty vector.

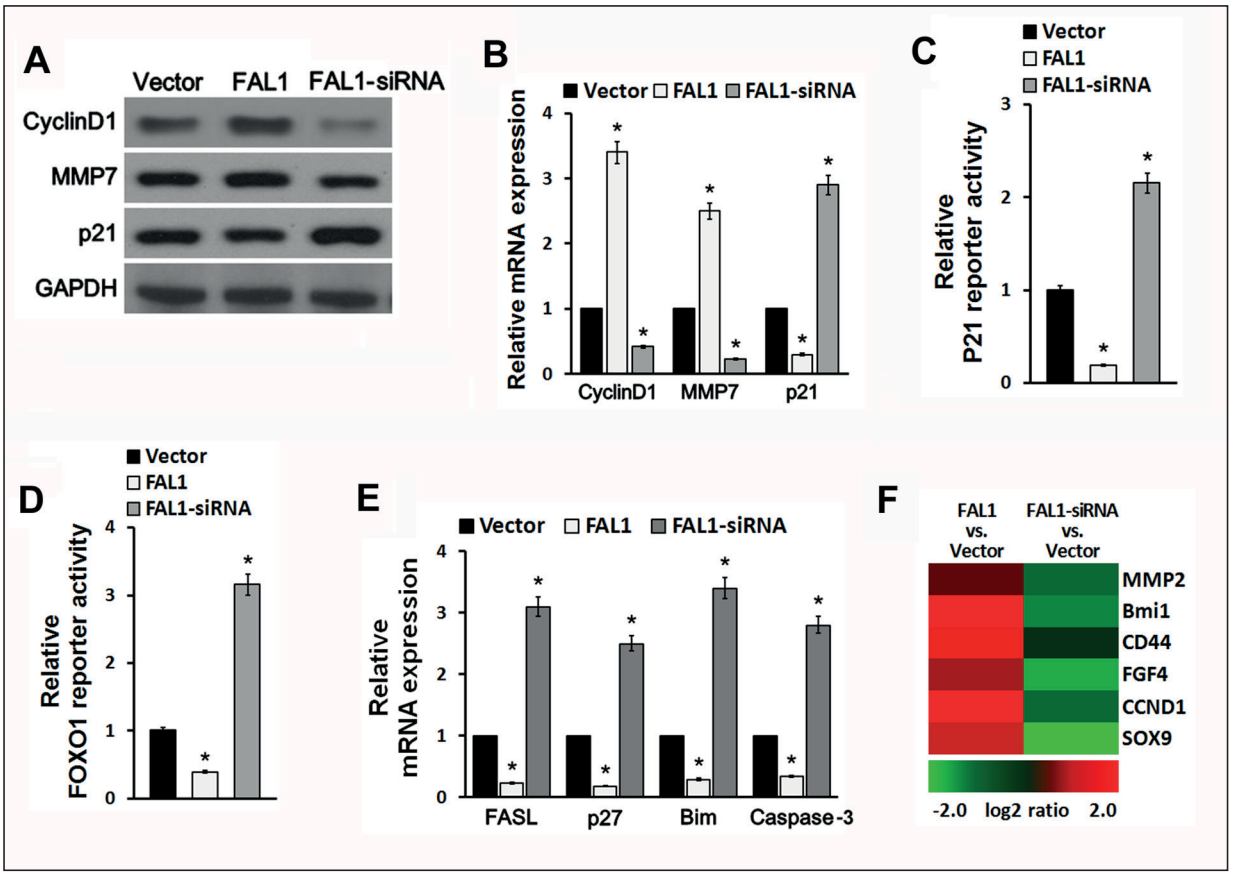

Figure 3. Effect of lncRNA FAL1 on the expression of related genes. **A-B,** The protein and mRNA levels of several cell cycle-related genes including cyclinD1, MMP7, and p21 are measured by western blot and RT-PCR, respectively. **C-D,** Luciferase reporter assay was performed to confirm the effect of FAL1 on p21 and FOXO1, respectively. **E,** The mRNA levels of several cell cycle and cell apoptosis-related genes were measured by RT-PCR. **F,** Several genes which are related to tumor growth and metastasis, such as MMP2, Bmi1, CD44, FGF4, CCND1, and SOX9 were determined by RT-PCR. The pseudocolors represented the intensity scale of gene expressions in FAL1-overexpressing vector *vs.* empty vector transfected cells, or FAL1-siRNA *vs.* empty vector transfected cells. *$p < 0.05$ *vs.* cells transfected with empty vector.

But FAL1-siRNA would increase the mRNA levels of FASL, p27, Bim, and caspase-3 (Figures 3E). Moreover, several genes, related to tumor growth and metastasis (such as MMP2, Bmi1, CD44, FGF4, CCND1, and SOX9), were found to be accelerated in the FAL1 overexpressing cells (Figures 3F). These findings denoted that FAL1 promoted cell proliferation, invasion ability and cell cycle, and inhibited cell apoptosis of Eca109 cells.

LncRNA FAL1 Activated AKT Pathway Via Targeting PDK1

To assess whether Akt pathway was involved in the effect of FAL1, the expression levels of Akt and p-Akt were measured by Western blot. As shown in Figure 4A, the p-Akt expression was markedly enhanced in the cells transfected with FAL1-overexpressing vector, while it was reduced in the cells transfected with FAL1-siRNA. But the expression level of Akt was not altered. The results indicated that FAL1 overexpression facilitated the phosphorylation of Akt, and activated Akt pathway. PDK1, an upstream molecule of Akt pathway, is charging of activating phosphorylation of Akt. Thus, we speculated that FAL1 may interact with PDK1, and CHIP assay was applied to verify the conjecture. The results of CHIP assay showed that FAL1 directly targeted to PDK1 (Figure 4B).

Discussion

FAL1 has been proved to possess oncogenic activity and associate with human cancers.

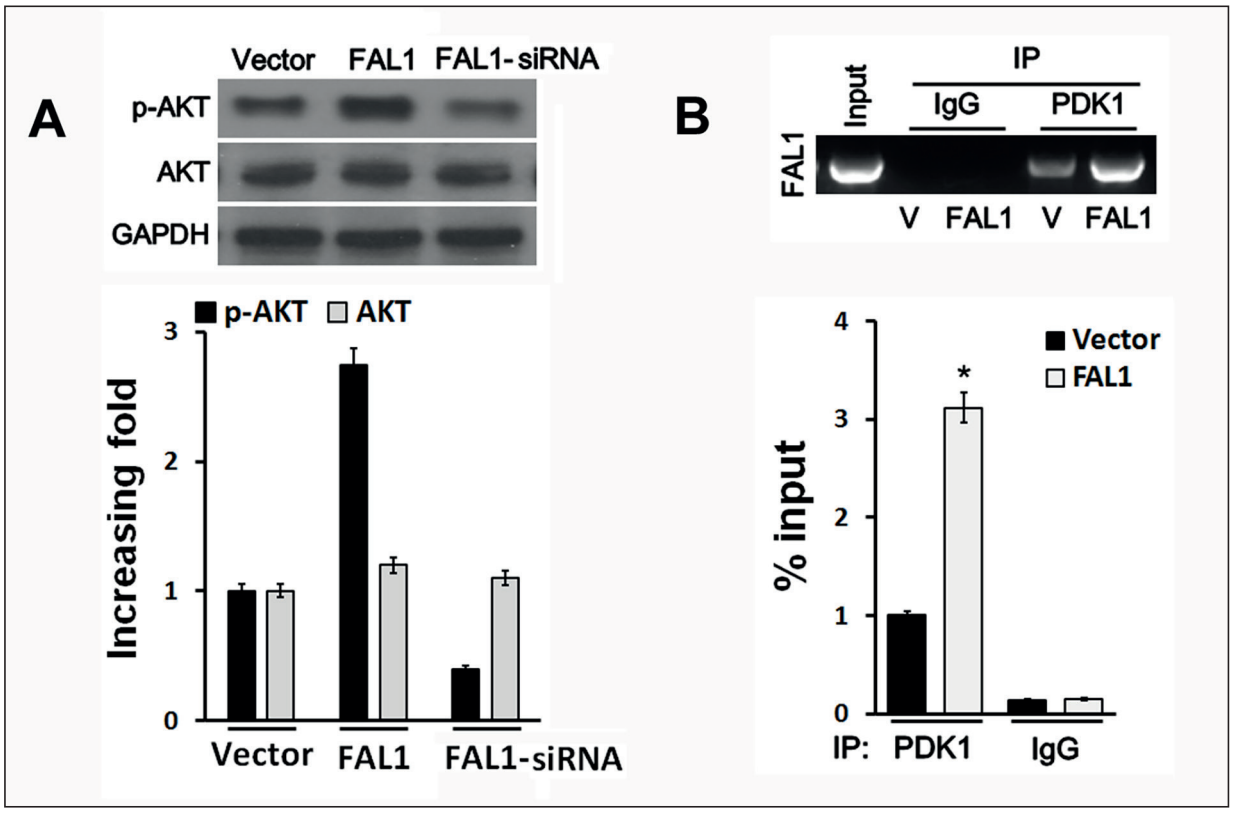

Figure 4. LncRNA FAL1 activated AKT pathway via targeting PDK1. **A,** The expression levels of Akt and p-Akt were measured by western blot. **B,** The interaction effect between FAL1 and PDK1 was evaluated by chromatin immunoprecipitation (ChIP) assay. *$p < 0.05$ *vs.* cells transfected with empty vector.

Pan et al[13] proved that FAL1 is up-regulated in human non-small cell lung cancer tissues compared to the adjacent normal tissues. The FAL1 expression is also increased in non-small cell lung cancer cell lines when compared with normal cell line[13]. They also found that FAL1 level is closely correlated with histological grade, lymph node metastasis, tumor size, and TNM stage, which suggests that FAL1 may participate in tumorigenesis of non-small cell lung cancer[13]. Further investigations denoted that FAL1 facilitates cell proliferation, cell cycle, invasion, and migration of non-small cell lung cancer cell lines[13]. Moreover, FAL1 also contributed to lung cancer metastasis via promoting epithelial-mesenchymal transition (EMT)[13]. Jeong et al[14] reported that FAL1 expression is clearly higher in papillary thyroid cancer tissues than that in normal thyroid tissues. The results of the multivariate analysis revealed that patients with high FAL1 expression exhibited high risk of multifocality[14]. They also proved that FAL1 plays an important role in facilitating cell cycle progression and overexpressed FAL1 is closely related with the aggressive behavior of papillary thyroid cancer[14]. To evaluate the role of FAL1 in EC, we first detected the FAL1 expression level in EC tissues, adjacent normal tissues, EC cell lines, and normal cell lines. The results indicated that FAL1 was over-expressed in EC tissues and EC cell lines. Also, we found that FAL1 significantly promoted cell proliferation, invasion ability, and cell cycle, and suppressed cell apoptosis in EC cell lines.

Akt pathway is an intracellular signaling pathway which is crucial for regulating cell cycle, cell proliferation, and cell apoptosis[16]. Akt pathway is usually over-activated in various cancer progresses to reduce cell apoptosis and promote cell proliferation[16]. Activated Akt inhibits antiproliferative proteins p27 and p21, and then promotes cell proliferation[17,18]. Akt also suppresses FOXO, thus regulating cell apoptosis[19]. In the present study, we found that FAL1 inhibited expressions of FOXO1 and p21 by targeting the promoter of FOXO1 and

p21. Besides, FAL1 also inhibited expressions of p27 and cell apoptosis-related gene FASL which participates in the Akt signaling pathway[20]. Therefore, we speculated that Akt pathway may be involved in the effect of FAL1 on cell cycle and cell apoptosis. Western blot was performed to detect the expression of p-Akt and Akt. The results indicated that FAL1 overexpression facilitated the phosphorylation of Akt, suggesting that Akt pathway was activated by FAL1.

PDK1 is one of the downstream effectors of PI3K, and activates numerous proteins including Akt[21,22]. PDK1 is essential for Akt activation and responsible for the phosphorylation of Akt on the activation loop[23]. PDK1 has been demonstrated to contribute to regulate several physiological processes, such as cell migration, cell cycle, cell invasion, and cell apoptosis[24]. Published researches[24,25] revealed that PDK1 is oncogenic, and alteration of PDK1 is observed in many cancers. PDK1 plays a crucial role in tumor invasiveness and dissemination[24]. In our work, it has been proved that FAL1 activated Akt pathway in EC cell lines by directly targeted to PDK1. Increasing evidence validated that PDK1 can be considered as a therapeutic target for cancer treatment. And the inhibitors of PDK1 may be developed to prevent tumor progression[26]. As we show in the present investigation, FAL1 inhibitor might be useful for preventing the progression of EC.

Conclusions

The role of FAL1 in EC was investigated in the present study. We found that FAL1 was significantly up-regulated in EC tissues and human EC cell lines including Eca109, KYSE150, Eca9706, Kyse30, and TE-1 cells. FAL1 overexpression promoted cell proliferation, invasion ability and cell cycle, and inhibited cell apoptosis of EC cell lines. FAL1 overexpression activated Akt pathway via interacting with PDK1 in EC cell lines. These findings revealed that FAL1 exhibited oncogenic activity in EC, and further inhibiting FAL1 might be useful to prevent the progression of EC.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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