Ellagic Acid Attenuates BLM-Induced Pulmonary Fibrosis via Inhibiting Wnt Signaling Pathway

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Abstract

Idiopathic pulmonary fibrosis (IPF) is a progressive lung disease with high mortality, which characterized by epithelial cell damage and fibroblasts activation. Ellagic acid (EA) is a natural polyphenol compound widely found in fruits and nuts which has demonstrated multiple pharmacological activities. Herein we showed that Ellagic acid significantly alleviated bleomycin(BLM)-induced pulmonary fibrosis in mice, and also inhibited the Wnt/β-catenin signal in primary pulmonary fibroblasts. *In vitro* experiments indicated that Ellagic acid apparently suppressed Wnt3a-induced myofibroblasts activation and ECM accumulation mainly via inhibiting the phosphorylation of Erk2 and Akt. Further studies showed that Ellagic acid could induce autophagy formation of myofibroblasts mainly by suppressing mTOR signaling and promote apoptosis of myofibroblasts. *In vivo* experiments reveled that Ellagic acid significantly inhibited myofibroblasts activation and promoted autophagy formation. Taken together, our results showed that Ellagic acid effectively attenuated BLM-induced pulmonary fibrosis in mice by suppressing myofibroblasts activation, promoting autophagy and apoptosis of myofibroblasts mainly via inhibiting Wnt signaling pathway.

Background

Idiopathic pulmonary fibrosis (IPF) is a progressive, fatal and age-associated disease, and the average survival time for the IPF patients is only 2 to 5 years after diagnosis [1–3]. In Europe and North America, the incidence range is 3 to 9 cases per 100000 per a year [4]. Although the pathogenesis is not completely understood, many researchers believe that myofibroblasts activation, autophagy and apoptosis are essential factors in fibrotic progression[5].

The Wnt/β-catenin signal transduction pathway is essential for adult life of human beings, and could control myriad biological phenomena throughout development[6]. The Wnt signal also plays an essential role in fibrotic disease, and inhibiting this pathway could effectively suppress the fibrotic progression[7]. In lung epithelial cells and fibroblasts, β-catenin is over-expressed in fibrotic condition[8]. Many researches reveal that Wnt ligands induced fibroblasts activation and collagen synthesis, and blockading Wnt/β-catenin signal attenuates BLM-induced pulmonary fibrosis[9]. Overactive fibroblasts could produce abundant extracellular matrix(ECM) proteins, and inducing apoptosis of overactive fibroblasts was regarded as an effective method to alleviate fibrotic diseases[10]. In breast cancer cells, the block of Wnt signaling significantly induces the cellular apoptosis[11]. Hence, inhibiting fibroblasts activation and promoting myofibroblasts apoptosis could attenuate pulmonary fibrosis via suppressing Wnt/β-catenin signal.

There is an intimate relationship between autophagy and pulmonary fibrosis. The three main types of autophagy are chaperone-mediated autophagy, microautophagy and macroautophagy[12]. Here, we focus on macroautophagy(from here after referred to as autophagy), and the autophagic pathway is the catabolic mechanism for cytoplasmic organelles and degrading long-lived cellular proteins[13]. Recent researches indicate that the disruption of beclin1-BCL2 complex is an effective mechanism to promote

autophagy formation, which prevent premature ageing and improve health span in mammals[14]. Insufficient autophagy might result in the senescence of pulmonary epithelial cells and the activation of myofibroblasts in pulmonary fibrosis[15], and autophagy was inhibited in IPF patients[16]. In lung epithelial cells, bleomycin could induce pulmonary fibrosis in mice by impeding TFEB-mediated autophagic flux[17]. The AMPK-dependent activation of autophagy enhances collagen turnover to deactivate myofibroblasts, which attenuates BLM-induced pulmonary fibrosis[18]. The mTOR is a key regulator of growth in animals, and regulates the balance apoptosis and autophagy when cells were exposed to physiological stimulation, and also is the downstream Wnt signal[19]. Sirolimus, is a mTOR (mammalian target of rapamycin) inhibitor, attenuates BLM-induced pulmonary fibrosis in rats[20]. Therefore, inhibiting Wnt/mTOR signal could promote autophagy formation and this is an effective method to attenuate pulmonary fibrosis.

Ellagic acid (EA) is generated by hydrolysis of complex polyphenolic compounds named ellagitannins, and found in a wide variety of fruits and nuts such black currants, grapes, raspberries and strawberries[21, 22]. Ellagic acid has shown multiple protective effects during fibrotic diseases such as liver fibrosis, pancreatic fibrosis and cardiac fibrosis[23–25]. Ellagic acid promoted apoptosis and autophagy, suppressed Wnt/β-catenin and mTOR pathways in tumor cells[26–28]. In addition, previous studies have reported that Ellagic acid could attenuate BLM-induced pulmonary fibrosis in rats[29, 30], but the therapeutic effect of BLM-induced pulmonary fibrosis in mice and its mechanism are not clear. In our studies, we demonstrated that Ellagic acid could alleviate BLM-induced pulmonary fibrosis in mice mainly via inhibiting fibroblasts activation, inducing myofibroblasts autophagy and apoptosis and its main mechanism is regulating Wnt pathway.

Methods And Materials

1. **BLM-induced animal model of pulmonary fibrosis**

Thirty-five 7-8 weeks male C57BL/6J mice were purchased from Charles River(Beijing, China). All mice were housed and cared for in a pathogen-free facility at Nankai University. The mice were acclimatized in a room with constant temperature(25 ± 2℃) and relative humidity(60 ± 2%) and allowed free access to food and water. All animal experiments were approved by the Animal Care and Use Committee at Nankai University. The mice were randomly divided into 5 groups(n=7 per group): Control group, BLM group, Pirfenidone-treated group(200mg/kg), Low-Ellagic acid-treated group(10mg/kg), High-Ellagic acid-treated group(20mg/kg), Pirfenidone were purchase from Dalian Meilun Biotechnology(Dalian, China), Ellagic acid were purchased from Macklin Biochemical(Shanghai, China). The mice were orally exposed to Ellagic acid, Pirfenidone and water is once a day for 7-13 day. For BLM administration, mice were anesthetized with 10% chloral hydrate(Sangon, Shanghai, China) and then intratracheally injected with bleomycin(Nippon Kayaku Co., Ltd., Tokyo, Japan) at a dose of 2U/kg body weight for analysis of the fibrotic response. The sham-operated group received intratracheal injections of the same amount of saline. The drug administration group began to be administered daily from the seventh day of modeling

to 13th day. Mice were sacrificed on day 14 and lung tissue was harvested for the following experiments to evaluate the degree of pulmonary fibrosis.

2. **The isolation and culture of pulmonary fibroblasts**

Primary pulmonary fibroblasts(PPF) were isolated from and treated with 0.9% saline solution or BLM wild-type C57BL/6J mice, Mouse lung fibroblasts cell line(Mlg), and Mouse embryonic fibroblasts cell line(NIH-3T3), human lung fibroblasts (HLF-1) (kindly supplied by Professor Wen Ning, Nankai University) were cultured in DMEM medium (Solarbio, Beijing, China) or F12K medium (Gibco, New York, USA) supplemented with 10% fetal bovine serum (FBS, Biological Industries, Israel) and antibiotics(100mg/ml streptomycin, and 100 U/ml penicillin G) in a 37 ℃ atmosphere of 95% humidified air and 5% CO2. Chloroquine(CQ) were purchase from Macklin (Shanghai, China), Bafilomycin a1(Baf a1) were purchased from Cayman Chemical (Wuhan, China).

3. **Western blot**

The sample of lung tissues and cells were homogenized in RIPA lysis (Beyotime Biotechnology, Shanghai, China) buffer with PMSF and NaF (phosphatase inhibitor; need to add when extracting the phosphorylating protein), then centrifuged(10000 rpm, 10min) to obtain supernatants. The total protein concentration was measured by BCA Protein Assay kit(Beyotime Biotechnology, Shanghai, China). Secondary antibodies: goat anti-rabbit IgG-HRP(Abcam, Share, UK), goat anti-mouse IgG-HRP(Abcam, Share, UK). Relative density of each band was analyzed by Image J. The following first antibodies were used:

Antibody	Company &Item No.	Antibody	Company &Item No.
GAPDH	Affinity, AF7021	P62	Proteintech, 8420-1-AP
β-tubulin	Affinity, T0023	Atg16L1	CST, 8089T
Lamin B1	Affinity, DF6687	Beclin1	CST, 3495S
β-catenin	CST, 8480T	LC3A/B	CST, 12741S
p-β-catenin(s33/37/Thr41)	CST, 9561T	mTOR	Abcam, ab32028
p-β-catenin(Thr41/Ser45)	CST, 9565T	p-mTOR(S2448)	Abcam, ab109268
α-SMA	Affinity, BF9212	S6 ribosomal protein(S6RP)	Affinity, AF7831
Collagen I	Affinity, AF7001	p-S6 ribosomal protein(S235/S236)	CST, 4858T
Fibronectin	Affinity, AF5335	Caspase3	CST, 9662S
Akt	SANTA CRUZ, sc-56878	Cleaved-Caspase3	CST, 9664T
p-Akt(Ser473)	SANTA CRUZ, sc-514032	Caspase9	CST, 9009T
Erk1/2	SANTA CRUZ, sc-514302	Cleaved-Caspase9	CST, 9504T
p-Erk1/2(Thr202/Thy204)	SANTA CRUZ, sc-81492		

4. **Quantitative real-time PCR (qRT-PCR)**

The total RNA was extracted using TRIzol Reagent(Invitrogen, Carlsbad, CA, USA). The cDNA was obtained from total RNA through reverse transcribed. qRT-PCR was performed by using SYBR GreenER qPCR SuperMix Universal(Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocols. The relative quantification of gene expression (a-SMA, Collagen I, Fibronectin, Cyclin D1, MMP7, Wisp1) was measured relative to the endogenous reference gene β-actin using the comparative CT method in the experiment. Sequences of the specific primer sets are as follows:

α-SMA (NM_007392.2), 5-GCTGGTGATGATGCTCCCA-3 and 5-GCCCATTCCAACCATTACTCC-3;

Col1a1 (NM_007742.3), 5-CCAAGAAGACATCCCTGAAGTCA-3 and 5-TGCACGTCATCGCACACA-3;

Fn (NM_010233.1), 5-GTGTAGCACAACTTCCAATTACGAA-3 and 5-GGAATTTCCGCCTCGAGTCT-3;

Cyclin D1 (NM_007631), 5-GCGTACCCTGACACCAATCT-3 and 5-CAGGTCTCCTCCGTCTTGAG-3;

MMP7 (NM_010810), 5-CTTACCTCGGATCGTAGTGGA-3 and 5-CCCCAACTAACCCTCTTGAAGT-3;

Wisp1 (NM_018865), 5-CAGCACCACTAGAGGAAACGA-3 and 5-CTGGGCACATATCTTACAGCATT-3;

β-actin (NM_007393.3), 5-AGGCCAACCGTGAAAAGATG-3 and 5-AGAGCATAGCCCTCGTAGATGG-3.

5. **Hematoxylin-eosin staining (HE staining)**

Left lungs were fixed in 10% formalin for 24 hours and embedded in paraffin. Then lung sections(5μm) were prepared and stained with hematoxylin-eosin staining. HE staining images were collected using an upright transmission fluorescence microscope and opened in Image-Pro Plus Version 6.0 (Media Cybernetics, Inc, USA). Quantification of pulmonary fibrosis was performed as described previously[31]. In brief, the software selection tool can select the entire lung tissue area and automatically calculate the total pixel (Pw) of the region, and then use the same method to calculate the total pixel (Pf) of the fibrosis region, fibrosis ratio = fibrosis area pixel (Pf)/total lung pixel (Pw).

6. **GFP-LC3 and Cherry-GFP-LC3 transfections and Immunofluorescence**

Two mg of GFP-LC3B(mouse) and Cherry-GFP-LC3B(mouse) was transfected into NIH-3T3 cells using PEI according to the supplier's protocol (Sino Biolgical Inc., Beijing, China). Cells were fixed in 4% paraformaldehyde for 20 min, washed with PBS, permeabilized with 0.2% Triton X-100 in PBS, blocked with 5% BSA and incubated with α-SMA antibodies. Cells were washed with PBS, and FITC were used for immunofluorescence visualization. Next, tissues were incubated with anti-a-SMA, anti-Col1 antibody overnight at 4 °C and then secondary FITC or Rhodamine (TRITC)-labelled antibody for 30 min. Fluorescein (FITC) AffiniPure Goat Anti-Mouse IgG (H+L) and Rhodamine (TRITC) Affinipure Goat Anti-Rabbit IgG (H+L) were purchased from Jackson ImmunoResearch(Pennsylvania, USA). Nuclei were stained with DAPI (Solarbio, Beijing, China), Cells and Tissues were photographed with a TCS SP8 confocal microscope(Leica).

7. **Dual luciferase assay**

TCF/LEF promoters were cloned into the pGL4.49 luciferase reporter vector, and NIH-3T3 cells were transfected with luciferase reporter plasmids using PEI. Renilla-luciferase was used as an internal control. Cells were treated 18 h after transfection with a series of EA for 8 hous. Cells were harvested, and the luciferase activity of cell lysates was determined using a luciferase assay system (Promega, USA) as described by the manufacturer. Total light emission was measured using a GloMax®-Multi Detection System (Promega, USA).

8. **Immunohistochemistry and Masson staining**

The tissue sections were pretreated in a microwave, blocked and incubated using a series of antibodies, and stained with DAB and hematoxylin. The results were captured using a microscope(Nikon, Japan). The experimental procedure of Masson refers to Masson's Trichrome Stain Kit(Solarbio, Beijing, China).

The method of counting the positive area is as follows: 1)Using Image J to open a picture, click “Image” and “type”, change “RGB Color” to “RGB stack”; 2)Click “Image”, drop down and click “adjust”, change the B&W in the “Default” column to “Red”; 3)adjust the upper and lower pulleys to select the positive signal area, click “set”, click “ok”; 4) Then click “analyze”, click “set measurement”, choose “area fraction”; 5)Began to count positive results, click “Control+M” on the keyboard, the number of “the% Aera” is the result.

9. **Flow cytometric analysis of apoptosis**

1X10 NaCl-PPF or PPF-BLM cells/mL were seeded in six-well plate and left for 24 hours in incubator to resume exponential growth. The extent of apoptosis was measured through annexinV-FITC apoptosis detection kit (Beyotime, Shanghai, China) as described by the manufacture's instruction. Cells were exposed to drug and incubated for 24h. then they collected and washed with PBS twice, gently resuspended in annexin-V binding buffer and incubated with annexinV-FITC/PI in dark for 15min. The number of apoptosis was detected by CytoFLEX S(Beckman Coulter, USA).

10. **Hydroxyproline assay**

The collagen contents in right lungs of mice were measured with a conventional hydroxyproline method. [32], In brief, the right lungs were dried and acid hydrolyzed, then the residue was filtered and the PH value was adjusted to 6.5-8.0. The hydroxyproline analysis was performed using chloramine-T spectrophotometric absorbance as previously describe.

11. **Evaluation of pulmonary function**

The C57BL/6J mice were administrated by BLM(2U/Kg) on day 0, and were orally exposed to Ellagic acid, Pirfenidone and water are once a day for day 7-13, and finally were sacrificed on day 14. The mice were anesthetized with 10% chloral hydrate in saline (i.p.), If you recognized that the breath of mice is more quickly, you need to add anesthetic or wait for a moment until the mice breathe slowly. Carefully cut the neck skin with a scalpel (try not to bleed, otherwise it will affect the experimental data of lung functions), and subsequently using the surgical line to fix the cannula. The mice were transferred into the plethysmography chamber and analyzed functions using Anires2005 system (Biolab, Beijing, China). This system automatically calculates and displays pulmonary function parameters, including forced vital capacity (FVC), dynamic compliance (Cydn), inspiratory resistance (Ri) and expiratory resistance (Re).

12. **Statistical Analysis**

Statistical analysis was performed using GraphPad Prism 6.0 software. 2-tailed Student t test was used to analyze statistical differences. Pearson correlation and linear regression were used to determine the concordance. Data were shown as means ± s.d. and significance was described as follows: *,P <0.05; **, P < 0.01; ***, P < 0.001, #,P <0.05; ##, P < 0.01; ###, P < 0.001.

Results

1. **Ellagic acid attenuates BLM-induced pulmonary fibrosis in mice.**

To determine the therapeutic impact of the Ellagic acid in pulmonary fibrosis in mice, the BLM-induced pulmonary fibrosis mice model was established and mice were treated with Ellagic acid after being exposed to BLM. Pirfenidone is one of the listed drugs for the treatment of IPF, and was used as a positive drug. We treated C57BL/6J mice with Ellagic acid(10mg/kg, 20 mg/kg) from day 7 to day 13 after administrating BLM, and mice were sacrificed on day 14. Ellagic acid improved alveolar structure distortion and decreased fibrotic percent(Fig.1A-B). The Masson staining result indicates that the collagen level of lung section was decreased after treating Ellagic acid(Fig.1A,C). The dead ratio of mice sharply increased and reached up to 60% when the mice were exposed to BLM, and Ellagic acid were able to increase the survival percent of mice(Fig.1D). In addition, in the right lung tissue, Ellagic acid reduced the level of hydroxyproline(Fig.1E). Lung functions are a key indicator of treating efficiency in clinic trials, Ellagic acid has positive impacts on lung functions such as forced vital capacity(FVC), dynamic compliance(Cydn), expiratory resistance(Re), inspiratory resistance(Ri)(Fig.1F-I). These data indicated that Ellagic acid effectively attenuated BLM-induced pulmonary fibrosis in mice and the therapeutic effect of Ellagic acid was better than positive drug pirfenidone.

2. **Ellagic acid inhibits Wnt/β-catenin signaling pathway**

Ellagic acid has attenuated Wnt/β-catenin in other disease models, such as brain injury, HBP carcinomas and skin photoaging[33-35], but it has not been definitely proved whether Ellagic acid inhibited Wnt/β-catenin signal in pulmonary fibrosis. So our next works were to focus on Wnt/β-catenin after treating Ellagic acid in pulmonary fibroblasts. We tested its ability to inhibits β-catenin/TCF reporter activity, which was measured with reporter genes harboring TCF/LEF-binding sites. The result showed that Ellagic acid dose-dependently inhibited Wnt3a-induced TOPFlash activity in NIH-3T3 cells(Fig.2A). The accumulation of nuclear β-catenin is a hallmark for activation canonical Wnt signaling, Fig.2B showed that the nuclear β-catenin level were decreased in Ellagic acid-treated NaCl-PPF(NaCl-primary pulmonary fibroblasts) cells and the cytoplasmic β-catenin level was not change, these results meant that Ellagic acid inhibited the entry of β-catenin into pulmonary fibroblast nuclei. To further verify whether Ellagic acid could promote phosphorylation of β-catenin at serine/threonine, we performed the immunoblots assays of phosphorylation levels of β-catenin. As expected, expression levels of p-β-catenin(Ser33/37/Thr41) and p-β-catenin(Thr41/Ser45) were significantly up-regulated by Ellagic acid in NaCl-PPF cells(Fig.2C). We treated NaCl-PPF cells with Ellagic acid and also found that Ellagic acid inhibited the expression of the Wnt signal target genes, including CyclinD1, Wisp1 and MMP7 (Fig.2D-F). Therefore, these data above demonstrated that Ellagic acid suppressed Wnt/β-catenin signaling pathway in pulmonary fibroblasts.

3. **Ellagic acid suppressed Wnt3a-induced fibroblasts activation and ECM accumulation mainly via regulating the phosphorylation of Erk and Akt.**

We further explored whether Ellagic acid could decreased Wnt3a-induced myofibroblasts activation and ECM production and its mechanism. Ellagic acid could significantly decreased Wnt3a-induced Fn and Col 1 expression levels in Mlg cells, Col 1 and α-SMA expression levels in NaCl-PPF cells (Fig.3A-B), and the results of immunofluorescence also revealed that Ellagic acid suppressed Wnt3a-induced the expression level of α-SMA in Mlg and NaCl-PPF cells (Fig.3C-D). Ellagic acid could down-regulate Wnt3a-induced the mRNA levels of α-SMA, Col1 a1 and Fn in Mlg cells and NaCl-PPF cells (Fig.3E-F). To explore further mechanism, we detected whether Ellagic acid could affect the downstream Wnt signal. As expected, Ellagic acid significantly decreased Wnt3a-induced the proportions of p-Erk2(Tyr204)/Erk2 and p-Akt(Ser473)/Akt(Fig.3G), but p-Erk1(Thr202)/Erk1 was not significant(the densitometric analysis was not shown), and BLM-PPF(BLM-primary pulmonary fibroblasts) cells incubated with Ellagic acid also showed similar results(Fig.4H). These results showed that Ellagic could significantly decrease Wnt3a-induced pulmonary fibroblasts activation and ECM production mainly via inhibiting the activation of Erk2(Tyr204) and Akt(Ser475) in pulmonary fibroblasts.

4. **EA promotes pulmonary fibroblast autophagy mainly via inhibiting Wnt-mTOR signaling pathway.**

The Wnt pathway is a well-known pathway and involves the regulation of autophagy[36-38]. In order to explore whether Ellagic acid have impacts on autophagy and the downstream Wnt signal(mTOR sigal), and we have established the cellular model of inhibitory autophagy. The p62 protein accumulation suggested that the autophagy activity is decreased[39]. The two autophagy inhibitors, Chloroquine (CQ) and Bafilomycin a1 (Baf a1), could increase the expression levels of p62 (Fig.4A-B), and Ellagic acid could significantly down-regulate Baf a1 and CQ-induced the p62 expression level in Mlg cells (Fig.4C-D). The plasmids of GFP-LC3B and mCherry-GFP-LC3B were used to detect autophagic flux, Ellagic acid enabled to increase Wnt3a-induced the number of GFP-LC3B puncta in NIH-3T3 cells (Fig.4E). To corroborate these findings, we used mCherry-GFP-LC3B reporters to measure the formation of autophagosomes ($Cherry^+$ GFP^+ signal) and autolysosomes ($Cherry^+$ GFP^- signal) in NIH3T3 cells. As expected, Ellagic acid treatment significantly increased Wnt3a-induced the number of $Cherry^+$ GFP^- puncta(autolysosomes) (Fig.4F). In addition, we found that Ellagic acid promoted the expression of autophagy related proteins such as Atg16L, Beclin1, LC3-II(LC3 lipidation) (Fig.4G). The mTOR pathway is the downstream Wnt signal and relates to autophagy formation, so we examined protein expression levels of mTOR, S6RP(S6 ribosomal protein) and their phosphorylation by Western blot. Our studies revealed that the administration of Ellagic acid reduced Wnt3a-indued phosphorylation levels of mTOR(S2248) and S6RP(S235/S236) in Mlg cells, and Ellagic acid exposing to BLM-PPF cells also showed the similar results (Fig.4H-I). Therefore, these data suggested that Ellagic acid induced autophagy formation mainly by suppressing Wnt-mTOR signaling pathway in pulmonary fibroblasts.

5. **Ellagic acid promotes the apoptosis of pulmonary myofibroblasts.**

In order to detect whether Ellagic acid contributed to the cells of apoptosis, analyzing the number of apoptosis cells via using flow cytometry has established. Treatment of Ellagic acid resulted in an increase in annexin V^+/PI^- cells (early apoptosis), but no change in annexin V^+/PI^+(late apoptosis) or

annexin V^-/PI^+ cells (necrosis) (Fig. 5A). In addition, Caspase proteins tightly related to cellular apoptosis, our experiments indicated that Ellagic acid increased the expression levels of Cleaved-Caspase9 and Cleaved-Caspase3 in PPF cells (Fig.5B) and Wnt3a-induced the expression levels of Cleaved-Caspase9 in Mlg cells(Fig.5C). Hence, Ellagic acid could promote the apoptosis of pulmonary myofibroblasts.

6. **Ellagic attenuates BLM-induced fibroblasts activation and ECM accumulation *in vivo.***

The mice were treated with Ellagic acid on day 7 to day 13, and we employed immunohistochemistry in order to identify that Ellagic acid have negative impact on expression levels of α-SMA, Col 1, Fn and p62 in lung sections which were equal results to a positive drug Pirfenidone (Fig.6A). Our results associated with immunofluorescence also showed inhibitory expression levels of α-SMA and Col1 in lung sections(Fig.6B-C). In addition, we used a tissue homogenizer to break up lung tissues, and α-SMA, p-mTOR, mTOR and LC3 protein were analyzed by using Western bolt. Ellagic acid decreased the expression level of α-SMA and the ratio of p-mTOR/mTOR, and increased the ratio of LC3-II/LC3-I (Fig.6D). Together, these data identified that Ellagic acid significantly inhibited myofibroblasts activation and mTOR signal, promoted autophagy formation *in vivo.*

Discussion

Idiopathic pulmonary fibrosis is a chronic, fatal lung disease, and finally many IPF patients were respiratory failure[3]. The importance of Wnt signaling pathway have been identified in pulmonary fibrosis, and the main reason is that aberrant Wnt signal could induce fibroblasts activation and ECM production[40, 41]. When Wnt signal was abnormal activated, β-catenin was translocated into nuclear to upregulate related-target genes such as Cyclin D1, MMP7 and Wisp1[42–44]. In our studies, Ellagic acid could inhibit Wnt/β-catenin signaling pathway mainly via decreasing the expression of Cyclin D1, MMP7, Wisp1 and nuclear β-catenin and increase the phosphorylation of β-catenin.

Collagen, a main component of extracellular protein, could maintain the basic structure of the lung. However, there are overactivated fibroblasts and overexpressed ECM proteins in fibroblastic foci, and inhibiting this condition could effectively attenuate pulmonary fibrosis[45]. Excessive ECM proteins resulted in the formation of lung scar, which might accelerate the decline of force vital capacity[46, 47]. Previous studies showed that Wnt ligands promoted fibroblasts activation and collagen synthesis[9] and increased the phosphorylation levels of Akt, Erk[48, 49]. Therefore, there is an effective method to inhibit fibroblasts activation and ECM accumulation via down-regulating Wnt3a-induced the activation of Akt, Erk. Our experiment results revealed that Ellagic acid significantly inhibited fibroblasts activation and ECM production via decreasing the translation and transcription levels of α-SMA, Col 1 and Fn *in vivo or in vitro*, and the underlying mechanism mainly was the inhibitory Wnt/Akt, Erk signal.

The insufficient autophagy plays an essential role in the pathogenesis of IPF. Many researchers pay attention to fibroblasts activation because selected autophagy could promote collagen turnover[18]. The PB1 domain of p62 protein has involved to degrade polyubiquitinated, misfolded, aggregated proteins

and dysfunctional organelles via regulating autophagy formation in mammalian cells[50]. Our studies showed that Ellagic acid could increase the expression of p62 in pulmonary fibroblasts. LC3-II, a processed form of LC3, was localized in the membrane of autophagosomes and regarded as a powerful marker for autophagosomes[51]. During the formation of early autophagosomes, some complexes formed by LC3-II and other autophagy-related proteins was required[52]. In addition, Atg16L1 and beclin1 were essential factors to autophagy flux and its biological function[53, 54]. In BLM-primary pulmonary fibroblasts, Ellagic acid treatment could increase LC3-II, Atg16L1 and beclin1 expression levels. The mTOR pathway also played an important role in regulating the balance growth and autophagy in response to environmental stress, and this signal was regulated by Wnt signal[55]. Interestingly, our results showed that Ellagic acid significantly inhibited Wnt3a-induced the activation of mTOR signal in pulmonary fibroblasts. Hence, Ellagic acid could induce autophagy and this process might be regulated by Wnt/mTOR signal pathway in pulmonary fibrosis. Inhibition of autophagy (by knockout of Atg3, Atg5, Atg7 and mutation of Atg1, Atg13, vsp34) suppresses the induction of apoptosis[56–58]. Autophagy could degrade catalase and promote necrotic cell death[59]. In chronic lymphocytic leukemia cells, down-regulating the Wnt signaling pathway promoted the cells apoptosis[60]. Ellagic acid induced apoptosis by increasing the number of early apoptosis cells and promoting the activation of Caspase3 and Caspase9 in Mlg and PPF cells.

Conclusion

In conclusion, we have identified that Ellagic acid alleviated BLM-induced pulmonary and improved lung functions in mice. *In vitro* experiments revealed that Ellagic acid could inhibit fibroblasts activation, ECM production and promoted myofibroblasts autophagy and apoptosis mainly via down-regulating Wnt signaling pathway. Ellagic acid also inhibited myofibroblasts activation and promoted autophagy formation *in vivo*. Therefore, our studies provides a powerful proof for the idea that Ellagic acid might be used as a candidate compound for anti-pulmonary fibrosis drugs and provide more potential therapeutic options for IPF patients.

Abbreviations

BLM
Bleomycin; IPF:Idiopathic pulmonary fibrosis; ECM:Extracellular matrix; EA:Ellagic acid; mTOR:mammalian target of rapamycin; BCL2:B-cell lymphoma-2; TFEB:Transcription factor EB; PPF:Primary pulmonary fibroblasts; Mlg:Mouse lung fibroblasts cell; HLF-1:human lung fibroblasts; DMEM:dulbecco's modified eagle medium; NIH-3T3:Mouse embryonic fibroblasts cell line; FBS:etal bovine serum; CQ:Chloroquine; Baf a1:Bafilomycin a1; PMSF:Phenylmethylsulfonyl fluoride; PEI:Polyethylenimine; FVC:forced vital capacity; Cydn:dynamic compliance; Ri:inspiratory resistance; Re:expiratory resistance; Pf:fibrosis area pixel; Pw:total lung pixel; H&E:Hematoxylineosin; TGF-β:Transforming growth factor β; Fn:Fibronectin; Col 1:Collagen I; α-SMA:α-smooth muscle actin; LC3:Microtubules associated protein 1 light chain 3; MMP:Matrix metalloproteinase

Declarations

Ethics approval and consent to participate

All animal experiments were approved by the experimental animal ethics committee of Nankai University (No.: SYXK 2019-0001).

Availability of data and materials

Data available on request from the authors.

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Competing interests

The authors state no conflict of interest.

Authors' contributions

HZ and CY contributed to the conception and design. XL, KH carried out the immunoassays. XL and HR carried out in vivo experiments. LM, YC, YW and HL contributed to the collection and assembly of data. YW, JL, and ZL contributed to the data analysis and interpretation. BY, JG and SW contributed to the data revision. XL and GY contributed to the manuscript writing. HZ and CY contributed to the administrative support. All authors read and approved the final manuscript. All authors read and approved the final manuscript.

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Figures

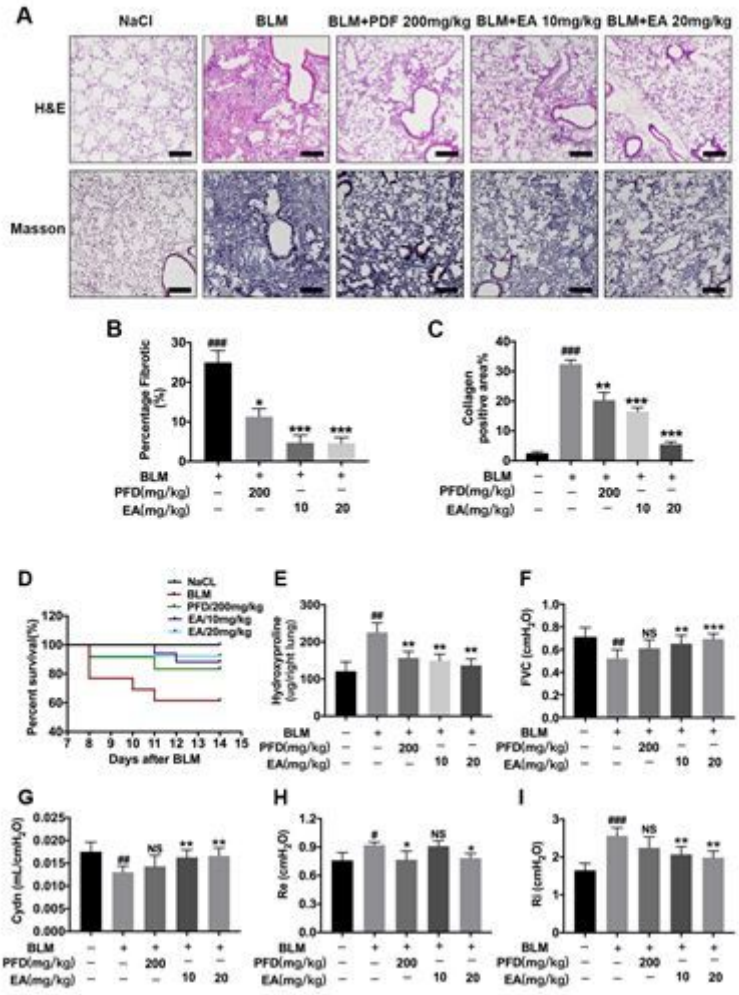

Figure 1

Ellagic acid attenuates BLM-induced pulmonary fibrosis in mice. Ellagic acid (10 mg/kg, 20 mg/kg) and pirfenidone (200mg/kg) were given orally once a day from day 7-13 after BLM-treatment and lungs were harvested on day 14. (A-C)Representative images of hematoxylin and eosin (H&E) and Masson staining of lung tissue sections. Scale bars: 50μM. (D)Percentages of surviving mice were plotted from day 7-13 after BLM treatment. (E)Hydroxyproline contents in right lung tissues. (G-I)Parameters of lung function such as forced vital capacity(FVC), dynamic compliance(Cydn), expiratory resistance(Re) and inspiratory resistance(Ri). Data(Fig.1B-C, F-I) are means ± standard deviation (SD), n=7, *P<0.05, **P<0.01, ***P<0.001,#P<0.05, ##P<0.01, ###P<0.001, NS: nonsignificant(Student t test).

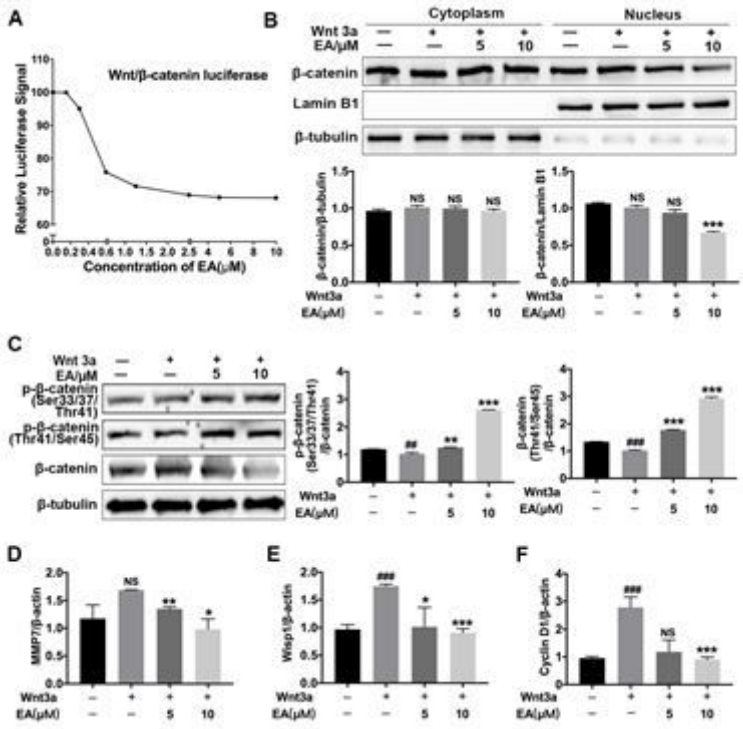

Figure 2

EA inhibits Wnt/β-catenin signaling pathway. (A)NIH-3T3 cells were transfected with the TOPFlash plasmids. After 18 hours of transfection, the cells were treated with Wnt3a(100ng/mL) and/or Ellagic acid at various concentrations for 8 h and then lysed for luciferase assays. (B)NaCl-PPF cells were treated with Ellagic acid (5μM,10μM) and/or Wnt3a(100ng/mL) for 4 hours, and cell lysates were used to analyze the expression levels of β-catenin in nucleus and cytoplasm. Densitometric analyses were shown below. (C)NaCl-PPF cells were treated with Ellagic acid (5μM,10μM) and/or Wnt3a(100ng/mL) for 4 hours, p-β-catenin(Ser33/37/Thr41) and p-β-catenin(Thr41/Ser45) were detected by Western blot. Densitometric analyses were shown beside. (D-F)NaCl-PPF cells were incubated with Ellagic acid (5μM,10μM) and/or Wnt3a(100ng/mL) for 12 hours to analyze the mRNA levels of Cyclin D1, Wisp1 and MMP7 by using quantitative real-time PCR(n=3). Data(Fig.2B-F) are means ± standard deviation(SD), *P<0.05, **P<0.01, ***P<0.001, ##P<0.01, ###P<0.001, NS: nonsignificant(Student t test). β-tubulin was used as a cytosolic loading control and Lamin B1 was used as a nuclear loading control.

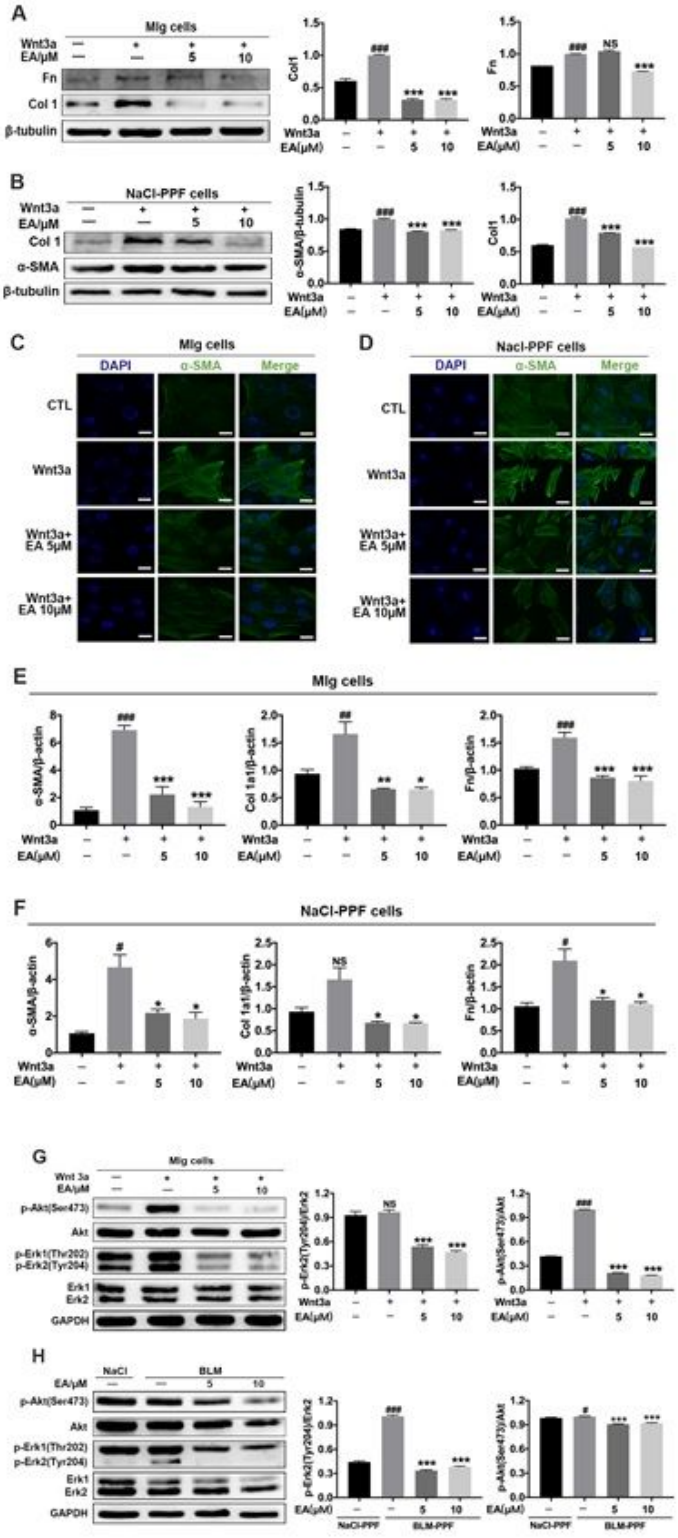

Figure 3

Ellagic acid decreases Wnt3a-induced pulmonary fibroblasts activation and ECM accumulation. (A-D)Mlg and NaCl-PPF cells were exposed to Wnt3a(100ng/mL) and/or Ellagic acid(5μM,10μM) 24 hours to detect expression levels of α-SMA, Col1 and Fn by using Western blot or immunofluorescence. Densitometric analyses were shown beside. (E-F)Wnt3a(100ng/mL) and/or Ellagic acid(5μM,10μM) were incubated with Mlg and NaCl-PPF cells for 12 hours, and cell lysate was used to analyze the mRNA levels

of α-SMA, Col1 and Fn by using quantitative real-time PCR(n=3). (G)Mlg and NaCl-PPF cells were exposed to Wnt3a(100ng/mL) and/or Ellagic acid(5μM,10μM) for 12 hours, (H)BLM-PPF cells were treated with Ellagic acid(5μM,10μM) for 12 hours to evaluate protein expression levels of p-Akt(Ser473), Akt, p-Erk1/2 (Thr202/Tyr204)and Erk1/2 by western blot. Densitometric analyses were shown beside. Data(Fig.4A-B, E-H) are means ± standard deviation (SD), *P<0.05, **P<0.01, ***P<0.001, #P<0.05, ###P<0.001, NS: nonsignificant(Student t test). β-tubulin or GAPDH were used as a loading control.

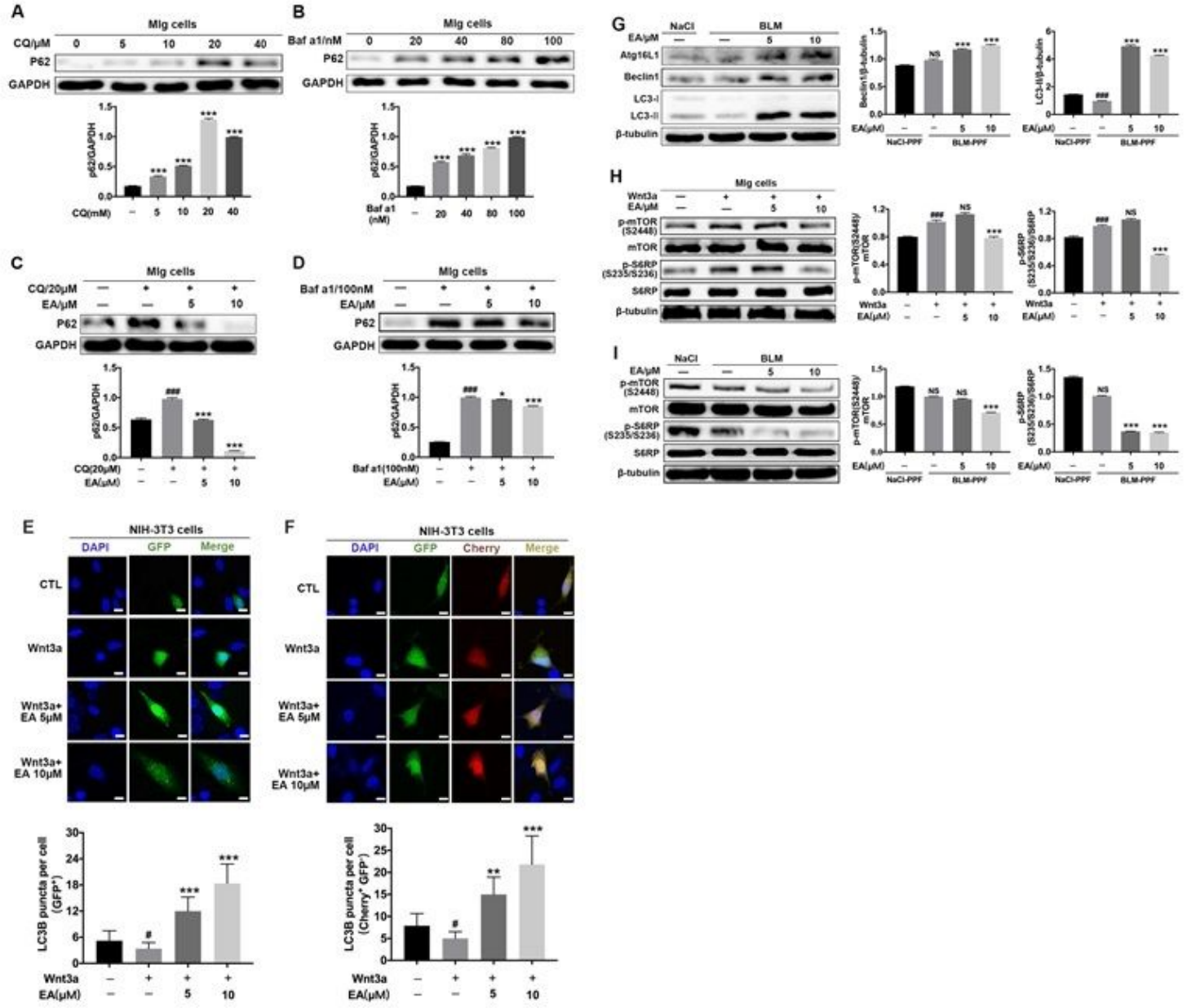

Figure 4

EA promotes pulmonary fibroblast autophagy mainly via inhibiting Wnt-mTOR signaling pathway. (A-B)Mlg cells were exposed to CQ and Baf a1 in a serious concentration for 24 hours, (C-D)Mlg cells were exposed to CQ(20μM) and Baf a1(100nM) with or without Ellagic acid(5μM, 10μM) to analyze the p62 expression level by using western blot. Densitometric analyses were shown below. (E-F)The plasmids of GFP-LC3B and mCherry-GFP-LC3B were transfected to NIH3T3 cells with PEI, and these cells were

subsequently exposed to Ellagic acid (5μM, 10μM) and/or Wnt3a (100ng/mL) for 12 hours(n=3). DNA was counterstained with DAPI (blue). Quantitative analyses are showed below. Scale bars: 50μM. (G)BLM-PPF cells were treated with Ellagic acid (5μM, 10μM) for 24 hours, and the Atg16L1, Beclin1 and LC3-II/I expression levels were detected by Western blot. Densitometric analyses were shown beside. (H)Mlg cells were treated with Ellagic acid (5μM, 10μM) and/or Wnt3a(100ng/mL) for 12 hours. Densitometric analyses were shown beside. (I)BLM-PPF cells were treated with Ellagic acid(5μM, 10μM) for 12 hours, and protein expression levels of mTOR, S6RP and their phosphorylation were detected by Western blot. Densitometric analyses were shown beside. Data are means ± standard deviation (SD), *P<0.05, **P<0.01, ***P<0.001, #P<0.05, ###P<0.001, NS: nonsignificant(Student t test). β-tubulin or GAPDH were used as a loading control.

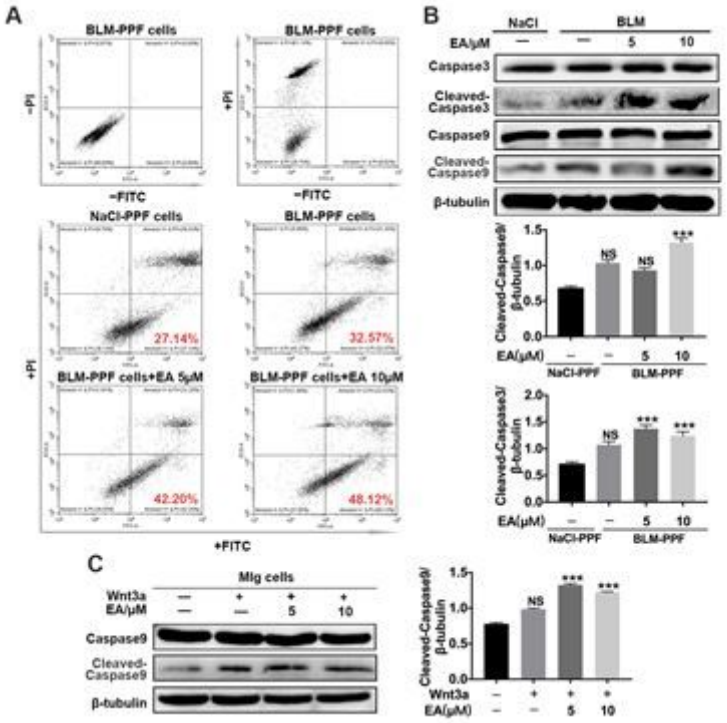

Figure 5

Ellagic acid promotes the apoptosis of pulmonary myofibroblasts. (A)Mlg cells incubated with Ellagic acid(5μM, 10μM) and/or Wnt3a(100ng/mL) for 24 hours, and Annexin V/PI staining was subsequently performed to estimate early, late apoptosis and necrosis by flow cytometry(n=3). (B)BLM-PPF cells were treated with Ellagic acid (5μM, 10μM) for 24 hours, Lysates were immunoblotted for Caspase3, Cleaved-Caspase3, Caspase9 and Cleaved-Caspase9. Densitometric analyses were shown below. (C)Mlg cells were treated with Ellagic acid (5μM, 10μM) and/or Wnt3a(100ng/ mL) for 24 hours, Lysates were immunoblotted for Caspase9 and Cleaved-Caspase9. Densitometric analysis were shown beside. Data in (B-C) are means ± standard deviation (SD), ***P<0.001, NS: nonsignificant(Student t test). β-tubulin was used as a loading control.

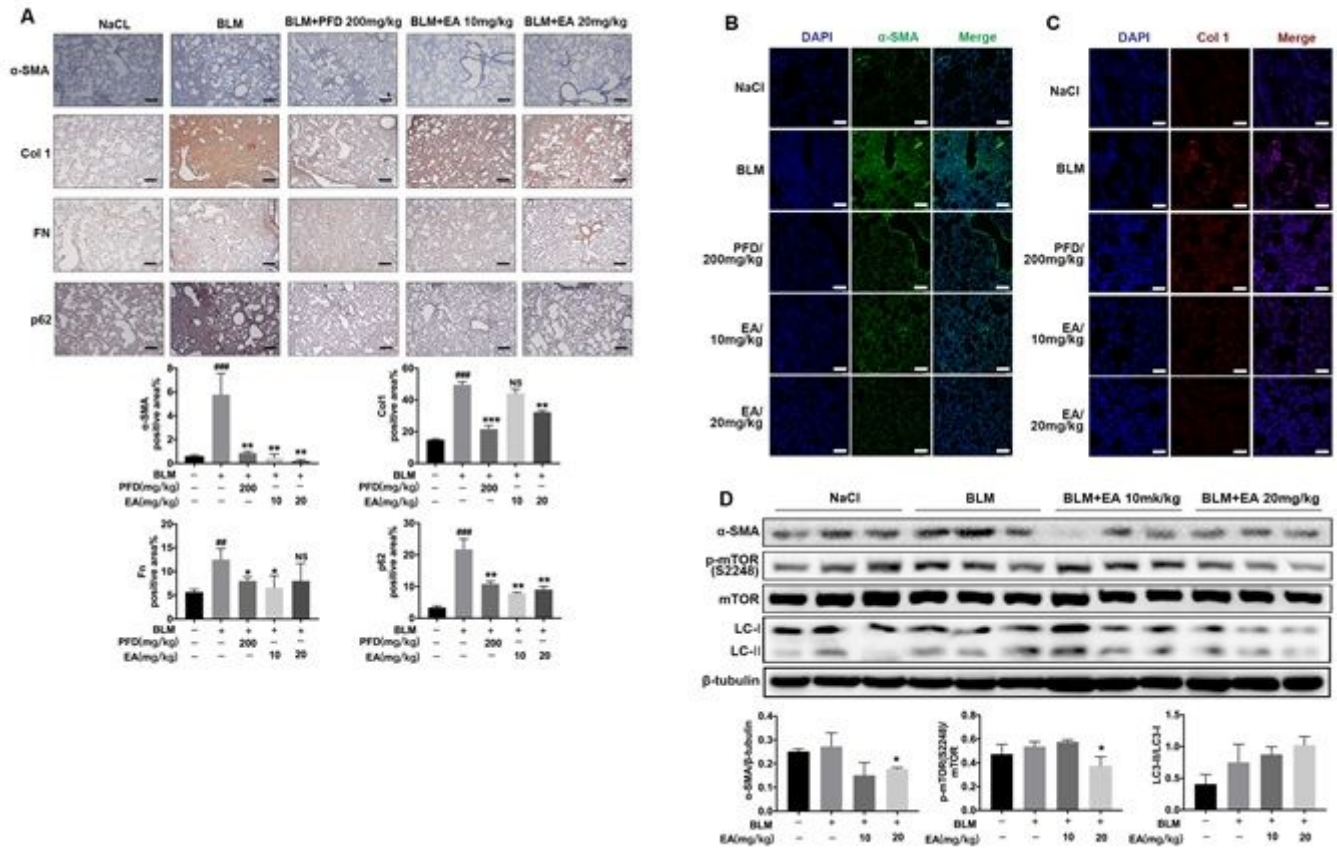

Figure 6

Ellagic attenuates BLM-induced fibroblasts activation and ECM accumulation in vivo. The mice were treated with Ellagic acid (10mg/kg, 20mg/kg) from day 7-13 after administrating BLM. (A)Immunohistochemistry was used to analyze the expression levels of α-SMA, Col 1, Fn and p62(n=3). Quantitative analysis were shown below. Scale bars: 50μM (B-C)The expression levels of α-SMA and Col1 were detected by immunofluorescence in lung sections. Scale bars: 50μM. (D)Lung homogenization was used to analysis the α-SMA, p-mTOR(S2248), mTOR and LC3-II/I expression levels by Western blot(n=3). Densitometric analyses were shown below. Data in (A, D) are means ± standard deviation (SD), *P < 0.05, **P < 0.01, ***P < 0.001, ##P < 0.01, ###P < 0.001, NS: nonsignificant(Student t test). β-tubulin was used as a loading control.