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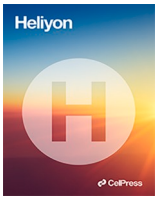

Research article

β-elemene alleviates esophageal fibrosis after endoscopic submucosal dissection via the FAP-mediated PTEN-PI3K/AKT signaling pathway

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ABSTRACT

Esophageal stricture caused by fibrosis is a serious complication after esophageal Endoscopic submucosal dissection (ESD). Myofibroblasts play a crucial role in esophageal fibrosis, so inhibiting activated myofibroblasts is a promising approach for treating esophageal fibrosis. β-Elemene, a natural product with anti-tumor and anti-fibrotic properties, has not been thoroughly examined in esophageal fibrosis. Additionally, fibroblast activation protein (FAP) and PTEN-PI3K/AKT signaling pathway are both notably linked to fibrotic diseases. Therefore, we investigated the potential mechanisms of β-elemene in esophageal fibrosis by treating primary human esophageal granulation fibroblasts (PHEGFs) with gradient concentrations of β-elemene. Our findings demonstrated that β-elemene inhibited the activity of PHEGFs in a dose-dependent manner, accompanied by downregulation of FAP, p-PI3K, and p-AKT protein expression, along with upregulation of p-PTEN protein expression. In addition, we substantiated the potential correlation between FAP and the PTEN-PI3K/AKT signaling pathway by establishing models of FAP overexpression and silencing. These results provide a new perspective on the potential mechanism of β-elemene in relieving esophageal fibrosis and offer novel therapeutic strategies for managing post-esophageal ESD stricture in clinical practice.

1. Introduction

In recent years, with the development of digestive endoscopy technology, endoscopic submucosal dissection (ESD) as a diagnostic and treatment method for early esophageal cancer has been widely accepted worldwide [1,2]. Esophageal stricture caused by fibrosis is one of the serious complications after ESD, which leads to different degrees of dysphagia and seriously affects the quality of life of patients. Previous research has indicated that lesions encompassing more than three-quarters of the esophageal circumference or tumor invasion depth exceeding the submucosal layer of the esophageal mucosa are risk factors for postoperative stricture. Specifically, when the area of circumferential mucosal defect caused by ESD exceeds 71 %, it demonstrates a specificity and sensitivity for stricture occurrence reaching 100 % and 97.1 % respectively [3,4]. These is due to the exacerbation of inflammatory response caused by factors such as a large defect site, food, digestive juice reflux, and wound infection, leading to an expanded healing range and

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ultimately resulting in esophageal stricture. Myofibroblasts produce pathological extracellular matrix (ECM) proteins and collagen deposition to promote tissue fibrosis, then rapidly restore the defective tissue's structural integrity. However, excessive or continuous myofibroblast activity can cause excessive tissue fibrosis and loss of its original function [5]. Therefore, repressing the activity of myofibroblasts to mitigate ECM and collagen deposition represents a promising therapeutic strategy for esophageal fibrosis.

Fibroblast activation protein (FAP) is a marker specifically expressed after activation of fibroblasts. It significantly correlates with inflammatory and fibrotic conditions, such as chronic inflammation, rheumatoid arthritis, osteoarthritis, liver cirrhosis, and pulmonary fibrosis [6–9]. It is noteworthy that numerous studies have demonstrated the pivotal role of phosphatase and tensin homolog deleted on chromosome ten (PTEN) - phosphatidylinositol 3-kinase/protein kinase B (PI3K/AKT) signaling pathway in the pathogenesis of tumors and fibrotic diseases, functioning as a central signaling hub in tumor progression and fibrosis development [10–13]. Furthermore, recent research has shown that the inhibitor FAPi or FAPα targeted silencing can effectively suppress the PI3K/AKT pathway, thereby inhibiting the activation of myofibroblasts and ultimately ameliorating the progression of skin fibrosis [14]. Therefore, it is plausible that FAP and the PTEN-PI3K/AKT signaling pathway are intricately linked to the progression of esophageal fibrosis.

In previous studies, systemic or local administration of corticosteroids has shown positive effects in patients with esophageal stenosis [15,16]. However, the systemic administration of corticosteroids poses a potential risk of adverse events, and the local injection of corticosteroids exhibits limited efficacy [17–19]. β-elemene, a terpene compound derived from the traditional Chinese herb, is frequently utilized as adjunctive therapy in anti-tumor protocols. Furthermore, it has the potential to function as a nano-delivery drug to counteract tamoxifen-induced immunosuppression and thereby enhance chemoimmunotherapy [20]. Interestingly, β-elemene has been demonstrated to possess the efficacy of mitigating fibrosis in various tissues and organs, including esophageal fibrosis [21–24]. However, its specific mechanism in esophageal fibrosis remains unclear. Recent studies have shown that β-elemene effectively suppresses the aberrant activation of the PI3K-AKT pathway induced by FMS-like tyrosine kinase 3 (FLT3) mutations, leading to the inhibition of Acute myeloid leukemia (AML) cell proliferation [25–27]. It can be inferred that the PI3K/AKT signaling pathway may represent a downstream signaling cascade of for β-elemene. Therefore, to elucidate the impact of β-elemene on esophageal stricture and investigate its molecular mechanisms, we cultured primary human esophageal granulation fibroblasts (PHEGFs) *in vitro* and treated them with different concentrations of β-elemene.

2. Methods

2.1. Tissue collection

A total of 10 patients who underwent ESD surgery for high-grade intraepithelial neoplasia or early esophageal cancer at the Digestive Endoscopy Center of the Second Affiliated Hospital of Fujian Medical University from June to September 2022 and whose mucosa resection scope exceeded 2/3 of the circumference were selected, 2–3 granulation tissues were extracted by gastroscope and biopsy forceps during routine endoscopic review one month after surgery.

2.2. Drugs and reagents

The β-elemene compound was procured from MedChemExpress (USA). 0.25 % Trypsin-EDTA, Phosphate buffered solution (PBS), fetal bovine serum (FBS), Dulbecco's modified Eagle's medium (DMEM), and 4 % paraformaldehyde were obtained from Gibco (USA). Endogenous Peroxidase Blocking Buffer, diaminobenzidine (DAB), and Vimentin antibody were purchased from MXB Biotechnologies (Fuzhou, China). alpha-smooth muscle actin (*α*-SMA*)* antibody (AF1032), *FAP* antibody (AF0739), *p-PTEN* antibody (AF3351), *p-PI3K* antibody (AF3242), *p-AKT* antibody (AF0016), and *β-actin* antibody (AF7018) were acquired from Affinity (USA). Rabbit Anti-Keratin (bsm-60235R) was sourced from Bioss (Beijing, China). CCK-8 viability assay kits and Annexin V-FITC/PI apoptosis detection kits were obtained from Meilunbio (Dalian, China). The secondary antibody (A0108) was procured from Beyotime (Shanghai, China). HE

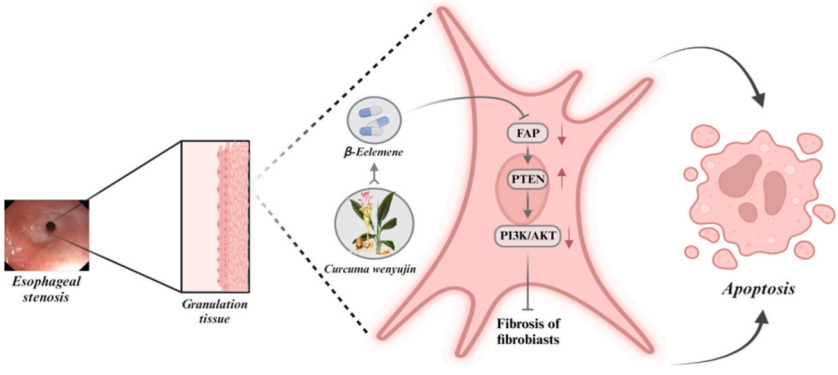

Fig. 1. β-elemene mediates FAP to negatively regulate PTEN/PI3K/AKT signaling pathway to alleviate esophageal stenosis after ESD.

Staining Solution (BL735A) was purchased from Biosharp (Beijing, China). The plasmids were designed, constructed, sequenced, and packaged for transduction by Hanbio (Shanghai, China). All plasmids contained green fluorescent protein(see Fig. 1).

2.3. Culture fibroblasts in vitro

The granulation tissue, which appeared after ESD, was retrieved using sterile biopsy forceps during electronic gastroscopy. It was washed thrice with pre-cooled PBS containing 100 U/mL of penicillin and streptomycin to eliminate blood stains. Subsequently, the tissue was fragmented into ≤ 1 mm^3 pieces using ophthalmic scissors (after undergoing high-pressure sterilization) in DMEM supplemented with 20 % FBS. Following this, 0.25 % Trypsin-EDTA replaced DMEM, and the fragments were digested at 37 °C for 8 min before terminating the digestion process by adding DMEM. The resulting fragments and liquid were collected in an EP tube, centrifuged at 1000r/min to remove supernatant, and resuspended in DMEM containing 20 % FBS. Finally, they were transferred to a 25 cm_2 culture flask while maintaining a certain distance between tissue fragments. The culture flasks are placed in a culture incubator, and the medium is refreshed every 4–5 days (Fig. 2). Once more than 90 % of cell fusion occurred, cells were passaged according to a ratio of 1:2. After three passages, the concentration of fetal bovine serum was reduced to 10–15 %. As dominant cells, PHEGFs continued their growth, while other cells, such as epithelial cells, gradually diminished or even died out until PHEGFs became purified.

2.4. Immunocytochemistry

The fourth generation of PHEGFs were selected for subsequent identification. Following adherence of PHEGFs to the cover glass, the PHEGFs were fixed with 4 % paraformaldehyde. Afterward, the PHEGFs were immersed in a 0.5 % Triton X-100 solution for 20 min and blocked with Endogenous Peroxidase Blocking Buffer for 10 min. Next, PHEGFs were incubated with primary anti-vimentin antibody reagent (rabbit anti-human, 1:50), α-SMA (rabbit anti-human, 1:100) or Keratin antibody (rabbit anti-human, 1:50) at 25 °C for 2 h, followed by 1-h incubation at room temperature with a secondary antibody (1:50). After incubation with DAB and staining with hematoxylin, the PHEGFs were observed and photographed under an optical microscope.

2.5. β-elemene intervention and cell transfection

We divided PHEGFs into five groups: control group (PHEGFs conventionally cultured), β-E−160 μM group (160 μg/ml β-elemene intervention PHEGFs), β-E−320 μM group (320 μg/ml β-elemene intervention PHEGFs), β-E−480 μM group (480 μg/ml β-elemene intervention PHEGFs), and β-E−640 μM group (640 μg/ml β-elemene intervention PHEGFs). Additionally, to investigate the association between FAP and the PTEN-PI3K/AKT pathway, we established the following groups: FAP-oe group (PHEGFs transfected with FAP overexpressing plasmids), FAP-sh group (PHEGFs transfected with FAP shRNA1 plasmids), and NC group (PHEGFs transfected with FAP NC plasmids). PHEGFs were transfected following the protocols of lipofectamine 3000 for subsequent experiments.

2.6. Cell viability analysis

According to the grouping above, PHEGFs were diluted in a culture medium to a concentration of (4–5) × 10^3/ml and subsequently seeded into 96-well plates at 100 μL per well. Then, the cells were cultured in DMEM medium supplemented with 10 % FBS for

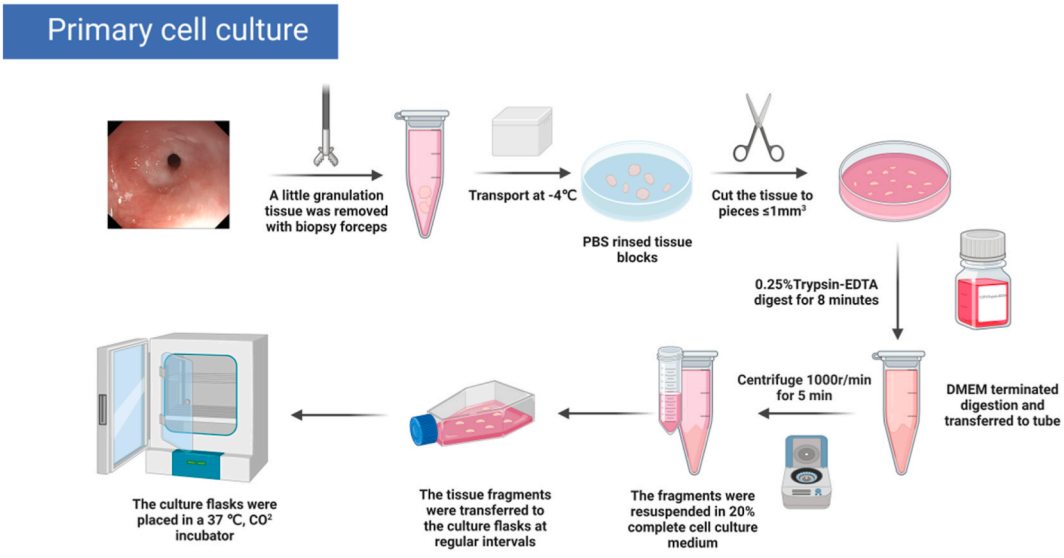

Fig. 2. *In vitro*, PHEGFs were cultured using the "tissue adhesion method" and "trypsin digestion method."

24, 48, and 72 h. Next, each well was replaced by adding DMEM supplemented with 10 % CCK-8 at a volume of 100 μL. Additionally, a blank control without PHEGFs were included using DMEM alone. After incubation for 1.5 h, each well's optical density (OD) was measured at a wavelength of 450 nm.

2.7. Flow Cytometry

After 24 h of administration and transfection, PHEGFs were digested with 0.25 % Trypsin-EDTA and subsequently resuspended with a binding buffer. Following this, the cell concentration was adjusted to 1 × 10^3/mL. The PHEGFs were incubated with AnnexinV-FITC (5 μL) and PI (10 μL) for 15 min in a light-free environment at room temperature. Finally, the apoptosis rate of the PHEGFs at each time point was identified by flow cytometry.

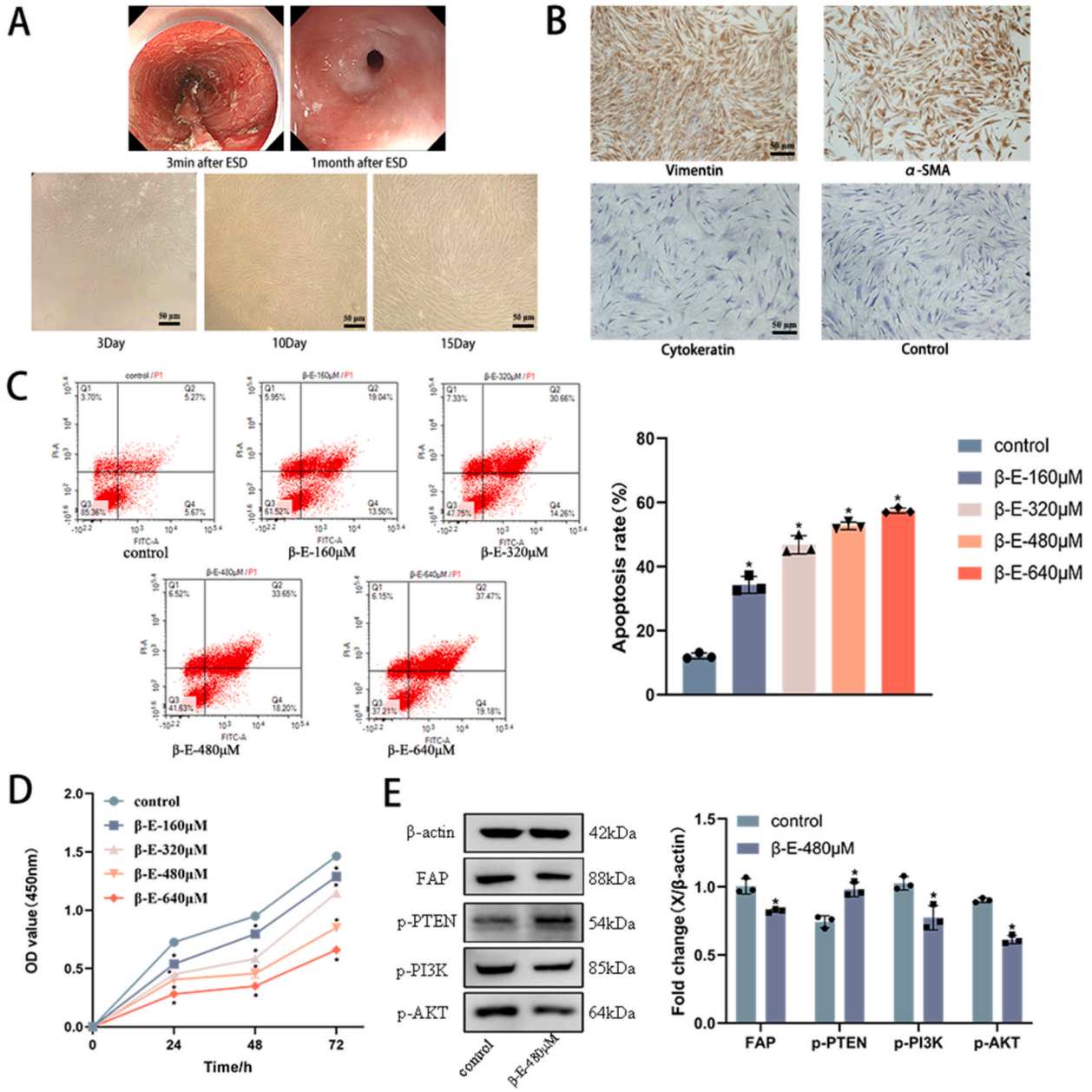

Fig. 3. β-elemene suppressed the proliferation and promoted the apoptosis of PHEGFs. (A) Endoscopic view after ESD and PHEGFs culture at different stages. (B) The primary cells were identified as fibroblasts by immunocytochemistry. (C) Flow cytometry detection of PHEGFs apoptosis rate after β-elemene intervention (n = 3). (D) CCK-8 to assess the proliferation of PHEGFs after β-elemene intervention (n = 3). (E) Flod change of FAP, p-PTEN, p-PI3K, and p-AKT (n = 3).*$P < 0.05$ versus the control group.

2.8. Western blotting

The expression levels of β-actin, FAP, p-PI3K, p-PTEN, and p-AKT proteins in PHEGFs were detected via Western blotting. Following protein extraction, the samples were loaded onto SDS-PAGE and subjected to electrophoresis at 80 V for 30 min, followed by 120 V, until bromophenol blue reached the bottom and electrophoresis was halted. The total duration of electrophoresis was approximately 1 h. Then, the sample was transferred to the PVDF membrane. The protein blots were incubated with β-actin (rabbit anti-human, 1:10,000), FAP (rabbit anti-human, 1:1000), p-PTEN (rabbit anti-human, 1:1000), p-PI3K (rabbit anti-human, 1:1000) and p-AKT (rabbit anti-human, 1:1000) antibodies overnight at 4 °C, and incubating with the secondary antibodies for 1 h at 25 °C temperature. Visualization of the protein blots was achieved using an ECL chemiluminescence reagent. Finally, the protein blots were observed using ImageQuant LAS4000mini and quantified by ImageJ software.

2.9. Statistical analysis

The statistical analyses were conducted using GraphPad Prism software (version 9.0, USA). T-tests were used for comparisons between groups. One-way analysis of variance (ANOVA) was performed for multiple comparisons. The measurement data were presented as ($x \pm s$). $^{*}P < 0.05$ and $^{**}P < 0.001$ were considered statistically significant.

3. results

3.1. Purification and culture of PHEGFs in vitro

The granulation tissue was obtained using biopsy forceps one month after ESD, and primary esophageal granulation fibroblasts were successfully cultured *in vitro* by combining the "tissue adherent method" and "trypsin digestion method." Under microscopic observation, primary fibroblasts could be observed migrating from the tissue mass and undergoing division (Fig. 3A). Immunocytochemistry results demonstrated positive staining for Vimentin and α-SMA, with evenly distributed brown particles in the cytoplasm. Conversely, cytokeratin showed negative staining without any brown particles in the cytoplasm (Fig. 3B), confirming their identification as myofibroblasts rather than epithelial cells.

3.2. β-elemene exerts inhibitory effects on the proliferation of PHEGFs and facilitates its apoptosis

The flow cytometry results demonstrated that treatment of PHEGFs with 160 μM β-elemene significantly increased the apoptosis rate, which was also dose-dependent as evidenced by the increasing apoptosis rate with higher drug doses (Fig. 3C). Moreover, the CCK-8 assay revealed a decrease in OD values of the experimental groups compared to the control group ($P < 0.05$), indicating that β-elemene could attenuate PHEGFs proliferation (Fig. 3D). Considering subsequent protein extraction requirements and drug intervention effects, Table 1 showed that a concentration of 480 μM β-elemene approximated the half maximal inhibitory (IC 50) concentration and thus was chosen as the optimal drug intervention concentration for further experiments. To further clarify the mechanism of β-elemene inhibiting PHEGFs activity, we determined the protein expression levels of FAP, p-PTEN, p-PI3K, and p-AKT by using Western blotting analysis (Fig. 3E). The β-E−480 μM group showed decreased protein expressions of FAP, p-PI3K, and p-AKT while enhanced expression of p-PTEN compared with the control group ($P < 0.05$). In conclusion, β-elemene negatively regulates the PTEN-PI3K/AKT signaling pathway by regulating FAP, thereby slowing down esophageal fibrosis.

3.3. FAP negatively regulates the PTEN-PI3K/AKT signaling pathway to inhibit PHEGFs activity

The above results suggest that β-elemene may inhibit PTEN-PI3K/AKT signaling pathway through FAP. To further verify the association between FAP and PHEGFs activity, as well as PTEN-PI3K/AKT signaling pathway, we constructed FAP overexpression and FAP silencing cell lines. As shown in Fig. 4A, the PHEGFs exhibited green fluorescence in all groups following transient transfection for 24 h. Subsequently, the expression of FAP protein was detected by WB after protein extraction. The results revealed that the FAP-oe group exhibited a statistically significant increase in FAP protein expression compared to the NC group ($P < 0.05$). In contrast, it was down-regulated in the FAP-sh group, indicating the successful establishment of transiently transfected PHEGFs cell line for subsequent functional studies (Fig. 4B). CCK-8 assay demonstrated enhanced proliferation ability of PHEGFs upon FAP overexpression, while

Table 1
Inhibition rate of PHEGFs after intervention with β-elemene at different concentrations and for different times (X ± S, n = 3).

Group	24 h	48 h	72 h
control	0	0	0
β-E-160μM	24.16 ± 3.02**	15.15 ± 0.22**	11.98 ± 2.67**
β-E-320μM	36.74 ± 1.37**	37.86 ± 1.21**	21.52 ± 1.75**
β-E-480μM	45.47 ± 1.42**	51.26 ± 4.17**	41.9 ± 2.17**
β-E-640μM	60.53 ± 0.22**	62.69 ± 0.59**	54.91 ± 1.93**

**$P < 0.001$ compared with control group

silencing of FAP resulted in reduced cell proliferation (Fig. 4D). In addition, Flow cytometry analysis revealed that FAP overexpression inhibited apoptosis of PHEGFs, whereas silencing of FAP promoted apoptosis (Fig. 4C). Furthermore, WB results showed that p-PTEN expression was significantly downregulated and the phosphorylation levels of PI3K and AKT were significantly upregulated in the presence of FAP overexpression, while FAP silencing was the opposite (Fig. 4E). These findings suggest that regulation of PHEGFs activity by FAP occurs through modulation of the PTEN-PI3K/AKT signaling pathway.

4. Discussion

Esophageal stricture resulting from ESD surgery significantly impacts patients' quality of life and is generally attributed to fibrosis and scar formation in the esophagus. Currently, it is widely accepted that circumferential dissection exceeding three-quarters of the esophageal circumference and pathological infiltration depth beyond the mucosal layer are established risk factors for postoperative stricture [28]. In the pathogenesis of esophageal fibrosis, the activation and proliferation of myofibroblasts play a pivotal role.

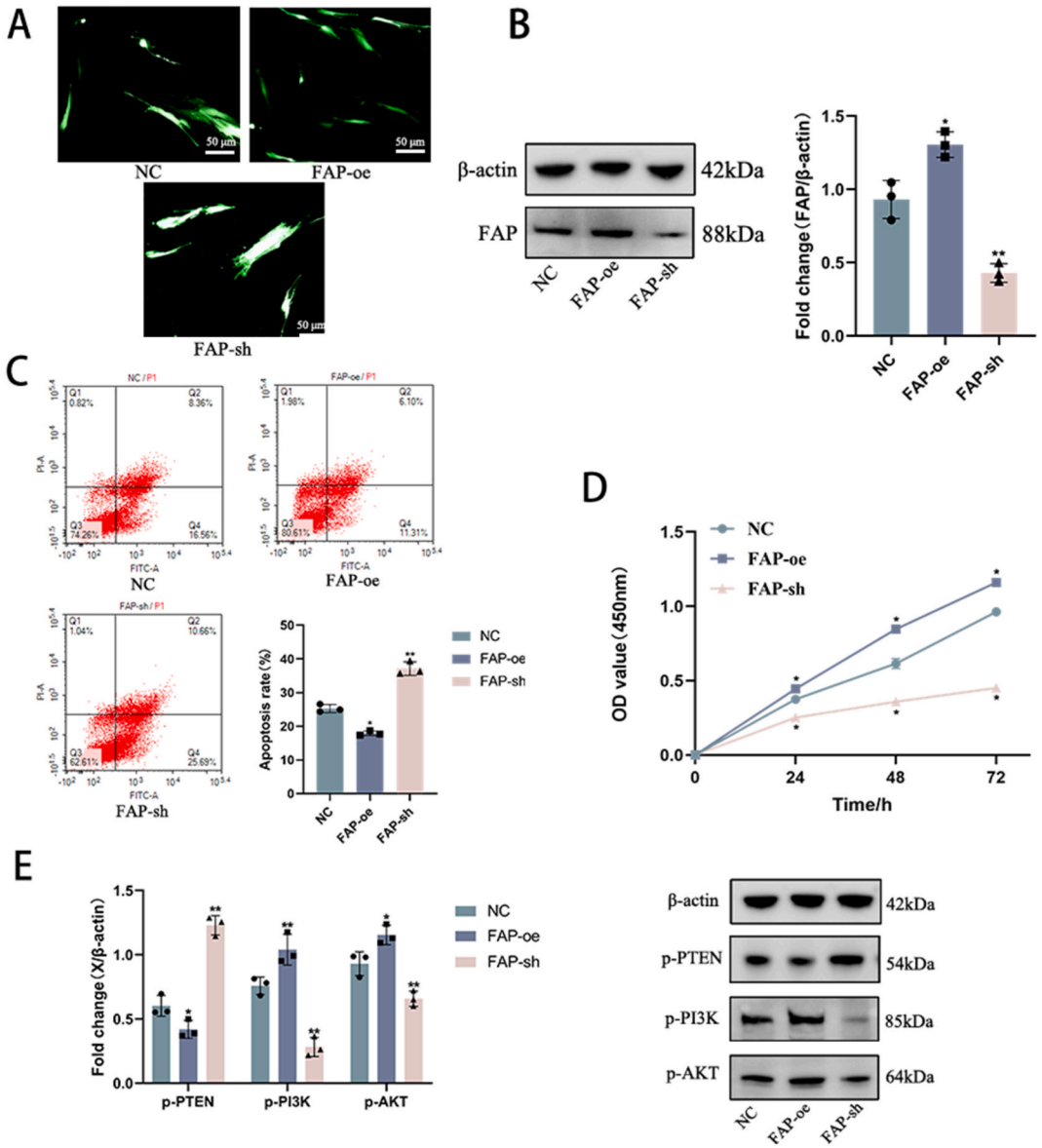

Fig. 4. Overexpression and silencing of FAP regulated PHEGFs activity through PTEN-PI3K/AKT signaling pathway. (A) The transient transfected PHEGFs emit green fluorescence. (B) The fold change of FAP after transient transfection (n = 3). (C) Flow cytometry detection of PHEGFs apoptosis rate after transient transfection (n = 3). (D) CCK-8 to assess the proliferation of PHEGFs after transient transfection (n = 3). (E) The overexpression and interference of FAP regulate of p-PTEN, p-PI3K, and p-AKT (n = 3).*$P < 0.05$, **$P < 0.001$ versus the NC group. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Sustained inflammatory responses drive excessive myofibroblast proliferation, resulting in aberrant extracellular matrix and collagen deposition, ultimately leading to the development of esophageal fibrosis [5,29]. Although studies have indicated drugs are a promising approach for preventing and treating esophageal fibrosis, developing safe and effective medications is still ongoing [30–33]. The anti-tumor drug β-elemene, derived from Curcuma wenyujin, a traditional Chinese herb known for its blood circulation activation and blood stasis removal properties, exhibits significant therapeutic efficacy against various tumors [34,35]. Our findings revealed that β-elemene can inhibit the proliferation of PHEGFs and induce their apoptosis in a concentration-dependent manner. This finding is in line with previous research indicating that β-elemene has the ability to suppress the proliferation of tracheal granulation fibroblasts and mitigate airway stenosis [36,37]. The PI3K/AKT signaling pathway, a classic intracellular signal transduction pathway, governs diverse biological processes, including cell survival, apoptosis, migration, proliferation, and inflammatory responses [38]. It has been reported that *FLT3* mutation activates the downstream PI3K/AKT pathway, thereby promoting AML cell proliferation [39]. In contrast, β-elemene demonstrates significant cytotoxicity against *FLT3* ITD-mutated AML cells [26]. In this study, we confirmed that β-elemene attenuates the activity of PHEGFs by suppressing FAP expression and the activation of the PTEN-PI3K/AKT pathway.

In addition, the involvement of FAP in tissue fibrosis development has been well established [40]. To further elucidate the role of FAP in PHEGFs, we conducted investigations involving both overexpression and silencing of FAP. The results revealed that FAP overexpression promoted cell proliferation and suppressed apoptosis, whereas the silencing of FAP yields opposite effects. Furthermore, recent reports have highlighted the regulatory impact of FAP on the PTEN-PI3K/AKT signaling pathway [14,41]. In this research, we observed that FAP overexpression downregulated phosphorylation activation of PTEN, thereby facilitating the activation of the PI3K/AKT signaling pathway. Conversely, FAP silencing resulted in the upregulation of PTEN phosphorylation activation and subsequent inhibition of the PI3K/AKT signaling pathway.

These findings collectively indicate that β-elemene exerts a negative regulatory effect on the PTEN-PI3K/AKT signaling pathway through inhibition of FAP expression, thereby impeding the PHEGFs and promoting their apoptosis. Consequently, this effectively retards the progression of esophageal fibrosis. In conclusion, our study has made a significant contribution to the existing literature on the occurrence and development of esophageal stricture. Additionally, It has elucidated a novel molecular mechanism for β-elemene in anti-esophageal fibrosis and proposed innovative strategies for clinical prevention and treatment of post-ESD esophageal stricture. In the future, it is necessary to further elucidate the potential mechanism of β-elemene in ameliorating esophageal fibrosis based on this groundwork. Further, the therapeutic efficacy of β-elemene warrants exploration through animal and clinical trials.

5. Study limitation

However, this study still has certain deficiencies and limitations. Firstly, it should be noted that this study is confined to *in vitro* experiments, and further investigation through future clinical trials is necessary to determine the impact of β-elemene on clinical application and its potential effect on wound healing. In addition, the results presented in this study are preliminary, and there may be additional regulatory mechanisms of β-elemene on granular fibroblasts after ESD that remain unexplored. Furthermore, it is important to mention that this study solely focused on verifying the protein level without conducting relevant experiments at the gene level to investigate potential underlying mechanisms. Therefore, more extensive research in this field is warranted to establish a solid foundation for future clinical translation.

Data availability statement

Data will be made available on request.

Funding

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Conflict of interest disclosure

The authors declare no conflicts of interest.

Ethics statement

This study was conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee of the Second Affiliated Hospital of Fujian Medical University (approval No.297). Written informed consent was obtained from all patients.

CRediT authorship contribution statement

Jingju Wu: Writing – original draft, Methodology, Conceptualization. **Caifa Hong:** Visualization, Methodology, Conceptualization. **Ting Qiu:** Formal analysis. **Weitao Hu:** Methodology. **Jiangmu Chen:** Data curation. **Taiyong Fang:** Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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