

# Pterostilbene Alleviates Pulmonary Fibrosis by Regulating ASIC2

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## Research

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# Abstract

**Background:** Idiopathic pulmonary fibrosis (IPF) is a serious chronic disease of the respiratory system, and its current treatment have certain shortcomings and adverse effects. In this study, we evaluate the anti-fibrotic activity of pterostilbene (PTE) using an IPF model induced by TGF- $\beta$ 1 in vitro.

**Methods:** A549 and AEC cells were incubated with 10 ng/ml TGF- $\beta$ 1 to induce lung fibroblast activation. 30  $\mu$ mol/L PTE was used to treat the cells. The epithelial-mesenchymal transition (EMT), accumulation of extracellular matrix (ECM) and autophagy of cells were suggested by western blot. The apoptosis was proved by flow cytometry analysis and western blot. Transcriptome high-throughput sequencing on A549 cells incubated with TGF- $\beta$ 1 alone or TGF- $\beta$ 1 and PTE (TGF- $\beta$ 1+PTE) was performed, and differentially expressed genes caused by PTE were identified. The ASIC2 overexpression plasmid was used to rescue the protein level of ASIC2 in A549 and AEC cells.

**Results:** TGF- $\beta$ 1 caused the EMT and accumulation of ECM, and blocked the autophagy and apoptosis of A549 and AEC cells. Most importantly, 30  $\mu$ mol/L PTE inhibited the pulmonary fibrosis induced by TGF- $\beta$ 1. Compared with cells treated with TGF- $\beta$ 1, PTE treatment inhibited the EMT and accumulation of ECM, and rescued cell apoptosis and autophagy. The results of transcriptome high-throughput sequencing performed that PTE greatly reduced the protein level of ASIC2. In addition, compared with the TGF- $\beta$ 1+PTE group, the transfection of ASIC2 overexpression plasmid stimulated the EMT and accumulation of ECM, and inhibited apoptosis and autophagy, suggesting that PTE inhibited pulmonary fibrosis by down-regulating ASIC2.

**Conclusions:** In conclusion, our study suggests that PTE and ASIC2 inhibitors may benefit future IPF treatments.

## Background

Idiopathic pulmonary fibrosis (IPF) is a chronic, progressive and fibrotic lung disease, which is characterized by sustained reproduction of lung fibroblasts and accumulation of extracellular matrix (ECM), ultimately leading to breathing system failure and death[1, 2]. IPF occurs worldwide, of which prevalence seems to be increasing[3]. It is not clear whether this is due to improved cognition, or the real increase in morbidity. IPF is more common in men and people with a history of smoking[4]. As a typical senile disease, the median age at diagnosis of it is 65 years[5, 6]. The course of this disease is uncertain and unpredictable, and prognosis is extremely poor. The median survival of patients after diagnosis is 3–5 years. Currently, lung transplantation remains the only treatment that can significantly improve the survival rate of carefully selected patients[7]. Although, two molecules used to treat IPF (pirfenidone and nintedanib) have been approved by the FDA[8, 9]. These two drugs have been shown to slow down the decline of lung function in IPF patients, but are ineffective in reversing the process of fibrosis or even stabilizing lung function, and cannot improve the survival rate or quality of life of patients[10, 11].

The main feature of IPF is the accumulation of fibroblasts. Fibroblasts respond to secreted transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1) and differentiate into myofibroblasts[12]. Myofibroblasts express  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA) and a large amount of ECM (such as collagen), and accumulate in fibrous hyperplasia foci to inhibit cell exchange. In IPF, the scattered accumulation of myofibroblasts in fibrotic lesions and the deposition of ECM lead to irreversible destruction of lung structure, respiratory failure and death[12]. TGF- $\beta$ 1 treatment of human type II alveolar epithelial cells in vitro can induce fibrosis.

Increasing studies have revealed that naturally active compounds isolated from plants or herbs have the potential to treat organ fibrosis, including pulmonary fibrosis[13–15]. Pterostilbene (PTE; trans-3,5-dimethoxy-4-hydroxystilbene) (Fig. 1A) is a structural analogue of resveratrol, mainly derived from the Pterocarpus plants, such as grapes and blueberry[16]. It has been demonstrated that PTE possesses anti-oxidation, anti-inflammatory, anti-cancer, anti-hypertensive, and anti-aging properties[17, 18]. Emerging evidence shows that PTE can intervene in the fibrosis process of various organs. PTE can attenuate the liver fibrosis and renal fibrosis induced by excessive fructose intake by inhibiting TGF- $\beta$ 1/Smads signal transduction[19, 20]. It can also reduce fructose-induced myocardial fibrosis by inhibiting the Pitx2c/miR-15b pathway driven by ROS[21]. PTE also alleviates renal fibrosis in a mouse model of severe hyperuricemia nephropathy (HN) by inhibiting the activation of TGF- $\beta$ 1/Smad3, Src and STAT3 signaling pathways[22, 23]. Our previous study used intratracheal injection of bleomycin to induce pulmonary fibrosis in SD rats, and found that PTE (30 mg/(kg/d)) can reduce the degree of bleomycin-induced pulmonary fibrosis[24].

Here, the effect of PTE on the IPF process induced by TGF- $\beta$ 1 was evaluated, and the underlying mechanism was further investigated. The results showed that PTE suppressed TGF- $\beta$ 1-induced cell proliferation, epithelial-mesenchymal transition (EMT) and accumulation of ECM, and promoted TGF- $\beta$ 1-inhibited autophagy and apoptosis. Moreover, our data showed ASIC2 as a downstream effector of PTE involved in the anti-fibrosis of PTE on IPF.

## Materials And Methods

### Cell culture and treatment

Human normal alveolar epithelial cells A549 and AEC were purchased from the American Type Culture Collection (ATCC; Rockville, USA) and cultured in DMEM medium (Hyclone, USA) containing 10% FBS (Gibco, USA) in a humidified atmosphere of 5% CO<sub>2</sub> at 37 °C. The cells were cultured in serum-free medium for 12 h for synchronization, and further incubated with TGF- $\beta$ 1 (10 ng/ml; R&D Systems, USA) to induce lung fibroblast activation. PTE (Sigma-Aldrich, USA) was dissolved in DMSO and further diluted before use. The cells were treated with TGF- $\beta$ 1, TGF- $\beta$ 1 with PTE (TGF- $\beta$ 1 + PTE), PTE for 24 h, and cells without any treatment was used as negative control (NC) group. pcDNA3.1-ASIC2 plasmid was constructed and transfected into cells using Lipofectamine 2000 (Invitrogen, USA) to overexpress ASIC2 expression.

## Dose-dependent assay

Cells were planted in 96-well plates at a density of  $5 \times 10^3$  cells per well and treated with 10 ng/ml TGF- $\beta$ 1 and various concentrations of PTE (0, 10, 20, 30, 40, 60, 80, 100  $\mu$ mol/L) for 24 h. Then, cells were incubated with CCK8 reagent (10  $\mu$ l/well; Solarbio) for another 1.5 h. Finally, the OD value at 450 nm of each well was detected using a microplate reader.

## Western blot analysis

The cells were collected and lysed in RIPA (Beyotime), and the extracted protein concentration was determined using BCA kit (Beyotime). Proteins of each sample was separated with 10% SDS-PAGE and then transferred onto PVDF membranes (Bio-Rad). After blocked in 5% milk for 1 h at room temperature, the membranes were incubated with primary antibodies at 4 °C overnight and secondary antibodies for 2 h at room temperature. The blots were visualized using an enhanced chemiluminescence kit (CWBio, Beijing, China) and quantified by ImageJ software (NIH, USA). The primary antibodies used in this study were as follows: E-cadherin (3195, CST), N-cadherin (207608, Abcam), Vimentin (5741, CST), Collagen I (sc-293, Santa Cruz), Fibronectin (15613-1-AP, Proteintech),  $\alpha$ -SMA (5694, Abcam), Bcl2 (ab32124, Abcam), Bax (ab32503, Abcam), Caspase 3 (ab13847, Abcam), LC3-I/II (14600-1-AP, Proteintech), Beclin1 (ab207612, Abcam), p62 (18420-1-AP, Proteintech), p21 (ab109520, Abcam) and GAPDH (60004-1-Ig, Proteintech).

## Flow cytometry assay

Cell apoptosis was measured using the Annexin-V-FITC/PI kit (BioVision, USA) according to the instructions. Cells ( $1 \times 10^5$ ) treated with TGF- $\beta$ 1 or PTE for 24 h were cultured in serum-free medium for another 24 h and stained with Annexin-V-FITC/PI for 10 min in the dark. Subsequently, the rate of apoptotic cells was detected using a flow cytometer (BD FACSCanto II, BD Biosciences, USA) and analyzed using the FlowJo software (Tree Star, USA).

## High-throughput sequencing

Transcriptome high-throughput sequencing was performed on A549 cells incubated with TGF- $\beta$ 1 (TGF- $\beta$ 1) alone or TGF- $\beta$ 1 and PTE (TGF- $\beta$ 1 + PTE) for 24 h. Isolate the cross-linked RNA fragments, convert them into cDNA libraries, and perform high-throughput sequencing with Illumina HiSeq. The original image data file obtained by sequencing was converted into raw data of the sequencing sequence by CASAVA base calling analysis. The raw data was filtered to get clean reads. The clean reads were aligned to a reference genome using HISAT[25]. The PoissonDis algorithm is used for differential gene detection.  $|\log_2(\text{Fold Change})| > 1$  & q value < 0.001 genes are regarded as differentially expressed genes.

## Statistical analysis

Data were expressed as Mean  $\pm$  SD from three separate experiments and statistically analyzed using GraphPad prism 7.0 (GraphPad software, UAS). Student's t test or one-way analysis of variance (ANOVA) was used to compare the differences between groups.  $P < 0.05$  was considered significant.

# Results

## PTE inhibits TGF- $\beta$ 1-induced cell proliferation, EMT and accumulation of ECM

The chemical structure of PTE was shown in Fig. 1A. As exhibited in Fig. 1B, PTE at a dose of 30–100  $\mu$ mol/L significantly reduced the cell viability of 10 ng/ml TGF- $\beta$ 1 exposed A549 and AEC cells (Fig. 1B). Furthermore, our preliminary experiments showed that 10 ng/ml TGF- $\beta$ 1 caused EMT after 48 h exposure in A549 and AEC cells (Fig. 1C and D). Here, the N-cadherin and Vimentin expression in cells were significantly increased after incubation with 10 ng/ml TGF- $\beta$ 1 (Fig. 1C and D), while E-cadherin protein levels were decreased (Fig. 1C and D). 30  $\mu$ mol/L PTE suppressed N-cadherin and Vimentin protein levels, and rescued E-cadherin expression in TGF- $\beta$ 1-exposed cells (Fig. 1C and D). For accumulation of ECM, the results from western blot analysis confirmed the increased protein levels of  $\alpha$ -SMA and fibronectin, as well as collagen 1, which induced by TGF- $\beta$ 1 in A549 and AEC cells (Fig. 1E and F). PTE significantly reduced the  $\alpha$ -SMA, fibronectin, and collagen 1 expression (Fig. 1E and F). In addition, incubation with PTE alone, without TGF- $\beta$ 1, seemed to have no effect on the EMT and accumulation of ECM compared into normally cultured A549 and AEC cells (Fig. 1C-F).

## PTE promotes apoptosis and autophagy in TGF- $\beta$ 1-induced cells

Some studies have reported that PTE induces apoptosis in ovarian[26] and pancreatic cancer cells[27]. In this study, we found that the treatment of TGF- $\beta$ 1 significantly decreased the proportion of apoptosis cell (Fig. 2A and B), and inhibited the expression of Bax and caspase-3, and induced the expression of Bcl2 (Fig. 2C and D). The treatment of PTE rescued the apoptosis which inhibited by TGF- $\beta$ 1 (Fig. 2A and B), elevated Bax and caspase 3 protein levels, and degraded Bcl2 in A549 and AEC cells (Fig. 2C and D). We also focused on PTE in cell autophagy. As shown in Fig. 2E and F, TGF- $\beta$ 1 significantly inhibited LC3-II, Beclin-1 and p21 protein expression, and enhanced LC3-I and Beclin-1 protein expression in A549 and AEC cells, which all were reversed by PTE. Moreover, incubation with PTE alone, without TGF- $\beta$ 1, had no effect on the apoptosis and autophagy compared into normally cultured A549 and AEC cells (Fig. 2).

## Identification of the differentially expressed genes in PTE-treated cells

We further performed transcriptome high-throughput sequencing on A549 cells incubated with TGF- $\beta$ 1 alone (TGF- $\beta$ 1), or TGF- $\beta$ 1 and PTE (TGF- $\beta$ 1 + PTE) for 24 h to identify differentially expressed genes caused by PTE. The cross-linked RNA fragments were isolated, converted into cDNA libraries, and performed to high-throughput sequencing with Illumina HisEq. TGF- $\beta$ 1 samples produced 8.4430 Gb clean bases, and 95.74% reads were compared to the reference genome, and measured 14848 genes. TGF- $\beta$ 1 + PTE samples produced 8.4045 Gb clean bases, and 95.54% reads were compared to the reference genome, and measured 15451 genes. Compared with PTE samples, 2898 differentially expressed genes were detected in TGF- $\beta$ 1 + PTE samples (Fig. 3). Among them, 2037 genes were differentially up-regulated and 861 genes were differentially down-regulated.

## PTE inhibits pulmonary fibrosis by down-regulating ASIC2

Among the 861 differentially down-regulated genes, we found that PTE inhibited pulmonary fibrosis by down-regulating ASIC2. As shown in Fig. 4A and B, PTE significantly reduced the protein levels of ASIC2 compared with TGF- $\beta$ 1 group. To explore the role of ASIC2, the ASIC2 overexpression plasmid was used to rescue the protein levels of ASIC2 compared with TGF- $\beta$ 1 + PTE group. Furthermore, the restoration of ASIC2 protein levels stimulated the EMT (Fig. 4C and D) and accumulation of ECM (Fig. 4E and F), and inhibited apoptosis and autophagy (Fig. 5) compared with TGF- $\beta$ 1 + PTE group.

## Discussion

IPF is one of the most serious chronic diseases of the respiratory system with the characteristics of progressive development and poor prognosis. The current treatment of IPF has certain shortcomings and adverse effects[28]. Therefore, finding new treatments for IPF has become the focus of current research. It is reported that PTE has antifibrotic activity in the fibrosis process of various organs, including fructose-induced liver fibrosis[29], renal fibrosis[30, 31], and myocardial fibrosis[32]. In the present study, we established a TGF- $\beta$ 1-induced IPF model to evaluate the antifibrotic activity of PTE in vitro. Our data demonstrated that PTE alleviated TGF- $\beta$ 1-induced IPF by inhibiting cell proliferation, EMT and accumulate of ECM, and promoting autophagy and apoptosis, indicating that PTE may be served as a promising strategy for IPF treatment.

TGF- $\beta$ 1 is considered to be the master profibrotic cytokine in the process of fibrosis, which is commonly used to induce cellular pulmonary fibrosis to establish an IPF model in vitro[33, 34]. TGF- $\beta$ 1 can stimulate the abnormal proliferation of lung fibroblasts, promote the formation of myofibroblasts and pro-fibrotic gene activation, leading to excessive accumulation of ECM between the lung interstitium and alveoli[35]. TGF- $\beta$ 1 is also the principal driver of fibrogenesis, a dynamic pathophysiologic process that involves cell injury and apoptosis[36]. In this study, 10 ng/ml TGF- $\beta$ 1 caused EMT and accumulate of ECM in A549 and AEC cells, which suggested by the results of western blot. TGF- $\beta$ 1 also impeded the autophagy and apoptosis of A549 and AEC cells, which proved by the results of flow cytometry analysis and western blot. Most importantly, we found that PTE inhibited pulmonary fibrosis which induced by TGF- $\beta$ 1. The treatment of PTE inhibited the EMT and accumulation of ECM, and rescued the apoptosis and autophagy compared with TGF- $\beta$ 1-treated cells.

We further performed transcriptome high-throughput sequencing on A549 cells incubated with TGF- $\beta$ 1 alone or TGF- $\beta$ 1 and PTE, and identified differentially expressed genes caused by PTE. We found that PTE significantly reduced the protein levels of ASIC2. Furthermore, the ASIC2 overexpression plasmid was used to rescue the protein levels of ASIC2 in A549 and AEC cells, which stimulated the EMT and accumulation of ECM, and inhibited apoptosis and autophagy compared with TGF- $\beta$ 1 + PTE group, suggesting that PTE inhibited pulmonary fibrosis by down-regulating ASIC2.

ASICs are a group of proton-gated ion channels belonging to the degenerin/epithelial sodium channel (DED/ENaC) family, which can be activated by extracellular protons. Although the cellular metabolism mechanisms during IPF are still poorly understood, lactate has recently been identified as a metabolite

that is elevated in the lung tissue of IPF patients[37]. Then, acidification or low pH can activate ASICs[38]. ASICs are encoded by four genes (*ASIC1-4*). These genes generate 6 subtypes (ASIC1a, ASIC1b, ASIC2a, ASIC2b, ASIC3 and ASIC4) through alternative splicing. Each subtype has different biophysical properties[39]. The initial study suggests that ASICs are widely distributed in the central and peripheral nervous system[40]. However, the latest research shows that multiple subunits of ASICs are also present in vascular smooth muscle and endothelial cells of various vascular beds[41]. Su et al. find the expression of ASIC2 and ASIC3 in human submucosal (Calu-3), bronchial (16HBE14o), pancreas (CFPAC) and colon (T84) epithelial cells, as well as the expression of lung tissue slices in alveolar tissue[42]. One study has also determined the expression of ASIC1, ASIC2 and ASIC3 in A549 cells[43]. Acidic extracellular medium or overexpression of ASIC1a promotes the proliferation and migration of A549 cells[43].

Ion channels are transmembrane proteins that have physiological and pathological functions across biological membranes. Currently, about 13% of drugs used to treat various human diseases, including cardiovascular and neurological diseases, are mainly aimed at ion channels[44]. These channels are associated with many human diseases/pathologies, including IPF. For example, the classical transient receptor potential 6 (TRPC6) is a cation channel permeable to Na<sup>+</sup> and Ca<sup>2+</sup>, which can promote the differentiation of primary mouse lung fibroblasts into myofibroblasts. Its specific channel inhibitors may benefit future IPF treatment[45]. Our experimental results suggest that ASIC2 inhibitors may also benefit future IPF treatments.

## Conclusion

In sum, with the utilization of in vitro model of IPF, our data demonstrated that PTE could inhibit TGF- $\beta$ 1-induced EMT and accumulation of ECM, and promote autophagy and apoptosis, suggesting that PTE may be served as a promising strategy for IPF treatment. Moreover, we found that ASIC2 as a downstream effector of PTE may be involved in the activity of PTE to alleviate pulmonary fibrosis. Further investigation of the activity and mechanism of PTE in vivo are still required.

## Declarations

### Ethics approval and consent to participate

Not applicable.

### Consent for publication

Not applicable.

### Availability of data and materials

The datasets generated and/or analysed during the current study are not publicly available, but are available from the corresponding author on reasonable request.

### Competing interests

The authors declare that they have no competing interests.

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### Authors' contributions

Yanfang Peng and Yingwen Zhang have made substantial contributions to the conception and design of the work. Each author has made substantial contributions to the acquisition, analysis, and interpretation of data

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Not applicable.

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## Figures

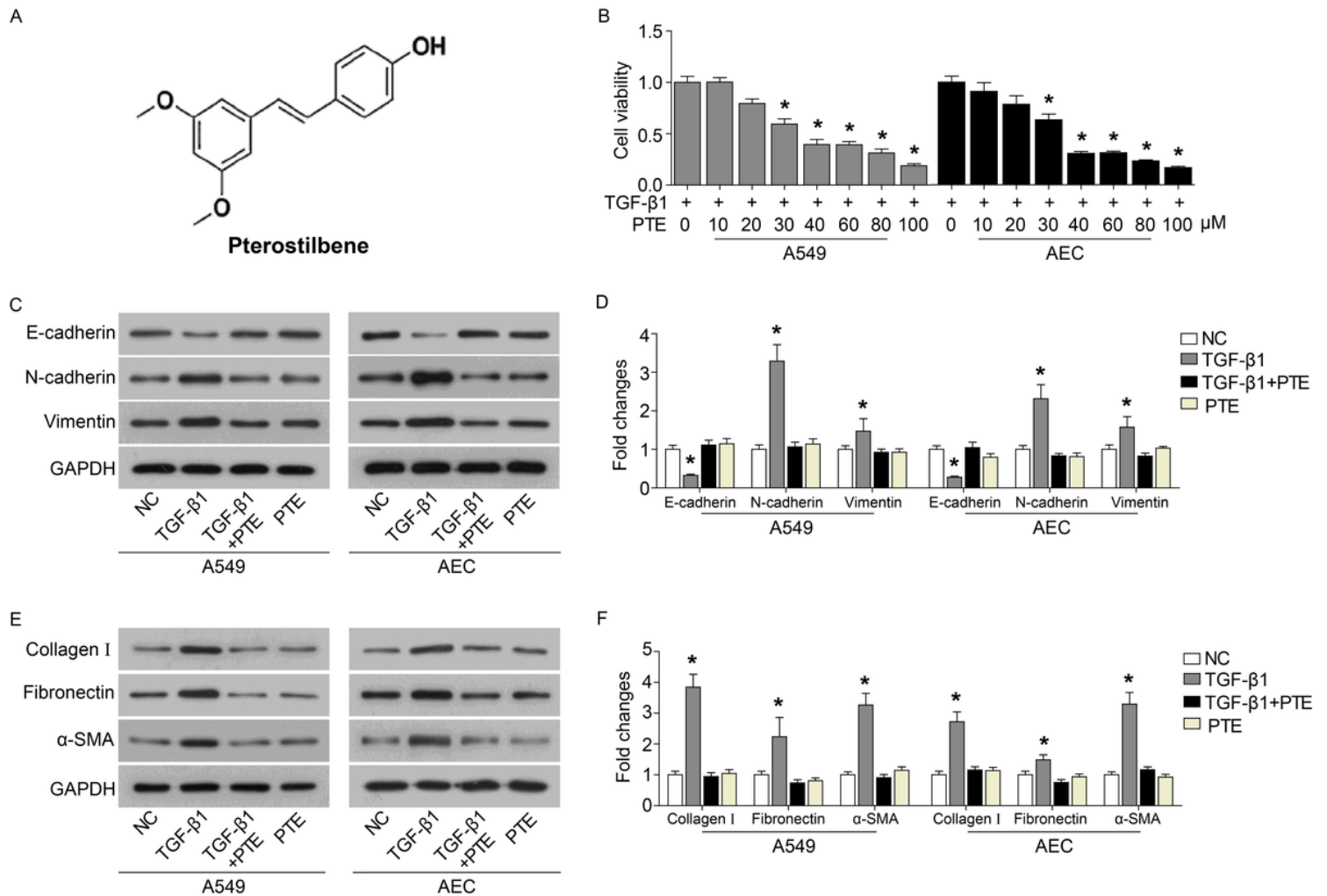


**Figure 1**

PTE inhibits TGF-β1-induced cell proliferation, EMT and accumulation of ECM. (A) Chemical structure of PTE. (B) A549 and AEC cells were treated with 10 ng/ml TGF-β1 and various concentrations of PTE (0, 10, 20, 30, 40, 60, 80, 100 μmol/L) for 24 h, and the cell viability was detected by CCK8 assay. A549 and AEC cells were treated with 10 ng/ml TGF-β1 and 30 μmol/L PTE for 24 h, the key protein of epithelial-mesenchymal transition (EMT) (C and D), α-smooth muscle actin (α-SMA) and extracellular matrix (ECM) (E and F) were detected by western blot. \*P<0.05.

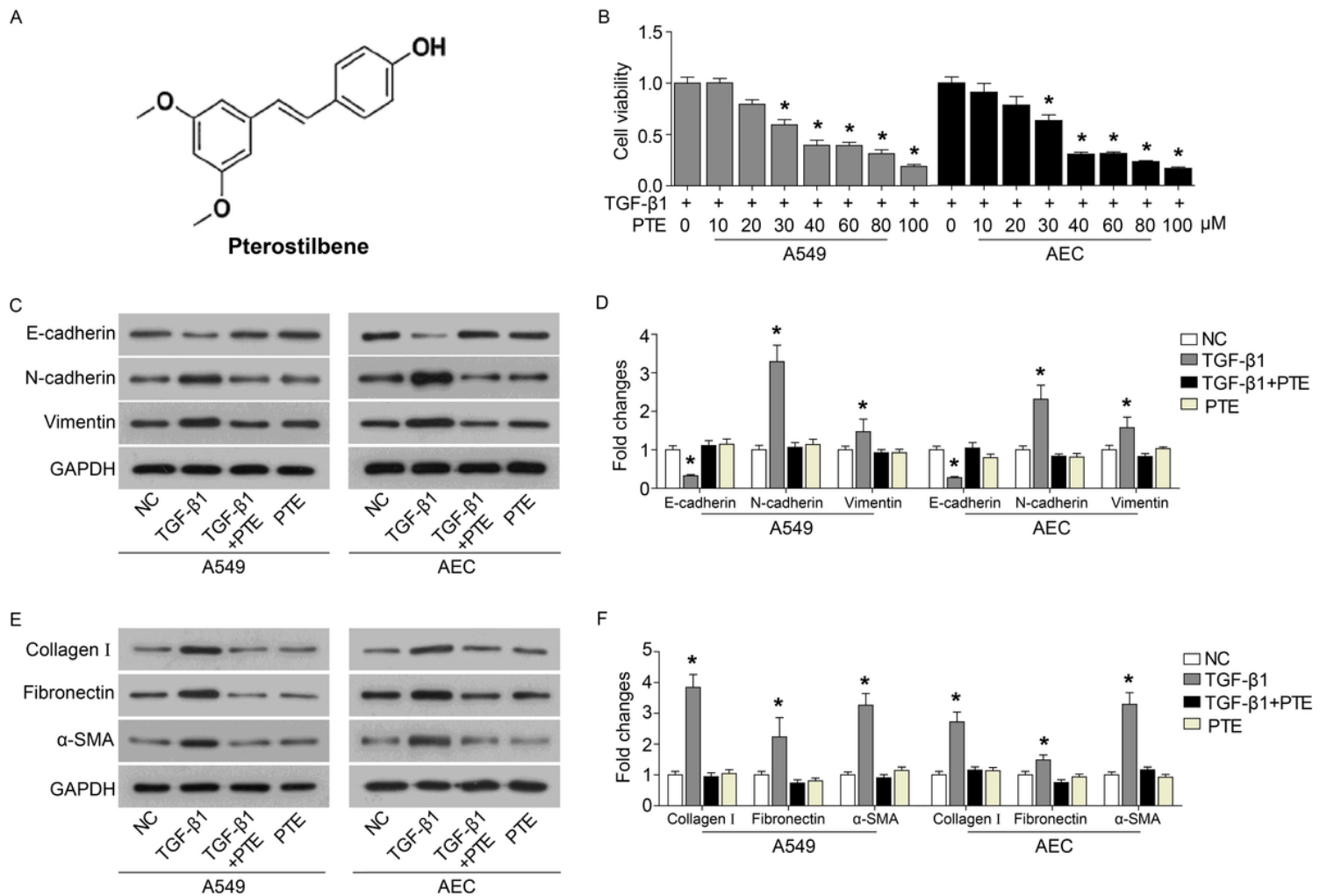


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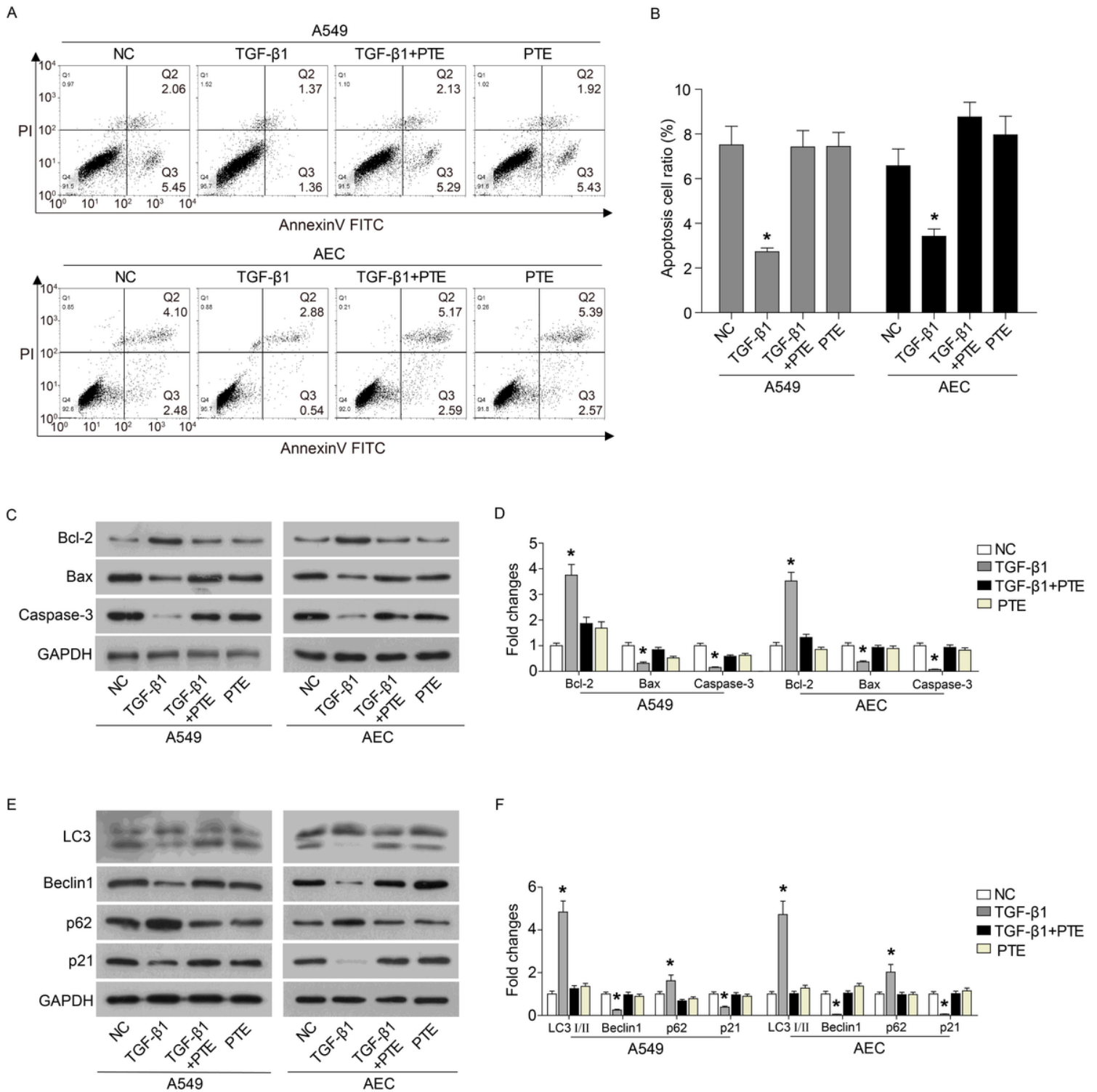


**Figure 2**

PTE promotes apoptosis and autophagy in TGF- $\beta$ 1-induced cells. A549 and AEC cells were treated with 10 ng/ml TGF- $\beta$ 1 and 30  $\mu$ mol/L PTE for 24 h, the apoptosis was analyzed by flow cytometry assay (A and B); the expression of apoptosis-related protein (C and D) and autophagy-related protein (E and F) were detected by western blot. \* $P < 0.05$ .

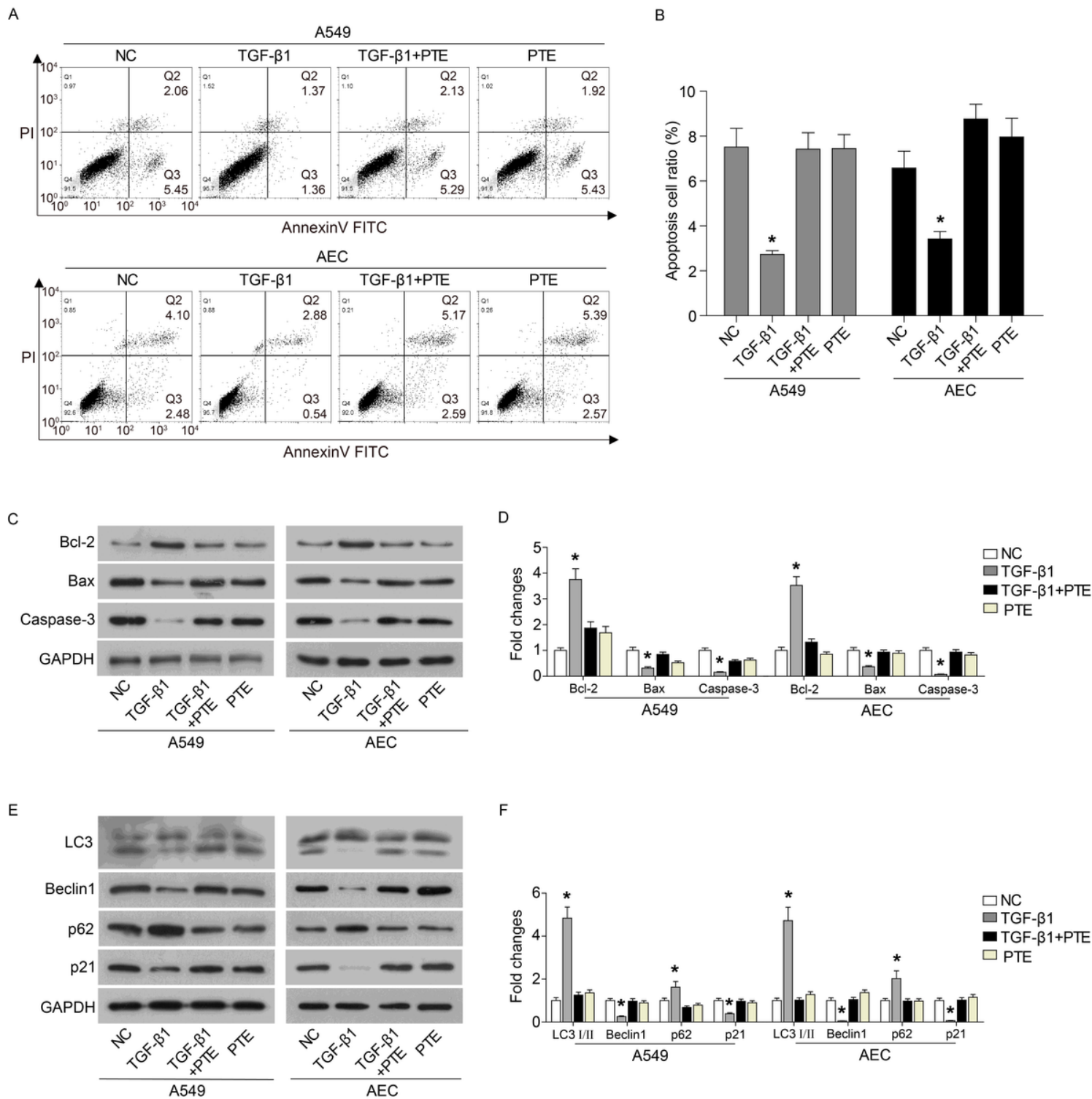


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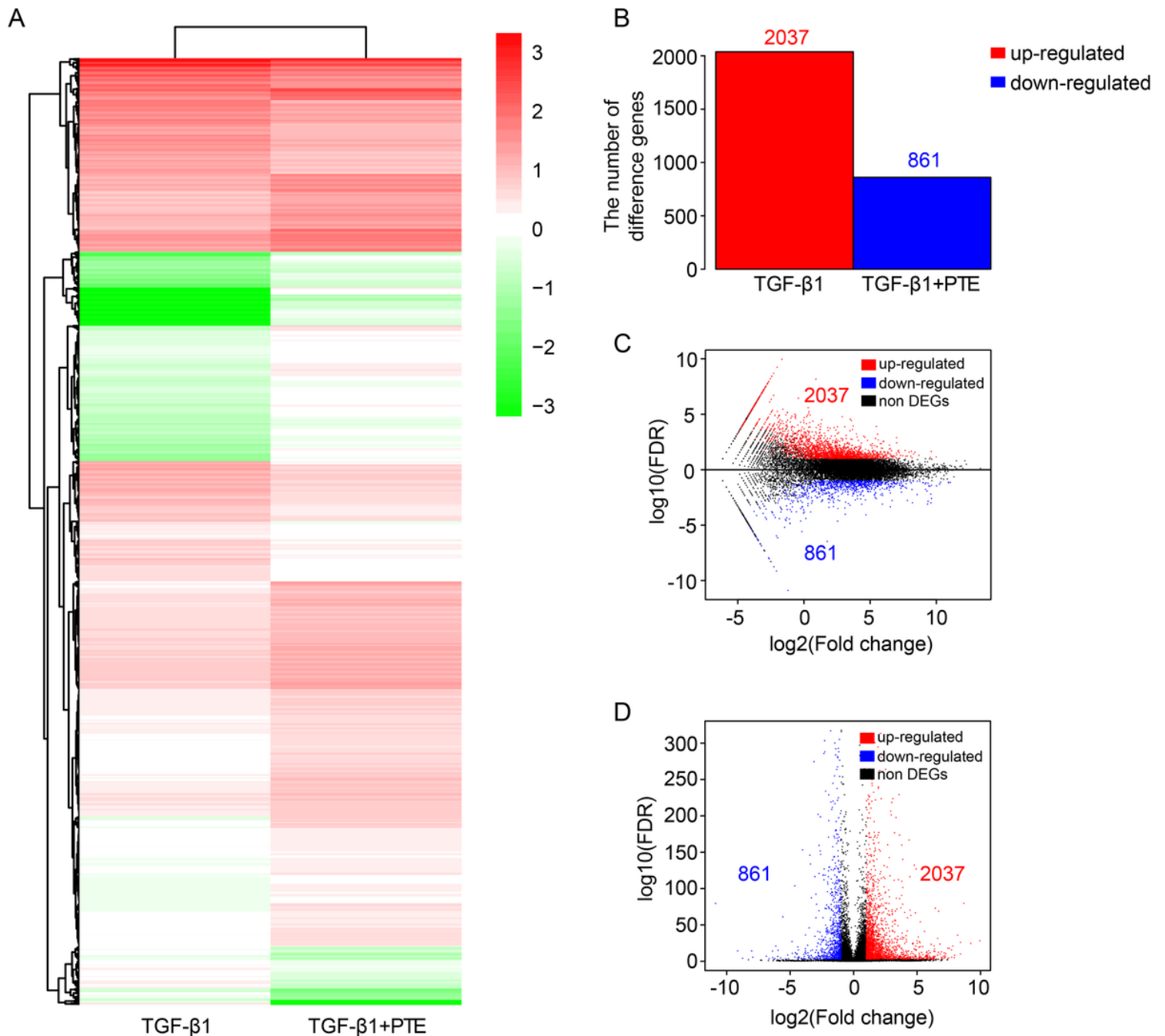


**Figure 3**

Identification of the differentially expressed genes in PTE-treated cells. Transcriptome high-throughput sequencing was performed on A549 cells incubated with TGF- $\beta$ 1 alone (TGF- $\beta$ 1), or TGF- $\beta$ 1 and PTE (TGF- $\beta$ 1+PTE) for 24 h. (A) Clustering heat map of differentially expressed genes. (B) Statistical graph of the number of differential genes. (C) Volcano map of differentially expressed genes. (D) MA map of differentially expressed genes.

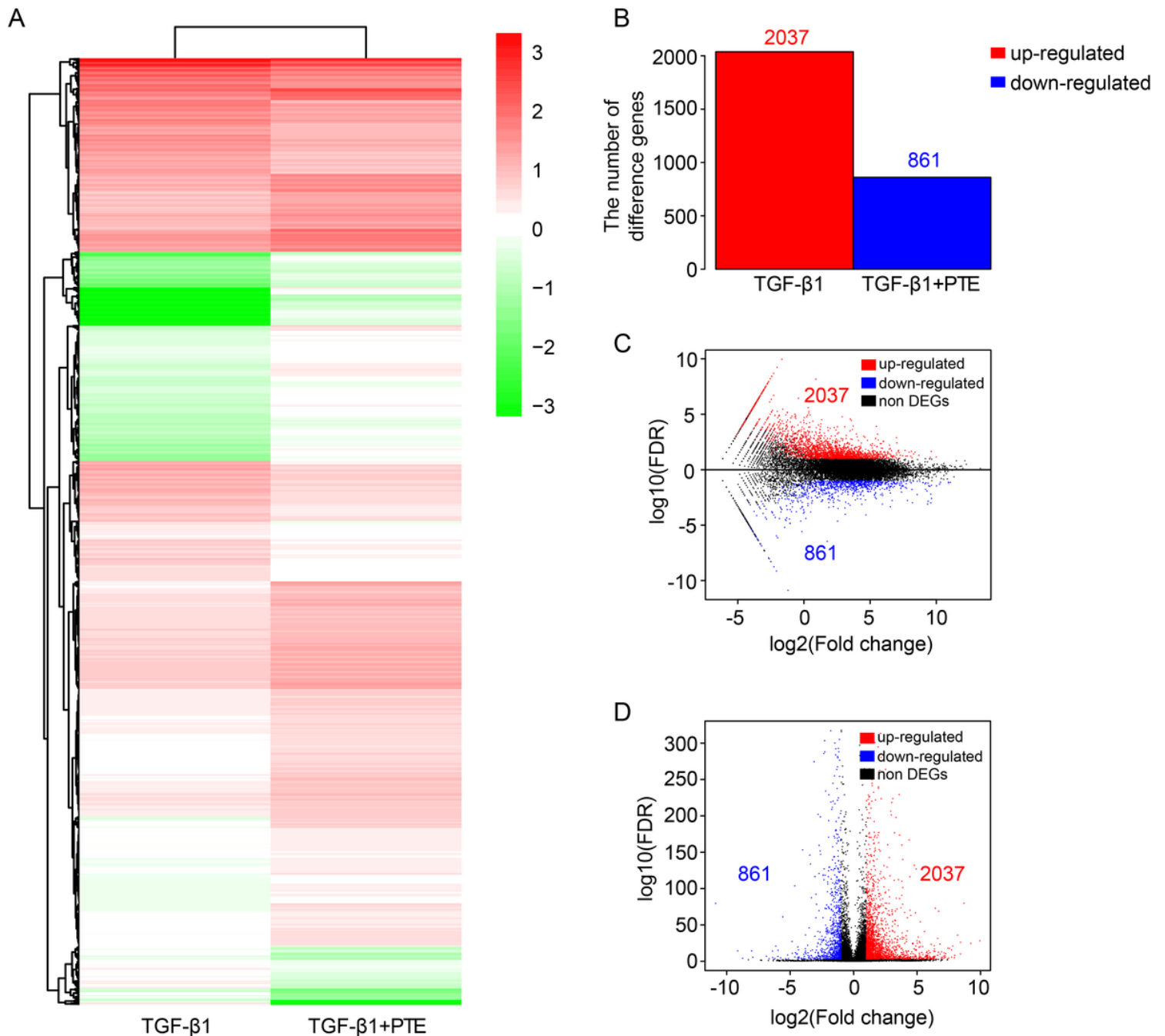


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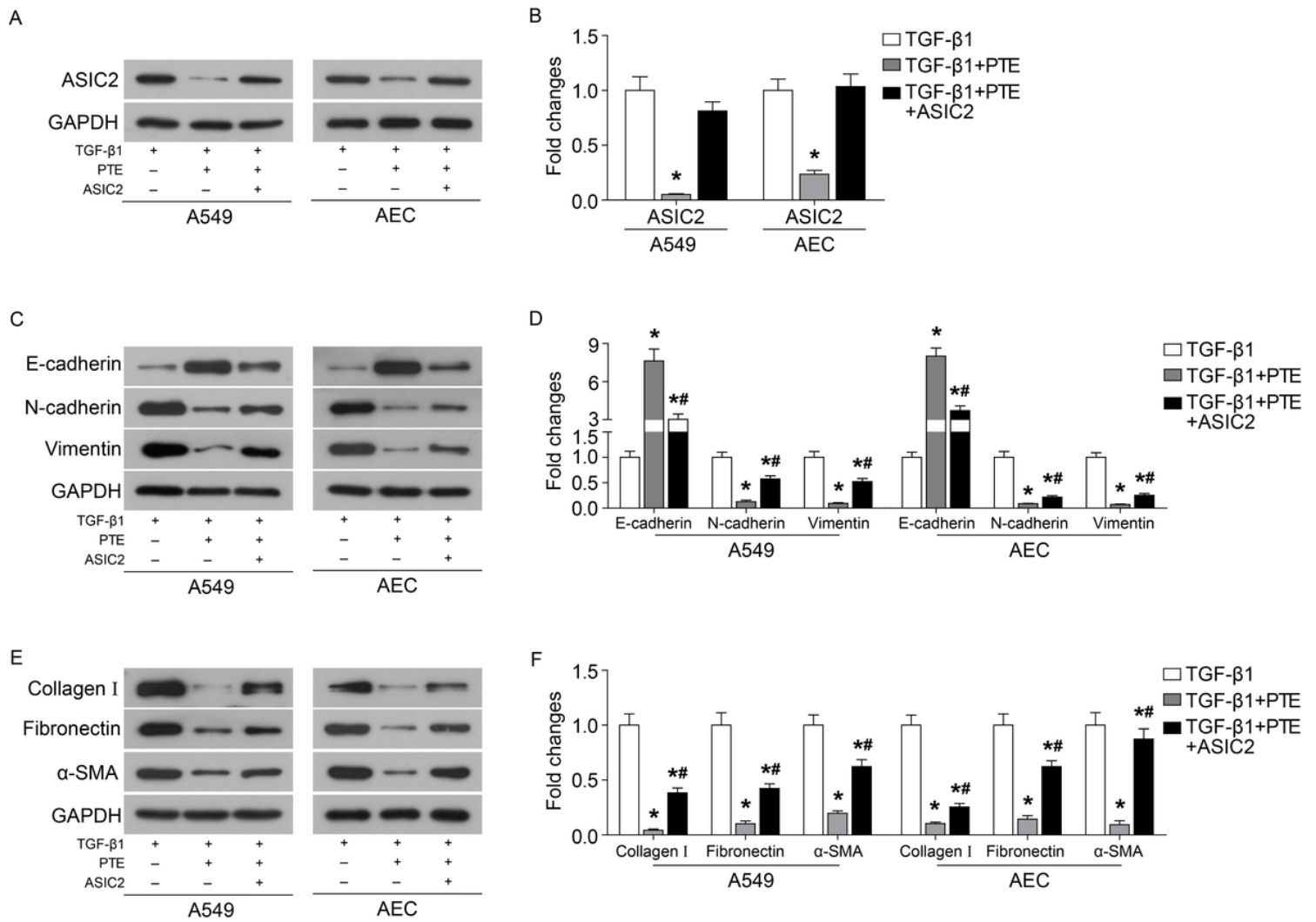


**Figure 4**

PTE inhibits TGF-β1-induced EMT and accumulation of ECM by down-regulating ASIC2. A549 and AEC cells which transfected with ASIC2 overexpression plasmid were treated with 10 ng/ml TGF-β1 and 30 μmol/L PTE for 24 h, the expression of ASIC2 (A and B), the key protein of epithelial-mesenchymal transition (EMT) (C and D), α-smooth muscle actin (α-SMA) and extracellular matrix (ECM) (E and F) were detected by western blot. \*P<0.05 compared with TGF-β1 group; #P<0.05 compared with TGF-β1+PTE group.

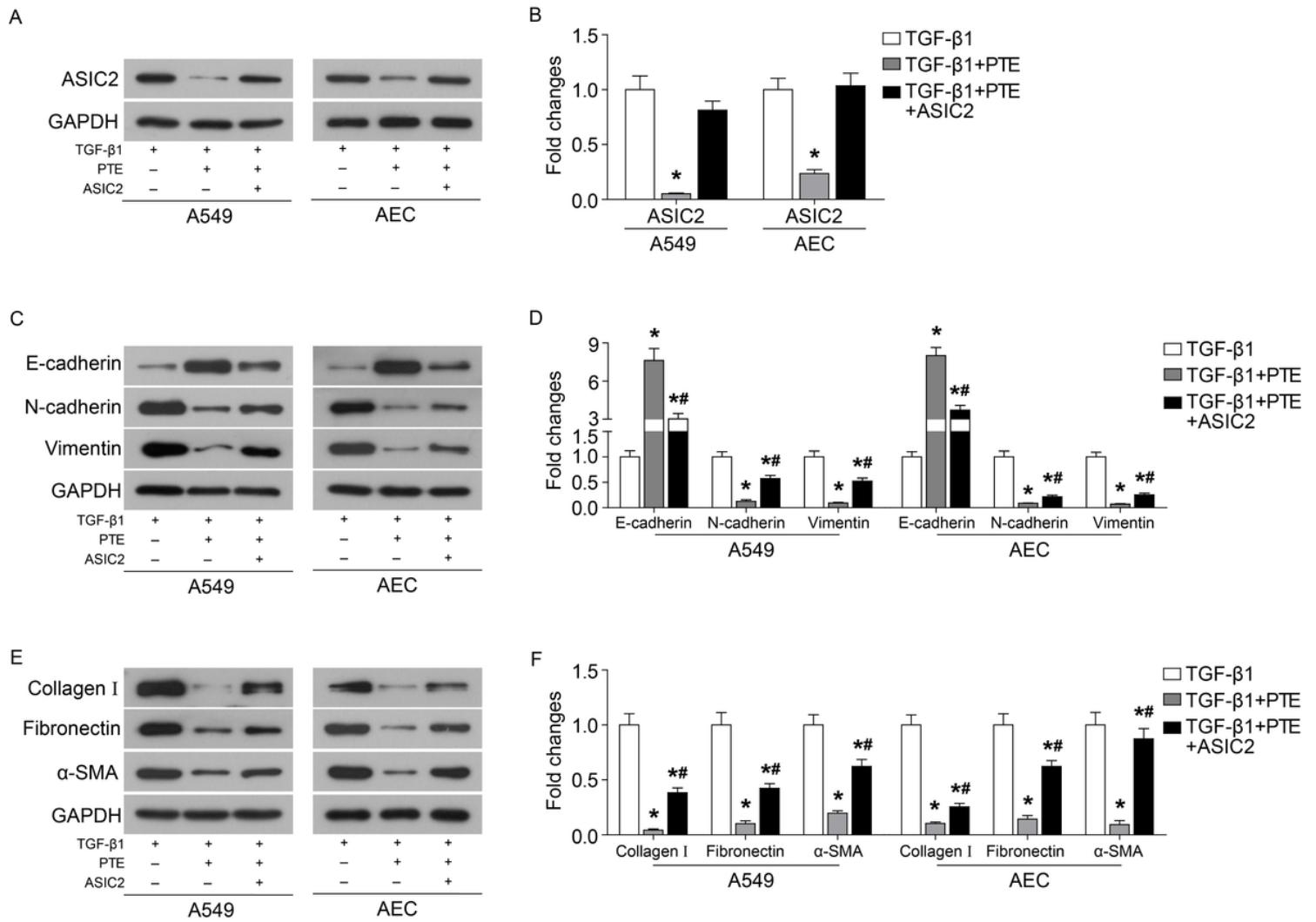


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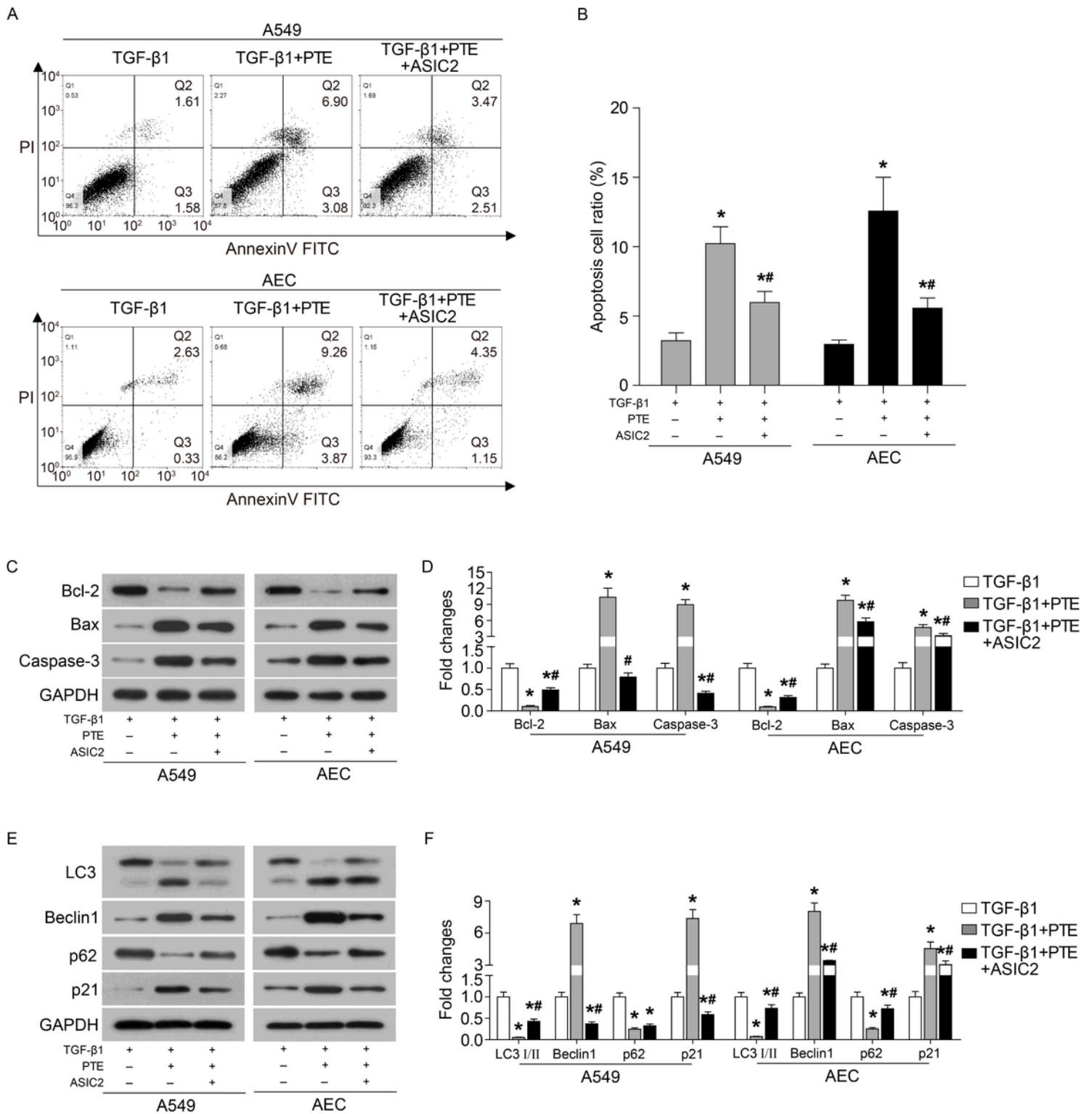


**Figure 5**

PTE promotes apoptosis and autophagy by down-regulating ASIC2 in TGF- $\beta$ 1-induced cells. A549 and AEC cells which transfected with ASIC2 overexpression plasmid were treated with 10 ng/ml TGF- $\beta$ 1 and 30  $\mu$ mol/L PTE for 24 h, the apoptosis was analyzed by flow cytometry assay (A and B); the expression of apoptosis-related protein (C and D) and autophagy-related protein (E and F) were detected by western blot. \* $P$ <0.05 compared with TGF- $\beta$ 1 group; # $P$ <0.05 compared with TGF- $\beta$ 1+PTE group.

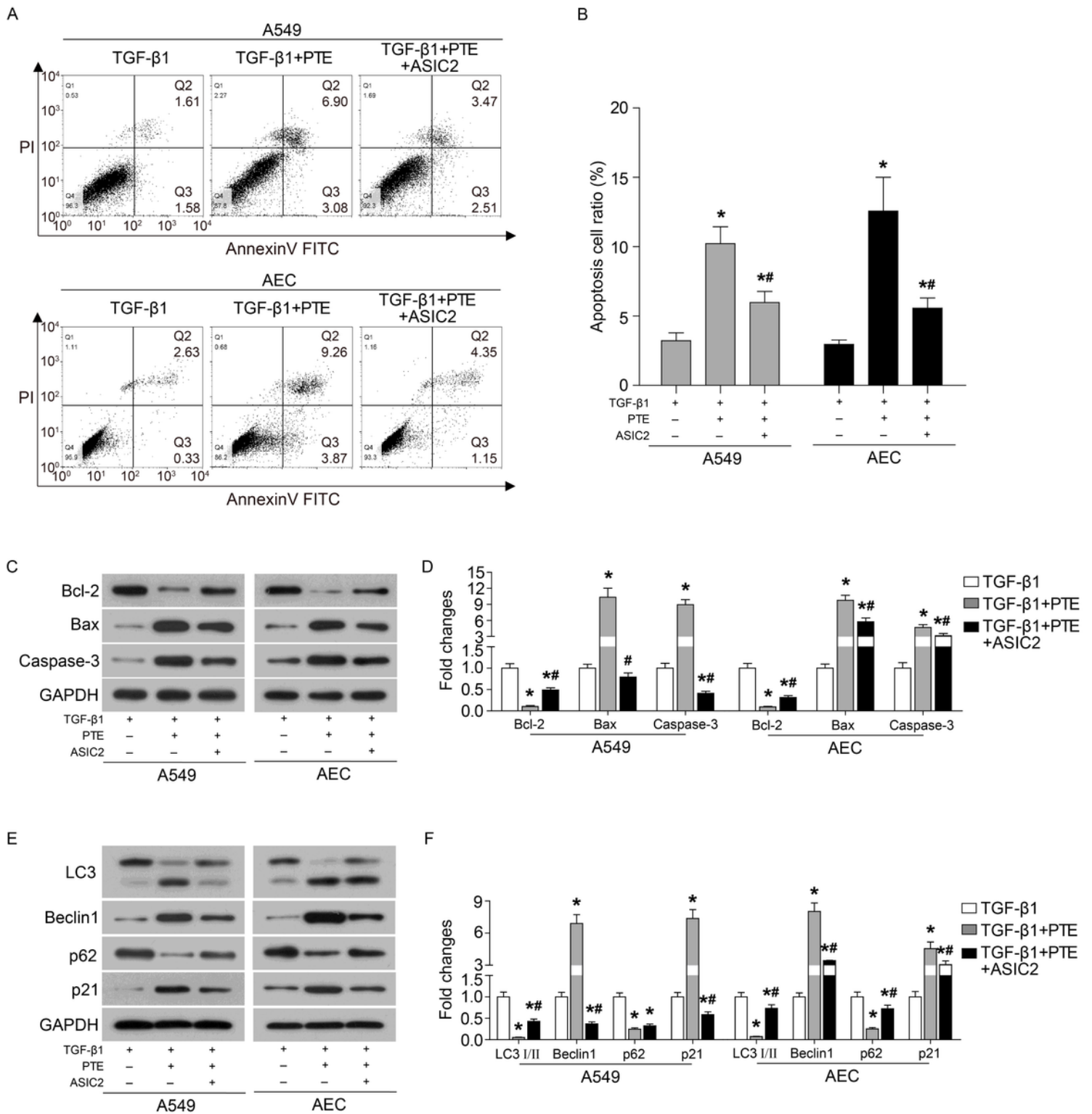


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